PUBLISHED AND SPONSORED BY:
- Publication of Royan Institute, Iranian Academic Center for Education Culture and Research (ACECR)

Abstract & Full Text Indexing to:
1- PubMed Central (PMC)
2- National Library of Medicine (NLM)
3- Index Medicus for the Eastern Mediterranean Region (IMEMR)
4- Index Copernicus International
5- EMBASE
6- Scopus
7- CINAHL Database
8- Google Scholar
9- Proquest
10- Directory of Open Access Journals (DOAJ)
11- Open Academic Journals Index (OAJI)
12- Directory of Research Journals Indexing (DRJI)
13- Scientific Information Database (SID)
14- Iranmedex
15- Regional Information Center for Sciences and Technology (RICeST)
16- Islamic World Science Citation Center (ISC)
17- Magiran

ACECR

Editorial Office Address: P.O.Box: 16635-148, Royan Institute, Tehran, Iran
(Mohammad Hossein Nasr Esfahani, Ph.D.)
Tel & Fax: +9821-22510895
Web: www.ijfs.ir
Emails: ijfs@royaninstitute.org & info@ijfs.ir

Printing Company:
Jurband Ghaemprint Co.
NO. 5, Jalil khoob alley, Niroo Havaei Street, Tehran, Iran

INTERNATIONAL JOURNAL OF FERTILITY AND STERILITY

Int J Fertil Steril, Vol 9, No 1, Apr-Jun 2015, Pages: 1-140

Contents

Original Article

Comparison of 25-hydroxyvitamin D and Calcium Levels between Polycystic Ovarian Syndrome and Normal Women

Ashraf Moini, M.D.[1, 2, 3], Nooshin Shirzad, M.D.[1, 4], Marzieh Ahmadzadeh, M.D.[1], Reihaneh Hosseini, M.D.[1]*, Ladan Hosseini, M.Sc.[1], Shahideh Jahanian Sadatmahalleh, Ph.D.[2, 5]

1. Department of Gynecology and Obstetrics, Arash Women's Hospital, Tehran University of Medical Sciences, Tehran, Iran
2. Department of Endocrinology and Female Infertility at Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
3. Vali-e-Asr Reproductive Health Research Center, Tehran University of Medical Sciences, Tehran, Iran
4. Endocrinology and Metabolism Research Center, Endocrinology and Metabolism Clinical Sciences Institute, Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences, Tehran, Iran
5. Department of Reproductive Health and Midwifery, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Abstract

Background: Given the relationship of vitamin D deficiency with insulin resistance syndrome as the component of polycystic ovary syndrome (PCOS), the main aim of this study was to compare serum level of 25- hydroxyvitamin D [25(OH)D] between PCOS patients and normal individuals.

Materials and Methods: A cross sectional study was conducted to compare 25(OH)D level between117 normal and 125 untreated PCOS cases at our clinic in Arash Hospital, Tehran, Iran, during 2011-2012. The obtained levels of 25(OH)D were classified as follows: lower than 25 nmol/ml as severe deficiency, between 25-49.9 nmol/ml as deficiency, 50-74.9 nmol/ml as insufficiency, and above 75 nmol/ml asnormal. In addition, endocrine and metabolic variables were evaluated.

Results: Among PCOS patients, our findings shows 3(2.4%) normal, 7(5.6%) with insufficiency, 33(26.4%) with deficiency and 82(65.6%) with severe deficiency, whereas in normal participants, 5(4.3%) normal, 4(3.4%) with insufficiency, 28(23.9%) with deficiency and 80(68.4%) with severe deficiency. Comparison of 25(OH)D level between two main groups showed no significant differences ($p= 0.65$). Also, the calcium and 25(OH)D levels had no significant differences in patients with overweight ($p=0.22$) and insulin resistance ($p=0.64$). But we also found a relationship between 25(OH)D level and metabolic syndrome ($p=0.01$). Furthermore, there was a correlation between 25(OH)D and body mass index (BMI) in control group ($p=0.01$), while the C-reactive protein (CRP) level was predominantly higher in PCOS group ($p<0.001$).

Conclusion: Although the difference of 25(OH)D level between PCOS and healthy women is not significant, the high prevalence of 25(OH)D deficiency is a real alarm for public health care system and may influence our results.

Keywords: Polycystic Ovary Syndrome, 25-Hydroxyvitamin D, Calcium

Citation: **Moini A, Shirzad N, Ahmadzadeh M, Hosseini R, Hosseini L, Jahanian Sadatmahalleh Sh. Comparison of 25-hydroxyvitamin D and calcium levels between polycystic ovarian syndrome and normal women. Int J Fertil Steril. 2015; 9(1): 1-8.**

Received: 15 Aug 2013, Accepted: 10 Feb 2014
*** Corresponding Address: Department of Gynecology and Obstetrics, Arash Women's Hospital, Tehran University of Medical Sciences, Tehran, Iran**
Email: arash_hosp@tums.ac.ir

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 1-8

Introduction

Polycystic ovarian syndrome (PCOS) is known as one of the most common endocrine disordersand affects 5-10% of women that is characterized by hyperandrogenism and chronic anovulation. PCOS as a multi-dimensional syndrome influences various systems. Infertility, irregular menses, acanthosis nigricans, insulin resistance, and hirsutism are known as some of symptoms of PCOS (1). Also, it has some long term consequences such as hypertension coronary artery diseases and type II diabetes. Therefore, PCOS seems a real dilemma for gynecologists and endocrinologists to reveal its basic phathophysiology and offer a reasonable treatment.

There are many evidence showing the relationship between serum level of 25- hydroxyvitamin D [25(OH)D] and PCOS. Furthermore, the strong association between PCOS and insulin resistance indicates that insulin directly influences ovarian function (2), while impaired glucose tolerance and insulin secretion have been shown to be associated with vitamin D deficiency (3). Additionally, recent data have suggested that both calcium and vitamin D supplements may improve insulin sensitivity in PCOS women (4).

There are suggestions that calcium has important role in activation and maturation of oocyte in animals (5); therefore, abnormalities in calcium metabolism may play an important role in pathogenesis of PCOS.

In this study, we tried to investigate the correlation between vitamin D levels and PCOS in our population in order to make a decision about screening programs or supplement therapy in PCOS patients. Also, we aimed to find a correlation between body mass index (BMI), hyperandrogenism and metabolic syndrome with serum level of 25(OH)D in PCOS patients.

Materials and Methods

This cross sectional study and was performed on 242 women, 125 patients with PCOS and 117 healthy individuals, during 2011-2012. All women in 16-44 age group (reproductive age) were recruited from our clinic in Arash Hospital, Tehran, Iran, consecutively. PCOS was diagnosed based on the presence of two of following Rotterdam criteria: oligo and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries in ultrasound, meaning presence of 12 or more follicles measuring 2-9 mm in diameter in each ovary and/or ovarian volume more than 10 cm^3. The main problems of PCOS patients who attended our clinic were abnormal uterine bleeding and infertility.

A healthy woman was defined as woman in reproductive age with regular cycles. They came to our clinic for annual check-up, or their partners had male fertility problems. Women with congenital adrenal hyperplasia, hyperprolactinemia, hyperparathyroidism, and androgen secretory tumors were excluded using specific laboratory tests to verify the concentrations of 17 OH progesterone, dehydroepiandestronsulfate (DHEA-S), and prolactin. Furthermore, women who used medications suspicious to affect carbohydrate metabolism or calcium/vitamin D concentrations during 6 months prior to the study, who had a history of chronic disease or endocrinophaties, and who had a history of smoking or drug abuse were excluded. All participants were living in Tehran, Iran, and they had no history of calcium or vitamin D supplementation.

A morning blood sample was taken after 12 hours fasting during the follicular phase (3-5 days of spontaneous or progesterone-induced menstrual cycle). The levels of calcium, vitamin D, insulin, high density lipoprotein- cholesterol (HDL-C), fasting blood sugar (FBS), triglyceride (TG), thyroid stimulating hormone (TSH), prolactin, testosterone, dehydroepiandrosterone sulfate (DHEAS), and C-reactive protein (CRP) were measured. All samples were obtained during fall and winter seasons.

The PCOS patients were divided into two subgroups as follows: metabolic syndrome group (n=39) and non-metabolic syndrome group (n=86). The metabolic syndrome was defined by the National Cholesterol Education Program (NCEP) and the adult treatment panel III (ATP III) after observing three of the four following criteria: systolic blood pressure >130 mmHg and diastolic blood pressure >85 mmHg, TG level >150 mg/dl, HDL <50 mg/dl, fasting glucose >100 mg/dl, and waist circumference >88 cm. Also, we used the homeostatic model assessment

of insulin resistance (HOMA-IR) to evaluate insulin resistance based on the following formula: fasting plasma glucose (mmol/L)×fasting plasma insulin (μU/L) divided by 22.5.

Next 25(OH)D was measured using enzyme-linked immunosorbent assay (ELISA, IDS, Boldon, UK) with normal range of 75-100 nmol/ml with 5.4 coefficient of variation (CV). Furthermore, insulin (2.0-25.0 μIU/ml), prolactin (2.8-29.2 μg/ml), TSH (0.1-4 mIU/ml), total testosterone (0.14-0.9 ng/ml), and DHEAS (up to 5.8 μg/ml) concentrations were measured using ELISA (Monobind, USA). Fasting glucose (≤100 mg/dl), TG (≤150 mg/dl), HDL (>40 mg/dl), calcium (8.3-10.5 mg/dl) and CRP (≤8 mg/ml) concentrations were determined using photometry (Parsazmoon, Iran). Hyperandrogenism was defined as the clinical presence of hirsutism (Ferriman-Gallway score ≥8), acne or alopecia and/or elevated androgen levels, meaning as testosterone level above 0.9 ng/ml and/or DHEAS level above 5.8 μg/ml. The measurement of 25(OH)D concentration was done using serum assay and at least 1 cc of patient's serum was stored in freezer (-40˚C) for maximum 30 days.

The approval was obtained from the Ethic Committee of Endocrinology and Metabolism Research Institute, Tehran University of Medical Science, Tehran, Iran, and an informed consent was obtained from all participants.

Statistical analysis

We used Kolmogorov-Smirnov test (K-S test) for evaluating data distribution. To analyze differences between groups, Student's t test was used for normally distributed samples and nonparametric Mann-Whitney U-test was appliedfor abnormally distributed samples. Relationship between variables were evaluated using Pearson's correlation coefficient. All analyses were performed by SPSS version 16 (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was considered as statistically significant.

Results

Totally, all data were collected from 125 PCOS patients and 117 healthy women. The mean age was 27.85 in PCOS group and 30.82 in normal subjects. Also, the mean weight in PCOS and healthy women were 83.08 and 82.97, respectively. Table 1 shows the means of various factors in two main groups.

Table 1: Comparison of different biological and biochemical values between two main groups

	PCOS	Non-PCOS	P value
	Mean ± SD	Mean ± SD	
Age (Y)	28.2 ± 8.4	30.82 ± 7.12	0.02
BMI (kg/m²)	25.92 ± 4.71	24.41 ± 3.88	0.01
FBS (mg/dl)	90.33 ± 16	90.99 ± 9.47	0.61
TG (mg/dl)	119.41 ± 66.73	111.12 ± 49.72	0.77
HDL-C (mg/dl)	48.24 ± 13.7	47.7 ± 9.49	0.82
Ca (mg/dl)	9.34 ± 0.92	9.43 ± 0.69	0.88
CRP (mg/ml)	1.77 ± 1.97	1.50 ± 2.08	<0.001
Testosterone (ng/ml)	0.82 ± 0.37	0.82 ± 0.41	0.46
TSH (mIU/ml)	2.55 ± 2.02	2.30 ± 1.87	0.163
DHEAS (μg/ml)	1.59 ± 0.80	1.47 ± 0.76	0.18
Insulin (μIU/ml)	16.66 ± 17.3	15.34 ± 11.19	0.96
Prolactin (ng/ml)	15.96 ± 10.2	16.55 ± 9.45	0.41
25-OH vitamin D (nmol/ml)	8.92 ± 6.43	9.29 ± 7.35	0.41

BMI; Body mass index, FBS; Fasting blood sugar, TG; Triglyceride, HDL; High density lipoprotein, CRP; C-reactive protein, TSH; Thyroid stimulating hormone and DHEAS; Dehydroepiandrosterone sulfate.

We stratified level of 25(OH)D as follows: lower than 25 nmol/ml as severe deficiency, between 25-49.9 nmol/ml as deficiency, 50-74.9 nmol/ml as insufficiency, and above 75 nmol/ml as normal (6). Among PCOS patients, our findings shows that 3 (2.4%) normal, 7 (5.6%) with insufficiency, 33 (26.4%) with deficiency and 82 (65.6%) with severe deficiency, whereas in normal participants, 5 (4.3%) normal, 4 (3.4%) within sufficiency, 28 (23.9%) with deficiency and 80 (68.4%) with severe deficiency. Totally, 162 women (66.9%) had severe deficiency, 61women (25%) had deficiency and 11 women (4.5%) had insufficiency, whereas only 8 women (3.3%) were normal. Comparison between two main groups showed no statistically differences. The mean of calcium was 9.34 mg/dl in PCOS subjects and 9.43 mg/dl in normal group. None of them implied statistical significant differences. Table 2 shows the relationships between 25(OH)D and other parameters in PCOS and non-PCOS groups.

Table 2: The correlation of vitamin D and other biochemical levels between two main groups

	25-hydroxyvitamin D					
	PCOS group			Non-PCOS group		
	R	P	OR	R	P	OR
BMI	-0.63	0.50	-0.02	0.10	0.25	-0.13
FBS	-0.56	0.55	-0.06	-0.05	0.52	0.23
TG	-0.08	0.36	0.24	0.18	0.04	-0.14
HDL-C	-0.10	0.25	-0.08	-0.16	0.06	0.00
Ca	-0.01	0.90	0.08	0.10	0.24	0.19
CRP	-0.06	0.52	-0.08	-0.03	0.69	-0.05
Testosterone	-0.05	0.55	-0.04	0.00	0.95	0.19
TSH	0.06	0.49	0.09	0.03	0.66	-0.07
DHEAS	0.00	0.99	0.22	0.17	0.92	0.01
Insulin	-0.11	0.23	-0.11	0.00	0.94	-0.07
Prolactin	0.06	0.51	-0.17	-0.14	0.10	-0.31

BMI; Body mass index, FBS; Fasting blood sugar, TG; Triglyceride, HDL; High density lipoprotein, CRP; C-reactive protein, TSH; Thyroid stimulating hormone, DHEAS; Dehydroepiandrosterone sulfate, P; P value, R; Pearson correlation coefficient and OR; Odd ratio.

We chose consecutive patients in our clinic, and the age and BMI values were different between two main groups in final analysis. We used logistic regression to control confounding factors and in this analysis, PCOS was not correlated with 25(OH)D level (OR: 1, 95% CI=0.97- 1, p=0.65, Fig.1).

Furthermore, in PCOS group, 39 women belonged to metabolic syndrome group, while86 women belonged to non-metabolic syndrome group. The mean of 25(OH)D and calcium concentrations were 28.15 nmol/ml and 9.27 mg/dl in the metabolic syndrome group in comparison with 22.57 nmol/ml and 9.39 mg/dl in the non-metabolic syndrome group, indicating no significant difference between two sub-groups (p_{vitD}=0.18, p_{Ca}=0.35). Also, in control group, the levels of Ca and 25(OH)D showed no statistically difference (p_{vitD}=0.59, p_{Ca}=0.17). But in total population of our study, there was a relationship between metabolic syndrome and the level of 25(OH)D (p_{vitD}=0.01, p_{Ca}=0.35). According to BMI, 59 out of 125 PCOS patients (47.2%) were lean, 39 patients (31.2%) were overweight, and 27 women were obese (21.6%). The mean of calcium and vitamin D concentrations were 25.57 nmol/ml and 9.33 mg/dl in normal weight women, 24.34 nmol/ml and 9.34 mg/dl in overweight women, and 15.29 nmol/ml and 9.39 mg/dl in obese women with presenting no significant differences between these sub-groups(p_{vitD}=0.87, p_{Ca}=0.52). Although, the level is lower in obese women. In control group, the level of 25(OH)D is significantly lower in obese women (p_{vitD}=0.01, p_{Ca}=0.47). In total population, the level of 25(OH)D showed no significant correlation with BMI (p=0.22).

We had 62 patients with insulin resistance in PCOS (HOMA-IR >2.5) group and the mean of their 25(OH)D and calcium concentrations were 25.08 ng/ml and 9.32 mg/dl,respectively. Besides, 63 women were non-insulin resistant with the mean of 23.95 ng/ml for 25(OH)D level and 9.38 mg/ml for calcium level, showing there were no significant differences between these two sub-groups (p_{vitD}=0.37, p_{Ca}=0.74).

A total of 30 patients had hyperandrogenism and the mean of their calcium and 25(OH)D concentrations did not differ significantly with the related values of non-hyperandrogenism sub-group.

C-reactive protein, as an inflammatory factor, was measured and showed no correlation with 25-hydroxyvitamin D levels; however, it was significantly higher in PCOS patients.

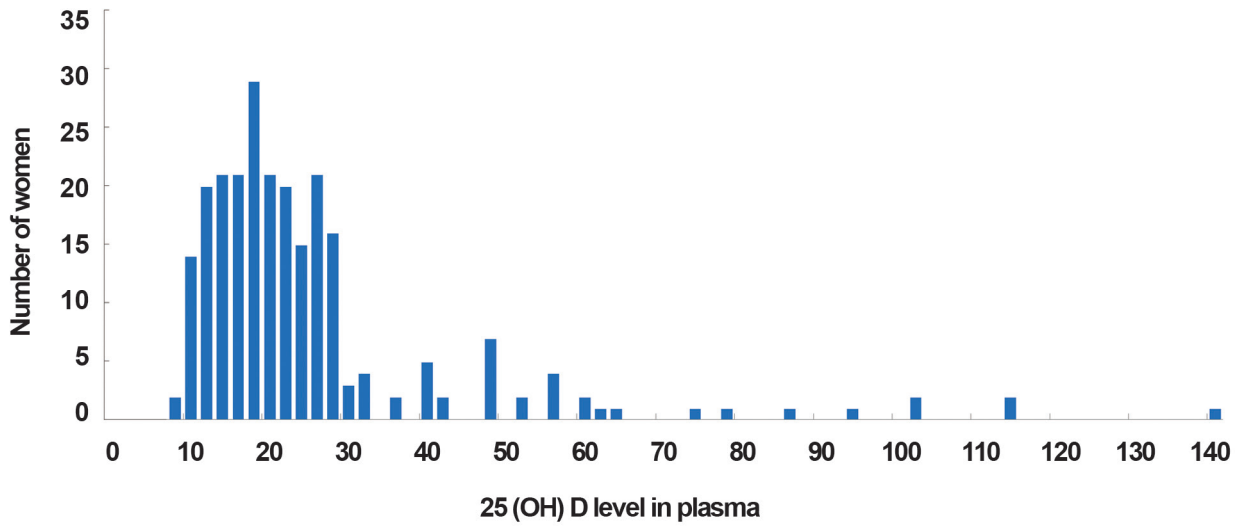


Fig.1: Distribution of 25(OH)D level in women.

Discussion

In overall, the most prominent point in our study was the high percentage of moderate to severe 25(OH)D deficiency in over than 70% of our population. It seems that this high prevalence influenced other aspects of our study. This proportion is lower in other studies, like 26.7% in PCOS patients in Germany (7), 2.9% in Austria (8), 27% in Caucasian infertile women (9), and 64.9% for female population in Iran (10).

Animal researches have demonstrated the role of calcium in oocyte activation and maturation (5) and hypothesized that disturbances in calcium homeostasis may mediate the pathogenesis of PCOS. In our study, there was a high incidence of 25(OH)D deficiency in both PCOS and normal groups. Although the PCOS group had lower levels of calcium and 25(OH)D, the difference was not considerable. Also, Yildizhan et al. (11) evaluated 100 patients with PCOS and found no correlation between 25(OH)D level and PCOS, but they suggested a negative correlation between BMI and 25(OH)D levels. In our study, this negative correlation was found only in control group. It may be due to confounding effects of other factors such as higher rate of insulin resistance and metabolic syndrome in PCOS group.

In another study from Hahn et al. (7) who evaluated 120 PCOS patients, there was a significant negative correlation between 25(OH)D levels and BMI, but there was no difference in calcium level. In addition, Asheim et al. (12) studied consecutively 76 PCOS women and 30 healthy ones and found lower levels of 25(OH)D in morbidly obese women.

25(OH)D deficiency has diverse effects in human bodies. There is evidence that demonstrate a correlation between 25(OH)D and insulin resistance. Although the mechanisms underlying these associations are not fully understood (13, 14), vitamin D has some effects on beta-cell function and may have a beneficial effect on insulin action by stimulating the expression of insulin receptors (8). In a study by Hahn et al. (7), they showed that 25(OH)D was associated with higher BMI values and body fat. In severely obese patients, Manco et al. (15) has illustrated that the fat mass is the best predictor for serum level of 25(OH)D. However, the mechanisms mediating these finding remain unclear.

Despite these facts, in this study, the level of 25(OH)D did not differ significantly between two sub-groups (obese and non-obese), in total. It may be due to high percentage of severe vitamin D deficiency in our subjects that influenced our outcomes.

Furthermore, our result imply a negative correlation between metabolic syndrome and 25(OH)D level which is in agreement with mentioned mechanisms of 25(OH)D. According to study of Wehr et al. (8) which included 205 PCOS women, they found a strong association between low serum levels of vitamin D and the metabolic syndrome. Also, we saw a positive correlation between TG and 25(OH)D levels in PCOS group ($p=0.002$). It may be due to the common nutritional sources, but this result has not been reported in other articles and needs more precise studies.

Another study in Iran also shows that 64.9% of women in Tehran have mild to moderate 25(OH) D deficiency (11). Also, several studies from the Middle East have implied high prevalence of vitamin D deficiency in this area (16-19). Possible explanation for high proportion of vitamin D deficiency is different level of sun exposure as a result of urban life style or different levels of calcium intake. Low-calcium, high-phytate diets, pregnancy, and winter-related reduced sunlight exposure have been reported as probable causes (16).

Furthermore, many studies have reported negative correlation between 25(OH)D level and HOMA-IR (8, 11, 20). 25(OH)D is believed to have some roles in insulin release, expression of insulin receptors, and suppression of cytokines that are possible mediators for insulin resistance (21). But in this study, although the HOMA-IR (as insulin resistance) was higher in severe deficient group, the difference was not statistically significant. It may be due to the small sample size or high percentage of deficiency in all groups.

Whereas obesity and insulin resistance aggravate hyperandrogenism (7), there are no significant differences in testosterone and DHEAS levels between PCOS and non-PCOS women in our study. Therefore, the correlation between 25(OH) D and testosterone levels in our PCOS group is not reliable. Perhaps this result can be due to the fact that we didnot measure the sex hormone binding

globulin (SHBG) and free androgen index (FAI) in this study. Some data revealed a significant correlation between levels of 25(OH)D with SHBG and FAI (7, 20). The total androgen level is not significantly higher in PCOS groups. Some PCOS studies in Iran have reported 20-40% hyperandrogenism in native population with PCOS (22, 23) and some other studies from Middle East have found lower androgen levels than expected (24). But we need to reevaluate the androgen level of our PCOS patients in another study with different methods of measurement.

Besides, in our study, PCOS group showed higher levels of CRP level and BMI that have been known as risk factors for cardiovascular disease (CVD), leading to elevated risk of CVD and/or stroke in this population. Women with PCOS are characterized by a prothrombotic state, as reflected by increased plasminogen activator inhibitor-1(PAI-1) activity and fibrinogen concentration. The inflammatory markers such as CRP together with low heart rate explain high fibrinogen levels in women with PCOS (25). In other studies, this elevation has been also reported (26, 27). In a study by Li et al. (20), they reported that 25(OH)D concentration was negatively correlated with CRP. However, in this study, we did not find this correlation (e.g. higher level of CRP in sever vitamin D deficiency group).

There are increasing evidence indicating the role of vitamin D deficiency as a risk factor for multiple sclerosis (28), type 1 diabetes (29), CVD (30), and several malignant tumors (31). So, this pandemic of 25(OH)D deficiency is an alarm for public health care system and implies an emergent need to interfere. Consequently, we should try to change people's life style and to design practical plans for food fortification and screening programs. Our study had some limitations such as relatively small sample size and no information available with respect to dietary calcium intake.

It seems that there is an emergent need for supplement therapy and screening programs among our women in reproductive age-PCOS and -non-PCOS groups. Although we did not find any difference in 25(OH)D level between two groups in our study, high prevalence of vitamin D deficiency may influence these results and a clinical trial with vitamin D supplement therapy can be the next step of our study.

Conclusion

The first aim of the study was to find a relationship between vitamin D deficiency and PCOS, whereas the final result implicates to not only an association between vitamin D and metabolic syndrome, but also a real peril of pandemic of severe vitamin D deficiency which is considered as real threats for women of reproductive age. Although a direct association between PCOS and vitamin D was not found, it may need another study after the correction of vitamin D level.

Acknowledgements

This study was financially supported by Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences, Tehran, Iran. The authors reported no conflict of interest.

References

1. Franks S. Polycystic ovary syndrome. N Engl J Med. 1995; 333(13): 853-861.
2. Nestler JE, Barlascini CO, Matt DW, Steingold KA, Plymate SR, Clore JN, et al. Suppression of serum insulin by diazoxide reduces serum testosterone levels in obese women with polycystic ovary syndrome. J Clin Endocrinol Metab. 1989; 68(6): 1027-1032.
3. Ortlepp JR, Metrikat J, Albercht M, von Kroff A, Hanrath P, Hoffmann R. The vitamin D receptor gene variant and physical activity predicts fasting glucose levels in healthy young men. Diabetic Medicine. 2003; 20(6): 451-454.
4. Kosta K, Yavropoulou MP, Anastasiou O, Yavos JG. Role of vitamin D treatment in glucose metabolism in polycystic ovary syndrome. Fertil Steril. 2009; 92(3): 1053-1058.
5. DeFelici M, Dolci S, Siracusa G. An increase of intracellular free Ca^{2+} is essential for spontaneous meiotic resumption by mouse oocytes. J Exp Zool. 1991; 260(3): 401- 405.
6. Holick MF. Vitamin D deficiency. N Engl J Med. 2007; 357(3): 266-281.
7. Hahn S, Haselhorst U, Tan S, Quadbeck B, Schmidt M, Roesler S, et al. Low serum 25-hydroxyvitamin D concentrations are associated with insulin resistance and obesity in women with polycystic ovary syndrome. Exp Clin Endocrinol Diabetes. 2006; 114(10): 577-583.
8. Wehr E, Pilz S, Schweighofer N, Giuliani A, Kopera D, Pieber TR, et al. Association of hypovitaminsis D with metabolic disturbances in polycystic ovary syndrome. Eur J Endocrinol. 2009; 161(4): 575-582.
9. Pal L, Shu J, Zeitlian G, Hickmon C. Vitamin D insufficiency in reproductive years may be contributory to ovulatory infertility and PCOS. Fertil Steril. 2008; 90 Suppl 1: S14.
10. Moradzadeh K, Larijani B, Keshtkar AA, Hossein-Nezhad A, Rajabian R, Nabipour I, et al. Normative values of vitamin D among Iranian population: a population based study. Int J Osteoporosis Metab Disorders. 2008; 1(1): 8-15.
11. Yildizhan R, Kurdoglu M, Adali E, Kolusari A, Yildizhan B, Guler Sahin H, et al. Serum 25-hydroxyvitamin D concentrations in obese and non-obese women with polycystic ovary syndrom. Arch Gynecol Obstet. 2009; 280(4): 559-

563.
12. Ashiem ET, Hofso D, Hjelmesaeth J, Birkeland KI, Bohmer T. Vitamin status in morbidly obese patients: a cross sectional study. Am J Clin Nutr. 2008; 87(2): 362-369.
13. Paanidis D, Balaris C, Farmakiotis D, Rousso D, Kourtis A, Balaris V, et al. Serum parathyroid hormone concentrations are increased in women with polycystic ovary syndrome. Clin Chem. 2005; 51(9): 1691-1697.
14. Chiu KC, Chu A, Go VL, Saad MF. Hypovitaminosis D is associated with insulin resistance and beta cell dysfunction. American J Clin Nutr. 2004; 79(5): 820-825.
15. Manco M, Calvani M, Nanni G, Greco AV, Iaconelli A, Gasbarrini G, et al. Low 25-hydroxyvitamin D does not affect insulin sensitivity in obesity after bariatric surgery. Obes Res. 2005; 13(10): 1692-1700.
16. Goswami R, Gupta N, Goswami D, Marwaha RK, Tandon N, Kochupillai N. Prevalence and significance of low 25-hydrxyvitamin D concenterations in healthy subjects in Delhi. Am J Clin Nutr. 2000;72(2): 472-475.
17. Dawodu A, Agarwal M, Hossain M, Kochiyil J, Zayed R. Hypovitaminosis D and vitamin D deficiency in exclusively breast-feeding infants and their mothers in summer: a justification for vitamin D supplementation of breast-feeding infants. J Pediatr. 2003; 142(2): 169-173.
18. Azizi F, Rais-Zadeh F, Mir Said Ghazi A. Vitamin D deficiency in a group of Tehran population. J Res Med Sci. 2000; 4(38): 291-303.
19. Kazemi A, Sharifi F, Jafari N, Mousavinasab N. High prevalence of vitamin D deficiency among pregnant women and their newborns in an Iranian population. J Womens Health (Larchmt). 2009; 18(6): 835-839.
20. Li HW, Brereton RE, Anderson RA, Wallace AM, Ho CK. Vitamin D deficiency is common and associated with metabolic risk factors in patients with polycystic ovary syndrome. Metabolism. 2011; 60(10):1475-1481.
21. Teegarden D, Donkin SS. Vitmin D: emerging new roles in insulin sensitivity. Nutr Resh Rev. 2009; 22(1): 82-92.
22. Mehrabian F, Khani B, Kelishadi R, Ghanbari E. The prevalence of polycystic ovary syndrome in Iranian women based on different diagnostic criteria. Endokrynologia Pol. 2011; 62(3): 238-242.
23. Tehrani FR, Simbar M, Tohidi M, Hosseinpanah F, Azizi F. The prevalence of polycystic ovary syndrome in a community sample of Iranian population: Iranian PCOS prevalence study. Reprod Biol Endocrinol. 2011; 9: 39.
24. Guzel AI, Kuyumcuoğlu U, Celik Y. Factors affecting the degree of hirsutism in patients with polycystic ovary syndrome. Arch Gynecol Obstet. 2012; 285(3):767-770.
25. Manneras-Holm L, Baghaei F, Holm G, Janson PO, Ohlsson C, Lonn M, et al. Coagulation and fibrinolytic disturbances in women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2011; 96(4): 1068-1076.
26. Moran LJ, Cameron JD, Strauss BJ, Teede HJ. Vascular function in the diagnostic categories of polycystic ovary syndrome. Hum Reprod. 2011; 26(8): 2192-2199.
27. Boulman N, Levy Y, Leiba R, Shachar S, Linn R, Zinder O, et al. Increased C-reactive protein levels in the polycystic ovary syndrome: a marker of cardiovascular disease.J Clini Endocrinol Metab. 2004; 89(5): 2160-2165.
28. Cantorna MT, Hayes CE, DeLuca HF. 1,25- Dihydroxyvitamin D_3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. Proc Natl Acad Sci USA. 1996; 93(15): 7861-7864.
29. Hypponen E, Laara E, Reunanen A, Jarvelin MR, Virtanen SM. Intake of vitamin D and risk of type 1 diabetes: a birth cohort study. Lancet. 2001; 358(9292): 1500-1503.
30. Zittermann A, Schleithoff SS, Tenderich G, Berthold HK, Korfer R, Stehle P. Low vitamin D status: a contributing factor in the pathogenesis of congestive heart failure?. J Am Coll Cardiol. 2003; 41(1): 105-112.
31. Ahonen MH, Tenkanen L, Teppo L, Hakama M, Tuohimaa P. Prostate canser risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland). Cancer Causes Control. 2000; 11(9): 847-852.

Original Article

Comparison between Unilateral and Bilateral Ovarian Drilling in Clomiphene Citrate Resistance Polycystic Ovary Syndrome Patients: A Randomized Clinical Trial of Efficacy

Ziba Zahiri Sorouri, M.D.[1], Seyede Hajar Sharami, M.D.[1]*, Zinab Tahersima, M.D.[1], Fatemeh Salamat, M.Sc.[2]

1. Reproductive Health Research Center, Department of Obstetrics and Gynecology, Alzahra Hospital, Guilan University of Medical Sciences, Rasht, Iran
2. Research Vice Chancellorship, Guilan University of Medical Sciences, Rasht, Iran

Abstract

Background: Laparoscopic ovarian drilling (LOD) is an alternative method to induce ovulation in polycystic ovary syndrome (PCOS) patients with clomiphene citrate (CC) resistant instead of gonadotropins. This study aimed to compare the efficacy of unilateral LOD (ULOD) versus bilateral LOD (BLOD) in CC resistance PCOS patients in terms of ovulation and pregnancy rates.

Materials and Methods: In a prospective randomized clinical trial study, we included 100 PCOS patients with CC resistance attending to Al-Zahra Hospital in Rasht, Guilan Province, Iran, from June 2011 to July 2012. Patients were randomly divided into two ULOD and BLOD groups with equal numbers. The clinical and biochemical responses on ovulation and pregnancy rates were assessed over a 6-month follow-up period.

Results: Differences in baseline characteristics of patients between two groups prior to laparoscopy were not significant ($p>0.05$). There were no significant differences between the two groups in terms of clinical and biochemical responses, spontaneous menstruation (66.1 vs. 71.1%), spontaneous ovulation rate (60 vs. 64.4%), and pregnancy rate (33.1 vs. 40%) ($p>0.05$). Following drilling, there was a significant decrease in mean serum concentrations of luteinizing hormone (LH) ($p=0.001$) and testosterone ($p=0.001$) in both the groups. Mean decrease in serum LH ($p=0.322$) and testosterone concentrations ($p=0.079$) were not statistically significant between two groups. Mean serum level of follicle stimulating hormone (FSH) did not change significantly in two groups after LOD ($p>0.05$).

Conclusion: Based on results of this study, ULOD seems to be equally efficacious as BLOD in terms of ovulation and pregnancy rates (Registration Number: IRCT138903291306N2).

Keywords: Bilateral, Unilateral, Ovarian Induction, Polycystic Ovary Syndrome

Citation: Zahiri Sorouri Z, Sharami SH, Tahersima Z, Salamat F. Comparison between unilateral and bilateral ovarian drilling in clomiphene citrate resistance polycystic ovary syndrome patients: a randomized clinical trial of efficacy. Int J Fertil Steril. 2015; 9(1): 9-16.

Introduction

The most common cause of anovulatory infertility is polycystic ovary syndrome (PCOS) (1-4). Induction of ovulation with clomiphene citrate (CC) is the first line of treatment in these patients (1, 5-8). CC resistant is defined as failure to ovulate after receiving a maximum dosage of 150 mg per day for five days beginning on the third day of menstrual cycle (1, 9) Laparoscopic ovarian drilling (LOD) is an alternative method to induce ovu-

Received: 4 Nov 2013, Accepted: 8 Mar 2014
* Corresponding Address: P.O. Box: 4144654839, Reproductive Health Research Center, Department of Obstetrics and Gynecology, Alzahra Hospital, Guilan University of Medical Sciences, Rasht, Iran
Email: sharami@gums.ac.ir

lation in these patients instead of administration of gonadotropins (1, 2, 10-14). Despite minimal morbidity associated with this method, LOD has some benefits. The benefits consist of the elimination of cycles monitoring, decreasing the risk of ovarian hyperstimulation syndrome (OHSS), multifetal pregnancy associated with gonadotropins (2, 14-21), as well as occurring spontaneous ovulation in some patients without further treatments (16). Two disadvantages of LOD are the probability of tubo-ovarian adhesion (TOA) (12, 22-31) and risk of premature ovarian failure (POF) (24, 25). Reducing the potential damage to ovarian surface epithelium (OSE) leads to a significantly decreased risk for TOA and POF (24, 25). A few studies have compared unilateral LOD (ULOD) and bilateral LOD (BLOD) and concluded that ULOD is equally efficacious as BLOD in inducing ovulation and achieving pregnancy besides minimizing the risk of adhesion and POF (24, 25, 32-34). Therefore, changing the usual method of LOD for both ovaries to only one ovary may minimize those risks. This study was done prospectively to compare the efficacy of ULOD versus BLOD in CC resistant patients in terms of ovulation and pregnancy rates.

Materials and Methods

This prospective parallel randomized clinical trial was conducted in Al-Zahra Hospital in Rasht, Guilan Province, Iran, from June 2011 to July 2012. Among PCOS women attending the infertility clinic with CC resistant ovaries, 121 patients with CC resistance PCOS were initially examined. Before laparoscopy, five patients had other endocrine abnormally, four patients had mechanical factors abnormally such as unilateral or bilateral tubal blockages in hysteroscopy (HSC), seven patients had concomitant male infertility, and five patients refused to participate in the study; therefore, 100 patients were included in this study (Fig.1).

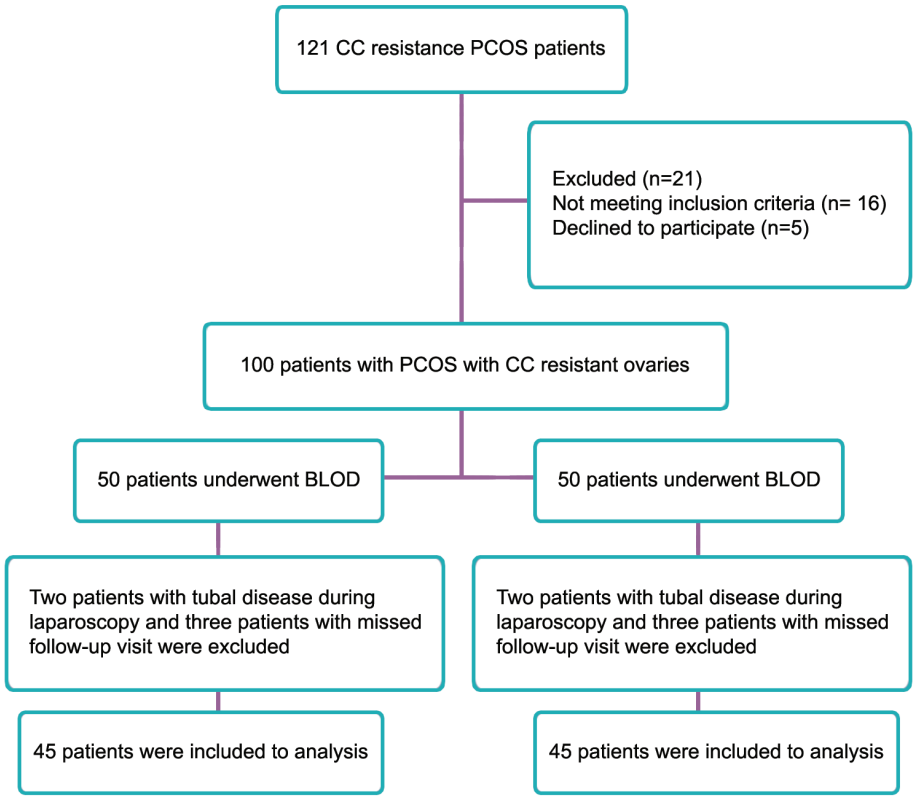


Fig.1: Flowchart of randomized clinical trial for comparing ULOD versus BLOD in CC resistance PCOS patients.
ULOD; Unilateral laparoscopic ovarian drilling, BLOD; Bilateral laparoscopic ovarian drilling, CC; Clomiphene citrate and PCOS; Polycystic ovary syndrome.

Given that few studies have been done in this field, this study was considered as a pilot study after considering the attrition coefficient, so an equal number (n=50) were allocated to each ULOD (group I) and BLOD (group II) groups. PCOS patients were diagnosed based on presence two out of three Rotterdam 2003 criteria, including: oligomenorrhea and/or anovulation, hyper androgenism (biochemical or clinical) and PCOS. We used transvaginal ultrasound to diagnose PCOS, after ruling out other causes, like congenital adrenal hyperplasia (CAH), Cushing syndrome, administration of androgen, and androgen secreting tumor (AST). CC resistant is defined as failure to ovulate after receiving a maximum dosage of 150 mg per day for five days beginning on the third day of menstrual cycle (1, 9). All patients had normal hysterosalpingography and their partners had normal spermiogram using criteria of World Health Organization (WHO). Also all patients had normal uterus in ultrasound scan. Normal uterus was defined as normal size and shape with regular endometrium without any polyp or myoma. According to laparoscopic findings, patients with evidence of tubo-peritoneal diseases, such as tubal obstruction and peritoneal adhesion to tubes or ovaries and endometriosis were also excluded. Among 50 patients in group I undergoing ULOD, two patients were excluded because of tubal disease diagnosed during laparoscopy, and three patients were excluded due to the missed follow-up visit. Among 50 patients in group II, one patient was excluded because of endometriosis diagnosed during laparoscopy, and 4 patients were excluded due to the missed follow-up visit. Finally 45 patients in each group were included for analysis (Fig.1). The cycles of all patients were oligomenorrhea or amenorrhea.

This study was approved by the Ethics Committee of Guilan University of Medical Sciences, Guilan, Iran. All patients provided a written informed consent before entering the study. A randomization list was generated using blocked sample randomization. The permuted block randomization method was used in order to give a block size of four. Assignment proceeds by randomly selecting one of the orderings and allocating the next block of subjects to groups according to the specific sequence. Prior to the laparoscopic procedure, all the patients were tagged in the changing room before entering the operation room by an operating room (OR) nurse, in a blocked randomization design, and the surgeon were not aware of the type of the tag, before entering the operation room.

All 100 PCOS patients with CC resistance were randomly assigned into ULOD (group I) and BLOD (group II) groups. The group I, right ovary, and group II, both ovaries, underwent electrocauterization. We chose right ovary in ULOD because most of the studies have concluded that ovulation occurs more frequently (about 55% of the time) in the right ovary as compared with the left one, and oocytes from the right ovary have a higher potential for pregnancy (35). Besides the probability of adhesion is more in left ovary than right one (31). For all patients, triple puncture laparoscopy was done by a gynecologist. After establishing tubal patency with methylene blue, LOD was performed using unipolar diathermy needle (Karl Storz, Germany). The penetration was about in depth of 8 mm, a setting of 60W, and 5 points per ovary. Ovaries were cooled by normal saline immediately after cauterization, and about 300-500 ml of normal saline was left in pelvic cavity for prevention of adhesion. In the cases of any complications during surgery such as anesthetic problems or injury to organs, the operation was discontinued and the cases were dropped out of the study. The variables, including: age, infertility duration, cycle characteristic (oligomenorrhea or amenorrhea), body mass index (BMI), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone level on day 3 of spontaneous or induced menstruation, were assessed before and after laparoscopy.

The day after laparoscopy, the women were asked to keep their menstrual calendar. If the patients started a menstrual period within 6 weeks after LOD, a blood sample for measurement of LH, FSH and testosterone levels was taken on days 2-3 of menstrual cycle. If spontaneous menstruation did not occur within 6 weeks, an intramuscular injection of 100 mg progesterone (Iran hormone,

Iran) was prescribed. After excluding pregnancy, on days 2-3 of menstrual cycle, hormonal measurements were done.

Ovulation was assessed on day 21 by measurement of progesterone in patients who had spontaneous menstruation. Progesterone level >3 ng/mL is considered as ovulation. If there was no ovulation as evidenced by progesterone level or lack of menstruation, the patients was advised to use CC with starting dose of 50 mg/day up to 150 mg/day from days 3-7 that was monitored by ultrasound. Patients were followed-up until they conceived or 6 mount after LOD. The clinical, defined as menstrual resumption, spontaneous or induced, and biochemical, defined as FSH, LH and free testosterone levels before and after surgery, responses on ovulation and pregnancy rates were measured. In this study, pregnancy was defined as detection of fetal heart on transvaginal ultrasound.

Statistical analysis

Data were analyzed in IBM SPSS software, version 11.5. (SPSS, SPSS Inc., Chicago, IL, USA). We used descriptive and analytic statistics. For numeric variables, data were described as mean and standard deviation, while for categorical variables, data were shown as number and percentage. For statistical analysis, independent t test (two-tailed) was used to compare mean values between two groups, while paired t test was used to compare mean values of FSH, LH, and testosterone levels before and after LOD. Also Fisher's exact test was used to compare relative proportions of variables between two groups. Differences were considered significant at $p<0.05$.

Results

Total of 100 patients with PCOS who underwent LOD were included in this study. These patients were divided into two groups of ULOD or BLOD equally. Among 50 patients in group I undergoing ULOD, two patients were excluded because of tubal disease diagnosed during laparoscopy, and three patients were excluded due to the missed follow-up visit. Among 50 patients in group II undergoing BLOD, one patient was excluded because of endometriosis diagnosed during laparoscopy, and 4 patients were excluded due to the missed follow-up visit. Finally 45 patients in each group were included for analysis (Fig.1).

The baseline characteristics of the women in two groups are shown in table 1. There were no significant differences between patients of two groups in terms of clinical and endocrinologic characteristics and cycle history. After LOD, 30 (66.7%) of patient in group I and 32 (71.1%) in group II had spontaneous menstruation within 6 weeks. But this difference was not statistically significant (p=0.820). To induce menstrual period, an intramuscular injection of 100 mg progesterone was prescribed for remaining women (Table 2).

The ovulation rate after the first menstruation (spontaneous or induced) was assessed with mid-lutealprogesterone level. Overall 27 (60%) patients in group I and 29 (64.4%) patients in group II had spontaneous ovulation. CC was used by starting dose of 50 mg/day up to 150 mg/day for 5 days from third day of cycle, while the findings showed that 11 (24.4%) women in group I and 11 (24.4%) women in group II ovulated successfully.

There were no significant differences between two groups in term of spontaneous (p=0.82) or CC-induced (p=0.70) ovulation (Table 2). Fourteen women (31.1%) in group I and 18 women (40%) in group II were pregnant within 6 month of follow-up visit, but this difference was not statistically significant. In both groups, after LOD, means serum levels of LH (p=0.0001) and testosterone (p=0.001) were decreased significantly.

Also there were no significant differences in means serum levels of LH (p=0.322), testosterone (p=0.079) and FSH (p=0.758) between two groups after drilling (Table 3). Two cases in group II were aborted after detection of fetal heart and one case of triplet was seen in group I.

Table 1: Baseline characteristics of 90 CC-resistant PCOS patients prior to laparoscopy

Screening parameter	Group I (n=45)	Group II (n=45)	P value
Clinical			
Mean age (Y)	27.60 ± 4.25	28.02 ± 4.27	0.644
Mean of infertility duration (Y)	3.04 ± 2.78	4.11 ± 2.61	0.064
Mean of menarche (Y)	12.86 ± 1.84	12.64 ± 1.70	0.649
BMI (%)			
>30	35.6%	48.9%	0.286
≤30	64.4%	51.1%	
Cycle history (%)			
Amenorrhea	20%	13.3%	0.573
Oligomenorrhea	80%	86.7%	
Endocrinologic: (mean)			
LH (IU/L)	11.1 ± 0.6	11.4 ± 1.4	0.601
FSH (IU/L)	5.7 ± 1.7	5.8 ± 2.5	0.840
Testosterone (pg/ml)	1.7 ± 0.8	1.9 ± 1.3	0.455

CC; Clomiphene citrate, PCOS; Polycystic ovary syndrome, BMI; Body mass index, LH; Luteinizing hormone and FSH; Follicle stimulating hormone.

Table 2: Clinical response on ovulation and pregnancy rates in 90 CC-resistant PCOS patients after laparoscopy

	Group I n (%)	Group II n (%)	P value
Menstrual resumption			
Spontaneous	30 (66.7)	32 (71.1)	0.820
Induced	15 (33.3)	13 (28.9)	
Ovulation rate			
Spontaneous	27 (60)	29 (64.4)	0.828
Induced	11 (24.4)	11 (24.4)	0.715
Pregnancy rate	14 (31.1)	18 (40)	0. 350

CC; Clomiphene citrate and PCOS; Polycystic ovary syndrome.

Table 3: Comparison among mean serum levels of FSH, LH, and testosterone before and after LOD

Mean serum level		Before LOD	After LOD	P value
FSH (IU/L)	Unilateral	5.7 ± 1.7	5.7 ± 2.1	0.940
	Bilateral	5.8 ± 2.5	6 ± 2.6	0.577
T test p value		0.840	0.758	
LH (IU/L)	Unilateral	11.1 ± 0.6	6.1 ± 3.4	<0.001
	Bilateral	11.4 ± 1.4	7 ± 2.5	<0.001
T test p value		0.601	0.322	
Testosterone (pg/ml)	Unilateral	1.7 ± 0.8	1.2 ± 0.75	0.001
	Bilateral	1.9 ± 1.3	1.5 ± 1.7	0.001
T test p value		0.455	0.079	

LOD; Laparoscopic ovarian drilling, LH; Luteinizing hormone and FSH; Follicle stimulating hormone.

Discussion

In this study, we have evaluated the effect of ULOD versus BLOD on the ovulation and pregnancy rates of 90 CC resistant PCOS patients. We found that there are no significant differences between groups in terms of ovulation and pregnancy rates.

PCOS women who are CC resistant can be treated with gonadotropins, but there are risks of OHSS and multiple pregnancies in this method. Also gonadotropines are expensive and time-consuming treatment requiring intensive monitoring. Surgical therapy is an alternative method for ovulation induction in these patients to overcome the disadvantages of gonadotropins (1, 2, 9, 14, 36, 37). Ovarian wedge resection surgery was an accepted method of ovulation induction over 40 years (38). However, it was abandoned because of adhesion formation (39-41). LOD was first described by Gjonnaess in 1984 (42).

The mechanism of LOD is similar to ovarian wedge resection surgery. Destruction of androgen-producing ovarian tissue leads to a decrease in the peripheral conversion of androgen to estrogen. Decreased serum levels of androgen and LH and increased FSH level have been demonstrated after ovarian drilling (40, 43, 44). A change in endocrine function converts the androgen-dominant intrafollicular environment to estrogenic one (45). It affects ovarian-pituitary feedback mechanism (46), so both local and systemic effects may induce ovulation in these patients. Due to ovulation and pregnancy success rates, mentioned in various studies, LOD is an accepted method for ovulation induction in CC resistant PCOS patients (25).

Two important potential adverse effects of LOD are peri-ovarian adhesions and reduced ovarian function (47, 48). The rate of peri-ovarian adhesion is very different in various studies, from 19 to 43%, and with greater damage to the ovaries, the risk become higher (42, 49-51). Furthermore POF is another concern of LOD that is dependent on the number of puncture made (>4-6) (52). Therefore, the risk of peri-ovarian adhesion and the rate of POF can be minimized by decreasing the number of punctures (24, 25).

The idea of ULOD instead of BLOD for minimizing these two side effects was first introduced by Ballen and Jacobs (53). They showed that ULOD can result in bilateral ovarian activity due to local cascade of growth factors, such as insulin-like growth factor-1 (IGF-1), which interacts with FSH, leading to a decrease in the serum LH concentration (53, 54).

Nowadays BLOD is a standard method of LOD. Few studies compared ULOD and BLOD and concluded that ULOD is as effective as BLOD and minimizes the risk of adhesion and POF (24, 25, 31-34, 54, 55).

In this study, after performing LOD, we found significant decreases in serum levels of LH and testosterone in both groups that were similar in both groups. Also there were no significant differences between groups in terms of ovulation and pregnancy rates. Youssef and Atallah (25) in 2007 evaluated 87 patients with ovulation failure as a result of PCOS who were randomly allocated into ULOD (n=43) and BLOD (n=44). In patients who ovulated after drilling, there was a significant fall in serum LH concentration, while ovulation, pregnancy and miscarriage rates were similar between both groups. Roy et al. (24) in 2009 evaluated the effect of ULOD versus BLOD in 22 patients. The clinical and biochemical responses on ovulation and pregnancy rates over a 1-year follow-up period were compared. They also evaluated tubo-ovarian adhesion rate during cesarean section or a second-look laparoscopy. They found no significant differences between two groups in terms of clinical and biochemical responses, ovulation and pregnancy rates, and tubo-ovarian adhesions. They concluded that ULOD may be a suitable option in CC resistant infertile patients of PCOS which can replace BLOD with the potential advantage of decreasing the chance of adhesion formation. Abdelhafeez et al. (55) in 2013 reported that ULOD is as effective as BLOD in terms of restoration of regular menstrual pattern and ovulation rate. Sunj et al. (31) in 2013 represented that the results of applied method can be improved when using less thermal energy in volume-adjusted ULOD in comparing to BLOD.

Conclusion

Based on the results of this study, ULOD seems to be equally efficacious as BLOD in terms of ovulation and pregnancy rates.

Acknowledgements

This study was based on a thesis submitted by the third author to the Guilan University of Medical Sciences, Rasht, Iran. We would like to thanks the Vice Chancellor for Research of Guilan University of Medical Sciences for funding this project. Also we gratefully thank Mr. Davoud Pourmarzi and Mrs. Nazli Peiravian for their kind collaboration. We sincerely appreciate the patients participating in this project.

The authors state that they have no conflict of interest.

References

1. Abu Hashim H, Al-Inany H, De Vos M, Tournaye H. Three decades after Gjönnaess's laparoscopic ovarian drilling for treatment of PCOS; what do we know? An evidence-based approach. Arch Gynecol Obstet. 2013; 288(2): 409-422.
2. Costello MF, Misso ML, Wong J, Hart R, Rombauts L, Melder A, et al. The treatment of infertility in polycystic ovary syndrome: a brief update. Aust N Z J Obstet Gynaecol. 2012; 52(4): 400-403.
3. Hull MG. Epidemiology of infertility and polycystic ovarian: endocrinological and demographic studies. Gynecol Endocrinol. 1987; 1(3): 235-245.
4. Adam J, Polson DW, Franks S. Prevalence of polycystic ovaries in women with anovulation and idiopathic hirsutism. Br Med J (Clin Res Ed). 1986; 293(6543): 355-359.
5. Zolghadri J, Motazedian S, Dehbashi S, Tavana Z. Laparoscopic ovarian drilling inclomiphene resistant pco patients: a preferred method of treatment. Med J Islam Repub Iran. 2005; 19(3): 237-240.
6. Legro RS, Barnhart HX, Schlaff WD, Carr BR, Diamond MP, Carson SA, et al. Clomiphene, metformin, or both for infertility in the polycystic ovary syndrome. N Engl J Med, 2007; 356(6): 551-566.
7. Moll E, Bossuyt PM, Korevaar JC, Lambalk CB, van der Veen F. Effect of clomifene citrate plus metformin and clomifene citrate plus placebo on induction of ovulation in women with newly diagnosed polycystic ovary syndrome: randomised double blind clinical trial. BMJ. 2006; 332(7556): 1485.
8. Gysler M, March CM, Mishell DR Jr, Bailey EJ. A decade's experience with an individualized clomiphene treatment regimen including its affect on postcoital test. Fertil Steril 1982; 37(2): 161-167.
9. Ott J, Kurz C, Nouri K, Wirth S, Vytiska-Binstorfer E, Huber JC, et al. Pregnancy outcome in women with polycystic ovary syndrome comparing the effects of laparoscopic ovarian drilling and clomiphene citrate stimulation in women pre-treated with metformin: a retrospective study. Reprod Biol Endocrinol. 2010; 8: 45.
10. Bayram N, van Wely M, Kaaijk EM, Bossuyt PM, van der Veen F. Using an electrocautery strategy or recombinant follicle stimulating hormone to induce ovulation in polycystic ovary syndrome: randomised controlled trial. BMJ. 2004; 328(7433): 192.
11. Kaya H, Sezik M, Ozkaya O. Evaluation of a new surgical approach for the treatment of clomiphene citrate-resistant infertility in polycystic ovary syndrome: laparoscopic ovarian multi-needle intervention. J Minim Invasive Gynecol. 2005; 12(4): 355-358.
12. Greenblatt EM, Casper RF. Adhesion formation after laparoscopic ovarian cautery for polycystic ovarian syndrome: lack of correlation with pregnancy rate. Fertil Steril. 1993; 60(5): 766-770.
13. Dabirashrafi H, Mohammad K, Tabrizi NM, Zandinejad K. Comparison of adhesion formation in open wedge resection (OWR) with microsurgical wedge resection (MWR). J Am Assoc Gynecol Laparosc. 1994; 1(4, Part 2): S8-9.
14. Hameed N, Ali MA. Laparoscopic ovarian drilling for polycystic ovarian syndrome: treatment outcome. J Ayub Med Coll Abbottabad. 2012; 24(3-4): 90-92.
15. Flyckt RL, Goldberg JM. Laparoscopic ovarian drilling for clomiphene-resistant polycystic ovary syndrome. Semin Reprod Med. 2011; 29(2): 138-146.
16. Fernandez H, Morin-Surruca M, Torre A, Faivre E, Deffieux X, Gervaise A.Ovarian drilling for surgical treatment of polycystic

ovarian syndrome: a comprehensive review. Reprod Biomed Online. 2011; 22(6): 556-568.
17. Salah IM. Office microlaparoscopic ovarian drilling (OMLOD) versus conventional laparoscopic ovarian drilling (LOD) for women with polycystic ovary syndrome. Arch Gynecol Obstet. 2013; 287(2): 361-367.
18. Ombelet W, Martens G, De Sutter P, Gerris J, Bosmans E, Ruyssinck G, et al. Perinatal outcome of 12,021 singleton and 3108 twin births after non- IVF-assisted reproduction: a cohort study. Hum Reprod. 2006; 21(4): 1025-1032.
19. Jacobs HS, Agrawal R. Complications of ovarian stimulation. Baillieres Clin Obstet Gynecol. 1998; 12(4): 565-579.
20. Homburg R, Howles CM. Low dose FSH therapy for anovulatory infertility associated with polycystic ovarian syndrome: rationale, results, reflections, and refinements. Hum Reprod Updates. 1999; 5(5): 493-499.
21. Li TC, Saravelos H, Chow MS, Chisabingo R. Factors affecting the outcome of laparoscopic ovarian drilling for polycystic ovarian syndrome in women with anovulatory infertility. Br J Obstet Gynaecol. 1998; 105(3): 338-344.
22. Farquhar C, Lilford RJ, Marjoribanks J, Vandekerckhove P. Laparoscopic "drilling" by diathermy or laser for ovulation induction in anovulatory polycystic ovary syndrome. Cochrane Database Syst Rev. 2005; 20(3): CD001122.
23. Strowitzki T, von Wolff M. Laparoscopic ovarian drilling (LOD) in patients with polycystic ovary syndrome (PCOS): an alternative approach to medical treatment?. Gynecological Surgery. 2005; 2(2): 71-79.
24. Roy KK, Baruah J, Moda N, Kumar S. Evaluation of unilateral versus bilateral ovarian drilling in clomiphene citrate resistant cases of polycystic ovarian syndrome. Arch Gynecol Obstet. 2009; 280(4): 573-578.
25. Youssef H, Atallah MM. Unilateral ovarian drilling in polycystic ovarian syndrome: a prospective randomized study. Reprod Biomed Online. 2007; 15(4): 457-462.
26. Wang XH, Wang JQ, Xu Y, Huang LP. Therapeutic effects of metformin and laparoscopic ovarian drilling in treatment of clomiphene and insulin-resistant polycystic ovary syndrome. Arch Gynecol Obstet. 2014. (In press).
27. Gurgan T, Kisnisci H, Yarali H, Develioglu O, Zeyneloglu H, Aksu T. Evaluation of adhesion formation after laparoscopic treatment of polycystic ovarian disease. Fertil Steril. 1991; 56(6): 1176-1178.
28. Liguori G, Tolino A, Moccia G, Scognamiglio G, Nappi C. Laparoscopic ovarian treatment in infertile patients with polycystic ovarian syndrome (PCOS): endocrine changes and clinical outcome. Gynecol Endocrinol. 1996; 10(4): 257-264.
29. Felemban A, Tan SL, Tulandi T. Laparoscopic treatment of polycystic ovaries with insulated needle cautery: a reappraisal. Fertil Steril. 2000; 73(2): 266-269.
30. Weinstein D, Polishuk WZ. The role of wedge resection of ovary as a cause for mechanical sterility. Surg Gynecol Obstet. 1975; 141(3): 417-418.
31. Sunj M, Canic T, Baldani DP, Tandara M, Jeroncic A, Palada I. Does unilateral laparoscopic diathermy adjusted to ovarian volume increase the chances of ovulation in women with polycystic ovary syndrome?. Hum Reprod. 2013; 28(9): 2417-2424.
32. Al-Mizyen ES, Grudzinskas JG. Ultrasonographic observations following unilateral and bilateral laparoscopic ovarian diathermy in infertile women with clomiphene citrate resistant polycystic ovarian syndrome (PCOS). Middle East Fertil Soc J. 2007; 12(3): 207-212.
33. Sharma M, Kriplani A, Agarwal N. Laparoscopic bipolar versus unipolar ovarian drilling in infertile women with resistant polycystic ovarian syndrome: a pilot study. J Gynecol Surg. 2006; 22(3): 105-111.
34. Al-Mizyen E, Grudzinskas JG. Unilateral laparoscopic ovarian diathermy in infertile women with clomiphene citrate-resistant polycystic ovary syndrome. Fertil Steril. 2007; 88(6): 1678-1680.
35. Fritz MA, Speroff L. Clinical Gyncologic Endocrinology and Infertility. In: Regulation of the menstrual cycle. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2011; 228.
36. Mehrabian F, Eessaei F. The laparoscopic ovarian electrocautery versus gonadotropin therapy in infertile women with clomiphene citrate-resistant polycystic ovary syndrome; a randomized controlled trial. J Pak Med Assoc. 2012; 62(3 Suppl 2): S42-44.
37. Safia S Munir, Dawood Amin, Misbah Sultana, Tariq Saeed. Ovulation induction using laparoscopic ovarian drilling in women with polycystic ovarian syndrome: predictors of success. Biomedica. 2010; 26(2): 130-134.
38. STEIN IF Sr. Duration of fertility following ovarian wedge resection--stein-leventhal syndrome. West J Surg Obstet Gynecol. 1964; 72: 237-242.
39. Adashi EY, Rock JA, Guzick D, Wentz AC, Jones GS, Jones HW Jr. Fertility following bilateral ovarian wedge resection: a critical analysis of 90 consecutive cases of the polycystic ovary syndrome. Fertil Steril. 1981; 36(3): 320-325.
40. Buttram VC Jr, Vaquero C. Post-ovarian wedge resection adhesive disease. Fertil Steril. 1975; 26(9): 874-876.
41. Lunde O, Djoseland O, Grottum P. Polycystic ovarian syndrome: a follow-up study on fertility and menstrual pattern in 149 patients 15-25 years after ovarian wedge resection. Hum Reprod. 2001; 16(7): 1479-1485.
42. Gjonnaess H. Polycystic ovarian syndrome treated by ovarian electrocautery through the laparoscope. Fertil Steril. 1984; 41(1): 20-25.
43. Armar NA, McGarrigle HH, Honour J, Holownia P, Jacobs HS, Lachelin GC. Laparoscopic ovarian diathermy in the management of anovulatory infertility in women with polycystic ovaries: endocrine changes and clinical outcome. Fertil Steril. 1990; 53(1): 45-49.
44. Greenblatt E, Casper RF. Endocrine changes after laparoscopic ovarian cautery in polycystic ovarian syndrome. Am J Obstet Gynecol. 1987; 156(2): 279-285.
45. Aakvaag A, Gjønnaess H. Hormonal response to electrocautery of the ovary in patients with polycystic ovarian disease. Br J Obstet Gynaecol. 1985; 92(12): 1258-1264.
46. Balen AH. Hypersecretion of luteinizing hormone and the polycystic ovary syndrome. Hum Reprod. 1993; 8 Suppl 2: 123-128.
47. Gurgan T, Urman B, Aksu T, Yarali H, Develioglu O, Kisnisci HA. The effect of short-interval laparoscopic lysis of adhesions on pregnancy rates following Nd-YAG laser photocoagulation of polycystic ovaries. Obstet Gynecol. 1992; 80(1): 45-47.
48. Farquhar C, Brown J, Marjoribanks J. Laparoscopic drilling by diathermy or laser for ovulation induction in anovulatory polycystic ovary syndrome. Cochrane Database Syst Rev. 2012; 6: CD001122.
49. Dabirashrafi H, Mohamad K, Behjatnia Y, Moghadami-Tabrizi N. Adhesion formation after ovarian electrocauterization on patients with polycystic ovarian syndrome. Fertil Steril. 1991; 55(6): 1200-1201.
50. Naether OG, Fischer R, Weise HC, Geiger-Kötzler L, Delfs T, Rudolf K. Laparoscopic electrocoagulation of the ovarian surface in infertile patients with polycystic ovarian disease. Fertil Steril. 1993; 60(1): 88-94.
51. Naether OG, Fischer R. Adhesion formation after laparoscopic electrocoagulation of the ovarian surface in polycystic ovary patients. Fertil Steril. 1993; 60(1): 95-98.
52. Thessaloniki ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Consensus on infertility treatment related to polycystic ovary syndrome. Hum Reprod. 2008; 23(3): 462-477.
53. Balen AH, Jacobs HS. A prospective study comparing unilateral and bilateral laparoscopic ovarian diathermy in women with the polycystic ovary syndrome. Fertil Steril. 1994; 62(5): 921-925.
54. Adashi EY, Resnick CE, Hernandez ER, May JV, Knecht M, Svoboda ME, et al. Insulin-like growth factor-I as an amplifier of follicle-stimulating hormone action: studies on mechanism(s) and site(s) of action in cultured rat granulosa cells. Endocrinology. 1988; 122(4): 1583-1591.
55. Abdelhafeez MA, Ali MS, Sayed SN. Unilateral versus bilateral laparoscopic ovarian drilling in clomiphene citrate resistant polycystic ovary syndrome. Life Sci J. 2013; 10(1): 3057-3060.

Original Article

A Randomized Clinical Trial on Comparing The Cycle Characteristics of Two Different Initiation Days of Letrozole Treatment in Clomiphene Citrate Resistant PCOS Patients in IUI Cycles

Nayereh Ghomian, M.D., Ashraf Khosravi, M.D., Nezhat Mousavifar, M.D.*

Ovulation Dysfunction Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Abstract

Background: There are still many questions about the ideal protocol for letrozole (LTZ) as the commonest aromatase inhibitor (AI) used in ovulation induction. The aim of this study is to compare the ultrasonographic and hormonal characteristics of two different initiation times of LTZ in clomiphene citrate (CC) failure patients and to study androgen dynamics during the cycle.

Materials and Methods: This randomized clinical trial was done from March to November 2010 at the Mashhad IVF Center, a university based IVF center. Seventy infertile polycystic ovarian syndrome (PCOS) patients who were refractory to at least 3 CC treatment cycles were randomly divided into two groups. Group A (n=35) receiving 5 mg LTZ on cycle days 3-7 (CD3), and group B (n=35) receiving the same amount on cycle days 5-9 (CD5). Hormonal profile and ultrasonographic scanning were done on cycle day 3 and three days after completion of LTZ treatment (cycle day 10 or 12). Afterward, 5,000-10,000 IU human chorionic gonadotropin (hCG) was injected if at least one follicle ≥18 mm was seen in ultrasonographic scanning. Intrauterine insemination (IUI) has been done 36-40 hours later. The cycle characteristics, the ovulation and pregnancy rate were compared between two groups. The statistical analysis was done using Fisher's exact test, t test, logistic regression, and Mann-Whitney U test.

Results: There were no significant differences between two groups considering patient characteristics. The ovulation rate (48.6 vs. 32.4% in group A and B, respectively), the endometrial thickness, the number of mature follicles, and length of follicular phase were not significantly different between the two groups.

Conclusion: LTZ is an effective treatment in CC failure PCOS patients. There are no significant differences regarding ovulation and pregnancy rates between two different protocols of LTZ starting on days 3 and 5 of menstrual cycle (Registration Number: IRCT201307096467N3).

Keywords: Letrozole, Clomiphene Citrate, Polycystic Ovarian Syndrome (PCOS)

Citation: Ghomian N, Khosravi A, Mousavifar N. A randomized clinical trial on comparing the cycle characteristics of two different initiation days of letrozole treatment in clomiphene citrate resistant PCOS patients in IUI cycles. Int J Fertil Steril. 2015; 9(1): 17-26.

Introduction

Clomiphene citrate (CC) is known as one of the oldest drugs that has remained the standard choice for ovulation induction (1). CC has been an appropriate, non-expensive, and highly effective agent for inducing ovulation since 1963 (2). However, it certainly has not been successful in all patients; about 15-20% of women do not ovulate on CC, labeled as CC-resistant group (3). There are also other problems reported about CC, such as the an-

Received: 8 Jul 2013, Accepted: 28 Jan 2014
* Corresponding Address: P.O. Box: 9137913316, Ovulation Dysfunction Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
Email: mosavifarn@mums.ac.ir

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 17-26

ti-estrogenic mucosal and endometrial changes (2) that lead to higher rate of abortion and miscarriage in ovulatory women (3, 4).

Letrozole (LTZ), the prominent drug in the aromatase inhibitor (AI) family, has been introduced as a new choice for ovulation induction in the past decade, especially in polycystic ovarian syndrome (PCOS) patients who have failed to respond to CC. LTZ also seems to be very efficient in pregnancy rates, equivalent to injectable gonadotropins, at lower cost and with fewer adverse effects (5).

Furthermore, there are extra advantages for LTZ-therapy in comparison to CC, including: normal negative feedback mechanism for follicle-stimulating hormone (FSH) in the brain, more mono-follicular cycles, no negative anti-estrogenic effects on the endometrial and cervical mucus, lower risk of ovarian hyperstimulation syndrome (OHSS), and lesser need for cycle monitoring (6).

By reviewing the literature, we found 2000 articles published related to CC since 1963, whereas, there is only about 200 articles published related to LTZ since 2000 (7).

Since LTZ is a new agent in the era of ovulation induction, there are several questions regarding the best protocol for administering. The usual doses for LTZ are mentioned as 2.5 and 5 mg. Doses higher than 5 mg per day for 5 days may result in persistence of aromatase inhibition that is followed by low estrogen level for normal endometrial development by the time of ovulation. Some researchers have suggested different LTZ protocols as follows: single dose of 20 mg given on cycle day 3, extended dose for up to 7-10 days, and step-up protocol including an escalating dose of 2.5 mg on day 3 along with 10 mg on day 6. The suggested starting day of LTZ administration is on cycle days 3-7 (6).

Hormonal profile of LTZ cycles in infertility literature is a nowadays matter of challenge. It has been shown that LTZ can induce a marked decrease in plasma concentrations of estradiol (E_2) and estrone, with approximately no effect on other steroidal hormones. No accumulation of androgens, androgen precursors, luteinizing hormone (LH), FSH, thyroid-stimulating hormone (TSH) or renin was reported in pharmacodynamics studies of LTZ (4, 8).

On the other hand, Garcia-Velasco et al. (9) in 2005 found significantly elevated follicular fluid levels of testosterone and androstenedione with LTZ therapy during ovarian stimulation for *in vitro* fertilization (IVF). Another study has reported significant higher LH, testosterone, androstenedione, and postovulatory progesterone (P) levels in LTZ treated patients compared to natural cycles (10). Also, in another research, some minor changes have been found in follicular phase hormonal profiles (P, LH, and E_2) compared to natural cycles (11).

It seems that there are many unknown aspects of using aromatase inhibitors for ovulation induction. Thus, it is reasonable to do more studies. The aims of our study were to evaluate the cycle characteristics, including: follicular phase length, endometrial thickness, monofollicular response, and pregnancy rate, of LTZ in CC failure PCOS patients in order to compare fixed dose of LTZ between cycle days 3 and 5 and to evaluate the hormonal changes during these two protocols.

Materials and Methods

Based on Rotterdam criteria, 70 PCOS patients were enrolled in this randomized clinical trial. A written informed consent was taken from all women participating in this study. The diagnosis of PCOS was made when two of the following three criteria existed: oligomenorrhea or amenorrhea, clinical hyperandrogenism, and polycystic ovaries on ultrasonography. The inclusion criteria were as follows: i. Previous diagnosis of PCOS according to Rotterdam criteria, ii. Age between 20-30 years, iii. No previous history of ovarian surgery and iv. lack of ovulation with CC in at least 3 previous cycles (lack of follicle ≥18 mm on ultrasound scan). The exclusion criteria were as follows: i. No other infertility factors, ii. Exposure to cytotoxic drugs and iii. Pelvic radiation therapy. The study was performed from March to November 2010 at the Mashhad IVF center, a university based infertility center.

The patient's age, her partner's age, duration of infertility, type of infertility (primary and secondary), history of previous intrauterine insemination (IUI) cycles, pattern of ovary (PCO and non-PCO), pattern of menstruation (regular, oligo-menorrhea and amenorrhea), body mass index (BMI) and basal LH/FSH ratio were recorded for each patient.

The patients were randomly assigned into two groups: Group A (n=35) receiving 5 mg LTZ (Letrofem; Iran Hormone, Iran) on cycle days 3-7 (CD3), and group B (n=35) receiving the same amount on cycle days 5-9 (CD5) (Fig.1).

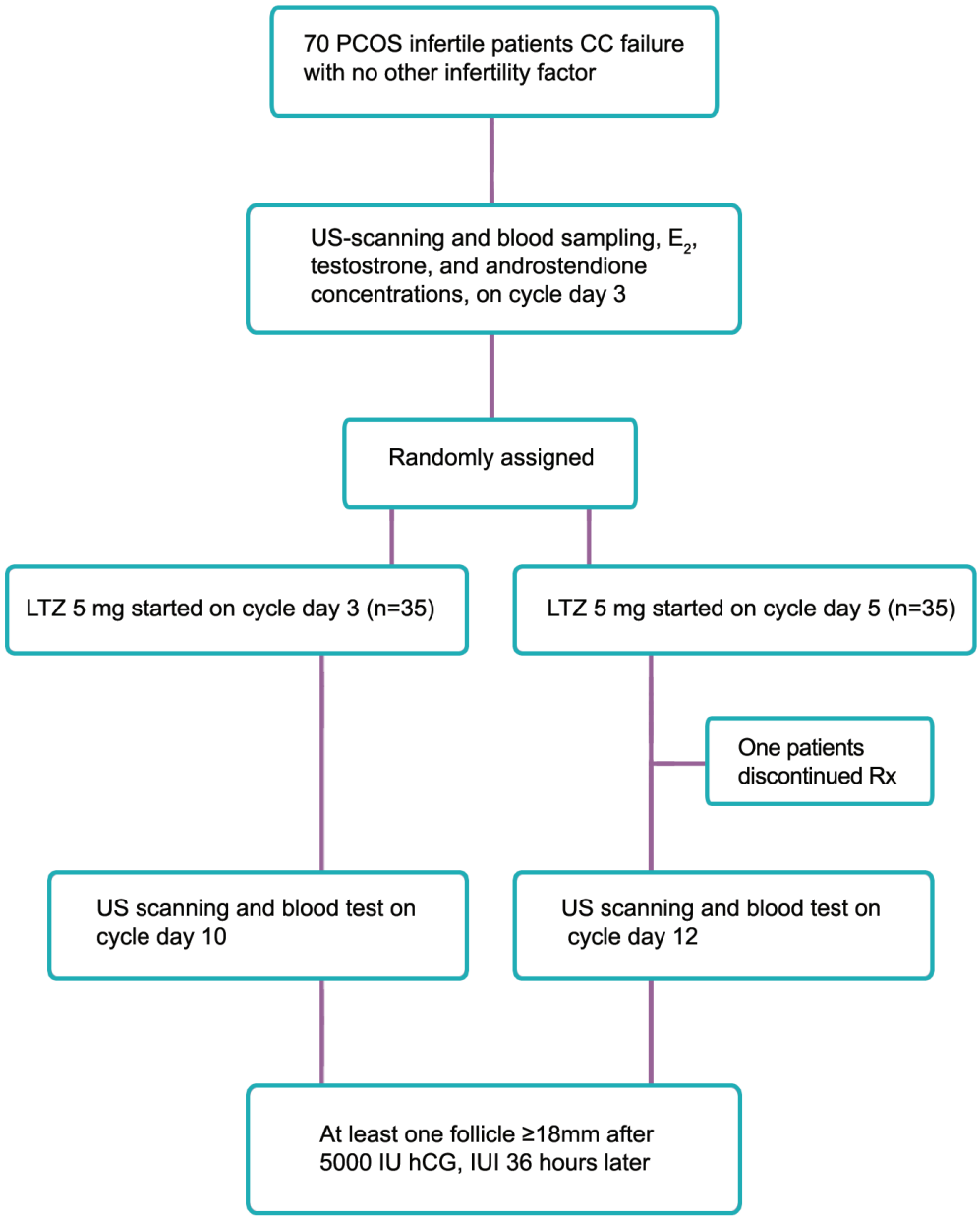


Fig.1: PCOS; Polycystic ovary syndrome, CC; Clomiphene citrate, E_2; Estradiol , LTZ; Letrozole, hCG; Human chorionic gonadotropin and IUI; Intrauterine insemination.

Both groups underwent a vaginal ultrasonographic (US) scan (probe 7.5 MHz; Ultrasonix, USA) and a blood analysis just before the first dose of LTZ and three days after the last dose of LTZ therapy. US scanning were continued if indicated.

By observing at least one follicle ≥18 mm, the patient was considered ovulatory and 5,000 -10,000 IU human chorionic gonadotropin (hCG) was injected followed by a single IUI 36-40 hours later. Pregnancy was documented by observing fetal pole 2 weeks after missed period.

The number of follicles ≥18 mm and the endometrial thickness were measured on US scanning. The length of follicular phase and the hormonal levels of LH, E_2, testosterone, and androstendione were registered for all the patients before and three days after completion of LTZ.

We used t test, chi-square, Fisher's exact test, and Mann-Whitney U test by SPSS (SPSS Inc., Chicago, IL, USA) version 12.0 for statistical analysis. A p value less than 0.05 was considered significant. A comparison was done between demographic characteristics in ovulatory (n=28) and anovulatory patients (n=41). Follicle numbers, endometrial thickness, follicular phase length and pregnancy rate were compared between CD3 and CD5 patients. The hormonal levels were compared between two groups before and after receiving LTZ. The hormonal levels were compared in ovulatory and anovulatory patients before and after treatment. This study was approved by Ethical Committee of Mashhad University of Medical Sciences.

Results

The total number of recruited PCOS patient in this study was 70 (n=35/each group). There was no significant difference between two therapeutic groups considering patient's characteristics, like: age, duration of infertility, pattern of ovary, BMI, and basal LH/ FSH ratio (Table 1).

The ovulation rate (presence of at least 1 follicle ≥18 mm during ovarian stimulation) in CD3 and CD5 groups were 48.6% (17/35) versus 32.4% (11/35), respectively, whereas the difference was not statistically significant (p=0.17).

The age, duration of infertility, pattern of ovary, BMI, and basal LH/FSH ratio were not statistically significant difference between ovulatory and anovulatory patients (Table 2).

By comparing the basal androgen level, we found that the patients with a successful ovulation as compared to anovulatory patients had a significant lower androstenedione (94.76 ± 59.42 vs. 181.95 ± 239.58 ng/dl, p=0.02) and testosterone (33.76 ± 13.26 vs. 42.10 ± 18.90 ng/dl, p=0.02) levels before treatment (Table 3).

The E_2 concentration was similar between ovulatory and anovulatory patients before the treatment (56.66 ± 29.02 vs. 65.54 ± 26.93 pg/ml, respectively, p=0.32), but it was significantly higher in ovulatory patients after treatment (118.35 ± 72.89 vs. 56.18 ± 46.13 pg/ml respectively, p=0.01, Table 3).

There was no significant difference between two therapeutic protocols regarding the cycle characteristics. Thus, follicular phase length (14.1 ± 3.8 in CD3 and 14.7 ± 1 days in CD5 patients), endometrial thickness (8.0 ± 1.16 mm in CD3 and 7.8 ± 1.3 mm in CD5 patients) and mono-follicular response (76% in CD3 and 82% in CD5 patients) were similar between groups (p>0.05, Table 4).

Between two groups, testosterone significantly increased during the treatment (36.55 ± 34.55 ng/dl for CD3 and 24.71 ± 28.20 ng/dl for CD5, p<0.01 for both). Although this increase was higher in CD3 patients, there is no statistically significant difference between two groups (p=0.15, Table 5).

Androstenedione was also increased during treatment course (75.72 ± 151.92 ng/ml for CD3 and 119.50 ± 116.27 ng/dl for CD5, p=0.01 for both). Although this increase was higher in CD5 patients, there is no statistically significant difference between two groups (p=0.21, Table 5).

The E_2 pattern was different between the groups. Although there was a significant increase in the CD3 patients, (the change from baseline=31.24 ± 75.05 pg/ml, p=0.03), there was a decrease among the CD5 (the change from baseline=-8.96 ± 42.64 pg/ml, p=0.24). So, E_2 changes in two protocols showed statistically significant difference (p=0.01, Table 5).

The positive clinical pregnancy outcome was also higher in CD3 patients (12.1% in CD3 versus 9.4% in CD5 patients), but the difference was not statistically significant (p~1, Table 6).

Table 1: Basic and demographic characteristics in study groups

Variable		Treatment group		P value
		CD3	CD5	
Age (Y)		25.3 ± 4.4	25.6 ± 3.5	0.777
BMI (kg/m²)		27.0 ± 3.8	26.4 ± 4.81	0.674
Types of infertility	Primary	31 (91.2%)	34 (97.1%)	0.356
	Secondary	3 (8.8%)	1 (2.9%)	
Pattern of ovary	PCOS	24 (68.6%)	23 (65.7%)	1
	non-PCOS	11 (31.4%)	12 (34.3%)	
Familial history of PCOS	Yes	1 (2.9%)	3 (8.6%)	0.614
	No	34 (97.1%)	32 (91.4%)	
LH /FSH (mIU/ml)		1.35 ± 2.43	1.94 ± 1.91	0.082
TSH (µu/ml)		2.95 ± 3.03	3.29 ± 4.17	0.376[a]
PRL (ng/ml)		24.5 ± 41.38	29.2 ± 65.43	0.672[a]
The number of previous treatment cycles		1.09 ± 0.39	1.25 ± 0.52	0.096[a]

CD3; Cycle day 3, CD5; Cycle day 5, BMI; Body mass index, PCOS; Polycystic ovary syndrome, LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, TSH; Thyroid-stimulating hormone, PRL; Prolactin and [a]; Mann-Whitney U test results.

Table 2: Basic and demographic characteristics in ovulatory and non-ovulatory patients

Variable		Treatment group		P value
		Ovulatoty N=28	Anovulatory N=41	
Age (Y)		25.40 ± 3.4	23.75 ± 0.54	0.971
BMI (kg/m²)		26.51 ± 4.61	26.76 ± 4.34	0.870
Types of infertility	Primary	28 (100%)	37 (90.5%)	0.303
	Secondary	0 (0%)	4 (9.5%)	
PCO pattern in ovary by ultrasonography	PCOS	20 (71.0%)	32 (78.0%)	0.600
	non-PCOS	8 (29.0%)	9 (22.0%)	
Familial history of PCOS	Yes	1 (4.0%)	2 (5.0%)	0.303
	No	27 (96.0%)	39 (95.0%)	
LH (mIU/ml)		10.17 ± 7.37	10.30 ± 6.49	0.942
FSH (mIU/ml)		7.18 ± 3.62	6.28 ± 2.73	0.267
Menstrual pattern(n)	Oligo-menorrhea	25 (90.5%)	28 (72.3%)	0.096
	Amenorrhea	3 (9.5%)	13 (27.7%)	

BMI; Body mass index, PCOS; Polycystic ovary syndrome, LH; Luteinizing hormone and FSH; Follicle-stimulating hormone.

Table 3: Comparison of follicular phase testosterone, androstendione, and estradiol dynamics in CD3 and CD5 patients, before and after treatment

Hormone	Blood sample	Ovulatory	Anovulatory	P value
Testosterone (ng/dl)	Before treatment	33.76 ± 13.26	42.10 ± 18.90	0.02
	After treatment	63.41 ± 30.22	69.29 ± 37.15	0.52
Androstendione (ng/dl)	Before treatment	94.76 ± 59.42	181.95 ± 239.58	0.02
	After treatment	176.35 ± 90.92	289.159 ± 207.78	0.00
Estradiol (pg/ml)	Before treatment	56.66 ± 29.02	65.54 ± 26.93	0.32
	After treatment	118.35 ± 72.89	56.18 ± 46.13	0.01

CD3; Cycle day 3 and CD5; Cycle day 5.

Table 4: Comparison of some cycle parameters in CD3 and CD5 patients

Cycle characteristics	CD3	CD5	P value
Follicular phase length (day)	14.1 ± 3.8	14.7 ± 1	0.093
Endometrial thickness (mm)	8.0 ± 1.16	7.8 ± 1.3	0.721
Monofollicular response (among ovulatory patients)	13 (76%)	9 (82%)	0.322

CD3; Cycle day 3 and CD5; Cycle day 5.

Table 5: Comparison of follicular phase testosterone, androstendione, and estradiol dynamics in CD3 and CD5 patients

Type of hormone	Group	Before treatment	After treatment	Difference	P value
Testosterone (ng/dl)	CD3	36.00 ± 15.49	72.55 ± 35.94	36.55 ± 34.55	0.147
	CD5	43.87 ± 24.10	68.59 ± 39.88	24.71 ± 28.20	
Androstendione (ng/dl)	CD3	153.24 ± 176.29	228.96 ± 116.63	75.72 ± 151.92	0.209
	CD5	169.78 ± 244.52	289.28 ± 232.78	119.50 ± 116.27	
Estradiol (pg/ml)	CD3	64.00 ± 29.36	95.24 ± 76.94	31.24 ± 75.05	0.012
	CD5	64.03 ± 25.55	55.06 ± 31.96	-8.96 ± 42.64	

CD3; Cycle day 3 and CD5; Cycle day 5.
P values were calculated for the differences in hormonal values between CD3 and CD5.

Table 6: Comparison of cycle outcome in CD3 and CD5 groups

		CD3	CD5	P value
Ovulation rate (at least one follicle more than 18 mm)	Positive	17 (48.6%)	11 (32.4%)	0.174
Clinical pregnancy	Positive	4 (12.1%)	3 (9.4%)	1

CD3; Cycle day 3 and CD5; Cycle day 5.

Discussion

In two different protocols of LTZ starting on cycle days 3 and 5, there were no significant differences in follicular phase length, endometrial thickness, monofollicular response, and pregnancy rate.

AIs were originally developed for the treatment of advanced breast cancer (12); however, it has been also introduced as reproductive medicine by Mitwally and Casper (7). They showed that LTZ was effective in inducing ovulation in women with PCOS. LTZ is rapidly and completely absorbed from gastrointestinal (GI) tract with absolute bioavailability of 99.9%. The terminal elimination half-life in plasma is about 2 days and the maximal suppression of estrogen concentration is achieved in 48-78 hours after single oral dose administration.

Since only a decade has passed since the introduction of LTZ in the field of ovarian stimulation, there is still debate about the optimal protocol to use. The dosage of LTZ, therefore, differs between studies. The majority of researchers have used 2.5-5.0 mg LTZ daily based on the dosage used for the treatment of patients with breast cancer (12). In one study, the effect of a single dose of 20 mg LTZ on cycle day 3 was compared to 2.5 mg on cycle days 3-7, which was not significantly different in pregnancy rate (13). In another study, 7.5 mg LTZ for 5 days was compared to 150 mg CC that was proved to be more efficacious in terms of ovulation and pregnancy rates (14).

The duration of stimulation in most studies was similar to CC, namely 5 days in the early follicular phase, although longer stimulation for 10 days has been also tested (15).

To induce ovulation, FSH is necessary in the early phase of the cycle to recruit and to select follicles. In ovulation cycles by gonadotropins, the earlier time for FSH administration is started during the cycles (prior to the selection phase) and more follicles are then recruited (11).

CC is usually administered on day 5 of menstruation. This is based on the theory that on cycle day 5, a physiologic decrease in FSH concentration provides the means for selection of the dominant follicle. Initiation of the drug on cycle day 2 induces earlier ovulation which is analogous to the physiologic events of the normal menstrual cycle. In one study, ovulation and conception rates and pregnancy outcome were similar when CC treatment started anywhere between cycle days 2 and 5 (16). In another study, CC was started on cycle days 3, 4, 5, or 7 in IVF cycles. The researchers concluded that protocol of cycle day 5 had more oocytes recovered, fertilized and transferred (17). In another study, CC commenced on day 1 of the menstrual cycle rather than day 5 resulted in more rapid follicular growth and higher pregnancy rate in IUI cycles (18). Treatment with CC was associated with higher rate of pregnancy if started early (days 1 through 5 than 5 through 9) in the menstrual cycle in the study by Dehbashi et al. (19).

Based on experiences on CC, the starting day for LTZ in most studies has been found on day 3 of spontaneous or induced menstruation. In one

study, 5 mg LTZ administered on cycle days 1-10 showed higher pregnancy rate compared to same amount administered from cycle days 1-5 (1). In another study, they compared the effect of 2.5 mg LTZ administered on cycle days 2-6 to placebo and their findings showed 33.3% ovulation rate compared to 0.00% for the placebo (20).

In our study, we compared the cycle characteristics for two different starting days including cycle days 5 and 3 in CC resistant PCOS patients who developed no dominant follicle during their previous cycles with CC, examined by ultrasound scanning. Our findings did not show significant difference in ovulation rate between CD3 and CD5 groups. The overall ovulation rate was 40.6%. The monofollicular response showed no significant differences between two groups. In different studies, this rate has been reported between 33.3% (20) to 84.4% (21).

In our study, the endometrial thickness was not significantly different between CD3 and CD5 groups. The overall endometrial thickness in both groups was 7.9 mm compared to 11.2 mm in a study by Bedaiwy et al. (11) and 7.1 mm in a study by Al-Fozan et al. (14).

There was 4 (12.1%) clinical pregnancies in CD3 group and 3 (9.4%) in CD5 group. The total pregnancy rate was 10.8%. The pregnancy rate in cycles of LTZ treatment in literature is 5.6% (20) to 40.6% 9 (22).

In our research, we studied the hormonal dynamics as well. In both groups, testosterone and androstenedione concentrations were increased from baseline three days after termination of LTZ treatment. The change from baseline was 30.5 ng/dl for testosterone and 98.7 ng/dl for androstenedione. It could be realized that the patients with lower serum androgens level would get more benefit from LTZ treatment.

Serum E_2 concentration was increased from baseline for 31.2 pg/ml in CD3 group and decreased for 8.96 pg/ml in CD5 group. This difference should be related to E_2 secretion from the ovum.

Reviewing the pharmacokinetic studies of LTZ, there was no effect on plasma concentration of testosterone and androstenedione after single doses of 0.1-5 mg (8). On the other hand, Garcia-Velasco et al. (9) have shown a significant increase in intrafollicular androgen levels in IVF cycles treated by LTZ. Cortinez et al. (10) have also shown an increase in serum androgen level and a decrease in E_2 levels on the last day of LTZ treatment compared to natural cycle.

In our study, the androgen levels were increased significantly 3 days after termination of LTZ, days 10 and 12 for CD3 and CD5 groups, respectively. The follicular phase length was shown in both groups for about 14 days, and high androgen level at the time of conception was also considered.

PCOS patients experience a higher incidence of miscarriage, preterm delivery and low birth weight infants (23) that has been attributed to hyperandrogenic state of this syndrome, indicated by many authors (24-26). The other effect of hyperandrogenism in PCOS pregnant patients at the early embryonic stage has been proposed as a developmental etiology for PCOS in female fetus (27).

Therefore, it seems logical that an increase in androgen level after receiving LTZ in hyperandrogenic PCOS patients at the time of conception should become a major concern.

Although we have not measured free androgen index (FAI) in our patients, but our finding showed that anovulatory patients in both groups had significantly higher basal androgen levels and this was in accordance with Imani et al. (28), in which they proved FAI as an important predictor of ovulation in CC protocol.

Conclusion

LTZ is an alternative treatment in PCOS patients. Two different protocols of LTZ starting on cycle days 3 and 5 showed no significant differences in follicular phase length, endometrial thickness, monofollicular response, and pregnancy rate. Androgen levels were significantly increased after treatment. Lower basal androgen levels showed significantly better result. More studies are needed to evaluate different initiation day, length, and dose for LTZ administration.

Acknowledgements

The study was the result of a thesis presented for the specialty degree in Obstetrics & Gynecology, number 2327-T. The authors would like to thank to Mashhad University of Medical Sciences for financial support. There was no conflict of interest in this article.

References

1. Badawy A, Mosbah A, Tharwat A, Eid M. Extended letrozole therapy for ovulation induction in clomiphene-resistant women with polycystic ovary syndrome: a novel protocol. Fertil Steril. 2009; 92(1): 236-239.
2. Casper RF, Mitwally MF. Review: aromatase inhibitors for ovulation induction. J Clin Endocrinol Metab. 2006; 91(3): 760-771.
3. Franks S, Adams J, Mason H, Polson D. Ovulatory disorders in women with polycystic ovary syndrome. Clin Obstet Gynaecol. 1985; 12(3): 605-632.
4. Bhatnagar AS, Hausler A, Schieweck K, Lang M, Bowman R. Highly selective inhibition of estrogen biosynthesis by CGS 20267, a new non-steroidal aromatase inhibitor. J Steroid Biochem Mol Biol. 1990; 37(6): 1021-1027.
5. Polyzos NP, Tzioras S, Badawy AM, Valachis A, Dritsas C, Mauri D. Aromatase inhibitors for female infertility: a systematic review of the literature. 2009; 19(4): 456-471.
6. Casper RF, Mitwally MF. Use of the aromatase inhibitor letrozole for ovulation induction in women with polycystic ovarian syndrome. Clin Obstet Gynecol. 2011; 54(4): 685-695.
7. Mitwally MF, Casper RF. Aromatase inhibition: a novel method of ovulation induction in women with polycystic ovary syndrome. Reproductive Technologies. 2001; 10(5): 244-247.
8. Buzdar AU, Robertson JF, Eiermann W, Nabholtz JM. An overview of the pharmacology and pharmacokinetics of the newer generation aromatase inhibitors anastrozole, letrozole, and exemestane. Cancer. 2002; 95(9): 2006-2016.
9. Garcia-Velasco JA, Moreno L, Pacheco A, Guillen A, Duque L, Requena A, et al. The aromatase inhibitor letrozole increases the concentration of intraovarian androgens and improves in vitro fertilization outcome in low responder patients: a pilot study. Fertil Steril. 2005; 84(1): 82-87.
10. Cortinez A, De Carvalho I, Vantman D, Gabler F, Iñiguez G, Vega M. Hormonal profile and endometrial morphology in letrozole-controlled ovarian hyperstimulation in ovulatory infertile patients. Fertil Steril. 2005; 83(1): 110-115.
11. Bedaiwy MA, Abdelaleem MA, Hussein M, Mousa N, Brunengraber LN, Casper RF. Hormonal, follicular and endometrial dynamics in letrozole-treated versus natural cycles in patients undergoing controlled ovarian stimulation. Reprod Biol Endocrinol. 2011; 9: 83.
12. Bajetta E, Zilembo N, Dowsett M, Guillevin L, Di Leo A, Celio L, et al. Double-blind, randomised, multicentre endocrine trial comparing two letrozole doses, in postmenopausal breast cancer patients. Eur J Cancer. 1999; 35(2): 208-213.
13. Mitwally MF, Casper RF. Single-dose administration of an aromatase inhibitor for ovarian stimulation. Fertil Steril. 2005; 83(1): 229-231.
14. Al-Fozan H, Al-Khadouri M, Tan SL, Tulandi T. A randomized trial of letrozole versus clomiphene citrate in women undergoing superovulation. Fertil Steril. 2004; 82(6): 1561-1563.
15. Badawy A, Abdel Aal I, Abulatta M. Clomiphene citrate or letrozole for ovulation induction in women with polycystic ovarian syndrome: a prospective randomized trial. Fertil Steril. 2009; 92(3): 849-852.
16. Wu CH, Winkel CA. The effect of therapy initiation day on clomiphene citrate therapy. Fertil Steril. 1989; 52(4): 564-568.
17. Marrs RP, Vargyas JM, Shangold GM, Yee B. The effect of time of initiation of clomiphene citrate on multiple follicle development for human in vitro fertilization and embryo replacement procedures. Fertil Steril. 1984; 41(5): 682-685.
18. Biljan MM, Mahutte NG, Tulandi T, Tan SL. Prospective randomized double-blind trial of the correlation between time of administration and antiestrogenic effects of clomiphene citrate on reproductive end organs. Fertil Steril. 1999; 71(4): 633-638.
19. Dehbashi S, Vafaei H, Parsanezhad MD, Alborzi S. Time of initiation of clomiphene citrate and pregnancy rate in polycystic ovarian syndrome. Int J Gynaecol Obstet. 2006; 93(1): 44-48.
20. Kamath MS, Aleyamma TK, Chandy A, George K. Aromatase inhibitors in women with clomiphene citrate resistance: a randomized, double-blind, placebo-controlled trial. Fertil Steril. 2010; 94(7): 2857-2859.
21. Al-Omari WR, Sulaiman WR, Al-Hadithi N. Comparison of two aromatase inhibitors in women with clomiphene-resistant polycystic ovary syndrome. Int J Gynaecol Obstet. 2004; 85(3): 289-291.
22. Begum MR, Ferdous J, Begum A, Quadir E. Comparison of efficacy of aromatase inhibitor and clomiphene citrate in induction of ovulation in polycystic ovarian syndrome. Fertil Steril. 2009; 92(3): 853-857.
23. Van der Spuy ZM, Dyer SJ. The pathogenesis of infertility and early pregnancy loss in polycystic ovary syndrome. Best Pract Res Clin Obstet Gynaecol. 2004; 18(5): 755-771.
24. Qiao J, Feng HL. Extra- and intra-ovarian factors in polycystic ovary syndrome: impact on oocyte maturation and embryo developmental competence. Hum Reprod Update. 2011; 17(1): 17-33.
25. Okon MA, Laird SM, Tuckerman EM, Li TC. Serum androgen levels in women who have recurrent miscarriages and their correlation with markers of endometrial function. Fertil Steril. 1998; 69(4): 682-690.
26. Tuckerman EM, Okon MA, Li T, Laird SM. Do androgens have a direct effect on endometrial function? An in vitro study. Fertil Steril. 2000; 74(4): 771-779.
27. Abbott DH, Barnett DK, Bruns CM, Dumesic DA. Androgen excess fetal programming of female re-

production: a developmental aetiology for polycystic ovary syndrome?. Hum Reprod Update. 2005; 11(4): 357-374.
28. Imani B, Eijkemans MJ, te Velde ER, Habbema JD, Fauser BC. Predictors of chances to conceive in ovulatory patients during clomiphene citrate induction of ovulation in normogonadotropic oligoamenorrheic infertility. J Clin Endocrinol Metab. 1999; 84(5): 1617-1622.

Original Article

Age-Specific Serum Anti-Mullerian Hormone and Follicle Stimulating Hormone Concentrations in Infertile Iranian Women

Alireza Raeissi, M.Sc.[1], Alireza Torki, M.Sc.[1], Ali Moradi, Ph.D.[1]*, Seyed Mehdi Mousavipoor, M.Sc.[2], Masoud Doosti Pirani, M.Sc.[1]

1. Department of Biochemistry and Molecular Biology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
2. Department of Biochemistry, International Campus of Shahid Sadoughi University of Medical Sciences, Yazd, Iran

Abstract

Background: Anti-Müllerian hormone (AMH) is secreted by the granulosa cells of growing follicles during the primary to large antral follicle stages. Abnormal levels of AMH and follicle stimulating hormone (FSH) may indicate a woman's diminished ability or inability to conceive. Our aim is to investigate the changes in serum AMH and FSH concentrations at different age groups and its correlation with ovarian reserves in infertile women.

Materials and Methods: This cross-sectional study analyzed serum AMH and FSH levels from 197 infertile women and 176 healthy controls, whose mean ages were 19-47 years. Sample collection was performed by random sampling and analyzed with SPSS version 16 software.

Results: There were significantly lower mean serum AMH levels among infertile women compared to the control group. The mean AMH serum levels from different ages of infertile and control group (fertile women) decreased with increasing age. However, this reduction was greater in the infertile group. The mean FSH serum levels of infertile women were significantly higher than the control group. Mean serum FSH levels consistently increased with increasing age in infertile women; however mean luteinizing hormone (LH) levels were not consistent.

Conclusion: We have observed increased FSH levels and decreased AMH levels with increasing age in women from 19 to 47 years of age. Assessments of AMH and FSH levels in combination with female age can help in predicting ovarian reserve in infertile women.

Keywords: Anti-Müllerian Hormone, Follicle Stimulating Hormone, Infertility, Women, Age

Citation: Raeissi A, Torki A, Moradi A, Mousavipoor SM, Doosti Pirani M. Age-specific serum anti-mullerian hormone and follicle stimulating hormone concentrations in infertile Iranian women. Int J Fertil Steril. 2015; 9(1): 27-32.

Introduction

One of the main causes for infertility is decreased ovarian reserve. Ovarian reserve is the number of good quality oocytes that remain within the ovaries. As a woman's age increases, her ovarian reserves decline (1-5). This decline may also result from autoimmune, genetic and iatrogenic conditions (6). In addition, autoimmune endocrinopathies, radiation therapy, or pelvic surgery may lead to decreased ovarian reserve (7-9). There are different tests to diagnose reductions in ovarian reserve such as elevated serum follicle stimulating hormone (FSH) levels on days 2 or 3 of the menstrual cycle FSH is mostly used in routine laboratories), low ovar-

Received: 28 Oct 2013, Accepted: 9 Feb 2014
* Corresponding Address: P.O. Box: 8915173149, Department of Biochemistry and Molecular Biology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
Email: morady2008@gmail.com

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 27-32

ian volume, an antral follicle count of <5 per ovary, low inhibin B levels, and <5 oocytes retrieved during an assisted reproductive technology (ART) cycle. A new test for the assessment of decreased ovarian reserve analyzes anti-Müllerian hormone (AMH) levels (10-14). AMH, a member of the transforming growth factor superfamily, is secreted in the human ovary by granulosa cells of primary growing follicles until the early antral stage (15-19). In females AMH regulates the growth of primary follicles by inhibiting further recruitment of other follicles during folliculogenesis (20). Serum AMH levels decline with increasing age in women and the levels are undetectable after menopause (21). Serum levels of AMH decrease prior to any increase in baseline FSH. FSH mostly indicates follicular maturation during the previous two weeks when gonadotropin follicles become sensitive. However AMH levels indicate pre-antral follicles to the post-primary pool that pass through stages before folliculogenesis (22-24).

Few studies have assessed the levels of FSH and AMH at various ages in infertile women, therefore our aim was to investigate the changes in serum concentrations of AMH and FSH at different ages and its correlation with ovarian reserves in infertile women.

Materials and Methods

This cross-sectional study was performed at the Research Center of Infertility, Shahid Sadoughi University, Yazd, Iran between May 2010 and September 2012. We assessed serum AMH and FSH levels on days 2 or 3 of the menstrual cycles of 197 infertile women with problem decreasing ovarian reserve and 176 healthy controls, without decreasing ovarian reserve and age 19-47 years, who were admitted to infertility clinic to investigate infertility. Inclusion criteria were: no history of gynecological surgical procedures, presence of a regular menstrual cycle, no signs of hyper-androgenemia, and normal sonographic appearance of the ovaries. Infertile women were excluded if they were using fertility drugs or had any autoimmune, genetic, or iatrogenic conditions, autoimmune endocrinopathies, radiation therapy or pelvic surgery, or polycystic ovary syndrome as these factors have been shown to alter serum AMH levels (6-10).

Patients were stratified into the following age categories: <25, 25-29.9, 30-34.9, 35-39.9, 40-45 and ≥45 years. Ethical approval for the study was received from the Women's and Medical Ethics Committee. This approval allowed for measurement of serum AMH levels in stored routine clinical samples without the need for the patient's written permission in order to produce an age-related normal range data for AMH levels. All 197 patients and 176 healthy controls were asked to provide their consent in order to link their AMH results with IVF outcome.

Serum AMH levels were assessed in serum by the enzyme linked immunosorbent assay (ELISA) method (Beckman Coulter, USA). The sensitivity of the assay is 0.08 ng/ml with a reference range of 12.6 ng/ml. Inter- and intra-assay coefficients of variation (CV) are <7.7% and <5.8%, respectively. Luteinizing hormone (LH) and FSH levels were assessed in serum by the electrochemiluminescent immunoassay (ECLIA) method (Cobas, England). The assay sensitivity for FSH is <0.1 mIU/ml with inter- and intra-assay CVs of <7.7% and <5.8%, respectively. Sample collection was performed by random sampling at days 3-5 of a spontaneous menstrual cycle. The serum was separated one hour after sampling and frozen at -20˚C until assayed.

Data analysis

The data were presented as mean ± standard deviation as calculated in each group by SPSS software version 16 (SPSS Inc., Chicago, IL, USA). The student's t test was used to assess differences between mean values of AMH, FSH and LH in the infertility and control groups with a confidence level of 95% and p value <0.05.

Results

Table 1 shows the mean AMH, FSH and LH levels according to the six age categories. Overall there was a significantly lower mean AMH serum level in infertile women compared to the control group (Fig.1, p=0.002). Although the mean serum AMH levels consistently decreased with increasing age in both the infertile and control groups, the reduction seen in the infertile group was more (Fig.2, p<0.05). Overall, the mean FSH serum level in infertile women was significantly higher than the control group (Fig.1, p=0.03). Mean serum FSH levels consistently increased with increasing age in both the infertile and control groups. However, this increase was higher in the infertile group (Fig.3, p<0.05). The mean LH serum level in infertile women was higher than the control group (Fig.1, p=0.32). The mean LH serum level in both groups with increasing age was not consistent (Fig.4).

Table 1: Changes in anti-Mullerian hormone (AMH), follicle stimulating hormone (FSH) and luteinizing hormone (LH) serum concentrations for different ages of women in the infertile and control groups

Age (Y)		Infertile	Fertile	P value
		Mean ± SD	Mean ± SD	
		n=197	**n=176**	
	BMI (kg/m^2)	27 ± 2	27 ± 6	
19-47 Total sample	AMH (ng/ml)	0.32 ± 0.2	1.85 ± 0.19	0.002
	FSH (mIU/ml)	21.36 ± 4.28	9.79 ± 4.38	0.03
	LH (mIU/ml)	11.68 ± 2.05	8.66 ± 2.16	0.32
		n=17	**n=15**	
	BMI (kg/m^2)	28 ± 3	27 ± 5	
<25	AMH (ng/ml)	0.55 ± 0.16	2.04 ± 0.21	0.000
	FSH (mIU/ml)	17.27 ± 2.36	5.02 ± 4.39	0.002
	LH (mIU/ml)	9.2 ± 1.25	5.54 ± 1.23	0.2
		n=29	**n=27**	
	BMI (kg/m^2)	26 ± 2	27 ± 4	
25-29.9	AMH (ng/ml)	0.39 ± 0.23	2.06 ± 0.24	0.001
	FSH (mIU/ml)	20.41 ± 4.49	6.7 ± 3.35	0.04
	LH (mIU/ml)	12.32 ± 2.54	8.98 ± 1.35	0.27
		n=38	**n=32**	
	BMI (kg/m^2)	28 ± 1	26 ± 7	
30-34.9	AMH (ng/ml)	0.41 ± 0.21	1.91 ± 0.17	0.000
	FSH (mIU/ml)	29.3 ± 3.24	13.21 ± 5.26	0.02
	LH (mIU/ml)	14.76 ± 1.81	9.39 ± 2.49	0.35
		n=22	**n=57**	
	BMI (kg/m^2)	27 ± 5	28 ± 1	
35-39.9	AMH (ng/ml)	0.34 ± 0.18	1.78 ± 0.21	0.000
	FSH (mIU/ml)	27.48 ± 5.62	7.02 ± 1.66	0.004
	LH (mIU/ml)	12.38 ± 2.53	6.89 ± 3.39	0.55
		n=61	**n=31**	
	BMI (kg/m^2)	28 ± 6	26 ± 3	
40-44.9	AMH (ng/ml)	0.29 ± 0.25	1.72 ± 0.26	0.032
	FSH (mIU/ml)	29.32 ± 3.59	13.23 ± 6.34	0.023
	LH (mIU/ml)	7.22 ± 2.3	6.32 ± 2.12	0.34
		n=30	**n=14**	
	BMI (kg/m^2)	27 ± 2	28 ± 4	
≥45	AMH (ng/ml)	0.25 ± 0.22	1.35 ± 0.14	0.012
	FSH (mIU/ml)	32.54 ± 6.42	17 ± 5.32	0.043
	LH (mIU/ml)	10.61 ± 1.87	8.34 ± 2.43	0.21

BMI; Body mass index.

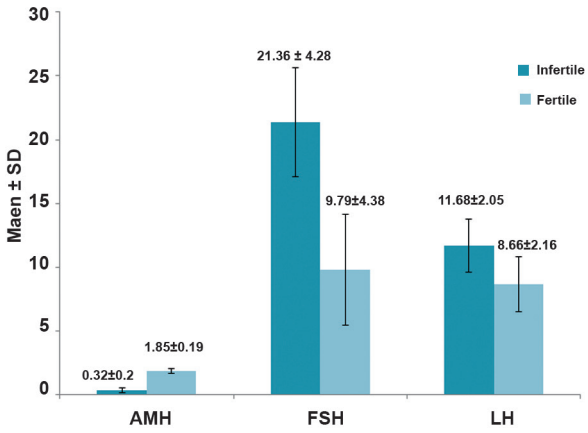


Fig.1: Mean ± standard deviation in anti-Müllerian hormone (AMH), follicle stimulating hormone (FSH) and luteinizing hormone (LH) in both fertile and infertile women.

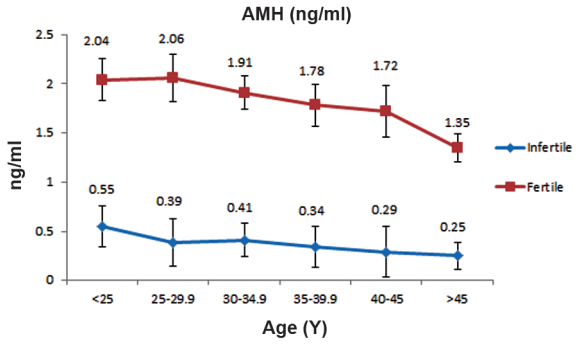


Fig.2: Mean ± standard deviation values for anti-Müllerian hormone (AMH) over the reproductive age range in control group and infertile women.

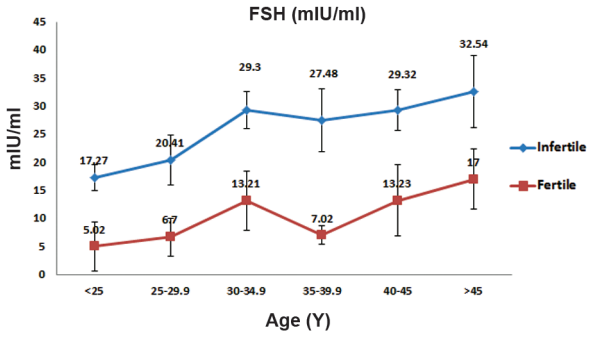


Fig.3: Mean ± standard follicle stimulating hormone (FSH) values for the reproductive age range in control and infertile group women.

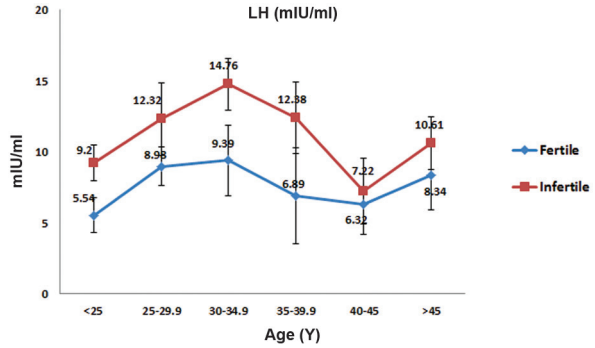


Fig.4: Mean ± standard deviation values for follicle luteinizing stimulating hormone (LH) over the reproductive age range in fertile and infertile women.

Discussion

In this study infertile women had higher FSH levels and lower AMH levels than fertile women. The range of AMH observed in infertile women was <1 whereas in the control group it was approximately 1 to 3. Mean serum AMH levels steadily decreased with increasing age in the age range of 19 to 47 years. In addition, mean FSH level approximately increased with increasing age in this range (19 to 47 years) and was attributed to reduced ovarian reserve. Since AMH are produced by preantral and antral follicles (15-18, 24, 25), hence with increasing age, levels of pre-antral follicles decrease, causing a reduction in the amount of AMH. Thus, lower levels of AMH and higher FSH show declining ovarian reserve. Increased levels of FSH and decreased AMH can be considered as a marker for reduced fertility potential. This fluctuation on the third day FSH levels makes it difficult to predict ovarian reserve. Hence, the most appropriate factor for the assessment of ovarian reserve is an evaluation of AMH levels, which are independent of the cycle.

We observed good correlation between serum AMH and FSH levels with ovarian reserve. These results supported those of previous studies in terms of the connections between low AMH serum levels and poor ovarian response (26, 27). Several studies examined decreased AMH with age. Seifer et al. (25) reported that mean AMH levels decreased steadily with increasing age, in the range from 24 to 50 years, which was similar to the results of the current study.

La Marca et al. (28), in a study on 277 healthy women (aged 18-50 years) reported that serum AMH levels progressively declined with increasing age. Mulders et al. evaluated serum AMH levels in 98 infertile and 48 healthy control women. They reported that serum AMH levels decreased over time in both infertile and healthy control women, which was similar to the results of our study (29).

Conclusion

With increasing age AMH levels decrease due to reduced ovarian reserves. Hence AMH can be used as a marker for the assessment of ovarian reserves in the follicular and luteal phases.

Acknowledgements

The authors wish to express their gratitude to the staff of the Research and Clinical Infertility Center in Yazd, Iran. There is no financial support for this article. The authors declare that they have no conflict of interests.

References

1. Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson JF. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Hum Reprod. 1992; 7(10): 1342-1346.
2. Klein NA, Battaglia DE, Fujimoto VY, Davis GS, Bremner WJ, Soules MR. Reproductive aging: accelerated ovarian follicular development associated with a monotropic follicle-stimulating hormone rise in normal older women. J Clin Endocrinol Metab. 1996; 81(3): 1038-1045.
3. Gougeon A. Ovarian follicular growth in humans: ovarian ageing and population of growing follicles. Maturitas. 1998; 30(2): 137-142.
4. Gougeon A, Ecochard R, Thalabard JC. Age-related changes of the population of human ovarian follicles: increase in the disappearance rate of non-growing and early-growing follicles in aging women. Biol Reprod. 1994; 50(3): 653-663.
5. te Velde ER, Scheffer GJ, Dorland M, Broekmans FJ, Fauser BC. Developmental and endocrine aspects of normal ovarian aging. Mol Cell Endocrinol. 1998; 145(1-2): 67-73.
6. Sullivan AK, Marcus M, Epstein MP, Allen EG, Anido AE, Paquin JJ, et al. Association of FMR1 repeat size with ovarian dysfunction. Hum Reprod. 2005; 20(2): 402-412.
7. Bath LE, Wallace WH, Shaw MP, Fitzpatrick C, Anderson RA. Depletion of ovarian reserve in young women after treatment for cancer in childhood: detection by anti-Mullerian hormone, inhibin B and ovarian ultrasound. Hum Reprod. 2003; 18(11): 2368-2374.
8. Iwase A, Hirokawa W, Goto M, Takikawa S, Nagatomo Y, Nakahara T, et al. Serum anti-Mullerian hormone level is a useful marker for evaluating the impact of laparoscopic cystectomy on ovarian reserve. Fertil Steril. 2010; 94(7): 2846-2849.
9. Papaleo E, De Santis L, Fusi F, Doldi N, Brigante C, Marelli G, et al. Natural cycle as first approach in aged patients with elevated follicle-stimulating hormone undergoing intracytoplasmic sperm injection: a pilot study. Gynecol Endocrinol. 2006; 22(7): 351-354.
10. Coccia ME, Rizzello F. Ovarian reserve. Ann N Y Acad Sci. 2008; 1127: 27-30.
11. Bukman A, Heineman MJ. Ovarian reserve testing and the use of prognostic models in patients with subfertility. Hum Reprod Update. 2001; 7(6): 581-590.
12. Dennis NA, Houghton LA, Jones GT, van Rij AM, Morgan K, McLennan IS. The level of serum anti-Mullerian hormone correlates with vitamin D status in men and women but not in boys. J Clin Endocrinol Metab. 2012; 97(7): 2450-2455.
13. Maheshwari A, Fowler P, Bhattacharya S. Assessment of ovarian reserve--should we perform tests of ovarian reserve routinely?. Hum Reprod. 2006; 21(11): 2729-2735.
14. van Rooij IA, Broekmans FJ, Hunault CC, Scheffer GJ, Eijkemans MJ, de Jong FH, et al. Use of ovarian reserve tests for the prediction of ongoing pregnancy in couples with unexplained or mild male infertility. Reprod Biomed Online. 2006; 12(2): 182-190.
15. Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA, et al. Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. Mol Hum Reprod. 2004; 10(2): 77-83.
16. Durlinger AL, Visser JA, Themmen AP. Regulation of ovarian function: the role of anti-Mullerian hormone. Reproduction. 2002; 124(5): 601-609.
17. Freeman EW, Sammel MD, Lin H, Gracia CR. Anti-mullerian hormone as a predictor of time to menopause in late reproductive age women. J Clin Endocrinol Metab. 2012; 97(5): 1673-1680.
18. Josso N. Anti-mullerian hormone. Clin Endocrinol (Oxf). 1986; 25(3): 331-345.
19. Li HW, Ng EH, Wong BP, Anderson RA, Ho PC, Yeung WS. Correlation between three assay systems for anti-Mullerian hormone (AMH) determination. J Assist Reprod Genet. 2012; 29(12): 1443-1446.
20. Visser JA, de Jong FH, Laven JS, Themmen AP. Anti-Mullerian hormone: a new marker for ovarian function. Reproduction. 2006; 131(1): 1-9.
21. de Vet A, Laven JS, de Jong FH, Themmen AP, Fauser BC. Antimullerian hormone serum levels: a putative marker for ovarian aging. Fertil Steril. 2002; 77(2): 357-362.
22. Feyereisen E, Mendez Lozano DH, Taieb J, Hesters L, Frydman R, Fanchin R. Anti-Mullerian hormone: clinical insights into a promising biomarker of ovarian follicular status. Reprod Biomed Online. 2006; 12(6): 695-703.
23. La Marca A, Volpe A. Anti-Mullerian hormone (AMH) in female reproduction: is measurement of circulating AMH a useful tool?. Clin Endocrinol (Oxf). 2006; 64(6): 603-610.
24. Fanchin R, Schonauer LM, Righini C, Guibourdenche J, Frydman R, Taieb J. Serum anti-Mullerian hormone is more strongly related to ovarian follicular status than serum inhibin B, estradiol, FSH and LH on day 3. Hum Reprod. 2003; 18(2): 323-327.
25. Seifer DB, Baker VL, Leader B. Age-specific serum anti-Mullerian hormone values for 17,120 women presenting to fertility centers within the United States. Fertil Steril. 2011; 95(2): 747-750.
26. Seifer DB, MacLaughlin DT, Christian BP, Feng B, Shelden RM. Early follicular serum mullerian-inhibiting substance levels are associated with ovarian response during assisted reproductive technology cycles. Fertil Steril. 2002; 77(3) : 468-471.

27. van Rooij IA, Broekmans FJ, te Velde ER, Fauser BC, Bancsi LF, de Jong FH, et al. Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. Hum Reprod. 2002; 17(12): 3065-3071.
28. La Marca A, Sighinolfi G, Giulini S, Traglia M, Argento C, Sala C, et al. Normal serum concentrations of anti-Mullerian hormone in women with regular menstrual cycles. Reprod Biomed Online. 2010; 21(4): 463-469.
29. Mulders AG, Laven JS, Eijkemans MJ, de Jong FH, Themmen AP, Fauser BC. Changes in anti-Mullerian hormone serum concentrations over time suggest delayed ovarian ageing in normogonadotrophic anovulatory infertility. Hum Reprod. 2004; 19(9): 2036-2042.

Original Article

The Outcomes of Intracytoplasmic Sperm Injection and Laser Assisted Hatching in Women Undergoing *In Vitro* Fertilization Are Affected by The Cause of Infertility

Hsin-Fen Lu, M.D.[1], Fu-Shiang Peng, M.D.[1], Shee-Uan Chen, M.D.[2], Bao-Chu Chiu, B.Sc.[1], Szu-Hsing Yeh, B.Sc.[1], Sheng-Mou Hsiao, M.D.[1]*

1. Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Far Eastern Memorial Hospital, New Taipei, Taiwan
2. Department of Obstetrics and Gynecology, National Taiwan University Hospital, Taipei, Taiwan

Abstract

Background: We sought to determine the association between factors that affected clinical pregnancy and live birth rates in patients who underwent *in vitro* fertilization (IVF) and received intracytoplasmic sperm injection (ICSI) and/or laser assisted hatching (LAH), or neither.

Materials and Methods: In this retrospective cohort study, the records of women who underwent IVF with or without ICSI and/or LAH at the Far Eastern Memorial Hospital, Taipei, Taiwan between January 2007 and December 2010 were reviewed. We divided patients into four groups: 1. those that did not receive ICSI or LAH, 2. those that received ICSI only, 3. those that received LAH only and 4. those that received both ICSI and LAH. Univariate and multivariate analyses were performed to determine factors associated with clinical pregnancy rate and live birth rate in each group.

Results: A total of 375 women were included in the analysis. Oocyte number (OR=1.07) affected the live birth rate in patients that did not receive either ICSI or LAH. Maternal age (OR=0.89) and embryo transfer (ET) number (OR=1.59) affected the rate in those that received ICSI only. Female infertility factors other than tubal affected the rate (OR=5.92) in patients that received both ICSI and LAH. No factors were found to affect the live birth rate in patients that received LAH only.

Conclusion: Oocyte number, maternal age and ET number and female infertility factors other than tubal affected the live birth rate in patients that did not receive ICSI or LAH, those that received ICSI only, and those that received both ICSI and LAH, respectively. No factors affected the live birth rate in patients that received LAH only. These data might assist in advising patients on the appropriateness of ICSI and LAH after failed IVF.

Keywords: Assisted Reproduction Technology, *In Vitro* Fertilization, Intracytoplasmic Sperm Injection

Citation: Lu HF, Peng FS, Chen SU, Chiu BC, Yeh SH, Hsiao SM. The outcomes of intracytoplasmic sperm injection and laser assisted hatching in women undergoing in vitro fertilization are affected by the cause of infertility. Int J Fertil Steril. 2015; 9(1): 33-40.

Received: 18 Sep 2013, Accepted: 28 Jan 2014
* Corresponding Address: Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Far Eastern Memorial Hospital, New Taipei City 220, Taiwan
Email: smhsiao2@gmail.com

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 33-40

Introduction

Since the introduction of assisted reproductive technology (ART), *in vitro* fertilization (IVF) has enabled countless couples to achieve pregnancy. However, failure to conceive after multiple attempts with different methods imparts a significant emotional and financial burden on patients (1-4). It has been estimated that up to 85% of embryos do not implant (5, 6). Many attempts have been made to identify factors that can predict the success of IVF and it is generally accepted that female age, duration of subfertility, baseline follicle stimulating hormone (FSH) levels, and number of oocytes are predictors of pregnancy after IVF (7, 8). In our prior study, we have identified that the number of embryos transferred, the presence of ovarian hyperstimulation syndrome, female infertility factors other than tubal factors, and embryo quality were correlated with the failure to achieve birth emphasizing a successful singleton at term (BESST) (i.e., the singleton, term gestation and live birth) (9, 10). Other studies have shown that IVF success is associated with the diagnosis after an infertility workup, the number of previous unsuccessful IVF attempts, and a prior successful pregnancy; however, no truly useful model for predicting the success of IVF exists (11).

Depending on the reasons for infertility in a particular couple, numerous techniques such as intracytoplasmic sperm injection (ICSI) and assisted hatching (AH) have been developed to increase the probability of pregnancy and a live birth (5, 12, 13). ICSI is typically used for male factor infertility and in cases where eggs cannot easily be penetrated by sperm. Despite the concern for genetic abnormalities, it is a proven technique for achieving successful pregnancy and live birth (14, 15). It is well known that a proportion of euploid embryos fail to implant because of hatching difficulties (15) and AH involves artificial disruption of the zona pellucida with the intent of increasing implantation potential (16). Many methods have been developed to disrupt the zona pellucida and laser AH (LAH) has been found to be more effective in some subgroups of patients (12, 17). However, a recent analysis by Myers et al. (18) has concluded that there is relatively little high-quality evidence to support the choice of specific interventions.

The purpose of this study was to determine the association of factors that affected the clinical pregnancy and live birth rates in patients that underwent IVF who received both ICSI and LAH, neither ICSI or LAH, or only ICSI or LAH.

Materials and Methods

In this retrospective cohort study the outcomes of women who underwent IVF with or without ICSI at the Far Eastern Memorial Hospital, Taipei, Taiwan between January 2007 and December 2010 were reviewed. Cases in which estradiol levels exceeded 50 pg/mL on the second day of the menstrual cycle were excluded. The study was approved by the Research Ethics Review Committee of the Far Eastern Memorial Hospital. Due to the retrospective nature of the study the requirement for informed consent was waived.

Causes of reduced female fertility included tubal causes, endometriosis, anovulation, polycystic ovary syndrome (PCOS), decreased ovarian reserve, uterine disorders, age >35 years (advanced maternal age) and unidentified reasons. Females might have had one or multiple factors. Male causes of infertility were decreased sperm concentration ($<2\times10^7$/ml), decreased sperm motility (<50%) and azoospermia. Patients with one or more of the following criteria underwent ICSI: 1. fertilization rate below 50% in a prior IVF attempt and 2. male factor infertility. In cases of azoospermia, sperm for ICSI was obtained by microsurgical epididymis sperm aspiration (MES) or testicular sperm extraction (TESE). Patients with one or more of the following criteria underwent LAH: 1. zona pellucida >15 μm, 2. maternal age over 38 years and 3. at least three failed IVF attempts.

LAH was performed in a standard manner. Briefly, a 1.48 μm infrared diode laser (OCTAX Laser Shot™ System, Medical Technology Vertriebs-GmbH, Germany) in a computer-controlled non-contact mode was used. After positioning the embryo, the laser was focused at the equatorial level of the zona pellucida. A pulse length of 2.8 ms was used and the LAH procedure was performed until 25% of the zona pellucida was drilled.

The method of ovulation induction used in the study center was previously published (9). In brief, gonadotropin-releasing hormone agonist (Supremon, Aventis Pharma Deutschland, Frankfurt, Germany) was administered from the third day of the menstrual cycle via nasal spray, daily, in 4 doses of 200 μg. FSH (Gona-F, Serono, Geneva, Switzerland), 150-225 IU, was administered daily from the fifth day of the menstrual cycle via subcutaneous injection into the abdomen. Luteinizing hormone (LH) and estradiol levels were measured from the seventh day of the cycle, and transvaginal ultrasonography was performed every

two days in order to adjust dosages until complete follicular growth was achieved. When appropriate follicular growth was detected, 10000 IU of human chorionic gonadotropin (hCG, Pregnyl, NV Organon, Oss, The Netherlands) was injected and oocyte retrieval was performed 35 hours later. At four hours after oocyte retrieval, IVF was carried out, with or without ICSI. Two to five days later, embryos at the 4-cell to blastocyst stage were transferred; the remainder were frozen and stored in liquid nitrogen.

Pregnancy was defined as a βhCG level greater than 50 mIU/mL 14 days after day 2 embryo transfer (ET). Clinical pregnancy was defined by the ultrasound observation of fetal cardiac activity. We defined live birth as the birth of a newborn, irrespective of the duration of gestation that exhibited any signs of life.

For analysis, patients were divided into four groups: 1. those that did not receive either ICSI or LAH, 2. those that received ICSI only, 3. those that received LAH only and 4. those that received both ICSI and LAH.

Statistical analysis

For comparability among the four groups we used one-way analysis of variance (ANOVA) for normally distributed continuous variables and the chi-square test for categorical variables. If the data was non-normally distributed, Kruskal-Wallis tests were used to determine the difference among the four groups. When significance among group differences were apparent, multiple comparisons of means were performed using the Bonferroni procedure with type-I error adjustment. Parametric variables were represented as mean and standard deviation (SD) and categorical data were represented by number (n) and percentage (%). Nonparametric variables were represented as median (inter-quartile range). Univariate logistic regression analysis was performed to analyze the odds ratio (OR) of significant factors associated with successful pregnancy and live birth. Variables having a p value <0.05 in the univariate analysis were selected and evaluated by multivariate logistic regression models with the conditional forward selection method. All statistic assessments were two-sided and evaluated at the 0.05 level of significance. Statistic analyses were performed using SPSS 15.0 statistics software (SPSS Inc., Chicago, IL, USA).

Results

After applying the inclusion and exclusion criteria, a total of 375 women who underwent IVF between January 2007 and December 2010 were included in the analysis. The mean age of patients was 34.1 ± 4.7 years, and the mean age of their partners was 37.3 ± 5.4 years. In total, 121 patients (32.2%) did not receive either ICSI or LAH, 176 patients (46.9%) received ICSI only, 22 patients (5.9%) had LAH only, and 56 patients (14.9%) underwent both ICSI and LAH. The demographic and clinical characteristics of the patients are shown in table 1. There were significant differences in the age of partners, age of the patients, duration of infertility, the reason for infertility (tubal factor, other female factors, and male factor), number of previous IVF courses, oocyte number, and embryo number among the four groups (p<0.05).

In total, 179 (47.7%) women became pregnant. Of these, 126 (33.6%) had subsequent live births. The results of the univariate and multivariate analyses for factors that affected clinical pregnancy rate are shown in tables 2 and 3, respectively. Multivariate logistic regression indicated that only advanced maternal age affected clinical pregnancy rate in those that did not receive either ICSI or LAH (OR=0.87, 95% CI: 0.78 to 0.96, p=0.005). In patients that received ICSI only, advanced maternal age (OR=0.93, 95% CI: 0.86 to 0.99, p=0.044), female factors other than tubal (OR=3.37, 95% CI: 1.26 to 19.05, p=0.016) and embryo number (OR: 1.10, 95% CI: 1.03 to 1.18, p=0.007) affected the clinical pregnancy rate. In patients that received LAH only, only embryo number (OR=3.26, 95% CI: 1.24 to 8.57, p=0.017) affected the clinical pregnancy rate. In those that received both ICSI and LAH, only male factor (OR=0.32, 95% CI: 0.11 to 0.97, p=0.044) affected the clinical pregnancy rate.

The results of univariate and multivariate analyses of factors influencing the live birth rate are shown in tables 4 and 5, respectively. Multivariate logistic regression analysis indicated that oocyte number (OR=1.07, 95% CI: 1.01 to 1.13, p=0.031) affected the live birth rate in patients that did not receive either ICSI or LAH. In patients that received ICSI only, advanced maternal age (OR=0.89, 95% CI: 0.82 to 0.96, p=0.004) and ET number (OR=1.59, 95% CI:1.05 to 2.418, p=0.027) affected the live birth rate. In patients that received both ICSI and LAH, female factors other than tubal affected the live birth rate (OR=5.92, 95% CI:1.14 to 30.73, p=0.016). No factors were found to affect the live birth rate in patients that received LAH only.

Table 1: Patient demographic clinical characteristics (n=375)

	Total (n=375)	Neither ICSI or LAH (n=121)	ICSI (n=176)	LAH (n=22)	Both ICSI and LAH (n=56)	P value
Age of partner (Y)[1]	37.30 ± 5.40	36.54 ± 5.34	37.10 ± 5.47	37.48 ± 3.94	39.49 ± 5.36†‡	0.007*
Age of patient (Y)[1]	34.08 ± 4.66	33.43 ± 4.08	33.43 ± 4.77	35.81 ± 4.46	36.83 ± 4.50†‡	<0.001*
Duration of infertility (Y)[2]	4 (2, 6)	4 (2, 6)	3 (2, 5.5)	4 (2, 7)	6 (4, 10) †‡	<0.001*
Cause of infertility[3]						
Tubal factor	83 (22.1)	46 (38.0)	25 (14.2)†	5 (22.7)	7 (12.5)†	<0.001*
Female factors other than tubal (e.g., endometriosis, PCOS, uterine disorders)	96 (25.6)	35 (28.9)	33 (18.8)	9 (40.9)‡	19 (33.9)	0.020*
Male factor	136 (36.3)	15 (12.1)	97 (55.1)†	1 (4.5)‡	23 (41.1)†§	<0.001*
Combined female and male factors	23 (6.1)	5 (4.1)	13 (7.4)	2 (9.1)	3 (5.4)	0.634
Previous IVF course[2]	0 (0, 1)	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 1.5)†‡	0.021*
Oocyte number[2]	9 (4, 14)	8 (4, 13)	10 (5.5, 15)	6.5 (2, 14)‡	6 (4, 12)‡	0.005*
Embryo number[2]	5 (3, 9)	4 (2, 9)	6 (3, 10)	4 (1, 7)	4 (3, 6)	0.045*
ET number[2]	3 (2, 4)	3 (2, 4)	3 (3, 4)	3 (1, 4)	3 (2, 4)	0.110
Reason for ICSI						
Maternal age >35 years	138 (40.2)	0	94 (64.3)	0	44 (82.6)	NA
Fertilization rate <50% in prior IVF attempt	61 (17.8)	0	39 (25.8)	0	22 (42.3)	NA
Male factor	121 (35.3)	0	98 (64.9)	0	23 (44.2)	NA
Reason for LAH						
Maternal age >38 years	63 (16.9)	0	0	18 (81.8)	45 (80.4)	NA
Zona pellucida >15 µm	23 (6.2)	0	0	8 (36.4)	15 (26.8)	NA

ET; Embryo transfer, ICSI; Intracytoplasmic sperm injection, IVF; *In vitro* fertilization, LAH; Laser assisted hatching, PCOS; Polycystic ovary syndrome, *; Indicates a significant difference,p<0.05, †; Indicates a statistically significant difference between the indicated group and the group that did not receive ICSI or LAH group, ‡; Indicates a statistically significant difference between the indicated group and the ICSI group, §; Indicates a statistically significant difference between the LAH and both ICSI and LAH groups, p values are based on [1]; ANOVA, [2]; Kruskal-Wallis test and [3]; Chi-square test.
Data are presented as mean ± standard deviation, number (percentage), or median (interquartile range).
Pair-wise multiple comparisons between groups were determined using Bonferroni's test with α=0.008 adjustment.

Table 2: Results of univariate analysis for factors that affected clinical pregnancy rates in the four groups

	Neither ICSI or LAH	ICSI	LAH	Both ICSI and LAH
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
Age of partner (Y)	0.90 (0.84- 0.97)*	0.98 (0.93-1.04)	0.83 (0.65-1.07)	0.95 (0.86-1.05)
Age of female (Y)	0.87 (0.76- 0.96)*	0.89 (0.83-0.95)*	0.94 (0.77-1.15)	0.97 (0.86-1.09)
Duration of infertility (Y)	0.95 (0.83-1.07)	0.97 (0.87-1.08)	0.89 (0.67-1.18)	1.00 (0.87-1.14)
Tubal factor (no vs. yes)	1.58 (0.74-3.34)	1.56 (0.67-3.64)	0.59 (0.08-4.50)	0.45 (0.08-2.56)
Female factors other than tubal (no vs. yes)	0.95 (0.43-2.09)	4.20 (1.63-10.78)*	7.88 (1.01-56.12)*	3.29 (0.98-11.03)
Male factor (no vs. yes)	1.55 (0.52-4.59)	0.64 (0.35-1.18)	-	0.32 (0.11-0.97)*
Multiple factors (no vs. yes)	1.98 (0.32-12.30)	0.30 (0.09-1.02)	-	1.66 (0.14-19.39)
Previous IVF course	1.05 (0.87-1.28)	0.56 (0.35-0.97)*	-	0.89 (0.65-1.22)
Oocyte number	1.09 (1.02-1.15)*	1.07 (1.02-1.11)*	1.08 (0.93-1.26)	0.98 (0.90-1.07)
Embryo number	1.09 (1.01-1.18)*	1.14 (1.06-1.22)*	1.13 (0.93-1.38)	0.95 (0.83-1.09)
ET number	1.55 (1.05- 2.27)*	1.64 (1.19-2.27)*	3.26 (1.24-8.57)*	1.36 (0.79-2.33)

CI; Confidence interval, ET; Embryo transfer, ICSI; Intracytoplasmic sperm injection, IVF; *In vitro* fertilization, LAH; Laser assisted hatching, OR; Odds ratio and *; Significance: p<0.05.

Table 3: Results of multivariate analysis for factors that affected clinical pregnancy rates in the four groups

	Neither ICSI or LAH	ICSI	LAH	Both ICSI and LAH
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
Age of female (Y)	0.87 (0.78-0.96)*	0.93 (0.86-0.99)*	-	-
Female factors other than tubal (no vs. yes)	-	3.37 (1.26-19.05)*	-	-
Male factor (no vs. yes)	-	-	-	0.32 (0.11-0.97)*
Embryo number	-	1.10 (1.03-1.18)*	3.26 (1.24-8.57)*	-

CI; Confidence interval, ICSI; Intracytoplasmic sperm injection, IVF; *In vitro* fertilization, LAH; Laser assisted, OR; Odds ratio and *; Significance: p<0.05.

Table 4: Results of univariate analysis for factors that affected live birth rate in the four groups

	Neither ICSI or LAH	ICSI	LAH	Both ICSI and LAH
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
Age of partner (Y)	0.94 (0.87-1.01)	0.96 (0.90-1.03)	0.82 (0.64-1.06)	0.92 (0.82-1.03)
Age of female (Y)	0.91 (0.83-1.01)	0.87 (0.80-0.94)*	0.86 (0.69-1.07)	0.89 (0.78-1.02)
Duration of infertility	0.92 (0.81-1.06)	0.94 (0.83-1.07)	0.92 (0.69-1.22)	0.87 (0.74-1.03)
Tubal factor (no vs. yes)	0.73 (0.35-1.55)	0.56 (0.23-1.34)	1.05 (0.14-8.02)	1.33 (0.23-7.58)
Female factors other than tubal (no vs. yes)	1.57 (0.68-3.61)	3.55 (1.18-10.69)*	4.08 (0.60-27.65)	7.23 (1.46-35.84)*
Male factor (no vs. yes)	1.31 (0.42-4.11)	0.57 (0.29-1.12)	-	0.25 (0.77-0.79)*
Multiple factors (no vs. yes)	0.95 (0.15-5.91)	1.39 (0.37-5.28)	0.67 (0.04-12.27)	1.03 (0.09-12.12)
Previous IVF course	0.93 (0.74-1.16)	0.54 (0.28-1.04)	-	0.88 (0.62-1.25)
Oocyte number	1.07 (1.01-1.13)*	1.06 (1.02-1.11)*	1.05 (0.91-1.21)	1.02 (0.93-1.12)
Embryo number	1.06 (0.98-1.14)	1.12 (1.04-1.19)*	1.04 (0.87-1.24)	1.01 (0.88-1.14)
ET number	1.47 (0.99-2.19)	1.84 (1.23-2.73)*	1.97 (0.87-4.42)	2.23 (1.12-4.25)*

CI; Confidence interval, ET; Embryo transfer, ICSI; Intracytoplasmic sperm injection, IVF; *In vitro* fertilization, LAH; Laser assisted hatching, OR; Odds ratio and *; Significance: p<0.05.

Table 5: Results of multivariate analysis for factors that affected live birth rate in the four groups

	Neither ICSI or LAH	ICSI	LAH	Both ICSI and LAH
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
Age of female (Y)	-	0.89 (0.82-0.96)*	-	-
Female factors other than tubal (no vs. yes)	-	-	-	5.92 (1.14-30.73)*
Oocyte number	1.07 (1.01-1.13)*	-	-	-
ET number	-	1.59 (1.05-2.41)*	-	-

CI; Confidence interval, ET; Embryo transfer, ICSI; Intracytoplasmic sperm injection, IVF; *In vitro* fertilization, LAH; Laser assisted hatching, OR; Odds ratio and *; Significance p<0.05.

Discussion

The results of this study showed that different factors affected the clinical pregnancy rate and live birth rate in patients who underwent IVF that received ICSI and LAH, neither ICSI or LAH, and ICSI or LAH only. In patients that received LAH only, only embryo number (OR=3.26, 95% CI: 1.24 to 8.57, p=0.017) affected the clinical pregnancy rate, and in those that received both ICSI and LAH only male factor (OR=0.32, 95% CI: 0.11 to 0.97, p=0.044) affected the clinical pregnancy rate. Furthermore, in patients that received only ICSI, advanced maternal age was associated with a decreased chance and ET number with an increased chance of live births; in patients that did not receive either ICSI or LAH oocyte number was associated with an increased chance of live birth.

Numerous attempts have been made to develop models that predict the success or failure of IVF, though few have been shown to be successful (11). While studies have clearly indicated that factors such as female age and baseline FSH levels are predictive of pregnancy after IVF, it remains difficult for physicians to advise patients on how to proceed after an IVF failure.

ICSI is commonly used to treat male factor infertility and in cases where the sperm cannot penetrate the egg. We have found that in patients that received only ICSI, only ET number was associated with an increased chance of having a live birth. Though ICSI has increased pregnancy and live birth rates in patients undergoing IVF, concerns remain regarding chromosomal abnormalities and some authors consider the procedure over used (19, 20). Tan et al. (21) compared the outcomes of IVF-ET (IVF) and ICSI in non-male infertility patients with low numbers of oocytes retrieved and reported that the rates of fertilization, normal fertilization, complete fertilization failure, cleavage, good embryo, implantation, and clinical pregnancy did not differ between the groups. The authors concluded that ICSI did not improve clinical outcomes in non-male infertility patients with a low number of oocytes retrieved. Hodes-Wertz et al. (22) studied the use of ICSI in couples who previously underwent ICSI at another institution and found that stringent criteria for ICSI did not compromise clinical outcomes and concluded that ICSI was over used.

We found that LAH alone was not associated with an increased live birth rate, but that the use of both ICSI and LAH was associated with an increased live birth rate in cases when female infertility factors other than tubal were not present. While AH and LAH are commonly used, a recent review by Hammadeh et al. (16) observed that routine use of AH was not appropriate as no evidence of a universal benefit existed and the procedure was not without potential risks. Ali et al. (17) reported that LAH was beneficial for women ≤36 years of age, embryos with a thin zona (≤16 μm), and for those with repeated IVF failures. It was not beneficial for women ≥37 years of age or in cases in which the zona was ≥17 μm. Mansour et al. (23) reported a benefit of AH in patients with a poor prognosis such as those with two or more failed IVF cycles, poor embryo quality, and women >38 years of age. Petersen et al. (24) reported that for patients with repeated implantation failures, the implantation rate in those who received laser-thinned embryos was significantly higher (10.9%) than in those whose embryos were not laser-thinned (2.6%). This difference, however, was not seen in patients with only one previous implantation failure. A recent systematic review by Carney et al. (25) examined the effectiveness of AH and concluded that the increased chance of achieving a clinical pregnancy by AH only just reached statistical significance. The data did not support an increase in live birth rate. In our study, LAH did not increase the pregnancy or live birth rates. However, in table 3 LAH did increase the clinical pregnancy rate as related to embryo number. Combined with ICSI, in table 3 the results showed that in cases where infertility of the couple was caused by male factor, the clinical pregnancy rate increased significantly. Thus the use of assistance should be considered according to the special circumstances of each couple.

There are some limitations in this study that should be considered. First, this was a retrospective study, with a heterogeneous patient population. In addition, the numbers of patients in the subgroup that received only LAH was small.

Conclusion

The results of this study indicate that the chance of a live birth in patients undergoing IVF and ICSI and/or LAH vary with the causes of infertility. Oo-

cyte number, maternal age and ET number and female infertility factors other than tubal have affected the live birth rate in patients that did not receive ICSI or LAH, those that received ICSI only and those that received both ICSI and LAH, respectively. No factors affected the live birth rate in patients that received LAH only. These data might assist in advising patients on the appropriateness of ICSI and LAH after failed IVF.

Acknowledgements

No financial support for this study was received from any company or organization. All authors report no conflict of interest related to this study.

References

1. Johansson M, Hellstrom AL, Berg M. Severe male infertility after failed ICSI treatment--a phenomenological study of men's experiences. Reprod Health. 2011; 8: 4.
2. Bleil ME, Pasch LA, Gregorich SE, Millstein SG, Katz PP, Adler NE, et al. Fertility treatment response: is it better to be more optimistic or less pessimistic?. Psychosom Med. 2012; 74(2): 193-199.
3. Johansson M, Adolfsson A, Berg M, Francis J, Hogström L, Janson PO, et al. Gender perspective on quality of life, comparisons between groups 4-5.5 years after unsuccessful or successful IVF treatment. Acta Obstet Gynecol Scand. 2010; 89(5): 683-691.
4. Lee GL, Hui Choi WH, Chan CH, Chan CL, Ng EH. Life after unsuccessful IVF treatment in an assisted reproduction unit: a qualitative analysis of gains through loss among Chinese persons in Hong Kong. Hum Reprod. 2009; 24(8): 1920-1929.
5. Edwards RG. Clinical approaches to increasing uterine receptivity during human implantation. Hum Reprod. 1995; 10 Suppl 2: 60-66.
6. Huisman GJ, Fauser BC, Eijkemans MJ, Pieters MH. Implantation rates after in vitro fertilization and transfer of a maximum of two embryos that have undergone three to five days in culture. Fertil Steril. 2000; 73(1): 117-122.
7. Kyrou D, Kolibianakis EM, Venetis CA, Papanikolaou EG, Bontis J, Tarlatzis BC. How to improve the probability of pregnancy in poor responders undergoing in vitro fertilization: a systematic review and meta-analysis. Fertil Steril. 2009; 91(3): 749-766.
8. van Loendersloot LL, van Wely M, Limpens J, Bossuyt PM, Repping S, van der Veen F. Predictive factors in in vitro fertilization (IVF): a systematic review and meta-analysis. Hum Reprod Update. 2010; 16(6): 577-589.
9. Lien YR, Lu HF, Peng FS, Chiu BC, Yeh SH, Yang YS. Factors associated with failure to achieve birth emphasizing a successful singleton at term following in vitro fertilization. Int J Gynaecol Obstet. 2010; 110(1): 57-60.
10. Min JK, Breheny SA, MacLachlan V, Healy DL. What is the most relevant standard of success in assisted reproduction? The singleton, term gestation, live birth rate per cycle initiated: the BESST endpoint for assisted reproduction. Hum Reprod. 2004; 19(1): 3-7.
11. Leushuis E, van der Steeg JW, Steures P, Bossuyt PM, Eijkemans MJ, van der Veen F, et al. Prediction models in reproductive medicine: a critical appraisal. Hum Reprod Update. 2009; 15(5): 537-552.
12. Hsieh YY, Huang CC, Cheng TC, Chang CC, Tsai HD, Lee MS. Laser-assisted hatching of embryos is better than the chemical method for enhancing the pregnancy rate in women with advanced age. Fertil Steril. 2002; 78(1): 179-182.
13. Tsai CC, Huang FJ, Wang LJ, Lin YJ, Kung FT, Hsieh CH, et al. Clinical outcomes and development of children born after intracytoplasmic sperm injection (ICSI) using extracted testicular sperm or ejaculated extreme severe oligoastheno-teratozoospermia sperm: a comparative study. Fertil Steril. 2011; 96(3): 567-571.
14. Tournaye H. Male factor infertility and ART. Asian J Androl. 2012; 14(1): 103-108.
15. Nyboe Andersen A, Carlsen E, Loft A. Trends in the use of intracytoplasmatic sperm injection marked variability between countries. Hum Reprod Update. 2008; 14(6): 593-604.
16. Hammadeh ME, Fischer-Hammadeh C, Ali KR. Assisted hatching in assisted reproduction: a state of the art. J Assist Reprod Genet. 2011; 28(2): 119-128.
17. Ali J, Rahbar S, Burjaq H, Sultan AM, Al Flamerzi M, Shahata MA. Routine laser assisted hatching results in significantly increased clincal pregnancies. J Assist Reprod Genet. 2003; 20(5): 177-181.
18. Myers ER, McCrory DC, Mills AA, Price TM, Swamy GK, Tantibhedhyangkul J, et al. Effectiveness of assisted reproductive technology (ART). Evid Rep Technol Assess (Full Rep). 2008; 167: 1-195.
19. Merchant R, Gandhi G, Allahbadia GN. In vitro fertilization/intracytoplasmic sperm injection for male infertility. Indian J Urol. 2011; 27(1): 121-132.
20. Verpoest W, Tournaye H. ICSI: hype or hazard?. Hum Fertil (Camb). 2006; 9(2): 81-92.
21. Tan H, Chen L, He L, Quan S. Outcomes of in vitro fertilization-embryo transfer and intracytoplasmic sperm injection for non-male infertility patients with a low number of oocytes retrieved. Nan Fang Yi Ke Da Xue Xue Bao. 2012; 32(2): 251-254.
22. Hodes-Wertz B, Mullin CM, Adler A, Noyes N, Grifo JA, Berkeley AS. Is intracytoplasmic sperm injection overused?. J Urol. 2012; 187(2): 602-606.
23. Mansour RT, Rhodes CA, Aboulghar MA, Serour GI, Kamal A. Transfer of zona-free embryos improves outcome in poor prognosis patients: a prospective randomized controlled study. Hum Reprod. 2000; 15(5): 1061-1064.
24. Petersen CG, Mauri AL, Baruffi RL, Oliveira JB, Massaro FC, Elder K, et al. Implantation failures: success of assisted hatching with quarter-laser zona thinning. Reprod Biomed Online. 2005; 10(2): 224-229.
25. Carney SK, Das S, Blake D, Farquhar C, Seif MM, Nelson L. Assisted hatching on assisted conception (in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI). Cochrane Database Syst Rev. 2012; 12: CD001894.

Original Article

Are Uterine and Ovarian Artery Doppler Velocimetry Values Good Pregnancy Predictors in Clomiphene Citrate Cycles?

Ali Irfan Guzel, M.D.*, Selcuk Erkılınc, M.D., Irfan Ozer, M.D., Aytekin Tokmak, M.D., Ayse Kurt Sahin, M.D., Mustafa Ugur, M.D.

Division of Infertility and Gynecological Endocrinology, Department of Obstetrics and Gynecology, Dr. Zekai Tahir Burak Women's Health Research and Education Hospital, Ankara, Turkey

Abstract

Background: We conducted this prospective study to evaluate the prognostic significance of uterine and ovarian artery Doppler velocimetry in clomiphene citrate (CC) cycles.

Materials and Methods: A total of 80 patients with unexplained infertility were given 100 mg/day of CC from day 3 to day 7 of their cycles in this current prospective study. On cycle day 3, before administration of CC, each patient underwent Doppler transvaginal ultrasonography. The Doppler velocimetries of the right and left uterine and ovarian arteries were recorded and analyzed in association with demographic and clinical parameters.

Results: TheThere were 6 out of 80 patients who became pregnant, the overall pregnancy rate in this population was 7.5% for the current study. The cases were divided into two groups according to whether they became pregnant or not. Demographic characteristics showed no statistically significant differences between these groups ($p>0.05$). However, the duration of infertility did show statistically significant differences between the groups. Doppler velocimetry was not statistically significantly different between the two groups.

Conclusion: Doppler velocimetry of the uterine and ovarian arteries is not a factor in the prognosis for pregnancy in CC cycles.

Keywords: Clomiphene Citrate, Infertility, Uterine Artery, Ovarian, Doppler Velocimetry

Citation: Guzel AI, Erkılınc S, Ozer I, Tokmak A, Kurt Sahin A, Ugur M. Are uterine and ovarian artery doppler velocimetry values good pregnancy predictors in clomiphene citrate cycles?. Int J Fertil Steril. 2015; 9(1): 41-46.

Introduction

Clomiphene citrate (CC) is an agent that has been used since the 1960s for ovulation induction. In appropriately selected anovulatory women, CC successfully induces ovulation in about 70-80% of cases; among these women the overall cycle fecundability is approximately 15% (1). CC is generally used as the first step in treating early stage endometriosis, anovulation that is related to unexplained infertility and borderline male factor infertility with decreased requirements for close monitoring (2). CC acts on the central nervous system as an anti-estrogen and increases the pulse frequency of follicle stimulating hormone (FSH) and luteinizing hormone (LH), in order to induce ovulation. This anti-estrogen effect also has negative impacts on the endometrium and cervical mucus (3, 4).

Factors that affect pregnancy rates in CC cycles have been reported in previous studies. The ages of the women, longer duration times of their infertility and decreased ovarian reserves have been described as factors that indicate poor prognosis for pregnancy in CC cycles (5). The diameter of the leading follicle on the day before human chorionic gonadotropin (hCG) administration and the endometrial thickness (ET) have also been evaluated in relation to the success of CC cycles (6).

Received: 18 Aug 2013, Accepted: 21 Dec 2013
* Corresponding Address: Division of Infertility and Gynecological Endocrinology, Department of Obstetrics and Gynecology, Dr. Zekai Tahir Burak Women's Health Research and Education Hospital, Ankara, Turkey
Email: aliirfanguzel77@gmail.com

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 41-46

In addition, Doppler velocimetry studies have been conducted to evaluate the changes in blood flow in the uterus and ovaries during the normal menstrual cycle (7, 8). Ng et al. (9) reported their findings on the effects of Doppler velocimetry on the blood supply to the entire endometrium and the sub-endometrial region. These authors evaluated the role of endometrial and sub-endometrial vascularity in women who underwent frozen-thawed embryo transfer (FET) cycles and found that neither of these blood flow measures was a good predictor of pregnancy in FET cycles. Nakai et al. (10) also discussed their findings on uterine artery Doppler velocimetry in cycles stimulated by CC. Their study found lower endometrial perfusion.

The current study evaluated the effectiveness of Doppler velocimetries of the uterine and ovarian arteries as a predictor of successful pregnancy in CC cycles.

Materials and Methods

Study design and participants

This interventional, cross-sectional study was undertaken from May 2013 to June 2013 in the Department of Obstetrics and Gynecology, Division of Infertility and Gynecological Endocrinology at Dr. Zekai Tahir Burak Women's Health Education and Research Hospital. This is a tertiary referral research hospital located in the central region of Turkey. It is a government-supported hospital where most of the health services are offered free of charge.

Ethical considerations

The study was designed according to the Helsinki Declaration (11) and all patients gave written informed consent. The study was also approved by the Ethics Research Committee of Dr Zekai Tahir Burak Women's Health Education and Research Hospital (Ankara, Turkey).

Data collection

Women who had had unexplained infertility (primary or secondary) for at least one year were included in the study. A total of 80 women received oral CC, 100 mg daily, from day 3 to day 7 of their menstrual cycles. All of the cases received regular ultrasound monitoring for follicle growth and ET through transvaginal ultrasonography on day 12 of their cycles and again every other day until the leading follicle reached 17-23 mm, triggered by hCG. Transvaginal Doppler velocimetry waveforms were also obtained from the bilateral uterine and ovarian arteries of each patient for analysis. The ultrasound examinations, performed on day 3 of the menstrual cycle while the patients were in the lithotomy position used color Doppler sonography with a 7.5 MHz pulse endovaginal Doppler system for blood flow analysis (Aloka Co., Tokyo, Japan). The women's bladders were emptied before their examinations. The uterine artery was located laterally to the isthmic region of the uterus and the ovarian artery was found at the lateral pole of the ovary near the infundibulopelvic ligament.

The following factors that affected patients' risk levels were recorded: age, infertility type, duration of infertility, gravidy, parity, number of pregnancy losses, number of living children, ET, cycle day 3 serum FSH, LH and estradiol (E_2) levels, uterine and ovarian artery Doppler velocimetries, number of antral follicules, dominant follicle sizes and pregnancy. The Doppler velocimetries measured were the right side uterine and ovarian artery pulsatility index (PI) and resistance index (RI) in addition to the left side uterine and ovarian artery PI and RI.

The cases were divided into two groups according to whether patients achieved pregnancy or not. At the end of the study, a total of 6 (7.5%) patients were pregnant. Pregnancy was determined by positive β-hCG and a visible intrauterine gestational sac.

Statistical analyses

The primary outcome measure was a clinical pregnancy. Continuous variables were not normally distributed and recorded as median (interquartile range) unless otherwise indicated. The normal distribution of the variables was analyzed by the Kolmogorov-Smirnov test. Statistical comparison was carried out by Chi-square (χ^2), Mann-Whitney and independent sample t tests where appropriate. The sample size was determined according to the results of the central limit theorem (12). Statistical analysis was performed using the Statistical Program for Social Sciences (SPSS, Version 15.0, Chicago, IL, USA). The two-tailed value of $p<0.05$ was considered statistically significant.

Results

The demographic and clinical characteristics of the cases are shown in table 1. The study included 80 patients who had had unexplained infertility for at least one year. The pregnancy rate was 7.5%. The mean age of the cases was 26.83 ± 4.95 years for the group that achieved pregnancy and 27.45 ± 5.53 years for the group that did not (p=0.789). There were 54 (67.5%) cases of primary infertility. The median (min-max) gravidy and parity of the cases were 0.41 (0-3) and 0.23 (0-2), respectively. The infertility type (primary or secondary) showed no statistically significant differences between the groups in terms of pregnancy rate (p=0.640). The mean duration of infertility was 2.58 ± 0.80 years for the group that achieved pregnancy and 3.93 ± 2.34 years for the group that did not. These values showed statistically significant differences between the groups (p=0.007). The ET on day 3 of the cycle was 1.83 ± 0.98 mm in the group that achieved pregnancy and 2.37 ± 1.02 mm in group that did not, with no statistically significant differences (p=0.241).

Table 1: The differences in demographic and clinical characteristics among the pregnant and non-pregnant groups

	Pregnant group n=6	**Non-pregnant group** n=74	**P (95% CI)**
Age (Y) $\overline{X} \pm SD$	26.83 ± 4.95	27.45 ± 5.53	0.789 (-5.27- 4.02)
***Gravidy ≤1**	69	6	0.912
***Parity ≤1**	73	6	0.732
Infertility type*			0.964
Primary	4 (66, 66)	50 (67.6)	
Secondary	2 (33, 33)	24 (32.4)	
Duration of infertility (Y)	2.58 ± 0.80	3.93 ± 2.34	0.007 (-3.28-0.57)
ET (mm)	1.83 ± 0.98	2.37 ± 1.02	0.241 (-1.41-0.32)
Antral follicle number	6.66 ± 3.72	6.76 ± 3.15	0.947
Leading follicle (mm)			0.501
Right	16.50 ± 6.44	15.16 ± 7.30	
Left	15.16 ± 4.51	14.77 ± 4.82	
FSH (mIU/ml)	6.47 ± 1.61	6.47 ± 2.19	0.992 (-1.82-1.83)
LH (mIU/ml)	7.19 ± 5.32	5.75 ± 3.73	0.380 (-1.81-4.70)
E_2 (pg/ml)	55.81 ± 18.72	46.34 ± 22.49	0.320 (-9.35-28.28)

CI; Confidence interval, *; P values calculated by χ^2 test, FSH; Follicle stimulating hormone, LH; Luteinizing hormone, E_2; Estradiol and ET; Endometrial thickness.

The differences between the basal blood hormone levels of the FSH, LH and E_2 values were also not statistically significant between the groups (p>0.05). However, the durations of infertility of the two groups showed statistically significant differences (p<0.007). The basal follicle numbers were 6.66 ± 3.72 for the right ovary and 6.75 ± 3.15 for the left ovary in the group that achieved pregnancy and 6.33 ± 4.08 (right ovary) and 6.67 ± 3.17 (left ovary) for the group that did not. The mean diameters of the leading follicle on the day that hCG was administered for the group that achieved pregnancy was 16.50 ± 6.44 mm and for the group that did not achieve pregnancy it was 15.16 ± 4.51 mm, for the right ovary. For the left ovary, this value was 15.16 ± 7.30 mm in the group that achieved pregnancy versus 14.77 ± 4.82 mm in the group that did not achieve pregnancy (p>0.05).

The measurement of Doppler velocimetry on day 3 of the cycle was as follows: the right side uterine artery mean (min-max) was PI: 3.33 ± 2.40, RI: 1.14 ± 0.59, the right side ovarian artery was PI: 2.30 ± 1.97, RI: 1.11 ± 0.87; the left side uterine artery was PI: 3.11 ± 1.91, RI: 1.21 ± 0.64 and the left side ovarian artery was PI: 3.29 ± 2.31, RI: 1.10 ± 0.72.

Table 2 shows the Doppler velocimetry values of the two groups. There were no statistically significant differences between the groups (p>0.05).

Table 2: Doppler velocimetries of uterine and ovarian arteries among the pregnant and non-pregnant groups

	Pregnant group n=6	Non-pregnant group n=74	P (95% CI)
Right uterine artery PI	2.46 ± 0.66	3.40 ± 2.48	0.294 (-2.98-1.08)
Right uterine artery RI	0.84 ± 1.16	0.85 ± 0.60	0.155 (-0.82-0.16)
Right ovarian artery PI	2.91 ± 1.83	2.30 ± 1.99	0.985 (-1.63-1.71)
Right ovarian artery RI	1.21 ± 0.56	1.11 ± 0.90	0.420 (-0.63-0.85)
Left uterine artery PI	2.59 ± 0.94	3.16 ± 1.97	0.406 (-2.18-1.06)
Left uterine artery RI	1.04 ± 0.20	1.23 ± 0.66	0.883 (-0.73-0.35)
Left ovarian artery PI	3.29 ± 1.87	3.29 ± 2.35	0.763 (-2.02-2.01)
Left ovarian artery RI	0.97 ± 1.12	0.98 ± 0.70	0.985 (-0.77-0.48)

CI; Confidence interval, PI; Pulsatility index and RI; Resistance index.

Discussion

In this study we examined 80 women who had unexplained infertility for at least one year with the intent to evaluate the uterine and ovarian artery Doppler velocimetries as predictors of a successfully achieved pregnancy. The patients received oral CC, 100 mg daily, from day 3 to day 7 of their menstrual cycles. At the end of the study, 6 (7.5%) of the patients had achieved pregnancy. We divided the cases into two groups based on whether the patient had achieved pregnancy or not and found that the demographic and clinical parameters showed no statistically significant differences between the groups. However, the differences between the groups in terms of the durations of infertility were statistically significant. The Doppler velocimetry values also showed no statistically significant differences between the groups.

Doppler velocimetry of the uterine, spiral and ovarian arteries has been previously investigated in different types of cases, various phases of the menstrual cycle, ovulation induction and in early pregnancy. First, Kupesic and Kurjak (13) reported that blood flow in the uterine, spiral and ovarian arteries has a predictive value in endometrial receptivity. Bassil et al. (14) have also demonstrated that the RI values of the ovarian artery are a good indicator of ovarian inhibition and may be used to assess the optimal timing for the beginning of human menopausal gonadotrophin. In addition, Dickey et al. (15) have found impedance of the spiral artery to be a predictive parameter in the early development of the embryo. Based on these findings, we decided to conduct a study to evaluate the Doppler velocimetry in CC cycles.

Nakai et al. (10) studied the Doppler velocimetry values for spontaneous and stimulated menstrual cycles in women with unexplained infertility and found that the uterine blood flow was higher in spontaneous cycles than in cycles stimulated with CC.

In addition, Ragni et al. (16) evaluated 318 cases that received mild controlled ovarian stimulation (COS) and intrauterine insemination (IUI). They assessed the vascularity of the dominant follicle by Doppler ultrasound. These authors reported that Doppler velocimetry of follicular vascularity did not predict the chances for pregnancy in COS and IUI cycles. The findings of the current study were similar to this study; we have also believed that Doppler velocimetry of the uterine and ovarian arteries are not good predictors for achieving pregnancy.

In a previous study, Ng et al. (17) reported that the Doppler velocimetry values of uterine and ovarian arteries showed no differences between the early follicular phase and an ensuing CC challenge test.

Baruah et al. (18) designed a study with COS patients using letrozole or CC and studied the Doppler velocimetry of the spiral arteries. The study found that the letrozole group showed significantly lower impedance than the CC group and determined that the Doppler velocimetry of spiral arteries was a supportive tool for evaluating endometrial response in the treatment of anovulatory polycystic ovary syndrome patients with infertility.

Conclusion

We believe that Doppler velocimetries of the uterine and ovarian arteries are not prognostic factors for pregnancy in CC cycles.

Acknowledgements

We would like to thank all of the women who participated in this research. We declare we have no conflict of interest.

References

1. Fritz MA, Speroff L. Induction of ovulation. Clinical gynecological endocrinology and infertility. 8th ed. Philadelphia: Lippincott Williams and Wilkins; 2011; 1293-1330.
2. Bayar U, Tanriverdi HA, Barut A, Ayoğlu F, Ozcan O, Kaya E. Letrozole vs. clomiphene citrate in patients with ovulatory infertility. Fertil Steril. 2006; 85(4): 1045-1048.
3. Al-Fozan H, Al-Khadouri M, Tan SL, Tulandi T. A randomized trial of letrozole versus clomiphene citrate in women undergoing superovulation. Fertil Steril. 2004; 82(6): 1561-1563.
4. Elkattan EA. Ultrasonographic markers of endometrial receptivity of letrozole and clomiphene citrate in unexplained infertile women. Middle East Fertil Soc J. 2013; 18: 84-88.
5. Houmard BS, Juang MP, Soules MR, Fujimoto VY. Factors influencing pregnancy rates with a combined clomiphene citrate/gonadotropin protocol for non-assisted reproductive technology fertility treatment. Fertil Steril. 2002; 77(2): 384-386.
6. Palatnik A, Strawn E, Szabo A, Robb P. What is the optimal follicular size before triggering ovulation in intrauterine insemination cycles with clomiphene citrate or letrozole? An analysis of 988 cycles. Fertil Steril. 2012; 97(5): 1089-1094.e1-3.

7. Sladkevicius P, Valentin L, Marsal K. Blood flow velocity in the uterine and ovarian arteries during the normal menstrual cycle. Ultrasound Obstet Gynecol. 1993; 3(3): 199-208.
8. Tan SL, Zaidi J, Campbell S, Doyle P, Collins W. Blood flow changes in the ovarian and uterine arteries during the normal menstrual cycle. Am J Obstet Gynecol. 1996; 175(3 Pt 1): 625–631.
9. Ng EH, Chan CC, Tang OS, Yeung WS, Ho PC. The role of endometrial and subendometrial vascularity measured by three-dimensional power Doppler ultrasound in the prediction of pregnancy during frozen-thawed embryo transfer cycles. Hum Reprod. 2006; 21(6): 1612-1617.
10. Nakai A, Yokota A, Koshino T, Araki T. Assessment of endometrial perfusion with Doppler ultrasound in spontaneous and stimulated menstrual cycles. J Nippon Med Sch. 2002; 69(4): 328-332.
11. World Medical Association. The World Medical Association, Decleration of Helsinki: ethical principles for medical research involving human subjects. JAMA. 2013; 310(20): 2191-2194.
12. Celik Y. Biostatistics, principles of research. Diyarbakir: Dicle University Press; 2007.
13. Kupesic S, Kurjak A. Uterine and ovarian perfusion during the periovulatory period assessed by transvaginal color Doppler. Fertil Steril. 1993; 60(3): 439-443.
14. Bassil S, Wyns C, Toussaint-Demylle D, Nisolle M, Gordts S, Donnez J. The relationship between ovarian vascularity and the duration of stimulation in in-vitro fertilization. Hum Reprod. 1997; 12(6): 1240-1245.
15. Dickey RP, Gasser RF, Hower JF, Matulich EM, Brown GT. Relationship of uterine blood flow to chorionic sac and embryo growth rates. Hum Reprod. 1995; 10(10): 2676-2679.
16. Ragni G, Anselmino M, Nicolosi AE, Brambilla ME, Calanna G, Somigliana E. Follicular vascularity is not predictive of pregnancy outcome in mild controlled ovarian stimulation and IUI cycles. Hum Reprod. 2007; 22(1): 210-214.
17. Ng EH, Tang OS, Chan CC, Ho PC. Ovarian stromal blood flow following clomiphene citrate challenge test in infertile women. J Clin Ultrasound. 2008; 36(7): 403-408.
18. Baruah J, Roy KK, Rahman SM, Kumar S, Sharma JB, Karmakar D. Endometrial effects of letrozole and clomiphene citrate in women with polycystic ovary syndrome using spiral artery Doppler. Arch Gynecol Obstet. 2009; 279(3): 311-314.

Original Article

Chromosomal Analysis of Couples with Repeated Spontaneous Abortions in Northeastern Iran

Saeedeh Ghazaey, M.Sc.[1, 2], Fatemeh Keify, M.Sc.[2], Farzaneh Mirzaei, M.Sc.[2], Masumeh Maleki, M.Sc.[2], Semiramis Tootian, B.Sc.[2], Mitra Ahadian, B.Sc.[2], Mohammad Reza Abbaszadegan, Ph.D.[2, 3*]

1. Medical Genetics Research Center, Medical School, Mashhad University of Medical Sciences, Mashhad, Iran
2. Pardis Clinical and Genetics Laboratory, Mashhad, Iran
3. Division of Human Genetics, Immunology Research Center, Avicenna Research Institute, Mashhad University of Medical Sciences, Mashhad, Iran

Abstract

Background: Cytogenetic study of reproductive wastage is an important aspect in determining the genetic background of early embryogenesis. Approximately 15 to 20% of all pregnancies in humans are terminated as recurrent spontaneous abortions (RSAs). The aim of this study was to detect chromosome abnormalities in couples with RSAs and to compare our results with those reported previously.

Materials and Methods: In this retrospective study, the pattern of chromosomal aberrations was evaluated during a six-year period from 2005 to 2011. The population under study was 728 couples who attended genetic counseling services for their RSAs at Pardis Clinical and Genetics Laboratory, Mashhad, Iran.

Results: In this study, about 11.7% of couples were carriers of chromosomal aberrations. The majority of abnormalities were found in couples with history of abortion, without stillbirth or livebirth. Balanced reciprocal translocations, Robertsonian translocations, inversions and sex chromosome aneuploidy were seen in these cases. Balanced reciprocal translocations were the most frequent chromosomal anomalies (62.7%) detected in current study.

Conclusion: These findings suggest that chromosomal abnormalities can be one of the important causes of RSAs. In addition, cytogenetic study of families who experienced RSAs may prevent unnecessary treatment if RSA are caused by chromosomal abnormalities. The results of cytogenetic studies of RSA cases will provide a standard protocol for the genetic counselors in order to follow up and to help these families.

Keywords: Chromosomal Abnormalities, Abortions, Cytogenetic Analysis

Citation: Ghazaey S, Keify F, Mirzaei F, Maleki M, Tootian S, Ahadian M, Abbaszadegan MR. Chromosomal analysis of couples with repeated spontaneous abortions in northeastern Iran. Int J Fertil Steril. 2015; 9(1): 47-54.

Introduction

Approximately 15 to 20% of all pregnancies in humans result in recurrent spontaneous abortions (RSAs) (1). There are different reasons for RSAs including genetic abnormalities, maternal and paternal age, endocrine dysfunction, autoimmune disorders, infectious diseases, environmental toxins and congenital or structural uterine anomalies (2). Chromosomal unbalance have important role in abnormal early human development. Nearly, 50 to 60% of first-trimester spontaneous miscarriages have abnormal karyotype (3). Although the frequency of chromosomal abnormalities in couples with RSA varies between populations, it has been found higher frequency in the general population (0.3-0.4%) (4, 5). Therefore, cytogenetic study of the parent with history of RSAs is an integral part of diagnostic clarification. Several cytogenetic

Received: 13 Apr 2013, Accepted: 28 Jan 2014
* Corresponding Address: P.O. Box: 9196773117, Division of Human Genetics, Immunology Research Center, Avicenna Research Institute, Mashhad University of Medical Sciences, Mashhad, Iran
Email: abbaszadeganmr@mums.ac.ir

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 47-54

investigations have been performed in various countries to determine the pattern of chromosome abnormalities in parents with fetal wastage. The studies revealed that the prevalence of chromosomal anomalies varies from 2 to 8% in couples who are affected by RSAs (6). Unequal crossing over during meiosis can lead to chromosomal rearrangements producing gametes with unbalanced chromosomal aberrations like duplications or deletions, therefore, structural chromosome abnormalities in parents can be the major cause of recurrent miscarriages (7). The clinical outcomes of such unbalances generally are lethal to the developing embryo, leading to RSAs or early neonatal deaths (8).

The objective of the current study was to determine the prevalence and types of chromosomal anomalies in couples living in Northeast of Iran, to compare our findings with those reported previously and to increase the awareness of physicians and gynecologists about the frequency and nature of chromosomal aberrations that contribute to recurrent miscarriages.

Materials and Methods

This retrospective study done over a 6-year period from 2005 to 2011 included 728 couples with history of abortions ranged 1-7 who were referred to the Genetic Counseling Services in Pardis Clinical and Genetics Laboratory (PCGL), Mashhad, Iran. All patients gave a signed informed consent and the study was approved by Ethics Committee of PCGL.

All the referred couples were thoroughly examined, and detailed clinical and obstetric histories were recorded in prepared forms. The age of the couples, number of RSA and the possible existence of other causes for the abortion such as uterine malformations, hormonal insufficiency, and previously induced abortion(s) were investigated.

For conventional cytogenetic study, 5 ml peripheral blood from each subject was collected into heparinized test tubes. Lymphocyte cultures were initiated according to Moorhead et al. (9). Next 400 µl whole blood cells were cultured in 5 ml RPMI 1640 medium (Gibco, USA), supplemented with 20% (v/v) fetal bovine serum (FBS, Gibco, USA) and 10 µg/ml phytohaemagglutinin (Gibco, USA) at 37˚C for 72 hours. Cultured cells were harvested by adding colcemid (Gibco, USA) for 10 minutes followed by treatment of hypotonic solution (0.075 M KCl, Merck, Germany) for 15 minutes, and the treated cells were then fixed using Carnoy's fixative (3:1 methanol-glacial acetic acid; Merck, Germany). The karyotype of the couples was prepared using G-banding technique with trypsin and Giemsa staining (GTG) (10) and C-banding technique with barium hydroxide (11). Images of well-banded metaphases were obtained using olympus photomicroscope (BX-40, Japan) and were analyzed by CytoVision software (Applied Imaging, USA) at 400-550 band resolution. Karyotyping of 30 metaphases was performed routinely, while in cases of mosaicism, 100 metaphase spreads were analyzed. Karyotyping of each couple was carried out according to the International System for Human Cytogenetics Nomenclature (ISCN) 2009 (12).

Results

Couples' ages ranged from 18 to 45, with a mean of 29.6. As mentioned in table 1, 11.7% showed abnormal karyotype. In 728 couples, we determined that 48 women and 37 men had chromosomal aberrations. Among chromosomal abnormalities, 52 structural and 7 numerical anomalies were detected. In addition, there were 27 cases with three types of polymorphic variants including constitutional fragility of chromosome 16, pericentric inversion of chromosome 9, and prominent satellites in chromosomes 13 and 15. There were two instances where both members of a couple had an abnormal karyotype (couples no.: 11, 12, 17, and 18, mentioned in Table 1).

Table 2 shows the distribution of couples according to the number of spontaneous abortions. As mentioned, 51.6% had two miscarriages and 14.6% had only one miscarriage. The remaining (33.8%) had 3 or more miscarriages. The structural chromosomal abnormalities we encountered were divided into balanced reciprocal chromosomal translocations (37/85), Robertsonian translocation (8/85) and inversions (7/85). Reciprocal translocations were the most prevalent abnormality. Inversions, marker chromosomes and Robertsonian translocations were seen with a trend of decreasing percentage, respectively. The highest percentage of chromosomal aberration was seen in couples with five or more RSAs.

Table 1: Cytogenetic study, number of abortions, and parental age in cases with structural abnormalities

	Karyotypes	No of cases	Age	No of abortions
	Robertsonian translocations			
1	45,XY,t(15;15)(q10;q10)	1	27	2
2	45,XX,t(13;14)(q10;q10)	2	30, 35	2, 2
3	45,XY,t(13;14)(q10;q10)	3	22, 25, 37	2, 2, 3
4	45,XX,t(14;15)(q10;q10)	2	27, 32	2, 3
	Reciprocal translocations			
1	46,XX,t(2;15)(q25;q26.1)	1	28	2
2	46,XX,t(3;6)(q29;p21.1)	1	31	3
3	46,XX,t(1;3)(q22.2;q25.2)	1	27	2
4	46,XY,t(7;18)(p21.3;q12.2)	1	41	4
5	46,XX,t(4;7)(q34.3;q21.3)	2	37, 42	4, 5
6	46,XX,t(7;14)(q36;q24.3)	1	24	3
7	46,XX,t(12;22)(q10;q10)	2	30, 33	3, 4
8	46,XY,t(12;22)(p11.2;p11.2)	1	34	2
9	46,XY,t(6;10)(p25;p11.2)	1	36	3
10	46,XX,t(10;21)(p21.1;q22.2)	1	25	2
11	46,XY,t(6;16)(q26;p12)	1	34	4
12	46,XX,t(6;16)(q26;p12)	1	26	4
13	46,XY,t(9;17)(q22.1;p13.1)	1	26	2
14	46,XY,t(4;20)(q32;p12)	1	34	3
15	46,XX,t(8;17)(q24.3;q21)	1	27	2
16	46,XX,t(3;7)(q22;q32)	1	34	3
17	46,XY,t(11;22)(q23;q11)	2	29, 39	2, 5
18	46,XX,t(11;22)(q23;q11)	1	26	2
19	46,XX,t(15;20)(p10;p10)	1	29	2
20	46,XY,t(8;11)(p23;q21)	1	45	5
21	46,XX,t(8;11)(p23;q21)	1	27	4
22	46,XY,t(16;22)(q23;q12)	1	27	2
23	46,XX,t(2;18)(p21;q11.2)	1	24	2

	Karyotypes	No of cases	Age	No of abortions
24	46,XX,t(8;10)(q13;q22.2)	1	31	3
25	46,XX,t(13;20)(q22;p13)	2	18, 22	1, 2
26	46,XY,t(4;5)(q25;p15.2)	1	42	4
27	46,XX,t(5;6)(q34;p21.2)	1	27	2
28	46,XX,t(2;7)(q34;q34)	1	29	3
29	46,XX,t(2;7)(q37.1;q32)	1	37	4
30	46,XY,t(6;8)(p23;q12.2)	1	40	5
31	46,XX,t(10;12)(q23.2;q21.3)	1	29	1
32	46,XY,t(4;6)(q23;q21)	1	35	3
33	46,XX,t(10;17)(p13;q21.3)	1	36	7
	Pericentric inversions			
1	46,XX,inv(5)(p15.3q15)	2	27, 37	2, 2
2	46,XY,inv(10)(p14q21)	2	30, 33	2, 2
3	46,X,inv(Y)(p11.2q11.22)	3	24, 33, 41	2, 2, 4
	Numerical abnormalities			
1	47,XYY	1	41	4
2	47,XXY/46,XY	2	27, 36	1, 2
3	45,X/46,XX/47,XXX	3	22, 24, 32	2, 2, 3
4	47,XXX	1	31	2
	Polymorphic variants			
1	46,XY,inv(9)(p11q13)	11	18, 36	2, 5
2	46,XX,inv(9)(p11q13)	9	20, 42	2, 7
3	46,XX, Frag16q21	2	25, 31	2, 3
4	46,XX/46,XX, Frag 16q21	1	28	2
	$13p^+$	2	28, 31	1, 2
	$15p^+$	1	20	1
	Total	85 (11.7%)		

t; Translocation, inv; Pericentric inversion, Frag; Constitutional fragility and p^+; Prominent satellite.

Table 2: Distribution of chromosomal abnormalities according to the number of spontaneous abortions

	No. of RSAs					
	1	**2**	**3**	**4**	**≥5**	**Total**
No. of couples	106	376	153	60	33	728
rob (no.)	-	6	2	-	-	7
rcp	2	13	9	8	5	37
inv	-	9	8	1	3	21
mar	3	13	2	1	-	19
% (couples)	4.7	11	15	15	21.2	11.7

RSAs; Recurrent spontaneous abortions, rob; Robertsonian translocation, rcp; Reciprocal translocation, inv; Pericentric inversion and mar; Supernumerary marker chromosome.

Discussion

The prevalence of chromosomal aberrations among PCGL referral couples was 11.7%, which is similar to previous reports from Iran (13, 14) and greater than reported by other authors (Table 3) (13-19). The variable prevalence in several studies might be related to the different sample size and variable criteria used for investigation of cases. It is also quite possible that selective populations vary in the incidence of carriers of chromosomal aberrations (20).

The ratio of abnormal female-to-male (1.3:1) was not different from that found in most other studies in Iran (13, 14) and other countries (15-17). A possible explanation for this difference is that chromosomal aberrations such as autosomal reciprocal translocations in male carriers may cause severe meiotic disturbances and spermatogenic arrest, but oogenesis usually is conserved and results in production of gametes with a high risk of presenting unbalanced chromosomal abnormalities (21).

Among couples, 25.4% (185/728) had a subsequent successful pregnancy outcome, which is nearly similar to the report by Pal et al. (15) in Malaysian couples and in contrast to a previous study where the incidence of a successful pregnancy outcome in couples who had miscarriages has been reported to be nearly 70 % (22). According to our results, the highest percentages of abnormal karyotypes were related to the couples experiencing recurrent miscarriages without stillbirth or live birth outcome, 58/85, 68.2%. Majority of cases with RSA had only one parent with chromosomal aberration.

Reciprocal translocations were the most frequent chromosomal anomalies, 37/85, 43.5% detected in the current study as has also been reported in other studies (21). In the present study, there were more subsequent miscarriages among carriers of translocation, compared to chromosomally-normal couples. Sugiura-Ogasawara et al. (23) predicted a poorer prognosis in carriers of translocation, with a higher rate of subsequent miscarriages and lower rates of viable pregnancies. One couple in the current study included a 34-years old man and his 26-year wife (cases no. 11 and 12, mentioned in table 1) with consanguineous marriage who had four miscarriages. This couple had a balance translocation between the long arm of chromosome 6 and short arm of chromosome 16 [46,XX(Y),t(6;16)(q26;p12)] (Fig.1). Unfor-

tunately, chromosomal analysis of families of the couple was unknown. They went through an extensive genetic counseling, and prenatal diagnosis was also strongly recommended, because there is a 50% chance of unbalanced translocation that will be inherited in every future generation from this family (24).

As we confirmed, numerical chromosomal aberrations are less frequent among abnormal couples with recurrent abortions, 7/85, 8.2%. This type of aberrations are usually in the form of sex chromosomal aneuploidy, and they occur in a low frequency (<0.15% of cases) (8). The current study showed that the incidence and distribution of chromosomal abnormalities among Iranian couples with recurrent abortions are comparable to that reported worldwide. The prevalence and type of chromosomal abnormalities is similar to that seen in other reports. Table 3 shows the similarity of distribution of structural chromosomal rearrangements in our study to that reported worldwide (14-19).

The role of polymorphic variants of chromosomes in RSAs has not yet verified. Autosomal constitutional fragility of a particular chromosome site results in frequent breakages of this point, but their role in the causation of miscarriages is very difficult to assess due to lack of reliable data for their frequencies in normal populations, indicting to be estimated very low (25). Pericentric inversions with breakpoints comparatively close to the centromere produce large duplication deficiencies, i.e. severely unbalanced gametes (26). It is not evident if this inversion is related to pregnancy loss; however, there are studies about association of inversion 9 with subfertility, recurrent abortions and abnormal phenotypes (27). The correlation between prominent satellite and recurrent abortion is unknown (28).

Table 3: Distribution of chromosomal rearrangements in Iran and other countries

Country	Authors	Chromosomal abnormalities %	No. of couples	Abnormal cases	rob %	rcp %	inv %	mar %
Iran (Mashhad)	Current study	11.7	728	85	9.4	43.5	31.8	15.3
Iran (Tehran)	Nirumanesh et al. (14)	12	100	13	23	30.7	30.7	15.5
Malaysia	Pal et al. (15)	8.9	56	5	20	60	-	20
Pakistan	Azim et al. (18)	5.3	300	16	12.5	31.2	31.3	25
France	Turleau et al. (17)	4.6	413	27	20	36	28	16
Saudi Arabia	Al Husain et al. (19)	6.7	193	15	6.7	66.7	13.3	6.7

Rob; Robertsonian translocation, rcp; Reciprocal translocation, inv; Pericentric inversion and mar; Supernumerary marker chromosome.

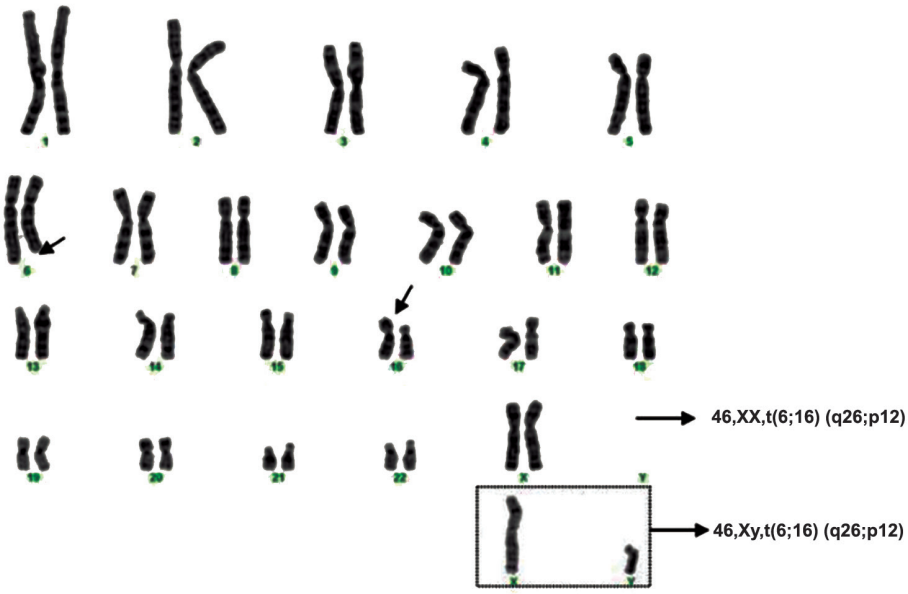


Fig.1: Karyotyping of a couple with balanced translocation between chromosomes 6 and 16. 46,XX/XY,t(6;16)(q26;p12). t; Translocation.

Conclusion

Present study confirmed that chromosomal abnormalities are common in Iranian couples having recurrent miscarriages. We discussed the significance of balance translocation, sex chromosome aneuploidy, and inversion in couples with RSA. These data would be useful for the physicians and gynecologists for better management of the couples with chromosomal aberrations that lead to their recurrent miscarriages. Therefore, it would be reasonable to recommend chromosome analysis to these couples.

Acknowledgements

This research was supported and funding by Pardis Clinical and Genetics Laboratory. All of authors declare that they have no conflict of interest.

References

1. Steer C, Campbell S, Davies M, Mason B, Collins W. Spontaneous abortion rates after natural and assisted conception. BMJ. 1989; 299(6711): 1317-1318.
2. Stephenson MD. Frequency of factors associated with habitual abortion in 197 couples. Fertil Steril. 1996; 66(1): 24-29.
3. Hassold TJ. A cytogenetic study of repeated spontaneous abortions. Am J Hum Genet. 1980; 32(5): 723-730.
4. Kajii T, Ferrier A. Cytogenetics of aborters and abortuses. Am J Obstet Gynecol. 1978; 131(1): 33-38.
5. Papp Z, Gardo S, Dolhay B. Chromosome study of couples with repeated spontaneous abortions. Fertil Steril. 1974; 25(8): 713-717.
6. Dutta UR, Rajitha P, Pidugu VK, Dalal AB. Cytogenetic abnormalities in 1162 couples with recurrent miscarriages in southern region of India: report and review. J Assist Reprod Genet. 2011; 28(2): 145-149.
7. Campana M, Serra A, Neri G. Role of chromosome aberrations in recurrent abortion: a study of 269 balanced translocations. Am J Med Genet. 1986; 24(2): 341-356.
8. Rao L, Murthy K, Babu A, Venkata P, Deenadayal M, Singh L. Chromosome inversions and a novel chromosome insertion associated with recurrent miscarriages in South India. Arch Gynecol Obstet. 2005; 272(4): 273-277.
9. Moorhead PS, Nowell PC, Mellman WJ, Battips DM, Hungerford DA. Chromosome preparation of leukocytes cultured from human peripheral blood. Exp Cell Res. 1960; 20: 613-616.
10. Seabright M. A rapid banding technique for human chromosomes. Lancet. 1971; 2(7731): 971-972.
11. Salamanca F, Armendares S. C bands in human metaphase chromosomes treated by barium hydroxide. Ann Genet. 1974; 17(2): 135-136.
12. Shaffer LG, Slovak ML, Campbell LJ, editors. ISCN 2009: An International System for Human Cytogenetic Nomenclature. Basel: S. Karger; 2009.
13. Firoozabadi RD, Klantar M, Seyed-Hasani M, Ghasemi N, Asgharnia M, Sheikhha MH. Cytogenetic analysis in couples with recurrent spontaneous abortion. Iran J Reprod Med. 2006; 4(1):13-17.
14. Niroumanesh S, Mehdipour P, Farajpour A, Darvish S. A

cytogenetic study of couples with repeated spontaneous abortions. Ann Saudi Med. 2011; 31(1): 77-79.
15. Pal S, Ma SO, Norhasimah M, Suhaida MA, Siti Mariam I, Ankathil R, et al. Chromosomal abnormalities and reproductive outcome in Malaysian couples with miscarriages. Singapore Med J. 2009; 50(10): 1008-1012.
16. Goud TM, Mohammed Al Harassi S, Khalfan Al Salmani K, Mohammed Al Busaidy S, Rajab A. Cytogenetic studies in couples with recurrent miscarriage in the Sultanate of Oman. Reprod Biomed Online. 2009; 18(3): 424-429.
17. Turleau C, Chavin-Colin F, de Grouchy J. Cytogenetic investigation in 413 couples with spontaneous abortions. Eur J Obestet Gynecol Repod Biol. 1979; 9(2); 65-74.
18. Azim M, Khan AH, Khilji ZL, Pal J A, Khurshid M. Chromosomal abnormalities as a cause of recurrent abortions: a hospital experience. J Pak Med Assoc. 2003; 53(3): 117-119.
19. Al-Hussain M, Al-Nuaim L, Abu Talib Z, Zaki OK. Cytogenetic study in cases with recurrent abortion in Saudi Arabia. Ann Saudi Med. 2000; 20(3-4): 233-236.
20. Stephenson MD, Awartani KA, Robinson WP. Cytogenetic analysis of miscarriages from couples with recurrent miscarriage: a case-control study. Hum Reprod. 2002; 17(2): 446-451.
21. Elghezal H, Hidar S, Mougou S, Khairi H, Saad A. Prevalence of chromosomal abnormalities in couples with recurrent miscarriage. Fertil Steril. 2007; 88(3): 721-723.
22. FitzSimmons J, Jackson D, Wapner R, Jackson L. Subsequent reproductive outcome in couples with repeated pregnancy loss. Am J Med Genet. 1983; 16(4): 583-587.
23. Sugiura-Ogasawara M. Reciprocal translocation carriers in recurrent miscarriage parents may yield an unbalanced fetal chromosome pattern. Hum Reprod. 2004; 19(9): 2171-2172.
24. Keify F, Zhiyan N, Mirzaei F, Tootian S, Ghazaey S, Abbaszadegan MR. Two novel familial balanced translocations t(8;11)(p23;q21) and t(6;16)(q26;p12) implicated in recurrent spontaneous abortion. Arch Iran Med. 2012; 152(4): 249-252.
25. Giraud F, Ayme S, Mattei JF, Mattei MG. Constitutional chromosomal breakage. Hum Genet. 1976; 34(2):125-136.
26. Fujimoto A, Towner JW, Turkel SB, Wilson MG. A fetus with recombinant of chromosome 8 inherited from her carrier father. Hum Genet. 1978; 40(3): 241-248.
27. Uehara S, Akai Y, Takeyama Y, Takabayashi T, Okamura K, Yajima A. Pericentric inversion of chromosome 9 in prenatal diagnosis and infertility. Tohoku J Exp Med. 1992; 166(4): 417-427.
28. Ward BE, Henry GP, Robinson A. Cytogenetic studies in 100 couples with recurrent spontaneous abortions. Am J Hum Genet. 1980; 32(4): 549-554.

Original Article

mRNA Expression of *VEGF* and Its' Receptors in Fallopian Tubes of Women with Ectopic Pregnancies

Nafise Zarezade, M.Sc.[1, 2#], Samane Saboori Darabi, M.Sc.[1, 2#], Fariba Ramezanali, M.D.[1], Elham Amirchaghmaghi, M.D., Ph.D.[1], Gholamreza Khalili, M.D.[3], Ashraf Moini, M.D.[1, 4, 5*], Reza Aflatoonian, M.D., Ph.D.[1*]

1. Department of Endocrinology and Female Infertility at Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
2. Department of Biochemistry, Payame Noor University, Tehran, Iran
3. Department of Epidemiology and Reproductive Health at Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
4. Department of Gynecology and Obstetrics, Arash Women's Hospital, Tehran University of Medical Sciences, Tehran, Iran
5. Vali-e-Asr Reproductive Health Research Center, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Background: Establishment of viable pregnancy requires embryo implantation and placentation. Ectopic pregnancy (EP) is a pregnancy complication which occurs when an embryo implants outside of the uterine cavity, most often in a fallopian tube. On the other hand, an important aspect of successful implantation is angiogenesis. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor responsible for vascular development that acts through its receptors, VEGF receptor 1 (VEGFR1) and VEGFR2. This study aims to investigate mRNA expression of *VEGF* and its receptors in fallopian tubes of women who have EP compared with fallopian tubes of pseudo-pregnant women. We hypothesize that expression of *VEGF* and its receptors in human fallopian tubes may change during EP.

Materials and Methods: This was a case-control study. The case group consisted of women who underwent salpingectomy because of EP. The control group consisted of women with normal fallopian tubes that underwent hysterectomies. Prior to tubal sampling, each control group subject received an injection of human chorionic gonadotropin (hCG) to produce a state of pseudo-pregnancy. Fallopian tubes from both groups were procured. We investigated *VEGF, VEGFR1* and *VEGFR2* mRNA expressions in different sections of these tubes (infundibulum, ampulla and isthmus) by reverse transcription polymerase chain reaction (RT-PCR) and quantitative PCR (Q-PCR).

Results: RT-PCR showed expressions of these genes in all sections of the fallopian tubes in both groups. Q-PCR analysis revealed that expressions of *VEGF, VEGFR1* and *VEGFR2* were lower in all sections of the fallopian tubes from the case group compared to the controls. Only *VEGFR2* had higher expression in the ampulla of the case group.

Conclusion: Decreased expressions of *VEGF, VEGFR1* and *VEGFR2* in the EP group may have a role in the pathogenesis of embryo implantation in fallopian tubes.

Keywords: Ectopic Pregnancy, Fallopian Tube, *VEGF Receptor-1, VEGF Receptor-2*, Gene Expression

Citation: Zarezade N, Saboori Darabi S, Ramezanali F, Amirchaghmaghi E, Khalili G, Moini A, Aflatoonian R. mRNA expression of VEGF and its' receptors in fallopian tubes of women with ectopic pregnancies. Int J Fertil Steril. 2015; 9(1): 55-64.

Received: 19 Dec 2013, Accepted: 11 Sep 2014

* Corresponding Address: P.O. Box: 16635-148, Department of Endocrinology and Female Infertility at Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
Emails: r.aflatoonian@gmail.com, a_moini@royaninstitute.org

# The first two authors contributed equally to this work.

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 55-64

Introduction

An ectopic pregnancy (EP) is a pregnancy complication which occurs when an embryo implants anywhere other than the intrauterine cavity. EP is a major cause of maternal morbidity and mortality in the first trimester of pregnancy (1-3) and its incidence has increased in the last two decades (4). Several risk factors for EP have been identified thus far which include pelvic inflammatory disease (PID) following sexually transmitted infections (STDs), damage and infection of the fallopian tubes, endometriosis, history of tubal surgery and previous EP. More than 95% of EPs occur in the fallopian tubes (tubal pregnancy) (5). The majority are located in the ampulla (80%), followed by the isthmus and infundibulum, respectively (6).

Establishment of a viable pregnancy requires implantation and placentation, both important and critical processes (7). An important aspect of successful implantation is the organization of angiogenesis which is mediated by a number of growth factors (8). Vascular endothelial growth factor (VEGF) is a potent angiogenic factor produced by different tissues of the female reproductive tract including the endometrium, ovaries (9, 10), trophoblast and corpus luteum (11). VEGF is a major modulator of vascular growth, remodeling and permeability in the endometrium, decidua, and trophoblast. It is responsible for vascular development in the embryo (11-15). In addition, VEGF stimulates endothelial cell proliferation, promotes cell migration and inhibits apoptosis. In mammals there are five isoforms of the VEGF family: VEGFA, B, C, D and placental growth factor (PLGF) (16). These isoforms are produced as a result of alternative splicing from VEGF mRNA (17). VEGFs act on endothelial cells through their receptors. These receptors include three receptor protein-tyrosine kinases (VEGFR1, VEGFR2 and VEGFR3) and two non-protein kinase receptors (neuropilin-1 and -2) (18). Different cytokines, growth factors and gonadotropins modulate VEGF expression. Factors such as fibroblast growth factor-4 (FGF-4) (19), platelet-derived growth factor (PDGF) (20), tumor necrosis factor-α (TNF-α) (21), transforming growth factor-β (TGF-β) (22), keratinocyte growth factor (KGF) (23), insulin-like growth factor-I (IGF-I) (24), interleukin-1β (IL-1β) (25) and IL-6 (26) result in up-regulation of VEGF expression. In addition there are two important inducers of VEGF expression, hypoglycemia and hypoxia (27).

Fallopian tubes play an essential role in successful human reproduction. They provide an appropriate environment for pre-implantation development of the embryo and its transportation to the uterus (28). Previously, Lam et al. (29, 30) have investigated the expression of *VEGF* and its receptors (*VEGFR1* and *VEGFR2*) in normal human fallopian tubes and reported expressions of *VEGF*, *VEGFR1* and *VEGFR2* in various regions of the fallopian tubes of fertile women. Additionally, these researchers studied the changes in mRNA expression throughout the menstrual cycle. Also Amirchaghmaghi et al. (31) showed the expression of *VEGF* and its receptors (*VEGFR1* and *VEGFR2*) in the endometrium of women with recurrent miscarriage.

The exact causes of embryo implantation in fallopian tubes during an EP are not definitely known (32-34). On the other hand, VEGF plays important roles in embryo implantation. Several investigators (13-15) have reported that *VEGF* expression in the endometrium and corpus luteum may be regulated by ovarian steroids and possibly beta human chorionic gonadotropin (β-hCG). This is somehow similar to the expression of Toll like receptors (TLRs) and G alpha(i2) in human endometrium and fallopian tubes (35, 36). Evans et al. (37) report that *VEGF* levels increase during the first trimester of a normal pregnancy and show a positive correlation with gestational age as well as β-hCG, estrogen (E2), and progesterone levels. The current research aims to study mRNA expressions of *VEGF* and its receptors in fallopian tubes with EP compared with fallopian tubes of pseudo-pregnant women as the control group.

Materials and Methods

Tissue collection for genomic studies

All procedures were approved by the Ethics Committee of Royan Institute and written informed consent was obtained prior to sample collections. All specimens were collected at the Arash Women's Hospital, Tehran, Iran.

Case group

In this case-control study, ten fallopian tubes were obtained from women who underwent salpingectomy because of EP.

Control group

Due to inaccessibility to normal fallopian tubes of pregnant women as the control group, we decided to investigate the fallopian tubes obtained from women who underwent hysterectomies because of benign gynecological conditions that did not affect the tubes. All women in the control group were fertile and had regular menstrual cycles with no evidence of any pathological tubal disorders. To induce hormonal conditions similar to a normal pregnancy, each woman from the control group received intramuscular injections of 5000 IU per injection of hCG. For this purpose, hCG was administered every 3 days beginning in the midluteal phase before total abdominal hysterectomy, for a 12-day period. This treatment creates a state of pseudo-pregnancy which is harmless and has been previously used by other research studies (38, 39).

Sampling and processing

In order to ensure the integrity of tubal morphology and function in the case group, we excised at least 1 cm away from the implantation site of the embryo in the fallopian tube to avoid collection of any embryonic or trophoblastic tissues.

After obtaining required samples from both case and control groups we cut each fallopian tube into three regions - infundibulum, ampulla and isthmus. Then, each of these regions was divided into small (1×1 cm) pieces and immediately placed in RNAlater solution (Ambion, Huntington, UK) followed by immediate immersion in liquid nitrogen after which samples were stored at -80˚C until processing.

Total RNA extraction, cDNA synthesis and reverse transcription polymerase chain reaction (RT-PCR)

Samples were removed from RNAlater and TRI reagent (Sigma, Pool, UK) was used for total RNA isolation. RNA concentrations were quantified by spectrophotometric analysis.

First-strand cDNA synthesis was performed using oligodT primers and the Superscript II Reverse Transcriptase System (Fermentas, Sanktleon-rot, Germany). Briefly, reverse transcription was performed according to the recommended method (incubation for 60 minutes at 42˚C and termination of the reaction by heating at 70˚C for 5 minutes). cDNA was amplified by RT-PCR using the prepared cDNA, forward and reverse primers of human *VEGF*, *VEGFR1*, *VEGFR2* and *β-actin* (Metabion, Martinsried, Germany), and Platinum Blue PCR Super Mix (Invitrogen, Paisley, UK). Primer sequences used in this study are shown in table 1. *β-actin* was used as the housekeeping gene.

Cycling conditions were continued for 40 cycles as follows: 5 minutes at 95˚C for initial denaturation, followed by 39 cycles of 45 seconds at 95˚C, 45 seconds at 60˚C and 45 seconds at 72˚C. Reactions were also amplified in the absence of reverse transcriptase as negative RT controls. Placenta samples were used as the positive control (40) while molecular water was used as the negative control. PCR products were subjected to electrophoresis on 1.7% agarose gel (Sigma, UK) that contained ethidium bromide and photographed under ultraviolet (UV) light.

Quantitative PCR (Q-PCR)

Q-PCR was used to quantify whether there was any difference in *VEGF* and *VEGFRs* mRNA expression levels in fallopian tubes of the case and control groups. In this procedure we used the prepared cDNA, Power SYBR Green Master Mix (Applied Biosystems, UK) and primers of human *VEGF*, *VEGFR1*, *VEGFR2* and *β-actin* (Table 1). All experiments included negative controls with no cDNA. Each reaction of the PCR plate contained 10 µl SYBR green, 6 µl molecular water, 1 µl of each primer (20 pmol/µl) and 2 µl cDNA. The amplification was performed under the following conditions: 10 minutes at 95˚C, 50 cycles at 95˚C for 15 seconds and 60˚C for 60 seconds. Q-PCR was performed

under standard conditions and all experiments were run in triplicate. Relative *VEGF* and *VEGFRs* expression quantities were compared between case and control groups. Q-PCR data were analyzed using the comparative cycle threshold (CT) method (41). The difference in CT (ΔCT) was determined as the difference between the number of cycles required for amplification of the test gene and the housekeeping gene, human *β-actin*. Then ΔΔCT was calculated by finding the difference between case and control groups.

Differences in normalized expression values between groups were analyzed using the analysis of variance (ANOVA) statistical test. The results were presented as mean ± standard error of mean (SEM). The level of statistical significance was set at $p<0.05$.

Results

Reverse transcription polymerase chain reaction (RT-PCR)

This study enrolled 20 women, 10 for the control and 10 for the case groups. The mean age of the control group was 47.5 ± 5.36 years which significantly differed from the case group (36 ± 5.69 years). The age range was 42-56 years old for the control group and 26-40 years for the case group.

RT-PCR showed that *VEGF* and *VEGFRs* mRNA were expressed in all regions of the fallopian tubes (infundibulum, ampulla and isthmus) in both case and control groups (Fig.1). All amplified products were at the predicted size of their respective genes. There was no amplified product in negative control samples which was indicative of the absence of genomic DNA contamination.

Quantitative PCR (Q-PCR)

The quantitative expression profiles of *VEGF*, *VEGFR1* and *VEGFR2* in all regions of the fallopian tubes in both groups are shown in figures 2-4. The results revealed that expressions of all studied genes differed between the two groups and were significantly higher in the pseudo-pregnant women (control group) compared to the EP women (case group). As an exception, we observed higher *VEGFR2* gene expression in the ampulla of the case group.

The expression of *VEGF* was the highest in the ampulla of the control group and infundibulum of the case group compared to the other areas of the same tube (Fig.2). In contrast to *VEGF, VEGFR1* and *VEGR2* expressions were highest in the ampulla of the case group and infundibulum of the control group (Figs.3, 4).

Table 1: Sequences of primers used for quantitative polymerase chain reaction (Q-PCR)

Gene	Forward primer (5´-3´)	Reverse primer (5´-3´)	Annealing temperature (˚C)	Product size (bp)	GenBank accession no.
VEGF	TGCAGATTATGCGGATCAAACC	TGCATTCACATTTGTTGTGCTGTAG	60	81	AB021221
VEGFR1	CAGGCCCAGTTTCTGCCATT	TTCCAGCTCAGCGTGGTCGTA	60	82	AF063657
VEGFR2	CCAGCAAAAGCAGGGAGTCTGT	TGTCTGTGTCATCGGAGTGATATCC	60	87	AF063658
ß-actin	CAAGATCATTGCTCCTCCTG	ATCCACATCTGCTGGAAGG	60	90	NM 001101

VEGF; Vascular endothelial growth factor, VEGFR1; VEGF receptor 1; VEGFR2; VEGF receptor and ß-actin; Beta actin.

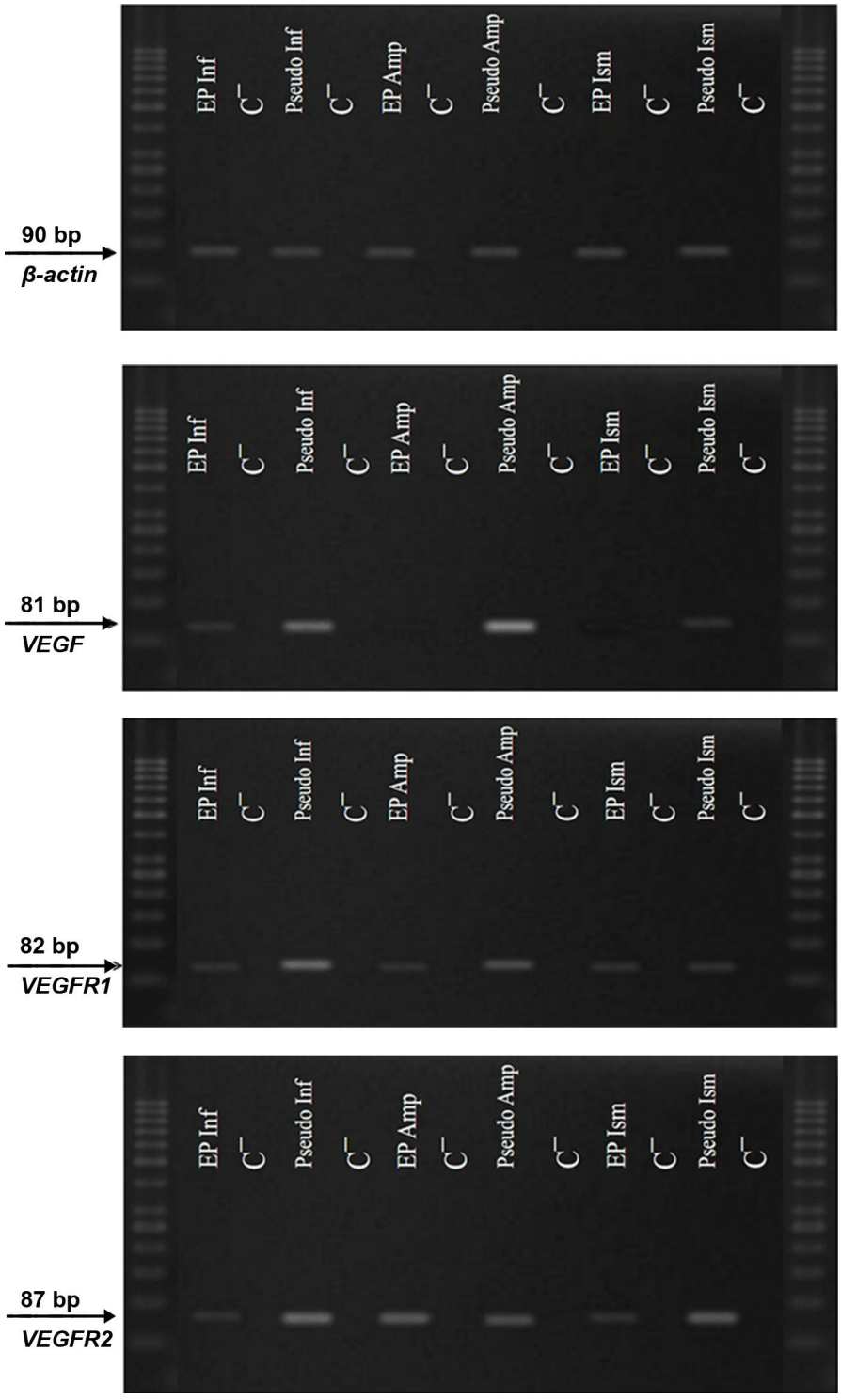


Fig.1: Expressions of *vascular endothelial growth factor (VEGF)* and its receptors, *VEGF receptor 1 (VEGFR1)* and *VEGFR2*, mRNA in the infundibulum (Inf), ampulla (Amp) and Isthmus (Ism) of fallopian tubes. These genes were expressed in all parts of the fallopian tubes in both case and control groups. There was no amplified product in negative control (C^-) samples.

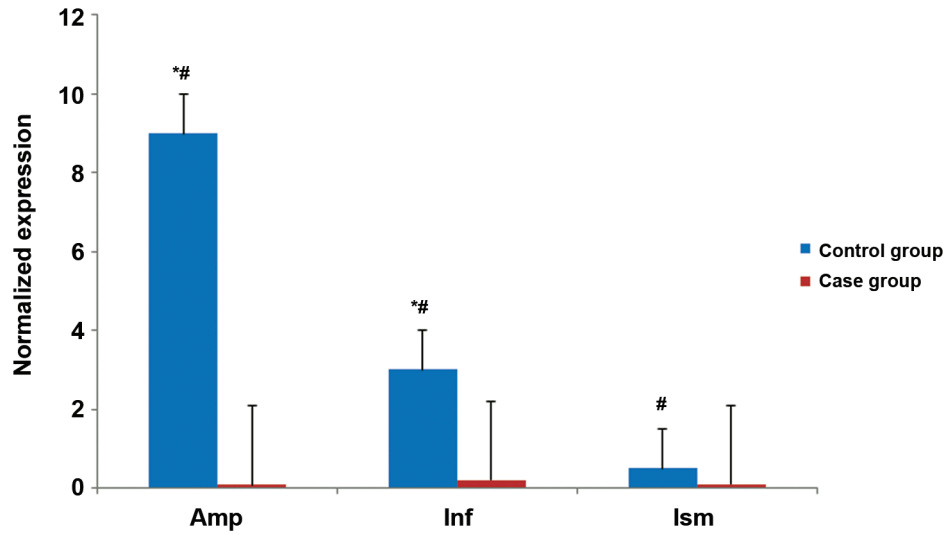


Fig.2: Quantitative PCR (Q-PCR) results of *vascular endothelial growth factor (VEGF)* mRNA expression.
*; Significantly different expression between the two groups, #; Significantly different expression between different parts of the control group, Amp; Ampulla, Inf; Infundibulum and Ism; Isthmus.
The level of statistical significance was set as at p<0.05.

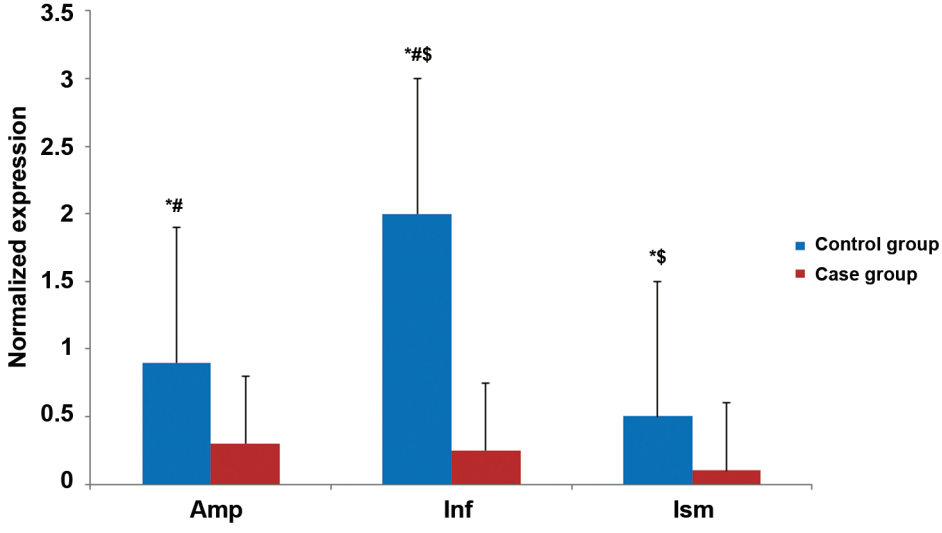


Fig.3: Quantitative PCR (Q-PCR) results of *vascular endothelial growth factor receptor 1 (VEGFR1)* mRNA expression.
*; Significantly different expression between the two groups, #; Significantly different expression between the ampulla and infundibulum of the control group, $; Significant different expression between the isthmus and infundibulum of the control group, Amp; Ampulla, Inf; Infundibulum and Ism; Isthmus.
The level of statistical significance was set as at p<0.05.

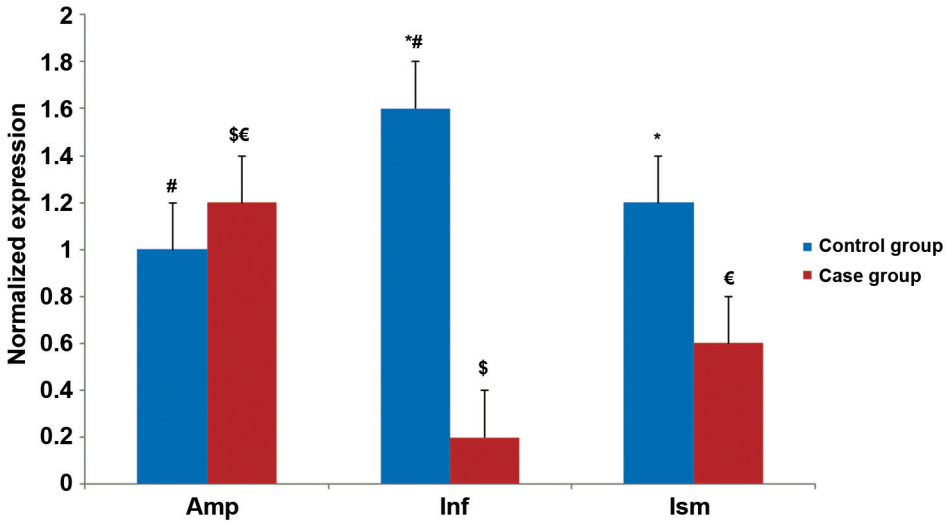


Fig.4: Quantitative PCR (Q-PCR) results of *vascular endothelial growth factor receptor 2 (VEGFR2)* mRNA expression.
*; Significantly different expression between two groups, #; Significantly different expression between the ampulla and infundibulum of the control group, $; Significantly different expression between the ampulla and infundibulum of the case group, €; Significantly different expression between the ampulla and isthmus of the case group, Amp; Ampulla, Inf; Infundibulum and Ism; Isthmus.
The level of statistical significance was set as at $p<0.05$.

Discussion

Embryo implantation is an essential process that occurs in the early stage of pregnancy and leads to the establishment of a functional placenta and pregnancy. Successful embryo implantation depends on proper interactions between the blastocyst and a receptive endometrium. For development of a receptive endometrium, ovarian hormones that include estrogen and progesterone act on epithelial cells of the endometrium leading to the establishment of an appropriate environment which supports blastocyst development, attachment and subsequent implantation events (42-44). The processes of implantation and trophoblast invasion are associated with growth of blood vessels coincident with decidualization, improvement of vascular membranes, and placenta formation (45). These processes accompanied by the formation of new blood vessels from pre-existing vasculature (angiogenesis) (46) and establishment of the embryonic vascular system (vasculogenesis) (47).

One of the key factors in regulation of angiogenesis is VEGF. It has been suggested that VEGF is an essential cytokine for embryo implantation. This cytokine plays a crucial role in maternal-fetal interactions as a local mediator which facilitates blastocyst implantation (37). Expressions of VEGF and its receptor mRNAs are induced by growth factors, cytokines and gonadotropins, and depend on local conditions such as hypoxia (27).

Although many cellular and molecular events during embryo implantation are unknown, however a study of these changes during normal human pregnancy is practically impossible because of ethical limitations. On the other hand, most women with EP undergo salpingectomy as treatment. Thus, EP can be used as an accessible model for human embryo implantation.

In the present study we investigated mRNA expression levels of *VEGF* and its receptors in fallopian tubes of women with EP (case group) compared to a control group of pseudo-pregnant women with normal fallopian tubes. Because of ethical limitations, accessibility to fallopian tubes

of normal pregnant women is impossible; therefore we have injected hCG for the induction of pseudo-pregnant conditions in control group women that underwent hysterectomies (5). RT-PCR showed that *VEGF* and *VEGFR1*, 2 mRNA expressed in all regions of the fallopian tubes of both groups. Q-PCR confirmed that the relative expression of these genes was significantly higher in fallopian tubes of pseudo-pregnant women compared with fallopian tubes from case group women, with the exception of *VEGFR2* mRNA expression.

Previously, other studies investigated the presence of *VEGF* and its receptors in normal fallopian tubes. Lam et al. (29, 30) conducted immunohistochemical analyses and showed that *VEGF*, *VEGFR1* and *VEGFR2* expressed at the protein level in the infundibulum, ampulla and isthmus of fallopian tubes in fertile women throughout the menstrual cycle. Using semi-quantitative RT-PCR, they observed that *VEGF, VEGFR1* and 2 mRNA expressions were highest in fallopian tubes in the periovulatory stage. Expressions in the ampullary and infundibular regions were higher than the isthmus. In addition, they reported a significant positive correlation between serum follicle stimulating hormone (FSH) and luteinizing hormone (LH) concentrations and *VEGF* and *VEGFR1* mRNA expressions in normal fallopian tubes. They hypothesized that *VEGF* in human fallopian tubes might play important roles related to early reproductive events, which occur predominantly in the ampulla during the peri-ovulatory phase when serum FSH and LH concentrations were high.

The finding of the present study in pseudo-pregnant women was consistent with a study by Lam et al. (30) who observed the highest expressions of *VEGF* and *VEGFR1* in the ampullary and infundibulary regions, respectively.

A study of *VEGF* family gene expression in EP conducted by Lam et al. (29), researched the implantation site of fallopian tubes with EP compared to other regions of same fallopian tube. In the present study we excluded the implantation site. In their study (29), *VEGF* and *VEGFRs* mRNA expressions increased at the implantation site of the fallopian tube with the EP compared to the rest of the same fallopian tube. The current study differed from the study by Lam et al. in the samples used for comparison. They compared the expression of these genes at the implantation site and other sections of same fallopian tube with EP. The current study collected fallopian tubes from normal women who received hCG to mimic the hormonal status of a normal pregnancy (pseudo-pregnant state) as the control group. (29).

Lower gene expression of *VEGF* and its receptors in the EP group compared to pseudo-pregnant women from the control group might be due to the effect of hCG on *VEGF* and *VEGFRs* expressions. The result of a study by Lam et al. (30) showed that mRNA expression of *VEGF* and its receptors in normal fallopian tubes was positively correlated with serum sex hormone concentrations.

Another potential explanation for the difference in gene expression between the case and control groups in the present study might be the differences in age between the studied women. The mean age of the control group was 47.5 years, whereas the case group was 36 years. The increased *VEGF* and *VEGFRs* mRNA expression in the control group might be secondary to changes in sex hormones, cytokines and growth factor expressions in older women (48, 49).

Despite limited studies with regards to *VEGF* gene expression, several studies investigated the concentration of VEGF in sera of pregnant women. Evans et al. (37) stated that maternal serum *VEGF* concentrations increased during the first trimester of pregnancy. In a study by Wheeler et al. (50) maternal serum VEGF concentrations remained elevated up to week-20 of pregnancy and was positively correlated with placental volume at mid-pregnancy, as well as to placental and fetal weight at delivery. Daniel et al. (51) reported that serum VEGF levels up-regulated in women with EP compared to those who had intrauterine pregnancies, although there was only borderline significance between tubal EP and failed intrauterine pregnancy.

Conclusion

Our results suggest that expressions of *VEGF* and *VEGFRs* mRNAs are lower in fallopian tubes that contain EP compared with normal fallopian tubes that receive hCG. Further studies are required with larger sample sizes that include a group of fertile women in the periovulatory phase.

Acknowledgements

We would like to thank the staff at Royan Institute for technical assistance as well as the staff at Arash Women's Hospital for recruitment of subjects. This study was financially supported by Royan Institute. There was no conflict of interest in this project.

References

1. Brown JK, Horne AW. Laboratory models for studying ectopic pregnancy. Curr Opin Obstet Gynecol. 2011; 23(4): 221-226.
2. Shaw JL, Dey SK, Critchley HO, Horne AW. Current knowledge of the aetiology of human tubal ectopic pregnancy. Hum Reprod Update. 2010; 16(4): 432-444.
3. Warma R, Gupta J. Tubal ectopic pregnancy. Clin Evid (Online). 2009. pii: 1406.
4. Kriebs JM, Fahey JO. Ectopic pregnancy. Midwifery womens health. 2006; 51(6): 431-439.
5. Refaat B, Amer S, Ola B, Chapman N, Ledger W. The expression of activin βA and βB subunits, follistatin, and actirin type II receptors in fallopian tubes bearing an ectopic pregnancy. J Clin Endocrinol Metab. 2008; 93 (1): 293-299.
6. Breen JL. A 21 year survey of 654 ectopic pregnancies. Am J Obstet Gynecol. 1970; 106(7): 1004-1019.
7. Nowacek GE, Meyer WR, McMahon MJ, Thorp JR, Wells SR. Diagnostic value of cervical fetal fibronectin in detecting extrauterine pregnancy. Fertil Steril. 1999; 72(2): 302-304.
8. Felemban A, Sammour A, Tulandi T. Serum vascular endothelial growth factor as a possible marker for early ectopic pregnancy. Hum Reprod. 2002; 17(2): 490-492.
9. Geva E, Jaffe RB. Role of vascular endothelial growth factor in ovarian physiology and pathology. Fertil Steril. 2000; 74 (3): 429-438.
10. Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, et al. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat Med. 1998; 4 (3): 336-340.
11. Clark DE, Smith SK, Sharkey AM, Charnock-Jones DS. Localization of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy. Hum Reprod. 1996; 11 (5): 1090-1098.
12. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 1996; 380 (6573): 435-439.
13. Torry DS, Holt VJ, Keenan JA, Harris G, Caudle MR, Torry RJ. Vascular endothelial growth factor expression in cycling human endometrium. Fertil Steril. 1996; 66 (1): 72-80.
14. Torry DS, Torry RJ. Angiogenesis and the expression of vascular endothelial growth factor in endometrium and placenta. Am J Reprod Immunol. 1997; 37(1): 21-29.
15. Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, et al. Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. J Clin Endocrinol Metab. 1996; 81(8): 3112-3118.
16. Takahashi H, Shibuya M. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. J Clin Sci (Lond). 2005; 109(3): 227-241.
17. Tischer E, Gospodarowicz D, Mitchell R, Silva M, Schilling J, Lau K, et al. Vascular endothelial growth factor: a new member of the platelet- derived growth factor gene family. Biochem Biophys Res Commun. 1989; 165(3): 1198-1206.
18. Ferrara N, Gerber Hp, Lecouter J. The biology of VEGF and its receptors. Nat Med. 2003; 9(6): 669-676.
19. Deroanne CF, Hajitou A, Calberg-Bacq CM, Nusgens BV, Lapiere CM. Angiogenesis by fibroblast growth factor 4 is mediated through an autocrine up-regulation of vascular endothelial growth factor expression. Cancer Res. 1997; 57(24): 5590-5597.
20. Finkenzeller G, Sparacio A, Technau A, Marme D, Siemeister G. Sp1 recognition sites in the proximal promoter of the human vascular endothelial growth factor gene are essential for platelet-derived growth factor-induced gene expression. Oncogene. 1997; 15(6): 669-676.
21. Ryuto M, Ono M, Izumi H, Yoshida S, Weich HA, Kohno K, et al. Induction of vascular endothelial growth factor by tumor necrosis factor alpha in human gliomacellspossible roles of SP-1. J Biol Chem. 1996; 271(45): 28220-28228.
22. Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O et al. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. J Biol Chem. 1994; 269(9): 6271-6274.
23. Frank S, Hubner G, Breier G, Longaker MT, Greenhalgh DG, Werner S. Regulation of vascular endothelial growth factor expression in cultured keratinocytes-implications for normal and impaired wound healing. J Biol Chem. 1995; 270(21): 12607-12613.
24. Goad DL, Rubin J, Wang H, Tashjian AH, Patterson C. Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. Endocrinology. 1996; 137(6): 2262-2268.
25. Li J, Perrella MA, Tsai JC, Yet SF, Hsieh CM, Yoshizumi M, et al. Induction of vascular endothelial growth factor gene expression by interleukin-1 beta in rat aortic smooth muscle cells. J Biol Chem. 1995; 270(1): 308-312.
26. Cohen T, Nahari D, Cerem LW, Neufeld G, Levi B. Interleukin-6 induces the expression of vascular endothelial growth factor. Biol Chem. 1996; 271 (2): 736-741.
27. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature. 1992; 359(6398): 843–845.
28. Jansen RP. Endocrine response in the fallopian tube. Endocr Rev. 1984; 5(4): 525-551.
29. Lam PM, Briton-Jones C, Cheung CK, Leung SW, Cheung LP, Haines C. Increased messenger RNA expression of vascular endothelial growth factor and its receptors in the implantation site of the human oviduct with ectopic gestation. Fertil Steril. 2004; 82(3): 686-690.
30. Lam PM, Briton-Jones C, Cheung CK, Lok IH, Yuen PM, Cheung LP, et al. Vascular endothelial growth factor in the human oviduct: localization and regulation of messenger RNA expression in vivo. Biol Reprod. 2003; 68(5): 1870-1876.
31. Amirchaghmaghi E, Rezaei A, Moini A, Roghaei MA, Hafezi M, Aflatoonian R. Gene expression analysis of VEGF and its receptors and assessment of its serum level in unexplained recurrent spontaneous abortion. Cell J. 2015; 16(4): 538-545.
32. Ghasemi N, Amjadi F, Salehi E, Shakeri M, Aflatoonian A, Aflatoonian R. Expression of Toll-like receptors 7-10 in

human fallopian tubes. Iran J Reprod Med. 2014; 12(6): 389-394.
33. Amjadi F, Salehi E, Mehdizadeh M, Aflatoonian R. Role of the innate immunity in female reproductive tract. Adv Biomed Res. 2014; 3: 1.
34. Amirchaghmaghi E, Taghavi SA, Shapouri F, Saeidi S, Rezaei A, Aflatoonian R. The role of toll like receptors in pregnancy. Int J Fertil Steril. 2013; 7(3): 147-154.
35. Aflatoonian R, Tuckerman E, Elliott SL, Bruce C, Aflatoonian A, Li TC, et al. Expression of toll-like receptors in endometrium during the menstrual cycle. J Reprod Immunol. 2006: 71 (Suppl 2): 152.
36. Monkkonen KS, Aflatoonian R, Lee KF, Yeung WS, Tsao SW, Laitinen JT, et al. Localization and variable expression of G alpha(i2) in human endometrium and fallopian tubes. Hum Reprod. 2007; 22(5): 1224-1230.
37. Evans PW, Wheeler T, Antony FW, Osmond C. Alongitudinal study of maternal serum vascular endothelial growth factor in early pregnancy. Hum Reprod. 1998; 13(4): 1057-1062.
38. Illingworth PJ, Reddi K, Smith K, Baird DT. Pharmacological 'rescue' of the corpus luteum results in increased inhibin production. Clin Endocrinol (Oxf). 1990; 33(3): 323-332.
39. Tay PY, Lenton EA. Corpus luteum response to exogenous HCG during the mid-luteal phase of the menstrual cycle. Clin Endocrinol (Oxf). 2000; 53(3): 345-350.
40. Chung JY, Song Y, Wang Y, Magness RR, Zheng J. Differential expression of vascular endothelial growth factor (VEGF), endocrine gland derived-VEGF, and VEGF receptors in human placentas from normal and Pre-eclampticpregnancies. J Clin Endocrinol Metab. 2004; 89(5): 2484-2490.
41. Psychoyos A. Uterine receptivity for nidation. Ann N Y Acad Sci. 1986; 476: 36-42.
42. Paria BC, Huet-Hudson YM, Dey SK. Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. Proc Natl Acad Sci USA. 1993; 90(21): 10159-10162.
43. Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T, et al. Molecular cues to implantation. Endocr Rev. 2004; 25(3): 341-373.
44. Ramsey EM, Donner NW. Placental vasculature and circulation. Stuttgart (NY): Thieme; 1980.
45. Folkman J, Shing Y. Angiogenesis. J Biol Chem. 1992; 267(16): 10931-10934.
46. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling- in control of vascular function. Nat Rev Mol Cell Biol. 2006; 7(5): 359-371.
47. Sharkey A. Cytokines and implantation. Rev Reprod. 1998; 3(1): 52-61.
48. Aflatoonian R, Fazeli A. Toll-like receptors in female reproductive tract and their menstrual cycle dependent expression. J Reprod Immunol. 2008; 77(1): 7-13.
49. Monkkonen KS, Aflatoonian R, Lee KF, Yeung WS, Tsao SW, Laitinen JT, et al. Hormonal regulation of Galphai2 and mPRalpha in immortalized human oviductal cell line OE-E6/E7. Mol Hum Reprod. 2007; 13(12): 845-851.
50. Wheeler T, Evans PW, Antony FW, Godfrey KM, Howe DT, Osmond C. Relationship between maternal serum vascular endothelial growth factor concentration in early pregnancy and fetal and placental growth. Hum Reprod. 1999; 14(6): 1619-1623.
51. Daniel Y, Geva E, Lerner-Geva L, Eshed-Englender T, Gamzu R, Lessing JB, et al. Levels of vascular endothelial growth factor are elevated in patients with ectopic pregnancy: is this a novel marker?. Fertil Steril. 1999; 72(6): 1013-1017.

Original Article

The Effects of Post-Mating Administration of Anti-IL-10 and Anti-TGFß on Conception Rates in Mice

Ali Risvanli, Ph.D., D.V.M.[1]*, Ahmet Godekmerdan, Ph.D., M.D.[2]

1. Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, University of Firat, Elazig, Turkey
2. Deparment of Microbiology, Faculty of Medicine, University of Yildirim Beyazit, Ankara, Turkey

Abstract

Background: In fertility studies, it has been shown that transforming growth factor β (TGFβ) and interlukin 10 (IL-10) play very important roles in implantation, maternal immune tolerance, placentation and fetal development, and the release beginning of release for fetal and postnatal death. The present study aims to determine the effects of the post-mating administration of neutralizing antibodies against IL-10 and TGFβ, which significantly impact pregnancy in females and the conception rates in mice via assessments of blood serum and uterine fluid concentrations of IL-2, IL-4, IL-6, IL-10, IL-17, interferon γ (IFNγ), Tumor necrosis factor α (TNFα), and TGFβ.

Materials and Methods: In this experimental study, 21 BALB/c strain female mice were mated and randomly divided into three groups. The mice in the first group were selected as the control group. The second group of animals was injected with 0.5 mg of anti-IL-10 after mating, while those in the third group were intraperitoneally injected with 0.5 mg of anti-TGFβ. The animals in all groups were decapitated on the 13th day after mating and their blood samples were taken. The uteri were removed to determine pregnancy. The mice's uterine irrigation fluids were also obtained. We used the multiplex immunoassay technique to determine the cytokine concentrations in uterine fluid and blood serum of the mice.

Results: We observed no intergroup difference with respect to conception rates. A comparison of the cytokine concentrations in the uterine fluids of pregnant mice revealed higher TGFβ concentrations ($p<0.01$) in the second group injected with the anti-IL-10 antibody compared with the other groups. There was no difference detected in pregnant animals with regards to both uterine fluid and blood serum concentrations of the other cytokines.

Conclusion: Post-mating administration of anti-IL-10 and anti-TGFβ antibodies in mice may not have any effect on conception rates.

Keywords: Pregnancy, Mouse, Cytokine

Citation: Risvanli A, Godekmerdan A. The effects of post-mating administration of anti-IL-10 and anti-TGFβ on conception rates in mice. Int J Fertil Steril. 2015; 9(1): 65-70.

Introduction

The maternal immune system plays an important role in establishment of pregnancy. It is generally believed that cellular immune response is inhibited while humoral immune response becomes dominant during gestation. In this regard, Treg cells and their secreted cytokines such as interlukin 10 (IL-10) and transforming growth factor β (TGFβ) may have important roles.

The significant role played by Treg lymphocytes during pregnancy has been first shown in mice in 2004. Treg cells can be detected in lymph nodes that drain the uterus as early as two days after mating. It is also argued that there is an increase in the number of these cells within the days following mating in mice (1). Many different mechanisms have been suggested for the immunosuppressive effect of Treg lymphocytes. IL-10 and TGFβ are

Received: 27 May 2013, Accepted: 21 Dec 2013
* Corresponding Address: Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, University of Firat, 23119, Elazig, Turkey
Email: arisvanli@firat.edu.tr

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 65-70

known to increase during this immunosuppression. IL-10 and TGFβ play very important roles in conception rates and the pregnancy period (2).

For a pregnancy, IL-10 and its receptors must be found in the endometrium and decidual cells in early pregnancy under normal conditions. This cytokine leads to the proliferation of decidual cells and the secretion of Tumor necrosis factor α (TNFα) due to the autocrine effect, while also causing a maternal immune response due to the paracrine effect (3).

TGFβ is known to play a role in providing maternal immune tolerance during implantation and in *in vitro* regulation of various implantation-related molecules such as vascular endothelial growth factor (VEGF), matrix metallopeptidase 9 (MMP-9), insulin-like growth factor-binding protein 1 (IGFBP-1), and leukemia inhabitory factor (LIF) (4, 5). Early embryonic deaths or postpartum deaths have been reported in TGFβ knockout mice (6). Other studies demonstrate mRNA expression in TGFβ type 1 and type 2 receptors in rat endometria during the estrous cycle and in early pregnancy, claiming that functional TGFβ signals are linked to the beginning of implantation and trophoblast invasion (7, 8).

Mating triggers a temporary inflammatory response in the uterus, particularly for reasons related to seminal plasma, which might start with blastocyst hatching and continue to implantation. This response is believed to originate from the immunopermissive effect of the lymphocytes in the uterus. Specific factors in the seminal vesicles such as TGFβ trigger cytokines and chemokines such as granulocyte macrophage colony-stimulating factor (GM-CSF) in the uterine epithelium and leukocytes in mice (9, 10). In this context, mating is also argued to cause immunomodulation in the long run. Prior to implantation, the embryo needs a suitable cytokine environment for survival. In particular, IL-10 with immunosuppressive activity has a role in the emergence of a mating-induced inflammatory response (11). Robertson et al. (12) have reported that IL-6 and GM-CSF levels also increased after mating.

The Treg lymphocyte population increases in regional lymph nodes and in the uterus of females exposed to seminal plasma as a result of mating. This increase is believed to play a role in the development of fetal immune tolerance prior to implantation. Moreover, TGFβ-involved immune deviation and antigenic stimulation should exist for this reaction to take place. This process requires both sperm and seminal plasma in the same environment (9, 13). The present study aims to determine the effects of post-mating administration of antibodies developed against IL-10 and TGFβ with roles in conception and continuation of pregnancy upon conception rates in mice. The blood serum and uterine fluid concentrations of IL-2, IL-4, IL-6, IL-10, IL-17, interferon γ (IFNγ), TNFα, and TGFβ cytokines are also analyzed.

Materials and Methods

Animals

Female BALB/c mice, 3-4 months old, that weighed 25-30 g were used in this experimental study. The animals were obtained from the Experimental Research Center at Firat University. During the study period, the animals were kept in cages of seven animals per cage and were exposed to a light regimen of 12 hours dark and 12 hours light. They were given free access to food and water. The maintenance and care of experimental animals complied with the National Institutes of Health Guidelines for the Humane Use of Laboratory Animals or those of our Institute. A report was obtained from the Ethics Board for Experimental Animals of Firat University to conduct the study (FUHADEK No: 28/24.03.2010).

Treatment groups

One male mouse was placed in each cage and the animals' mating was monitored. Only females with vaginal plugs or those with spermatozoa in vaginal smears were considered to be mated females. The floors of all cages were covered with black material to facilitate the detection of vaginal plug formation. Vaginal plug monitoring was carried out at 2 hour intervals. This method was the preferred technique to avoid missing the vaginal plugs as an indication that mating had occurred. Then, the animals were randomly placed into three groups.

The animals in group 1 (n=7) were intraperitoneally injected with 0.5 ml of saline solution immediately after the detection of vaginal plugs or spermatozoa in vaginal smears.

The animals in group 2 (n=7) were intraperitoneally injected with 0.5 mg of monoclonal mouse anti-IL-10 antibody (eBioscience) immediately after the detection of vaginal plugs or spermatozoa in vaginal smears (14).

The animals in group 3 (n=7) were intraperitoneally injected with 0.5 mg of monoclonal mouse anti-TGFβ antibody (GeneTex) immediately after the detection of vaginal plugs or spermatozoa in vaginal smears (14).

The animals in all groups were decapitated 13 days after the detection of vaginal plugs or spermatozoa in vaginal smears. Before decapitation, their intracardiac blood was taken. Blood samples were centrifuged at 3000 rpm for 5 minutes and the resultant sera were stored at -80˚C until assayed. The uteri of the decapitated animals were removed to identify whether they were pregnant.

Collection of uterine fluid

After the animals were killed, their uterine fluid was obtained as described by Harris et al. (15) and Orsi et al. (11). Accordingly, the cornua of the removed uteri were ligatured and then irrigated with mineral oil to collect uterine fluids. We used 1 ml of mineral oil for irrigation purposes. Subsequently these samples were microcentrifuged at 9000 rpm for 5 minutes to remove cell debris. The resultant uterine fluids were kept at -80˚C until the measurements were carried out.

Cytokine analyses

The IL-2, IL-4, IL-6, IL-10, IL-17, IFNγ, TNFα, and TGFβ concentrations in blood serum samples and uterine fluids obtained from the animals were determined by multiplex immunoassay (Procarta® Cytokine Assay Service, Diax, Italy) based on xMAP® technology. The multiplex assay is a test capable of a large number of simultaneous measurements. It is used in cases of small volumes of sera. All cytokines tested with immunoassay multiplex analysis can be made with a single plate. Since TGFβ kits can only determine the active form of TGFβ, acid assays have been performed to separate the active form of TGFβ in the samples. A separate plate was used for TGFβ measurements (16).

Statistical analysis

The chi-squared test was used to compare intergroup conception rates while the Kruskal-Wallis test was performed to compare intergroup cytokine concentrations. In cases where the test showed significance, the Mann-Whitney U test was employed to determine the significance level. All statistical analyses were performed using SPSS 11.5 software.

Results

Table 1 summarizes the conception rates on day 13 after mating in the light of the data obtained. No intergroup difference was detected in terms of conception rates ($p>0.05$).

A comparison of the cytokine concentrations in the uterine fluids from conceived animals revealed a significantly higher TGFβ concentration in group 2 injected with the anti-IL-10 antibody (12.30 ± 2.67 pg/ml) compared to the other groups ($p<0.01$, Table 2). However, no significant difference was found between conceived animals with regard to the concentrations of other cytokines both in uterine fluid and blood serum ($p>0.05$, Tables 2 , 3).

Statistical computations were not performed for intergroup comparisons because the number of non-conceived animals was low in all groups. No comparison could be made between the cytokine concentrations of conceived and non-conceived animals due to the same reason.

Table 1: Distribution of pregnancy rates according to groups

Group 1 * Pregnancy		Group 2 * Pregnancy		Group 3 * Pregnancy	
Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
N (%)	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**
6 (85.71)	1 (14.29)	**5 (71.43)**	2 (28.57)	**5 (71.43)**	2 (28.57)

No significant differences between groups ($p>0.05$), *; N=7.

Table 2: Distribution of uterine fluid cytokine concentrations according to groups

Cytokine (pg/ml)	Group 1 (n=7) Pregnancy		Group 2 (n=7) Pregnancy		Group 3 (n=7) Pregnancy		P
	+ (n=6)	- (n=1)	+ (n=5)	- (n=2)	+ (n=5)	- (n=2)	
IL-2	4.33 ± 0.49	4.0	3.30 ± 0.49	4.5	2.80 ± 0.20	3.0	-
IL-4	3.83 ± 0.65	4.0	4.00 ± 0.77	3.0	3.80 ± 0.37	3.5	-
IL-6	5.50 ± 1.12	5.0	5.00 ± 0.89	5.5	4.80 ± 0.97	4.5	-
IL-10	3.33 ± 0.21	3.0	4.30 ± 0.44	4.25	5.20 ± 0.97	3.5	-
IL-17	7.33 ± 0.71	6.0	7.00 ± 0.32	7.00	6.40 ± 1.02	6.0	-
IFNγ	38.83 ± 5.87	53.0	48.17 ± 3.32	48.75	49.00 ± 4.37	60.5	-
TNFα	4.67 ± 0.49	6.0	4.60 ± 0.51	4.5	3.90 ± 0.40	4.75	-
TGFβ	4.66 ± 0.33[b]	6.0	12.30 ± 2.67[a]	17.5	5.50 ± 0.61[b]	4.75	*

-; No significant differences between groups (p>0.05).
[a, b]; The difference between different letter-carrying averages is significant, *; P<0.01, IL; interlukin, IFNγ; interferon γ, TNFα; Tumor necrosis factor α and TGFβ; transforming growth factor β.

Table 3: Distribution of serum cytokine concentrations according to groups

Cytokine (pg/ml)	Group 1 (n=7) Pregnancy		Group 2 (n=7) Pregnancy		Group 3 (n=7) Pregnancy		P
	+ (n=6)	- (n=1)	+ (n=5)	- (n=2)	+ (n=5)	- (n=2)	
IL-2	6.17 ± 1.04	14.0	4.4 ± 0.43	6.5	5.41 ± 2.22	3.75	-
IL-4	10.92 ± 5.29	31.0	7.00 ± 1.17	9.0	9.34 ± 13.17	6.5	-
IL-6	63.11 ± 7.88	87.0	38.40 ± 25.05	12.0	74.80 ± 17.44	127.75	-
IL-10	5.0 ± 0.73	4.0	4.8 ± 0.2	4.0	5.2 ± 0.58	3.5	-
IL-17	287.73 ± 11.12	195.0	170.90 ± 56.58	168.5	218.80 ± 27.60	396.0	-
IFNγ	26.33 ± 5.12	18.0	24.20 ± 4.0	19.0	5.33 ± 5.21	14.75	-
TNFα	31.25 ± 8.52	14.5	39.33 ± 12.33	32.5	19.50 ± 4.84	14.5	-
TGFβ	2403.08 ± 410.56	2830.5	3798.50 ± 962.25	4177.5	2287.60 ± 710.70	4147.25	-

-; No significant differences between groups (p>0.05), IL; interlukin, IFNγ; interferon γ, TNFα; Tumor necrosis factor α and TGFβ; transforming growth factor β.

Discussion

Studies that examine the role of cytokines in reproductive processes have argued that IL-10 has a role in fertility, implantation, maternal immune tolerance, placentation, and fetal development (3), while the same roles are also played by TGFβ and fetal or postpartum deaths are claimed to occur due to the defects in its release (6).

Immunosuppressive IL-10 and TGFβ secreted by Treg cells during pregnancy inhibit the effector functions of activated leukocytes (17, 18). Slager et al. (19) have reported a considerable decrease in implantation rates after the neutralizing antibodies specific for TGFβ-2 were injected into the cavity of mouse blastocysts 3.5 days after mating. After one day in culture, embryos were transferred to pseudopregnant females. In order to evaluate the physiologic roles of IL-10, BALB/c mice were continuously treated with neutralizing anti-IL-10 antibodies from birth until the eighth week as follows: three times per week mice received 0.2 mg/injection for week one, 0.5 mg/injection for week two, and 1.0 mg/injection for weeks three through eight. As a result, their endogenous IFNγ and TNFα levels increased (20). In a study aimed at eliminating the protective effects of Treg cells in pregnant mice (14), anti-IL-10 antibodies were used as a 1 mg single intraperitoneal dose. Very high abortion rates were observed in the animals injected with these antibodies. In the same study, the researchers blocked TGFβ in pregnant animals by using neutralizing antibodies administered as a single 1 mg intraperitoneal dose. They observed high abortion rates in these animals as well, although not as high as in the groups treated with anti-IL-10 antibodies. This result has demonstrated the importance of TGFβ and particularly IL-10 secreted by Treg cells during pregnancy in protecting the allogeneic fetus. The results of the present study were not consistent with the results of these studies. These differences might arise from issues such as the method antibody administration, breed discrimination, and dosage.

There are certain publications asserting views that are contrary to those presented above. For instance, Rijhsinghani et al. (21) have treated pregnant mice with anti-IL-10 monoclonal antibodies to neutralize IL-10. As a result they found that this treatment did not affect pregnancy duration and there were no adverse effects on fetal development. However, certain problems in the newborn at later stages were observed. It has been reported that in early pregnancy and during implantation, inflammatory responses develop in endometrial tissues of IL-10 null mutant mice following mating despite inadequate IL-10. The implantation rates in these animals and survival rates of their newborns are higher when compared with normal mice. Thus, these studies have claimed that IL-10 is not required in mice for maternal immune tolerance and for a healthy pregnancy. In the present study, we observed that the post-mating anti-TGFβ and anti-IL-10 treatments in mice did not have any effect on conception rates. In this respect, the data obtained were consistent with the results reported in the above mentioned studies.

Conflicting results have been obtained in various studies with regard to the impacts of anti-IL-10 and anti-TGFβ treatments on the formation and continuation of pregnancy. Nevertheless, fetal formation and survival requires the development of maternal immunotolerance during pregnancy and this requires an immunosuppressive environment, also including IL-10 and TGFβ. At this point, neutralizing antibodies that develop against IL-10 and TGFβ may adversely affect the formation and continuation of pregnancy by suppressing the functions of the cytokines in question. Studies have been conducted by researchers opposing this hypothesis such as Rijhsinghani et al. (21) and White et al. (22) that failed to mention whether the antibodies used sufficiently neutralized IL-10 and TGFβ. They also varied in their methods used as well as the number and types of their subjects. Researchers who proposed positive opinions about the subject used different concentrations of anti-IL-10 and anti-TGFβ during pregnancy. No study has been found on the post-mating use of these antibodies.

In the present study, despite the lack of any statistical difference, the blood serum TNFα and TGFβ concentrations were found to be higher in the group treated with anti-IL-10 antibodies compared with the other groups, whereas IL-2, IL-4, IL-6, IL-10, and IL-17 were lower in this group. Even though there was no statistical difference, this result could be interpreted to demonstrate that treatment of these antibodies in mice partially influenced blood serum cytokine concentrations in pregnant animals. However, since a limited number of animals were used in the study for ethical reasons we could not compare cytokine concentrations in conceived and non-conceived animals. Hence, we did not have any findings on this topic. Similar interpretations also apply to the group treated with anti-TGFβ antibodies and could be extended to comparisons of both uterine fluid and blood serum

cytokine concentrations between conceived and non-conceived animals.

One of the main objectives of reproductive biology is a healthy early pregnancy and, in particular, implantation period. The present study has aimed at demonstrating the importance of IL-10 and TGFβ in the formation of pregnancy and attempted to prevent pregnancy formation in mice by the post-mating use of neutralizing antibodies developed against these cytokines. In this way, this study also aimed to obtain data to develop a new immunocontraceptive method to control reproduction. However, in our study, we observed that either post-mating anti-TGFβ and or anti-IL-10 treatments in mice did not influence conception rates. Therefore, we concluded that it would be useful to support these results with further studies that use a greater number of subjects and/or other species.

Conclusion

In the present study, we observed that cytokine concentrations obtained from the uterine fluid were significantly lower than the blood serum cytokine concentrations in all groups. However, our results were consistent with the findings of Orsi et al. (11) and these conclusions should be considered by studies that aim to determine cytokine concentrations and their effects in organs with lumen, such as the uterus. The lower cytokine concentrations in uterine fluid compared with blood serum could be attributed to the fact that cytokine concentrations might have been diluted during the irrigation process.

Acknowledgements

This study was supported by the Scientific and Technological Research Council of Turkey (110S232). There is no conflict of interest in this article.

References

1. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nat Immun. 2004; 5(3): 266-271.
2. Kahna DA, Baltimore D. Pregnancy induces a fetal antigen-specific maternal T regulatory cell response that contributes to tolerance. Proc Natl Acad Sci USA. 2010; 107(20): 9299-9304.
3. Vigano P, Somigliana E, Mangioni S, Vignali M, Vignali M, Di Blasio AM. Expression of interleukin-10 and its receptor is up-regulated in early pregnant versus cycling human endometrium. J Clin Endocrinol Metab. 2002; 87(12): 5730-5736.
4. Dimitriadis E, White CA, Jones RL, Salamonsen LA. Cytokines, chemokines and growth factors in endometrium related to implantation. Hum Reprod Update. 2005; 11(6): 613-630.
5. Herrler A, von Rango U, Beier HM. Embryo-maternal signaling: how the embryo starts talking to its mother to accomplish implantation. Reprod Biomed Online. 2003; 6(2): 244-256.
6. Kulkarni AB, Karlsson S. Transforming growth factor-beta 1 knockout mice. A mutation in one cytokine gene causes a dramatic inflammatory disease. Am J Pathol. 1993; 143(1): 3-9.
7. Lin HY, Wang HM, Li QL, Liu DL, Zhang X, Liu GY, et al. Expression of Smad2 and Smad4, transforming growth factor-beta signal transducers in rat endometrium during the estrous cycle, pre-, and peri-implantation. Anim Reprod Sci. 2004; 80(3-4): 303-316.
8. Lin HY, Qian D, Zhang X, Liu GY, Wang HM, Zhu C. Gene expression of transforming growth factor-beta receptors types I and II in rat endometrium during the estrous cycle and early pregnancy. Life Sci. 2006; 78(23): 2669-2675.
9. Robertson SA, Allanson M, Mau VJ. Molecular regulation of uterine leukocyte recruitment during early pregnancy in the mouse. Placenta. 1998; 19(Suppl 1): 101-119.
10. Tremellen KP, Seamark RF, Robertson SA. Seminal transforming growth factor beta1 stimulates granulocyte-macrophage colony-stimulating factor production and inflammatory cell recruitment in the murine uterus. Biol Reprod. 1998; 58(5): 1217-1225.
11. Orsi NM, Ekbote U, Walker JJ, Gopichandran N. Uterine and serum cytokine arrays in the mouse during estrus. Anim Reprod Sci. 2007; 100(3-4): 301-310.
12. Robertson SA, Ingman WV, O'Leary S, Sharkey DJ, Tremellen KP. Transforming growth factor beta–a mediator of immune deviation in seminal plasma. J Reprod Immunol. 2002; 57(1-2): 109-128.
13. Weiner HL. Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta secreting regulatory cells. Microbes Infect. 2001; 3(11): 947-954.
14. Schumacher A, Wafula PO, Bertoja AZ, Sollwedel A, Thuere C, Wollenberg I, et al. Mechanisms of action of regulatory T cells specific for paternal antigens during pregnancy. Obstet Gynecol. 2007; 110(5): 1137-1145.
15. Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM. Nutrient concentrations in murine follicular fluid and the female reproductive tract. Theriogenology. 2005; 64(4): 992-1006.
16. de Jager W, Prakken B, Rijkers GT. Cytokine multiplex immunoassay: methodology and (clinical) applications. Methods Mol Biol. 2009; 514: 119-133.
17. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. J Immunol. 2001; 166(6): 3789-3796.
18. Josien R, Douillard P, Guillot C, Muschen M, Anegon I, Chetritt J, et al. A critical role for transforming growth factor-beta in donor transfusion-induced allograft tolerance. J Clin Invest. 1998; 102(11): 1920–1926.
19. Slager HG, Van Inzen W, Freund E, Van den Eijnden-Van Raaij AJ, Mummery CL. Transforming growth factor-beta in the early mouse embryo: implications for the regulation of muscle formation and implantation. Dev Genet. 1993; 14(3): 212-224.
20. Ishida H, Hastings R, Kearney J, Howard M. Continuous anti-interleukin 10 antibody administrations depletes mice of Ly-1 B cells but not conventional B cells. J Exp Med. 1992; 175(5): 1213-1220.
21. Rijhsinghani AG, Thompson K, Tygrette L, Bhatia SK. Inhibition of interleukin-10 during pregnancy results in neonatal growth retardation. Am J Reprod Immunol. 1997; 37(3): 232-235.
22. White CA, Johansson M, Roberts CT, Ramsay AJ, Robertson SA. Effect of interleukin–10 null mutation on maternal immune response and reproductive outcome in mice. Biol Reprod. 2004; 70(1): 123-131.

Original Article

Effect of Human Umbilical Cord Mesenchymal Stem Cells Transplantation on Nerve Fibers of A Rat Model of Endometriosis

Yan Chen, M.Sc.[1], Dong Li, Ph.D.[2], Zhe Zhang, M.Sc.[3], Natsuko Takushige, Ph.D.[4], Bei-Hua Kong, Ph.D.[3], Guo-Yun Wang, Ph.D.[3]*

1. Department of Obstetrics and Gynecology of the Third Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China
2. Cryomedicine Laboratory, Qilu Hospital of Shandong University, Jinan 250012, China
3. Department of Obstetrics and Gynecology, Qilu Hospital of Shandong University, Jinan 250012, China
4. Department of Obstetrics and Gynaecology, University of Sydney, Sydney, NSW, 2006, Australia

Abstract

Background: Endometriosis is a common, benign, oestrogen-dependent, chronic gynaecological disorder associated with pelvic pain and infertility. Some researchers have identified nerve fibers in endometriotic lesions in women with endometriosis. Mesenchymal stem cells (MSCs) have attracted interest for their possible use for both cell and gene therapies because of their capacity for self-renewal and multipotentiality of differentiation. We investigated how human umbilical cord-MSCs (hUC-MSCs) could affect nerve fibers density in endometriosis.

Materials and Methods: In this experimental study, hUC-MSCs were isolated from fresh human umbilical cord, characterized by flow cytometry, and then transplanted into surgically induced endometriosis in a rat model. Ectopic endometrial implants were collected four weeks later. The specimens were sectioned and stained immunohistochemically with antibodies against neurofilament (NF), nerve growth factor (NGF), NGF receptor p75 (NGFRp75), tyrosine kinase receptor-A (Trk-A), calcitonin gene-related peptide (CGRP) and substance P (SP) to compare the presence of different types of nerve fibers between the treatment group with the transplantation of hUC-MSCs and the control group without the transplantation of hUC-MSCs.

Results: There were significantly less nerve fibers stained with specific markers we used in the treatment group than in the control group ($p<0.05$).

Conclusion: MSC from human umbilical cord reduced nerve fiber density in the treatment group with the transplantation of hUC-MSCs.

Keywords: Endometriosis, Mesenchymal Stem Cells, Nerve Fibers, Immunohistochemistry

Citation: Chen Y, Li D, Zhang Z, Takushige N, Kong BH, Wang GY. Effect of human umbilical cord mesenchymal stem cells transplantation on nerve fibers of a rat model with endometriosis. Int J Fertil Steril. 2015; 9(1): 71-81.

Introduction

Endometriosis is defined as the presence of tissues which somewhat resembles endometrial glands and stroma outside the uterine cavity, most commonly implanted over visceral and peritoneal surfaces within the female pelvis. Endometriosis exhibits disturbances of cellular proliferation, cellular invasion and neoangiogenesis (1). Although the exact prevalence of endometriosis in the general population is not clear, the prevalence in women of reproductive age is estimated to range between 10 and 15%

Received: 18 Feb 2013, Accepted: 6 Jan 2014
* Corresponding Address: Department of Obstetrics and Gynecology, Qilu Hospital of Shandong University, Jinan 250012, China
Email: wangguoy@sdu.edu.cn

(2). Endometriosis is a chronic, benign, oestrogen-dependent multifactorial and gynaecological disease, with pain being the most common and specific symptom. To date, the cardinal treatments for endometriosis are medical and surgical therapies. Pain symptoms may persist despite seeming adequate medical or/and surgical treatment of the disease (3).

Stem cell therapy as a promising and unprecedented strategy has the potential to be more effective than single-agent drug therapies (4). Mesenchymal stem cells (MSCs) are especially well suited for cell therapy owing to their ability to differentiate into different lineages and secrete a number of cytokines (5). Human umbilical cord-MSCs (hUC-MSCs) have become strong candidates for a cell-based therapy because of their key characteristics of long-term self-renewal and capacity to differentiate into diverse tissues. In addition, they can be easily obtained and cultured without raising ethical issues (6), as well as being an excellent alternative to bone marrow as a source of MSCs for cell therapies (6, 7). Furthermore, hUC-MSCs are a subset of primitive stem cells. HUC-MSCs neither induce teratomas nor result in acute rejection after being transplanted into non-immune-suppressed animals (8). In various animal disease models, transplantation of hUC-MSCs was reported to improve neurobehavioral functions following ischemic stroke (9), ameliorate mouse hepatic injury (10), and show effectiveness in apomorphine-induced rotations in a rodent model of Parkinson's disease (6, 11). Nevertheless, currently little is known about the application of hUC-MSCs to endometriosis.

Some researchers have identified nerve fibers in endometriotic lesions in women with endometriosis (12-14). Berkley et al. (15) and Oosterlynck et al. (16) have reported that endometriotic implants developed a sensory and sympathetic nerve supply both in rats and in women, similar to that of the healthy rat uterus. The present study demonstrated the existence of a much greater density of nerve fibers in deep infiltrating endometriosis than in peritoneal endometriotic lesions (17). These nerve fibers in endometriotic lesions could possibly exert their functions on the pathogenesis or symptoms of endometriosis.

As a consequence, we established surgically induced endometriosis in a rat model to investigate the effects of the hUC-MSCs transplantation on nerve fibers and the pathogenesis of the disease.

Materials and Methods

Generation and administration of hUC-MSC

The study protocol was approved by the Research Ethics Committee of Qilu Hospital of Shandong University (Shandong, P. R. China). HUCs (n=10, clinically normal pregnancies) were excised and washed in a 0.1 mol/L phosphate buffer saline (PBS, pH=7.4, Gibco-BRL, Grand Island, NY, USA) to remove excess blood (6). The cords were dissected and the blood vessels were removed. The remaining tissues were cut into small pieces (1-2 mm^3) and placed in plates with low-glucose Dulbecco-modified Eagle medium (L-DMEM, Gibco-BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Grand Island, NY, USA), 2 ng/mL vascular endothelial growth factor (VEGF, R&D Systems, Minneapolis, MN), 2 ng/mL epidermal growth factor (EGF, R&D Systems, Minneapolis, USA), 2 ng/mL fibroblast growth factor (FGF, R&D Systems, Minneapolis, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco-BRL, Grand Island, USA). Cultures were maintained at 37˚C in a humidified atmosphere with 5% CO_2. The media were changed every 3-4 days. Adherent cells proliferated from individual explanted tissues 7-12 days after initiating incubation. At this time, the small tissue pieces were removed from the culture and the adherent fibroblast-like cells were cultured to confluence, which subsequently took 2-3 weeks in culture. The cells were then trypsinized using 0.25% trypsin (Gibco-BRL, Grand Island, USA) and passaged at 1×10^4 cells/cm^2 in the medium described above. The cells were used after five or more passages.

Cell surface antigen phenotyping

Fifth- to seventh-passage cells were collected and treated with 0.25% trypsin. The cells

were stained with either fluorescein isothiocyanate-conjugated or phycoerythrin-conjugated monoclonal antibodies in 100 µL PBS for 15 minutes at room temperature, as suggested by the manufacturer. The antibodies used were against human antigens cluster of differentiation 34 (CD34), CD29, CD44, CD45, CD105, and CD106 (SeroTec, Raleigh, NC, USA). Cells were analyzed using flow cytometry (Cytometer 1.0, CytomicsTM FC500, Beckman Coulter Inc., USA). Positive cells were counted and compared to the signal of corresponding immunoglobulin isotypes.

Differentiation capacity

To investigate the differentiation potential of the fibroblast-like cells, the fourth passage cells were cultured under conditions appropriate for inducing the differentiation of each lineage.

Cells were seeded at a density of 2×10^4 cells/cm^2 and the differentiation media were changed every 3-4 days. The osteogenic differentiation medium consisted of L-DMEM supplemented with 10% FBS, 0.1 µM dexamethasone, 50 mM β-glycerol phosphate, and 0.2 mM ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA). The adipogenic differentiation medium consisted of high-glucose DMEM supplemented with 0.25 mM 3-isobutyl-1-methylxanthine, 0.1 µM dexamethasone, 0.1 mM indomethacin (Sigma-Aldrich, USA), 6.25 µg/mL insulin (PeproTech, UK), and 10% FBS. Cells were kept in the normal growth medium served as the control.

Animal model and cell transplantation

All animal procedures were conducted in accordance with the institutional guidelines of Qilu Hospital of Shandong University (Shandong, P. R. China). Adult female Wistar rats, weighing 180-210 g, were housed in cages in an air-conditioned room at 25 ±1˚C with a 12 hours dark/light cycle. The oestrous stage was monitored daily by vaginal smear every morning, beginning at least 2 weeks before surgery and continued until the day of death. Only rats with a regular 4-day cycle both before and after surgery were used. Surgically induced endometriosis in a rat model was done as previously described (18) and surgery was done under aseptic precautions. Rats in estrus were anesthetized with 3% pelltobarbitalum natricum (Solarbio, Beijing Solarbio Science & Technology Co., Ltd. China) at a dose of 0.2 mL /200 g by means of intraperitoneal injection. A midline abdominal incision exposed the uterus, and a 1-cm segment of the middle of the left uterine horn was removed and placed in warm sterile saline. Four pieces of uterine horn (≈2×2 mm) were cut from this segment and sewn with 4.0 nylon sutures around the alternate cascade mesenteric arteries that supply the caudal small intestine, starting from the caecum. The incision was closed in layers, and the rats were allowed to recover from anesthesia under close observation. Hereafter the endometriosis model rats were randomly divided into two groups (12 rats each), namely the treatment group and the control group. Two weeks later, the treatment group received hUC-MSCs by injection of 1×10^6 cell/mL normal saline into the tail vein every 5 days for 15 days. Meanwhile, the control group only received the same volume of normal saline. Four weeks later, ectopic implants were collected and fixed in 10% neutral buffered formalin for 18~24 hours.

Immunohistochemistry

We examined the presence of different types of nerve fibers in endometriotic implants in a rat model by immunohistochemistry using highly specific markers. We used neurofilament (NF), nerve growth factor (NGF), NGF receptor p75 (NGFRp75), tyrosine kinase receptor-A (Trk-A), calcitonin gene-related peptide (CGRP) and substance P (SP) to differentiate types of nerve fibers.

These implants were fixed with formalin, processed and embedded in paraffin according to a standard protocol. Each section was cut at 4 um and mounted onto slides. These sections were routinely stained with haematoxylin and eosin (H&E, Gibco-BRL, Grand Island, NY, USA) staining. For immunohistochemistry, the slides were submitted to antigen retrieval by boiling in citrate buffer (0.01 mol/L, pH=6.0) for 15

minutes using a micro-wave oven.

Endogenous peroxidase activity was prevented by incubating in 0.3% hydrogen peroxide for 15 minutes. Nonspecific binding was blocked by 10% goat serum (Zhongshan Golden Bridge Biotecnology Co., Ltd., China) for 20 minutes at room temperature. The sections were immunostained overnight at 4˚C using antibodies for monoclonal mouse anti-NF (dilution 1:150; Abcam, UK), a highly specific marker for myelinated nerve fibers, as follows: polyclonal rabbit anti-NGF (dilution 1:200; Abcam, UK), monoclonal mouse anti-NGFRp75 (dilution 1:200; Abcam, UK), polyclonal rabbit anti-TrkA (dilution 1:500; Abcam, UK), polyclonal mouse anti-SP (dilution 1:250), and polyclonal rabbit anti-CGRP (dilution 1:300, Abcam, UK), which are sensory fiber markers, and they can be present in both Ad and C nerve fibers. The slides were washed and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 30 minutes.

Peroxidase activity was visualized by exposure to diaminobenzidine tetrahydrochloride solution (DAB kit, Zhongshan Golden Bridge Biotecnology Co., Ltd., China) for 3-5 minutes. The sections were then washed, counterstained with hematoxylin for 1 minute, dehydrated, and mounted with coverslips. We used normal rat skin as a positive control as it reliably contains myelinated and unmyelinated nerve fibers expressing NF, NGF, NGFRp75, Trk-A, SP, and CGRP.

Quantification of nerve fiber density

The images were captured using an Olympus DP72 camera (Tokyo, Japan). The assessment of the mean density of nerve fibers was performed by Image Pro Plus software (Media Cybernetics, MD, USA). The integrated optical density (IOD) and area of the images were calculated using Image Pro Plus software. The area was divided by integrated optical density to obtain the mean density of nerve fibers. All lighting conditions and magnifications were held constant. Moreover, the investigator was unaware of the experimental groups from which the slices were obtained.

Statistical analysis

The results were expressed as the mean ± SD. All analyses were performed using the SPSS (SPSS Inc., Chicago, IL, USA) version 17.0. The comparison between two groups was performed using non-parametric 2-tailed t test (Mann-Whitney test). Statistical significance was defined as a p value of less than 0.5.

Results

After several passages, adherent cells from UC could form a monolayer of typical fibroblastic cells. Flow cytometry results showed that UC-derived cells shared most of their immunophenotype with MSCs, including positive expression for stromal markers (CD29, CD44, CD105, and CD106), but negative expression for hematopoietic markers (CD34 and CD45) (Fig.1A, B).

MSC differentiation was assessed using the fourth passage cells. When being induced to differentiate under osteogenic conditions, MSC congregation increased with increasing induction time and formed a mineralized matrix, as confirmed by alizarin red staining (Fig.1C). Most of the MSC-like cells became alkaline-phosphatase-positive by the end of 14 days (Fig.1D). No mineralized matrix was observed in the control cells kept in the normal growth medium. The spindle shape of the MSCs flattened and broadened after 1 week of adipogenic induction. Small oil droplets gradually appeared in the cytoplasm. By the end of the second week, almost all cells contained numerous oil-red-O-positive lipid droplets (Fig.1E). The control cells maintained in the regular growth medium did not stain positive for oil red O.

The mean density values of nerve fibers are given in table 1. Nerve fibers stained with kinds of special markers in ectopic endometriotic lesions were shown in figure 2. In summary, there were significant differences ($p<0.05$) in the mean density of nerve fibers in endometriotic implants stained with most of the specific markers which we used between the treatment group and the control group.

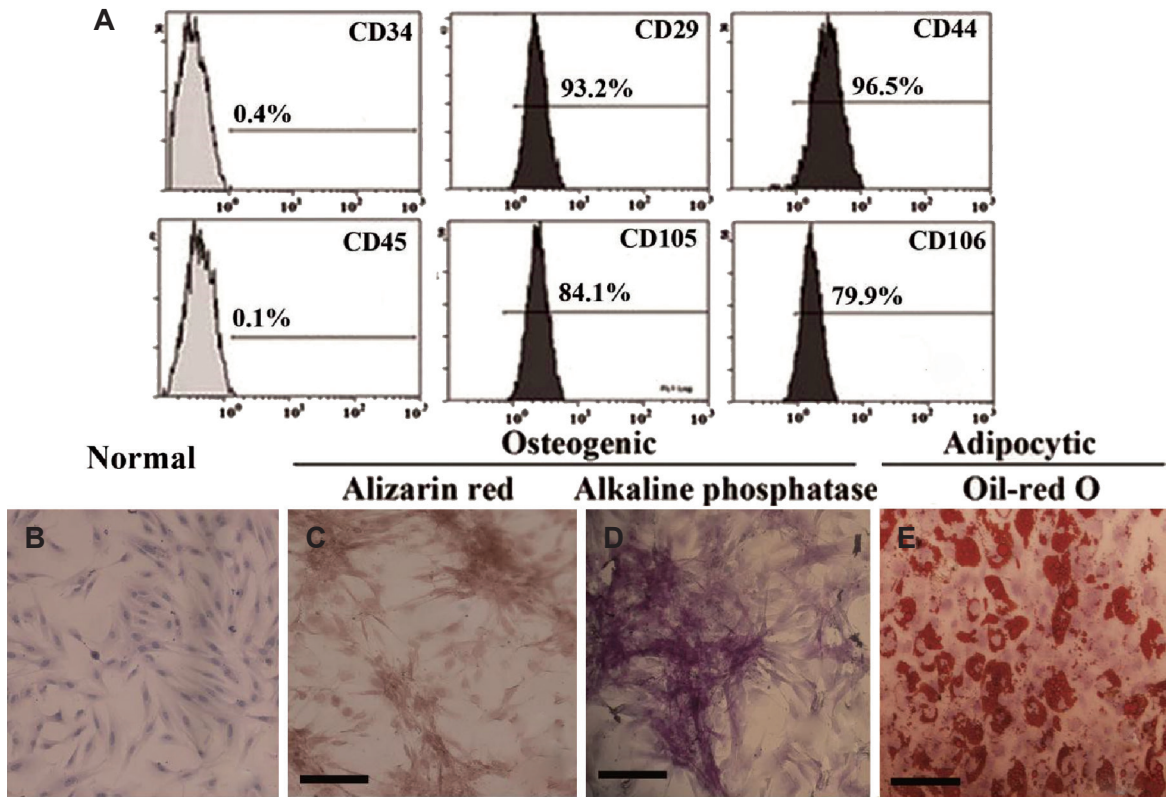
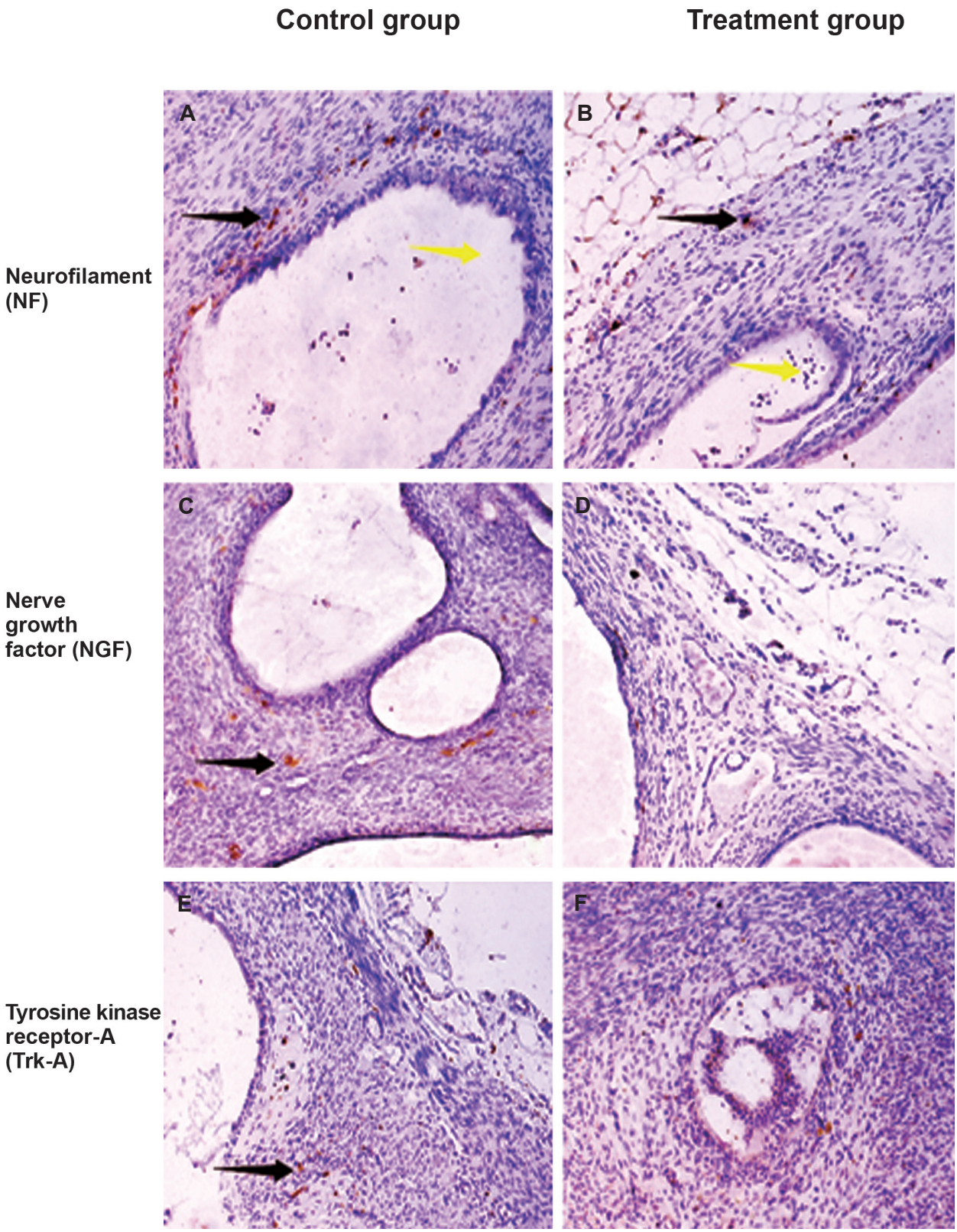


Fig.1: HUC-derived MSC-like cells in passaged cultures. Immunophenotype (A) and H&E staining of UC-derived MSC-like cells (B). Osteogenic differentiation as indicated by the formation of mineralized matrix shown by alizarin red staining (C) and alkaline phosphatase expression (D). Adipocytic differentiation was noted by the presence of broadened morphology and formation of lipid vacuoles (E) (positive oil-red O staining). Scale bars=80 µm.
hUC; Human umbilical cord, MSCs; Mesenchymal stem cells, H&E; Haematoxylin and eosin and CD; Cluster of differentiation.

Table 1: Quantitative assessment of the endometrial mean nerve fiber density stained against different neural markers in model rat with endometriosis

Marker	Treatment group (n=12) Mean ± SD (range)	Control group (n=12) Mean ± SD (range)
NF	0.40 ± 0.20 (0.19~0.90)	1.50± 1.27* (0.33~4.28)
NGF	0.27 ± 0.23 (0.00~0.77)	1.23± 0.72** (0.39~2.59)
Trk-A	0.19 ± 0.11 (0.00~0.44)	1.64± 0.95** (0.26~3.74)
NGFRp75	0.24 ± 0.13 (0.00~0.55)	0.99± 1.04* (0.17~4.07)
CGRP	0.32 ± 0.35(0.08~1.08)	1.45± 1.58* (0.33~5.64)
SP	0.24 ± 0.11 (0.00~0.45)	1.32± 1.23** (0.15~5.18)

Data are represented by mean density ± SD.
NF; Neurofilament, NGF; Nerve growth factor, Trk-A; Tyrosine kinase receptor-A, NGFRp75; NGF receptor p75, CGRP; Calcitonin gene-related peptide, SP; Substance P, *; P<0.01 and **; P<0.001.


Control group
Treatment group
Neurofilament (NF)
Nerve growth factor (NGF)
Tyrosine kinase receptor-A (Trk-A)
A
B
C
D
E
F

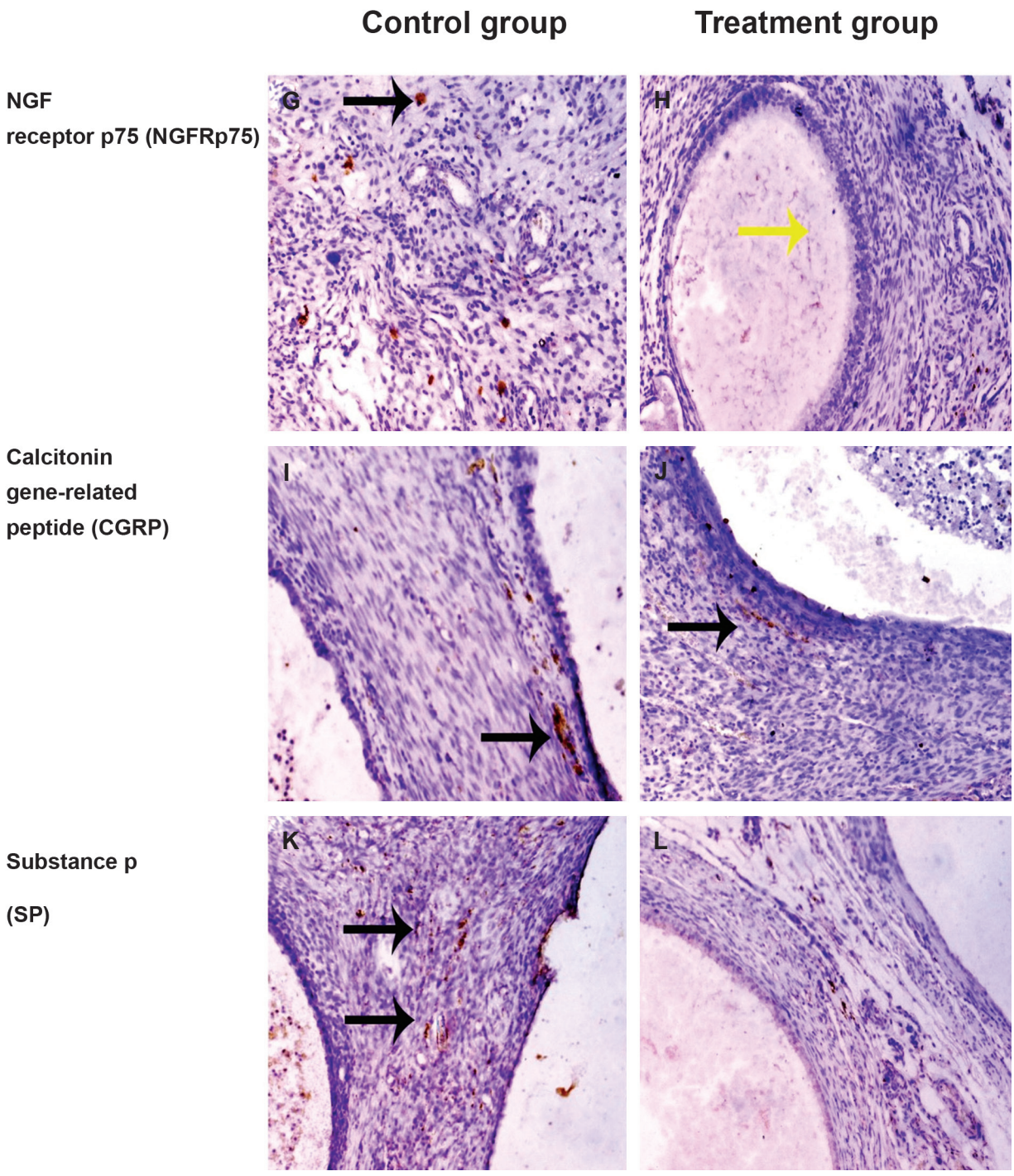


Fig.2: Nerve fibers in ectopic endometriotic lesions. A. Nerve fibers stained with NF from the control group without the transplantation of hUC-MSCs. B. Nerve fibers stained with NF from the treatment group with the transplantation of hUC-MSCs. C. Nerve fibers stained with NGF from the control group. D. Nerve fibers stained with NGF from the treatment group. E. Nerve fibers stained with Trk-A from the control group. F. Nerve fibers stained with Trk-A from the treatment group. G. Nerve fibers stained with NGFRp75 from the control group. H. Nerve fibers stained with NGFRp75 from the treatment group. I. Nerve fibers stained with CGRP from the control group. J. Nerve fibers stained with CGRP from the treatment group. K. Nerve fibers stained with SP from the control group. L. Nerve fibers stained with SP from the treatment group. Scale bars represent 50 μm in A-R (magnification ×200). Black arrows represent nerve fibers and yellow arrows represent endometrial glands.

Discussion

NF as a highly specific marker for myelinated nerve fibers stains Aα, Aβ, Aγ, Aδ and B fibers. Both SP and CGRP are sensory nerve fiber markers that can be present in both Aδ and C nerve fibers. In the present study, statistically significant difference was observed in the mean density of the NF-immunoactive nerve fibers between the treatment and control groups. Lower number of nerve fibers stained with NGF, TrkA, and NGFRp75 existed in the treatment group than in the control group. The mean densities of the CGRP- and SP- immunoreactive nerve fibers were lower in the treatment group, which indicates that the sensory nerve fibers were reduced. To sum up, our results showed that there were less nerve fibers stained with most of the specific markers used in this study in the treatment group compared with the control group.

It is believed that rich innervation in endometriosis may be involved in pain generation (17, 19). Patients with the highest pain scores displayed significantly more neural invasion into endometriosis than those with lower pain scores (20). Therefore, less innervation may ameliorate the symptoms of disease. Tokushige et al. (21) reported that the nerve fiber density in peritoneal endometriotic lesions from women with endometriosis who were on hormone treatment with progestogens and combined oral contraceptives was statistically significantly lower than in peritoneal endometriotic lesions from untreated women with endometriosis. In the present study, our results showed that there was lower number of nerve fibers in the treatment group, which is consistent with the findings of previous studies.

The pathogenesis of endometriosis and pathophysiological basis for endometriosis–associated pain are still unclear. Endometriosis is believed to be a chronic inflammatory state, with disturbances of both cell-mediated and humoral immunity (16). In women with endometriosis, the peritoneal fluid has high concentrations of cytokines, growth factors, and angiogenic factors (16, 22-24), derived from the lesions themselves; secretory products of macrophages and other immune cells; and follicular fluid after follicle rupture in ovulating women. Once endometriotic lesions are formed, they secrete several pro-inflammatory molecules (23, 24).

These nerve fibers in endometriotic lesions probably play an important role in the pathogenesis of pain and hyperalgesia. The nerve endings of nerve fibers can potentially be stimulated by many inflammatory substances, including histamine, serotonin, bradykinin, prostaglandins, leukotrienes, interleukins (ILs), acetylcholine, VEGF, tumor necrosis factor-α (TNF-α), epidermal growth factors, transforming growth factor-β (TGF-β), platelet-derived growth factor and NGF. Many of the above substances can be secreted by macrophages as well as from endometriotic lesions (25, 26), and are found in high concentration in the peritoneal fluid of endometriosis patients. Moreover, macrophages and their products may play important roles in the growth and repair of nerve fibers. The growth of nerve fibers is regulated by many substances, including NGF, brain-derived neurotropic factor (BDNF) and VEGF, and the synthesis of these substances is also affected by macrophage activities.

HMSCs, first described by Fridenstein et al. (27) in 1974, have extensive proliferative potential and the capacity to differentiate into various cell types. The bone marrow has been considered as the major source of MSCs. Transplantation of bone marrow-MSCs (BM-MSCs), however, may not be acceptable because of the variations in cell numbers and the proliferative potential of these cells from different donors (28). Other sources of MSCs have been considered and currently the presence of MSCs has been confirmed in the placenta, amniotic fluid, peripheral blood, lungs and teeth (29). Because there are large numbers of MSCs in neonates (30), human umbilical cords may be an ideal source for these cells. Supporting their potential as a source of cells, MSCs have been isolated from human umbilical cord (9, 27, 31). MSCs are poor antigen-presenting cells and do not express major histocompatibility complex class II or costimulatory molecules. HMSCs suppress T-lymphocyte proliferation induced by cellular or non-specific mitogenic stimuli and inhibit the response of naive and memory antigen-specific T cells to their cognate peptide (32). HMSCs altered the cytokine secretion profile of dendritic cells (DCs), naive and effector T cells [T helper 1 (T(h)1) and T(h)2], and natural killer (NK) cells to induce a more anti-inflammatory or tolerant phenotype (33). MSCs have potent anti-inflammatory effects in multiple

disease states (34). Some researchers have reported that MSCs administered by intravenous injections potently inhibit systemic levels of inflammatory cytokines and chemokines in the serum of treated animals (35). In addition, MSCs were able to modulate the immune system through the release of anti-inflammatory cytokines, prostaglandin E2 in many models (36). Aggarwal and Pittenger (33) reported that through the interactions of hMSCs with the various immune cells, hMSCs could inhibit or limit inflammatory responses and promote the mitigating and anti-inflammatory pathways. They demonstrated that when hMSCs are present in an inflammatory environment (such as that artificially created by activating DCs, macrophages, NK cells, or T cells using various stimuli), they may alter the outcome of the on-going immune response by altering the cytokine secretion profile of DC subsets (DC1 and DC2) and T-cell subsets (TH1, TH2, or TRegs), thereby resulting in a shift from a proinflammatory environment toward an anti-inflammatory or tolerant cell environment.

There was significantly lower number of nerve fibers stained with specific markers we used in the treatment group than in the control group. Endometriosis is a benign oestrogen-dependent inflammatory disease and hUC-MSCs could attune inflammatory effects of inflammatory factors such as cytokines, growth factors, and angiogenic factors. Other underlying mechanisms such as the differentiation of hUC-MSCs and/or the paracrine mediator secreted by hUC-MSCs may be also involved. A recent study also suggested that hUC -MSCs may serve as a promising treatment approach to ameliorate endometrial damage (37). Our study was the preliminary exploration of hUC –MSC treatment with endometriosis. The exact mechanism and outcome of hUC-MSCs remain to be elucidated in future studies.

Conclusion

We demonstrated that hUC-MSCs could reduce nerve fibers density in the treatment group and may provide a new potential therapeutic modality to endometriosis.

Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (81270671).

There is no conflict of interest in this article.

References

1. Giudice LC, Tazuke SI, Swiersz L. Status of current research on endometriosis. J Reprod Med. 1998; 43(3 Suppl): 252-262.
2. Lebovic DI, Mueller MD, Taylor RN. Immunobiology of endometriosis. Fertil Steril. 2001; 75(1): 1-10.
3. Kennedy S, Bergqvist A, Chapron C, D'Hooghe T, Dunselman G, Greb R, et al. ESHRE guideline for the diagnosis and treatment of endometriosis. Hum Reprod. 2005; 20(10): 2698-2704.
4. Togel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C. Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. Am J Physiol Renal Physiol. 2007; 292(5): F1626-1635.
5. Cao H, Qian H, Xu W, Zhu W, Zhang X, Chen Y, et al. Mesenchymal stem cells derived from human umbilical cord ameliorate ischemia/reperfusion-induced acute renal failure in rats. Biotechnol Lett. 2010; 32(5): 725-732.
6. Fu YS, Cheng YC, Lin MY, Cheng H, Chu PM, Chou SC, et al. Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopaminergic neurons in vitro: potential therapeutic application for Parkinsonism. Stem Cells. 2006; 24(1): 115-124.
7. Jomura S, Uy M, Mitchell K, Dallasen R, Bode CJ, Xu Y. Potential treatment of cerebral global ischemia with Oct-4+ umbilical cord matrix cells. Stem Cells. 2007; 25(1): 98-106.
8. Weiss ML, Troyer DL. Stem cells in the umbilical cord. Stem Cell Rev. 2006; 2(2): 155-162.
9. Ding DC, Shyu WC, Chiang MF, Lin SZ, Chang YC, Wang HJ, et al. Enhancement of neuroplasticity through upregulation of beta1-integrin in human umbilical cord-derived stromal cell implanted stroke model. Neurobiol Dis. 2007; 27(3): 339-353.
10. Yan Y, Xu W, Qian H, Si Y, Zhu W, Cao H, et al. Mesenchymal stem cells from human umbilical cords ameliorate mouse hepatic injury in vivo. Liver Int. 2009; 29(3): 356-365.
11. Weiss ML, Medicetty S, Bledsoe AR, Rachakatla RS, Choi M, Merchav S, et al. Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. Stem Cells. 2006; 24(3): 781-792.
12. Tokushige N, Markham R, Russell P, Fraser IS. High density of small nerve fibres in the functional layer of the endometrium in women with endometriosis. Hum Reprod. 2006; 21(3): 782-787.
13. Tamburro S, Canis M, Albuisson E, Dechelotte P, Darcha C, Mage G. Expression of transforming growth factor beta1 in nerve fibers is related to dysmenorrhea and laparoscopic appearance of endometriotic implants. Fertil Steril. 2003; 80(5): 1131-1136.
14. Tokushige N, Markham R, Russell P, Fraser IS. Different types of small nerve fibers in eutopic endometrium and myometrium in women with endometriosis. Fertil Steril. 2007; 88(4): 795-803.
15. Berkley KJ, Rapkin AJ, Papka RE. The pains of endometriosis. Science. 2005; 308(5728): 1587-1589.
16. Oosterlynck DJ, Cornillie FJ, Waer M, Vandeputte M, Koninckx PR. Women with endometriosis show a defect in natural killer activity resulting in a decreased cytotoxicity to autologous endometrium. Fertil Steril. 1991; 56(1): 45-51.
17. Wang G, Tokushige N, Markham R, Fraser IS. Rich in-

nervation of deep infiltrating endometriosis. Hum Reprod. 2009; 24(4): 827-834.
18. Berkley KJ, Dmitrieva N, Curtis KS, Papka RE. Innervation of ectopic endometrium in a rat model of endometriosis. Proc Natl Acad Sci USA. 2004; 101(30): 11094-11098.
19. Tokushige N, Markham R, Russell P, Fraser IS. Nerve fibres in peritoneal endometriosis. Hum Reprod. 2006; 21(11): 3001-3007.
20. Anaf V, Chapron C, El Nakadi I, De Moor V, Simonart T, Noel JC. Pain, mast cells, and nerves in peritoneal, ovarian, and deep infiltrating endometriosis. Fertil Steril. 2006; 86(5): 1336-1343.
21. Tokushige N, Markham R, Russell P, Fraser IS. Effect of progestogens and combined oral contraceptives on nerve fibers in peritoneal endometriosis. Fertil Steril. 2009; 92(4): 1234-1239.
22. Chegini N. Peritoneal molecular environment, adhesion formation and clinical implication. Front Biosci. 2002; 7: e91-115.
23. Gazvani R, Templeton A. Peritoneal environment, cytokines and angiogenesis in the pathophysiology of endometriosis. Reproduction. 2002; 123(2): 217-226.
24. Bedaiwy MA, Falcone T. Peritoneal fluid environment in endometriosis. Clinicopathological implications. Minerva Ginecol. 2003; 55(4): 333-345.
25. Bergqvist A, Bruse C, Carlberg M, Carlstrom K. Interleukin 1beta, interleukin-6, and tumor necrosis factor-alpha in endometriotic tissue and in endometrium. Fertil Steril. 2001; 75(3): 489-495.
26. Anaf V, Simon P, El Nakadi I, Fayt I, Simonart T, Buxant F, et al. Hyperalgesia, nerve infiltration and nerve growth factor expression in deep adenomyotic nodules, peritoneal and ovarian endometriosis. Hum Reprod. 2002; 17(7): 1895-1900.
27. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol. 1974; 2(2): 83-92.
28. Wu KH, Zhou B, Mo XM, Cui B, Yu CT, Lu SH, et al. Therapeutic potential of human umbilical cord-derived stem cells in ischemic diseases. Transplant Proc. 2007; 39(5): 1620-1622.
29. Bobis S, Jarocha D, Majka M. Mesenchymal stem cells: characteristics and clinical applications. Folia Histochem Cytobiol. 2006; 44(4): 215-230.
30. Fibbe WE, Noort WA, Schipper F, Willemze R. Ex vivo expansion and engraftment potential of cord blood-derived CD34+ cells in NOD/SCID mice. Ann N Y Acad Sci. 2001; 938: 9-17.
31. Ma L, Feng XY, Cui BL, Law F, Jiang XW, Yang LY, et al. Human umbilical cord Wharton's Jelly-derived mesenchymal stem cells differentiation into nerve-like cells. Chin Med J (Engl). 2005; 118(23): 1987-1993.
32. Nauta AJ, Kruisselbrink AB, Lurvink E, Willemze R, Fibbe WE. Mesenchymal stem cells inhibit generation and function of both CD34+-derived and monocyte-derived dendritic cells. J Immunol. 2006; 177(4): 2080-2087.
33. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005; 105(4): 1815-1822.
34. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. Nat Rev Immunol. 2008; 8(9): 726-736.
35. Pati S, Gerber MH, Menge TD, Wataha KA, Zhao Y, Baumgartner JA, et al. Bone marrow derived mesenchymal stem cells inhibit inflammation and preserve vascular endothelial integrity in the lungs after hemorrhagic shock. PLoS One. 2011; 6(9): e25171.
36. Qian H, Yang H, Xu W, Yan Y, Chen Q, Zhu W, et al. Bone marrow mesenchymal stem cells ameliorate rat acute renal failure by differentiation into renal tubular epithelial-like cells. Int J Mol Med. 2008; 22(3): 325-332.
37. Yang X, Zhang M, Zhang Y, Li W, Yang B. Mesenchymal stem cells derived from Wharton jelly of the human umbilical cord ameliorate damage to human endometrial stromal cells. Fertil Steril. 2011; 96(4): 1029-1036.

Original Article

Exploration of Infertile Couples' Support Requirements: A Qualitative Study

Fatemeh Jafarzadeh-Kenarsari, Ph.D.[1], Ataollah Ghahiri, M.D.[2], Mojtaba Habibi, Ph.D.[3], Ali Zargham-Boroujeni, Ph.D.[4*]

1. Nursing and Midwifery Student Research Center, Faculty of Nursing and Midwifery, Isfahan University of Medical Sciences, Isfahan, Iran
2. Department of Obstetrics and Gynecology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
3. Family Research Institute, Shahid-Beheshti University, Tehran, Iran
4. Nursing and Midwifery Care Research Center, Faculty of Nursing and Midwifery, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: Due to high prevalence of infertility, increasing demand for infertility treatment, and provision of high quality of fertility care, it is necessary for healthcare professionals to explore infertile couples' expectations and needs. Identification of these needs can be a prerequisite to plan the effective supportive interventions. The current study was, therefore, conducted in an attempt to explore and to understand infertile couples' experiences and needs.

Materials and Methods: This is a qualitative study based on a content analysis approach. The participants included 26 infertile couples (17 men and 26 women) and 7 members of medical personnel (3 gynecologists and 4 midwives) as the key informants. The infertile couples were selected from patients attending public and private infertility treatment centers and private offices of infertility specialists in Isfahan and Rasht, Iran, during 2012-2013. They were selected through purposive sampling method with maximum variation. In-depth unstructured interviews and field notes were used for data gathering among infertile couples. The data from medical personnel was collected through semi-structured interviews. The interview data were analyzed using conventional content analysis method.

Results: Data analysis revealed four main categories of infertile couples' needs, including: i. Infertility and social support, ii. Infertility and financial support, iii. Infertility and spiritual support and iv. Infertility and informational support. The main theme of all these categories was assistance and support.

Conclusion: The study showed that in addition to treatment and medical needs, infertile couples encounter various challenges in different emotional, psychosocial, communicative, cognitive, spiritual, and economic aspects that can affect various areas of their life and lead to new concerns, problems, and demands. Thus, addressing infertile couples' needs and expectations alongside their medical treatments as well as provision of psychosocial services by development of patient-centered approaches and couple-based interventions can improve their quality of life and treatment results and also relieve their negative psychosocial consequences.

Keywords: Crisis, Infertility, Needs, Qualitative Research, Support

Citation: Jafarzadeh-Kenarsari F, Ghahiri A, Habibi M, Zargham-Boroujeni A. Exploration of infertile couples' support requirements: a qualitative study. Int J Fertil Steril. 2015; 9(1): 81-92.

Received: 20 Apr 2014, Accepted: 28 Sep 2014
* Corresponding Address: P.O. Box: 6699398, Nursing and Midwifery Care Research Center, Faculty of Nursing and Midwifery, Isfahan University of Medical Sciences, Isfahan, Iran
Email: zargham@nm.mui.ac.ir

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 81-92

Introduction

Infertility is a stressful experience and complicated crisis for infertile couples (1, 2) which can coincide with various social, psychological, physical and financial stresses (3). Researchers have compared the stress induced by infertility with the feeling when losing a child or a spouse (4). Infertility was defined, in the International Conference of Infertility in Bangkok in 1998, as a global health problem with physical, psychological and social dimensions (5). The prevalence of infertility ranges from 5 to 30% in different countries (6). The prevalence of primary infertility has been reported as 24.9% among Iranians in 2004. In addition, about a fourth of Iranian couples have experienced primary infertility at some point in their lives (7).

Inability to achieve pregnancy is usually coupled with low confidence, depression, sexual problems, feeling of shame or guilt, lack of communication with friends and family members and occupational challenges. These conditions can be deteriorated in different socio-cultural contexts, especially when being concurrent with medical, emotional and communicative challenges triggered by infertility (8). Infertility can negatively affect social, personal and marital relations that results in a mental imbalance leading to divorce (6). The socio-cultural context can be an important factor in one's conceptualization of and response to, infertility (9).

Nowadays, there are tremendous changes in family values, but parenthood has still been an undeniable importance for women and men (10). Infertility carries much social stigma in many communities, leading to many social problems for infertile couples (11). In Iranian culture, infertility coincides with numerous psychological and social challenges, especially for women, and affects all aspects of infertile couples' life (10, 12), including their emotional, psychological, physical and social performances (11).

Diagnosis and treatment of infertility is usually coupled with noticeable stress (13). Assisted reproductive technology (ART) has been regarded as one of the most stressful infertility treatment method. The application of these technologies is coupled with emotional and physical burden and a high level of depression and stress (1, 14, 15). Thus, infertility and its treatment are considered as significant medical issues affecting quality of life of infertile couples (11, 16).

Various studies have demonstrated that infertile couples have a variety of needs (12, 15, 17-20) that require emotional support, professional psychosocial services, couple-centered interventions and patient-centered approaches (21). Due to the diversity of needs and problems of infertile patients, this necessitates to identify and address infertile patients' needs along with their regular medical treatments. Provision of fertility care based on patients' needs and expectations (patient-centered fertility care) is considered as one of their fundamental right (22). Patient-centered fertility care not only fulfills patients' needs and expectations, but also has significant clinical benefits such as improving patients' quality of life and emotional health as well as decreasing their distress (12, 15). In addition, it is recommended to provide infertility treatment alongside patient-centered care in order to promote patients' well being during treatment and increase treatment success rates (23). To this end, healthcare professionals are in a good position to sympathize with infertile patients during their treatment period and encourage them to express their experiences, needs and concerns (24).

Different aspects of infertility have been studied in various quantitative research works in Iran. However, qualitative studies are so limited and the nature of Iranian infertile couples' needs has not been completely justified yet. Exploration of infertile couples' viewpoints on their needs can be a prerequisite for planning effective supportive treatment interventions (3, 24, 25) for fulfilling these needs and improving treatment capabilities of healthcare professionals. On one hand, need is a complicated phenomenon and is rooted in the cultural, social and economic context of communities (26). On the other hand, quantitative approaches lack the required capability for providing a detailed description of the phenomena and patients' viewpoints. Thus, qualitative researches seem to be appropriate for exploration of needs and experiences regarding fertility care (15). They also offer exclusive capability to provide a deeper

understanding of phenomena (27). In addition, they can provide descriptions of life experiences, human interpretations and perceptions in the respective cultural and social contexts (27, 28). Thus, considering the existing gap, the current qualitative study has been conducted in an attempt to explore and understand infertile couples' experiences and viewpoints with regard to their needs.

Materials and Methods

Participants and data gathering methods

This study was part of a larger sequential exploratory mixed methods research with the purpose of identifying infertile couples' needs, development and validation of a tool for measuring couples' needs. The first phase of this mixed methods research was a qualitative study with a content analysis approach. The main purpose of this qualitative phase was exploration of infertile couples' needs and identifying the primary items of the needs assessment tool. The second phase of the research was a quantitative study aimed at validation of the needs assessment tool. Part of the data obtained from qualitative interviews with infertile couples and key informants was reported in this paper. Another part of the data obtained from qualitative interviews with 17 infertile couples was reported in another published paper (22).

This study was conducted in settings and places, like a state-run infertility center in an university hospital, private offices of infertility specialists and private infertility centers in Isfahan and Rasht, Iran, during 2012-2013, where infertile couples were accessible. Due to insignificant of the study population in qualitative research projects (29), there was no limitation for places as research setting for the current study. Participants of the study included 26 infertile couples.

The researchers interviewed each infertile couple using a couple-based approach. This approach is appropriate because infertility is a condition involving the two partners in a joint relationship (30). A dyadic approach were applied for 17 couples to establish a better interaction between spouses and interviewer, while to provide a clearer picture of couples' needs and viewpoints. Due to husbands' busy schedules (n=3), their unwillingness to participate in the interviews (n=4), becoming ill at the time of interview (n=2) and only wives (n=9) as representatives of the couples were individually interviewed.

In addition, interviews with seven medical professionals (3 gynecologists and 4 midwives) as key informants were carried out to obtain more comprehensive and deep information regarding infertile couples' needs that may be overlooked by couples. Interviews were carried out in infertility care centers or the private offices.

Infertile couples were selected through purposive sampling method with maximum variation, including different causes of infertility, different types of infertility (primary and secondary), a wide range of age, at different stages of infertility treatment and different durations of infertility. The inclusion criteria for infertile couples were as follows: at least one year after marriage, confirmation of their infertility by the specialist, Iranian nationality, proficiency in Farsi, willingness to participate in the study, no history of mental disorders (during last 12 months) or physical disabilities and ability to express their feelings and experiences. Also the medical personnel were selected through purposive sampling method and their inclusion criteria were as follows: willingness to participate in the study, adequate experience in infertility treatment, and caring for infertile couples. All participants were interviewed in one or two sessions lasting approximately 20 to 60 minutes.

In-depth, unstructured interviews and field notes were used for data gathering among infertile couples. The general question of the research posed for the infertile couples was "Tell me about your infertility experiences and the problems (concerns) you have encountered in this regard." In proportion to the received answers, explorative questions were asked for expansion of findings such as, "Can you mention some more examples? and What does that mean?". Demographic information were also asked in addition to questions related to the experience of infertility, such as: age, gender, education, causes of infertility and its duration, number of children in couples with secondary infertility, type of treatments received, etc. Researchers also used

field note-taking for data gathering to this end, the first author attended the infertility treatment clinics and infertility specialists' offices so as to observe the interaction between patients, their families and medical personnel.

Semi-structured interviews with medical personnel were carried using questions extracted from main subjects and ideas mentioned by infertile couples in their interviews. Some of the questions asked during interview sessions with the medical personnel were the following: "In your opinion, what issues and problems are infertile couples facing?", "What problems are these couples facing during diagnosis and treatment procedures?", "What are their most significant needs?", "What solutions do you recommend for fulfilling their needs?", "Is the quality of behavior of medical team important for motivating patients to continue their treatment process?", "Are patients usually complaining about the presence of other patients in the office during their check-up time?", and "What is your idea on this?".

Interviews and field note-takings were continued up to data saturation. All interviews were conducted in Farsi by the first author and then translated into English. All interviews were recorded, transcribed verbatim, and then analyzed concurrently. The interview data were analyzed using conventional content analysis.

Data analysis

Drawing on steps recommended by Graneheim and Lundman (31), the following procedure was employed for analysis of the collected data: i. Transcribing all interviews immediately after each interview, ii. Reading the whole transcription for general comprehension of the content, iii. Determining the number of meaning units and primary codes, iv. Categorizing similar primary codes in more comprehensive categories and v. Determining the latent content (themes) in the data.

Trustworthiness of data

To ensure the credibility, feedback was obtained from participants (member checking) and the number of interviews with some participants was increased. To increase the transferability of findings to other settings and groups, participants with various experiences in the realm of the subject under study and with maximum variation were selected. Also, confirmability and dependability of findings were established through peer checking, peer debriefing, reviewing of transcripts by some participants, researchers' interest in the phenomenon under study and their prolonged engagement in data.

Ethical considerations

This study were confirmed by the Ethical Committee of Isfahan Universtiy of Medical Sciences, Isfahan, Iran. Also, prior to interviews, participants were made aware of the objectives of the research and an informed consent was obtained. Oral and written permissions were obtained from them for recording their interviews and they were assured that the gathered data would only be used for research objectives. It was also announced to the participants that they could withdraw the research anytime they wish and their information would remain confidential during and after the research.

Results

Out of 26 couples (n=43) participating in the study, females' age ranged 20 to 47 with a mean age of 31.36, while males' age ranged 25 to 55 with a mean age of 36.5. Out of 7 members of medical personnel, two were female, one was a male gynecologist and four were midwives. Twenty couples (76.9%) had primary infertility and the rest experienced the secondary type. Duration of the couples' infertility ranged 1 to 21 years (Mean: 4.44 years) and their treatment ranged 1 month to 20 years. Infertility was of female-related in 15 couples, of male-related in 5 couples and of mixed causes in 4 couples. Moreover, two couples had unexplained infertility. All couples with secondary infertility, except one who had no children (with a history of still birth in 28th week of the first pregnancy in 3 years ago), had one child. Educational level of female and male participants ranged from secondary school to higher education (university).

Data analysis yielded four main categories of infertile couples' needs including: i. Infertility and social support, ii. Infertility and financial support, iii. Infertility and spiritual support and iv. Infertility and informational support. Assistance and support constituted the main theme of all the categories (Table 1).

Table 1: Themes, categories and sub-categories

Theme	Categories	Sub-categories
A need for assistance and support	Infertility and social support	-Spouse's support
		-Familial and social support
	Infertility and financial support	-Efficient medical insurance
		-Support from authorities of governmental and non-governmental agencies
	Infertility and spiritual support	-Hope in God
		-Communication with God
	Infertility and informational support	-Need to information on disease
		- Need for educating and preparing the family and society

Infertility and social support

Spouse's support

A demand for mutual understanding between husband and wife, their emotional support for one another, the compassion and love shared between them, encouraging persistence with treatment, accompanying each other during the treatment and respect for spouse's opinion about the method of treatment were among the instances emphasized by participants in their interviews. It can be inferred from the most participants' statements that spouse's full support can be encouraging and can inspire self-confidence, security and equanimity in the onerous path of infertility experience. One of the women (couple number: 15) stated in this regard:

I was so upset when I found out about my problem. I felt so desperate and completely hopeless. I really needed my husband's support. Fortunately, my husband was there by my side supporting me.

This woman's husband added:

I didn't want her to think that I am pretending in front of her. In our marriage the most important things for me are her health and peace of mind. I have already told her many times and am telling her now, that we have no problem. She should not get disappointed. This is surely God's will not to have any kids for now.

As the burden of infertility falls principally on women, they are mainly involved in infertility diagnosis and treatment processes; therefore, female participants showed a greater need for the spouse's accompaniment during the treatment process. Most of female participants preferred their spouse by their side when attending infertility clinics. One of the participants (couple number: 1) stated in this regard:

I like to have my husband by my side to empathize and support me when coming to clinic, but men do not like to sit somewhere idle, they are very impatient and always in rush to get back to work.

This woman's husband added:

She is right, it is difficult to be alone. I know that she needs my support, but I am so busy and not able to leave my office to accompany her for coming to the clinic in the morning. When I have to be here, I would do my best. But she must to understand my work condition.

But some of the female participants had no tendency for having their husband by their side during the whole process of treatment. One of the participants (woman number: 24) said in this regard:

I would like my husband to be with me in some places. But also I prefer not to involve him in some certain places. Sometimes I think I can decide bet-

ter when I am alone. Because I think it is my problem. It is better not to inform him initially. I will break the news to him little by little and I believe this makes it easier to get his acceptance. Hopefully, he will response positively.

"They don't let husbands into the doctor's office as they send three women together and they say that men are 'namahram' (i.e. men or women other than one's blood relatives and spouse). Islam bans any relationship with a Namahram person that involves seeing any part of the body except the face, hands and feet. Therefore, I didn't come with my wife anymore." one of the men (couple number: 10) said and tried to explain why he did not accompany his wife to gynecologist's office. Also this man's wife added:

Anyhow, doctor's office is far away from where we live, so it gets dark by the time we get back home. As he said, he refuses to accompany me, so I had to ask my sister to be with me to avoid being alone.

One of the gynecologists participating in the study said:

This is one of the major problems we are all facing in this regard. This is due to overloaded doctors' office. We are not like developed countries where the government mandates that only 4 patients must be visited in an hour. If I visit four patients in an hour, I would be at my office until next day. Therefore, I must visit patients in group of four per office visit.

Another gynecologist stated in this regard: "I believe this is necessary and this is considered as patient's right. Scientifically speaking, both men and women need to be visited in their first referral. But this is impossible due to overloaded doctor's office." Another participant (couple number: 4) mentioned why her husband refused to accompany her to the gynecologist's office, although she admitted that she didn't like him to do so:

My husband supports me financially during the treatment, but he prefers not to be with me during the visits. He thinks that this is a women issue to handle and I must do it by myself.

This woman's husband added:

A gynecologists' office is always full of patients, and there is not enough room to sit, even for pregnant women. To be honest, men should not be there.

Although most of the participating couples asserted that infertility had no negative effect on their interpersonal relations with their partner, they believed their attempts for having a baby were to ease the loneliness they felt, to preserve the sweetness of their life, to strengthen their relationship and to value loyalty and faithfulness of their spouses. This was more evident in infertile women as they saw the continuation of infertility and a childless life as a threat to their marital life and a trigger for separation and divorce. One of the participants (couple number: 5) said: "I had always the feeling of fear that my husband would decide to marry another woman because I couldn't get pregnant. I used to have this fear". This woman's husband said:

I don't know how these thoughts came to her mind. I have told her several times that I love my life. I believe that, it's God's will to have a kid or not.

Some of the female participants (couples numbers: 12, 14, 15, and 17) also believed that if the infertility problem could not be treated and they would have no children of their own, the husbands should understand their ever-greater needs for pregnancy, child bearing or having children for easing their loneliness. They also should cooperate and agree on alternative solutions such as donated embryo or adoption. In this regard, some of husbands disagreed with oocyte or egg donation and adoption. Some also chose adoption over egg donation. Some also said that they had not thought about these and had no idea. Some women (women numbers: 8, 20 and 22) also were not willing to adopt any kids or receive any donated oocyte or embryo. One of the gynecologists said:

Something must be done in this regard, so receiving embryo and oocyte of someone else become more understandable. One of the current problems is that assisted reproductive methods such as egg donation or gestational surrogacy are not culturally accepted for infertile couples or their families.

Familial and social support

Other important points in participants' experiences were as follows: i. Sympathy and emotional support provided by their family, friends, and society, ii. Respect for couples' privacy, iii. Encouragement for continuation of treatment, iv. Offer-

ing hope, v. Social acceptance and vi. Vocational support for the employed infertile couples. Due to judgment made by others and families, especially those of husband's, their direct and indirect interventions, the social stigma attached to infertile patients, discouragement and hopelessness in continuing the treatment, most of the patients (who were mainly women) tried to restrict their social relations and tell white lies in response to others' curiosity with regard to their infertility."Currently, my husband and I have tried to limit our relationship with his family for this issue, once in every two or three months." one of the couples (couple number: 20) said in this regard. One of the male participants (couple number: 11) stated:

Others used to ask me whether we had problem to get pregnant, but I reply that there was no problem and we did not want to have any kids. As we are living independently, we don't let others interfere with our privacy to cause further problems.

Two of female participants (couples numbers: 16, 23) whose infertility was male-related stated that they preferred to let their husband's family know about the cause of the problem to remove any doubts for infertility. One of the male participants (couple number: 10) who had an interesting opinion stated:

Our families know that our problem is female related infertility. I wanted them to know the true cause of the problem. Otherwise, they would doubt me and think it's my fault. I didn't want them to have such an idea about me.

"I don't know. It was his idea and I had to tell our families the truth. Although it was a bit difficult for me, my husband wants the other do not blame him for infertility," this man's wife mentioned.

The life of infertile couples is affected considerably by reactions and behaviors of families and friends. Unnecessary interventions and some behaviors, like pity, made by families annoy the couples (especially women), disrupt their equanimity and often destabilize their relationships. "We expect family and acquaintances to show less pity and to refuse to say such things, 'they have no kids, or our brother does not have any children yet,' that is so annoying," one of participants (couples number: 13) mentioned. "I think family support is very important. Both families of the husband and wife should participate in this issue. They should not look for someone to blame," one of the participating gynecologists stated.

Few couples also expressed their satisfaction and happiness with the supportive and positive role of their families in encouraging them to continue the treatment, maintain their equanimity during the treatment and bear the problems until achieving their goal.

Some couples were annoyed by the behavior and negative approach toward infertility that exerted by the society. "I heard from one of my students' parents saying that they should not enroll their kids in so-and-so's class as she is nervous and impatient with kids (tearful eyes)," one of the participants (couple number: 8) stated in this respect. "It is not like this at all. Both my wife and I love children dearly. Although we do not have any children, it doesn't mean that we treat people and their children badly," this woman's husband added.

As women are more involved in infertility treatment process, the employed women were concerned to leave the office in order to participate in treatment programs. "I am unable to leave the office, easily. This is also one source of stress and anxiety," one of the participants (woman number: 24) stated in this regard.

Infertility and financial support

Enormous expenses of infertility diagnosis and treatment, the long and iterative nature of these processes and patients' financial limitations were among patients' gravest concerns that were underlined frequently in their interviews. Most of the participants counted the enormous expenses and their financial problems as the cause of delayed commencement, or probable discontinuity of treatment procedures in the case of failed initial efforts. A demand for financial support from family and acquaintances, insurance companies, as well as cooperation from governmental authorities and nongovernmental entities were emphasized repeatedly in participants' statements and field notes taken during the study. One of the couples (couple number: 9) in this regard mentioned:

We mostly borrow some money for our treatment. We are not in a good financial condition. Nobody in our families could help us. In short, authorities should support more those who are in bad financial situation.

One of the gynecologists participating in the study said:

I think one of the major problems is their financial challenges. We witness patients who practically sell their houses and get large loans, so they can pay for infertility treatment expenses.

Another couple (couple number: 3) said: "We wish infertility were treated like special diseases and would get the same degree of attention. We wish insurance would pay for the treatment and medical expenses." "Government should help and pay for part of treatment cost. It would be great if we could have an infertility committee like Emdad Committee." Another couple (couple number: 5) mentioned.

Infertility and spiritual support

A need for a superior power (God), faith in God's will, connection with God and asking for His help were among other issues which were emphasized by most of participants. One of couples (couple number: 6) said in this regard: "We are sure that we are under supervision of God compassion. We have faith in God's will. We are satisfied with His will and decision. Doctors are just a means that will help us." Another female participant (couple number: 7) added:

I used to cry and be severely depressed, but praying gives me hope and helps to imagine myself having a kid. I ask God to help me to tolerate not having the child. Praying can lift me into a new lightness of heart.

"I believe it is not late at all. My wife is not too old to have a kid. Anyhow, we have faith in God's will. First God and then the doctor will help us. Nothing happens if God does not give us any kids," added by this woman's husband. With regard to the significance of infertile couples' religious and spiritual values, one of the midwives participating said:

All people live with hope. Trusting God and prophets can help the patients a lot in their life. It might be better to have a religious counselor in infertility centers, so these couples could receive some religious consultation before and after treatment, so they may find some peace.

Some of the participants believed that their infertility was a kind of divine trial and they should be satisfied with God's will and should not be ungrateful as God knows their interest better. Some also viewed infertility as a divine punishment for their past ungratefulness. "I was not interested in kids when I was single. I sometimes think my husband's infertility is a kind of punishment by God," one of the participants (woman number: 23) mentioned. "We tell ourselves we may have committed a sin that is why we have such problems. Perhaps God is testing us," another couple (couple number: 2) mentioned.

Infertility and informational support

The other issue emphasized in participants' interviews was a demand for obtaining comprehensive information from the medical and treatment teams during diagnosis and treatment of infertility. Educating society with regard to infertility and new available treatments were other aspects of the issue emphasized by participants. Patients' inadequate knowledge on the nature of the condition, the outcomes of a diagnostic and treatment method and ignorant behavior of medical teams to patients' questions were mentioned by participants as the current problems in the treatment process. Educating and providing information to patients lead to their trust and cooperation during the treatment. "We are so unhappy because they refuse to explain what the problem is, what the cause is, how long the treatment takes, or how hopeful we can be on the success of treatment. We have to search the web to find some answers," one of the couples (couple number: 2) mentioned in this regard. Another couple (couple number: 3) said:

If the doctor gives some information about our condition, we would worry less. Sometimes medical personnel answer some questions may cross our mind. But the answers are uttered too fast that we don't understand a word they are saying.

One of midwives participating in this study recommended as the following on the necessity of information dissemination and training on infertility:

It may be better to have informative and training courses for the youth on the issue of infertility, its preventive methods and treatment in universities. It is not a bad idea to acquaint the youth with this phenomenon before marriage.

One of the gynecologists participating in the study said:

This must be cultivated in our culture. Educational movies should be made and broadcast in public media for expanding the general knowledge on this issue. People should know that, due to the scientific progress made recently, there is no such thing as infertility anymore, but there are solutions.

Discussion

The current study is the first qualitative study trying to explore the Iranian infertile couples' needs. A close overview of the research findings from interviews showed that a need for support and assistance is among infertile couples' main demands, so that they can cope with the stress caused by infertility. This need includes four main categories as follows: i. Infertility and social support, ii. Infertility and financial support, iii. Infertility and spiritual support and iv. Infertility and informational support.

The findings of the study showed that spouse's support is one of the main sources of support for patients, especially for female infertile patients. Even when family and acquaintances fail to play a supportive and positive role, spouse's empathy, affection, loyalty and adequate support can provide the necessary emotional support for the partner to keep her/his hope alive and to be confidence in order to continue the treatment program. Results obtained by Akizuki and Kai's study on Japanese infertile women suggested that partner's support plays a vital role and decreases the need for others' support (32). Abbasi-Shavazi et al. (33) realized that the infertile women who were supported by the good behavior of their husbands during the treatment processes, despite having no children, could manage their life and activities more efficiently. Infertile couples need each other's support to better cope with their issue, so if one partner evades his/her responsibilities, the other partner who is usually the woman (not always) would be hurt (34).

Another interesting finding in this study was attitudes expressed by most participants about spouse's accompaniment during diagnosis and treatment of infertility. As mentioned in the results section, in Iran, like other developing countries, treatment of infertility is mainly taken up by women without taking the causes of infertility into consideration. This issue adds to their burden of responsibility and increases their physical and psychological stress (35). Thus, most female participants considered spouse's accompaniment as an important source of support, and interpreted their spouse's absences as: busyness, lack of enough space in clinics (especially in gynecologists' offices), socio-cultural limitations, the great number of patients attending these centers and time limitations for patient visit. To encourage and improve men's cooperation in infertility treatment programs, effective policies are required for removing cultural, religious, ethical and social barriers rooted in the society. Apart from basic facilities, persistence, more supervision by national health care authorities, adequate staff training, facilitating flexibility in clinics organization and educating the society are also required.

Considering the significance of spouse's support and its effect on the relationship between the couples as well as the stress induced by infertility (especially in women), infertility should be considered as an issue involving both men and women in clinical settings (36). Thus, healthcare professionals should consider an infertile couple as a unit and pave the way for husbands' participation in diagnostic-treatment programs. These programs help husbands to change their views and interests and facilitate their cooperation during the treatment process and consequently improve the relationship between the wife and the husband (37).

The other finding of the current study was couples' various viewpoints on donated embryo and oocyte, gestational surrogacy, and adoption. This challenge is rooted in cultural and religious beliefs and attitudes of participants. This is in keeping with the findings obtained by previous studies conducted on this subject (12, 38).

The findings of the current study also showed the support from the family, acquaintances and society as the other important need, expressed by the infertile couples. Evidence has shown that positive social interactions and socio-emotional support have a salutary effect on infertile couples' psychosomatic health, ultimately leading to a decrease in the negative impacts of stress. In addition, they psychologically adapt better and take a proper action against infertility and accept the situation more easily (4, 36, 39). Adequate social support and understanding from family and friends help infertile men and women feel better about themselves, establish a better relationship with others,

and respond better to the treatment (4, 40). Evidence has shown that women more than men tend to have social support. The results of other studies have shown that family support affect infertility stress in women, directly and indirectly. Family support not only decreases social problems of infertility, but also leads indirectly to a decrease in infertility stress in four aspects of communicative problems, sexual problems, non-acceptance of a childfree lifestyle and a need for parenthood (36). It is also worth to note that people tend to have their family and friends' support for adapting to the issue of infertility. Some others tend to hide their infertility and want to receive no support from others (41).

Due to the specific cultural and social structure in Iran, the issue of infertility takes a deeper meaning in the Iranian social context. Hence, the role of different tribes, acquaintances and friends proves to be significant and even vital, in infertile couples' life (20). To this end, the findings of our study indicated that most infertile couples, especially infertile women, preferred to keep their infertility issue as a secret and avoid mentioning it, especially to their in-law's family. This finding is consistent with those obtained by other studies (12, 20, 33). Mollaiy nezhad et al. (42), have mentioned that the responsibility of infertility is on women's shoulder in most of the communities and her infertility is usually rebuked by her in-law family. This issue ultimately leads to the concern that woman's infertility is an adequate cause for divorce and man's marriage with another woman.

In addition, the current study showed that some of the participants had to tell the truth to their in-law families due to financial dependence, living with them, avoidance of misjudgment and husband's request. It is noted that some of the participants confirmed the positive role of their families in giving hope, providing financial support and consolidating couple's relationship. This has also been reported in the study conducted by Khodakarami et al. (20).

The findings of the present study showed that the overlap between office hours and treatment schedule with required arrangements for paid and medical leave are among concerns for some infertile couples who hold an office job. This issue jeopardized some patients' job position as well. The results obtained in India also indicated that many of employers were not much familiar with infertility. Thus, employers need to acquaint themselves with special needs of employees dealing with infertility and arrange the required facilities, such as flexibility in their work schedule in order to attend the treatment programs (43).

Other significant concerns during treatment were enormous treatment expenses and inefficacy of the insurance program for their treatment. Due to financial challenges, some of couples were forced to postpone their treatment or worried about its continuation. In countries where treatment expenses are mainly paid by the patients, financial problems usually play the most effective role in patients' decision whether to continue or abort their treatment program (44). Most of the couples deal with many financial problems as a part of infertility treatment programs that are not usually covered by insurance agencies. These results are consistent with those obtained by other studies (12, 20, 42). Most of diseases are covered by insurance, but infertility expenses are exceptions to insurance coverage and such discrimination is unfair (43). In this regard, the participated infertile couples requested support from the health care system and governmental authorities and non-governmental agencies. Patients participating in the study conducted by Fahami et al. (12) had similar requests as the participants of current study.

The results obtained in this study showed that spiritual and religious beliefs played an important role in infertile couples' equanimity and could be considered as a source of support for their adaptation with infertility stress. The results obtained by other studies also indicated that religious beliefs effectively decreased the stress of infertile couples (12, 38).

The results of the present study also showed that information support is considered as another requirement of infertile couples. This has also been reported in the study conducted by Akizuki and Kai (32). Evidence has shown that healthcare providers sometimes tend to underestimate and to ignore a patient's need for information acquisition (45). Inefficiencies in providing information force patients to obtain information from other sources such as Internet, books or other patients. While providing adequate information to patients by the medical team is considered as patients' natural right, it ultimately results in gaining patients' trust and satisfaction and reducing stress of infertile

couple (12).

Another finding of the present study was the necessity of educating society and attempting to raise the awareness about infertility and the new treatment methods. One of the main reasons why people surrounding an infertile couple do not know how to treat them is due to their unawareness about different aspects of infertility (40). Educating the society and infertile couples' families can reduce the psychological burden of these couples (20).

There are some strong points in the current study. Firstly, it studied both infertile men and women and did not focus solely on infertile women. In comparison with individual interviews, we used a dyadic approach and conducted interviews with husband and wife in a joint session that is of great value in infertility-related studies as infertility is a dyadic issue and not an individual one (30). Secondly, all interviews were conducted by an interviewer (the first author), while analysis was carried out by all four researchers, adding to the credibility of the research findings (46). Thirdly, to attain more comprehensive information on infertile couples' needs, several key informants, such as gynecologists and midwives who had experience with infertile couples, were also interviewed.

A few limitations need to be mentioned. First, as a dyadic approach was used in the interviews with participants, identification of gender differences with regard to the needs experienced by infertile men and women was not possible. Despite the fact that many findings indicate that women, in comparison with their husbands, bear greater negative impacts due to infertility (30), more studies are required to identify infertile men and women's needs, individually, by conducting dyadic and individual interviews with infertile couples. Second, due to the qualitative nature of the present study, purposive sampling of participants and the limited number of participants, the results of this study cannot be applied to all Iranian infertile couples. Further studies including a larger number of participants categorized in terms of their gender, age, type of infertility, different stages of treatment and different outcomes of treatment are recommended to assess the needs of these patients as per above criteria.

Conclusion

This study described the four main categories of infertile couples' needs, part of their challenges and concerns and necessity for cooperative assistance and support. Considering the complete descriptions provided on infertile couples and their needs, the health care professionals and authorities ought to attempt to provide support and consultative programs suiting the infertile couples' needs. Also it is required to encourage the quality improvement of the healthcare services by development of patient-centered approaches and couple-based interventions so as to reduce infertile patients' psychological stress induced by fertility problems.

Acknowledgements

This study is part of first author's Ph.D. dissertation in Reproductive Health that was ratified and funded by Isfahan University of Medical Sciences, Isfahan, Iran, under the Research Number: 391159. The authors would like to thank the supporting authorities. In addition, the authors would like to express their gratitude to all authorities of infertility treatment clinics, specialists and midwives participating in this study and all infertile couples who contributed to this study. The authors declare no conflict of interest in this study.

References

1. Hammerli K, Znoj H, Berger T. What are the issues confronting infertile women? a qualitative and quantitative approach. Qual Rep. 2010; 15 (4): 766-782.
2. Ramazanzadeh F, Noorbala AA, Abedinia N, Rahimi Forooshani A, Naghizadeh MM. Psychiatric intervention improved pregnancy rates in infertile couples. Malays J Med Sci. 2011; 18(1): 16-24.
3. Simbar M, Hashemi S, Shams J, Alavi-Majd H. Association between infertile women's anxiety with art success rates. J Reprod Infertil.2009; 10(4):279-285.
4. Torrez BL. Psychological stress of infertility: A conceptual analytic study. Presented for the Ph.D., San Francisco. Alliant International University. 2010.
5. Abbasi Molid H, Rezazadeh Varaghchi J. Psychological meta-analysis of the infertility researches in Iran. IJOGI. 2012; 15(13): 26-33.
6. Baghiani Moghadam MH, Aminian AM, Abdoli AM, Seighal N, Falahzadeh H, Ghasemi N. Evaluation of the general health of the infertile couples. Iran J Reprod Med. 2011; 9(4): 309-314.
7. Vahidi S, Ardalan A, Mohammad K. Prevalence of primary infertility in the Islamic Republic of Iran in 2004-2005. Asia Pac J Public Health. 2009; 21(3): 287-293.
8. Burnett JA. Cultural considerations in counseling couples who experience infertility. J Multicult Couns Devel. 2009; 37 (3): 166-177.
9. Callister LC. The pain and the promise of unfilled dreams: infertile couples. In: Crane DR, Marshal ES, editors. Handbook of families and health: interdisciplinary perspectives. CA: SAGE publications; 2005; 96-112.
10. Riahi ME, Zarezade Mehrizi E. A study on the gender

differences in psycho-social consequences of infertility: infertile couples using Yazd' Infertility Center Services. Women's Strategic Studies. 2012; 14(56): 155-210.
11. Aarts JW, van Empel IW, Boivin J, Nelen WL, Kremer JA, Verhaak CM. Relationship between quality of life and distress in infertility: a validation study of the Dutch FertiQoL. Hum Reprod. 2011; 26(5): 1112-1118.
12. Fahami F, Hosseini-Quchani S, Ehsanpour S, Zargham-Boroujeni A. Lived experience of infertile men with male infertility cause. IJNMR. 2010; 15(Special Issue): 265-271.
13. Latifnejad Roudsari R, Rasoulzadeh Bidgoli M, Mousavifar N, Modarres Gharavi M. The effects of participatory consultations on perceived stress of infertile women undergoing IVF. Iran J Obstet Gynecol Infertil. 2011; 14 (4): 22-31.
14. Dancet EAF, van Empel IWH, Rober R, Nelen WLDM, Kremer JAM, D'Hooghe TM. Patient-centred fertility care: a qualitative study to listen to the patient's voice. Hum Reprod. 2011; 26 (4): 827-833.
15. Van Empel IWH, Nelen WLDM, Tepe ET, van Laarhoven EA P, Verhaak CM, Kremer JAM. Weaknesses, strengths and needs in fertility care according to patients. Hum Reprod. 2010; 25(1): 142-149.
16. Yaghmaei F, Mohammadi S, Alavimajd H. Developing "quality of life in infertile couples questionnaire" and measuring its psychometric properties. J Reprod Infertil. 2009; 10(2): 137-143.
17. Schmidt L. Infertile couples' assessment of infertility treatment. Acta Obstet Gynecol Scand. 1998; 77(6): 649-653.
18. Daniluk JC. "If we had it to do over again...": couples' reflections on their experiences of infertility treatments. Fam J Alex Va. 2001; 9(2): 122-133.
19. Schmidt L, Holstein BE, Boivin J, Sangren H, Tjørnhøj-Thomsen T, Blaabjerg J, et al. Patients' attitudes to medical and psychosocial aspects of care in fertility clinics: findings from the Copenhagen Multi-centre Psychosocial Infertility (COMPI) Research Programme. Hum Reprod. 2003; 18(3): 628- 637.
20. Khodakarami N, Seddiq S, Hashemi S, Hamdieh M, Taheripanah R. The disregarded rights of infertility: a phenomenological study. IJME. 2009; 2(3): 39-50.
21. Schmidt L. Psychosocial burden of infertility and assisted reproduction. Lancet. 2006; 367(9508): 379-380.
22. Zargham-Boroujeni A, Jafarzadeh-Kenarsari F, Ghahiri A, Habibi M. Empowerment and sense of adequacy in infertile couples; a fundamental need in treatment process of infertility: a qualitative study. Qual Rep. 2014; 19(11): 1-14.
23. Gameiro S, Canavarro MC, Boivin J. Patient centred care in infertility health care: direct and indirect associations with wellbeing during treatment. Patient Educ Couns. 2013; 93 (3): 646-654.
24. Paterno MT. Families of two: meeting the needs of couples experiencing male infertility. Nurs Womens Health. 2008; 12(4): 300-306.
25. van Empel IW, Hermens RP, Akkermans RP, Hollander KW, Nelen WL, Kremer JA. Organizational determinants of patient-centred fertility care: a multilevel analysis. Fertil Steril. 2011; 95(2): 513-519.
26. Shahhosseini Z, Simbar M, Ramezankhani A. Female adolescents' health needs: the role of family. Payesh. 2012; 11(3): 351-359.
27. Straubert HJ,Carpenter CR. Qualitative research in nursing: advancing the humanistic imperative.[CD-ROM]. Philadelphia: Lippincott Williams & Wilkins; 2010.
28. Munhall PL. Nursing research: A qualitative perspective. [CD-ROM]. Sudbury, MA: Jones & Bartlett; 2007.
29. Polit DF, Beck CT. Nursing research: generating and assessing evidence for nursing practice.[CD-ROM]. Philadelphia: Lippincott Williams & Wilkins; 2012.
30. Read SC, Carrier ME, Boucher ME, Whitley R, Bond S, Zelkowitz P. Psychosocial services for couples in infertility treatment: What do couples really want?. Patient Educ Couns. 2014; 94(3): 390-395.
31. Graneheim UH, LundmanB. Qualitative content analysis in nursing research: concepts, procedures and measures to achieve trustworthiness. Nurse Educ Today. 2004; 24(2): 105-112.
32. Akizuki Y, Kai I. Infertile Japanese women's perception of positive and negative social interactions within their social networks. Hum Reprod. 2008; 23(12): 2737–2743.
33. Abbasi-Shavazi MJ, Asgari-Khanghah A, Razeghi-Nasrabad H. Women and infertility experience: a case study in Tehran. Journal of Women Studies. 2005; 3(3): 91-114.
34. Soltani M, Shaeiri M, Roshan R. The effects of emotional focused therapy on increasing the marriage suitability of barren couples. Journal of Behavioral Sciences. 2012; 6 (4): 23-24.
35. Johnson KM, Fledderjohann J. Revisiting "her" infertility: medicalized embodiment, self-identification and distress. Soc Sci Med. 2012; 75(5): 883-891.
36. Martins MV, Peterson BD, Almeida VM, Costa ME. Direct and indirect effects of perceived social support on women's infertility-related stress. Hum Reprod. 2011; 26(8): 2113-2121.
37. Pasch LA, Dunkel-Schetter C, Christensen A. Differences between husbands' and wives' approach to infertility affect marital communication and adjustment. Fertil Steril. 2002; 77(6): 1241-1247.
38. Dhont N, van de Wijgert J, Coene G, Gasarabwe A, Temmerman M. 'Mama and papa nothing': living with infertility among an urban population in Kigali, Rwanda. Hum Reprod. 2011; 26(3): 623-629.
39. Ezzati A, Nouri R, Hasani J. Structural relationship model between social support, coping strategies, stigma and depression in infertile women in Tehran, Iran, 2010. IJOGI. 2013; 16(45): 20-28.
40. Mahlstedt PP. Coping with infertility: how family and friends can help, Fact Sheet 6. RESOLVE Fact Sheet Series. 2007; 1-6.
41. Alesi R. Infertility and its treatment--an emotional roller coaster. Aust Fam Physician. 2005; 34(3): 135-138.
42. Mollaiy nezhad M, Jaaferpour M, Jahanfar Sh, Jamshidi R. Infertility related stress and marital life in Iranian infertile women who referred to Isfahan infertility treatment clinic. J Reprod Infertil. 2001; 2(1): 26-39.
43. Malpani A, Malpani A. How to have a baby: overcoming infertility. India: USB Publishers' Distributors; 2003.
44. Troude P, Ancelet S, Guibert J, Pouly JL, Bouyer J, de La Rochebrochard E. Joint modeling of success and treatment discontinuation in in vitro fertilization programs: a retrospective cohort study. BMC Pregnancy Childbirth. 2012; 12: 77.
45. Rejeh N, Heravi-Karimooi M, Foroughan M. The needs of hospitalized elderly patients: a qualitative study. Iran Journal of Ageing. 2010; 5(1): 42-52.
46. Wilkes S, Hall N, Crosland A, Murdoch A, Rubin G. Patient experience of infertility management in primary care: an in-depth interview study. Fam Pract. 2009; 26(4): 309-316.

Original Article

Effect of Phosalone on Testicular Tissue and *In Vitro* Fertilizing Potential

Amir Amniattalab, D.V.M, Ph.D.[1*], Mazdak Razi, D.V.M, Ph.D.[2]

1. Department of Pathology, Faculty of Veterinary Medicine, Urmia Branch, Islamic Azad University, Urmia, Iran
2. Department of Comparative Histology and Embryology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

Abstract

Background: The current study aimed to evaluate the effects of phosalone (PLN) as an organophosphate (OP) compound on testicular tissue, hormonal alterations and embryo development in rats.

Materials and Methods: In this experimental study, we divided 18 mature Wistar rats into three groups-control, control-sham and test (n=6 per group). Animals in the test group received one-fourth the lethal dose (LD50) of PLN (150 mg/kg), orally, once per day for 45 days. DNA laddering and epi-fluorescent analyses were performed to evaluate testicular DNA fragmentation and RNA damage, respectively. Serum levels of testosterone and inhibin-B (IN-B) were evaluated. Testicular levels of total antioxidant capacity (TAC), total thiol molecules (TTM) and glutathione peroxidase (GSH-px) were analyzed. Finally, we estimated sperm parameters and effect of PLN on embryo development. Two-way ANOVA was used for statistical analyses.

Results: There was severe DNA fragmentation and RNA damage in testicular tissue of animals that received PLN. PLN remarkably ($p<0.05$) decreased testicular TAC, TTM and GSH-px levels. Animals that received PLN exhibited significantly ($p<0.05$) decreased serum levels of testosterone and IN-B. Reduced sperm count, viability, motility, chromatin condensation and elevated sperm DNA damage were observed in the test group rats. PLN resulted in significant ($p<0.05$) reduction of *in vitro* fertilizing (IVF) potential and elevated embryonic degeneration.

Conclusion: PLN reduced fertilization potential and embryo development were attributed to a cascade of impacts on the testicles and sperm. PLN promoted its impact by elevating DNA and RNA damages via down-regulation of testicular endocrine activity and antioxidant status.

Keywords: Phosalone, DNA Fragmentation, *In Vitro* Fertilization, Sperm, Testicular Tissue

Citation: Amniattalab A, Razi M. Effect of phosalone on testicular tissue and in vitro fertilizing potential. Int J Fertil Steril. 2015; 9(1): 93-106.

Introduction

Phosalone (PLN) or O-diethyl-s-(6-chloro-1-3 ben 30XO3ol-2(3H)-O-methyl) phosphorodithiote is known as an organophosphate (OP) compound used as a replacement for dichlorodiphenyltrichloroethane (DDT) in the agriculture and domestic animal fields (1). OP compounds exert their toxic impacts by blocking acetyl cholinesterase (an enzyme which dissipates acetyl choline) activities, which in turn result in accumulation of excessive amounts of acetyl choline in nervous tissue (2-4). Although these compounds have been known for their effect on the nervous tissue, several reports indicate that OP combinations adversely impact

Received: 6 Oct 2013, Accepted: 28 Jan 2014
* Corresponding Address: P.O. Box: 969, Department of Pathology, Faculty of Veterinary Medicine, Urmia Branch, Islamic Azad University, Urmia, Iran
Email: a.amniattalab@iaurmia.ac.ir

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 93-106

the genital system in both males and females. Lastly we have shown that glyphosate (an OP agent) reduced sperm quality and promoted energy dependent degeneration in germinal cells (5). Previous reports showed that the OP agents diazinon and malathion down-regulated expression of gene coding proteins involved in protein transcription (6, 7). Fattahi et al. (8) reported that administration of diazinon resulted in remarkable decreases in gonadotropin synthesis in rats and this mechanism significantly down-regulated testicular endocrine activity.

It has been shown that OP compounds partially exert their pathological impacts via promotion of oxidative stress in reproductive tissue (9,10). Accordingly, OP agents increase oxidants by disrupting enzymatic and/or non-enzymatic antioxidant defenses as well as enhancing high energy consumption coupled with inhibition of oxidative phosphorylation (5, 9). In addition, oxidative stress may cause degenerative alterations in sperm cells due to the high levels of polyunsaturated fatty acids (PUFA) in their plasma membrane (11). Imbalanced generation of oxidants affects the integrity of the sperm's DNA by causing elevated frequencies of single strand DNA (SS-DNA) and double-strand DNA (DS-DNA) breaks (12).

However, the effect of PLN on testicular tissue, sperm parameters and *in vitro* fertilization (IVF) potential are unknown. Thus, we have aimed to estimate the effect of PLN on RNA and DNA damages in testicular tissue. The semen quality, sperm DNA fragmentation and IVF potential of sperm were analyzed. We also sought to analyze the testicular level of glutathione peroxidase (GSH-px), total antioxidant capacity (TAC) and total thiol molecules (TTM) in order to clarify any pathological alterations in testicular antioxidant capacity as well as illustrate the relationships of these factors with testicular degeneration and semen quality following PLN administration.

Materials and Methods

Chemicals

We obtained technical grade (9.8% purity) PLN [O, O- diethyl-s-(6-chloro-1-3 ben 30XO3ol-2(3H)-O-methyl) phosphorodithiote] from Azma Chemical Ltd. (Tehran, Iran). Acridine-orange staining powder (Merck, Germany) and 3% hydrogen peroxide were purchased from Elim Teb Laboratory Kits Co., Ltd. (Urmia, Iran). The Ransol Detection Kit (Rondax Lab., Crumlin, BT 29, UK) for the GSH-px assay was also purchased from Elim Teb Laboratory Kits Co., Ltd. (Urmia, Iran).

Animals and experimental design

To follow-up the current experimental study, 18 Wistar rats (200-220 g) were obtained from the animal resource of the Faculty of Veterinary Medicine, Urmia University. The animals were acclimatized for one week and had free access to food and water. The experimental protocols were approved by the Ethical Committee of Islamic Azad University, Urmia Branch in accordance with the Principles of Laboratory Animal Care.

The animals were randomly divided into three groups: control, control-sham and test. The animals in the control group received no chemical, whereas animals in the control-sham group received corn oil (1 cc daily by oral gavages). Lethal dose (LD_{50}) values were determined by the Probit method (13). Animals in the test group received one fourth the LD_{50} of PLN (150 mg/kg dissolved in 1 cc corn oil) by oral gavages daily for 45 days (14).

Histological analyses

After 45 days the animals were weighed and euthanized by a special CO_2 device (Uromadaco, Iran). The testicular tissues were dissected free from surrounding tissues under high magnification (×40) stereo zoom microscope (model TL2, Olympus Co., Tokyo, Japan) and their weight was recorded. Dissected testes samples were washed with chilled normal saline and half of the specimens were fixed in Bouin's fixative and kept for further histological analyses. The remaining samples were immediately frozen and stored at -70˚C for further biochemical analyses. Sections (5-6 µm) were stained with iron-Weigert Hematoxylin (Pajohesh Asia, Iran) for detection of germinal cell nuclei in the testis. The histological slides were analyzed under light microscope at two magnifications (×400 and ×1000). The tubular differentiation (TDI), repopulation (RI) and spermiogenesis (SPI) indices were evaluated from 20 sections of each sample. The results for percentage of tubules with positive TDI, RI and SPI were reported.

Fluorescent analyses for RNA damage

RNA damage was assessed based on the Darzynkiewicz method (15). In brief, the testes were washed out with ether alcohol and cut by a cryostat (8 µm). The prepared sections were fixed by different degrees of alcohol for 15 minutes. Then, sections were briefly rinsed in 1% aqueous acetic acid followed by washing in distilled water. The specimens were subsequently stained in acridine-orange (Sigma Aldrich, Germany) for 3 minutes and re-stained in phosphate buffer, followed by fluorescent color differentiations in calcium chloride. The degenerated cells were characterized by loss of RNA and/or by a faint red stained RNA. The normal cells were marked with bright red RNA close to the nucleolus. In order to reduce the bias problems, 20 sections for each sample were analyzed. We used an epi-fluorescent microscope (Model GS7, Nikon co., Japan) for imaging and analysis of the slides.

Assessment of serum levels of testosterone and inhibin-B (IN-B)

After 45 days the blood samples were collected directly from the heart and allowed to clot at room temperature for 1 hour. Samples were centrifuged at 3000×g for 10 minutes to obtain the serum. The serum samples were stored at -80˚C for subsequent assays. Testosterone was assessed by a competitive chemiluminescent immunoassay kit (DRG Co, Germany). The serum level of inhibin-B (IN-B) was evaluated by an enzyme immunometric assay using a commercial kit (Pishtaz Teb, Iran).

DNA laddering test

In order to examine for the presence of any DNA damage, we performed the qualitative DNA fragmentation assay on the frozen testis samples as previously described (16). Briefly, 0.2-0.3 g of frozen testis samples (pooled from at least 4 rats) from each individual group was homogenized in 3 ml lysis buffer (0.1 M tris-HCl/10 mM EDTA that contained 0.5% Triton X-100, pH=8.0). Following a short centrifugation (1200×g, 5 minutes at 4˚C), the pellets were treated with a mixture that contained buffer-saturated phenol, chloroform and isoamyl alcohol (25:24:1, v/v/v). After centrifugation (1500×g, 10 minutes at 4˚C), the supernatants were treated with a chloroform-isoamyl alcohol mix (49:1, v/v) to remove protein and fatty materials. Thereafter, to precipitate DNA, the solution was mixed with pre-chilled ethanol (absolute) and sodium acetate (3.5 M, pH=4.0), respectively. DNA samples were washed with ethanol (66%) and re-dissolved in buffer that contained tris-HCl (0.1 M) and EDTA (20 mM). DNA fragmentation was analyzed by loading the extracted DNA samples onto an agarose gel (1.6%) that contained ethidium bromide and electrophoresis was conducted at 60 V for 75 minutes. DNA fragmentation was imaged using a Gel Doc 2000 system (Bio-Rad).

Evaluating epididymal sperm characteristics

The epididymis was carefully separated from the testicles under a ×20 magnification under a stereo zoom microscope (model TL2, Olympus Co., Tokyo, Japan). The epididymis was divided into three segments: caput, corpus and cauda. The epididymal cauda was trimmed and minced in 5 mL Ham's F10 medium. After 20 minutes the minced epididymal tissue was separated from the released spermatozoa. The sperm count was performed according to standard hemocytometric lam method as described previously by Pant and Srivastava (17). We performed eosin-nigrosin staining to evaluate sperm viability. The sperm with stained head pieces were considered nonviable. Aniline-blue staining was performed in order to analyze the sperm chromatin condensation. For this purpose, we prepared 20 smeared slides from each sperm sample of animals from different groups. The percentage of dead sperm was compared between different groups.

Evaluating sperm motility

In order to evaluate sperm motility, the World Health Organization (1999) standard method for manual examination of sperm motility was used (18). Briefly, sperm samples were diluted (1:8) in Ham's F10 prior to examination. A total of 20 µl of the sperm sample was placed on the sperm examination area and examined by a ×10 magnification loop. Only the motile sperm with forward progression was counted within ten boxes and recorded. Finally, motility was evaluated based on the following equation:

$$\text{Motility (\%)} = [\text{motile sperm}/\text{motile+non-motile sperm}] \times 100$$

Evaluating sperm DNA damage

To evaluate DNA double-strand breaks, air

dried slides were stained with an acridine-orange staining kit (Sigma Co., St. Louis, MO, USA) after which the cover-slip was placed on the slides. The slides were evaluated on the same day using an epi-fluorescent microscope (model GS7, Nikon co., Japan). In all preparations at least 100 spermatozoa were evaluated at ×40 magnification. Spermatozoa with green fluorescence were considered to have native DS-DNA, the spermatozoa with yellow fluorescence were marked as having partly denatured SS-DNA (PSS-DNA), and with red fluorescence as completely denatured SS-DNA. Percentages of green, yellow, and red spermatozoa were assessed and compared between the groups.

Sperm processing for in vitro fertilization

Samples that contained spermatozoa were prepared from the sperm suspensions as mentioned earlier. The samples were incubated at 37˚C under 5% CO_2 (CO_2 Incubator, LEEC, England) for 3 hours. Then, as previously described, 0.1 ml from superficial sperm and/or 0.1 ml from sediment sperm of suspensions in one tube were added to 150 µl of tissue culture medium (TCM) that contained the oocytes (19).

Collection of oocytes and insemination

Eight mature female rats were injected subcutaneously with 7.5 IU pregnant mare's serum (Netherlands) 48 hours prior to an intra-peritoneal injection of 100 IU human chorionic gonadotropin (hCG, Teikoku Zohki Co., Korea). Rats were euthanized with a special CO_2 device 24 hours after the hCG injection. The oviducts were removed and the ampullar portion was placed into a plastic dish that contained phosphate buffered saline (pH=7.2). The oocytes in the cumulus masses were dissected out of the oviducts and introduced into TCM 199 (Sigma Co., USA). A drop of medium with 2 oocytes was allocated with a 10 µl sperm suspension (total: 80000 sperm) and incubated at 37˚C in 5% CO_2 .

Assessment of fertilization ratio and embryonic development

For this purpose, the appearance of pronuclei and polar bodies were checked under ×200 magnification using an inverted microscope (model NA100, Nikon CO., Japan). After 24 hours the two-cell embryo rate was assessed. *In vitro* embryonic development was evaluated at 120 hours by phase-contrast microscopy (model IX50, Olympus CO., Germany). Intact, fragmented and/or lysed embryos which did not develop were recorded as "arrested embryos". In the present study, the rate of cell lyses was recorded as follows: type I: fully lysed, necrotic and/or fragmented embryos, type II: embryos with partially lysed/fragmented blastomeres and type III: embryos with some lysed/fragmented blastomeres and/or cytoplasmic vesicles (20).

Assessment of serum total antioxidant capacity (TAC)

To determine the effect of PLN on oxidative stress, TAC of the testicular tissue from the control-sham and test groups were measured. The assessment was performed based on the ferric reduction antioxidant power (FRAP) assay (21). Briefly, at low pH (acetate buffer, 300 mM, pH=3.6), reduction of the FeIII-TPTZ complex to the ferrous form would produce an intensive blue color measurable at 593 nm. The intensity of the complex following addition of the appropriate volume of serum to the reducible solution of FeIII-TPTZ is directly related to the total reducing power of the electron donating antioxidant. An aqueous solution of FeII ($FeSO_4.7H_2O$) and appropriate concentration of freshly prepared ascorbic acid are used as blank and standard solutions, respectively.

Measurement of serum total thiol molecules (TTM)

The total sulfhydryl level in testicular tissue was measured according to a method by Hu and Dillared (22). Briefly, 0.3-0.4 g of the testes samples were homogenized in ice-cold KCl (150 mM) after which the mixture was centrifuged at 3000×g for 10 minutes. Thereafter 0.5 ml of the supernatant was added to 0.6 ml tris-EDTA buffer (tris base 0.25 M, EDTA 20 mM, pH=8.2) followed by the addition of 40 µl DTNB (10 mM in pure methanol) in a 10 ml glass test tube. The final volume of the mentioned mixture was made up to 4.0 ml by extra addition of methanol. After incubation for 15 minutes at room temperature, the samples were centrifuged at 3000×g for 10 minutes and ultimately the absorbance of the supernatant was assessed at 412 nm.

Assessment of glutathione peroxidase (GSH-px)

For this purpose, the testicular tissue was washed three times with 0.9% NaCl solution and 1.15% KCl was liquefied to the amount of 9 ml for each tissue. The homogenate of the tissues was prepared with a Teflon end on homogenizer (Elvenjem Potter, Newton, CT) and centrifuged at 4000 rpm for 5 minutes. The GSH-px activity was evaluated using a commercial Ransol measurement kit (Rondax-lab., Crumlin, BT 29, UK).

Statistical analysis

Statistical analyses were performed using SPSS software version 13.00. The comparisons between groups were made by analysis of variance (two-way ANOVA) followed by the Bonferroni post-hoc test. A p value <0.05 was considered significant. All values were expressed as mean ± SD.

Results

Phosalone (PLN) reduced the total body and testicular weights

At the end of the study, observations demonstrated that the administration of PLN significantly ($p<0.05$) reduced the total body and testicular weight gains compared to control and control-sham groups. The testicular to body weight ratio in PLN-administered animals showed a remarkable ($p<0.05$) decrease compared to control and control-sham groups. No significant differences ($p>0.05$) were observed for total body and testicular weight gains between control and control-sham animals (Fig.1A-B).

Phosalone (PLN) administration resulted in histological damages in testicular tissue

Histological analyses showed that the PLN-administered animals exhibited highly degenerated testicular tissue, an elevated percentage of tubules with arrested spermatogenesis (10.32 ± 4.31%), severe edema in connective tissue and atrophied seminiferous tubules. Distribution of the Leydig cells remarkably ($p<0.05$) decreased and the percentage of hypertrophied Leydig cells increased per one mm^2 of the interstitial tissue (Fig.2A-C). The PLN-treated animals showed an increased percentage of tubules with negative TDI, RI and SPI versus control and control-sham groups. No histopathological alterations were observed in control-sham animals. The data for histomorphometric analyses are presented in table 1.

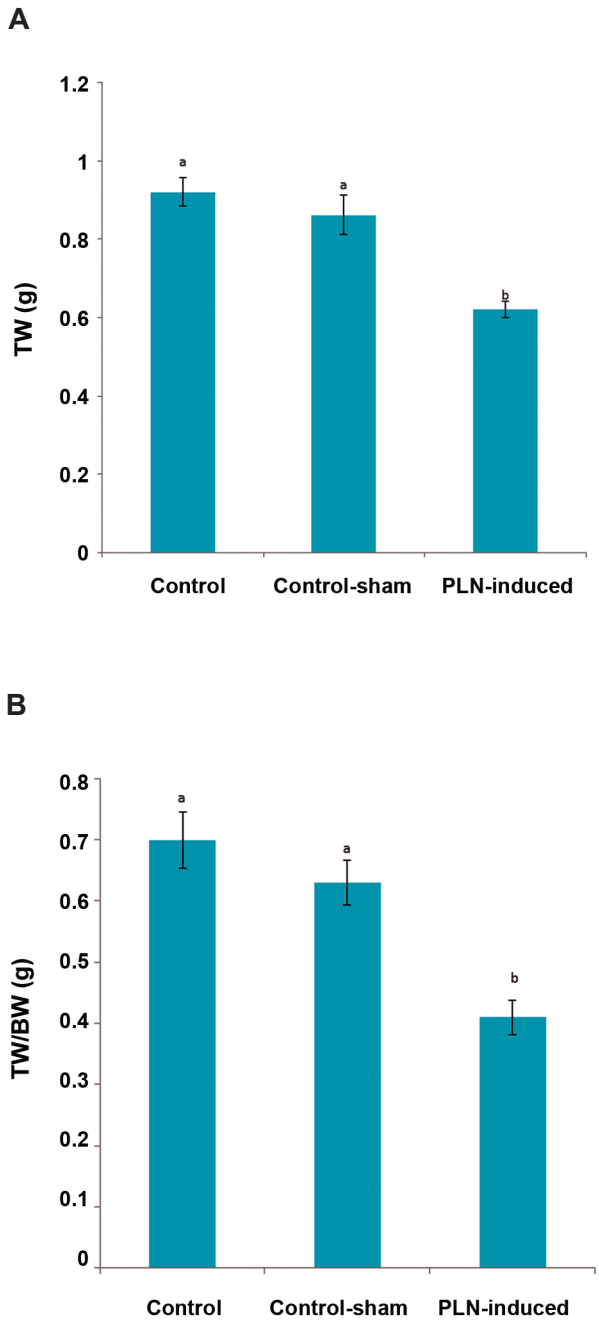


Fig.1: Effect of phosalone (PLN, 150 mg/kg) on (A) total testicular weight (TW) and on (B) testicular to body weight (BW) compared to control and control-sham groups. Data are mean ± SD.
[a]; No significant and [b]; Significant differences ($p<0.05$) between data for PLN-administered group with control and control-sham.

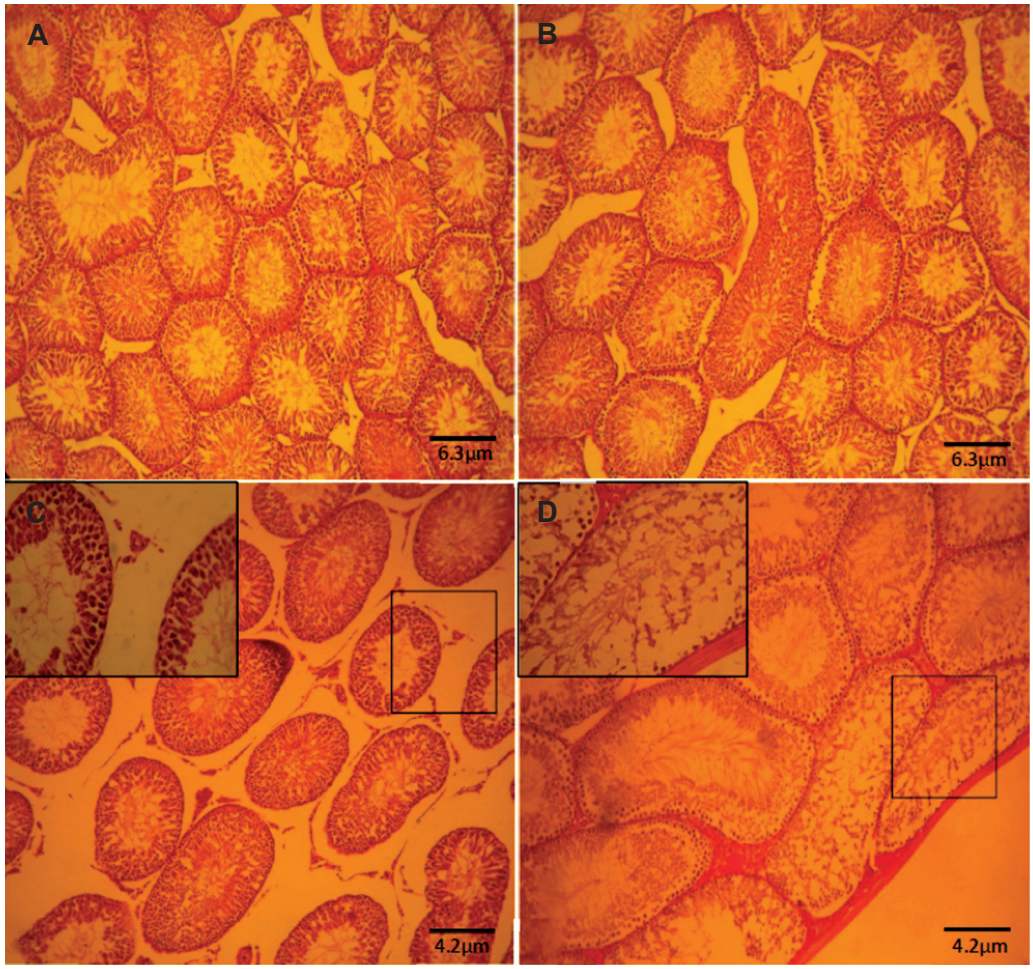


Fig.2: Cross-section from testis. (A) Control group. (B) Control-sham group: Note the normal seminiferous tubules with normal spermatogenesis. (C) and (D) Phosalone (PLN)-induced group: severe edema in connective tissue associated with tubular atrophy. Note the seminiferous tubules with negative tubular differentiation index (TDI) (magnified in figure C) and tubular depletion (TD) close to the capsule (magnified in figure D). Haematoxylin and eosin (H&E) staining (×400).

Table 1: Histomorphometric alterations in different groups

	Control	Control-sham	PLN-induced
T.D (µm)	234.48 ± 20.36[a]	228.52 ± 19.07[a]	188.21 ± 18.88[b]
G.E.H (µm)	136.17 ± 20.15[a]	137.8 ± 15.62[a]	106.07 ± 9.21[b]
Negative TDI (%)	14.20 ± 2.07[a]	16.18 ± 1.66[a]	34.50 ± 4.52[b]
Negative RI (%)	11.09 ± 1.04[a]	13.17 ± 0.98[a]	28.74 ± 6.01[b]
Negative SPI (%)	9.58 ± 2.00[a]	8.33 ± 1.26[a]	37.94 ± 3.03[b]

PLN; Phosalone (150 mg/kg), T.D; Tubular diameter, G.E.H; Germinal epithelium height, TDI; Tubular differentiation index, RI; Repopulation index, SPI; Spermiogenesis index and [a, b]; Significant differences ($p<0.05$) between PLN-induced group with control and control-sham groups (n=6 for each group).
Data are mean ± SD.

Phosalone (PLN) significantly decreased serum levels of testosterone and inhibin-B (IN-B)

The serum levels of testosterone and IN-B significantly ($p<0.05$) reduced in PLN-treated animals versus control and control-sham groups. No significant alterations were observed in serum levels of testosterone and IN-B in the control-sham group compared to control animals (Fig.3).

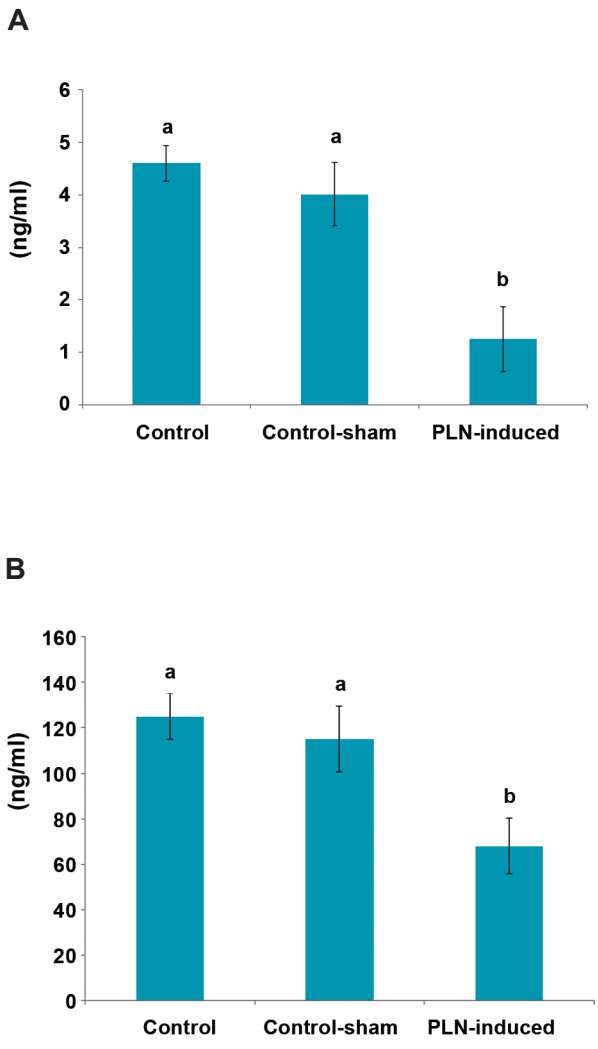


Fig.3: Effect of phosalone (PLN, 150 mg/kg) on serum levels of testosterone (A) and inhibin-B (IN-B) (B) compared to control and control-sham groups (n=6 rats for each group). Data are mean ± SD.
[a]; No significant and [b]; Significant differences ($p<0.05$) between data for PLN-administered group with control and control-sham.

Exposure to phosalone (PLN) elevated the DNA and RNA damage in testicular tissue

Agarose gel electrophoresis was performed to examine for apoptotic DNA laddering. An accumulative dose of PLN after 45 days resulted in DNA degradation as characterized by a smear shape (Fig.4). Comparing the bands in the control group (lane 2, two relatively high molecular weight bands) with those in the PLN-treated group indicated that in the animals which received PLN, there was no proper high molecular weight DNA (lane 4). Epi-fluorescent analyses for RNA damage showed that chronic administration of PLN resulted in severe RNA damage in spermatocytogenesis and spermatogenesis cell lineages (Fig.5A-C). Accordingly, the animals that received PLN showed a significantly ($p<0.05$) higher percentage of seminiferous tubules with damaged RNA content in germinal cells (Fig.5D).

Phosalone (PLN) reduced sperm quality

The PLN-administered animals showed a significant ($p<0.05$) decrease in sperm count ($48.25 \pm 6.21\times10^6$) compared to control-sham ($63.51 \pm 3.70\times10^6$) and control ($66.12 \pm 4.41\times10^6$) groups. The animals in the PLN-treated group exhibited a significantly ($p<0.05$) higher percentage of dead sperms versus the control-sham and control animals. The percentage of sperms with PSS-DNA and SS-DNA were remarkably ($p<0.05$) elevated in PLN-administered animals in comparison to control and control-sham animals. Moreover, the PLN-administered animals showed a significant ($p<0.05$) reduction in the percentage of sperms with condensed chromatin (Fig.6). The data for sperm parameters are presented in table 2.

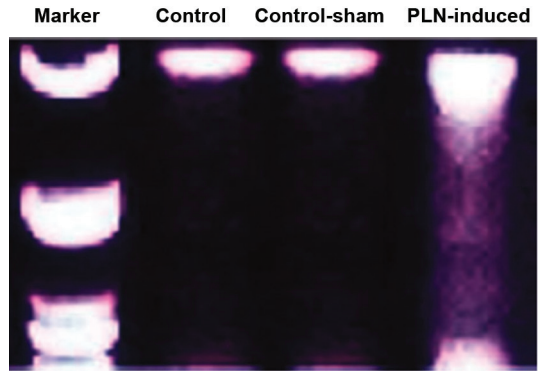


Fig.4: Phosalone (PLN)-induced DNA fragmentation in testicular tissue. PLN-induced DNA damage is shown as a smear shape in lane 4. No clear DNA fragmentation was observed in control and control-sham testicles, (DNA fragmentation assay).

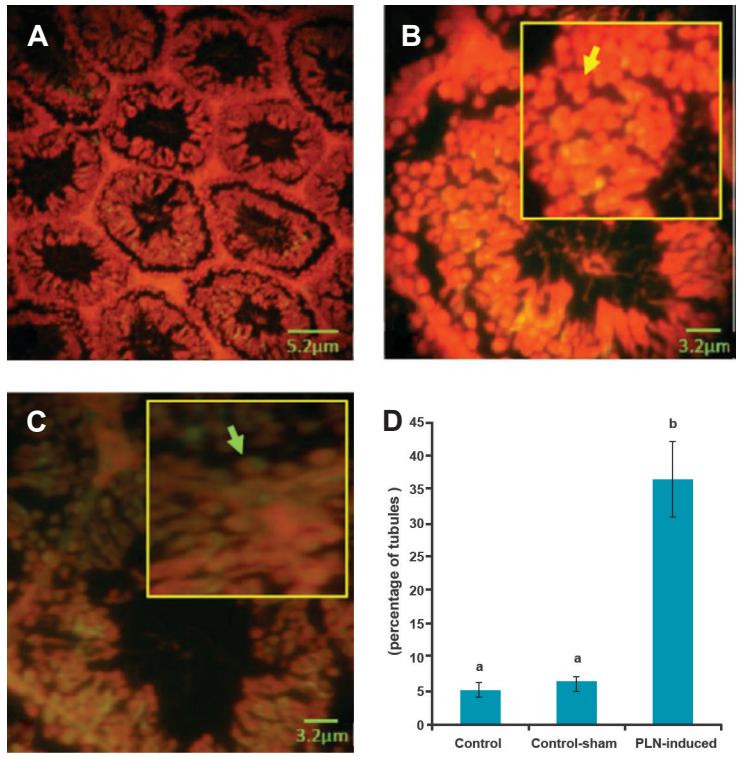


Fig.5: Cross-section from testis. (A) Control group. (B) Higher magnification from normal seminiferous tubule. Note the normal RNA content marked with bright, intensive fluorescent red in the germinal epithelium (yellow arrow). (C) Phosalone (PLN)-induced testis: remarkable RNA damage was shown by the faint fluorescent reaction (green arrow). The germinal cells exhibited lower RNA content versus normal cells in figure B. Epi-fluorescent analysis for RNA damage (A: ×400 and B, C: ×600). (D) Mean percentage of seminiferous tubules with RNA damage in different groups (n=6 rats per group). Data are mean ± SD.
[a]; No significant and [b]; Significant differences ($p<0.05$) between data for PLN-administered (150 mg/kg) group with control and control-sham groups.

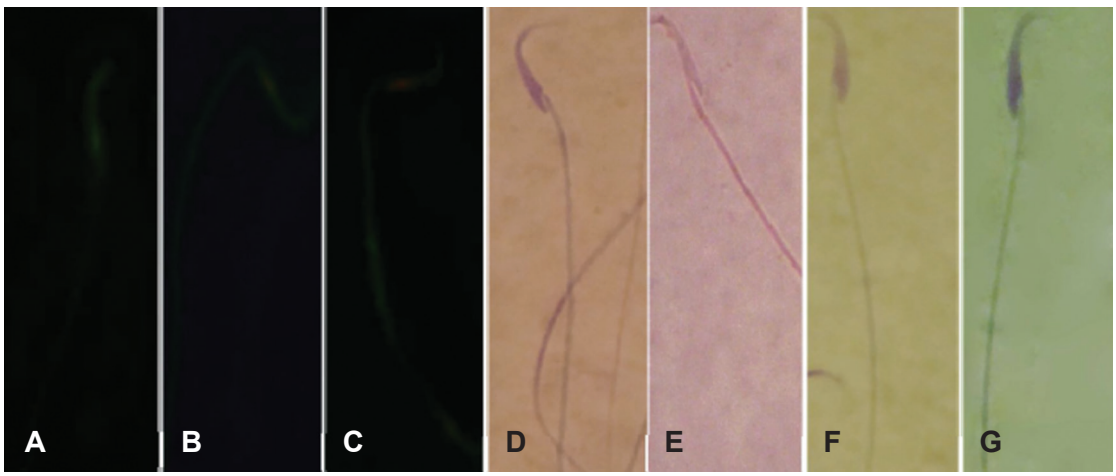


Fig.6: A. Sperm with double-strand DNA (DS-DNA). B. Sperm with partly denatured single-strand DNA (PSS-DNA). C. Sperm with single-strand DNA (SS-DNA). D. Nonviable sperm with stained cytoplasm. E. Live sperm with colorless cytoplasm. F. Sperm with condensed chromatin. G. Sperm with immature chromatin condensation. A-C. Acridine-orange stain, D, E. Eosin-nigrosin stain and F, G. Aniline-blue stain.

Table 2: Effect of phosalone (PLN) on sperm parameters in different groups

	Control	Control-sham	PLN-induced
Count ($\times 10^6$)	66.12 ± 4.41[a]	63.51 ± 3.70[a]	48.25 ± 6.21[b]
Viability (%)	83.45 ± 11.10[a]	80.00 ± 8.12[b]	40.15 ± 6.45[b]
Motility (%)	85.12 ± 9.21[a]	82.35 ± 10.12[a]	34.41 ± 6.52[b]
Chromatin condensation (%)	86.42 ± 6.74[a]	80.32 ± 4.63[a]	38.64 ± 3.10[b]
DS-DNA (%)	82.32 ± 6.14[a]	81.11 ± 7.33[a]	38.21 ± 5.12[b]
PSS-DNA (%)	10.07 ± 1.01[a]	12.31 ± 2.10[a]	27.81 ± 3.71[b]
SS-DNA (%)	8.33 ± 0.78[a]	10.28 ± 0.84[a]	34.63 ± 2.01[b]

PLN; Phosalone (150 mg/kg), DS-DNA; Double-strand DNA, PSS-DNA; Partial single-strand DNA, SS-DNA; Single-strand DNA and [a, b]; Significant differences ($p<0.05$) between PLN-induced group with control and control-sham groups (n=6 for each group) in the same row. Data are mean ± SD.

Phosalone (PLN) reduced in vitro fertilizing (IVF) potential

The results for IVF of oocytes by sperm collected from PLN-administered animals were remarkably ($p<0.05$) lower than control and control-sham animals. Interestingly, the significantly ($p<0.05$) higher percentage of 2-cell embryos stopped division in animals that received PLN and did not continue. Comparing the percentage of blastocysts between different groups showed that the PLN-treated animals exhibited a significantly ($p<0.05$) lower percentage of blastocyst versus the control and control-sham animals. No significant differences ($p>0.05$) were observed between control and control-sham animals (Fig.7). The data for IVF results are presented in table 3.

Phosalone (PLN) reduced antioxidant status and elevated oxidative stress

Observations demonstrated that the tissue levels of TAC and TTM significantly down-regulated in the PLN-administered group compared to control and control-sham animals. Biochemical analyses for GSH-px revealed that the animals in the group that received PLN exhibited a remarkable ($p<0.05$) decrease in testicular GSH-px levels (Fig.8A-C).

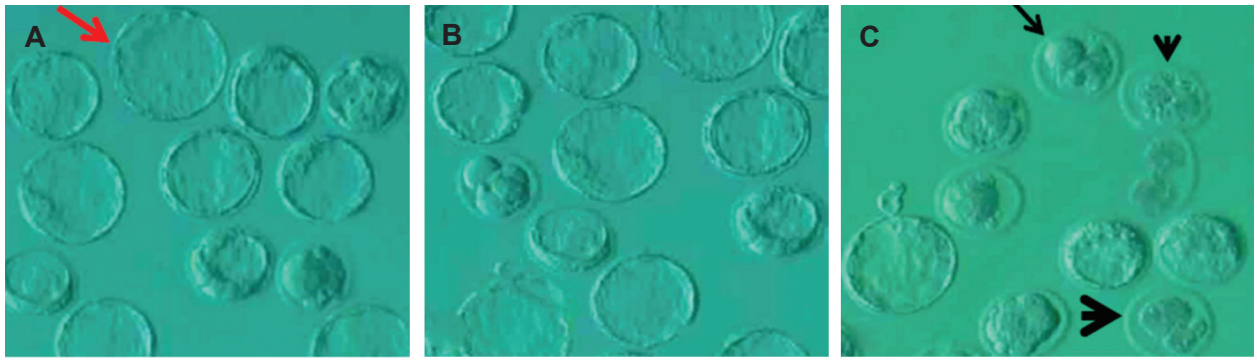


Fig.7: Embryonic development in (A) control, (B) control-sham and (C) phosalone (PLN)-induced animals. Control and control-sham groups exhibited significantly higher normal blastocysts (red arrow). The PLN-induced animal exhibited remarkably higher type I (head arrow), type II (big head arrow) and type III arrests (arrow).

Table 3: Effect of phosalone (PLN) on *in vitro* fertilizing (IVF) potential and embryo development

	Control	Control-sham	PLN-induced
Total oocytes (NO)	203	277	281
Appropriate oocyte (NO)	170	162	63
Fertilized oocyte (NO)	148	146	37
2-cell embryos (%)	81.03 ± 7.14[a]	78.64 ± 5.17[a]	64.30 ± 6.85[a]
Blastocysts (%)	54.28 ± 3.70[a]	47.21 ± 4.01[a]	20.22 ± 2.90[b]
Arrested embryos (%)	42.51 ± 5.20[a]	48.63 ± 4.21[a]	68.33 ± 4.83[b]
Arrest type I (%)	4.21 ± 0.98[a]	4.68 ± 1.01[a]	27.35 ± 4.43[b]
Arrest type II (%)	8.63 ± 1.41[a]	7.36 ± 1.33[a]	23.72 ± 3.10[b]
Arrest type III (%)	31.21 ± 2.84[a]	28.40 ± 4.71[a]	18.70 ± 2.69[b]

[a, b]; Significant differences ($p<0.05$) between PLN-induced group with control and control-sham groups (n=6 for each group) in the same row.
Data are mean ± SD.

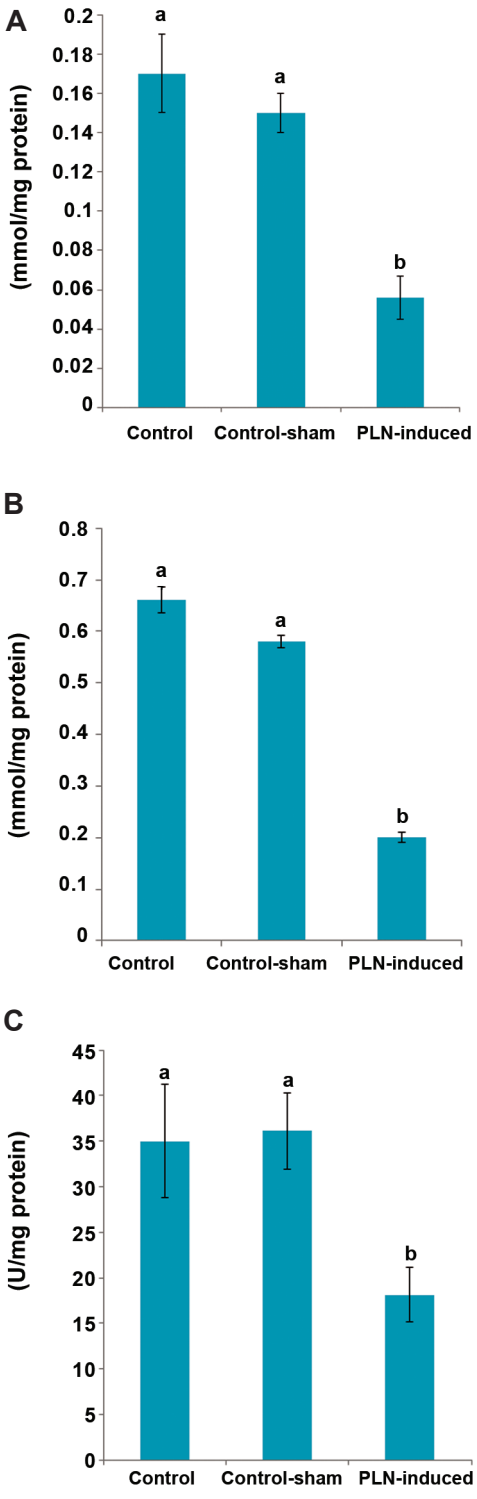


Fig.8: Effect of phosalone (PLN) on tissue (A) total antioxidant capacity (TAC), (B) total thiol molecules (TTM) and (C) glutathione peroxidase (GSH-px) compared to control and control-sham groups (n=6 rats for each group). Data are mean ± SD.
[a]; No significant and [b]; Significant differences ($p<0.05$) between data for PLN-administered group with control and control-sham groups.

Discussion

The results of the present study showed that chronic administration of PLN resulted in severe damages to testicular tissue. The animals that received PLN had remarkable DNA fragmentation, RNA damage and down-regulated intra-testicular endocrine activities. The enzymatic and non-enzymatic antioxidant potentials were down-regulated after 45 days. Finally, the PLN-administered animals exhibited a remarkable reduction in IVF outcomes. PLN-treated animals showed significantly higher embryonic arrests versus control and control-sham animals.

Previous reports indicated that chronic administration of different OP compounds (glyphosate, diazinon and malathion) resulted in severe reductions in gonad weights in rats (5-7). Severe testicular damages were considered to be a possible explanation for this impairment. Animals in the PLN-treated group had a significant reduction in total body weight gain. This alteration might be related to the effect of PLN on the central structures involved in the control of feed intake such as the ventromedian nucleus of the hypothalamus (23).

Previous reports showed that OP combinations affected the spermatogenesis process and impacted the male reproductive system via interruption of endocrine activities (24, 25). In physiologic conditions, the interactions between Leydig and Sertoli cells promote the spermatogenesis process (26). Accordingly, Leydig cells control the Sertoli cell endocrine activities via synthesis of testosterone (26, 27). In this regard, PLN-treated animals have shown a significant reduction in Leydig cell numbers per one mm^2 of the interstitial tissue as well as decreased serum levels of testosterone and IN-B. We can suggest that PLN has influenced the testicular endocrine status by decreasing distribution of Leydig cells, which in turn disrupted the Leydig stimulatory impact on Sertoli cell endocrine interactions. The elevated testicular damages such as negative RI, TDI and SPI reflect the functional derangement of the Sertoli cells. The decreased testicular to body weight ratio in animals that received PLN could be attributed to severe degeneration of testicular tissue.

Because of the high concentration of unsaturated fatty acids in mammalian germinal cells, these cells are susceptible to oxidative stress (28,

29). The produced oxidative stress results in considerable damages at lipids, proteins, DNA and RNA levels (12, 30, 31). In order to evaluate the effect of PLN on testicular antioxidant status, the testicular levels of TTM, GSH-px and TAC have been analyzed. Observations showed remarkable reductions in TTM, GSH-px and TAC levels in PLN-treated animals compared to control and control-sham groups. The intracellular antioxidant enzymes GSH-px and superoxide dismutase are known for the first line of cellular defense that prevent oxidative stress-induced damages (11, 28). GSH-px plays an important role in maintenance of the thiol-disulfide balance. Any lack in GSH-px physiological interactions and/or reduction in GSH-px synthesis will affect the enzymatic defense line and ultimately promote damages on biological macromolecules such as DNA and RNA (11, 28, 32). Our analyses have shown elevated DNA fragmentation and RNA damage as well as decreased testicular GSH-px levels in PLN-administered animals. Increased apoptotic DNA fragmentation and severe RNA damage in PLN-treated animals enabled us to conclude that remarkable depletion in GSH-px associated with decreased TTM level could promote the oxidative stress-induced damages at DNA and RNA levels.

It has been well established that sperm DNA integrity is very important for its fertilizing potential. The DNA integrity of sperm mainly depends on its compaction after chromatin condensation processes (11, 33). The deficiency in protamine expression and/or replacement with nucleosomal histones caused by environmental toxicants promotes DNA disintegrity because decondensed DNA of the sperm are susceptible to free radicals (32). Our observations have demonstrated that the percentage of sperms with condensed chromatin decreased and there was increased DNA damage in animals treated with PLN. Therefore, we concluded that PLN appended the sperm DNA disintegrity both by affecting the chromatin condensation process and by down-regulating antioxidant status. More analyses showed that PLN administration resulted in diminished sperm motility and viability. The sperm cell membrane contains high amounts of PUFA which are susceptible to exogenous free radicals (24). Therefore, it is logical to suggest that reduced sperm viability reflects severe oxidative stress in PLN-administered animals. Beside reduced viability, the PLN-induced oxidative stress can inhibit axonemal protein phosphorylations (34, 35) and consequently reduce sperm motility.

The cascade of events - PLN-increased percentage of dead sperms associated with elevated DNA damages, as well as immobility and nuclear immaturity are able to enhance oxidative stress. Accordingly, the damaged sperm are considered as the sources of radicals (36). It has been shown that incubation of abnormal and/or damaged spermatozoa lead to oxidative damages at the DNA level in oocytes and embryos (37). It is reported that oocytes have the ability to correct small scale DNA damage upon fertilization. If this increases above a certain level it may be difficult for the oocyte to cope and lead to fertilization failure or impaired embryo development (38). In corroboration with this finding we have observed that embryo development significantly decreased in PLN-treated animals. A possible explanation may be that the increased oxidants (produced from abnormal sperms) possibly led to remarkable DNA damage in oocytes, which in turn resulted in lower blastocyst generation. On the other hand, PLN resulted in an elevated percentage of sperm with abnormal chromatin condensation. Previous reports indicated that using sperm with decondensed DNA resulted in low *in vitro* embryo development, particularly at the 2-cell embryo level (38).Therefore, it could be suggested that decreased embryo development in PLN-administered animals could be attributed to increased damage at sperm levels such as reduced sperm motility, viability, chromatin condensation and increased DNA damage.

Conclusion

The results of the current study showed that chronic exposure to PLN resulted in enhanced DNA fragmentation and RNA damage in testicular tissue and reduced testicular endocrine activities. Our observations demonstrated that PLN exerted its impact via down-regulation of the antioxidant status and enhanced oxidative stress. Finally, we showed that PLN-induced problems in sperm parameters resulted in considerable embryo toxicity.

Acknowledgements

The authors wish to express their appreciation to the Vice President of Research, Urmia Branch, Islamic Azad University for financial support and Dr. Najafi for his kind assistance. There is no conflict of interest between all authors.

References

1. Singh AK. Kinetic analysis of inhibition of brain and red blood cell acetylcholinesterase and plasma cholinesterase by acephate or methamidophos. Toxicol Appl Pharmacol. 1985; 81(2): 302-309.
2. Harlin KS, Dellinger JA. Retina, brain and blood cholinesterase levels in cats treated with oral dichlorvos. Vet Hum Toxicol.1993; 35(3): 201-203.
3. Bustos-Obregon E, Gonzalez JR, Espinoza O. Melatonin as protective agent for the cytotoxic effects of diazinon in the spermatogenesis in the earthworm Eisenia foetida. Ital J Anat Embryol. 2005; 110(2 Suppl 1): 159-165.
4. Pina-Guzman B, Solis-Heredia MJ, Quintanilla-Vega B. Diazinon alters sperm chromatin structure in mice by phosphorylating nuclear protamines. Toxicol Appl Pharmacol. 2005; 202(2): 189-198.
5. Razi M, Najafi G, Feyzi S, Karimi A, Shahmohamadloo S, Nejati V. Histological and histochemical effect of Gly-Phosate on testicular tissue and function. Iran J Reprod Med. 2012; 10(3): 181-192.
6. Bonilla E, Hernández F, Cortés L, Mendoza M, Mejía J, Carrillo E, et al. Effects of the insecticides malathion and diazinon on the early oogenesis in mice in vitro. Environ Toxicol. 2008; 23(2): 240-245.
7. Adamkovicova M, Toman R, Cabaj M. Diazinon and Cadmium acute testicular toxicity in rats examined by histopathological and morphological methods. Slovak J Anim Sci. 2010; 43(3): 134-140.
8. Fattahi E, Parivar K, Jorsaraei SGA, Moghadamnia AA. The effects of diazinon on testosterone, FSH and LH levels and testicular tissue in mice. Iran J Reprod Med. 2009; 7(2): 59-64.
9. Harris C, Lee E, Hiranruengchok R, McNutt TL, Larson SJ, Akeila S, et al. Characteristics of glutathione redox and antioxidant status in post implantation rat embryos: response to oxidative stress. Toxicology. 1996; 30: 2.
10. Milatovic D, Gupta RC, Aschner M. Anticholinesterase toxicity and oxidative stress. ScientificWorldJournal. 2006; 6: 295-310.
11. Agarwal A, Allamaneni S. Oxidative stress and human reproduction. In: Singh K, editor. Oxidative stress, disease and cancer. Singapore: Mainland Press; 2006; 687-703.
12. Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM, Ames BN. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. Mutat Res. 1996; 351(2): 199-203.
13. Finney DJ. Probit analysis. London: Cambridge University Press; 1971.
14. Zama D, Meraihi Z, Boubekri N, Amrani A, Tebibel S, Baali N. Assesment of the changes in some diagnostic enzymes and other parameters in wistar albino rats treated with pesticides during gestation. Sci Technol. 2005; 23: 51-56.
15. Darzynkiewicz Z. Differential staining of DNA and RNA in intact cells and isolated cell nuclei with acridine orange. Methods Cell Biol. 1990; 33: 285-298.
16. Patel N, Joseph C, Corcoran GB, Ray SD. Silymarin modulates doxorubicin-induced oxidative stress Bcl-xL and p53 expression while preventing apoptotic and necrotic cell death in the liver. Toxicol Appl Pharmacol. 2010; 245(2): 143-152.
17. Pant N, Srivastava SP. Testicular and spermatotoxic effect of quinaphos in rats. J Appl Toxicol. 2003; 23(4): 271-274.
18. World Health Organization: WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge: Cambridge University Press; 2000.
19. Toyoda Y, Chang MC. Fertilization of rat eggs in vitro by epididymal spermatozoa and the development of eggs following transfer. J Reprod Fertil. 1974; 36(1): 9-22.
20. Cebral E, Carrasco I, Vantman D, Smith R. Preimplantation embryotoxicity after mouse embryo exposition to reactive oxygen species. Biocell. 2007; 31(1): 51-59.
21. Benzie IF, Strain JJ. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 1999; 299: 15-27.
22. Hu M, Dillared CJ. Plasma SH and GSH measurement. Method Enzymol. 1994; 33: 385-387.
23. Psychoyos A. La reproduction. In: Meyer P, editor. Physiologie Humaine. Paris: Flammarion Médecine-Sciences; 1983; 471-493.
24. Kamijima M, Hibi H, Gotoh M, Taki K, Saito I, Wang H, et al. A survey of semen indices in insecticide sprayers. J Occup Health. 2004; 46(2): 109-118.
25. Krause W, Hamm K, Weissmulter J. The effect of per orally administered DDVP and Malathion on spermatogenesis and Leydig cell in the Juvenile rat. Andrologia. 1975; 7(2): 109-116.
26. Skinner MK, Fritz IB. Testicular peritubular cells secrete a protein under androgen control that modulates Sertoli cell function. Proc Natl Acad Sci USA. 1985; 82(1): 114-118.
27. Walker W, Cheng J. FSH and testosterone signaling in Sertoli cells. Reproduction. 2005; 130(1): 15-28.
28. Yamanaka K, Hasegawa A, Sawamura R, Okada S. Cellular response to oxidative damage in lung induced by the administration of dimethylarsinic acid, a major metabolite of inorganic arsenics, in mice. Toxicol Appl Pharmacol. 1991; 108(2): 205-213.
29. Vernet P, Aitken RJ, Drevet JR. Antioxidant strategies in the epididymis. Mol Cell Endocrinol. 2004; 216(1-2): 31-39.
30. Sega GA. Adducts in sperm protamine and DNA vs. mutation frequency. Prog Clin Biol Res.1991; 372: 521-530.
31. Recio R, Robbins WA, Borja-Aburto V, Moran-Martınez J, Froines JR, Hernandez RM, et al. Organophosphorous pesticide exposure increases the frequency of sperm sex null aneuploidy. Environ Health Perspect. 2001; 109(12): 1237-1240.
32. Usoh IF, Akpan EJ, Etim EO, Farombi EO. Antioxidant actions of dried flower extract of Hibiscus sabdariffa L. On sodium arsenite-induced oxidative stress in rats. PJN. 2005; 4(3): 135-141.
33. Sakkas D, Urner F, Bizzaro D, Manicardi G, Bianchi

PG, Shoukir Y, et al. Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. Hum Reprod. 1998; 13 Suppl 4: 11-19.
34. Green DR, Reed JC. Mitochondria and apoptosis. Science.1998; 281(5381): 1309-1312.
35. Twigg J, Irvine DS, Houston P, Fulton N, Michael L, Aitken RJ. Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. Mol Hum Reprod. 1998; 4(5): 439-445.
36. Said TM, Agarwal A, Sharma RK, Thomas AJ, Sikka SC. Impact of sperm morphology on DNA damage caused by oxidative stress induced by beta-nicotinamide adenine dinucleotide phosphate. Fertil Steril. 2005; 83(1): 95-103.
37. Nasr-Esfahani MH, Aitken JR, Johnson MH. Hydrogen peroxide levels in mouse oocytes and early cleavage stage embryos developed in vitro or in vivo. Development. 1990; 109(2): 501-507.
38. Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U. Origin of DNA damage in ejaculated human spermatozoa. Rev Reprod. 1999; 4(1): 31-37.

Original Article

Predictive Factors of Successful Microdissection Testicular Sperm Extraction in Patients with Presumed Sertoli Cell-Only Syndrome

Tahereh Modarresi, M.Sc.[1], Hani Hosseinifar, M.Sc.[1], Ali Daliri Hampa, M.D.[1], Mohammad Chehrazi, M.Sc.[2], Jalil Hosseini, M.D.[1], Faramarz Farrahi, M.D.[1], Farid Dadkhah, M.D.[1], Marjan Sabbaghian, Ph.D.[1], Mohammad Ali Sadighi Gilani, M.D.[1, 3*]

1. Department of Andrology at Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
2. Department of Epidemiology and Reproductive Health at Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
3. Department of Urology, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Background: To evaluate predictive factors of successful microdissection-testicular sperm extraction (MD-TESE) in patients with presumed Sertoli cell-only syndrome (SCOS).

Materials and Methods: In this retrospective analysis, 874 men with non-obstructive azoospermia (NOA), among whom 148 individuals with diagnosis of SCOS in prior biopsy, underwent MD-TESE at Department of Andrology, Royan Institute, Tehran, Iran. The predictive values of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T) levels, testicular volume, as well as male age for retrieving testicular sperm by MD-TESE were analyzed by multiple logistic regression analysis.

Results: Testicular sperm were successfully retrieved in 23.6% men with presumed SCOS. Using receiver operating characteristic (ROC) curve analysis, it was shown that sperm retrieval rate in the group of men with FSH values >15.25% was 28.9%. This was higher than the group of men with FSH ≤15.25 (11.8%).

Conclusion: Sperm retrieval rate (SRR) was 23.6% in men with presumed SCOS and FSH level can be a fair predictor for SPR at MD-TESE. MD-TESE appears to be recommendable in such cases (SCOS with high FSH concentration) with reasonable results.

Keywords: Follicle Stimulating Hormone, Luteinizing Hormone, Sperm Retrieval, Azoospermia, Nonobstructive

Citation: **Modarresi T, Hosseinifar H, Daliri Hampa A, Chehrazi M, Hosseini J, Farrahi F, Dadkhah F, Sabbaghian M, Sadighi Gilani MA. Predictive factors of successful microdissection testicular sperm extraction in patients with presumed sertoli cell-only syndrome. Int J Fertil Steril. 2015; 9(1): 107-112.**

Introduction

For men with a zero sperm count (azoospermia), testicular biopsy is done to determine if a blockage is present (obstructive azoospermia), or if primary testicular failure [non-obstructive azoospermia (NOA)] is the cause (1, 2). Primary testicular failure affects approximately 1% of the population and 10% of those seeking fertility evaluations (3, 4). The general histological patterns of the patients with non-obstructive azoospermia are hypospermatogenesis, maturation arrest and Sertoli cell–only syndrome (SCOS) (4).

For many azoospermic men, *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) has become the major reproductive treatment op-

Received: 15 May 2013, Accepted: 30 Dec 2013
*** Corresponding Address: P.O. Box: 16635-148, Department of Andrology at Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran**
Email: masadighi@gmail.com

tion if testicular sperm can be retrieved (1). Microdissection testicular sperm extraction (MD-TESE) is an effective sperm retrieval procedure for men with NOA due to higher sperm retrieval rate (5, 6). Based on microscopical scale, MD-TESE identifies the most advanced pattern, not necessarily the predominant pattern of spermatogenesis in the testis (5). In contrast to the predominant spermatogenic pattern, the most advanced pattern appears to affect sperm retrieval results.

The sperm retrieval rates (SRRs) by MD-TESE for patients with hypospermatogenesis were 81 (7) to 100% (8, 9) of attempts, whereas in those with maturation arrest spermatozoa were retrieved in only 44 (7) to 75% (8, 9) of MD-TESE attempts. In SCOS patients, SRRs were between 22.5 (8) and 41% (7). Since sperm extraction is often scheduled in addition to oocyte retrieval after ovarian stimulation and monitoring, failed ME-TESE can have significant emotional and financial implications for the couples involved. So it would be valuable to predict the success of sperm retrieval using non-invasive parameters (5, 10).

Serum FSH is an indirect reflection of the spermatogenic function of the testis (5, 10). Clinically, testicular volume is correlated with spermatogenesis. Age may also affect the outcome of sperm extraction (10). Since one of the most frequent histological patterns characterizing absence of sperm is SCOS, the primary aim of this study was to evaluate the outcomes of ME-TESE, primarily the sperm retrieval, in patients with diagnosis of SCOS in prior biopsy. We also analyzed the predictive values of follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (T) levels, testicular volume, as well as male age for retrieving testicular sperm by MD-TESE in these patients.

Materials and Methods

Patients

In this a retrospective analysis, 874 men with NOA, among whom 148 patients with diagnosis of SCOS in prior biopsy, underwent MD-TESE at Royan Institute, Tehran, Iran, between April 2008 and March 2009. Azoospermia was confirmed by at least two semen analyses according to World Health Organization (WHO) guidelines (11). We excluded patients with karyotype abnormalities and Y chromosome microdeletion. Testicular volume was measured by physical examination using an orchidometer. Analysis of serum FSH, LH, and T levels was done by electrochemiluminescence assay in the morning. The reference ranges for FSH level is 1.0-10.5 mIU/mL, LH level is 1.0-9.5 mIU/mL and T level is 2.0-12 ng/mL. The men were divided into four main groups based on FSH level in increments of 10 IU/mL as follows: i. <10 mIU/mL, ii. 10-20 mIU/mL, iii. 20-30 mIU/mL, and iv. >30 mIU/mL. In other classification, the patients were classified into two following subgroups based on the best cut point of FSH values (see statistical analysis): i. the group with unsuccessful sperm retrieval and ii. the group with successful sperm retrieval. Approval was obtained from Ethics Committee of Royan Institute for this particular study, and participants provided a written informed consent.

Microdissection testicular sperm extraction (MD-TESE)

MD-TESE was performed under general anesthesia according to the technique previously described by Schlegel (12). In brief, the scrotum was incised along the scrotal raphe and testis was opened from the mid-part. Using an operating microscope with ×25-40 magnification, enlarged and opaque seminiferous tubules were removed and evaluated by one of our three expert lab technicians in operating room. Each sample was placed in a petri dish filled with 1 mL Ham's F10 medium (Biochrom, Germany), and was mechanically cut, dispersed and examined under an inverted microscope (Nikon, Japan) at ×400 magnification. If no spermatozoa were seen, microdissection of additional areas of that testicle and contralateral testicle were carried out and subsequent samples were taken. If no sperm was seen in the operating room, all testicular samples were subjected to centrifugation at 3000 rpm with 5 mL Ham's F10 and examined to determine the presence of even a single sperm.

Statistical analysis

Descriptive statistics are presented as mean ± SD and percent. Student's t test (Unpaired) was used to compare mean age, while Mann-Whitney U test was applied to compare testis volume as well as FSH, LH and T levels for outcome of sperm re-

trieval. Multiple logistic regression analysis was used to evaluate the association between FSH, LH and T levels and success of sperm retrieval, adjusting for potential confounding variables (testicular volume and age). Two tailed p values less than 0.05 were considered statistically significant. We performed receiver operating curve (ROC) analysis for a final model. The area under a curve (AUC) is a measure of predictive power called concordance index which is generated to evaluate the predictive accuracy of selected predictors on probability of retrieving sperm. The value of 0.5 means that predictions are no better than random guessing and the value of 1.0 indicates a (theoretically) perfect test (i.e., 100% sensitive and 100% specific). Moreover, we used ROC analysis to determine the best cut point of FSH level for outcome of sperm retrieval, and sensitivity and specificity were measured 64.3 and 15.4%, respectively.

Results

Testicular sperm was successfully retrieved in 23.6% (35/148) of the patients with SCOS. Successful rates for ICSI and clinical pregnancy were 57.1% (20/35) and 9%, respectively. The mean values of age, testicular volume; as well as serum FSH, LH and T levels were compared between those patients with unsuccessful sperm retrieval and the group with successful sperm retrieval. There were no significant differences between two groups (Table 1).

Findings were shown that there was an inverse linear correlation between FSH and testicular volume ($r=-0.37$, $p<0.001$). Sperm retrieval rates in the groups of men with FSH values of 10-20, 20-30, and >30 mIU/mL were 39, 37, and 24%, respectively, and this was higher than the group of men with FSH<10 mIU/mL (10%), but this difference was not significant ($p>0.05$).

We performed multiple logistic regression analysis with serum FSH, LH and T levels, testis volume and age to predict sperm retrieval during MD-TESE. Adjusted association from the model showed that chance of retrieving sperm during MD-TESE cannot be predicted by any variable.

Also we used ROC analysis to determine the best cut point of FSH levels for outcome of sperm retrieval. Our results showed that 15.25 was a cut point of FSH for sperm retrieval. SRR in men with FSH>15.25 was 28.9% and in men with FSH≤15.25 was 11.8%. Odds of SRR in men with FSH>15.25 was higher than men with FSH≤15.25 and was significant at level 10%. A logistic regression analysis based on this cut point showed a fair prediction model (AUC=0.68) for FSH (Fig.1). LH level, testosterone level, testicular volume and male age cannot predict presence of sperm with MD-TESE (Tables 2, 3).

Table 1: Values of age; testicular volume; as well as serum FSH, LH and T levels between two groups (the group with unsuccessful sperm retrieval and the group with successful sperm retrieval)

	Fail		Success		
	Mean ± SD	**CI (%95)**	**Mean ± SD**	**CI (%95)**	**P value**
Age (Y)	33 ± 6	31.9-34.2	33 ± 5	31.7-35.6	0.64
Testicular volume (mL)	11 ± 4.7	10.1 ± 11.9	9.9 ± 4.3	8.3-11.4	0.24
FSH (mIU/mL)	23.7 ± 15.9	20.3-27.2	23.8 ± 9.5	20-27.7	0.36
LH (mIU/mL)	9.5 ± 9.1	7.5-11.5	8.6 ± 7.4	5.6-11.6	0.89
T (ng/mL)	3.8 ± 2.3	3.3-4.3	4.1 ± 3.5	2.7-5.6	0.84

LH; Luteinizing hormone, T; Testosterone, FSH; Follicle stimulating hormone and CI; Confidence interval.

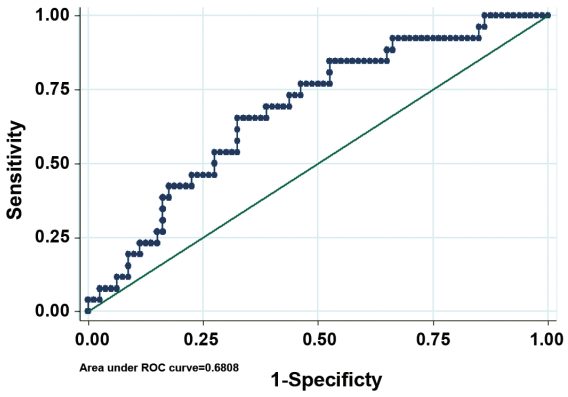


Fig.1: ROC curve of pertinent preoperative parameters to discriminate successful and failed MD-TESE (AUC=0.68). ROC; Receiver operating characteristic, MD-TESE; Microdissection-testicular sperm extraction and AUC; Area under a curve.

Table 2: Baseline characteristics of men with NOA and SCOS

	Serum FSH (mIU/mL)	
	≤15.25	**>15.25**
%	31%	69%
Male age (Y)	32 ± 4	34 ± 6
Mean FSH (mIU/mL)	11.2	29.4
Mean Testosterone (ng/mL)	4.6 ± 2.6	3.6 ± 2.6
Mean LH (mIU/mL)	5.7 ± 6.2	10.2 ± 9.3
Avg. vol. of testis (mL)	12.8 ± 4.1	9.6 ± 4.7

LH; Luteinizing hormone, T; Testosterone, SCOS; Sertoli cell-only syndrome, NOA; Non-obstructive azoospermia and FSH; Follicle stimulating hormone.

Table 3: Results of multivariable adjusted model of pertinent variables

Variable	P value	OR (95%CI)
FSH (IU/mL)		
≤15.25		Reference group
>15.25	0.067	2.96 (0.92-9.4)
Male age (Y)	0.648	1.01 (0.94-1.1)
LH (mIU/mL)	0.224	0.96 (0.89-1.02)
T (ng/mL)	0.262	1.1 (0.92-1.3)
Testes size (mL)	0.103	0.9 (0.8-1.02)

*Adjusted OR represents the estimates from full model adjusted for male age, testes size, LH; Luteinizing hormone, T; Testosterone, FSH; Follicle stimulating hormone levels, CI; Confidence interval and OR; Odds ratio.

Discussion

MD-TESE has become a recognized procedure for men with NOA. Simultaneous sperm extractiontesticular volume-ICSI exposes the couple to an emotional burden, so it would be beneficial to predict the success of sperm retrieval before treatment. Diagnostic biopsy, hormones levels, volume of testis and age are potentially predictive factors for sperm retrieval (3).

The diagnosis of NOA can only be definitely made on testicular biopsy, but the prognostic value of random biopsy to detect sperm production in these patients is unknown (3). Diagnostic biopsy has limited prognostic value for prediction of sperm retrieval in MD-TESE. Tsujimura et al. (8) reported that SRRs by MD-TESE for SCOS were 22.5%. Gul et al. (13) demonstrated that SRRs for these patients were 27.6%. Okada et al. showed that SRRs by MD-TESE for SCOS were 33.9% (9) and Ramasamy et al. (7) reported excellent SRRs of 41% for SCOS. Our study showed that SRRs by MD-TESE were 23.6% for SCOS.

An important serum parameter in the first years of TESE was the FSH level. In general, the serum concentration of FSH is inversely correlated with impairment of spermatogenesis. Earlier studies showed that elevated FSH levels were associated with a low probability for the retrieval of spermatozoa in TESE (14), but Ramasamy et al. (6) showed that after using MD-TESE, sperm retrieval was higher in NOA men with FSH>15 mIU/mL than those men with FSH<15 mIU/mL. In other study, Ramasamy et al. (15) showed that FSH (and testicular volume) at the repeat MD-TESE appeared to have predictive value to determine the success of a second attempt. Our study confirmed the results of their study. The present study showed sperm retrieval was higher in NOA men with FSH>15.25 mIU/mL than those men with FSH≤15.25 mIU/mL. Also we showed FSH could be a fair predictor of sperm retrieval. Ramasamy et al. (6) have described the reason of the conflicting evidence between TESE and MD-TESE.

We also studied the effect of serum LH and T levels on sperm retrieval, but these factors didn't have any effect on SRR. This is similar to the results of other studies (13, 16).

Clinically testicular volume is correlated with

spermatogenesis. Testicular volume has been found to have poor predictive value for successful TESE (10, 13, 14). Ramasamy et al. (6) reported that testicular volume didn't have predictive value to determine the success of MD-TESE, but there was an inverse linear correlation between FSH and testicular volume. Our study confirmed that study and showed that sperm retrieval rate was also higher in smaller testis, but testicular volume didn't have predictive value to determine the success of MD-TESE. Moreover, in this study, there was an inverse linear correlation between FSH and testicular volume.

Many studies have shown a relationship between testicular histopathologic findings and testicular sperm retrieval by TESE (14, 17). Histopathologic finding are generally the most useful predictive factor for successful TESE (6). However, it is still controversial whether invasive examination such as testicular biopsy should be performed due to possible presence of inflammatory change, hematoma, fibrosis and devascularization of testes (3, 6, 9). On the other hand, MD-TESE has fewer postoperative complications than random biopsy (10, 16). MD-TESE appears to be recommendable in cases of atrophied testicles and elevated amount of FSH concentration (16).

The p value for association between FSH (as continuous variable) and sperm retrieval was 0.98. Since FSH is a continuous variable, the odds of sperm retrieval do not change significantly per unit increase in FSH. This conclusion may be clinically expected. So we tried to find a cut point which discriminated sperm retrieval result extremely. ROC analysis was used to find the best cut point for sensitivity and specificity. The association between categorical FSH and sperm retrieval was significant at level of 10%. The p value is near 0.05 in this case. The border p values may be due to sampling errors or small sample size and need further study with larger sample size to find the best cut point.

Conclusion

Sperm retrieval rate was 23.6% in men with SCOS and FSH can be a fair predictor of sperm retrieval at MD-TESE. MD-TESE appears to be recommendable in such cases (SCOS with high FSH concentration) with reasonable results.

Acknowledgements

The authors thank Mehdi Lotfi Panah, Library Officer of Royan Institute, for his kind assistance in providing resources. The authors express their gratitude to Royan Institute for its financial support. There is no conflict of interest in this study.

References

1. Cerilli LA, Kuang W, Rogers D. A practical approach to testicular biopsy interpretation for male infertility. Arch Pathol Lab Med. 2010; 134(8): 1197-1204.
2. Khazamipour N, Noruzinia M, Fatehmanesh P, Keyhanee M, Pujol P. MTHFR promoter hypermethylationin testicular biopsies of patients with non-obstructive azoospermia: the role of epigeneticsin male infertility. Hum Reprod. 2009; 24(9): 2361-2364.
3. Ramasamy R, Schlegel PN. Microdissection testicular sperm extraction: effect of prior biopsy on success of sperm retrieval. J Urol. 2007; 177(4): 1447-1449.
4. Anniballo R, Ubaldi F, Cobellis L, Sorrentino M, Rienzi L, Greco E, et al. Criteria predicting the absence of spermatozoa in the Sertoli cell-only syndrome can be used to improve success rates of sperm retrieval. Hum Reprod. 2000; 15(11): 2269-2277.
5. Turunc T, Gul U, Haydardedeoglu B, Bal N, Kuzgunbay B, Peskircioglu L, et al. Conventional testicular sperm extraction combined with the microdissection technique in nonobstructive azoospermic patients: a prospective comparative study. Fertil Steril. 2010; 94(6): 2157-2160.
6. Ramasamy R, Lin K, Gosden LV, Rosenwaks Z, Palermo GD, Schlegel PN. High serum FSH levels in men with nonobstructive azoospermia does not affect success of microdissection testicular sperm extraction. Fertil Steril. 2009; 92(2): 590-593.
7. Ramasamy R, Yagan N, Schlegel PN. Structural and functional changes to the testis after conventional versus microdissection testicular sperm extraction. Urology. 2005; 65(6): 1190-1194.
8. Tsujimura A, Matsumiya K, Miyagawa Y, Tohda A, Miura H, Nishimura K, et al. Conventional multiple or microdissection testicular sperm extraction: a comparative study. Hum Reprod. 2002; 17(11): 2924-2929.
9. Okada H, Dobashi M, Yamazaki T, Hara I, Fujisawa M, Arakawa S, et al. Conventional versus microdissection testicular sperm extraction for nonobstructive azoospermia. J Urol. 2002; 168(3): 1063-1067.
10. Tsujimura A. Microdissection testicular sperm extraction: prediction, outcome, and complications. Int J Urol. 2007; 14(10): 883-889.
11. World Health Organization. WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction. Cambridge: Cambridge University Press; 1987.
12. Schlegel PN. Testicular sperm extraction: microdissection improves sperm yield with minimal tissue excision. Hum Reprod. 1999; 14(1): 131-135.
13. Gul U, Turunc T, Haydardedeoglu B, Yaycioglu O, Kuzgunbay B, Ozkardes H. Sperm retrieval and live birth rates in presumed Sertoli-cell-only syndrome in testis biopsy: a single centre experience. Andrology. 2013; 1(1): 47-51.
14. Tournaye H, Verheyen G, Nagy P, Ubaldi F, Goossens A, Silber S, et al. Are there any predictive factors for successful testicular sperm recovery in azoospermic pa-

tients?. Hum Reprod. 1997; 12(1): 80-86.
15. Ramasamy R, Ricci JA, Leung RA, Schlegel PN. Successful repeat microdissection testicular sperm extraction in men with nonobstructive azoospermia. J Urol. 2011; 185(3): 1027-1031.
16. Ghalayini IF, Al-Ghazo MA, Hani OB, Al-Azab R, Bani-Hani I, Zayed F, et al. Clinical comparison of conventional testicular sperm extraction and microdissection techniques for non-obstructive azoospermia. J Clin Med Res. 2011; 3(3): 124-131.
17. Seo JT, Ko WJ. Predictive factors of successful testicular sperm recovery in non-obstructive azoospermia patients. Int J Androl. 2001; 24(5): 306-310.

Original Article

Study on The Effect of Royal Jelly on Reproductive Parameters in Streptozotocin-Induced Diabetic Rats

Elham Ghanbari, M.Sc.[1]*, Vahid Nejati, Ph.D.[1], Gholamreza Najafi, Ph.D.[2], Mozafar Khazaei, Ph.D.[3], Mohammad Babaei, D.V.M.[2]

1. Department of Biology, Faculty of Science, Urmia University, Urmia, Iran
2. Department of Basic Science, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
3. Fertility and Infertility Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran

Abstract

Background: Diabetes mellitus has a variety of structural and functional effects on the male reproductive system. Diabetes results in reduced sperm parameters and libido. The present study aims to investigate the effects of royal jelly (RJ) on reproductive parameters of testosterone and malondialdehyde (MDA) production in diabetic rats.

Materials and Methods: This experimental study was conducted on adult male Wistar rats. The animals were divided into four groups (n=8 per group): control, RJ, diabetic and diabetic treated with RJ. Diabetes was induced by intraperitoneal injection of 60 mg/kg body weight (BW) of streptozotocin (STZ). RJ, at a dose of 100 mg/kg BW was given by gavage. The duration of treatment was six weeks. After the treatment period the rats were sacrificed. The testes were weighed and changes in sperm count, motility, viability, deformity, DNA integrity and chromatin quality were analyzed. Serum testosterone and MDA concentrations of testicular tissue were determined. Data were analyzed by one-way ANOVA with $p<0.05$ as the significant level.

Results: STZ-induced diabetes decreased numerous reproductive parameters in rats. Testicular weight, sperm count, motility, viability and serum testosterone levels increased in the diabetic group treated with RJ. There was a significant decrease observed in sperm deformity, DNA integrity, chromatin quality, and tissue MDA levels in diabetic rats treated with RJ compared to the diabetic group ($p<0.05$).

Conclusion: RJ improved reproductive parameters such as testicular weight, sperm count, viability, motility, deformity, DNA integrity, chromatin quality, serum testosterone and testicular tissue MDA levels in diabetic rats.

Keywords: Diabetes Mellitus, Male Rat, Royal Jelly, Sperm

Citation: Ghanbari E, Nejati V, Najafi G, Khazaei M, Babaei M. Study on the effect of royal jelly on reproductive parameters in streptozotocin-induced diabetic rats. Int J Fertil Steril. 2015; 9(1): 113-120.

Introduction

One of the major health problems in life is infertility; male factors comprise approximately 30% of this problem (1). Several factors can affect the spermatogenesis process and decrease sperm quality and quantity. Diabetes mellitus, liver and coronary heart diseases, air pollutants, chronic smoking and vitamin deficiency affect spermatogenesis (2). Diabetes leads to vacuolization in Sertoli cells, raises apoptosis in spermatogonia cells and spermatocytes in seminiferous tubules of male rats (3). According to research, male reproductive dysfunctions in animal models of diabetes include decreased semen quality, testicular weight, sperm count and motility, and testosterone levels in addition to increased abnormal sperm and oxidative

Received: 28 Oct 2013, Accepted: 25 Feb 2014
* Corresponding Address: Department of Biology, Faculty of Science, Urmia University, Urmia, Iran
Email: e_ghanbari90@yahoo.com

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 113-120

damage in the testes (4).

Oxidative stress has been described as an important factor in many diseases such as diabetes (5). Although the precise mechanism for diabetes mellitus is not well understood, increased production of free radicals is the major mechanism that causes damage (6). Diabetes mellitus has an extensive and close association with oxidative stress induced by exacerbation of oxygen free radical formation. Hyperglycemia is associated with increased oxidative stress and leads to many complications in different tissues (7). Evidence indicates that free radicals, membrane lipid peroxidation and protein oxidation are increased in diabetic patients and diabetic animals. The production of reactive oxygen species (ROS) is a normal physiological event in the testes. Excessive production of ROS can be detrimental to sperm, being associated with male infertility. The spermatozoa plasma membrane contains a high amount of unsaturated fatty acids. Therefore, it is susceptible to peroxidative damage (8). The lipid peroxidation destroys the structure of the lipid matrix of spermatozoa membranes and disturbs sperm motility (9).

Royal jelly (RJ), a food item secreted by the hypopharyngeal glands of worker honeybees is a mixture that contains lipid, glucose, protein, vitamins and minerals. RJ is widely used as a commercial medical product. Previous studies have shown that RJ has many chemical and physical properties such as anti-tumor, antioxidant, anti-inflammatory and immune-modulatory functions in animals (10). Estrogenic activity similar to other exogenous steroid hormones (11), higher testosterone content and intensive spermatogenesis in hamster testes (12), and increased serum testosterone levels in heat-stressed male rabbits are reported for RJ (13). Further, research has shown that alkaline and water extracts of RJ have high scavenging ability and antioxidative activity against active oxygen species (14).

Another study has shown that RJ collected 24 hours after larval transfer has the strongest antioxidative action (15). Various *in vitro* experimental models on rats have also proven the antioxidative activity of RJ (16) and protection against oxidative stress has been confirmed in experiments on laboratory animals (17). The present study aims to investigate the effect of RJ on reproductive parameters and malondialdehyde (MDA) levels of testicular tissue in diabetic male rats.

Materials and Methods

Animals

In this experimental study, 32 healthy adult male Wistar rats (200 ± 10 g) were used. The animals were obtained from the animal house at the Faculty of Science, Urmia University. The rats were kept under specific conditions on a constant 12-hour light/dark cycle and at a controlled temperature of 21 ± 2˚C. They were fed with standard diet pellets and allowed food and water ad libitum for an acclimation period of one week. The animals were housed in polypropylene cages and maintained in a strictly controlled environment. This study was conducted in accordance with the Guidelines of the Ethical Committee for Research on Laboratory Animals at Urmia University.

Experimental design

We randomly divided 32 male Wistar rats into four groups (n=8 per group): control [1 cc of distilled water (DW) day]; RJ (100 mg RJ/kg of BW/day); diabetic (1 cc of DW/day) and diabetic treated with RJ (100 mg RJ/kg of BW/day).

Fresh RJ was obtained from a local beekeeping association (Urmia, Iran) and stored at −20 ˚C until use. The RJ was confirmed by an expert academic member (Urmia University). RJ was dissolved in distilled water and administered orally to the RJ and diabetic treated RJ groups for 42 consecutive days. The diabetic and diabetic treated RJ groups were given a single intraperitoneal administration of streptozotocin (STZ, S0130-500 MG, Sigma-Aldrich Co, St. Louis, MO, USA) at 60 mg/kg BW dissolved in 0.1 M citrate buffer, pH=4.6 (18). At 72 hours after administration, the rats were allowed to fast for 18 hours after which their blood sugar levels were measured by tail puncture using a glucometer (IGM-0002A, GP5EAKFO9548). Rats that had blood sugar levels above 300 mg/dl were considered to be diabetic and included in this study.

On the 42nd day of treatment, the rats were sacrificed by intraperitoneal injection of 80 mg ketamine and a laparotomy was conducted. The testes and epididymides were collected. The cauda region of the epididymis was used for evaluation of sperm parameters. The right testes were processed for histopathological studies whereas the left testes were homogenated for biochemical estimations of MDA.

Sperm count

Epididymal sperm were collected by slicing the cauda region of the epididymis into small pieces in 1 ml human tubal fluid (HTF)+4 mg/ml bovine serum albumin (BSA) and incubated for 30 minutes at 37˚C in 5% CO_2 to allow the sperm to swim out of the epididymal tubules. Sperm count was performed with a hemocytometer. Results were expressed as millions of sperm/ml. A few drops of the diluted sperm suspension, as a sample, was transferred onto a Neubauer's improved counting chamber (depth: 0.1 mm) and allowed to remain for 5 minutes (19).

Sperm morphology

We evaluated sperm morphology by analyzing sperm smears made from the left cauda epididymides. An aliquot of the sample was used for preparation of the smears in order to evaluate deformities to the spermatozoa (18). Eosin/nigrosin stain was used to estimate spermatozoa morphology. To test, one drop of eosin/nigrosin was added to the suspension and mixed gently. The slides were then viewed under a light microscope at ×400 magnification. A total of 300 spermatozoa were analyzed on each slide for abnormalities of the head and tail (19).

Sperm viability

To assess sperm viability, 10 µl of eosin/nigrosin was added to an equal volume of spermatozoa suspension. After 2 minutes of incubation at room temperature, slides were viewed at ×400 magnification. Sperms with altered plasma membranes appeared pink and those with intact plasma membranes remained unstained. In each sample, 400 sperm cells were counted and the percentages of sperm viability (ratio of sperm with intact/altered plasma membranes) were calculated (19).

Sperm motility

The percentage of sperm motility was evaluated visually by a light microscope (Olympus Co., Tokyo, Japan) at ×400 magnifications. For this process, one drop of sperm suspension was placed on a glass slide which was then covered with a lamella. The number of sperm that had rapid progressive forward movement (RPFM), slow progressive forward movement (SPFM), circumferential motion (CM) and those which remained motionless (ML) were counted in several microscopic fields of vision and the percentages of motile and non-motile sperm were obtained. Motility estimates were obtained from ten different fields in each sample (18).

Body and testes weights

Animals were weighed to monitor their general health. The testes and epididymides were removed. The testes were weighed and processed for biochemical analysis.

Acridine orange (AO) DNA denaturation assay

The AO test is a simplified microscopic sperm chromatin structure assay which reflects sperm chromatin denaturation. A drop of the sperm suspension was spread on the glass slides and allowed to air-dry. All smears were fixed in methanol-acetic acid at 1:3 v/v for 2 hours. The slides were then stained with 2-3 cc of 19% AO solution in phosphate citrate for 5 minutes, then rinsed with deionized water. The sperm were evaluated by a fluorescence microscope (Zeiss Company, Germany). Two types of staining patterns were identified-green (double-stranded DNA) and yellow (single-stranded DNA) sperm (20).

Aniline blue (AB) chromatin quality assay

A drop of spermatozoa suspension was spread on glass slides and allowed to air-dry. All smears were fixed in 3% glutaraldehyde in phosphate buffered saline. The slides were then stained with 5% aqueous AB and mixed with 4% acetic acid (pH=3.5) for 5 minutes. Sperm heads that contained immature nuclear chromatin stained blue whereas those with mature nuclei did not stain. The percentage of spermatozoa that stained AB was determined by counting 400 spermatozoa (21).

Hormone assay

Blood serum was separated by centrifuge (3000 g for 15 minutes) and serum samples were directly frozen at –70˚C until biochemical analyses. Serum testosterone concentrations were measured by an electrochemiluminescence testosterone kit (Demeditec Diagnostics GmbH, Kiel, Germany). The amount of testosterone was expressed as ng/dL (22).

Malondialdehyde (MDA) level assay

Fresh tissue samples were minced and homogenized under ice-cold conditions. The testicular tissues

were homogenized into an ice-cold 1.15% solution of KCl to obtain a 10% (w/v) homogenate. Then, 300 µl of 10% trichloroacetic acid (TCA) was added to 150 µl of the homogenized sample and centrifuged at 1000 rpm for 10 minutes at 4˚C. The supernatant was transferred to a test tube with 300 µl of 67% thiobarbituric acid (TBA) and incubated at 100˚C for 25 minutes. After 5 minutes of cooling, a pink color appeared because of the MDA-TBA reaction. Absorbance was evaluated using a spectrophotometer (Pharmacia, Novaspec II, Biochrom, England) at wavelength of 535 nm (23). The level of lipid peroxides was expressed as µmol MDA/mg protein.

Statistical analysis

Data were presented as mean ± SEM and analyzed by one-way ANOVA followed by the Tukey test using SPSS package (version 18) and $p<0.05$ as the significance level.

Results

Body weight (BW) and testes weight

The initial BW of the rats did not significantly differ between the groups. A significant difference in the final BW of the rats was observed. The diabetic rats showed reduced BW but diabetic rats treated with RJ had a significant increase in BW (p=0.000, Table 1).

The testicular weights of the rats are shown in table 1. Diabetes caused a statistically significant decrease in testes weights compared to the control group (p=0.000). The administration of RJ caused an increase in testes weight compared to the control group. The testes/BW ratio revealed a significant decrease in the diabetic group compared to the control group. The group treated with RJ had a significant increase in the testes/BW ratio. However, significant differences were not observed in other groups (p=0.000, Table 1).

Sperm parameters

Diabetes caused a significant decrease in sperm count compared with the control group (p=0.000). Treatment with RJ significantly increased cauda epididymal sperm count. However, administration of RJ to treated diabetic rats significantly prevented the STZ-induced negative effects on sperm count compared with the diabetic group (p=0.000, Table 2).

There was a significantly lower percentage of sperm viability in diabetic rats than those of control and RJ groups (p=0.000). The diabetic group treated with RJ showed a significant increase in percentage of sperm viability in compared with the diabetic group (p=0.000, Table 2, Fig.1).

The percentage of sperm deformity increased significantly in the diabetic group compared with the control and RJ groups (p=0.000). The diabetic group treated with RJ showed a significant decrease in percentage of deformed sperm compared with the diabetic group (p=0.000, Table 2).

In terms of sperm motility, RPFM decreased significantly in the diabetic group when compared with the control group (p=0.010). Furthermore, SPFM and ML increased in the diabetic group. Daily administration of RJ caused a significant increase in sperm motility and type of RPFM compared to the diabetic group (p=0.010, Table 3).

Acridine orange (AO) DNA denaturation

We observed a significant increase in the percentage of spermatozoa with DNA damage in the diabetic group compared with the control and RJ groups (p=0.010, Table 4, Fig.2).

Aniline blue (AB) chromatin quality

Treatment with RJ showed a markedly significant decrease in the percentage of spermatozoa with chromatin abnormalities (p=0.010, Table 4, Fig.1).

Testosterone level

Statistical analysis showed that intraperitoneal administration of STZ to normal rats induced a significant decrease in serum testosterone to 3.93 ± 0.46 ng/dL versus 6.25 ± 0.13 ng/dL in the control group (p=0.000). Oral administration of RJ at 100 mg/kg BW for 42 days to diabetic rats caused a significant increase in serum testosterone levels compared with the diabetic group (p=0.000, Table 5).

Malondialdehyde (MDA) levels

Diabetes induced lipid peroxidation in the testis tissue as revealed by a significant rise of MDA in the diabetic group compared with the control and RJ groups (p=0.000). MDA contents in the diabetic treated with RJ group were lower than those in the diabetic group. Therefore, RJ administration caused a partial decline of testis tissue MDA levels in the diabetic group treated with RJ (p=0.000, Table 5).

Table 1: Effect of oral administration of royal jelly (RJ) for 42 days on weight characteristics of male rats (n=8 per group)

Groups	Initial BW (g)	Final BW (g)	Testis weight (g)	Testis/BW ratio (%)
Control	194.4 ± 4.91 [a]	225.33 ± 2.73 [a]	2.00 ± 0.01 [a]	0.89 ± 0.01 [a]
RJ	193.9 ± 6.43 [a]	212.47 ± 4.22 [a]	2.04 ± 0.08 [a]	0.96 ± 0.01 [b]
Diabetic	206.7 ± 9.79 [a]	123.86 ± 6.07 [b]	0.76 ± 0.07 [b]	0.61± 0.02 [c]
Diabetic/RJ	192.6 ± 7.40 [a]	173.93 ± 6.73 [c]	1.50 ± 0.05 [c]	0.86 ± 0.01 [a]

Data are presented as mean ± SEM. Values with different letters indicate significant differences among groups at p≤0.05. BW; Body weight.

Table 2: Effect of oral administration of royal jelly (RJ) on sperm parameters in male rats (n=8 per group)

Groups	Count (10^6/ml of suspension)	Viability (%)	Deformity (%)
Control	49.70 ± 1.17 [a]	88.89 ± 0.74 [a]	3.19 ± 0.12 [a]
RJ	50.08 ± 0.56 [a]	85.15 ± 3.58 [a]	3.28 ± 0.17 [a]
Diabetic	30.79 ± 1.11 [b]	55.48 ± 3.33 [b]	9.19 ± 0.53 [b]
Diabetic/RJ	49.00 ± 1.77 [a]	71.44 ± 2.34 [c]	4.34 ± 0.57 [a]

Data are presented as mean ± SEM. Values with different letters indicate significant differences among groups at p≤0.05.

Fig.1: Sperm with altered plasma membranes appeared pink whereas those with intact plasma membranes did not stain (EN ×400).

Table 3: Effects (%) of royal jelly (RJ) on sperm motility in male rats (n=8 per group)

Groups	Sperm motility (RPFM)	Sperm motility (SPFM)	Sperm motility (CM)	Sperm motility (ML)
Control	63.25 ± 1.69 [a]	17.15 ± 0.64 [a]	11.20 ± 0.77 [a]	7.43 ± 0.45 [a]
RJ	64.73 ± 0.71 [a]	17.81 ± 0.81 [a]	11.72 ± 0.69 [a]	10.02 ± 0.67 [a]
Diabetic	47.88 ± 6.01 [b]	23.57 ± 0.79 [b]	10.56 ± 0.83 [a]	10.89 ± 1.16 [b]
Diabetic/RJ	59.36 ± 0.98 [a]	15.55 ± 0.26 [a]	13.88 ± 0.97 [a]	8.95 ± 0.51 [a]

Data are presented as mean ± SEM. Values with different letters indicate significant differences among groups at p≤0.05. RPFM; Rapid progressive forward movement, SPFM; Slow progressive forward movement, CM; Circumferential movement and ML; Motionless.

Table 4: Effect of royal jelly (RJ) on DNA damage and chromatin abnormalities of sperm in male rats (n=8 per group)

Groups	AB^+ (%)	AO^+ (%)
Control	10.33 ± 0.88 [a]	9.67 ± 1.45 [a]
RJ	10.67 ± 0.89 [a]	10.33 ± 0.88 [a]
Diabetic	23.33 ± 2.03 [b]	22.67 ± 1.20 [b]
Diabetic/RJ	14.67 ± 1.20 [a]	14.67 ± 2.03 [a]

Data are presented as mean ± SEM. Values with different letters indicate significant differences among groups at p≤0.05.
AB; Aniline blue and AO; Acridine orange.

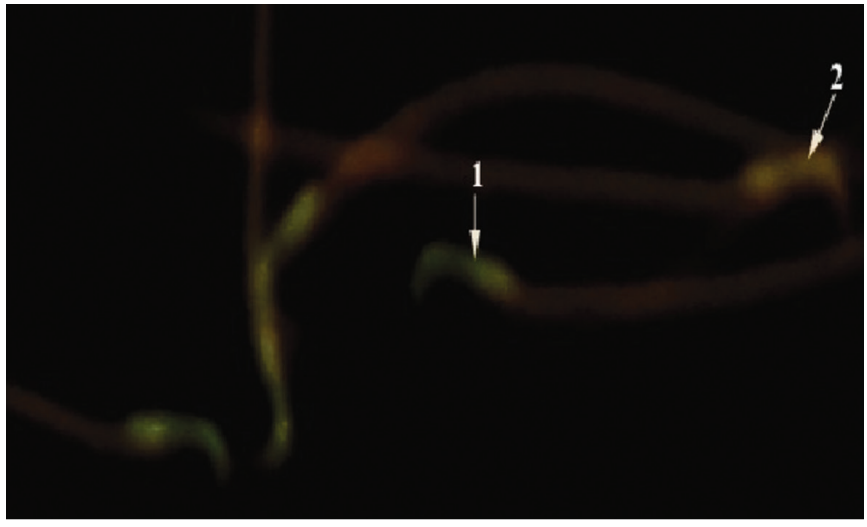


Fig.2: Diabetic treated royal jelly (RJ) group: 1. Sperm with normal DNA integrity showed green fluorescence and 2. Those with DNA damage stained orange-red (AO ×1000). AO; Acridine orange.

Table 5: Effect of oral administration of royal jelly (RJ) for 42 days on serum testosterone hormone and malondialdehyde (MDA) levels in male rats (n=8 per group)

Groups	Testosterone (ng/dL)	MDA (µmol/g tissue)
Control	6.25 ± 0.13 [a]	455.13 ± 7.40 [a]
RJ	7.13 ± 0.24 [a]	445.18 ± 4.73 [a]
Diabetic	3.93 ± 0.46 [b]	660.01 ± 12.61 [b]
Diabetic/RJ	6.32 ± 0.09 [a]	524.30 ± 19.96 [c]

Data are presented as mean ± SEM. Values with different letters indicate significant differences among groups at p≤0.05.

Discussion

The current study sought to determine the effect of RJ on reproductive parameters of diabetic male rats. We observed increased testicular weight, sperm count, sperm motility, number of viable spermatozoa and serum testosterone levels and decreased MDA level of testes tissue in the diabetic group treated with RJ. To our knowledge, this was the first report on the effect of RJ on diabetic male rats.

Diabetes decreased testicular weight and seminal vesicles, induced male reproductive dysfunctions, decreased serum testosterone levels and lowered semen quality and quantity. It is well-known that diabetes is positively associated with lowered male fertility and sexual disturbances (2). Previous studies have indicated that the neuropathy and vascular insufficiency caused by diabetes may be related to sexual dysfunction (24). Male sexual dysfunction in STZ-induced diabetic rats results from the alterations of the pituitary–testicular tract axis (25). Our results have clearly confirmed a decrease in testicular weight and serum testosterone levels in STZ-induced diabetic rats.

It has been demonstrated that oral administration of RJ improves the physiological status and a series of sperm parameters in heat-stressed male rabbits (13). Our study also showed that oral administration of 100 mg/kg BW of RJ to diabetic male rats for 42 days caused an increase in testes weight, viable sperm percentage, serum testosterone level and sperm motility, and decreased the number of abnormal sperm in diabetic rats. These results were attributed to the improvement of reproductive parameters in diabetic male rats by RJ, which was attributed to its antioxidant and estrogenic activities.

Diabetes is considered an important endocrine disease in the metabolism of carbohydrates. These changes result in an increase in free radical formation and LDL-oxidase production (6). Previous study have reported that diabetes causes a marked oxidative impact as evidenced by the significant increase in testicular lipid peroxidation as well as a significant decrease in testicular antioxidants, including CAT and GSH-R activities and GSH content (26). A number of studies have reported that intake of antioxidants and vitamins A, B, C, and E can increase stability of the testicular blood barrier and protect sperm DNA from oxidative stress caused by active free radicals (27).

RJ contains vitamins E and C (14) which have been reported to increase glutathione followed by decreased MDA levels in adult male rats (28). Vitamins E and C, whose antioxidant roles have been proven and shown to inhibit free radicals, induced damage to sensitive cell membranes of the testis and reduced lipid peroxidation in tissue estimation by MDA. Vitamins E and C significantly decreased MDA levels and increased glutathione levels (29). This study also showed that MDA levels in the testes decreased in diabetic rats treated with RJ. This effect could be attributed to vitamins C and E in RJ.

Studies have shown a direct link between blood sugar levels and sperm quality. In patients with high blood sugar levels, the incidence of non-viable sperm in seminal plasma is higher (6). Batchelder reported that RJ decreased blood sugar levels via the insulin-like material and other compounds (vitamins B3 and H and chromium). Also it has been noted that RJ is capable of sustaining a high level of blood sugar by participating in the oxidation of glucose to procure energy via the insulin-like material found in RJ. Furthermore, the insulin found in RJ highly resembles the insulin found in mammals (30). Therefore, our results have shown that RJ improved

reproductive parameters and decreased MDA levels in the testicular tissue of diabetic rats.

Conclusion

Oral administration of RJ to diabetic male rats decreased sperm deformity, DNA damage, chromatin abnormalities and testicular tissue MDA levels and increased testicular weight, sperm count, motility and viability. Therefore, this study has suggested that intake of RJ may be useful for diabetic patients who suffer from sexual impotency, as RJ produces anti-diabetic activity and exhibits fertility enhancing properties in male diabetic rats.

Acknowledgements

We thank the staff at Urmia University for their help and financial support in the preparation of this manuscript. The results of this paper are from Ghanbari's MSc thesis. There is no conflict of interest in this study.

References

1. Isidori AM, Pozza C, Gianfrilli D, Isidori A. Medical treatment to improve sperm quality. J Reprod Biomed. 2006; 12(6): 704 -714.
2. Mosher WD, Pratt WF. Fecundity and infertility in the United States: incidence and trends. J Fertil Steril. 1991; 56(2): 192-193.
3. Kanter M, Aktas C, Erboga M. Protective effect of quercetin against apoptosis and oxidative stress in streptozotocin-induced diabetic rat testis. Food Chem. Toxicol. 2012; 50(3-4): 719-725.
4. Amaral S, Moreno AJ, Santos MS, Seica R, Ramalho-Santos J. Effects of hyperglycemia on sperm and testicular cells of Goto-Kakizaki and streptozotocin-treated rat models for diabetes. Theriogenology. 2006; 66(9): 2056-2067.
5. Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R. Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. Circulation. 2005; 111(11): 1448-1454.
6. Khaki A, Nouri M, Fathiazad F, Ahmadi- Ashtiani HR, Rastgar H, Rezazadeh Sh. Protective effects of quercetin on spermatogenesis in streptozotocin-induced diabetic rat. J Med Plants. 2009; 8 Suppl 5: 57-64.
7. Pitocco D, Zaccardi F, Di Stasio E, Romitelli F, Santini SA, Zuppi C, et al. Oxidative stress, nitric oxide, and diabetes. Rev Diabet Stud. 2010; 7(1): 15-25.
8. Gharagozloo P, Aitken RJ. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. Hum Reprod. 2011; 26(7): 1628-1640.
9. Cande C, Cecconi F, Dessen P, Kroemer G. Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death?. J Cell Sci. 2002; 115(Pt 24): 4727-4734.
10. Guo H, Ekusa A, Iwai K, Yonekura M, Takahata Y, Morimatsu F. Royal jelly peptides inhibit lipid peroxidation in vitro and in vivo. J Nutr Sci Vitaminol (Tokyo). 2008; 54(3): 191-195.
11. Nakaya M, Onda H, Sasaki K, Yukiyoshi A, Tachibana H, Yamada K. Effect of royal jelly on bisphenol A-induced proliferation of human breast cancer cells. Biosci Biotechnol Biochem. 2007; 71(1): 253-255.
12. Kohguchi M, Inoue S, Ushio S, Iwaki K, Ikeda M, Kurimoto M. Effect of royal jelly diet on the testicular function of hamsters. Food Sci Technol Res. 2004; 10(4): 420-423.
13. Elnagar SA. Royal jelly counteracts bucks' "summer infertility". Animal Reprod Sci. 2010; 121(1-2): 174-180.
14. Nagaia T, Inoue R. Preparation and the functional properties of water extract and alkaline extract of royal jelly. Food Chem. 2004; 84: 181-186.
15. Liu JR, Yang YC, Shi LS, Peng CC. Antioxidant properties of royal jelly associated with larval age and time of harvest. J Agric Food Chem. 2008; 56(23): 11447-11452.
16. El-Nekeety AA, El-Kholy W, Abbas NF, Ebaid A, Amra HA, Abdel-Wahhab MA. Efficacy of royal jelly against the oxidative stress of fumonisin in rats. Toxicon. 2007; 50(2): 256-269.
17. Silici S, Ekmekcioglu O, Eraslan G, Demirtas A. Antioxidative effect of royal jelly in cisplatininduced testes damage. Urology. 2009; 74(3): 545-551.
18. Jin L, Xue HY, Jin LJ, Li SY, Xu YP. Antioxidant and pancreas-protective effect of aucubin on rats with streptozotocin-induced diabetes. Eur J Pharmacol. 2008; 582(1-3): 162-167.
19. Ghanbari M, Mortazavi SB, Khavanin A, Khazaei M. Simultaneous effects of exposure to microwaves and noise on male rat' sperm parameters and total antioxidant capacity. Health Scope. 2013; 1(4): 180-186.
20. Momeni HR, Daneshpajoh F. Protective effect of vitamin e on sperm parameters in adult rat treated with para-nonylphenol. Journal of Cell & Tissue. 2012; 2(4): 415-424.
21. Kazerooni T, Asadi N, Jadid L, Kazerooni M, Ghanadi A, Ghaffarpasand F, et al. Evaluation of sperm's chromatin quality with acridine orange test, chromomycin A3 and aniline blue staining in couples with unexplained recurrent abortion. J Assist Reprod Genet. 2009; 26(11-12): 591-596.
22. Nouri M, Khaki A, Fathi Azar F, Rashidi MR. The protective effects of carrot seed extract on spermatogenesis and cauda epididymal sperm reserves in gentamicin treated rats. Cell J. 2009; 11(3): 327-332.
23. Hosseinzadeh H, Sadeghnia HR. Safranal, a constituent of Corcus sativus (saffron), attenuated cerebral ischemia induced oxidative damage in rat hippocampus. J Pharm Pharm Sci. 2005; 8(3): 394-399.
24. Amaral S, Olivera PJ, Ramalho-Santos J. Diabetes and the impairment of reproductive functions: Possible role of mitochondria and reactive oxygen species. Curr Diabetes Rev. 2008; 4(1): 46-54.
25. Baccetti B, La-Marca A, Piomboni P, Capitani S, Bruni E, Petraglia F, et al. Insulin-dependent diabetes in men is associated with hypothalamo-pituitary derangement and with impairment in semen quality. Hum Reprod. 2002; 17(10): 2673-2677.
26. El-Missiry MA. Enhanced testicular antioxidant system by ascorbic acid in alloxan diabetic rats. Comp Biochem Physiol. 1999; 124(3): 233-237.
27. Jedlinska-Krakowska M, Bomba G, Jakubowski K, Rotkiewicz T, Jana B, Penkowski A. Impact of oxidative stress and supplementation with vitamins E and C on testes morphology in rats. J Reprod Dev. 2006; 52(2): 203-209.
28. Hassan AA. Effect of royal jelly on sexual efficiency in adult male rats. Iraqi Journal of Veterinary Sciences. 2009; 23 Supl 2: 155-160.
29. Ebisch IM, Pierik FH, DE Jong FH, Thomas CMG, Steeger-Theunissen RPM. Does folic acid and zinc sulphate intervention affect endocrine parameters and sperm characteristics in men?. Int J Androl. 2006; 29(2): 339-345.
30. Batchelder T. A novel mechanism of liver enhancement from a traditional bee product. Townsend Letter for Doctors and Patients. 2002; 233: 46-48.

Original Article

Oxidant and Antioxidant Status in Experimental Rat Testis after Testicular Torsion/Detorsion

Tatjana Cvetkovic, Ph.D.[1, 2], Jablan Stankovic, Ph.D.[3], Stevo Najman, Ph.D.[4], Dusica Pavlovic, Ph.D.[1], Dragana Stokanovic, M.D.[5*], Slobodan Vlajkovic, Ph.D.[6], Marija Dakovic-Bjelakovic, Ph.D.[6], Jovana Cukuranovic, M.D.[6], Vladimir Zivkovic, M.Sc.[6], Vladisav Stefanovic, Ph.D.[7]

1. Department of Biochemistry, School of Medicine, University of Nis, Nis, Serbia
2. Clinic of Nephrology, Clinical Centre Nis, Nis, Serbia
3. Clinic of Urology, Clinical Centre Nis, Nis, Serbia
4. Department of Biology, School of Medicine, University of Nis, Nis, Serbia
5. Department of Pharmacology and Toxicology, School of Medicine, University of Nis, Nis, Serbia
6. Department of Anatomy, School of Medicine, University of Nis, Nis, Serbia
7. School of Medicine, University of Nis, Nis, Serbia

Abstract

Background: The aim of this study was to determine oxidative stress (OS) parameters after testicular torsion/detorsion in adult rats.

Materials and Methods: In this experimental study, male adult Wistar rats were divided into four groups, each consisting of seven animals: group I-one hour right testicular torsion with subsequent orchiectomy, group II-one hour right testicular torsion followed by detorsion, group III-unilateral right-sided orchiectomy without previous torsion and group IV-control. After 30 days, bilateral orchiectomies were performed in rats with both testes and unilateral orchiectomies in rats with single testicles. Parameters of OS were determined in testicular tissue and in plasma.

Results: Plasma concentrations of advanced oxidation protein products (AOPP) and thiobarbituric acid reactive substances (TBARS) were higher ($p<0.05$ and $p<0.01$, respectively), whilst the plasma concentration of the total sulfhydryl (T-SH)-groups was lower ($p<0.05$) in group I compared to the control group. Group II had higher plasma concentrations of AOPP compared to group IV ($p<0.05$), as well as significantly increased TBARS and decreased T-SH-group levels compared to groups III ($p<0.05$ and $p<0.01$, respectively) and IV ($p<0.01$, for both parameters). There were significant differences in OS markers between the ipsilateral and contralateral testis, as well as significant correlations among levels of both plasma and tissue markers of OS.

Conclusion: The increase in TBARS levels seen throughout the experimental period indicated that OS development was caused by ischemia/reperfusion in the testicular tissue. The oxidant-antioxidant system of the testicular tissue was altered during torsion as well as detorsion.

Keywords: Oxidative Stress, Reperfusion Injury, Spermatic Cord Torsion, Testis, Advanced Oxidation Protein Products

Citation: Cvetkovic T, Stankovic J, Najman S, Pavlovic D, Stokanovic D, Vlajkovic S, Dakovic-Bjelakovic M, Cukuranovic J, Zivkovic V, Stefanovic V. Oxidant and antioxidant status in experimental rat testis after testicular torsion/detorsion. Int J Fertil Steril. 2015; 9(1): 121-128.

Received: 19 Oct 2013, Accepted: 28 Jan 2014
* Corresponding Address: Department of Pharmacology and Toxicology, School of Medicine, Bulevar dr Zorana Djindjica 81, 18000 Nis, Serbia
Email: dragana.stokanovic@medfak.ni.ac.rs

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 121-128

Introduction

Torsion of testis and spermatic cord is characteristic for adolescent and younger males, and requires emergency treatment. The incidence is 1:4000 in males aged less than 25 years (1). Urgent surgical treatment involves orchiectomy of the torsed testis or detorsion and its preservation if, on surgical exploration, the testicle is still viable. The two most important factors that determine testicular damage are the duration and degree of spermatic cord torsion (2, 3). The testis remains vulnerable to oxidative stress (OS) mainly due to the abundance of highly unsaturated fatty acids (4). Oxidative damage is the result of an imbalance between oxidative and antioxidative systems. It is suggested that the primary source of reactive oxygen species (ROS) are leukocytes that infiltrate testicular tissue, but they may originate from spermatozoa as well (5).

Increased expression of E-selectin and various cytokines is a stimulus for neutrophil accumulation and a subsequent rapid ROS generation (6). Oxidative phosphorylation in mitochondria is impaired by ischemia that consequentially leads to a decline in the level of cellular ATP and to the preservation of mitochondrial carriers in a reduced state (7). It has been demonstrated that reperfusion of the ischemic tissue promotes generation of ROS, which arise from activation of the xanthine oxidase system in parenchymal cells or from leukocytes that penetrate into interstitial tissue (8). Therefore, the treatment by detorsion may further damage the testis. Ipsilateral testis preservation leads to ischemic-reperfusion damage of both testes, primarily due to generation of ROS. Thus, reperfusion is beneficial for the ipsilateral testis by preventing ischemia-induced apoptosis and necrosis, but at the same time it may be deleterious for the contralateral testis. Testicular tissue is extremely susceptible to oxidative damage, due to high rate metabolism and cell replication, which also affects the contralateral testis (5). This may lead to functional impairment of both testes and infertility. In more than 35% of patients the spermatogram is abnormal and up to 25% become infertile (9). Beside ROS generation, there are other various theories of the mechanisms involved in sperm damage of both testes after detorsion, such as formation of antisperm antibodies, neutrophil infiltration, and decrease in contralateral blood flow.

ROS react with proteins, lipids, carbohydrates and nucleic acids leading to impaired cell function and apoptosis. Unfortunately both enzymatic [superoxide dismutase, glutathione peroxidase, catalase (CAT)] and non-enzymatic [glutathione (GSH), antioxidative vitamins] antioxidative defenses are limited. Therefore in pathologic conditions such as prolonged testicular torsion the damages can be irreversible (10).

The aim of this study was to determine OS parameters after testicular torsion/detorsion in plasma and both testes tissue of adult rats at 30 days after the surgical procedure.

Materials and Methods

Experimental animals

We conducted an experimental study on 28 adult male Wistar rats that weighed 150-190 g. Rats were obtained from the Institute of Biomedical Investigation, Faculty of Medicine, University in Nis, Serbia. All animals were treated humanely and the Ethical Committee of School of Medicine, University of Nis, Serbia approved all animal procedures. Rats were housed in a temperature-controlled room (23 ± 1˚C) on a 12-hour light and dark cycle, with ad libitum to food and water.

Experimental design

We randomly divided 28 male Wistar rats into four groups. The first group of rats was subjected to one hour right testicular torsion with subsequent orchiectomy (group I). The second group underwent right testicular torsion that lasted one hour, followed by detorsion (group II). In the third group, one hour after a scrotal incision, we performed a unilateral, right-side orchiectomy without previous torsion (group III). The fourth group served as a control and was not submitted to any surgical procedure (group IV). Thirty days later, bilateral orchiectomies were performed in the rats of groups II and IV, and left orchiectomies in groups I and III.

Surgical procedure

All surgical procedures were performed under

general anesthesia induced by an intraperitoneal one-shot injection of ketamine (8 mg/kg) and xylazine (10 mg/kg). The skin of the scrotal area was shaved and prepared with 10% povidone-iodine solution. A mid-scrotal longitudinal incision was performed for access to both testes. Torsion was created by twisting the right testis for 720° in a counterclockwise direction and maintained by fixing the testis to the scrotum with a 6-0 nylon suture passing through the tunica albuginea and dartos. The testis was left on top of the incised region, covered with a sterile gauze pad and kept moist with normal saline while the rat was kept under continuous anesthesia. After one hour of ischemia, we removed the suture; the right testis was either untwisted and removed or replaced in the scrotum for reperfusion. After each surgical intervention, the incision was closed by suture in two layers. Rats were allowed water and food. After 30 days, the rats were anesthetized and we removed their testes for further investigations. Animals were euthanized with an intracardiac barbiturate overdose injection.

Analytical procedure

Venous blood from the abdominal aorta was collected, centrifuged and stored at -20˚C until assay. Tissues were separately weighed and homogenized in ten volumes of cold 0.01M Tris-HCl buffer (pH=7.4) using an automatic homogenizer. The homogenates were then centrifuged at 20000 g for 15 minutes at 4˚C. Clear supernatants were used for measuring CAT activity, thiobarbituric acid reactive substances (TBARS) and GSH content. Tissue protein levels were also quantified at this step according to the method used by Lowry et al. (11).

We assayed the for the level of TBARS as a measure of lipid peroxidation in plasma and tissue according to the methods of Andreeva et al. (12) and Ohkawa et al. (13). Malondialdehyde (MDA) reacts with TBA under acidic conditions at 95˚C, forming a pink complex that absorbs at 532 nm.

The plasma advanced oxidation protein products (AOPP) assay was performed as described by Witko-Sarsat et al. (14). Each well of a 96-well microplate was filled with 200 µL of supernatant diluted at a ratio of 1:5 in PBS or chloramine-T standard solutions (0-100 µmol/L). Afterwards, 10 µL of 1.16 M potassium iodide (KI) was added followed by 20 µL of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm in a microplate reader against a blank that contained 200 µL of PBS, 10 µL of KI, and 20 µL of acetic acid. AOPP concentrations were expressed in µmol/L of chloramine-T equivalents.

The CAT activity was determined by the spectrophotometric method based on the ability of hydrogen peroxide to form a stable stained complex with molybdenum salts (15).

Improved method for the determination of tissue reduced GSH was based on the formation of a color product, monitored at 412 nm after the addition of Ellman reagent (5, 5´-dithiobis-2-nitrobenzoic acid) (16).

We determined the concentration T-SH groups in plasma by using Ellman's reagent (5, 5´-dithiobis-(2-nitrobenzoic acid), DTNB). Absorbance was measured at 412 nm against blank samples without DTNB and expressed as mol/L (17).

Statistical analysis

All data were expressed as mean and standard deviation. Statistical analysis was performed using SPSS 16.0 statistical software. Parametric group data was compared using ANOVA with Tukey post-hoc test and the Student's t test. Correlation significance was determined according to Pearson's coefficient. $p<0.05$ was considered statistically significant.

Results

Levels of plasma markers of OS in each experimental and control group are shown in table 1. Compared to the control group, there were higher concentrations of AOPP ($p<0.05$) and TBARS ($p<0.01$) whereas the concentration of T-SH-groups in plasma was lower ($p<0.05$) in the torsion/castration group. group II (torsion/detorsion) had higher plasma concentrations of AOPP compared to group IV ($p<0.05$), as well as significantly increased TBARS and decreased T-SH-groups compared to both groups III ($p<0.05$ and $p<0.01$, respectively) and IV ($p<0.01$, for both parameters).

Table 1: Markers of oxidative stress (OS) in rat plasma

	Group	I	II	III	IV	p
Plasma (mean ± SD)	AOPP (μmol/L)	46.88 ± 1.58 [a]	53.5 ± 12.86 [a]	37.07 ± 15.2	35.39 ± 5.77	0.050
	CAT (U/L)	175.13 ± 82.54	147.16 ± 101.25	245.37 ± 57.07	237.47 ± 82.2	0.358
	TBARS (μmol/L)	23.38 ± 4.22 [b]	28.54 ± 6.52 [b, c]	17.6 ± 7.67	13.67 ± 2.73	0.004
	T-SH (μmol/L)	255.72 ± 71.02 [a]	217.84 ± 24.21 [b, d]	327.57 ± 85.42	354.26 ± 57.77	0.006

[a]; P<0.05, [b]; P<0.01 (vs. group IV). [c]; P<.0.05 and [d]; P<0.01 (vs. group III), AOPP; Advanced oxidation protein products, CAT; Catalase, TBARS; Thiobarbituric acid reactive substances and T-SH; Total sulfhydryl groups.

In figures 1-3, differences in markers of OS between ipsilateral and contralateral testis can be seen. There were no statistically significant differences between groups III and IV (sham operated and control rats). In group I, CAT activity was higher in the contralateral testis ($p<0.05$) as well as the concentrations of TBARS and GSH ($p<0.01$). In the torsion/detorsion group, the levels of all three markers were significantly lower in the contralateral testis ($p<0.05$).

We observed significantly higher CAT activity ($p<0.05$), TBARS concentrations ($p<0.001$) and GSH levels ($p<0.05$) in the tissue of the ipsilateral testis in group II which underwent detorsion compared to group I where the torsed testis was castrated after 1 hour. Compared to the control group, all three OS markers increased in the torsion/detorsion group: CAT ($p<0.05$), TBARS ($p<0.001$) and GSH ($p<0.01$). TBARS and GSH concentrations significantly increased in the tissue of detorsed testis in group II compared to the sham operated group III ($p<0.001$).

Similar results were obtained for the contralateral testis. The concentration of TBARS significantly increased when detorsion was performed 1 hour after torsion, but not castration (group II vs. group I $p<0.05$). CAT activity increased in group I compared to group III ($p<0.01$). TBARS concentration was higher in group II than in both groups III and IV ($p<0.001$).

We observed statistically significant correlations among levels of both plasma and tissue markers of OS (Table 2). Plasma concentrations of TBARS directly correlated with plasma levels of AOPP ($r=0.434$, $p<0.05$) as well as with CAT activity in the torsed testis ($r=0.427$, $p<0.05$) and TBARS in the ipsilateral ($r=0.598$, $p<0.01$) and contralateral ($r=0.595$, $p<0.01$ contralateral) tissues of both testes. TBARS levels in the ipsilateral testis were found to be negatively correlated with the plasma concentration of the T-SH-groups ($r=-0.455$, $p<0.05$). An increase in TBARS levels in the right torsed testis was associated with increased CAT activity ($r=0.567$, $p<0.01$) and GSH content ($r=0.911$, $p<0.001$) in the same testis and TBARS levels in the contralateral testis ($r=0.659$, $p<0.001$). Similarly, we observed positive correlations between TBARS in the contralateral testis and the other markers of OS in the tissue of the same testis, CAT activity ($r=0.648$, $p<0.001$), GSH levels ($r=0.597$, $p<0.01$), as well as with plasma AOPP concentration ($r=0.595$, $p<0.01$) and GSH quantities ($r=0.580$, $p<0.01$) in the torsed testis. In both testes, ipsilateral and contralateral, two markers of antioxidative capacity (CAT, GSH) showed positive correlations ($r=0.588$, $p<0.01$; $r=0.615$, $p<0.001$, respectively). The increase in plasma AOPP concentration was associated with the increase in CAT activity in the tissue of the contralateral testis ($r=0.540$, $p<0.01$). These data showed direct correlation between plasma and tissue markers of OS in both testes.

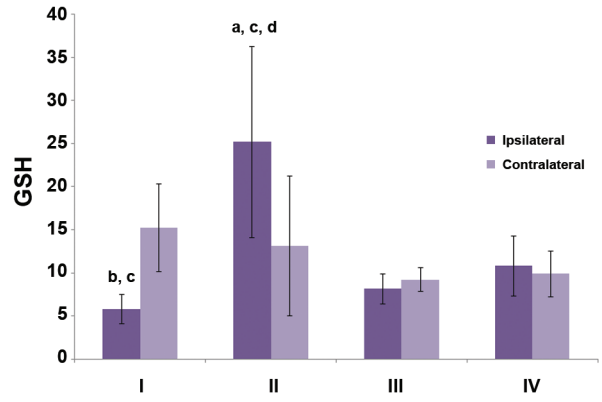


Fig.1: Concentration of glutathione (GSH) in testes tissues.
[a]; P<0.05 and [b]; P<0.01 (ipsilateral vs. contralateral testis), [c]; P<0.01 (vs. group IV) and [d]; P<0.001 (vs. group III).

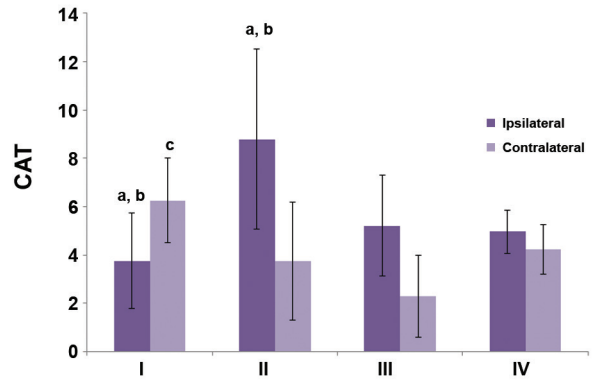


Fig.2: Concentration of catalase (CAT) in testes tissues.
[a]; P<0.05 (ipsilateral vs. contralateral testis), [b]; P<0.05 (vs. group IV), [c]; P<0.01 (vs. group III) and [d]; P<0.05 (vs. group II).

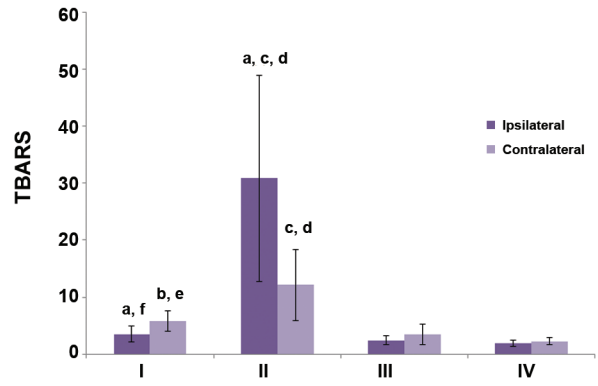


Fig.3: Concentration of thiobarbituric acid reactive substances (TBARS) in testes tissues.
[a]; P<0.05 (ipsilateral vs. contralateral testis), [b]; P<0.05 and [c]; P<0.001 (vs. group IV), [d]; P<0.001 (vs. group III), [e]; P<0.05 and [f]; P<0.001 (vs. group II).

Table 2: Correlation between plasma and tissue markers of oxidative stress (OS)

			Plasma				Ipsilateral testis			Contralateral testis		
			AOPP	**CAT**	**TBARS**	**T-SH**	**CAT**	**TBARS**	**GSH**	**CAT**	**TBARS**	**GSH**
Plasma	AOPP	r	-	-0.073	0.434	-0.266	0.095	0.357	0.223	0.540	0.595	0.384
		p	-	0.735	0.034	0.210	0.658	0.087	0.295	0.006	0.002	0.064
	CAT	r	-0.073	-	0.041	0.065	0.207	0.108	0.200	-0.137	0.114	-0.020
		p	0.735	-	0.850	0.761	0.333	0.616	0.348	0.522	0.595	0.925
	TBARS	r	0.434	0.041	-	-0.350	0.427	0.598	0.400	0.203	0.469	0.115
		p	0.034	0.850	-	0.094	0.037	0.002	0.053	0.340	0.021	0.592
	T-SH	r	-0.266	0.065	-0.350	-	-0.213	-0.455	-0.279	-0.063	-0.365	-0.029
		p	0.210	0.761	0.094	-	0.317	0.025	0.187	0.769	0.080	0.892
Ipsilateral testis	CAT	r	0.095	0.207	0.427	-0.213	-	0.567	0.588	-0.063	0.270	-0.101
		p	0.658	0.333	0.037	0.317	-	0.004	0.002	0.769	0.201	0.640
	TBARS	r	0.357	0.108	0.598	-0.455	0.567	-	0.911	0.338	0.659	0.116
		p	0.087	0.616	0.002	0.025	0.004	-	0.000	0.106	0.000	0.591
	GSH	r	0.223	0.200	0.400	-0.279	0.588	0.911	-	0.274	0.580	0.228
		p	0.295	0.348	0.053	0.187	0.002	0.000	-	0.196	0.003	0.284
Contralateral testis	CAT	r	0.540	-0.137	0.203	-0.063	-0.063	0.338	0.274	-	0.648	0.615
		p	0.006	0.522	0.340	0.769	0.769	0.106	0.196	-	0.001	0.001
	TBARS	r	0.595	0.114	0.469	-0.365	0.270	0.659	0.580	0.648	-	0.597
		p	0.002	0.595	0.021	0.080	0.201	0.000	0.003	0.001	-	0.002
	GSH	r	0.384	-0.020	0.115	-0.029	-0.101	0.116	0.228	0.615	0.597	-
		p	0.064	0.925	0.592	0.892	0.640	0.591	0.284	0.001	0.002	-

AOPP; Advanced oxidation protein products, TBARS; Thiobarbituric acid reactive substances, CAT; Catalase, T-SH; Total sulfhydryl groups and GSH; Glutathione.

Discussion

Prolonged testicular torsion leads to testicular ischemia and high levels of free radical production (18). An increase in ROS-induced OS has been shown in testicular tissue following detorsion, indicating reperfusion injury (19). OS induces poor sperm function since mammalian spermatozoa membranes are very sensitive to oxidative damage. Leydig cell mitochondria and microsomes of testes are known to contribute significantly to an increased generation of ROS (20). Ischemia also triggers the release of cytokines causing neutrophils to infiltrate testes and may represent yet another good source of uncontrolled free radical generation for mediating the pathophysiological consequences of temporary testicular ischemia (21). Measured after different time periods of reperfusion- one hour, 24 hours, 48 hours and one week, the increase in MDA levels were maintained compared to sham-operated controls (4). It has been reported that after testicular detorsion OS increases and impairs testicular functions, partially by disrupting the normal structure of seminiferous tubules and by diminishing the number of germ cells (22, 23). Thus, despite testicular torsion being repaired before infarction and necrosis, there is an occurrence of I/R injury that is a classic inducer of OS. The cutoff point for the preservation of torsed testis is 12 hours. In experimental animals permanent damage occurs after 4-6 hours, but in humans it is not before 12 hours of torsion that necrosis takes place (1, 9). If preserved, ischemic damage to the torsed testis is followed by reperfusion injury of both testes. In the first few hours the rapid increase in ROS generation may be compensated by natural antioxidative defenses, primarily GSH. Later, other mechanisms such as inflammation and inflammatory cell infiltration become involved and damage becomes irreversible (10).

Biochemical markers of OS are more sensitive indicators of tissue damage and can be detected much earlier than histological alterations. The most prominent tissue alterations are due to lipid peroxidation. Even if sperm count is not significantly impaired, infertility may result from low motility or DNA damage caused by OS (24). The time of reperfusion plays an important role as a determinant of the extension of reperfusion injury in both testes. Independent of torsion time, after one hour of reperfusion the content of GSH decreases and the CAT activity and levels of TBARS begin to increase in both the ipsilateral and contralateral testis (10, 25-27). After a short period of time (4, 19), a month or even more than a month after detorsion (28, 29), MDA levels drastically increase. The results of present study have shown that preservation of the detorsed testis increased oxidative damage in the contralateral one. There was a significant difference in TBARS concentrations between both torsion/castration (group I) and torsion/detorsion models (group II) on one side, and the sham-operated (group III) and control (group IV) groups on the other side. Additionally, lipid peroxidation was more intense in the contralateral testis when the detorsed testis was preserved compared to the model where castration was performed after one hour of ischemia. Increased levels of AOPP and TBARS, and decreased levels of T-SH-groups in plasma in both models of testicular torsion suggested that ROS generated in the torsed testis might have systemic effects including those on the contralateral testis.

The reduced availability of cellular GSH becomes a rate limiting factor for detoxification of oxygen metabolites, most likely hydrogen peroxide and lipid hydroperoxide. Simultaneously, an important accumulation and release of oxidized GSH occurs which causes further reduction of the GSH/GSSG ratio and a shift of the cellular thiol redox state toward oxidation. Ischemia that lasts for three hours or less increases OS and diminishes antioxidative GSH to a level sufficient to disrupt spermatogenesis. In the first hours of reperfusion, the level of reduced GSH has been shown to decrease. However after 48 hours its concentration was significantly above those observed in control groups and the ischemic model (4, 30, 31). In other short-term torsion/detorsion models (19), GSH diminished in the ipsilateral testis, while plasma total antioxidative capacity remained unchanged. In our experiment, ischemia did not change GSH levels in either of the testes. On the other hand, reperfusion significantly increased the concentration of GSH in the ipsilateral testis, which suggested an increase in antioxidative defenses.

In our experiment, plasma CAT activity was unchanged. Concerning testicular tissue CAT, we observed an increase in its activity in the castrated testis one hour after torsion, while in the contralateral testis there were no alterations. This result implied that in this model the contralateral testis was less affected by ischemia of the ipsilateral testis compared to the second model where detorsion was performed. In the second model, CAT activity significantly increased in the ipsilateral testis a month after detorsion due to increased ROS levels. Other studies' results were inconclusive; some confirmed the current study results (25, 30), whereas others had the opposite findings (26-29).

The correlations found between levels of various markers of OS in both testes and plasma directly im-

plicated a causal relationship between processes ongoing in torsed/detorsed testis and the effects on the contralateral testis.

In this experimental study we have used adult rats with the assumption that their testes are less vulnerable to OS than testes of young rats. The second difference was the extended period of reperfusion (30 days) and the evaluation of OS parameters in both plasma and testes tissues.

Since reperfusion potentiates ROS generation and subsequent impairment of both testes, a possible solution could be ischemic preconditioning. Numerous studies with various protocols have shown diminished testicular injury (25, 32). Preconditioning may also be pharmacological: various antioxidative, anti-inflammatory and immunosuppressive agents have been proposed as beneficial in preserving male fertility after unilateral testicular torsion.

Conclusion

The increase in TBARS levels seen throughout the experimental period indicated that ischemia followed by reperfusion of testicular tissue caused OS. The oxidant-antioxidant system of the testicular tissue changed during torsion as well as detorsion.

Acknowledgements

This study was supported by a grant from the Ministry of Science and Technological Development-projects number 41018 and 175092. The authors declare that there is no conflict of interest for this article.

References

1. Ellati RT, Kavoussi PK, Turner TT, Lysiak JJ. Twist and shout: a clinical and experimental review of testicular torsion. Korean J Urol. 2009; 50(12): 1159-1167.
2. Abuja PM, Albertini R. Methods for monitoring oxidative stress, lipid peroxidation and oxidation resistance of lipoproteins. Clin Chim Acta. 2001; 306(1-2): 1-17.
3. Vysser AJ, Heyns CF. Testicular function after torsion of the spermatic cord. BJU Int. 2003; 92(3): 200-203.
4. Elshaari FA, Elfagih RI, Sheriff DS, Barassi IF. Oxidative and antioxidative defense system in testicular torsion/detorsion. Indian J Urol. 2011; 27(4): 479-484.
5. Turner TT, Lydiak JJ. Oxidative stress: a common factor in testicular dysfunction. J Androl. 2008; 29(5): 488-498.
6. Turner TT, Bang HJ, Lysiak JL. The molecular pathology of experimental testicular torsion suggests adjunct therapy to surgical repair. J Urol. 2004; 172(6 Pt2): 2574-2578.
7. Guimaraes SB, Vasconcelos PR. In vivo acute changes in ATP and glucose concentrations in the testicles of prepubertal rats following unilateral torsion. Acta Cir Bras. 2002; 17(2): 110-115.
8. Granger DN, Korthuis RJ. Physiological mechanisms of postischemic tissue injury. Annu Rev Physiol. 1995; 57: 311-332.
9. Kapoor S. Testicular torsion: a race against time. Int J Clin Pract. 2008; 62(5): 821-827.
10. Filho DW, Torres MA, Bordin AL, Crezcynski-Pasa TB, Boveris A. Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia-reperfusion injury. Mol Aspects Med. 2004; 25(1-2): 199-210.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951; 193(1): 265-275.
12. Andreeva LI, Kozhemiakin LA, Kishkun AA. Modification of the method of determining lipid peroxidation in a test using thiobarbituric acid. Lab Delo. 1988; (11): 41-43.
13. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979; 95(2): 351-358.
14. Witko-Sarsat V, Friedlander M, Capeillere-Blandin C, Nguyen-Khoa T, Nguen AT, Zingraff J, et al. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney Int. 1996; 49(5): 1304-1313.
15. Goth L. A simple method for determination of serum catalase activity and revision of reference range. Clin Chim Acta. 1991; 196(2-3): 143-151.
16. Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med. 1963; 61: 882-888.
17. Sedlak J, Lindsay RH. Estimation of total protein bound and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem. 1968; 25(1): 192-205.
18. Taneli F, Vatansever S, Ulman C, Yilmaz O, Giray G, Genc A, et al. The effect of spermatic vessel ligation on testicular nitric oxide levels and germ cell-specific apoptosis in rat testis. Acta Histochem. 2005; 106(6): 459-466.
19. Guimaraes SB, Aragao AA, Santos JM, Kimura Ode S, Barbosa PH, Vasconcelos PR. Oxidative stress induced by torsion of the spermatic cord in young rats. Acta Cir Bras. 2007; 22(1): 30-33.
20. Mishra M, Acharya UR. Protective action of vitamins on the spermatogenesis in lead-treated Swiss mice. J Trace Elem Med Biol. 2004; 18(2): 173-178.
21. Tian WN, Braunstein LD, Apse K, Pang J, Rose M, Tian X. Importance of glucose-6-phosphate dehydrogenase activity in cell death. Am J Physiol. 1999; 276(5 Pt1): C1121-C1131.
22. Rodriguez MG, Rival C, Theas MS, Lustig L. Immunohistopathology of the contralateral testis of rats undergoing experimental torsion of the spermatic cord. Asian J Androl. 2006; 8(5): 576-583.
23. Lysiak JJ, Zheng S, Woodson R, Turner TT. Caspase-9-dependent pathway to murine germ cell apoptosis: Mediation by oxidative stress, BAX, and caspase-2. Cell Tissue Res. 2007; 328(2): 411-419.
24. Tremellen K. Oxidative stress and male infertility--a clinical perspective. Hum Reprod Update. 2008; 14(3): 243-258.
25. Shimizu S, Saito M, Dimitriadis F, Kinoshita Y, Shomori K, Satoh I, et al. Protective effect of ischaemic post-conditioning on ipsilateral and contralateral testes after unilateral testicular ischaemia-reperfusion injury. Int J Androl. 2011; 34(3): 268-275.
26. Raju AB, Challa SR, Akula A, Kirn K, Harinadh GB. Evaluation of oxidant and anti-oxidant balance in experimentally induced testicular injury by ischemia reperfusion in rats. Eur J Gen Med. 2011; 8(2): 117-121.
27. Payabvash S, Kiumehr S, Tavangar SM, Depour AR. Ethyl pyruvate reduces germ cell-specific apoptosis and oxidative stress in rat model of testicular torsion/detorsion. J Pediatr Surg. 2008; 43(4): 705-712.
28. Hamed GM, Ahmed RM, Emara MM, Mahmoud MH. Effects of erythropoietin on experimental unilateral testicular torsion detorsion in rat model. Life Sci J. 2011; 8(2):405-412.
29. Wei SM, Yan ZZ, Zhou J. Protective effect of rutin on testicular ischemia-reperfusion injury. J Pediatr Surg. 2011; 46(7): 1419-1424.
30. Hekimoglu A, Kurcer Z, Aral F, Baba F, Sahna E, Atessahin A. Lycopene, an antioxidant carotenoid, attenuates testicular injury caused by ischemia/reperfusion in rats. Tohoku J Exp Med. 2009; 218(2): 141-147.
31. Yildiz H, Durmus AS, Simsek H, Yaman M. Protective effect of sildenafil citrate on contralateral testis injury after unilateral testicular torsion/detorsion. Clinics (Sao Paulo). 2011; 66(1): 137-142.
32. Ozkisacik S, Yazici M, Gursoy H, Serter M, Culhaci N. The effects of short-interval postconditioning in preventing testicular ischemia-reperfusion injury in rats. J Pediatr Surg. 2011; 46(3): 546-550.

Original Article

In Vitro Effect of Cell Phone Radiation on Motility, DNA Fragmentation and *Clusterin* Gene Expression in Human Sperm

Adel Zalata, M.D.[1], Ayman Z El-Samanoudy, M.D.[1], Dalia Shaalan, M.D.[1], Youssef El-Baiomy, M.D.[2], Taymour Mostafa, M.D.[3]*

1. Department of Medical Biochemistry, Faculty of Medicine, Mansoura University, Mansoura, Egypt
2. Department of Dermatology and Andrology, Faculty of Medicine, Mansoura University, Mansoura, Egypt
3. Department of Andrology and Sexology, Faculty of Medicine, Cairo University, Cairo, Egypt

Abstract

Background: Use of cellular phones emitting radiofrequency electromagnetic field (RF-EMF) has been increased exponentially and become a part of everyday life. This study aimed to investigate the effects of *in vitro* RF-EMF exposure emitted from cellular phones on sperm motility index, sperm DNA fragmentation and seminal *clusterin (CLU)* gene expression.

Materials and Methods: In this prospective study, a total of 124 semen samples were grouped into the following main categories: i. normozoospermia (N, n=26), ii. asthenozoospermia (A, n=32), iii. asthenoteratozoospermia (AT, n=31) and iv. oligoasthenoteratozoospermia (OAT, n=35). The same semen samples were then divided into two portions non-exposed and exposed samples to cell phone radiation for 1 hour. Before and immediately after exposure, both aliquots were subjected to different assessments for sperm motility, acrosin activity, sperm DNA fragmentation and *CLU* gene expression. Statistical differences were analyzed using paired t student test for comparisons between two sub-groups where $p<0.05$ was set as significant.

Results: There was a significant decrease in sperm motility, sperm linear velocity, sperm linearity index, and sperm acrosin activity, whereas there was a significant increase in sperm DNA fragmentation percent, *CLU* gene expression and CLU protein levels in the exposed semen samples to RF-EMF compared with non-exposed samples in OAT>AT>A>N groups, respectively ($p<0.05$).

Conclusion: Cell phone emissions have a negative impact on exposed sperm motility index, sperm acrosin activity, sperm DNA fragmentation and seminal *CLU* gene expression, especially in OAT cases.

Keywords: Cell Phone, Spermatozoa, Electromagnetic Radiation, Sperm Motility

Citation: **Zalata A, El-Samanoudy AZ, Shaalan D, El-Baiomy Y, Mostafa T. In vitro effect of cell phone radiation on motility, DNA fragmentation and clusterin gene expression in human sperm. Int J Fertil Steril. 2015; 9(1): 129-136.**

Introduction

Nowadays, cell phone technology is an integral part of everyday life, and its use will continue to grow as their providers proceed to offer more liberal services and newer, better products. Generally, a growing concern for possible adverse effects of cell phones on human health has evoked a flurry of scientific activity. Several studies have shown the association between human health and exposure to radiofrequency electromagnetic field (RF-EMF), emphasizing on clinical conditions as childhood leukemia, brain tumors, neurodegenerative diseases and genotoxicity (1).

Received: 30 Oct 2012, Accepted: 24 Dec 2013
*** Corresponding Address: Department of Andrology and Sexology, Faculty of Medicine, Cairo University, Cairo 11562, Egypt**
Email: taymour.mostafa@yahoo.com

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 129-136

RF energy is a type of non-ionizing radiation, including electromagnetic radiation (EMR), produced by cell phones, but is not strong enough to cause ionization of atoms or molecules. Cellular phones emit low levels of RF in the micro-wave range while being used. Although high-level of RF causes adverse health effects through heating body tissues, exposure to low-level RF does not produce such effects. Several experimental studies demonstrated that exposure to electromagnetic or static magnetic fields had adverse effects on the reproductive system (2).

De Iuliis et al. (3) demonstrated that RF-EMF in both the power density and frequency range of mobile phones enhances mitochondrial reactive oxygen species (ROS) generation in human spermatozoa that leads to decreased sperm motility and vitality, while stimulates DNA base adduct formation and, ultimately sperm DNA fragmentation. Kang et al. (4) showed that cell phone radiation may cause structural and functional injuries of the testis, alter semen parameters, and reduce epididymal sperm concentrations. In May 2011, the international agency for research on cancer (IARC) at World Health Organization (WHO) has categorized the RF-EMF from mobile phones, and from other devices that emit similar non-ionizing electromagnetic fields, as a group 2B (possible) human carcinogen (5).

Sperm DNA fragmentation in the male germ line has been associated with impaired fertilization, poor embryonic development and high rates of miscarriage (6). Of course, the attention has been focused on the environmental and genetic factors that might be involved in the etiology of sperm DNA damage. One of these factors growing rapidly is the increased exposure to RF-EMF emitted from cell phones (7).

Clusterin (CLU), a 70-80 ku heterodimeric, disulfide-linked glycoprotein is over-expressed in a variety of tissues undergoing stress. *CLU* encoding clusterin appears to be a potential pathophysiologically gene having multiple functions related to apoptosis, inflammation, proliferation, and differentiation (8, 9).

This study aimed to assess the possible effects of *in vitro* RF-EMF exposure emitted from cell phones on sperm motility index, sperm DNA fragmentation and seminal *CLU* gene expression.

Materials and Methods

In this prospective study, semen samples were collected from 124 individuals presented to Mansoura University Hospital, Mansoura, Egypt, after Ethical Committee and Institutional Review Board approval with informed consent. Exclusion criteria were as follows: smoking, leukocytospermia, varicocele and abnormal karyotyping. Semen samples were collected by masturbation after an abstinence period of 4-5 days according to WHO guidelines (10). According to their semen analysis, they were grouped into the following main categories: normozoospermia (N, n=26), asthenozoospermia (A, n=32), asthenoteratozoospermia (AT, n=31) and oligoasthenoteratozoospermia (OAT, n=35).

Each semen sample were divided into two portions, non-exposed (control) and exposed (experimental). Experimental semen samples were exposed to electromagnetic waves (EMW) emitted from a commercially available cellular phone (850 MHz frequency, maximum power <1 W, specific absorption rates 1.46 W/kg) kept at 10 cm distance for 60 minutes. Unexposed semen aliquots were kept under the same conditions without RF-EMW exposure at room temperature to avoid the effect of temperature or reactive oxygen species (ROS) formation on semen parameters. After elapsed time, both aliquots were evaluated in terms of sperm motility, acrosin activity, sperm DNA fragmentation and seminal *CLU* gene expression before and immediately after exposure.

Sperm acrosin activity

It was assessed by gelatinolysis technique where gelatin-covered slides were prepared by spreading 20 µl of 5% gelatin (Merck, Darmstadt, Germany) in distilled water on the slides. The slides were air-dried, stored at 4˚C overnight, fixed and washed in phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA). Purified spermatozoa were diluted 1:10 in PBS containing 15.7 mMol α-D-glucose (Sigma-Aldrich, St. Louis, MO, USA). Semen samples were incubated in a moist chamber at 37˚C for 2 hours. The halo diameter around any 10 sperms was measured in phase contrast using an eyepiece micrometer (VWR, Radnor, PA,

USA). The halo formation rate was calculated/slide as the percentage of spermatozoa showing a halo after evaluating 100 spermatozoa (acrosin activity index=halo diameter×halo formation rate) (11).

Sperm DNA fragmentation analysis

It was performed in fresh semen using flowcytometry (DAKO-Cytomation, Glostrup, Denmark) by a kit supplied by Coulter (Beckman Coulter, Fulterton, CA, USA) based on the fluorescence emission from individual sperm that was stained with propidium iodide (PI) and excited at 488 nm with an argon laser. Semen samples were diluted with PBS (pH=7.4) to reach 2×10^6 sperm/ml where 50 µl were incubated with 100 µl lysing reagent for 15 seconds, and then 2 ml of PI were added and mixed. After staining, flowcytometry acquisition was performed where the intensity of its fluorescence emission corresponds to the DNA content. The analysis displays a constant and characteristic bimodal non-artifactual DNA pattern confirming the existence of two distinct populations. The main population is represented by a peak followed by a shoulder which is the marginal population representing a sperm group altered in the nuclear condensation (DNA damage), yielding unstable chromatin appearing more stainable. The Percentage of sperm cells with DNA damage is automatically calculated by the flowcytometer after acquisition of 5000 sperms (12).

CLU gene expression

Simultaneous isolation of total RNA and total proteins were done using Tri-Fast reagent kit (PeqLab Biotechnologie GmbH, Germany). The remaining DNA was digested using DNase I (Sigma-Aldrich, St. Louis, MO, USA). The concentration of isolated RNA was determined spectrophotometrically at optical density of 260 nm. Ten µl of each sample were added to 990 µl diethylpyrocarbonate (DEPC)-treated water and quantified by measuring the absorbance at 260 nm as follows: RNA yield (µg/ml)=A260×40×100 (dilution factor). Purity of RNA was assessed by gel electrophoresis through formaldehyde agarose gel electrophoresis (Sigma-Aldrich, St. Louis, MO, USA) and ethidium bromide staining (Sigma-Aldrich, St. Louis, MO, USA) to show 2 sharp purified bands representing 28S and 18S ribosomal RNA.

RT-PCR for extracted RNA

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using ready-to-go RT-PCR beads for first cDNA synthesis and PCR reaction (Amersham Biosciences, Piscataway, NJ, USA) utilizing Moloney-murine leukemia virus reverse transcriptase (M-MuLV RT) and Taq polymerase to generate PCR product from RNA template. Each bead is optimized to allow the first strand cDNA synthesis and PCR reaction to proceed sequentially as a single tube, single step reaction.

Synthesis of cDNA

The following items were added to the tube containing the beads: 2 µl the first strand primer, 3 µl (30 pmol) PCR gene-specific primer (sense), 3 µl (30 pmol) PCR gene-specific primer (anti-sense), 25 µl total template RNA containing 1 µg and 17 µl DEPC-treated water to reach a total volume of 50 µl. One tube was prepared as a negative control reaction to test DNA contamination. The dehydrated bead (without template and primers) was incubated at 95˚C for 10 minutes to inactivate M-MuLV RT where 50 µl mineral oil was added to overlay the reaction. The reactions were transferred to the thermal cycler in order to be incubated at 40˚C for 30 minutes for synthesis of cDNA followed by incubation at 95˚C for 5 minutes to inactivate reverse transcriptase and to denature the template. The sequence of oligonucleotide primers of clusterin gene were designed from GenBank sequences 5´-CTTGATGCCCTTCTCTCCGTA-3´ (sense) and 5´-AACGTCCGAGTCAGAAGTGTG-3´ (antisense), located at nucleotides 684 to 704 and 1194 to 1214 of *CLU* cDNA. Thermal cycling reaction was performed as follows: 30 cycles of denaturation at 95˚C for 1 minute, annealing at 55˚C for 1 minute, extension at 72˚C for 1 minute and final extension at 72˚C for 10 minutes. The products were subjected to agarose gel electrophoresis using 2% agarose, stained with ethidium bromide, visualized via a light UV transilluminator, (Clinx Science Instruments Co., Ltd, Shanghai, China) and photographed.

CLU protein was analyzed by Western blotting technique using rabbit anti-human CLU polyclonal unconjugated primary antibody against β- tubulin as a control. Goat anti-rabbit IgG antibody conju-

gated to horseradish peroxidase (HRP) was used as secondary antibody. Colorimetric immunodetection of the protein was used as an enzyme substrate (tetramethylbenzidine) that reacted with the reporter enzyme (HRP) and precipitated into the conjugated antibodies. The bands on the membrane were digitally photographed and analyzed with Scion image release alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA) performing bands detection and conversion to peaks. Area under each peak was calculated in square pixels and used for quantification. CLU gene expression and CLU protein levels were determined by calculating the ratio between the square pixel values of the target bands in relation to the control bands.

Statistical analysis

It was performed using SPSS program version 17 (SPSS Inc., Chicago, IL, USA). The data were expressed as mean ± standard deviation (SD). The statistical differences were analyzed using paired t-student test for comparison between two subgroups. P<0.05 was set as significant.

Results

The mean sperm concentration values in the N, A, AT and OAT groups were 54.34 ± 5.0, 38.85 ± 4.04, 23.52 ± 8.94 and 8.00 ± 3.77 (106/ml), respectively. The mean percentage values of abnormal sperm belonging to the investigated groups (A, AT and OAT groups) were 11.42 ± 2.61, 10.04 ± 3.7, 30.80 ± 7.22, 39.68 ± 5.6, respectively. Sperm motility, sperm linear velocity, sperm linearity index and sperm acrosin activity were significantly decreased (p<0.05). However, there is a significant increase in sperm DNA fragmentation percent, *CLU* gene expression and CLU protein levels in the exposed semen samples to RF-EMR compared with non- exposed samples in OAT>AT> A>N groups, respectively (p<0.05). Semen samples of N group demonstrated a non-significant decrease in sperm motility, sperm linear velocity, sperm linearity index, and sperm acrosin activity, whereas demonstrated a significant increase in sperm DNA fragmentation percent, sperm *CLU* gene expression and CLU protein levels (p<0.05) compared with the non-exposed samples (Table 1, Figs.1, 2).

Table 1: Estimated data in the exposed semen groups vs. non-exposed groups (mean ± SD)

	N (n=26)		A (n=32)		AT (n=31)		OAT (n=35)	
	Non-exposed	**Exposed**	**Non-exposed**	**Exposed**	**Non-exposed**	**Exposed**	**Non-exposed**	**Exposed**
Sperm motility %	60.8 ± 4.5	56.5 ± 4.2	30.9 ± 5.4	26.5 ± 5.0[a]	23.3 ± 9.4	18.4 ± 11.9[a]	17.7 ± 10.9	12.7 ± 7.9[a]
Sperm linear velocity %	59.6 ± 8.0	56.0 ± 8.4	44.9 ± 14.7	39.1 ± 12.8[a]	25.5 ± 11.7	20.67 ± 9.5[a]	23.8 ± 13.6	16.6 ± 9.4[a]
Sperm linearity index	79.0 ± 7.0	76.7 ± 6.8	64.9 ± 10.2	56.5 ± 8.9[a]	66.0 ± 11.4	51.23 ± 9.7[a]	58.5 ± 15.8	41.3 ± 11.4[a]
Sperm acrosin activity	13.2 ± 3.3	12.6 ± 3.2	10.0 ± 2.4	8.3 ± 2.0[a]	5.7 ± 3.1	4.05 ± 2.5[a]	2.5 ± 2.6	1.8 ± 1.9[a]
***CLU* - RNA expression**	0.4 ± 0.1	0.6 ± 0.1[a]	0.8 ± 0.3	1.5 ± 0.6[a]	1.2 ± 0.4	2.6 ± 0.8[a]	1.8 ± 0.5	4.0 ± 1.1[b]
CLU -protein expression	0.6 ± 0.2	0.8 ± 0.5[a]	0.8 ± 0.2	1.4 ± 0.4[a]	1.9 ± 0.4	4.1 ± 0.8[b]	3.2 ± 0.7	5.6 ± 2.1[b]
Sperm DNA fragmentation %	11.5%	30.8%[b]	18.8%	56.3%[c]	29%	71.0%[c]	40.0%	80%[c]

[a]; Significant difference compared with unexposed semen samples (p<0.05). [b]; Significant difference compared with unexposed semen samples (p<0.01), [c]; Significant difference compared with unexposed semen samples (p<0.001), N; Normozoospermia, A; Asthenozoospermia, AT; Asthenoteratozoospermia, OAT; Oligoasthenoteratozoospermia and *CLU*; *Clusterin*.

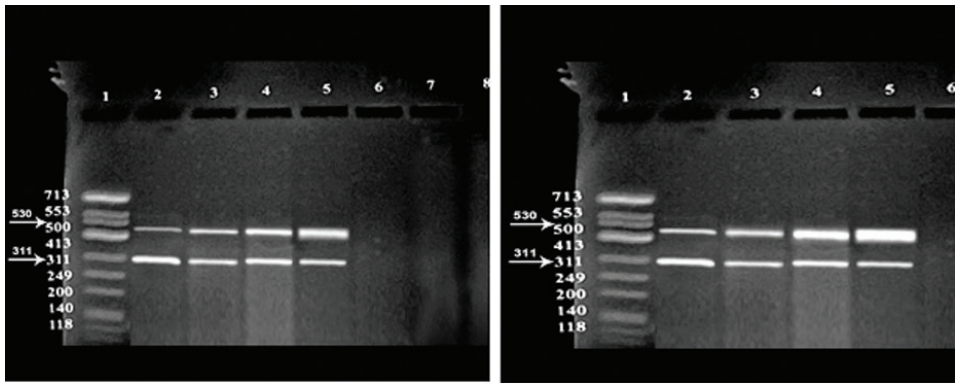


Fig.1: *CLU* gene expression of non-exposed groups (right) and exposed group (left) to mobile phone radiation. Lane1; DNA marker, Lane 2; N group, Lane 3; A group, Lane 4; AT group, Lane 5; OAT group, and Lane 6; Negative control, N; Normozoospermia, A; Asthenozoospermia, AT; Asthenoteratozoospermia, OAT; Oligoasthenoteratozoospermia and *CLU*; Clusterin.

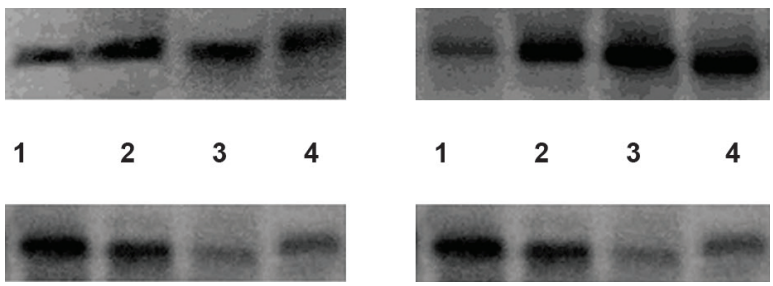


Fig.2: CLU protein expression by Western blotting (40 kd) in non-exposed groups (upper right) and exposed groups (upper left). Internal control; tubulin expression by Western blotting (50 kd) in the non-exposed groups (lower right) and exposed groups (lower left). Lane 1; N group, Lane 2; A group, Lane 3; AT group, Lane 4; OAT group, N; Normozoospermia, A; Asthenozoospermia, AT; Asthenoteratozoospermia, OAT; Oligoasthenoteratozoospermia and *CLU; Clusterin*.

Discussion

In the current study, semen exposure to RF-EMF led to a significant decrease in sperm motility compared to non-exposed semen samples. Previously, Fejes et al. (7) in an epidemiological study have pointed negative correlation between cell phone use and various attributes of semen quality, particularly sperm motility. This was followed by an experimental study involving exposure of male mice to RF-EMF that revealed a significant impact on the integrity of both the mitochondrial and nuclear genomes (13). Kilgallon and Simmons (14) demonstrated that storage of mobile phones close to the testes can decrease semen quality. Similarly, Erogul et al. (15) reported decreased sperm motility in the samples exposed to 900 MHz cell phone for 5 minutes where non-progressive and immotile sperm populations were increased after exposure. Agarwal et al. (16) confirmed such negative impact on semen quality correlating defects in sperm count, motility, viability and normal morphology, with longer duration of usage independent of the initial semen quality. Agarwal

et al. (17) added that exposed spermatozoa to mobile phone radiation for 1 hour leads to significant declines in sperm motility and vitality associated with increased cellular ROS generation coupled with decreased ROS-total antioxidant capacity score.

Such a significant decline in sperm motility was explained by intrinsic ROS generation reinforced with a significant increase in sperm DNA fragmentation in the exposed semen samples compared to the unexposed one in *in vitro* culture (18). Several lines of evidence suggested that oxidative stress (OS) plays a key role in the underlying etiology. Spermatozoa are sensitive to such stress as they possess limited endogenous antioxidant protection while presenting abundant substrates for free radical attack in the form of unsaturated fatty acids and DNA (19). Moskovtsev et al. (20) showed that EMF of cell phones may cause DNA breakage in spermatozoa in a low-frequency EMF that is likely due to stimulation of spermatozoa's plasma membrane redox system by ROS production. De Iuliis et al. (3) added that RF-EMF in both the power density and frequency range of mobile phones enhances mitochondrial ROS generation by human spermatozoa, decreasing its motility and vitality while stimulating DNA base adduct formation and, ultimately sperm DNA fragmentation.

It has been suggested that spermatozoa are particularly vulnerable to the induction of OS by RF-EMF, while a decrease in sperm motility and viability is expected to be linked to concentration of superoxide anion in semen that can oxidize sperm membrane phospholipids. In addition, these reported effects could be attributed to thermal insult induced by RF exposure (18). Aitken et al. (13) observed a significant impact on the integrity of both the mitochondrial and nuclear genomes after exposure of male mice to RF-EMF. In contrast, McNamee et al. (21), Tice et al. (22) and Stronati et al. (23) demonstrated non-significant DNA damage in human cells exposed to RF-EMF.

De Iuliis et al. (3) suggested that excess exposure to RF-EMF emitted from mobile phones is one of the key environmental factors involved in the stimulation of sperm mitochondria that results in producing high levels of ROS. Moreover, such stress is known to induce functional and structural lesions including loss of sperm motility mediated by peroxidative damage to the sperm plasma membrane, as well as to form DNA base adducts in the sperm nucleus leading to DNA fragmentation (24). Agarwal et al. (25) concluded that DNA damage due to EMW is significant, but this damage may be the result of cumulative effect of repeated exposure, not revealed after short term exposures.

Exposure to emitted radiation from mobile phones was demonstrated to have an up-regulation of both *CLU* mRNA and its full length protein in infertile semen samples compared with the normozoospermic samples. Strocchi et al. (26) hypothesized that increased levels of *CLU* mRNA in morphologically normal cells were due to cellular stress response in which cells attempt to protect themselves from local stress conditions. Therefore, increased *CLU* expression could be explained by the physiological defense to reduce cell damage and to maintain cell viability during periods of exposure exerted through *CLU*, ability to act as a scavenger. Trougakos and Gonos (8) proposed that *CLU* with its antioxidant properties is capable of protecting cells from apoptosis induced by ROS. Strocchi et al. (27) supported the notion that an increase in *CLU* expression may be a physiological defense mounted to reduce cell damage and to maintain cell viability during periods of increased OS.

Therefore, increased *CLU* expression was associated in parallel with increased sperm DNA fragmentation and decreased sperm acrosin activity being triggered by OS (28). It is suggested that OS plays a key role in the underlying sperm DNA fragmentation as well as acrosin activity. When ROS production by the sperm' mitochondria is excessive, the gamete's limited endogenous antioxidant defenses are rapidly overwhelmed, and oxidative damage is induced, leading to peroxidation of the sperm acrosomal membrane and diminished acrosin activity (29-32).

A point of limitation in this study is the inability to assess the effects of multiple exposures in addition to reversibility effects to know whether sperm affections are time related or not that is needed for further work. Also, a future study

is suggested to be conducted on the comparison of the effects of RF radiation between iPads and cell phones on sperm motility, sperm DNA fragmentation and seminal *CLU* gene expression.

Conclusion

Cell phone emissions have a negative impact on sperm motility, sperm acrosin activity, sperm DNA fragmentation and *CLU* gene expression, especially in OAT cases.

Acknowledgements

There is no funding and conflict of interest in this study.

References

1. Shum M, Kelsh MA, Sheppard AR, Zhao K. An evaluation of self-reported mobile phone use compared to billing records among a group of engineers and scientists. Bioelectromagnetics. 2011; 32(1): 37-48.
2. Hardell L, Sage C. Biological effects from electromagnetic field exposure and public exposure standards. Biomed Pharmacother. 2008; 62(2): 104-109.
3. De Iuliis GN, Newey RJ, King BV, Aitken RJ. Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. PLoS One. 2009; 4(7): e6446.
4. Kang N, Shang XJ, Huang YF. Impact of cell phone radiation on male reproduction. Zhonghua Nan Ke Xue. 2010; 16(11): 1027-1030.
5. Baan R, Grosse Y, Lauby-Secretan B, El Ghissassi F, Bouvard V, Benbrahim-Tallaa L, et al. Carcinogenicity of radiofrequency electromagnetic fields. Lancet Oncol. 2011; 12(7): 624-626.
6. Tawadrous GA, Aziz AA, Mostafa T. Effect of smoking status on seminal parameters and apoptotic markers in infertile men. J Urol. 2011; 186(5): 1986-1990.
7. Fejes I, Zavaczki Z, Szollosi J, Koloszar S, Daru J, Kovacs L, et al. Is there a relationship between cell phone use and semen quality?. Arch Androl. 2005; 51(5): 385-393.
8. Trougakos IP, Gonos ES. Regulation of clusterin/apolipoprotein J, a functional homologue to the small heat shock proteins, by oxidative stress in ageing and age-related diseases. Free Radic Res. 2006; 40(12): 1324-1334.
9. Zalata A, El-Samanoudy AZ, Shaalan D, El-Baiomy Y, Taymour M, Mostafa T. Seminal clusterin gene expression associated with seminal variables in fertile and infertile men. J Urol. 2012; 188(4): 1260-1264.
10. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Switzerland: WHO; 2010.
11. Mortimer D, Curtis EF, Camenzind AR. Combined use of fluorescent peanut agglutinin lectin and Hoechst 33258 to monitor the acrosomal status and vitality of human spermatozoa. Hum Reprod. 1990; 5(1): 99-103.
12. Erenpreiss J, Bars J, Lipatnikova V. Comparative study of cytochemical tests for sperm chromatin integrity. J Androl. 2001; 22(1): 45-53.
13. Aitken RJ, Bennetts LE, Sawyer D, Wiklendt AM, King BV. Impact of radio frequency electromagnetic radiation on DNA integrity in the male germline. Int J Androl. 2005; 28(3): 171-179.
14. Kilgallon SJ, Simmons LW. Image content influences men's semen quality. Biol Lett. 2005; 1(3): 253-255.
15. Erogul O, Oztas E, Yildirim I, Kir T, Aydur E, Komesli G, et al. Effects of electromagnetic radiation from a cellular phone on human sperm motility: an in vitro study. Arch Med Res. 2006; 37(7): 840-843.
16. Agarwal A, Deepinder F, Sharma RK, Ranga G, Li J. Effect of cell phone usage on semen analysis in men attending infertility clinic: an observational study. Fertil Steril. 2008; 89(1): 124-128.
17. Agarwal A, Desai NR, Makker K, Varghese A, Mouradi R, Sabanegh E, et al. Effects of radiofrequency electromagnetic waves (RF-EMW) from cellular phones on human ejaculated semen: an in vitro pilot study. Fertil Steril. 2009; 92(4): 1318-1325.
18. Falzone N, Huyser C, Fourie F, Toivo T, Leszczynski D, Franken D. In vitro effect of pulsed 900 MHz GSM radiation on mitochondrial membrane potential and motility of human spermatozoa. Bioelectromagnetics. 2008; 29(4): 268-276.
19. Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. J Clin Endocrinol Metab. 2008; 93(8): 3199-3207.
20. Moskovtsev SI, Willis J, White J, Mullen JB. Leukocytospermia: relationship to sperm deoxyribonucleic acid integrity in patients evaluated for male factor infertility. Fertil Steril. 2007; 88(3): 737-740.
21. McNamee JP, Bellier PV, Gajda GB, Miller SM, Lemay EP, Lavallée BF, et al. DNA damage and micronucleus induction in human leukocytes after acute in vitro exposure to a 1.9 GHz continuous-wave radiofrequency field. Radiat Res. 2002; 158(4): 523-533.
22. Tice RR, Hook GG, Donner M, McRee DI, Guy AW. Genotoxicity of radiofrequency signals. I. Investigation of DNA damage and micronuclei induction in cultured human blood cells. Bioelectromagnetics. 2002; 23(2): 113-126.
23. Stronati L, Testa A, Moquet J, Edwards A, Cordelli E, Villani P, et al. 935 MHz cellular phone radiation. An in vitro study of genotoxicity in human lymphocytes. Int J Radiat Biol. 2006; 82(5): 339-346.
24. Taha EA, Ezz-Aldin AM, Sayed SK, Ghandour NM, Mostafa T. Smoking influence on sperm vitality, DNA fragmentation, reactive oxygen species and zinc in oligoasthenoteratozoospermic men with varicocele. Andrologia. 2014; 46 (6): 687-691.
25. Agarwal A, Singh A, Hamada A, Kesari K. Cell phones and male infertility: a review of recent innovations in technology and consequences. Int Braz J Urol. 2011; 37(4): 432-454.
26. Strocchi P, Rauzi F, Cevolani D. Neuronal loss up-regulates clusterin mRNA in living neurons and glial cells in the rat brain. Neuroreport. 1999; 10(8): 1789-1792.
27. Strocchi P, Smith MA, Perry G, Tamagno E, Danni O, Pession A, et al. Clusterin up-regulation following sublethal oxidative stress and lipid peroxidation in human neuroblastoma cells. Neurobiol Aging. 2006; 27(11): 1588-1594.
28. Aitken RJ, De Iuliis GN, Finnie JM, Hedges A, McLachlan RI. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient

population: development of diagnostic criteria. Hum Reprod. 2010; 25(10): 2415-2426.
29. Zalata AA, Ahmed AH, Allamaneni SS, Comhaire FH, Agarwal A. Relationship between acrosin activity of human spermatozoa and oxidative stress. Asian J Androl. 2004; 6(4): 313-318.
30. Abdel Aziz MT, Mostafa T, Atta H, Kamal O, Kamel M, Hosni H, et al. Heme oxygenase enzyme activity in seminal plasma of oligoasthenoteratozoospermic males with varicocele. Andrologia. 2010; 42(4): 236-241.
31. Mostafa T, Anis T, El Nashar A, Imam H, Osman I. Seminal plasma reactive oxygen species-antioxidants relationship with varicocele grade. Andrologia. 2012; 44(1): 66-69.
32. Taha EA, Ez-Aldin AM, Sayed SK, Ghandour NM, Mostafa T. Effect of smoking on sperm vitality, DNA integrity, seminal oxidative stress, zinc in fertile men. Urology. 2012; 80(4): 822-825.

Case Report

Microlithiasis of Seminal Vesicles and Severe Oligoasthenospermia in Pulmonary Alveolar Microlithiasis (PAM): Report of An Unusual Sporadic Case

Giuseppe Castellana, M.D.[1]* , Domenico Carone, M.D.[2], Marco Castellana, M.D.[3]

1. District Health Center, ASL BA, via de Amicis, Conversano (70014), Bari, Italy
2. Center of Human Reproduction and Andrology (CREA), Via Scoglio del Tonno 79/81, Taranto (74120), Italy
3. University of Bari, Piazza Giulio Cesare 11, Bari (70124), Italy

Abstract

Pulmonary alveolar microlithiasis (PAM) is classified as an elective dysmetabolic thesaurotic pneumoalveolitis and characterized by the presence within the alveoli of the lungs of myriad of tiny calculi. The classic presentation of the chest radiography is unmistakable with multiple small "sand-like" opacities diffusely involving both lung fields.

We present a case of male infertility for hypoposia and severe oligoasthenospermia in a young patient with recurrent haematuria and small calcifications in the seminal vesicles similar to pulmonary microliths. PAM was diagnosed on routine chest radiography, computer tomography (CT), transbronchial biopsy and bronchoalveolar lavage (BAL).

Keywords: Seminal Vesicles, Microlithiasis, Oligoasthenospermia, Infertility, Pulmonary Alveolar Microlithiasis

Citation: **Castellana G, Carone D, Castellana M. Microlithiasis of seminal vesicles and severe oligoasthenospermia in pulmonary alveolar microlithiasis (PAM): report of an unusual sporadic case. Int J Fertil Steril. 2015; 9(1): 137-140.**

Introduction

Pulmonary alveolar microlithiasis (PAM) (OMIM 265100) is a rare disease caused by the mutation of the *SLC34A2* gene, encoding the type IIb sodium-phosphate co-transporter in alveolar type II cells. It is characterized by the diffuse presence in the pulmonary alveoli of microliths (calcified lamellar deposits measuring 0.01-3 mm made of calcium phosphate) and has a typical radiographic appearance featuring diffuse "sand-like" micronodules (1, 2). The extension and severity of the disease can best be assessed by high resolution computerized tomography scan (HRCT). A radiological classification of the disease, subdivided into four phases of evolution, has been proposed (3).

PAM is present worldwide and at the most recent review of the literature, a total of 576 cases had been reported. As regards distribution in the continents, it has most frequently been described in Europe, followed by Asia, especially Asia Minor. The nation with the highest number of reported cases is Turkey, followed by Italy and then the United States of America, India, Russia and Germany (4).

Cases of PAM are defined as "familial" when two or more members of a family (usually two or three, but exceptionally four, five or even six) are found to be affected by the disease, and "sporadic" when family history is negative. Sporadic cases are prevalent in the male sex, whereas familial cases are much more frequently observed in females. The transmission of the disease is autosomal recessive (5).

PAM is not easily described as regards its clinical course, including the initial phase, evolution and stabilization. The onset age varies from the neona-

Received: 10 Nov 2013, Accepted: 30 Dec 2013
*** Corresponding Address: District Health Center, ASL BA, via de Amicis, Conversano (70014), Bari, Italy**
Email: bepcaste@libero.it

Royan Institute
International Journal of Fertility and Sterility
Vol 9, No 1, Apr-Jun 2015, Pages: 137-140

tal period to the geriatric. On the admission, more than half the patients are asymptomatic, while others complain dyspnoea, cough, chest pain, fever or sputum, cyanosis and finger clubbing are also reported. The illness may remain static as regards both symptoms and radiographic findings, or it may worsen over time, leading to pulmonary fibrosis, respiratory failure and chronic pulmonary heart disease (4, 6).

In most of the cases of PAM described in the literature, the disease is confined to the lungs and similar lesions have almost never been described in other organs and tissues. The only exceptions are the following ones: pleura, sympathetic ganglia, epididymis, seminal vesicles and prostate (7-12).

Calcifications have also been reported in other organs, such as urinary and biliary tract, but they do not seem to have the typical aspects of microliths (11, 13-16).

Separate considerations should be made for testicular microlithiasis (TM), which is not rare, it has a prevalence of 0.6-9% in the population and is associated with 1% of idiopathic infertility cases. Analyzing 15 subjects with diffuse bilateral TM, Corut identified 2 patients with 2 rare variants in the heterozygous state for *SLC34A2*: the first variant was a synonymous, the second was noncoding. He also evaluated seven male patients with PAM, but none had positive findings when investigated for TM (1). These results seem to suggest a different aetiology for PAM and TM.

We report here a patient affected by microlithiasis of the seminal vesicles and severe oligoasthenospermia associated with PAM.

Case Report

A 32-year-old male underwent medical investigations for recurrent haematuria. Chest X-rays showed diffuse interstitial disease and the patient was referred for a pneumology examination. He denied ever suffering from respiratory troubles and was a non smoker. Routine blood tests were normal, as were spirometry and blood-gas analysis. Close examination of the chest X-rays (Fig.1) revealed a diffuse, fine, sand-like deposit involving both entire lungs, but especially the bases. HRCT (Fig.2), broncho-alveolar lavage (BAL) and transbronchial biopsy were performed that demonstrated the characteristic microliths. A genetic study on the *SLC34A2* gene was not carried. Screening by chest X-rays of the parents and two brothers yielded negative results.

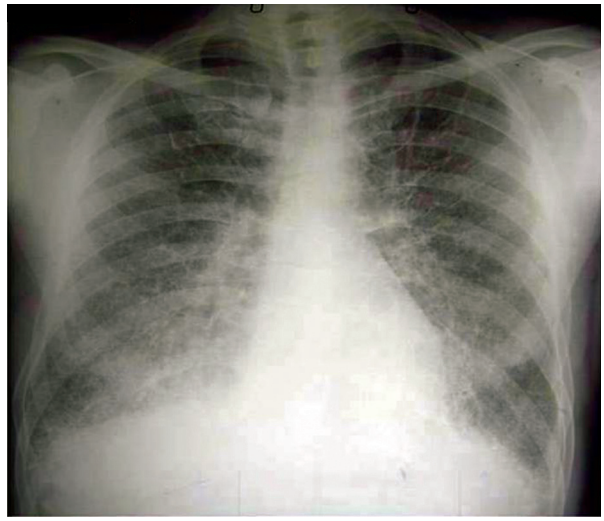


Fig.1: Chest X-ray: parenchyma puntiform shadows bilaterally diffuse in lungs, predominantly in the middle and lower fields.

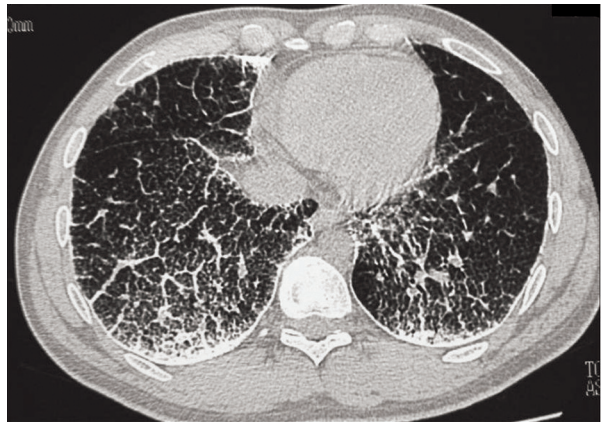


Fig.2: Chest HRCT: bilateral calcific micronodules diffusely involving the parenchyma and diffuse interstitial pattern (septal thickening). HRCT; High resolution computerized tomography scan.

Transrectal ultrasound showed minute calcifications of the seminal vesicles similar to those in the lungs and a calcification in the prostate. The patient was married and after two years of infertility despite complete unprotected sexual relations, he underwent a spermiogram that revealed evidence

of hypofertility with a low volume of ejaculate (< 1 ml) and a sperm count ranging from 3×10^6 to 15×10^6/ml, with low levels of fructose in the seminal plasma. Oligoasthenospermia and hypoposia due to distal obstruction of the seminal tract were diagnosed. Pelvic CT scans (Figs.3, 4) at the level of the seminal vesicles confirmed the ultrasonography (US) findings, demonstrating small radiopaque areas resembling the pulmonary microliths and a calcification in the prostate measuring 4 mm with a different appearance from the microliths. The patient gave an informed consent for the case report.

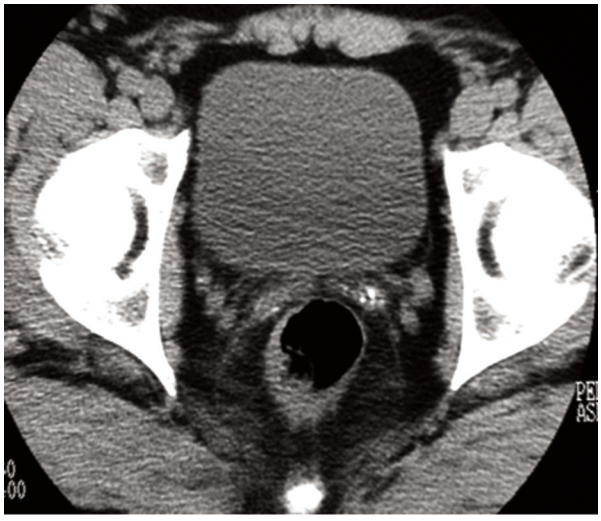


Fig.3: Pelvic HRCT: microliths in the seminal vesicles.
HRCT; High resolution computerized tomography scan.

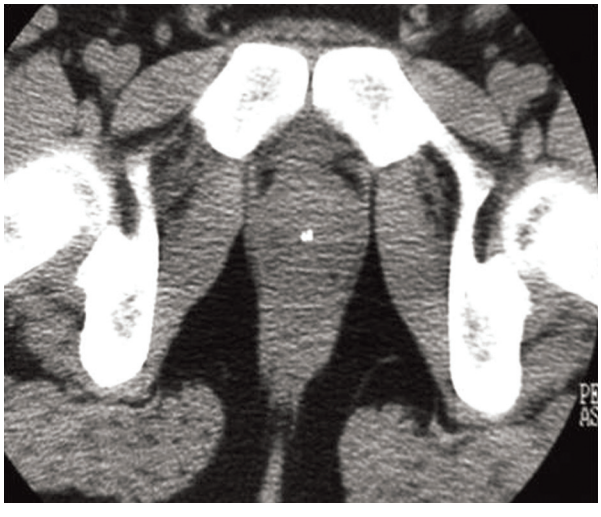


Fig.4: Pelvic HRCT: calcification in the prostate.
HRCT; High resolution computerized tomography scan.

Discussion

In this paper, we report the diagnosis of microlithiasis in seminal vesicles and PAM in a patient with recurrent haematuria, infertility and no respiratory symptoms.

For many years, the aetiology of PAM was a matter of debate, until the discovery of *SLC34A2* gene in 2006 (1). This finding raises a new question: could PAM really be a systemic disease and its localization to the male genital tract cause infertility? In 1967, O'Neill et al. reported a family pedigree showing relative infertility in one male member affected by PAM: he had only 2 children, in contrast to numerous progeny of the other siblings. Since he died at the age of 37 and no contraceptive practices were used, a large number of offspring would be expected in that setting (17). Sandhyamani et al. (18) reported a 27-year-old man with PAM and infertility caused by focal bilateral testicular atrophy related to seminal vesicle microliths. Chatterji et al. (19) reported a 40-year-old male with PAM and primary infertility caused by azoospermia. Kanat et al. (9) reported a 37-year-old man with PAM and epididymis and periurethral microliths causing obstructive azoospermia.

In the present case, small calcifications measuring approximately 1 mm, similar in appearance to those in the lungs, were diffusely distributed in the seminal vesicles. A remarkably variable sperm count with low level of fructose is considered as typical findings in forms of oligoasthenospermia due to distal obstruction of the seminal tract; therefore, it is assumed that there could be a correlation between the microlithiasis of the seminal vesicle and seminal data.

This clinical case is unusual inasmuch as it is only the third case of association of microlithiasis of the lungs and seminal vesicles. Therefore, it has prompted us to raise doubts about the exclusive localization of microlithiasis in the pulmonary alveoli, as has always been believed, and to support the hypothesis that the disease could be systemic or at any rate could also involve the male genital apparatus leading to infertility. This peculiar association needs to be further studied and suggests the need to make a particularly close examination of the chest X-ray in some cases of male infertility.

If PAM is suspected, the best diagnostic sched-

ule is the association of BAL and HRCT, as the first investigation can document the diagnosis, while the second provides further information about the degree of inflammation and/or fibrosis or calcification of the interstices. Nevertheless, standard radiography of the chest is quite reliable enough when there is already a known case in the family.

Identification of the *SLC34A2* gene mutation clinches the diagnosis. When the diagnosis is established, the examination of the family of the index patient is mandatory (2).

Acknowledgements

There is no conflict of interest in this article.

References

1. Corut A, Senyigit A, Ugur SA, Altin S, Ozcelik U, Calisir H, et al. Mutations in SLC34A2 cause pulmonary alveolar microlithiasis and are possibly associated with testicular microlithiasis. Am J Hum Genet. 2006; 79(4): 650-656.
2. Castellana G, Lamorgese V. Pulmonary alveolar microlithiasis. In: Laurent G, Shapiro S, editors. Encyclopedia of respiratory medicine. Waltham: Academic Press, Elsevier; 2006; 532-537.
3. Castellana G, Castellana R, Fanelli C, Lamorgese V, Florio C. Pulmonary alveolar microlithiasis: clinical and radiological course of three cases according to conventional radiology and HRCT. A hypothesis for radiological classification. Radiol Med. 2003; 106(3): 160-168.
4. Mariotta S, Ricci A, Papale M, De Clementi F, Sposato B, Guidi L, et al. Pulmonary alveolar microlithiasis : report on 576 cases published in the literature. Sarcoidosis Vasc Diffuse Lung Dis. 2004; 21(3): 173-181.
5. Castellana G, Gentile M, Castellana R, Fiorente P, Lamorgese V. Pulmonary alveolar microlithiasis : clinical features, evolution of the phenotype, and review of the literature. Am J Med Genet. 2002; 111(2): 220-224.
6. Castellana G, Lamorgese V. Pulmonary alveolar microlithiasis. World cases and review of literature. Respiration. 2003; 70(5): 549-555.
7. Arslan A, Yalin T, Akan H, Belet U. Pulmonary alveolar microlithiasis associated with calcifications in the seminal vesicles. J Belge Radiol. 1996; 79(3): 118-119.
8. Coetzee T. Pulmonary alveolar microlithiasis with involvement of the sympathetic nervous system and gonads. Thorax. 1970; 25(5): 637-642.
9. Kanat F, Teke T, Imecik O. Pulmonary alveolar microlithiasis with epididymal and periurethral calcifications causing obstructive azospermia. Int J Tuberc Lung Dis. 2004; 8(10): 1275.
10. Castellana G. Pulmonary alveolar microlithiasis. Case reports and clinical-radiological correlations. Arch Med Int. 1989; 41: 33-41.
11. Sosman MC, Dodd GD, Jones WD, Pillmore GU. The familial occurrence of pulmonary alveolar microlithiasis. Am J Roentgenol Radium Ther Nucl Med. 1957; 77(6): 947-1012.
12. Buenger P, Fassbender CW, Schuetze G. On alveolar pulmonary microlithiasis. Fortschr Geb Rontgenstr Nuklearmed. 1962; 97: 775-779.
13. Biressi PC, Casassa PM. Intraalveolar pulmonary microlithiasis. Minerva Med. 1956; 47(79): 930-939.
14. Pant K, Shah A, Mathur RK, Chhabra SK, Jain SK. Pulmonary alveolar microlithiasis with pleural calcifications and nephrolithiasis. Chest. 1990; 98(1): 245-246.
15. Badger TL, Gottlieb L, Gaensler EA. Pulmonary alveolar microlithiasis, or calcinosis of the lungs. N Engl J Med.1955; 253(1): 709-715.
16. Portnoy LM, Amadeo B, Hennigar GR. Pulmonary alveolar microlithiasis. an unusual case (associated with milk alkali syndrome). Am J Clin Pathol. 1964; 41: 194-201.
17. O'Neill RP, Cohn JE, Pellegrino ED. Pulmonary Alveolar Microlithiasis-- a family study. Ann Intern Med. 1967; 67(5): 957-967.
18. Sandhyamani S, Verma K, Sharma SK, Pande JN. Pulmonary alveolar microlithiasis. Indian J Chest Dis Allied Sci. 1982; 24(1): 33-35.
19. Chatterji R, Gaude GS, Patil PV. Pulmonary alveolar microlithiasis: diagnosed by sputum examination and transbronchial biopsy. Indian J Chest Dis Allied Sci. 1997; 39(4): 263-267.

International Journal of Fertility and Sterility (Int J Fertil Steril)
Guide for Authors

Aims and Scope: The ***"International Journal of Fertility & Sterility"*** is a quarterly English publication of Royan Institute of Iran. The aim of the journal is to disseminate information through publishing the most recent scientific research studies on Fertility and Sterility and other related topics. ***Int J Fertil Steril*** has been certified by Ministry of Culture and Islamic Guidance since 2007. It has also been accredited as a scientific and research journal by HBI (Health and Biomedical Information) Journal Accreditation Commission since 2008. **This open access journal holds the membership of the Committee on Publication Ethics (COPE).**

1. Types of articles
The articles in the field of Fertility and Sterility can be considered for publications in ***Int J Fertil Steril***. These articles are as below:
A. Original articles are scientific reports of the original research studies. The article consists of English Abstract (structured), Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, and References.
B. Review articles are the articles written by well experienced authors and those who have excellence in the related fields. The corresponding author of the review article must be one of the authors of at least three articles appearing in the references. The review article consists of English Abstract (unstructured), Introduction, Discussion, Conclusion, and References.
C. Short communications are the articles containing new findings. Submissions should be brief reports of ongoing researches. The short communication consists of English Abstract (unstructured), the body of manuscript (should not hold heading or subheading), Acknowledgements, and References.
D. Case reports are published if only the report is of exceptional interest. It consists of English Abstracts (Unstructured), Introduction, Case Report, Discussion, Acknowledgements, and References.
E. Editorial should be written by either the editor in chief or the editorial board.
F. Imaging in reproductive medicine should focus around a single case with an interesting illustration such as a photograph, histological specimen or investigation. Color images are welcomed. The text should be brief and informative.
G. Letter to the editors are comments made by our readers on recently published articles.

2. Submission Process
A. Cover letter
Each article should be accompanied by a cover letter, signed and dated by corresponding author specifying the following statement: The manuscript has been seen and approved by all authors and is not under active consideration for publication. It has neither been accepted for publication, nor published in another journal fully or partially (except in abstract form). I hereby assign the copyright of the enclosed manuscript to ***Int J Fertil Steril.*** Corresponding author can also suggest three peer reviewers in the field of their article.
B. Copyright
All listed authors must sign the "Copyright Transfer Agreement". To the same token, the responsibility for the article's content should be borne by each one of the authors. No article will be published without a copyright transfer agreement signed by all the listed authors. Any use, distribution, reproduction or abstract of this publication in any medium, with the exception of commercial purposes, is permitted if the original work is properly cited.
C. Manuscript preparation
Authors whose first language is not English, encouraged to consult a native English speaker in order to confirm his manuscripts to US or British (not a mixture) English usage and grammar. Manuscript should be prepared in accordance with the "International Committee of Medical Journal Editors (ICMJE)". Before publishing author's article, it would be the author's responsibility to pay for the expenses, if the editor feels the level of English used in the manuscript requires editing. The manuscript must be typed in a font size of at least 12 points, double spaced with margins of at least 2.5 cm (1 inch). Please send your article in two formats (word and Pdf). The abstract and text pages should have consecutive line numbers in the left margin beginning with title page and continuing through the last page of the written text. Each abbreviation must be defined in the abstract and text when they are mentioned for the first time. Avoid using abbreviation in title. Please use the international and standard abbreviations and symbols.
It should be added that an essential step toward the integration and linking of scientific information reported in published literature is using standardized nomenclature in all fields of science and medicine. Species names must be italicized (e.g., *Homo sapiens*) and also the full genus and species written out in full, both in the title of the manuscript and at the first mention of an organism in a paper.
It is necessary to mention that genes, mutations, genotypes, and alleles must be indicated in italics. Please use the recommended name by consulting the appropriate genetic nomenclature database, e.g., HUGO for human genes. In another words; if it is a human gene, you must write all the letters in capital and italic (e.g., *OCT4, c-MYC*). If not, only write the first letter in capital and italic (e.g., *Oct4, c-Myc*). **In addition, protein designations are the same as the gene symbol, but are not italicized**.
Each of the following manuscript components should begin in the following sequence:
Title is providing the full title of the research (do not use abbreviations in title), full name(s), highest awarded academic degree(s), email(s), and institutional affiliation(s) of all the authors in English. Also you must send moblie number and full postal address of corresponding author.
Running title is providing a maximum of 7 words (no more than 50 characters).
Abstract must include: Background, Materials and Methods, Results, and Conclusion.
Keywords, three to five, must be supplied by the authors at the foot of the abstract chosen from the Medical Subject Heading (MeSH). Therefore; they must be specific and relevant to the paper.
The following components should be identified after the abstract:
Introduction: This part includes the purpose and the rationale of the study. It should neither review the subject extensively, nor have data or conclusions of the study.
Materials and Methods: It should include the exact methods or observations of experiments. If an apparatus is used, its manufacturer's name and address should be stipulated in parenthesis. If the method is established, give reference but if the method is new, give enough information so that another author can perform it. If a drug is used, its generic name, dose and route of administration must be given. Standard units of measurements and chemical symbols of elements do not need to be defined.
Statistical analysis: Type of study and statistical methods should be mentioned and specified by any general computer program used.
Ethical considerations*:* Please state that informed consent was obtained from all human adult participants and from the parents or legal guardians of minors and include the name of the appropriate institutional review board that approved the project. It is necessary to indicate in the text that the maintenance and care of experimental animals complies with National Institutes of Health guidelines for the humane use of laboratory animals, or those of your Institute or agency.
Results: They must be presented in the form of text, tables and figures. The contents of the tables should not be all repeated in the text. Tables and figures must be numbered consecutively as appeared in the text and should be organized in separate pages at the end of article while their location should be mentioned in the main text. Long articles may need sub-headings within some sections (especially the Results and Discussion parts) to clarify their contents.
Legends of Tables: A short descriptive heading should be given above each table and any footnotes and explanations underneath.
Figures: They must be sent in color and also in GIF or JPEG format with 300 dpi resolutions.
Discussion: It should emphasize the present findings and the variations or similarities with other researches done by other researchers. The detailed results should not be repeated in the discussion again. Emphasize the new and important aspects of the study.
Conclusion: It emphasizes the new and important aspects of the study. All conclusions are justified by the results of the study. It must be mentioned whether the hypothesis mentioned in the article is true, false or no conclusions can be derived.
Acknowledgements: This optional part should include a statement thanking those who contributed substantially with work relevant to the study. It should include persons who provided technical help, writing assistance and name of departments that provided only general support. Grant support should be included in this section.

Conflict of Interest: Any conflict of interest (financial or otherwise) and sources of financial support must be listed in the Acknowledgements. It includes providers of supplies and services from a commercial organization. Any commercial affiliation must be disclosed, regardless of providing the funding or not.
References The references must be written based on the Vancouver style. Thus the references are cited numerically in the text and listed in the bibliography by the order of their appearance. The titles of journals should be abbreviated according to the style used in the list of Journals Indexed in PubMed. Write surname and initials of all authors when there are six or less. In the case of seven or more authors, the names of first six authors followed by "et al." should be listed. The reference of information must be based on the following order:
Article:
Surname(s) and first letter of name & middle name(s) of author(s). Manuscript title. Journal title (abbr).publication date (year); Volume (Issue): Page number.
Example: Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U, et al. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. Biol Reprod. 1995; 52(4): 864-867.
Book:
Surname(s) and first letter of name & middle name(s) of author(s). Book title. Edition. Publication place: publisher name; publication date (year); Page number.
Example: Edelman CL, Mandle CL. Health promotion throughout the life span. 2nd ed.ST Louis: Mosby; 1998; 145-163.
Chapter of book:
Surname(s) and first letter of name & middle name(s) of author(s). Chapter title. In: Surname(s) and first letter of name &middle name(s) of editor(s), editors. Edition. Publication place: publisher name; publication date (year); Page number.
Example: Phillips SJ, Whisnant JP. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York: Raven Press; 1995; 465-478.
Abstract book:
Example: Nabavi SM. Stem cell therapy for multiple sclerosis. Cell J. 2013; 5 Suppl 1: Os-13.
Thesis:
Name of author. Thesis title. Degree. City name. University. Publication date (year).
Example: Eftekhari Yazdi P. Comparison of fragment removal and co-culture with Vero cell monolayer's on development of human fragmented embryos. Presented for the Ph.D., Tehran. Tarbiyat Modarres University. 2004.
Conferences:
Name(s) of editor(s). Conference title; Holding date; Holding place. Publication place; Publisher name; Publication date (year).
Example: Harnden P, Joffe JK, Jones WG, editors. Germ cell tumors V.Proceedings of the 5th Germ Cell Tumors Conference; 2001 Sep 13-15; Leeds,UK. New York: Springer; 2002.
Internet References
Article:
Surname(s) and first letter of name & middle name(s) of author(s). Manuscript title. Journal title (abbr). publication date (year); Volume (Issue): Page number. Available from: URL link. (Observation date).
Example: Jahanshahi A, Mirnajafi-Zadeh J, Javan M, Mohammad-Zadeh M, Rohani M. Effect of low-frequency stimulation on adenosineA1 and A2A receptors gene expression in dentate gyrus of perforant path kindled rats. Cell J. 2008; 10 (2): 87-92. Available from: http://www.celljournal.org. (20 Oct 2008).
Book:
Example: Anderson SC, Poulsen KB. Anderson's electronic atlas of hematology.[CD-ROM]. Philadelphia: Lippincott Williams & Wilkins; 2002.
Law:
Example: Embryo donation law. Iran Judicature, Official Gazette of the Islamic Republic of Iran. Available from: http://www.dastour.ir/Brows/?lid=245069 .(20 Jul 2013).
D. Proofs: Proofs are sent by email as PDF files and should be checked and returned within 72 hours of receipt. It is the authors' responsibility to check that all the text and data as contained in the page proofs are correct and suitable for publication. The authors are requested to pay particular attention to author's names and affiliations as it is essential that these details be accurate when the article is published.
E. Pay for publication: Publishing any article is free of charge.
F. Ethics of scientific publication: Plagiarism of text from a previously published manuscript by the same or another author is a serious publication offence. Some parts of text may be used, only where the source of the quoted material is clearly acknowledged.
G. Clinical trial registration: All of the Clinical Trials performed in Iran must be registered in Iranian Registry of Clinical Trials (www.irct.ir), in order to be considered for publication even if they register in other registration site. The clinical trials performed abroad, could be considered for publication if the authors register in a website approved by WHO. This includes all of the clinical trials conducted.

3. General information
A. You can send your article by email to **ijfs@royaninstitute.org** or via online submission system which is available at our website: **http://www.ijfs.ir.** If the article is not prepared according to the format of ***Int J Fertil Steril***, it will be returned to authors.
B. The order of article appearance in the Journal is not demonstrating the scientific characters of the authors.
C. Int J Fertil Steril has authority to accept or reject the articles.
D. The received articles will be evaluated by one epidemiologist. Then associate editor will determine its reviewers. If three reviewers pass their judgments on the article, it will be presented to the editorial board of ***Int J Fertil Steril.*** If editorial board has a positive judgment about the article, reviewers' comments will be presented to corresponding author (the identification of the reviewers will not be revealed). The executive member of journal will contact the corresponding author directly within 7-8 weeks by email or mail. If authors do not receive any reply from journal office after the specified time, they can contact journal office. Executive manager will respond promptly to authors' message.
E. Three reprints of each article will be supplied for the corresponding author free of charge.
The Final Checklist
The authors must ensure that before submitting the manuscript for publication, they have to consider the following parts:
1. Title page should contain title, name of the author/coauthors, their academic qualifications, designation & institutions they are affiliated with, mailing address for future correspondence, email address, phone, and fax number.
2. Text of article and References prepared as stated in the "guide for authors" section.
3. Tables should be in a separate page. Figures must be sent in color and also in GIF or JPEG format with 300 dpi resolutions.
4. Covering Letter

The Editor-in-Chief: Mohammad Hossein Nasr Esfahani, Ph.D.
International Journal of Fertility and Sterility (Int J Fertil Steril)
P.O. Box: 16635-148, Iran
Tel/Fax: + 98-21-22510895
Emails: ijfs@royaninstitute.org
info@ijfs.ir