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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 12, No 1, Apr-Jun 2018, Pages: 1-91

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Comparison of Cabergoline and Quinagolide in Prevention of Severe Ovarian Hyperstimulation Syndrome among Patients Undergoing Intracytoplasmic Sperm Injection

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Abstract

Background: The aim of the current study is to compare quinagolide with cabergoline in prevention of ovarian hyperstimulation syndrome (OHSS) among high risk women undergoing intracytoplasmic sperm injection (ICSI).

Materials and Methods: This randomized clinical trial study was performed from March 2015 to February 2017. One hundred and twenty six women undergoing ICSI who were at high risk of developing OHSS (having over 20 follicles of >12 mm), were randomized into two groups. The first group received cabergoline 0.5 mg and the second group received quinagolide 75 mg every day for 7 days commencing on the day of gonadotropin-releasing hormone (GnRH) agonist administration. Then OHSS symptoms as well as their severity were assessed according to standard definition, 3 and 6 days after GnRH agonist administration. Ascites were determined by trans-vaginal ultrasound. Other secondary points were the number of oocytes and the number of embryos and their quality. Quantitative and qualitative data were analyzed using Student's t test, and Chi-square or fisher's exact test, respectively. A $P<0.05$ was considered statistically significant.

Results: The incidence of severe OHSS in the quinagolide-treated group was 3.1% while it was 15.8% in cabergoline-treated subjects ($P<0.001$). Ascites were less frequent after treatment with Quinagolide as compared to cabergoline (21.9 vs. 61.9%, respectively) ($P=0.0001$). There was no significant statistical deferences between the two groups in terms of mean age, number of oocytes, metaphase I and metaphase II oocytes, and germinal vesicles. There was a significant difference between cabergoline and quinagolide groups regarding the embryo number ($P=0.037$) with cabergoline-treated group showing a higher number of embryos. But, the number of good quality embryo in quinagolide-treated individuals was significantly higher than that of the cabergoline-treated group ($P=0.001$).

Conclusion: Quinagolide seems to be more effective than Cabergoline in prevention of OHSS in high-risk patients undergoing ICSI (Registration number: IRCT2016053128187N1).

Keywords: Dopamine Agonists, Dopamine D2, Ovarian Hyperstimulation Syndrome, Receptors

Citation: Taheripanah R, Vasef M, Zamaniyan M, Taheripanah A. Comparison of cabergoline and quinagolide in prevention of severe ovarian hyperstimulation syndrome among patients undergoing intracytoplasmic sperm injection. Int J Fertil Steril. 2018; 12(1): 1-5. doi: 10.22074/ijfs.2018.5259.

Introduction

Ovarian hyperstimulation syndrome (OHSS) could be a life-threatening complication of assisted reproduction treatment (ART) (1). The incidence of OHSS varies between 6 and 12% based on the studied population and classification of disease; also, severe cases have an incidence of 2-4% (2, 3). OHSS is characterized by the presence of multiple luteinized cysts within the ovaries that induce ovarian enlargement and increase capillary permeability with enhanced fluid shift to the third space (4). Recent findings have introduced vascular endothelial growth factor (VEGF) as the mainstay for increased capillary permeability (2, 5). OHSS has a broad spectrum of clinical manifestations ranging from mild to severe symptom. Subjects with mild disease presented with enlargement of ovaries, lower abdominal pain and discomfort, temporary nausea and vomiting, diarrhea, and abdominal distention. Persistent toxic symptoms or the presence of ascites indicates a progressive OHSS that requires treatment (3, 6, 7).

Raised serum estradiol levels to concentrations of >2,500 pg/mL, and observations of large numbers of

Received: 16 Mar 2017, Accepted: 15 May 2017
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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 1, Apr-Jun 2018, Pages: 1-5

small and intermediate-sized ovarian follicles, are signs of high risk necessitating to proceed with great caution (8, 9). Administration of cabergoline, a dopamine agonist as a prophylactic agent is associated with significant reductions in the incidence of symptoms and signs of moderate to severe OHSS. This drug inhibits vascular endothelial growth factor 2 phosphorylation (VEGFR-2) (9-12) and decreases the incidence of OHSS and cycle cancellation rate without having any adverse effects on gestation. Quinagolide (Norprolac™) is a non-ergot extract and dopamine agonist with a chemical structure similar to apomorphine. Binding of quinagolide to D2 dopamine receptors on the lactotroph cells in the anterior pituitary decreases adenylyl cyclase activity, reduces the intracellular cyclic adenosine monophosphate, and inhibits prolactin excretion (13). The specificity of quinagolide for D2-type dopamine receptors diminishes its side effects compared to dopamine agonists (6, 13, 14).

Several studies have indicated that quinagolide effectively reduces the development of OHSS (6, 15). Therefore, the aim of the present study was to compare the quinagolide and cabergoline effects in preventing severe OHSS in high risk female patients who undergo intracytoplasmic sperm injection (ICSI), and to evaluate quinagolide's effect on the oocyte and embryo quality.

Materials and Methods

The present study was a parallel single-blind randomized clinical trial (IRCT2016053128187N1) with a 1:1 allocation ratio, recruiting 126 patients, who had undergone assisted reproductive procedure and were at risk of severe OHSS. The patients were randomly allocated to one of the study groups according to a random allocation sequence generated by a statistician using a computer software. The sequence was built through generating block size of 4.

The study was conducted in Infertility and Reproductive Health Research Center and Imam Hussein Medical Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran, from March 2015 to February 2017. The project was approved by the Ethics Committee (IR.SBMU.RETECH.REC.1395.542) and institutional review board of Shahid Beheshti University of Medical Sciences, Tehran, Iran, and it was initiated after obtaining written informed consents from all participants. Randomization on the day of gonadotropin-releasing hormone (GnRH) agonist administration was based on a computer-generated random list which determined the random allocation of the subjects into the two groups.

Selection and randomization of the patients were performed by a nurse, using a series of sequentially numbered sealed envelopes; therefore, the sequence of allocation was hidden. The study was single-blinded, because the physicians were blind to the treatment group, but the patients were aware of the management option (Fig.1).

In this study, patients of 20-40 years old, who had 20 oocytes and serum estradiol levels of >3000 pg/ml on the day of GnRH agonist injection during ICSI cycles, were recruited. The inclusion criteria were being at high risk of developing OHSS and not having hepatic dysfunction, hypertension and a history of syncope. All participants underwent controlled ovarian hyperstimulation (COH) with gonadotropin/GnRH-antagonist protocol. Ovarian stimulation using recombinant-follicle-stimulating hormone (FSH, GONAL-f, Serono, Switzerland) was started on day 3 of cycle at a dose of 150 IU per day.

Transvaginal ultrasound was performed every 3 days to examine the follicular development. Also, serum estradiol levels were measured every 2-3 days using radioimmunoassay method. After 5 days of stimulation, when at least two follicles with diameters of 14 mm were observed, GnRH antagonist (Cetrotide, Merk, USA) or (Orgalutran, Organon, the Netherlands) was started with a daily dose of 0.25 mg until administration of GnRH agonist. Final oocyte maturation was triggered when at least two follicles with diameters of at least 17 mm were observed, using a single intramuscular injection of 0.2 mg GnRH agonist (Decapeptyl, Ferring GmbH, Germany). Oocytes were collected 36-38 hours later using transvaginal-guided follicle aspiration. All embryos were frozen after fertilization through ICSI. On day of GnRH agonist administration, patients were randomized using computer-generated random tables into two groups.

The first group comprised of 63 women, was treated with 0.5 mg cabergoline (Dostinex™, Pfizer, USA) every day for 7 days and the second group comprised of 63 women, was treated with quinagolide 75 mg (Norprolac™, Ferring, Denmark) every day for 7 days. Diagnosis of OHSS as well as determination of its severity was performed according to Golan's classification (16), on days 3 and 6 after GnRH agonist administration. The patients vital signs and weight were recorded at each visit. Transvaginal ultrasound was used to measure the ovarian volume and estimate the volume of pelvic free fluid. Data were extracted from the Checklist, clinical and laboratory notes and ultrasound reports. Age, body mass index (BMI), number of retrieved oocytes, number of metaphase I and II oocytes and germinal vesicles, number of embryos and number of high quality embryos were all recorded in specified data sheet. All patients were checked for any related symptoms or side effects of cabergoline and quinagolide.

Statistical analysis

This was a randomized clinical trial study. To detect 20% difference in OHSS rates that is considered significant (1, 2) with a power of 80% and α=0.05, 63 patients in each group were needed. Statistical analysis was done using SPSS 21.0 (SPSS Inc., Chicago, IL USA). Effect size for comparing two means was determined by computing the mean difference between the two groups, and then dividing the result by the pooled standard deviation, according to Cohen's d effect size. So, it is likely to have

a negative effect size. However, if just the magnitude was important, we could take the absolute difference so that the effect size would be positive (17). Quantitative data were presented as mean ± SD. Quantitative and qualitative data were analyzed using Student's t test, and Chi-square or fisher's exact test, respectively. A P<0.05 was considered statistically significant.

Results

A total of 130 women were recruited into the study. Four women were omitted from the research due to various reasons including declining to participate, having hypertension, hepatic dysfunction, or history of syncope, and discontinuing the treatment or loss to follow-up (Fig.1). The mean age of the patients in cabergoline and quinagolide groups were 31.05 ± 5.2 and 31.63 ± 4.4 years old, respectively. There was no significant differences between the mean ages of the two groups. Also, the two groups were not significantly different in terms of other major demographic characteristics such as type of infertility, menstrual cycle pattern, BMI and duration of infertility (Table 1). There was significant differences between quinagolide and cabergoline groups regarding the incidence of OHSS (22.2 vs. 47.6%, respectively) (P=0.001).

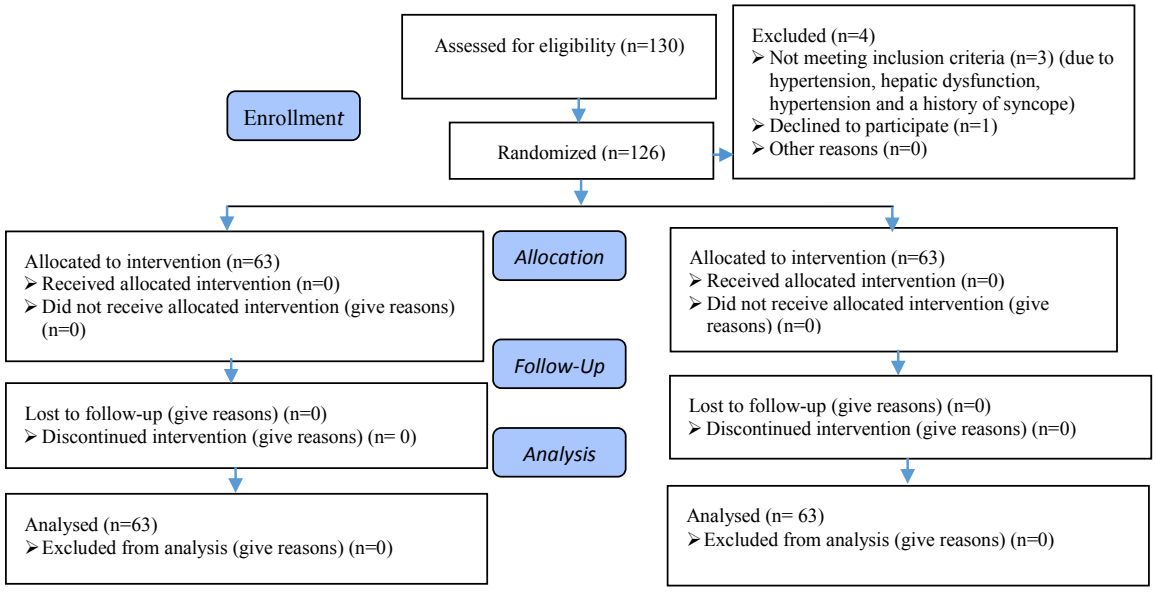

Fig.1: Flowchart of the trial.

Table 1: Clinical and hormonal characteristics of patients in two groups of patients entering the study

Variable	Quinagolide n=63	Cabergoline n=63	P value
Age (Y)	31.63 ± 5.2	31.05 ± 4.4	0.503
Body mass index (BMI)	26.4 ± 3.8	27.5 ± 3.2	0.174
Type of infertility			
Primary	20 (31.7)	21 (33.3)	0.762
Secondary	10 (15.8)	11 (17.46)	0.762
Cause of infertility			
Female factor	3 (4.76)	2 (3.17)	0.644
Male factor	11 (17.46)	10 (15.87)	0.644
Both (male+PCOS)	4 (6.34)	5 (7.93)	0.644
Unexplained	1 (1.58)	2 (3.17)	0.644

Data are presented as mean ± SD or n (%). PCOS; Polycystic ovary syndrome.

Table 2: The outcomes of ovarian stimulation in quinagolide and cabergoline-treated groups

Variable	Quinagolide n=63	Cabergoline n=63	Effect (95% CI)	P value
E2 on day of GnRH agonist (pg/ml)	3293.74 ± 3836.9	3615.79 ± 1473.5	0.11 (-0.24-0.46)	0.304
HB (g/dl)	12.10 ± 1.42	12.61 ± 1.17	-0.39 (-0.74-0.04)	0.034
HCT	36.85 ± 4.38	38.37 ± 3.77	-0.37 (-0.72-0.02)	0.045
Number of oocytes retrieval	29.02 ± 11.45	28.76 ± 6.46	0.03 (-0.32-0.38)	0.443
Number of GV	4.02 ± 2.93	3.60 ± 2.38	0.16 (-0.19-0.51)	0.834
Number of MI	2.81 ± 1.92	3.49 ± 2.15	-0.33 (-0.68-0.02)	0.786
Number of MII	22.58 ± 9.57	22.05 ± 7.88	0.06 (-0.29-0.41)	0.386
Number of embryo	6.22 ± 15.00	5.59 ± 17.23	-0.38 (-0.73-0.02)	0.037
Number of high quality of embryos	18.3 ± 5.1	14 ± 8.6	0.61 (0.25-0.96)	0.001
OHSS	14 (22.2)	30 (47.6)	0.46 (0.27-0.79)	0.001
Mild	6 (9.5)	9 (14.28)	0.66 (0.25-1.76)	0.432
Moderate	6 (9.5)	11 (17.46)	0.54 (0.22-1.38)	0.545
Severe	2 (3.1)	10 (15.8)	0.2 (0.04-0.87)	0.001
GI symptoms	38 (59.4)	40 (63.5)	0.95 (0.72-1.25)	0.857
Ascites	14 (21.9)	39 (61.9)	0.36 (0.22-0.59)	0.0001
Paracentesis	7 (10.9)	17 (27.0)	0.41 (0.18-0.92)	0.021
Admission	2 (3.1)	14 (22.2)	0.14 (0.03-0.60)	0.001

Data are presented as mean ± SD or n (%). E2; Estradiol, GnRH; Gonadotropin releasing hormone, HB; Hemoglobin, HCT; Hematocrit, GV; Germinal vesicle, MI; Metaphase I, MII; Metaphase II, OHSS; Ovarian hyper stimulation syndrome, GI; Gastrointestinal, and CI; Confidence interval.I.

The incidence of severe OHSS was considerably lower in the Quinagolide group (3.1% in quinagolide-treated group vs. 15.8% in the cabergoline-treated group, P<0.001). Ascites were less frequent after treatment with quinagolide as compared to cabergoline (21.9 vs. 61.9%, P=0.0001). Also, ascites paracentesis was significantly lower in quinagolide group compared to cabergoline group (10.9 and 27%, respectively, P=0.021). Hematocrit and hemoglobin were significantly lower after treatment with quinagolide as compared to cabergoline (P=0.045 and 0.034, respectively) and admission rate was significantly lower in quinagolide group compared to cabergoline (3.1 vs. 22.2%, P=0.001, Table 2). There was no statistically significant deferences between the two groups in terms of gastrointestinal symptoms, estradiol levels on the day of agonist administration, the number of oocytes, metaphase I and metaphase II oocytes and germinal vesicles. The number of embryos in cabergoline group was significantly higher in comparison to the quinagolide group (17.23 vs. 15.00%, P=0.037), but the number of good quality embryos in quinagolide group was significantly higher than the cabergoline group (P=0.001, Table 2).

Discussion

OHSS is a life-threatening complication induced by ART which is more frequently observed when a strong ovarian response occurs (1). This strong ovarian response is characterized by development of several ovarian follicles and high levels of serum estradiol (2, 4). Prophylactic administration of cabergoline and quinagolide as dopamine agonists, is associated with a significant decrease in incidence of signs and symptoms related to moderate or severe OHSS (1, 6).

This prospective randomized study showed that risk of OHSS is more markedly reduced following administration of quinagolide at a dose of 75 mg compared to cabergoline at a dose of 0.5 mg among high risk patients. In our study, the incidence of severe OHSS in quinagolide-treated group was significantly lower compared to that of cabergoline-treated group. Kamel et al. (18) compared quinagolide 75 mg with cabergoline 0.5 mg in prevention of OHSS among high-risk patients undergoing *in vitro* fertilization (IVF).

Patients received drugs for 8 days starting from the day of human chorionic gonadotropin injection. The number of patients who developed OHSS was similar in the two groups, which was not consistent with our findings. Busso et al. (2) in a randomized double-blind placebo-controlled trial, evaluated different doses of quinagolide in prevention of early OHSS. Their findings showed that quinagolide when given at three dose (50, 100, 200 mg/day), was effective in reducing the incidence of moderate and severe OHSS from 4-12% to 0-2. Their results were similar to ours regarding the incidence of moderate OHSS after prophylactic administration of quinagolide at the dose of 200 mg/day and severe OHSS at the dose of 50 mg/day.

According to our results, the number of patients with ultrasound evidence of ascites within the 6 days after GnRH agonist administration, was significantly reduced in quinagolide compared to cabergoline-treated group. Similarly,

Baumgarten et al. (6) in a randomized controlled prospective study on role of quinagolide in preventing OHSS among high risk ICSI patients, showed that the number of patients with ultrasound evidence of ascites within the initial 8 days after human chorionic gonadotropin (hCG) administration, was significantly lower in quinagolide-treated group than control group.

In our study, admission rate was significantly reduced in quinagolide-treated group as compared to cabergoline-treated group. Kamel et al. (18) found that hospitalization rate is similar in cabergoline and quinagolide-treated groups which was contrary to our results.

We found no significant statistical differences between the two groups in terms of the number of oocytes, metaphase I and metaphase II oocytes, and germinal vesicles. Although the number of embryos in cabergoline-treated group was significantly higher compared to quinagolide-treated group, the number of good quality embryos in quinagolide-treated group was significantly higher than that of the cabergoline-treated group. Kılıç et al. (19) evaluated the effects of cabergoline in prevention of OHSS in women at risk undergoing IVF treatment cycles and showed that in cabergoline-treated group, total number of embryos, number of total good quality embryos, and the fertilization rate were significantly higher than control group. In this study, cabergoline and quinagolide administration had no negative impact on oocyte and embryos numbers and their quality which was consistent to previously published data.

In our study, there was no statistically significant differences were observed between the two groups in terms of gastrointestinal symptoms. Busso et al. (2) noted that upper gastrointestinal symptoms, especially nausea and vomiting, were more frequent following administration of quinagolide compared to placebo, especially when quinagolide was given at high doses. One important limitation of the present study was the small sample size. In this regard, the small number of patients restricts the generalizability of the results of the present study. Advanced trials with adjusted doses are therefore required. There was also some potential sources of bias including interactions with other drugs.

Conclusion

Quinagolide seems to be more effective than cabergoline in preventing OHSS among high-risk patients undergoing ICSI. Further studies should be performed to compare quinagolide and cabergoline to achieve a firm conclusion.

Acknowledgements

This study was financially supported by Infertility and Reproductive Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Authors have no conflict of interest concerning the content of this manuscript.

Author's Contributions

R.T., M.Z.; Contributed to conception and design. M.V.; Contributed to all experimental work, wrote the manuscript, data and statistical analysis, and interpretation of data. A.T.; Participated in study design, data collection and evaluation. All authors read and approved the final manuscript.

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Original Article

Comparative Effectiveness of Antidepressant Medication versus Psychological Intervention on Depression Symptoms in Women with Infertility and Sexual Dysfunction

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Abstract

Background: Fertility loss is considered as a challenging experience. This study was conducted to compare the effectiveness of antidepressant medication and psychological intervention on depression symptoms in women with infertility and sexual dysfunctions (SD).

Materials and Methods: This randomized, controlled clinical trial study was completed from December 2014 to June 2015 in Babol, Iran. Of the 485 participants, 93 were randomly assigned in a 1:1:1 ratio to psychosexual therapy (PST), bupropion extended-release (BUP ER) at a dose of 150 mg/d, and control (no intervention) groups. The Beck Depression Inventory (BDI) was completed at the beginning and end of the study. Duration of study was eight weeks. Statistical analyses were performed by using paired-test and analysis of covariance.

Results: The mean depression score on the BDI was 22.35 ± 8.70 in all participants. Mean BDI score decreased significantly in both treatment groups (PST: P<0.0001, BUP: P<0.002) from baseline to end of the study, whereas intra-individual changes in BDI score were not significant in the control group. The decrease in mean BDI score was greater with PST compared to BUP treatment (P<0.005) and the control group (P<0.0001). The PST group showed greater improvement in depression levels (severe to moderate, moderate to mild) in comparison with the two other groups (P<0.001). Drug treatment was well tolerated by the participants in the BUP group.

Conclusion: PST can be a reliable alternative to BUP ER for relieving depression symptoms in an Iranian population of women with infertility and SD (Registration number: IRCT2015042721955N2).

Keywords: Bupropion, Depression, Infertility, Psychotherapy, Sexual Dysfunction

Citation: Pasha H, Basirat Z, Faramarzi M, Kheirkhah F. Comparative effectiveness of antidepressant medication versus psychological intervention on depression symptoms in women with infertility and sexual dysfunction. Int J Fertil Steril. 2018; 12(1): 6-12. doi: 10.22074/ijfs.2018.5229.

Introduction

Infertility is an emotionally challenging experience in women's lives. Fertility loss can cause various mental problems such as feeling of loss of control, low self-esteem, stress, depression, marital distress, and sexual dissatisfaction (1-5). The prevalence of depression ranges between 40-50% among women with infertility (1, 6). Also, sexual dysfunction (SD) is a common problem in women with infertility, where the rate is 46.6% in a sample of Iranian infertile women (7). Moreover, there is an association between SD and depression; namely, a review of the literature reveals that depression has been frequently associated with sexual impairments (8, 9).

Therefore, depression itself may contribute to SD and vice-versa (4, 10, 11). The studies showed that the depression leads to decreasing in success rate with *in vitro* fertilization (IVF). In addition, depression found to have an inversely correlation with pregnancy. The higher levels of depression is associated with lower rate of pregnancy (12).

Both cognitive behavioral therapy and drug therapy are effective in treating depression in women with infertility (3). A review of the literature revealed that psychosocial interventions had a beneficial effect on depression and the well-being of women with infertility (13). Psychotherapy or sex therapy had antidepressant effects and improved sexual function (3, 14, 15). For drug therapy, bupropion is used to treat depression related to sexual dysfunction. It is a norepinephrine and dopamine reuptake inhibitor (16), and is effective in the improvement of depression (17). Bupropion is an effective antidepressant and can be used as a supplemental treatment to reverse antidepressant-induced SD (18). Although one study showed that there was not a significant difference in depression rating scale between the bupropion users with placebo groups (19).

Receive: 16 Feb 2017, Accepted: 29 May 2017
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While behavioral and pharmacological treatments are effective in treating depression and SD in infertility, few studies have assessed these treatments for depression in infertile women with sexual dysfunction. To our knowledge, there are no published studies that have compared the effect of psychosexual therapy (PST) and bupropion, in the extended release (ER) formulation, in this population. Hence, the present study aimed to compare the effectiveness of bupropion extended-release versus psychological intervention on depression symptoms in depressed women with infertility and SD in Fatemeh Zahra Infertility and Reproductive Health Research Center of Babol University of Medical Sciences, Iran.

Materials and Methods

The authors carried out an open-label randomized controlled clinical trial between December 2014 to June 2015 at the Fatemeh Zahra Infertility and Reproductive Health Research Center, Babol, Iran. The study design was confirmed by the Ethics Committee (4930, 12 Jan 2014) and then registered in IRCT. Primary objective of this project was based on the treatment of SD with subsequent improvement of depression symptoms.

Women with infertility less than 45 years of age were eligible for the research under the following criteria: a score of ≥10 in beck depression index (BDI), a score of ≤26.55 on Iranian version of the female sexual function index (IV-FSFI), an infertility duration of greater than one year, were literate, ability to read and write, weren't undergone any fertility treatment in the next 2 months and were sexually active in the past four weeks. Subjects were excluded from the study if they had a history of seizures, were taking medications that could lower the seizure threshold or were known to effect sexual function, had a history of head trauma, had a major change in living conditions, or had psychological support. Exclusion criteria also included serious medical conditions and mental health problems under the treatment of a physician, having actively suicidal, having major depressive disorder (MDD) in the clinical interview by a female psychologist.

Subjects have been assessed for eligibility by two midwives with no clinical involvement in the study. Sample size was calculated in accordance with 22 subjects in each group, with accuracy=6.6, confidence interval (CI)=95%, and approximate SD=6.8 based on previous studies (3, 17, 20, 21) and power=90% for each group. After consideration of the corrected sample size formula ($n'=\sqrt{K}n$, k=2) (22), a total of 93 eligible infertile women were selected through computer-aid randomization in equal 3 groups (31 person in each group). A total of 93 depressed women with infertility and SD were randomly allotted in a 1:1:1 ratio to three equal groups as follows: i. PST, ii. Pharmacotherapy (BUP), and iii. Control (Fig.1). In this study both the researcher and the participants were not blinded.

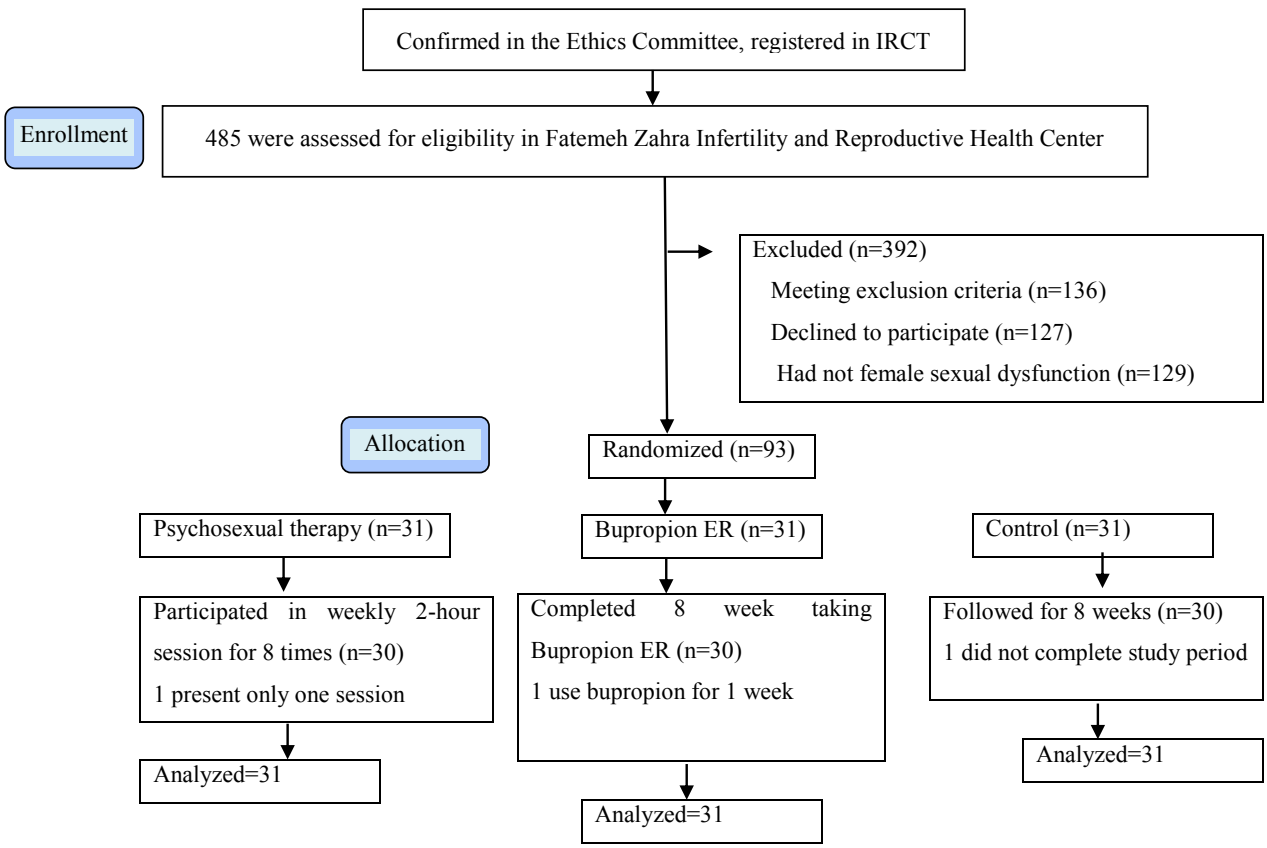

Fig.1: Flow diagram of participants through each stage of a randomized clinical trial.

The PST interventions focused on educational programs that mainly contained eight weeks of two-hour sessions including mindfulness-based cognitive therapy (MBCT), relaxation training, behavior sex therapy (Mixed method) based on the Crowe and Ridley model, and also booklet of tranquility, mindfulness and medication (23-25) in the shape of group discussions, questions and answers, lectures, booklets, and CD with groups of 9-13 members. The pharmacotherapy group was treated with bupropion ER at a dose of 150 mg/d (Wellban Extended release, Abidi Company, Iran) for up to eight weeks.

The control group did not receive intervention, but educational package was given to them, and were referred to a sex therapy clinic after the end of study. Study duration, and bupropion dosage were different in previous studies (4-24 week, and 100-450 mg/day) (3, 17, 18, 26, 27). Therefore, intervention period, and dosing for bupropion in this study was considered 8 week, and 150 mg orally once a day in patients with depression symptoms treated for sexual dysfunction. Adverse events (AEs), vital sign, and Anthropometric measurements (weight, height) were serially conducted for each of groups by researcher, were summarized at each data collection schedule assessment point. During these visits, the previous bag of bupropion was collected and new bags of bupropion were given to be used for the next two weeks. Participants were also contacted by phone biweekly to monitor for any complaint, or any change in their health status(for any reason). At the same time the researcher could be contacted by phone at any time.

A total of 90 participants completed the study. At first, the research protocol was described for participants, and then written informed consent was obtained from each subject. A secure and confidential environment was considered for collecting data. The researcher used a binder for each participant to keep individual information.

Demographic information was collected. All subjects completed the BDI at baseline and after treatment at the end of the study. The BDI is a self-reported measure consisting of 21 questions to assess the severity of depression symptoms, Created by Beck in 1961, it has an approved validity (0.89); reliability (0.96) during the first decade following its introduction. The translated and Persian of BDI had Cronbachs alpha 0.87 in the Iranian population.

The intensity of the item rates on a 4-point scale (0-3) and the test is scored by summing the ratings given to each of the 21 items. The total score range between 0 and 63 and the results range as follows: 0-9 as no depression, 10-18 as mild depression, 19-29 as moderate depression, and 30 and greater as severe depression (28-30). A score ≥10 in BDI was considered ‘‘at risk’’ for depression (3). The FSFI, used to measure sexual dysfunction. It contains 19 items in six different subscales of sexual desire, arousal, lubrication, orgasm, satisfaction, and pain. A score of ≤26.55 indicates sexual dysfunction. The validity and reliability of the Iranian version of FSFI is high (Cronbach’s alpha 0.70-0.9) (4, 7).

Statistical analysis

Data analyses were performed using Paired t tests, Pearson’s correlation, χ^2 test, analysis of covariance (ANCOVA), and Tukey’s test (SPSS software, version 21) in an ITT analysis, with $P<0.05$ indicating statistical significance. Paired t tests was applied to show significant inter individual changes in BDI score within each treatment group. The chi-square test was used for comparing categorical variables between three groups. The ANCOVA was used as a statistical technique to control for variability (with baseline BDI scores as a covariate variable).

Tukey’s test was used for pair wise comparisons. Subsequent tests included the homogeneity of variances, the linear relationship between the dependent variable and the covariate, and the normality of distributions (Skewness and Kurtosis test). The change of depression level from baseline to end of study was calculated for each group. Improvement in depression symptoms was defined as a pre- to post treatment decrease in BDI depression level (severe to moderate, moderate to mild, and mild to no depression). Worsening of depression was defined as an increase in BDI depression level from baseline to end of study (mild to moderate, moderate to severe).

Results

The demographic characteristics of subjects are showed in Table 1. The majority of participants were unemployed (78.5%), while the majority of the participants’ spouses were self-employed (44.1%). The mean age of participants was 29 ± 5.44 years. The economical situation in more than one third of participants was poor (38.7%). The type of housing for the majority of participants was private (65.6%). Nearly two thirds (62.4%) of participants had primary infertility. There were no statistically significant differences in baseline factors, occupation, husband occupation, educational level, economic status, type of housing, infertility type, and infertility etiology between the three groups (Table 1).

The mean BDI score of participants was 22.35 ± 8.70 at baseline. Mean BDI scores at baseline and at the end of study for the three groups are given in Table 2. Paired t tests showed significant inter individual changes in BDI score within each treatment group (PST, $P<0.0001$, BUP, $P<0.002$, Table 3). Depression symptoms decreased significantly in both PST and BUP groups from baseline to end of study. The intra-individual changes were not significant in control group ($P=0.105$).

Changes in depression level (pre to post treatment) showed that 79.1% of participants in the treatment groups(PST and BUP) improved from their baseline depression level, while 8.05% had a worse depression level, and 12.9% had no change. In the control group, 38.7% of participants improved from their baseline depression level, while 16.1% had a worse depression level, and 45.2% had no change. The improved depression levels were showed more in PST group compared to others groups (Fig.2).

Table 1: Distribution of the participarts according to the sociodemographic charecteristics

Variable	Treatment group		Control n=31 n (%)	P value	Total n=93 n (%)
	PST n=31 n (%)	BUPER n=31 n (%)			
Occupation				0.168	
Unemployed	21 (67.7)	27 (87.1)	25 (80.6)		73 (78.5)
Employed	10 (32.3)	4 (12.9)	6 (19.4)		20 (21.5)
Husband occupation				0.810	
Unemployed	1 (3.2)	1 (3.2)	1 (3.2)		3 (3.2)
Worker	8 (25.8)	10 (32.3)	5 (16.1)		23 (24.7)
Employee	7 (22.6)	8 (25.8)	11 (35.5)		26 (28.0)
Self-employed	15 (48.4)	12 (38.7)	14 (45.2)		41 (44.1)
Type of housing				0.716	
Private	20 (64.5)	19 (61.3)	22 (71.0)		61 (65.6)
Rental	11 (35.5)	12 (38.7)	9 (29.0)		32 (34.4)
Infertility type				0.242	
Primary	17 (54.8)	23 (74.2)	18 (58.1)		58 (62.4)
Secondary	14 (45.2)	8 (25.8)	13 (41.9)		35 (37.6)
Education				0.183	
0-12 (Y)	24 (77.4)	25 (80.6)	19 (61.3)		68 (73.1)
>12 (Y)	7 (22.6)	6 (19.4)	12 (38.7)		25 (26.9)
Infertility etiology				0.806	
Female	2 (6.5)	2 (6.5)	5 (16.1)		9 (9.7)
Male	11 (35.5)	11 (35.5)	9 (29)		31 (33.3)
Both	9 (29)	10 (32.2)	7 (22.6)		26 (28)
Unknown	9 (29)	8 (25.8)	10 (32.3)		27 (29)

PST; Psychosexual therapy and BUPER; Bupropion extended-release. χ^2 test was used for comparing categorical variables between three groups.

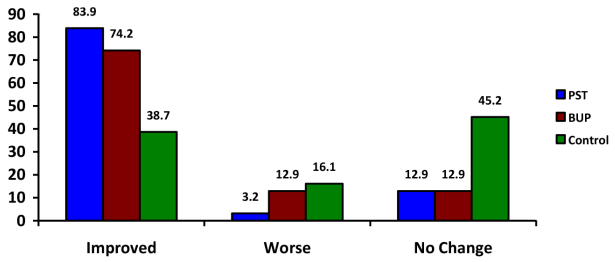

Fig.2: The changes of final levels of baseline depression in three groups. PST; Psychosexual therapy, BUP ER; Bupropion extended-release. n=93 (each group of 31 participants). P<0.001 (χ^2 test).

The change from baseline in BDI score was analyzed by ANCOVA and significant differences between the three groups were found (P<0.001). Pair wise comparisons on mean BDI showed that only PST decreased significantly compared to control group (P<0.0001). The decrease in mean BDI score in the BUP group was not significantly different compared to control group (P<0.282). There was a significant diference in mean BDI scores between PST and BUP groups (P<0.005) (post-hoc ANCOVA). Statistical power for this analysis was approximately than 95.4%. As a result, the assumption of research on the effects of therapeutic interventions on BDI score with a probability of 95.4% in infertile women was accepted (Table 2). There was statistically significant negative relationships between the mean of FSFI score with BDI score (P<0.001). Bupropion is well tolerated.

Table 2: Covariance analysis test for total score in beck depression and pair wise comparisons in the groups

Variable	Sum of squares	Mean square	df	F statistics	Observed power	P value
Depression	2114.060	2114.060	1	24.149	0.998	0.0001
Group	1427.404	713.702	2	8.153	0.954	0.001

BDI; Beck depression inventory, PST; Psychosexual therapy, BUP ER; Bupropion extended-release, n=93 (each group of 31 participants), df; Degrees of freedom, and F; Test statistic. ANCOVA test was used to compare the change from baseline in BDI score between the three groups; post-hoc ANCOVA was used for pair wise comparisons. There was a significant diference in mean BDI scores between PST and control groups (P<0.0001), PST and BUP ER groups (P<0.005); but not between BUP and control groups (P<0.282).

Table 3: The mean scores of BDI in three groups of infertile women at beginning and end of the study

Variable BDI	Pre-test Mean ± SD	Post-test Mean ± SD	P value
PST	24.59 ± 7.76	10.42 ± 9.01	0.0001
BUP ER	22.42 ± 10.70	16.09 ± 11.81	0.002
Control	20.06 ± 6.83	17.35 ± 10.46	0.105

BDI; Beck depression inventory, PST; Psychosexual therapy, BUP ER; Bupropion extended-release, n=93 (each group of 31 participants). Paired t test was used to compare the pre-to-post depression BDI mean score in each group.

Discussion

Study participants with SD showed where they were at risk for depression symptoms and had a high average of depression scores. This finding suggests that women with infertility and SD are more likely to experience symptoms of depression. Previous studies by Pasha et al. (1), Pakpour et al. (4) and Peyvandi et al. (31) show similar results, where female SD put women with infertility at high risk for depression. Sexual problems are severely distressing experiences, and may be important factors in the development of depression (7). Consistent with this, marital dissatisfaction is associated with an increase in severe depression (31).

We found that BDI scores decreased significantly from baseline at the end of study in each treatment group (PST and BUP). In both treatment groups combined, more than two thirds of participants showed improvement in their depressive symptoms levels (pre to post treatment). The similar studies showed that both psychosocial and pharmaceutical therapeutic strategies, such as psychotherapy and antidepressants, are well established in the treatment of depression (3, 17). A review of psychological interventions by Boivin (14) reported that psychotherapy decreased negative affect in infertile people. Psychotherapy or sex therapy had a beneficial effect on the management of psychological symptoms affecting sexual function in women (3, 15).

Also bupropion is an effective antidepressant medication, which is used to treat remission of depressive disorder. The effectiveness of bupropion in improving depression found in clinical trials with the drug (27, 32). Furthermore, this research indicated a statistically significant negative relationship between sexual function and depression symptoms at patients treated for sexual dysfunction. In line with finding, another studies found that higher scores of FSFI are correlated with lower depression scores (21). There was a negative correlation between depression with sexual function and marital satisfaction (11). It was associated with lower depression, higher marital satisfaction reverse antidepressant-induced SD (21, 27, 33). Therefore, it is important to note that the resolution of depression scores can be related to relieving sexual problem in depressed patients (8, 14, 18, 34). This is because the SD and depression are interchangeable, so that the treatment of one will change the others.

After adjusting for baseline values, data showed a significant improvement in depression symptoms for women exposed to the psychosexual intervention compared with women in the BUP and control groups. Group PST was better than bupropion treatment in improving depression symptoms. PST was not only a reliable treatment approach to improving depression symptoms, but also it was superior to bupropion treatment to alleviate depression symptoms in women with infertility and SD. These results suggest that PST may be more effective compared with pharmacological therapy to treat depression symptoms in women with infertility and SD.

Consistent with this; study conducted by Faramarzi et al. (3) reported that group psychotherapy was superior to drug therapy in improving the well-being of women with infertility suffering from depression. In addition, they found that cognitive behavioral therapy was better to pharmacotherapy in relieving depression; depression symptoms were reduced to a greater extent than the control group. A review of literature suggests that counseling service is associated with lower depression, leading to higher life satisfaction compared to control group. Finally, the use of cognitive behavioral model can be effective in reducing frustration and depression, increasing skills, and improving the marital relationship and sexual satisfaction. Psychological model was an effective therapy for depression (35, 36).

Our findings showed that the decrease in the mean BDI score with BUP dosage of 150 mg per day was not statistically significant compared to the control group, although BUP group showed statistically significant improvement in depression symptom from beginning to end of research. Many trials showed that bupropion was efficacious in treatment MDD (27, 32).

It was found as an important antidepressant, and used to treat MDD (21). However, a few studies have observed no significant differences between bupropion and placebo groups on depression scores (19, 20) and bupropion 150 failed to demonstrate significant difference (19). It would be of great important to mention that low dose of bupropion was employed in subjects. Therefore, it seems that bupropion dosage of 150 mg once a day may have limited benefit for detecting difference between BUP and control groups in resolution of depression symptom. Furthermore, excluding the subjects with diagnosed MDD from this study may decrease the response rate to depression. Therefore, the different results of present research with others may relate to this model, especially in the infertility field. It should be noted that psychological placebo effects due to communication of the researcher with the patients in frequent assessments during the study should not be ignored. Further study need to distinguish factors leading to the lack of an effect.

Conclusion

Psychosocial therapy was a superior treatment compared to bupropion for alleviating depression symptoms

in women with infertility and SD. Therefore, counseling services and social support to recognize and treat depression and SD are necessary to establish in fertility centers.

There were a few limitations in this study. First, the data were collected from a small sample size of Iranian women with infertility; therefore, the findings cannot be generalized to all women with infertility or other populations and would require to be investigated in future research of a larger sample size. Another weakness of study was leak of follow-up. The strengths of this research include its use of a validated, self-reported Iranian version of the BDI. Also, for more effective treatment methods suggested that future studies consider the PST plus bupropion compared to each of them individually.

Acknowledgements

The present research was funded by Babol University of Medical Sciences, Iran. Our team would like to thank all the infertile women who participated in present research and to Dr. Evangeline Foronda (Ph.D.) for English editing of the manuscript. There is no conflict of interest in present research that could inappropriately influence this project.

Author's Contributions

H.P., Z.B., M.F., F.K., Participated in the conception and design of the protocol. H.P., Z.B., M.F.; Managed the literature searches. H.P., M.F.; Acquainted of data, interpretation of data had done by H.P., Z.B., M.F., F.K. H.P., Z.B.; Wrote the first draft of the article. All authors participated to final approval of the completed manuscript.

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Original Article

Comparison of The Effects of A Positive Reappraisal Coping Intervention and Problem-Solving Skills Training on Depression during The Waiting Period of The Result of Intrauterine Insemination Treatment: A Randomized Control Trial

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Abstract

Background: The outcomes of fertility treatments are unpredictable, and levels of depressive symptoms increase in patients during the waiting period of the result of intrauterine insemination (IUI) treatment. The aim of this study was to compare the effects of a positive reappraisal coping intervention (PRCI) and problem-solving skills training (PSS) on depression during the waiting period of the result of IUI Treatment.

Materials and Methods: This randomized control clinical trial was done among 108 women undergoing IUI treatment. In the control group, the women received routine care. In the PRCI group, women attended two training sessions and were asked to complete coping thoughts cards and fill out daily monitoring forms during the waiting period. In the PSS group, PSS were taught over three sessions. The depression was measured by the beck depression inventory.

Results: On the 10th day of the IUI waiting period, there were significant differences between the control group (21.42 ± 11.42) and the PSS group (12.52 ± 8.05) and PRCI groups (13.14 ± 9.7) ($P<0.001$), but no significant difference between the PRCI group and the PSS group.

Conclusion: According to the results of this randomized control trial there is no difference between a PRCI and PSS on depression during the waiting period of the result of IUI treatment. This suggests that both interventions can be used to help infertile women combat depression during the waiting period of the result of fertility treatments (Registration number: IRCT2016020926490N1).

Keywords: Artificial Insemination, Depression, Problem-Solving

Citation: Ghasemi Gojani M, Kordi M, Asgharipour N, Esmaeili H. Comparison of the effects of a positive reappraisal coping intervention and problem-solving skills training on depression during the waiting period of the result of intrauterine insemination treatment: a randomized control trial. Int J Fertil Steril. 2018; 12(1): 13-18. doi: 10.22074/ijfs.2018.5155.

Introduction

Infertility is defined as the failure to achieve a clinical pregnancy after 12 months or more of unprotected sexual intercourse (1). Infertility can threaten the mental health of infertile couples. In a study by Peyvandi et al. (2), 62% of women who had been visiting infertility treatment had various degrees of depression: 27.5% suffered from mild depression, 25.5% moderate depression, and 9% severe depression. Intrauterine insemination (IUI) treatment combined with ovulation induction is usually considered the first-line treatment for many infertile couples and it is the most common of the treatment methods. Although with the advancement of science and new assisted reproductive techniques such as: *in vitro* fertilization (IVF), intra cytoplasm sperm injection (ICSI), and IUI, the hopes of infertile couples have increased, these methods are expensive and involve broad medical interventions and long periods of treatment (3).

The waiting period of the result of IUI treatment, refers to the time interval between the IUI operation and the time of the pregnancy test (4). This can be associated with severe distress in individuals, the outcomes of fertility treatments are often unpredictable and the infertile women are not able to control or predict the treatment outcome (5). According to a study

Received: 19 Dec 2016, Accepted: 10 Jun 2017
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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 1, Apr-Jun 2018, Pages: 13-18

by Osuna (6), the waiting period causes psychological reactions, stress, and anxiety in individuals as a result of their concerns about an event which will happen in the future, and which they are not able to predict or control. In a more recent study, Boivin and Lancastle (4) showed that the degree of anxiety and depression increased during the waiting period before fertility treatment.

One of the interventions designed to help people cope with the medical waiting period is the Positive Reappraisal Coping Intervention (PRCI). The PRCI is based on positive reappraisal coping strategies, and use of this intervention helps people emphasize the positive aspects of the situation (7). This technique has been used for the waiting period associated with IVF treatment, genetic tests, and recurrent miscarriage. In a study by Ockhuijsen et al. (8) use of positive coping thoughts cards led to an appreciation of the positive aspects of the situation and creation of a positive feeling in infertile women during the waiting period of the result of IVF treatment. Although research suggests an effect of the PRCI on positive feeling during the waiting period of the result of IVF treatment, there is disagreement about the effect of this intervention on negative emotions, anxiety, and depression in infertile women during waiting period (8, 9). It is also necessary to use other interventions to reduce depression during the period of waiting for fertility treatments.

Problem Solving Skills training (PSS) is a psychological intervention, which aims to help individuals adapt more effectively to stressful problems in life (10). Problem solving is one of the most important strategies in facing infertility (11). In a study by Kordi et al. (10), the severity of postpartum depression was significantly lower in the PSS group than in the control group. But no similar studies have been conducted on the effect of PSS on depression in infertile women. According to increased levels of depression during the waiting period and contradictions in the studies (8, 9). This randomized controlled trial was conducted with the aim of comparing the effects of a PRCI and PSS on depression during the waiting period of the result of IUI treatment in infertile women in the Milad Infertility Treatment Centre in Mashhad, Iran, during the years 2015 and 2016.

Materials and Methods

This randomized control clinical trial involved 108 women, referred to the Milad Infertility Treatment Centre in Mashhad for IUI treatment. Sampling for the trial was undertaken after the research had been approved by the Ethics Committee of Mashhad University of Medical Sciences (Registration number: IRCT2016020926490N1) and consent obtained from the officials of Milad Infertility Treatment Centre. In order to prevent the dissemination of information between the groups were considered at three different times, so that after the completion of the sampling in a group, sampling was started in the other group. The manner of assignment was in this way that first the groups' names were written on paper, then according to the draw, the first time interval was assigned to the control group, for the second period the PRCI group and for the third period PSS group was assigned. Available sampling method was applied in each group.

The sample size was calculated based on Cohen's (1987) table, and considering a power of 80%, a confidence level of 95%, and an effect size of 70%, we determined 33 individuals to be required in each group. To take into account a 10% loss, we determined that 36 individuals were required for each group. Inclusion criteria for the study were: Iranian nationality, 18-40 years of age, ability to read and write, primary infertility, and obtaining a score less than 28 on the General Health Questionnaire (GHQ 28). Exclusion criteria for the study were: consumption of any psychoactive drug, occurrence of any stressful and unpleasant incidents over the past 6 months, suffering from medical illness, obtaining a depression score higher than 28 on the Beck Depression Inventory, cancellation of IUI treatment cycle, failure to participate in all training sessions, and unwillingness to continue cooperation in the research.

The instruments used in this trial included: a questionnaire on demographic and infertility-related information, the beck depression inventory, GHQ-28, daily monitoring forms, positive coping thoughts cards, and a checklist for implementing problem-solving skills. The questionnaire on demographic and infertility-related information consisted of questions about: age, level of education, employment status, duration of marriage, family income, duration of infertility, duration of treatment, number of times the participant had undergone IUI and IVF, cause of infertility, treatment seeking, expectancy of successful treatment, and the cost of treatment. The beck depression inventory contains 21 questions with answers scored between 0 and 3. The minimum possible score on the depression questionnaire is zero and the maximum is 63, classified as follows: minor depression 0-13, mild depression 14-19, moderate depression 20-28, and severe depression 29-63.

The GHQ 28 is a questionnaire containing 28 questions that measure physical symptoms, anxiety, insomnia, social dysfunction, and severe depression. The answers are on a four-point Likert scale. The threshold score for this questionnaire is 28 and a score higher than 28 is a sign of susceptibility to mental disorders. The daily monitoring form was designed by Ockhuijsen et al. (8) to assess physical and mental changes in infertile women during the waiting period before fertility treatment. It consists of 46 questions related to the person's emotions, physical symptoms, including symptoms related to anxiety and to the failure or success of treatment, coping strategies, person's assessment during the period of waiting of the result of treatment and coping strategies during

this waiting period. This form is a part of the PRCI and was completed each day by the PRCI group during the waiting period.

The positive coping thoughts card contains 10 statements based on the positive reappraisal coping strategy. The PRCI group repeated the positive thoughts at least twice a day during the waiting period. The validity of the qualitative content of this trial was assessed as follows: after the preparation and translation of the questionnaires (demographic data and information related to infertility, daily monitoring forms, positive coping thoughts cards, and check list of problem solving skills) under the supervision and guidance of professors in counseling, the questionnaires were reviewed by seven experts and professors from the Mashhad University of Medical Sciences. The final tools used incorporated the necessary revisions suggested by the experts.

The reliability of the beck depression inventory (α=0.83), GHQ28 (α=0.83), and daily monitoring form (α=0.74) were ascertained using Cronbach's alpha. Infertile women who visited the centre to plan their IUI treatment and who fulfilled the inclusion criteria for the study were recruited into the trial. PSS sessions and the PRCI sessions were performed by the researcher after confirmation of the researcher's ability by a specialized consultant with a Ph.D. in clinical psychology.

In first session in the PSS group, which was on days 2-3 of the menstrual cycle, we discussed infertility and the IUI treatment process, the research objectives and how to conduct the sessions, and the role of using PSS in dealing with the problems of life. The participants were then asked to write a list of problems that they have had during the course of their IUI treatment and determine the most important issue. In the second session, on days 9-12 of the menstrual cycle, the participants were asked to suggest solutions to their problems using a brainstorming method which they were taught in the session and prepare a list of the solutions that came to mind. During the third session, on days 14-15 of the menstrual cycle, the participants discussed the disadvantages and advantages of implementing the solutions arrived at in the second session. Following the discussion they made a list of the disadvantages and advantages of implementing each of the solutions and chose the best solution. The participants were also taught how to evaluate the effectiveness of a solution and advised of the possibility of returning to the previous step in case a solution was deemed to be ineffective. They were asked to implement this PSS in dealing with their daily problems during the waiting period, and record their efforts in the checklist for implementing PSS.

The first session of the PRCI group was held on days 2-3 of the menstrual cycle. We discussed infertility, the IUI treatment process, the research objectives, types of coping strategy, and the positive reappraisal coping strategy. The second session was held on days 9-12 of the menstrual cycle. In this session we explained the ten statements on the positive coping thoughts card using examples and showed participants how to complete the daily monitoring form. The Participants were then asked to repeat the positive coping thoughts at least twice a day during waiting period. Control group participants received the center's routine care, and presented themselves at Milad Infertility Treatment Center on days 2-3, 9-12, and 14-15 of the menstrual cycle to undergo an ultrasonography and determine any remedial measures for the IUI treatment. The beck depression inventory was completed by all the three groups on the 10th day of the waiting period. Depression score means were compared between the three groups before the intervention and on the 10th day of the waiting period.

Statistical analysis

After collection and coding, the data were entered into the computer and analyzed using SPSS version 16, with $P<0.05$ considered statistically significant. The normality of the quantitative variables was determined using the Kolmogorov-Smirnov test. If the variables were normal parametric statistics were used, otherwise the non-parametric equivalent was used. Means, frequencies and standard deviations were used to describe the characteristics of the participants in each of the three groups. To compare depression between the three groups we used the ANOVA test and the paired t test for intra-group comparisons of depression if the data were normal. In the case of non-normal data the Kruskal-Wallis test and Wilcoxon tests were used.

Results

Data were obtained from 34 individuals in the control group, 34 individuals in the PSS group, and 35 individuals in the PRCI group. Two individuals in the control group and one individual in the PRCI group were excluded from the study due to the cancellation of their treatment program, and in the PSS group one individual was excluded from the study due to the cancellation of their treatment program, and one individual in the was excluded due to her unwillingness to continue participating in the research (Fig.1).

There was no significant difference between participants in the three groups in terms of level of education (P=0.853), the woman's occupation (P=0.364), cause of infertility (P=0.824), experience of using assisted reproductive techniques (P=0.410), and paying for the treatment (P = 0.392, Table 1). The mean GHQ28 score was 25.85 ± 3.93 in the control group, 25.28 ± 4.20 in the PSS three groups were homogeneous in terms of this variable (P=0.712, X^2=0.68).

According to the paired t test and comparison of mean scores for depression in the two time intervals; before the intervention and on the 10th day of the waiting period (a within-group comparison), the mean score for depression showed a significant decrease in the PSS group (P<0.001) and PRCI group (P=0.002), and a significant increase in the control group (P=0.007, Table 2).

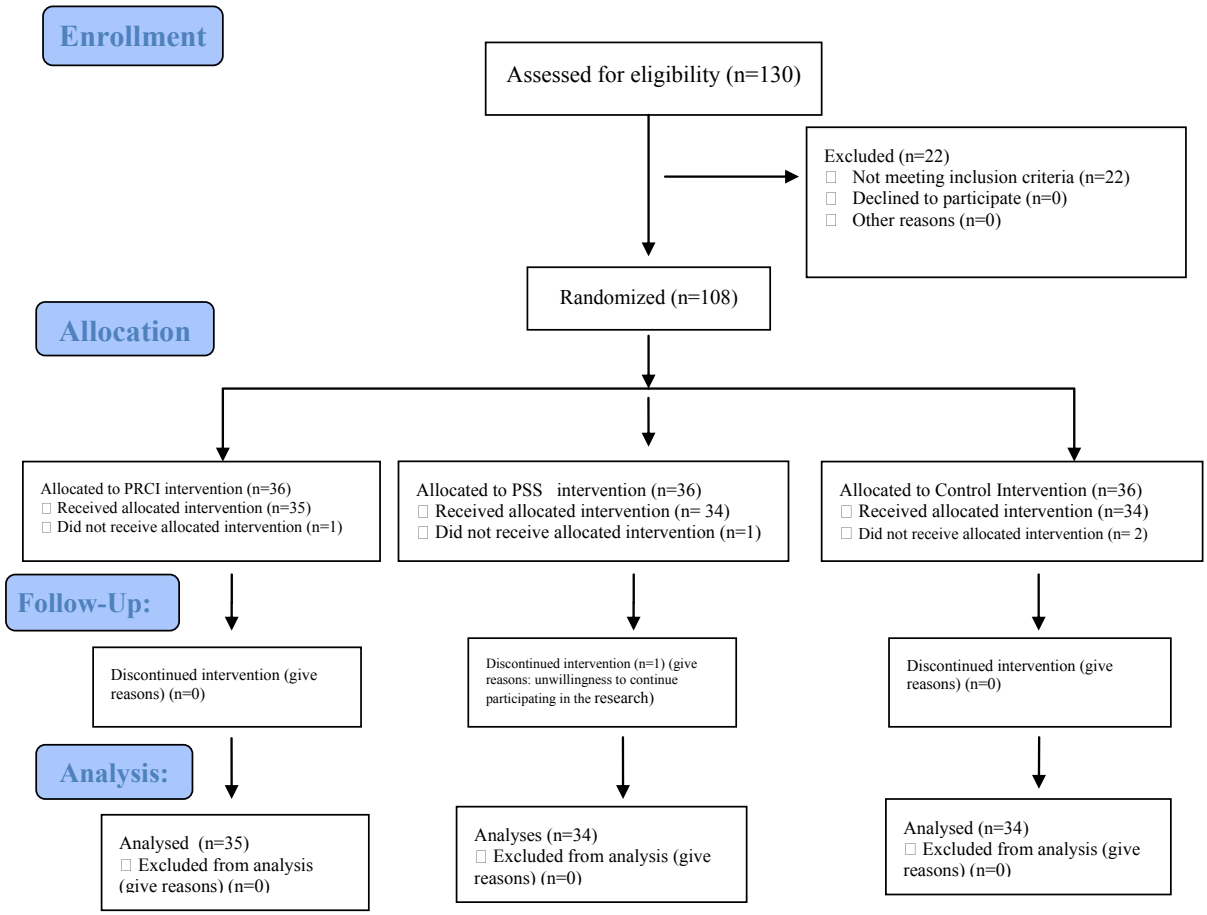

Fig.1: The stages of the intervention.

Table 1: Descriptive statistics for infertile women undergoing IUI treatment in the three intervention groups: control, PSS, and PRCI group.

Variable		Group			Test results
		Control n (%)	PSS n (%)	PRCI n (%)	
Education level	Elementary school	7 (19.4)	4 (11.1)	2 (5.6)	X^2=0.31
	Middle school	5 (13.9)	6 (16.7)	4 (11.1)	df=2
	High school	8 (22.2)	11 (30.6)	16 (44.4)	P=0.853[a]
	University	16 (44.5)	15 (41.6)	14 (38.9)	-
Woman's occupation	Housewife	30 (83.3)	30 (83.3)	25 (69.4)	ExactX^2=4.42
	Office employee	5 (13.9)	5 (13.9)	6 (16.7)	P=0.364[b]
	Student	1 (2.8)	1 (2.8)	5 (12.9)	-
Cause of infertility	Male factor	4 (11.1)	4 (11.4)	5 (16.7)	-
	Female factor	10 (27.8)	14 (40)	16 (41.7)	-
	Joint factor	9 (25)	8 (20)	7 (19.4)	ExactX^2=3.01
	Unknown factor	13 (36.1)	10 (28.6)	8 (22.2)	P=0.824[b]
Prior use of assisted reproductive techniques	None	17 (47.2)	20 (55.6)	19 (52.7)	-
	IUI	14 (38.9)	9 (25)	13 (36.1)	-
	IVF	0 (0.0)	0 (0.0)	1 (2.8)	ExactX^2=0.39
	Other	5 (13.9)	7 (19.4)	3 (8.4)	P=0.410[b]
Paying for treatment	Not at all	2 (5.6)	4 (11.1)	2 (5.6)	-
	Low	6 (16.7)	8 (22.2)	4 (11.1)	X^2=1.87
	Relatively high	13 (36.1)	12 (33.3)	15 (41.7)	df=2
	High	8 (22.2)	8 (22.2)	11 (30.6)	P=0.392[a]
	Very high	7 (19.4)	4 (11.1)	4 (11.1)	-

IUI; Intrauterine insemination, PSS; Problem-solving skills, PRCI; Positive reappraisal coping intervention, IVF; *In vitro* fertilization, [a]: Kruskal-Wallis, [b]; Fisher's exact test, and df; degrees of freedom.

Table 2: Comparing the mean and standard deviation of depression scores before the intervention and on the tenth day of the waiting period of the result of IUI treatment in the control, PSS, and PRCI groups

Depression	Group			One-way ANOVA test result
	Control Mean ± SD	PSS Mean ± SD	PRCI Mean ± SD	
Before the intervention	17.38 ± 9.96	19.13 ± 8.67	18.55 ± 9.00	F=0.33 P=0.716
Tenth day of waiting period of IUI treatment	21.7 ± 11.42	12.52 ± 8.05	13.14 ± 9.7	F=9.29 P<0.001
Mean changes before the intervention and on the tenth day of waiting period of IUI treatment	-4.88 ± 9.46	5.94 ± 7.38	2.19 ± 10.17	F=15.87 P<0.001
Results of paired-t test	t=-2.867 P=0.007	t=4.286 P<0.001	t=3.278 P=0.002	

IUI; Intrauterine insemination, PSS; Problem-solving skills, PRCI; Positive reappraisal coping intervention, t; Statistics of the test, and F; Statistics of the test.

The ANOVA test results showed that there was no significant difference in mean depression score between the three groups (P=0.716) before the intervention. However, on the 10th day of the waiting period of the result of IUI treatment, there were significant differences between the mean depression scores of the three groups (P<0.001). The results of the Tukey's post hoc test showed that there were significant differences in mean depression scores between the control and the PSS group and also the control and the PRCI group (P=0.001, Table 2).

Discussion

In the present study, depression increased in the control group during the waiting period of the result of IUI treatment. This is consistent with the results of studies conducted by Boivin and Lancastle (4) and Ockhuijsen et al. (8). Being in a medical waiting period causes psychological distress and increased levels of anxiety and depression in the infertile women (12). These feelings result from their concerns about an important event (the result of the pregnancy test) which will happen in the future and which (6) they are unable to change or control and about which there is little information through which they can predict the treatment outcome (4).

In our study, the mean depression score decreased in the PRCI group and PSS group. But, in a study by Ockhuijsen et al. (8), depression increased during the waiting period after IVF treatments in the PRCI group which is not consistent with the results of the present study. In this study the inclusion criteria for the study included being under IVF treatment and speaking the dutch language, but in the present study, those who obtained a score higher than 28 on the general health questionnaire and a depression score higher than 28 on the beck depression inventory, as well as cases who needed to be referred to a psychologist, were excluded.

In a study by Kordi et al. (10), after 5 sessions of PSS, the level of postpartum depression significantly decreased in the PSS group which is consistent with the results of the present study. Problem solving is an important coping strategy that enables an individual to appropriately control problematic situations (13, 14). PSS have an important role in mental and physical health, especially when people face unpleasant events and negative tensions in their lives (15). In a study by Talaei et al. (16), after 10 sessions of cognitive-behavioral therapy group training, the level of depression in infertile women significantly decreased in the cognitive-behavioral therapy group compared with controls which is consistent with the results of the present study.

Despite the fact that infertility treatments are stressful, infertile women expressed little desire to use the proposed psychological interventions. The reasons mentioned were as follows: fear of attending consultation sessions, loss of personal privacy, the cost of consultation, and ineffectiveness of the consultation process (17).

The PRCI is a new intervention based on positive reappraisal coping strategies for medical waiting periods, whose implementation does not need an in-person visit to the advisor, and which is affordable (5). Positive reappraisal coping strategies can help people adapt to unpredictable and long times, because positive reappraisal coping strategies lead to a reappraisal of the situation and emphasis on discovering the benefits and positive aspects during stressful conditions (8). The basis of this intervention is creating a positive thought through a cognitive process. The design of the PRCI is based on Lazarus and Folkman's theory of stress and coping. In their study, Lazarus and Folkman came to the conclusion that positive emotions play the most important role in encouraging people to continue making efforts to cope with stressful situations (5, 7, 8, 12, 18).

Among the strengths of this study are the adaptability of the training sessions to the therapeutic program intended for the participants. A limitation of our study is that the trial was not double-blind, which introduces the possibility of observer bias. However, the fact that our findings are consistent with those of other studies in the field means that this is unlikely to have had a major impact on our results.

Conclusion

As the results of this study found the impact of PRCI and PSS on depression during the waiting period of the result of IUI treatment were the same, it is suggested both interventions can be used, if facilities are available, to help infertile women reduce the depression generated by the waiting period and increase their adaptability.

Acknowledgements

Research Deputy of University, Mashhad University of Medical Sciences was Funding this article. This paper is part of a master's thesis in midwifery (code 940545) undertaken at Mashhad University of Medical Sciences. Hereby, thanks and appreciation are extended to the University's research deputy who sponsored this project, the University professors, the staff of the Milad Infertility Treatment Center, and all the women who participated in this study. The author declares that there is no conflict of interest regarding the publication of this paper.

Author's Contributions

M.Gh.G., M.K., N.A.; Contributed to concepts, definition of intellectual content. M.Gh.G., M.K., H.E.; Contributed to data analysis and statistical analysis. M.Gh.G., M.K.; Contributed to literature search. All authors contributed to design, data acquisition, manuscript preparation, editing, review and approved the final manuscript.

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Original Article

Lifestyle-Related Factors Associated with Reproductive Health in Couples Seeking Fertility Treatments: Results of A Pilot Study

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Abstract

Background: The objective of this pilot study was to evaluate the feasibility of conducting a larger prospective cohort study, which will aim at determining the independent contribution of male and female lifestyle-related factors to assisted reproductive technology (ART) success. The study also examined whether couples seeking fertility treatments present lifestyle-related factors that may interfere with their reproductive health.

Materials and Methods: This prospective pilot study was conducted in a fertility clinic between May 2015 and February 2016. Feasibility factors evaluated were recruitment rates, compliance with the protocol, retention rate and ART outcomes at six-month follow-up. Anthropometric profile and lifestyle habits of both partners were evaluated before the beginning of infertility treatments.

Results: We approached 130 eligible infertile couples. Among them, 32 (25%) agreed to participate and 28 (88%) complied with the protocol. At six-month follow-up, seven couples (25%) did not start, or stop, infertility treatments and 13 couples (62%) achieved a clinical pregnancy. Among the 28 couples included in the analyses, 16% of the partners were obese and 23% had abdominal obesity. The majority of the subjects were still drinking alcohol (84%). Sixty-eight percent of women needed improvement in their diet (vs. 95% of men, P=0.05) and none of them achieved the Canadian recommendations for physical activity (vs. 33% of men, P=0.001). Moreover, 35% of the partners had a poor sleep quality. Overall, women presented a worse reproductive health profile than men, with 3.1 and 2.4 out of seven adverse factors, respectively (P=0.04).

Conclusion: Conducting a large prospective cohort study in our fertility clinic will be feasible but recruitment and compliance with the protocol need to be improved. Many women and men seeking fertility treatments present unfavourable lifestyle-related factors that may explain, at least partially, their difficulties in conceiving.

Keywords: Infertility, Lifestyle, Sleep Quality

Citation: Piché ML, Babineau V, Robitaille J, Lachance É, Ruchat SM. Lifestyle-related factors associated with reproductive health in couples seeking fertility treatments: results of a pilot study. Int J Fertil Steril. 2018; 12(1): 19-26. doi: 10.22074/ijfs.2018.5135.

Introduction

It is well recognized that various lifestyle-related factors, such as obesity, smoking, other substance abuse and heavy alcohol consumption, have a negative impact on both male and female fertility, and the success of assisted reproductive technology (ART) (1, 2). Other lifestyle habits, such as mild-to-moderate alcohol consumption, caffeine intake, nutritional factors or exercise may also negatively affect reproductive health; however, the available evidence is inconclusive (2).

Obesity is associated not only with female infertility (3, 4) but also with decreased implantation and live birth rate after ART (1, 2). In men, obesity has been linked to an increased prevalence of azoospermia or oligozoospermia (5), a reduced ejaculate volume (6) and a higher risk of sperm DNA damage (7). Similarly, tobacco smoking and heavy alcohol intake affect both men and women reproductive health. In females, smoking is associated with an increased risk of infertility and lower success rate from ART (1, 2) whereas alcohol consumption has been linked to hormonal and menstrual dysfunction, and has a negative impact on embryo implantation (1, 2). In men, smoking is associated with impaired semen quality (8) and alcohol consumption contributes to testicular atrophy, reduced libido and alterations in semen parameters (1, 2). Finally, evidence also suggests that caffeine intake has a potential dose-response association with a longer time to conception (1). Other lifestyle habits, such as physical activity levels (9-11), nutritional factors (12-15) and sleep

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 1, Apr-Jun 2018, Pages: 19-26

quality (16) might also negatively affect female and/or male fertility and ART outcome.

The above-mentioned literature therefore suggests that infertile people can take non-medical actions, such as maintaining healthy body weight and lifestyle habits to improve their chance of conception, spontaneously or following ART. However, studies have shown that many women undergoing fertility treatments tend to make poor lifestyle choices that may affect their chance of conception. A significant proportion of these women continue to drink caffeine and alcohol (10, 17, 18) and do not make lifestyle changes to improve their chances of becoming pregnant (18). Importantly, the majority of the studies that evaluated lifestyle-related factors in fertility clinic settings, were conducted in women. Fewer studies were conducted in men and to the best of our knowledge, only one pilot study including 23 infertile couples documented lifestyle-related factors in both partners (19). Evaluating lifestyle-related factors in both partners and identifying those contributing to ART success, are essential to develop targeted recommendations to help infertile couples to conceive a child.

In this pilot study, we evaluated the feasibility of conducting a larger prospective cohort study that will aim at determining the independent contribution of male and female lifestyle-related factors to ART success. The study also examined whether couples seeking fertility treatments present unfavorable lifestyle-related factors that may interfere with their reproductive health and evaluated possible differences in these factors between men and women.

Materials and Methods

Heterosexual couples seeking fertility treatments for the first time and being able to understand, speak and write French were eligible to participate in this prospective pilot study. Recruitment took place at the fertility clinic of the Centre hospitalier affilié universitaire régional (CHAUR) de Trois-Rivières (Qc, Canada) between May 2015 and February 2016. Men and women who agreed to participate in our study were assessed prior to the initiation of infertility treatments. The couples were followed-up for six months to assess ART success, defined as the confirmation of a clinical pregnancy. This project was approved by the Centre intégré universitaire de santé et de services sociaux de la Mauricie et Centre-du-Québec (CIUSSS MCQ) and the Université du Québec à Trois-Rivières Ethics Committes. Written informed consent was obtained from all couples participating in the study.

Assessment of feasibility

To assess the feasibility of a larger prospective cohort study, recruitment rates, compliance with the protocol (defined as fulfilling the questionnaires and wearing the accelerometer as requested), as well as retention rate and ART outcomes at six-month follow-up were evaluated.

Assessment of anthropometric profile

Height was measured to the nearest millimetre using a standardized cloth tape measure, and body weight was measured to the nearest 0.1 kg on a calibrated balance after removing shoes (UM016 2202, Tanita Corporation, USA). Body mass index (BMI) was then calculated in kilograms per meter squared (kg/m^2). On the basis of international BMI cut-off values for adults, the prevalence of underweight ($<18.5\ kg/m^2$), normal weight (18.5-24.9 kg/m^2), overweight (25.0-29.9 kg/m^2) and obese ($\geq 30.0\ kg/m^2$) were calculated. Waist circumference (WC) was measured using a standardized cloth tape measure according to standard procedures (20). Abdominal obesity was defined as WC$\geq$102 cm in men and $\geq$88 cm in women (21).

Assessment of lifestyle habits

Each partner received an e-mail containing instructions for completing online questionnaires assessing their eating and sleeping habits. A web-based self-administered food frequency questionnaire (web-FFQ), containing a list of typical foods available in the province of Quebec, was used to assess dietary intakes over the last month. The test-retest method showed that this questionnaire has good reliability (mean R=0.72, 95% confidence interval 0.68; 0.76) (22). From the data collected by the web-FFQ, we calculated a diet quality index based on Kennedy's healthy eating index (HEI), adjusted to Canadian recommendations. The Kennedy's healthy eating index includes 10 components (grain products, vegetables and fruits, meat and alternatives, milk and alternatives, total fat, total saturated fatty acids, cholesterol, sodium and variety). A maximum of 100 points is possible, which would correspond to a perfect diet. We categorized partners as having a good diet (>80 points), a diet that needs improvement (50-80 points) and a poor diet (<50 points) (23). The web-FFQ also allowed assessing alcohol and caffeine consumption. Adverse behaviors related to reproductive health were defined as consuming more than two caffeinated drinks per day (>200 mg/day of caffeine) for women (1, 24) and consuming any alcohol for men and women (1, 2). Studies evaluating the relation between caffeine consumption and men reproductive health are limited, and therefore, no recommendations are available for men trying to conceive.

The pittsburgh sleep quality index (PSQI) was used to assess sleep quality over a month. PSQI consists of 19 items, each weighted on a 0-3 interval scale, generating seven "component" scores. The final score can vary from a minimum of 0 (no sleeping difficulty) to a maximum of 21 (significant sleeping difficulty). A score $\leq$5 is associated with good sleep quality, whereas a score >5 is associated with poor sleep quality (25).

To objectively assess current physical activity levels of the partners, we asked them to wear an accelerometer over their hip on an elastic belt from wake-up time to bedtime, for seven consecutive days. The participants were

asked to remove the accelerometer when sleeping, showering or performing water activities. Furthermore, they received a daily diary to document wear and non-wear time periods. We used the triaxial ActiGraph GT3X accelerometers (ActiGraph, Pensacola, FL). The ActiGraph GT3X measures data in a 60-s epoch and has been widely used in research for assessing physical activities in adults. The accelerometer provides measures such as activity intensity and duration, step counts, and energy expenditure and has been shown to reasonably correlate with doubly labeled water-derived, the gold standard to assess energy expenditure (26). Valid data were defined as four days of monitoring for 10 hours of wear time per day (27). Participants were asked to maintain their usual activities. Data were processed using the Actilife software version 6.13.2 (ActiGraph, LLC, FL, USA). The accelerometer data obtained were averaged across valid wear days. To derive the activity frequency, intensity and duration from the measured activity in counts per minute per day, the Freedson equation was used: sedentary (<100 counts), light (100-1951 counts), moderate (1952-5724), vigorous (5725-9498), and very vigorous (>9498) (28). Non-wear time was defined as previously suggested (27).

A global "reproductive health score" was calculated by attributing 1 point per adverse factor related to women and men reproductive health (for women: age≥35 years old, BMI ≥30 kg/m^2, waist circumference ≥88 cm, consuming alcohol (≥1 unit/week), HEI<50, <150 minutes of moderate to vigorous physical activity (MVPA) in bouts of ≥10 minutes, poor sleep quality (score>5); for men: age≥45 years old, BMI≥30 kg/m^2, waist circumference ≥102 cm, consuming alcohol (≥1 unit/week), HEI<50, <150 minutes of MVPA in bouts of ≥10 minutes, poor sleep quality (score>5).

Data on sociodemographic status, reproductive history, smoking and drug use, personal and family medical history, as well as causes of infertility, infertility treatments received and biochemical and clinical pregnancy were gathered from patients' medical records.

Statistical analysis

Means and standard deviations, as well as percentages, were computed for men and women for socio-demographic and anthropometric characteristics. The normality assumption was tested using the Shapiro-Wilk test. Because several variables were not normally distributed and our sample size was small, we used the Wilcoxon-Mann-Whitney non-parametric test to compare lifestyle-related factors between men and women. For categorical variables, we used the Fisher's exact test. Statistical analyses were performed by using SPSS statistical software (version 23.0) and results were considered to be significant at P≤0.05).

Results

Feasibility

Between May 2015 and February 2016, 130 eligible couples were approached and asked whether they were interested in participating in our pilot study. Thirty-two couples agreed to participate (25% recruitment rate). Reasons for not agreeing to participate were: not interested in the study, lack of time or overwhelmed by medical exams and treatments for infertility. Among the 32 couples, one left the study before having completed the questionnaires and worn the accelerometer. Three couples were excluded from the analyses because of non-compliance with fulfilling questionnaires by the man, leaving 28 couples for the analyses (Fig.1).

These 28 couples had missing data in the data set, especially for objective physical activity measures. Seven participants (12.5%) did not wear the accelerometer for at least four days of monitoring for 10 hours of wear time per day. Incomplete PSQI (n=5, 9%) was also another source of missing data. Seven couples (25%) did not start, or stopped, infertility treatments at six-month follow-up. Thirteen couples (13 out of 21, 62%) achieved a clinical pregnancy whereas 8 couples (8 out of 21, 38%) did not.

Characteristics and lifestyle-related factors of couples seeking fertility treatments

A description of the socio-demographic characteristics of the 28 couples included in the analyses, is provided in Table 1. According to this table, in general, the partners were in their thirties, were well educated and did not have a child. The cause of infertility of the couple was of female origin in 46.4% of the cases, of male origin in 17.9% of the cases, of male and female origin in 14.3% of the cases, and of unknown reasons for 21.4% of the cases.

Table 1: Socio-demographic characteristics of couples who underwent infertility treatments

Variable	Women n=28	Men n=28
Age (Y)	32.0 ± 4.4 (25.0-42.0)	35.6 ± 8.4 (25.0-58.0)
Women≥35 years old	10 (36)	-
Men≥45 years old	-	5 (18)
Maternity/Paternity		
No	22 (78)	19 (68)
Yes, with actual partner	3 (11)	2 (7)
Yes, with ex-partner	3 (11)	6 (21)
Yes, with actual and ex-partner	0 (0)	1 (4)
Educational level		
No-university degree	13 (46)	11 (39)
University degree	15 (54)	17 (61)
Cause of infertility		
Female	13 (46.4)	
Male	5 (17.9)	
Female and male	4 (14.3)	
Unknown	6 (21.4)	

Data are presented as means ± SD (minimum-maximum) or n (%).

Anthropometric profile and lifestyle habits related to reproductive health of the 28 couples (56 individuals) are presented in Table 2. Overall, 16% of them were obese and 23% had abdominal obesity. Only three individuals were smokers (one woman and two men); the two men who smoked tobacco also reported smoking marijuana on a weekly basis. Most partners (84%) were still drinking alcohol (≥ 1 drinks per week). No statistical difference in these lifestyle-related factors were found between men and women. Twenty-one percent of women were consuming more than the recommended 2 cups of caffeinated drinks per day. Eating habits were worse in men than in women, with 95% of them having a poor diet quality or a diet quality needing improvement (versus 68% of women, P=0.05). On the other hand, physical activity habits were better in men, with 33% of them achieving the Canadian recommendations for physical activity (versus 0% of women, P=0.001). A poor sleep quality was present in 35% of the partners with no difference between men and women.

When considering the seven lifestyle-related factors associated with reproductive health (age, BMI, WC, alcohol, diet, physical activity and sleep) in men and women for which all the data were available (n=44), we found that 9% of men and 41% of women presented at least four adverse factors (P=0.08), with a mean of 3.1 and 2.4 adverse factors observed in women and men, respectively (P=0.04, Table 3).

Table 2: Lifestyle-related factors associated with unfavorable reproductive health of couples about to undergo fertility treatments

Variable	All	Women	Men	P value
Anthropometric profile	n=56	n=28	n=28	
BMI (kg/m^2)	25.7 ± 4.9	29.9 ± 5.5	26.6 ± 4.3	0.08
UW	2 (3.6)	2 (7.1)	0 (0)	
NW	25 (44.6)	15 (53.6)	10 (35.7)	
OW	20 (35.7)	6 (21.4)	14 (50)	0.13
OB	9 (16.1)	5 (17.9)	4 (14.3)	
Abdominal obesity[a]	13 (23.2)	9 (32.2)	4 (14.3)	0.10
Smoking	n=56	n=28	n=28	
Yes	3 (5.4)	1 (3.6)	2 (7.1)	0.49
Drug use	n=56	n=28	n=28	
Yes	2 (3.6)	0 (0%)	2 (7.1)	0.15
Drinking/Eating habits	n=56	n=28	n=28	
Alcohol (unit/week)	6.1 ± 6.7	4.3 ± 3.7	7.9 ± 8.5	0.05
≥1 unit/week	47 (84)	23 (82.2)	24 (85.7)	0.57
Caffeine (mg/day)	153.8 ± 144.7	112.8 ± 88.0	194.8 ± 177.2	0.11
>200 mg/day[*]	-	6 (21.4)	-	
Diet quality index	69.2 ± 11.8	72.0 ± 12.4	66.4 ± 10.8	0.10
Good diet	11 (19.6)	9 (32.1)	2 (7.1)	0.05
Diet needing improvement	41 (73.2)	18 (64.3)	23 (82.2)	
Poor diet	4 (7.2)	1 (3.6)	3 (10.7)	
Physical activity habits	n=49	n=25	n=24	
Time spent at MVPA (minutes/day)	34.2 ± 38.8	24.3 ± 11.8	44.5 ± 52.8	0.05
Not achieving≥150 minutes of MVPA per week	16 (32.7)	10 (40)	6 (25)	0.36
Time spent at MVPA in bouts≥10 minutes (minutes/day)	13.5 ± 23.5	7.9 ± 6.3	19.3 ± 32.2	0.46
Not achieving ≥150 minutes of MVPA in bouts of ≥10 minutes	41 (83.7)	25 (100)	16 (66.7)	0.001
Time spent in sedentary activity (hours/day)	9.1 ± 1.7	9.2 ± 1.3	9.0 ±1.9	0.50
Sleeping habits	n=51	n=25	n=26	
Sleeping score	5.2 ± 2.7	5.16 ± 3.2	5.23 ± 2.3	0.49
Overall poor sleep quality	18 (35.3)	7 (28)	11 (42.3)	0.38

Data are presented as mean ± SD or n (%). P values indicate differences between women and men. BMI; Body mass index, MVPA; Moderate-to-vigorous intensity physical activity, OB; Obese, OW; Overweight, UW; Underweight, NW; Normal weight, [a]; Abdominal obesity was defined as: waist circumference≥88 cm in women, ≥102 cm in men, and [*]; No recommendations regarding caffeine intake are available for men trying to conceive.

Table 3: Overall number of adverse factors related to women and men reproductive health

	Overall n=44	Women n=22	Men n=22	P value
Number of factors[a]				
0	0	0	0	0.08
1	5 (11.4)	1 (4.5)	3 (13.6)	
2	15 (34.1)	7 (31.8)	9 (41.0)	
3	13 (29.5)	5 (22.7)	8 (36.4)	
≥4	11 (25.0)	9 (41.0)	2 (9.0)	
Global score[b]	2.8	3.1	2.4	0.04

BMI; Body mass index, MVPA; Moderate to vigorous intensity physical activity, HEI; Health eating index, [a]; Among the following factors: for women: age≥35 years old, BMI ≥30 kg/m², waist circumference≥88 cm, consuming alcohol (≥1 unit/week), HEI<50, <150 minutes/week of MVPA in bouts of ≥10 minutes, poor sleep quality (score>5). For men: age≥45 years old, BMI≥30 kg/m², waist circumference≥102 cm, consuming alcohol (≥1 unit/week), HEI<50, <150 minutes/week of MVPA in bouts of ≥10 minutes, poor sleep quality (score>5), and [b]; The global score was calculated by attributing 1 point per adverse factor related to women and men reproductive health.

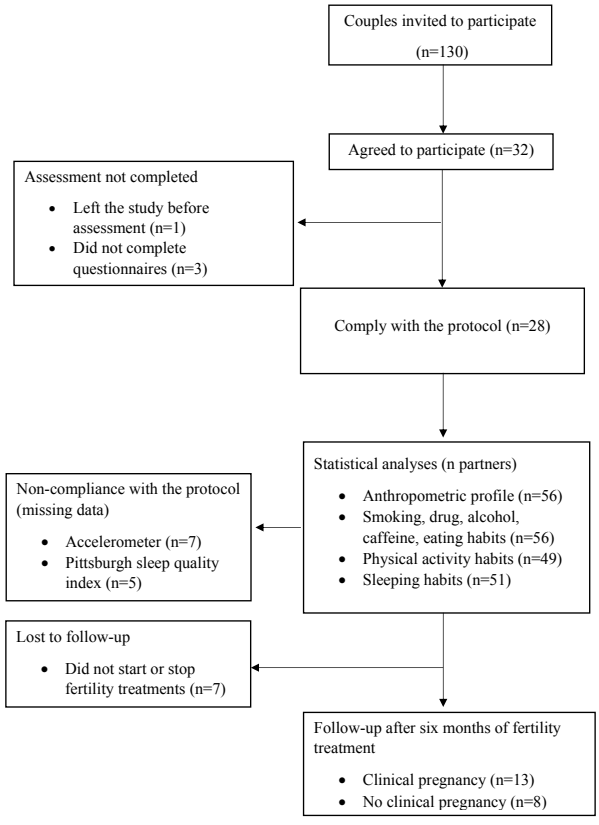

Fig.1: Flow diagram of recruitment, compliance with the protocol and retention of the study population.

Discussion

This pilot study demonstrated the feasibility of conducting a large prospective cohort study at the fertility clinic of the CHAUR of Trois-Rivières but also highlighted the need for improvement of several aspects of the protocol. First, recruitment rate was 25%. It is not possible to compare our recruitment rate with other similar studies because studies evaluating lifestyle-related factors in both partners, using detailed questionnaires and accelerometers, are inexistent. Nevertheless, recruitment was challenging and different explanations may be given. Men and women were recruited at the same time. Several men declined to participate in our study, which prevented us to recruit the couple. Working in close relationship with the medical team and delivering persuasive message to raise men interest in our study, will be essential to improve recruitment rate. In addition, the couple received a large amount of complex information about the medications, tests and procedures involved in infertility treatments on the day we invited them to participate in our study. They may have been overwhelmed and less inclined to participate in our study. Therefore, a better moment to approach the couples should be considered. Finally, the accelerometer to wear during seven days may have discouraged some couples to participate in our study.

Second, missing data were apparent in the data set in terms of sleeping and physical activity data. It will be essential to emphasize the importance of following the instructions provided in the questionnaires on how to respond to questions as well as wearing the accelerometer for at least 10 hours per day for four days in order to avoid missing data. Finally, at six-month follow-up, seven couples (25%) did not start, or stopped, infertility treatments for medical or personal reasons. This attrition rate will have to be taken into account when designing our larger prospective cohort study.

Our preliminary results also showed that many couples seeking infertility treatments present unfavourable lifestyle-related factors that may explain, at least partially, their difficulty in conceiving and affect future infertility treatment outcomes. Importantly, 41% of women and 9% of men presented at least four adverse factors that may have a negative impact on reproductive health. More specifically, 18% of women and 14% of men were obese, proportions similar to those reported by previous studies conducted in infertile populations (9, 11, 18, 29). In Canada, the prevalence of obesity in adults of reproductive age is slightly lower (22.4% in men and 16.6% in women).

Obesity is a well-known risk factor for female infertility, as it is related to ovulation and hormonal disorders (3, 4). In women who achieved a pregnancy, failure to achieve a live birth increased with higher BMI. In men, obesity may also affect fertility by altering sperm parameters (30). The localization of fat mass may also be important with regards to reproductive health. Abdominal obesity is associated with metabolic disorders, including insulin resistance, which may exert effects upon the hypothalamic-pituitary-ovarian (HPO) axis. Disturbances of the HPO axis may lead to alterations in sex hormone secretion and/or metabolism, which may in turn cause hyperandrogenism and polycystic ovaries syndrome (PCOS) in obese women and hypotestosteronemia in obese men.

Despite the recommendation that people trying to conceive should not drink alcohol (1), we found that 82% of women and 86% of men were still drinking alcohol

at the time they were seeking infertility treatments. Data from the 2012 Canadian Alcohol and Drug Use Monitoring Survey showed that 65.8% of adults (>25 years old) had drunk alcohol in the past month, which is lower than what we observed in our sample. Other studies also reported excessive alcohol intake by a significant number of women (any alcohol, 49%-73%) and men (>2 drinks/day, 17%) undergoing infertility treatments (17-19, 29). It has also been recommended to women who are trying to conceive to reduce their caffeine intake (≤200 mg per day) (1). In our study, 21% of the women consumed caffeine more than the recommended amounts, which is less than the previously reported rate (35-50%) (17, 29). In Canada, women of reproductive age (19-50 years old) consume 265 mg of caffeine per day (31), on average. This suggests that women of our study who are trying to conceive, slightly decrease their caffeine intake. Recommendations regarding caffeine intake in men trying to conceive are not available because the potential adverse effects of caffeine on male reproductive function have not been investigated extensively.

Finally, other lifestyle habits, such as nutrition, physical activity and sleeping habits may have a negative impact on fertility and ART outcome, but the currently available evidence is inconclusive. Ruder et al. (15) have reported that antioxidant intake was associated with shorter time to pregnancy, but this association varied according to BMI and age. A case-control study (n=61) found that fruit and vegetable intake could maintain or improve semen quality (14). A cohort study, conducted in men undergoing intracytoplasmic sperm injection cycles, has reported that semen parameters were negatively affected by the consumption of alcohol and red meat, but positively influenced by fruits and cereals consumption. The consumption of red meat also had a negative impact on fertilization and implantation rate (12). To the best of our knowledge, our study is the first to assess overall diet quality in infertile couples. We calculated a diet quality index based on Kennedy's healthy eating index (23) and found that although the number of women who had a good diet quality was higher than men, the majority of the partners needed to improve their eating habits.

Physical activity has been associated with improved ART outcome. Evenson et al. (9) have reported that women who have been active in the year preceding infertility treatments, were more likely to have favorable pregnancy outcome, whereas two studies have shown that women who remained active during infertility treatments had higher implantation and live birth rates (10, 32). Importantly, these three studies did not assess physical activity objectively. Only one study measured physical activity in infertile men using accelerometers and found an inverted U-shape association between the number of bouts of MVPA and semen quality (11). These findings suggest that too little or too much physical activity may be detrimental for male reproductive health. Current physical activity recommendations for adults are to accumulate 150 minutes per week of MVPA in bouts of ≥10 minutes (33). Although no specific recommendations are available for people trying to conceive, it is reasonable to think that these recommendations are also valid for them. Only 16% of our subjects were meeting these recommendations, which is similar to the data from the Canadian Community Health Measure Survey showing that 16% of adults of childbearing age reached these recommendations using accelerometer-derived data (27).

Finally, sleeping habits is increasingly recognized as an important factor of human health and well-being (34). However, the relationship between sleep quality and reproductive health is largely unknown. Possibly, sleep disturbances are related to high levels of stress and anxiety symptoms, which may be associated with fertility problems. Sleep may therefore indirectly affect reproductive health (16). In our study, we found that 28% of women and 42% of men had poor sleep quality. A previous study examining sleep quality in infertile women using the PSQI, reported that 35% of them had disturbed sleep (35). While there is evidence suggesting that sleep disturbances may affect testosterone production and semen parameters (16), no studies have examined sleep quality in infertile men.

While our data are interesting and appear to be feasible to collect, they should be considered preliminary and descriptive. The small sample size should be acknowledged, yet the primary objective of this pilot study was to evaluate the feasibility of a prospective cohort study. Consequently, we did not have the power to detect differences in lifestyle-related factors associated with reproductive health between men and women. Similarly, we did not have the power to compare baseline lifestyle-related factors between couples who achieved a clinical pregnancy and those who did not. Another limitation of our study is that the population was homogenous with respect to race/ethnicity and educational level, with the majority of recruited couples being highly educated.

We do not know whether lifestyle-related factors of the couples who agreed to participate were any different from those who did not agree to participate. The detailed questionnaires about eating and sleeping habits, as well as the accelerometer to wear during seven days, may have attracted more motivated and healthier couples. But, still, we observed a high proportion of unhealthy anthropometric profile and lifestyle habits despite having well-educated participants. These different factors suggest that a recruitment bias is likely to be present in our larger prospective cohort study, limiting the generalizability of our results to a wider population of infertile couples. Finally, although accelerometers provide a valid and objective measure of physical activity levels, non-waterproof accelerometers underestimate several type of physical activity, such as water activities. It is therefore possible that we underestimated the level of physical activity for some participants who removed the accelerometer to do water ac-

tivities but the underestimation would be minimal. Only 6 participants (11%) of our subjects reported doing water activities; however, data were considered invalid for three of them because the accelerometer was worn for less than 10 hours. The three other participants reported only one hour of water activities during the wearing period.

The literature shows that a number of lifestyle-related factors have unfavourable effects on reproductive success of infertile men and women; however, further prospective cohort studies assessing both partners' lifestyle-related factors, especially nutrition, physical activity and sleeping habits, will be needed to fully understand the independent contribution of male and female factors to ART success. Such large prospective cohort studies are essential to develop targeted recommendations to help infertile couples to conceive a child and this pilot study will help us to design such a prospective cohort study.

Conclusion

Though this pilot study had limitations, it provides us with key information that will help us to design a large prospective cohort study. Especially, improvement of recruitment strategies and directives to increase the compliance with the protocol will be essential to ensure its success. It also shows that a considerable proportion of men and women seeking infertility treatments present with several unfavourable lifestyle-related factors that may interfere not only with their fertility but also with future infertility treatment outcome. Conducting a large prospective cohort study will allow us to identify the independent contribution of male and female lifestyle-related factors to ART success. Such a study is essential to help designing interventions aimed at helping infertile couple to conceive a child.

Acknowledgements

The authors would like to acknowledge and thank A. Montplaisir, L. Arbiza, J. Pelletier and S. Drouin who assisted us to perform the study, J. Côté-Leclerc, M. Boisvert and C. Blanchet who assisted in data collection and all the couples who participated. This pilot study was funded by the Fonds institutionnel de recherche clinique de l'Université du Québec à Trois-Rivières (UQTR) and the Programme de soutien au démarrage de projets de recherche en collaboration CIUSSS-MCQ-UQTR. The authors have no potential conflicts of interest to report.

Author's Contributions

S.-M.R., V.B., É.L.; Participated to conception and design of the study. S.-M.R.; Was responsible for overall supervision of the project, revised statistical analysis and data interpretation and contributed extensively in drafting the paper. M.-L.P.; Coordinated the project, collected the data, conducted statistical analysis, interpreted the data and drafted the manuscript. J.R., É.L.; Contributed in interpretation of the nutrition and physical activity data and revised the paper. All authors performed editing and approved the final version of this paper for submission.

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Original Article

Modeling *In Vitro* Fertilization Data Considering Multiple Outcomes Observed among Iranian Infertile Women

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Abstract

Background: Women undergoing *in vitro* fertilization (IVF) cycles should successfully go through multiple points during the procedure (i.e., implantation, clinical pregnancy, no spontaneous abortion and delivery) to achieve live births. In this study, data from multiple cycles and multiple points during the IVF cycle are collected for each individual to model the effects of factors associated with success at different stages of IVF cycles in Iranian infertile women.

Materials and Methods: This historical cohort study includes 996 assisted reproductive technology (ART) cycles of 511 infertile women. Covariates considered in this study were women's age, type of cycle (fresh or frozen embryo transfer), number of embryos transferred and having polycystic ovarian syndrome during IVF cycles. Generalized estimating equations were used for calculation of odds ratio (OR) and 95% confidence intervals (95% CI) of success at different stages during IVF cycles. Cluster-weighted generalized estimating equations (CWGEE) was also fitted to handle informative cluster size.

Results: After adjusting for potential confounders, it was seen that receiving frozen embryo transfer was associated with higher odds of success compared to receiving fresh embryo transfer (adj OR: 2.26, 95% CI: 1.66-3.07); however, cycles with fresh embryo transfer exhibited better results in clinical pregnancy compared to those receiving frozen embryo. Being in the age category of 38 to 40 was associated with lower odds of success compared to the reference category (<35) in CWGEE model (adj OR: 0.67, 95% CI: 0.45-1.00). The number of embryos transferred was positively associated with the odds of success in CWGEE (adj OR: 1.21, 95% CI: 1.03-1.42) as well as the GEE model.

Conclusion: Receiving frozen embryo was positively associated with odds of success compared to cycles with fresh embryo. The number of embryos transferred and women's age were significantly associated with odds of success.

Keywords: Cluster Analysis, Infertility, *In Vitro* Fertilization

Citation: Ghaheri A, Rasekhi A, Omani Samani R, Hajizadeh E. Modeling in vitro fertilization data considering multiple outcomes observed among Iranian infertile women. 2018; 12(1): 27-30. doi: 10.22074/ijfs.2018.5187.

Introduction

In Iran, the average rate of infertility, primary and secondary infertility and current infertility is estimated to be 10.9% [95% confidence intervals (CI): 7.4-14.4], 10.6% (95% CI: 5.3-16.0), 2.7% (95% CI: 1.9-3.5) and 3.3% (95% CI: 2.7-3.8), respectively (1). Currently, assisted reproductive technology (ART) is increasingly used as a widely accepted treatment for infertile couples (2). The increase in popularity of ART, the factor influencing its outcome and the importance of success rate have motivated researchers towards modeling ART success rates and identifying factors that affect it in different ways (3-5).

An *in vitro* fertilization (IVF) process involves retrieving eggs (oocytes) and sperm from female and male, respectively and allowing sperm to fertilize the eggs; the resulting embryo(s) are then transferred to the uterus and hormones are administrated to aid embryo implantation (6). Women undergoing IVF should go successfully through multiple points during the procedure (i.e., chemical pregnancy, clinical pregnancy, having no spontaneous abortion (SAB) and a successful delivery) to achieve live births; therefore, in IVF data, success probabilities at each stage are conditional on success at the previous stage. Furthermore, pregnancy outcomes are believed to be correlated within different cycles of a woman and women's reproductive outcomes in previous ART cycles are believed to influence the outcomes of their current cycle; so, there is a need to consider previous cycles data rather than simply considering those of the current cycle.

Most studies on ART data have only inspected a part of infertile women's data (7-10). Multiple types of IVF failure and multiple IVF cycles experienced by each woman, have not simultaneously been considered in previous studies. Maity et al. (11) presented an approach based on ideas of discrete survival analysis of IVF data with multiple cycles and multiple failure types for each individual. Generalized estimating equations (GEE), which consider the correlation within clusters, can be used to fit the model

Received: 27 Jan 2017, Accepted: 13 May 2017
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Vol 12, No 1, Apr-Jun 2018, Pages: 27-30

presented in their study. In case of ART data, the cluster would be the woman and the cycles each woman undergoing the procedure would be the observation (subunit) within the cluster.

In the GEE analysis it is assumed that, the response is independent from the number of observations in the cluster (the cluster size) (12). However, in IVF data, the number of cycles that an infertile woman undergoes is believed to be associated with the success/failure of IVF outcome (known as informative cluster size). The model presented by Maity et al. (11) does not consider informative cluster size. In the present study, a cluster-weighted GEE (CWGEE) was used to model the factors associated with binary outcome of success/failure at different stages during IVF cycles while handling informative cluster size. The results were then compared with those of GEE model.

Materials and Methods

This historical cohort study includes 996 cycles of 511 infertile women who were enrolled in ART treatments between April 2011 and March 2012 in Royan institute, Iran. Only women who experienced embryo transfer were eligible to be included in the present analysis. All variables in this study were defined based data extracted from the medical record of the individuals, by trained nurses. The outcome variable was success or failure at four stages: i. Chemical pregnancy [a transient increase in serum beta-human chorionic gonadotropin (β-hCG)], ii. Clinical pregnancy (presence of an intrauterine gestational sac), iii. Spontaneous abortion (pregnancy loss before 20 completed weeks of pregnancy), and iv. Delivery (live birth of at least one baby).

Cycles resulted in failure types other than the four above-mentioned ones, were excluded from the study and couples who required donation or gestational carrier, were not eligible for enrollment. Covariates considered in this study were women's age (under 35, 35 to 37, 38 to 40, above 40), type of cycle (fresh or frozen embryo transfer), the number of embryos transferred and having polycystic ovarian syndrome (PCOS) during IVF cycles. Some other measured covariates were woman-specific, such as age at the first cycle while some others were cycle-specific, such as type of cycle or the number of embryos transferred.

The study was approved by the Ethics board of research of Royan institute (Ethical code: EC/90/1086). Informed consent was obtained from all subjects when they intended to start the treatment. Subjects were assured that the results would be published following statistical evaluations and no personal data would be disclosed.

Statistical analysis

The outcome at each stage (chemical pregnancy, clinical pregnancy, spontaneous abortion (SAB) and delivery) was considered as the binary response variable representing the success or failure of the stage. The probability of success occurrence at a specific stage of ART cycle, could be associated with the stage, cycle number, and covariates of interest. The main challenge is considering the correlations among repeated cycles of each woman, as well as correlations among the outcomes of multiple stages within each cycle. To consider these correlations, GEEs were used according to the model presented by Maity et al. (11), to assess the influence of covariates (women's age, type of cycle, number of embryos transferred and having PCOS) on the binary outcomes and calculation of odds ratio (OR) and 95% CI. In usual GEE analysis, it is assumed that the outcome is independent of the number of observations in each cluster. However, concerning IVF data, the cluster size is believed to be informative or non-ignorable. In this study, a CWGEE was also fitted to handle informative cluster size. Stata software, version 13 (Stata Corp, College Station, TX, USA) was used for statistical analyses.

Results

This study includes 511 women with a total of 996 IVF cycles, each woman having 1-3 cycles leading to embryo transfer. The mean (SD) age of women was 35.75 (5.12) years old and 86 (16.8%) of women had PCOS. Among the cycles included in this study, 585 (59%) were cycles with fresh embryo transfer and the median (inter quartile range) of the number of embryos ready for transfer was 3 (2-3).

Since the number of cycles that each woman experienced is reversely associated with the success/failure at different stages, conditional on other predictors (OR: 0.68, 95% CI: 0.52-0.89, P=0.005), cluster size is believed to be informative and CWGEE has been suggested for handling this situation (13).

GEE and CWGEE models used in this study incorporated the data from repeated IVF cycles and multiple stages, with a separate intercepts for stage (Table 1). According to this table, age was associated with odds of success in CWGEE model as women of 38-40 years old were less likely to have successful IVF outcome than women under 35 years old. However, this association was not statistically significant in the usual GEE model. Based on this table, higher number of transferred embryos is associated with an increase in the odds of success in a way that one unit increase in the number of transferred embryos is associated with 1.18 and 1.21-fold increase in the odds of success in unweighted and weighted GEE models, respectively. Having PCOS was associated with lower odds of success in IVF procedures but this association was not statistically significant in either models. Receiving frozen embryo transfers was associated with more than 2-fold increase in the odds of success in both models.

To explore the differing effect of fresh and frozen embryo transfer on the odds of success at various stages, the interaction term between type of embryo(s) transferred and failure type was included in the model. Although women receiving fresh embryo transfer showed significantly better results in clinical pregnancy, from then on, women receiving frozen embryo transfer could successfully continue in the same way as those receiving fresh embryos (Fig.1).

Table 1: Relationship between IVF outcomes and IVF/participants characteristics

IVF and participants characteristics	Unweighted GEE		Cluster weighted GEE	
	OR (95% CI)	P value	OR (95% CI)	P value
Intercepts				
Chemical pregnancy	1 (reference)	-	1 (reference)	-
Clinical pregnancy	2.11 (2.08, 2.15)	<0.001	2.12 (2.09, 2.18)	<0.001
SAB	2.20 (2.13, 2.29)	<0.001	2.22 (2.14, 2.34)	<0.001
Delivery	6.43 (3.42, 15.76)	0.010	8.69 (3.59, 30.03)	0.009
Embryos transferred number	1.18 (1.01, 1.38)	0.031	1.21 (1.03, 1.42)	0.021
PCOS				
Yes	0.74 (0.52, 1.06)	0.102	0.75 (0.52, 1.10)	0.138
No	1 (reference)	-	1 (reference)	-
Type of embryo(s) transferred				
Fresh	1 (reference)	-	1 (reference)	-
Frozen	2.50 (1.87, 3.35)	<0.001	2.26 (1.66, 3.07)	<0.001
Age categories (Y)				
<35	1 (reference)	-	1 (reference)	-
35-37	0.86 (0.38, 1.28)	0.460	0.87 (0.57, 1.31)	0.504
38-40	0.68 (0.46, 1.00)	0.052	0.67 (0.45, 1.00)	0.050
>40	0.74 (0.51, 1.07)	0.109	0.76 (0.52, 1.11)	0.161

IVF; *In vitro* fertilization, GEE; Generalized estimating equations, SAB; Spontaneous abortion, PCOS; Polycystic ovarian syndrome, CI; Confidence intervals, and OR; odds ratio.

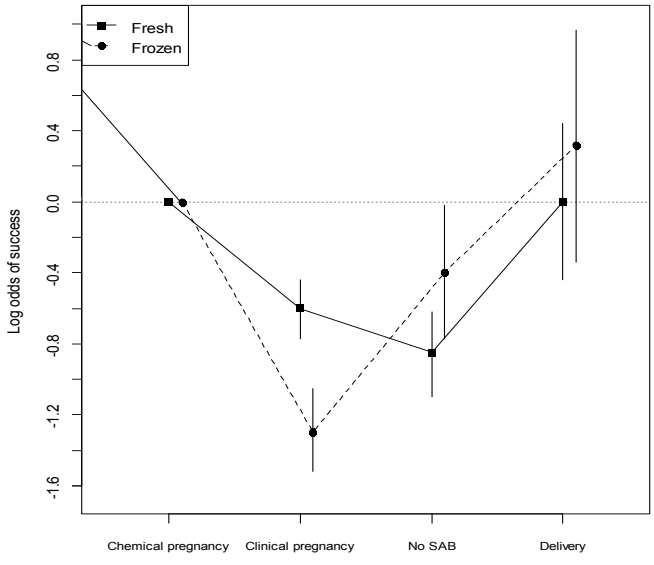

Fig.1: Log odds of success at multiple points during the IVF cycle with 95% confidence intervals.
IVF; *In vitro* fertilization and SAB; Spontaneous abortion.

Discussion

There are some existing approaches to model IVF data including multiple cycles with multiple failure types (9). Considering the whole existing IVF data set for each woman can lead to better estimations of the covariates effects than the standard approach which only consider the first IVF cycle or model each IVF outcome separately.

Since the number of cycles experienced by each infertile woman is believed to be associated with the success/failure of IVF outcome, studies on these type of data involve informative cluster size and GEE and CWGEE, might show different results as GEE assumes that cluster size is non-informative. This historical cohort study on Iranian infertile women also demonstrated strong reverse associations between the number of cycles and odds of success in IVF outcomes, indicating the presence of informative cluster size (12). Moreover, the result of this study showed that having more transferred embryos is significantly associated with higher odds of success which corroborates the findings of previous research in this field (14, 15).

Based on both GEE and CWGEE, our results also suggest that successful IVF outcomes seem to be associated with performing frozen embryo transfer compared to fresh embryo transfer. This could be explained by the fact that the endometrium is more receptive in frozen embryo transfer during the endometrial priming than in fresh embryo cycles; therefore, frozen embryo cycles could lead to a better embryo-endometrium synchrony (16). Despite the potential advantages of transferring frozen embryos, the effect of patient-specific variables or center-specific factors (e.g. laboratory setup and protocols), should be investigated in well-designed clinical trials (17). Exploring the differing effect of frozen embryo transfer on the odds of success at various stages showed that the likelihood of successful clinical pregnancy is significantly lower in frozen embryo transferred cycles which could be explained by the fact that usually the best-quality embryos are chosen for the fresh embryo transfer and this is in agreement with previous studies (18, 19). Continuing through the

cycles, the difference between frozen and fresh embryo transfer was not statistically significant which is probably due to the well-balanced embryo-endometrium interaction (16).

In our study, having PCOS was not significantly associated with odds of success in IVF procedures in either of the models which was not consistent with some previous research that found that women with PCOS have an increased prevalence of miscarriage, both after spontaneous and induced ovulation (10). However, this result is consistent with that of other studies which showed similar pregnancy and live birth rate per cycle in PCOS and non-PCOS women (20). Our limitation to include women's BMI in this study could influence the results as the impact of BMI on IVF outcomes and its interaction with PCOS was not considered.

A great deal of previous research has indicated significant associations between women age and fertility (21, 22). In this study, although this association was not significant in GEE model, CWGG model confirms that being in the age category of 38-40 years old was reversely associated with odds of success compared to women aging less than 35 years old. The difference between the women aged under 35 years and those of over 40 years was not statistically significant which could be due to the limited number of women aged over 40 years old in our study.

In this study, data from repeated IVF cycles was used by including the correlation among them; however, not including some variables of couples undergoing IVF, such as pretreatment variables, embryo quality, oocyte and sperm quality and also stimulation and laboratory variables is a limitation of this study. Data on previous cycles, which infertile women might have undergone in other infertility centers, was not included in this study due to lack of a national registry.

Conclusion

Frozen embryo transfer was positively associated with odds of success compared to cycles with fresh embryo transfer; but, cycles with fresh embryo transfer had better results in clinical pregnancy compared to frozen embryo transfer. The number of embryos transferred and women's age were significantly associated with odds of success.

Acknowledgements

Financial support of this study was provided by Royan Institute. The authors declare no conflict of interest.

Author's Contributions

A.G., E.H., R.O.S.; Participated in conception and study design. A.G., A.R., E.H.; Contributed to statistical analysis. A.G., R.O.S.; Contributed to interpretation of the results. A.G.; Drafted the manuscript which was revised by R.O.S. and A.R. All the authors approved the final manuscript.

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Original Article

Detection of *Chlamydia trachomatis* in Pap Smear Samples from South Khorasan Province of Iran

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Abstract

Background: *Chlamydia trachomatis* (CT), the most common bacterial sexually transmitted infection (STI), leads to pelvic inflammatory disease, infertility and chronic pelvic pain in women as well as an increased risk of vertical transmission, conjunctivitis and pneumonitis in infants. It may also be a co-factor along with human papillomavirus (HPV) in cervical cancer progression. We aimed to determine the prevalence of CT genotypes in genital specimens of women from South Khorasan, Iran and to test the association between CT and cytology statistics.

Materials and Methods: This was a cross-sectional study on 248 Pap smear samples from women who visited a gynecologist for routine Pap smear testing in South Khorasan province. Nested polymerase chain reaction (PCR) was used to test the residual fluids of Pap smears for CT-DNA after cytological examination. Direct sequencing, alignment and phylogenic analyses were performed on eight samples to identify their genotypes.

Results: The mean age of patients was 37.54 ± 5.21 years. Most samples had a normal cytology (214 cases, 86.29%). Overall, 31 samples were positive for CT infection (12.5%) of which 20 (9.34%) were normal and 11 (32.35%) were abnormal, with the frequency difference being significant (P=0.022). The co-infection of CT/HPV in total was identified in 14 cases (5.6%). The results of sequencing eight samples out of the 31 CT positive samples revealed the detection of genotypes D and E, each with four cases.

Conclusion: We show that a high prevalence of genital CT infection is present in women with both normal and abnormal cytology; however, the higher prevalence among women in the abnormal group may indicate its involvement in cervical neoplasia.

Keywords: Cervical Cancer, *Chlamydia trachomatis*, Iran, Pap Smear, Sexually Transmitted Infection

Citation: Javanmard D, Behravan M, Ghannadkafi M, Salehabadi A, Ziaee M, Namaei MH. Detection of Chlamydia trachomatis in pap smear samples from South Khorasan Province of Iran. Int J Fertil Steril. 2018; 12(1): 31-36. doi: 10.22074/ijfs.2018.5064.

Introduction

Chlamydia trachomatis (CT) is the most common sexually transmitted bacterial infection (1). Chlamydia species are aerobic obligate intracellular bacteria with a gram-negative cell wall. Because of their inability to produce ATP, they are dependent on their host energy (2). The major outer membrane protein (MOMP), a principle component of the CT cell wall, is encoded by the *omp1* gene, which includes four variable domains (VD) interspersed among 5 conserved domains (3). Based on minor variation in three VDs, which are exposed on the surface of the membrane, CT currently has 19 genotypes (A to K, L1 to L3, Ba, Da, Ia and L2a) (4), among which genotypes D to K are urogenital pathogens and responsible for neonatal conjunctivitis, genotypes A, B and C are related to trachoma, and L1 to L3 are responsible for the sexually transmitted infection, lymphogranuloma venereum (5).

Chlamydial infection in women can cause urogenital inflammations including urethritis, cervicitis and salpingitis (6). Also, infants born from mothers with active CT infection may develop conjunctivitis or pneumonia (2). Unfortunately, most CT infections are asymptomatic (70% in women) (7). This is challenging for early detection and treatment, and thus increases transmission. Risk factors that can be attributed to this infection are age (those aged 15-24 are most affected) and gender (women are more prone to infection than men) (8).

The co-infection of CT with other sexually transmitted pathogens may have complicated consequences. Genital CT infection may increase human immunodeficiency virus (HIV) viral shedding, therefore, identifying and treating patients with CT infection may reduce the genital transmission of HIV (9). Several studies have reported the coexistence of CT in cervical intraepithe-

Received: 30 Sep 2016, Accepted: 20 Jun 2017
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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 1, Apr-Jun 2018, Pages: 31-36

lial neoplasia (CIN) induced by human papillomavirus (HPV) infection (10). There are some reports of a higher prevalence of CT in HPV-positive populations (11), yet CT has been introduced as an independent risk factor for developing CIN (12).

There are several diagnostic methods for CT, such as isolation in cell lines, immunofluorescence, serologic assays and molecular testing methods such as polymerase chain reaction (PCR) (13). CT infection is easily treatable with accessible antibiotics, therefore, given the asymptomatic nature of most CT infections, the early detection of this sexually transmitted infection could enhance treatment and reduce the risk of a re-infection and/or transmission to others (9).

Although CT infection has been proven to be the most prevalent sexually transmitted infection (STI) (1), there is still no clear information on its prevalence in South Khorasan province in eastern Iran. In addition, there is a lack of data on the co-infection of CT/HPV and the prevalence of CT among different cytology groups in association with cervical malignancies in Iran. We therefore aimed to address these by undertaking cytological and sequencing analyses.

Materials and Methods

This was a cross-sectional study performed in Birjand, South Khorasan province of Iran from May 2015 to October 2016. The age of women ranged from 17 to 45 years, all of which were referred for routine Pap smear test. Those who had taken antibiotics within 3 weeks prior to their visit were excluded from the study. All patients signed an informed consent. This work was approved by the Ethics Committee of the Vice-chancellor for Research of Birjand University of Medical Sciences (#1393-12-07). Data collection and recording were performed based on questionnaires and forms. Total endocervical epithelial cells were collected from 248 women visiting different gynecologists in Birjand. These samples had been previously checked for HPV-DNA and their results were used in this study (14).

DNA extraction

On the same day of cytological examination, the residual fluids containing endocervical cells were processed for DNA isolation. A Bioneer DNA extraction kit (Bioneer Co, South Korea) was used according to the manufacturer's instructions with minor alterations including the preheating of samples and an additional round of centrifugation. The extracted DNA was checked using a Nanodrop Biophotometer (Eppendorf D30, Germany) at 260/280 nm, and samples with a ratio between 1.8- 2 were selected. An internal control gene (*β-globin*) was selected for amplification to confirm the process of cellular DNA extraction.

Amplification of *β-globin* and *Omp1* gene

The isolated DNA was subjected to PCR using primers beta 1 (5′-TCAACCCTACAGTCACCCAT-3′) and beta 2 (5′-CTAACAATTACGAACAGCAATGAG- 3′), as previously described, to assess its integrity (15). Positive samples were then selected for CT testing with nested-PCR as previously described (16). Briefly, in the first round, 5 µl of extracted DNA was added to a reaction tube containing 25 pmol of each outer primer (NLO: 5′-ATGAAAAAACTCTTGAAATCG-3′ and NRO: 5′-CTCAACTGTAACTGCGTATTT-3′), 0.2 mM of each dNTP, 1X PCR buffer, 2 mM $MgCl_2$ and 2 U Taq DNA polymerase (Cinaclone, Iran) in a volume of 50 µl. The nested step was performed on 3 µl of the first-round PCR product as a template with inner primers NLI (5′-TTTGCCGCTTTGAGTTCTGCT-3′) and NRI (5′-CCGCAAGATTTTCTAGATTTC-3′) (16) under reaction conditions identical to the first PCR except that the concentration of $MgCl_2$ was 1 mM. First- and second-round PCR reactions were performed using an Eppendorf thermocycler (Mastercycler Nexus, Eppendorf, Germany) under the following cycling conditions: 4 minutes preheating at 95ºC followed by 30 cycles of denaturation at 94ºC for 1 minutes, annealing at 57ºC for 1 minutes and extension at 72ºC for 1 minutes. A final extension step at 72ºC for 7 minutes was added to guarantee full-length products. The product of nested PCR was a 1050 base pair segment of the *Omp1* gene that was visualized on a 1% agarose gel and stained with DNA Green Viewer.

Genotyping

The products of nested PCR were sequenced bidirectionally using the same forward and reverse primers at Bioneer Company, South Korea (run on an ABI 3730XL DNA Analyzer). The obtained sequences were aligned using Mega BLAST to determine genotypes. The phylogenetic analysis in the given region of *Omp1* was performed using MEGA6; the Jukes-Cantor model was selected for nucleotide substitution with Gamma distributed rates among sites. Selected codon positions were 1st, 2nd, 3rd and noncoding sites. To assess the reliability of the phylogeny, 1,000 bootstrap replications were performed. The accession numbers of reference sequences of CT genotypes used in this study were KM369934 (E), X62918 (D), KM369939 (G), DQ064292 (J), KM369936 (F), AF202456 (Ia), DQ064282 (B), DQ064295 (L2), AF063204 (K), X16007 (H), FM872306 (A) and CP006945 (C).

Statistical analysis

The type of distribution was checked, and skewness and kurtosis were in the range of (2, -2). The Chi-square test (or Fisher's exact test when applicable) was used to test association and to compare between cytology and CT. The statistical significance was set at $P<0.05$. All statistical analyses were performed by Statistical Package for Social Sciences software version 17 (SPSS Inc, Chicago, IL, USA).

Results

Demographic population-based data

The demographic and clinical data of the 248 women

screened are shown in Table 1. The mean age of patients was 37.54 ± 5.21 years. Most participants had a normal cytology (214 cases; 86.3%), however, 34 cases (13.7%) had an abnormal cytology result. In the abnormal group, there were 20 cases (58.82%) with atypical squamous cell of undetermined significance (ASCUS) and 14 (41.17%) with low-grade squamous intraepithelial lesions (LSIL). There were no cases with high-grade squamous intraepithelial lesions (HSIL) and/or cervical neoplasia. Based on cytological examination, observation of inflammatory cells and clinical data, 38 of all cases (15.32%) were found to have cervicitis.

Prevalence of *Chlamydia trachomatis* among Pap smear samples

The results of PCR for the beta-globin gene demonstrated its amplification in all samples after agarose gel electrophoresis (Fig.1A). Based on PCR results, 31 cases (12.5%) were positive for CT (Fig.1B). The prevalence of CT among different groups is shown in Table 1. Among the samples with evidence of cervicitis, seven (18.42%) were positive for CT. The mean age of patients with CT infection was 36 ± 5.52 years. The distribution of CT in different age ranges is shown in Table 2. The modal age range was 21-30 years (130; 52.42%). The prevalence of CT and cervicitis was however higher in the first age range (18.18 and 27.27% respectively), and declined with age.

Genotyping of *Chlamydia trachomatis*

The forward and reverse sequences obtained from eight samples were assembled to a consensus using CLC software (CLC Genomics Workbench 7, https://www.qiagenbioinformatics.com/), trimmed in Bioedit software, and subsequently submitted to NCBI under accession numbers KY468517 to KY468523. In search for homology via BLAST, half of samples belonged to genotype D and the other half belonged to genotype E (Fig.2).

Demographic and clinical characteristics of these genotypes are in Table 3. The cases positive for genotype D were younger (28.45 ± 3.26 years), although it was not statistically significant. Interestingly, in the cases positive for genotype E, the co-infection of HPV and LISL were found more frequently.

In isolates of genotype E, there were no mutations at the amino acid level; however, there was a missense mutation in a case with genotype D (i.e. thr326ala). There were also two silent mutations, C915T in two cases of genotype E and T956A in two cases, with genotypes D and E. Overall, the nucleotide region 900-1000, a part of the variable domain- IV, was more prone to have a mutation.

Table 1: The prevalence of CT among different cytological groups, as well as co-infection with HPV

Cytology	n (%)	Age (Y) Mean ± SD	HPV DNA n (%)	CT DNA n (%)	HPV/CT co-infection	P value
Normal cytology	214 (86.29)	35.55 ± 4.66	33 (15.42)	20 (9.34)	11 (5.14)	
Total abnormal	34 (13.7)	38.45 ± 4.21	12 (35.29)	11 (32.35)	3 (8.82)	0.022*
ASCUS	20 (58.82)	37.1 ± 3.35	8 (40)	5 (25)	2 (10)	
LSIL	14 (41.17)	35.3 ± 5.6	4 (28.57)	6 (42.85)	1 (7.14)	0.056**
Total	248	37.54 ± 5.21	45 (18.14)	31(12.5)	14 (5.64)	

CT; *Chlamydia trachomatis*, HPV; Human papillomavirus, ASCUS; Atypical squamous cell of undetermined significance, LSIL; Low-grade squamous intraepithelial lesions, *; The prevalence of CT was significantly different between total abnormal and normal cytology groups, and **; The prevalence of CT was higher in the LISL group than ASCUS.

Table 2: The prevalence of *Chlamydia trachomatis* according to age and cervicitis test result

Age ranges (Y)	Total number (%)	CT+/each group n (%)	CT+/total (%)	Cervicitis+/each group n (%)	CT+and cervicitis+/each group n (%)
≤20	11 (4.43)	2 (18.18)	0.8	3 (27.27)	1 (9.09)
21-30	130 (52.42)	16 (12.3)	6.45	20 (15.38)	4 (3.07)
31-40	81 (32.66)	9 (11.11)	3.62	12 (14.81)	2 (2.46)
40>	26 (10.48)	4 (15.32)	1.6	3 (11.5)	0
Total	248	31 (12.5)	31 (12.5)	38 (15.32)	7 (2.82)

Table 3: Demographic and clinical characteristics of individuals with genotypes D and E identified in this study

Genotype	n	Mean age ± SD	Cervicitis n (%)	HPV n (%)	ASCUS n (%)	LISL n (%)
D	4	28.45 ± 3.26	2 (50)	1 (25)	3 (75)	0
E	4	34.51 ± 2.52	0	3 (75)	1 (25)	4 (100)
Total	8	31.48 ± 2.55	2 (25)	4 (50)	4 (50)	4 (50)

HPV; Human papillomavirus, ASCUS; Atypical squamous cell of undetermined significance, and LSIL; Low-grade squamous intraepithelial lesions.

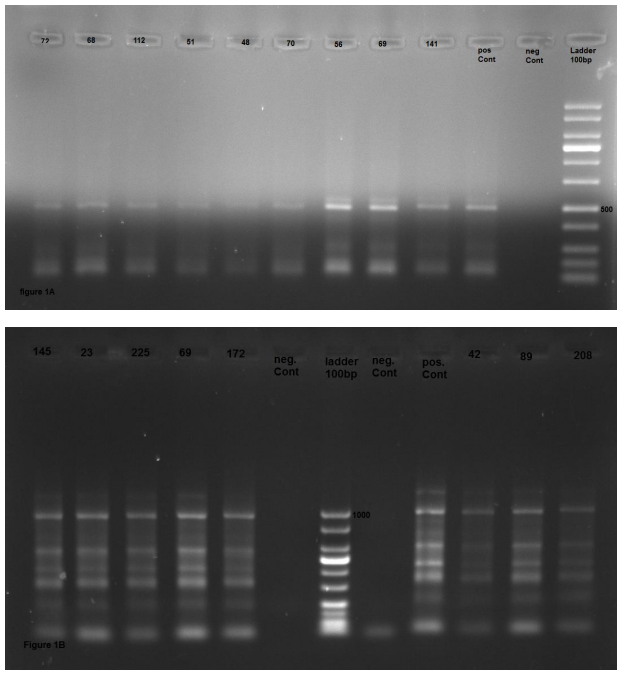

Fig.1: Agarose gel electrophoresis of polymerase chain reaction (PCR) products. **A.** The positive samples for amplification of human β-*globin* gene revealed a 500 bp fragment band, and **B.** Positive samples for *Chlamydia trachomatis* (CT) have a 1052 bp product.

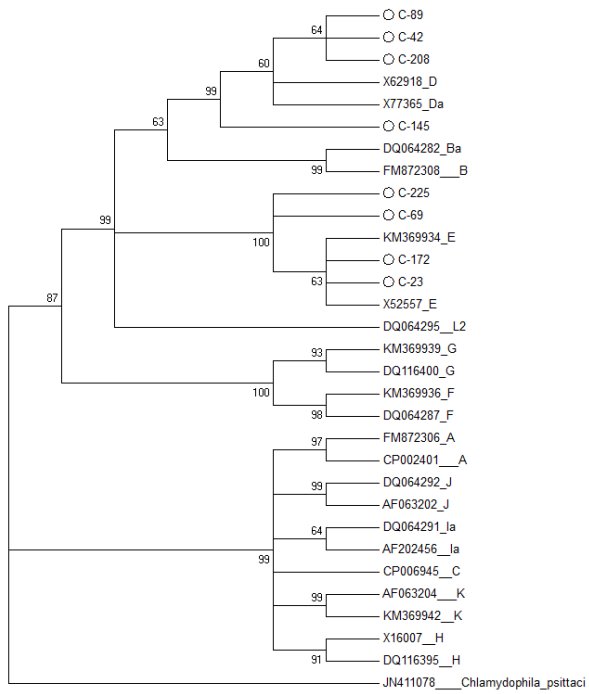

Fig.2: The phylogenic tree constructed based on the maximum likelihood method. Accession numbers and genotypes were given, and those in circle shape are the sequences reported here. *Chlamydia trachomatis* (CT) genotype D (X62918) was used as reference sequence and the *omp1* sequence of C. Pesittaci was used to root the tree.

Discussion

The Pap smear test is approved for screening cervical abnormalities and is performed routinely around the world. Therefore, a large and continuous sampling is in progress and is accessible. This study showed the capacity of the liquid Pap smear to enhance the molecular detection of genital CT infection, as other studies have also indicated (17). This study was the first to assess the frequency of CT infection and genotypes of CT among women from South Khorasan, Iran. The observed frequency of CT in South Khorasan (12.25%) is comparable to other studies in Iran by Chamani-Tabriz et al. (18), Zahirnia et al. (19) and Eslami et al. (20) which reported the prevalence of CT as 12.6, 13.2, and 13.25% respectively. Other studies in Iran have shown the molecular detection rate of CT from 2.6 to 21.25% (21), and according to a meta-analysis, the pooled prevalence for genital CT in Iran was 12.3% (22).

The large variance observed in the reported data may be due to sampling size, sample source, experimental test, socio-economic state of the population and other factors. The CT prevalence has been reported at variable rates in other parts of the world such as 6.2% in Australia (23), and 1.1-10.6% in other countries (24). In this work, the prevalence of CT was higher among ages lower than 20 years (18.18%) and showed a decreasing pattern with age increase, albeit it was relatively high at ages of 40 years and more (15.32%). Other studies from Iran have also indicated a declining prevalence of CT proportional to senescence (25, 26), however, some studies have shown the highest frequency is in the 30-40 age groups (18, 27). Also, in other countries, there is a higher prevalence of CT in late teens and early youth (24). The early incidence of CT infection and its different age distribution may be due to physiologic changes of the vagina in addition to social behavior and lower marriage age, a frequent phenomenon in this province.

We found that the incidence of CT infection was higher among patients with abnormal cytology (32.35%). Interestingly, this figure was 42.85% for the LISL group, 25% for the ASCUS group 9.34% for the normal group, indicating an ascending pattern toward malignancy. In a case-control serological survey in Iran, a strong association between CIN and CT was identified. In specific, in the CIN and healthy group, there were 45 and 12.9% seropositive individuals respectively (28). Nonetheless, others have reported no significant association between CT and CIN (29, 30). An investigation in Argentina revealed a rising prevalence of CT from low levels in normal cytology (11%) to 47% in those with HSIL (11), which is consistent with our results.

This result confirmed the shared risk of CT and HPV infection in the development of cervical cancer. The co-infection rate of HPV and CT was 14/248 (5.64%) in the total sample set and 14/31 (45.16%) among CT-infected patients reported here. A study in Italy showed that 58% of CT-infected women were also positive for HPV (31), which is somewhat consistent with our results. Panatto et al. (32) reported this as 2.7% of total women, and Bianchi et al. (33) showed that 1.5% of girls younger than 20 were co-infected with CT/HPV. This result is consistent with a meta-analysis that demonstrated the association between CT and the risk of cervical cancer (34).

HPV and CT share similar transmission routes, and

since CT may enhance the rate of other STI infections, it may have a role in the progression of cervical cancer. It may, however, be an independent co-risk factor of CIN with an unknown mechanism.

In the current study, the CT genotypes D and E were equally identified. These genotypes were also prominent in other genotyping surveys from Iran. Genotyping of CT from endocervical specimens in Shiraz identified genotype F (46.6%), E (33.3%) and D (13.3%) along with a singleton G (35). In a comprehensive genotyping study for genital CT in Ahvaz, genotype E was the most prevalent (31.5%), followed by F (23.1%), D (13%), K (9.2%), I (8.3%), G (7.5%), H (5.5%) and J (1.9%) (36). The lack of other genotypes in South Khorasan is interesting and shows a possible bottleneck effect. The insufficient number of samples genotyped may nevertheless have resulted in the absence of rarer genotypes.

Conclusion

The results of this study revealed a relatively high prevalence of genital CT in East Iran and underscore the benefit of liquid Pap smear samples for molecular assays. The association between the rate of CT and CIN grade merits further investigation. Determining the prevalence and genotypes can provide important epidemiologic knowledge for transmission patterns, prevention, and treatment programs for controlling STI infections. Further investigations in this region are also needed to obtain a more reliable prevalence of CT and to determine its relevance to any other genital infections or cervical carcinoma.

Acknowledgements

This project was fully financially supported by Birjand University of Medical Sciences. The authors greatly appreciate Dr. Haghighi for her kind donation of patient data. There is no conflict of interest in this study.

Author's Contributions

D.J.; Performed the experiment and drafted the manuscript. M.H.N.; Proposed the idea and supervised the project. M.B., M.Gh., A.S., M.Z.; All had equal roles in conducting the study, sampling and analyzing of results. All authors read and approved the final manuscript.

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Original Article

Overexpression of Endometrial Estrogen Receptor-Alpha in The Window of Implantation in Women with Unexplained Infertility

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Abstract

Background: Failure in the endometrial receptivity may account for a significant number of infertility cases including unexplained infertility in women. Reduction in the endometrial estrogen receptor-alpha (*ER-α*) expression during implantation may be a critical event that coincides with the expression of specific genes and the formation of a receptive endometrium. The aim of the present study was to assess the expression of *ER-α* in the mid-secretory phase in the endometrium of women with unexplained infertility.

Materials and Methods: This case-control study was carried out on randomly selected fertile (n=10) and infertile (n=16) women whose source of infertility remained unexplained. We evaluated the expression of ER-α and glycodelin-A (*GdA*) through mRNA level measurement with real-time polymerase chain reaction (PCR) in the endometrium of fertile women and patients suffering from unexplained infertility and fertile women. Endometrial biopsies of each subject were collected during a single menstrual cycle 7 days after the peak of luteinizing hormone (LH+7).

Results: Endometrial expression level of *ER-α* was significantly (P<0.05) higher in the patients with unexplained infertility compared to the control. Significantly (P<0.05) lower levels of *GdA* expression were seen in women with unexplained infertility. A statistically non-significant negative correlation was observed between *ER-α* and *GdA* mRNA expression.

Conclusion: Our findings demonstrate that reduction in the endometrial *GdA* expression is associated with elevated expression of *ER-α* in mid-luteal phase. Disruption in the endometrial *ER-α* expression, which leads to defects in uterine receptivity, may contribute to unexplained infertility.

Keywords: Estrogen Receptor-α, Glycodelin-A, Implantation

Citation: **Dorostghoal M, Ghaffari H, Moramezi F, Keikhah N. Overexpression of endometrial estrogen receptor-alpha in the window of implantation in women with unexplained infertility. Int J Fertil Steril. 2018; 12(1): 37-42. doi: 10.22074/ijfs.2018.5118.**

Introduction

Endometrial receptivity plays a key role in the establishment of a successful implantation and its impairment may contribute to infertility in women (1). A variety of molecules such as hormones, receptors, adhesion molecules, growth factors and cytokines mediate the embryo-maternal crosstalk and facilitate the reception of a blastocyst and the establishment of implantation (2). During the menstrual cycle uterine receptivity is regulated by the secretion of the ovarian steroids. Endometrial proliferation is induced by estrogen during the preovulatory phase, whereas progesterone causes secretory changes in the estrogen-primed endometrium (3).

Ligand-specific intracellular receptors located in stromal and epithelial endometrial cells mediate the actions of estrogen and progesterone (4). It is thought that the presence of progesterone after appropriate estrogen priming is required to stimulate key implantation-specific events in the mid-secretory phase of the menstrual cycle (5).

Estrogen receptor-alpha (*ER-α*) increases during the proliferative phase in response to estrogen and is down-regulated during the window of implantation in response to progesterone (6). The disappearance of *ER-α* at the time of implantation has been reported in most mammalian species (7). The decline in *ER-α* coincides with endometrial gene expression in the mid-luteal phase, and is a critical event in the establishment of endometrial receptivity (8). High levels of *ER-α* during implantation were observed in women with polycystic ovarian syndrome (PCOS) and endometriosis. Elevated expression of *ER-α* in both groups of patients was associated with the reduction in beta 3 integrin expression, a marker of endometrial receptivity (9). It has been suggested that the disappearance of *ER-α* at the time of implantation may disturb the expression pattern of proteins that regulate the endometrial receptivity.

Received: 11 Nov 2016, Accepted: 9 May 2017
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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 1, Apr-Jun 2018, Pages: 37-42

Glycodelin-A (*GdA*) is a progesterone-regulated glycoprotein with immunosuppressive properties that is highly upregulated in glandular epithelium at implantation and plays a role in the formation a receptive endometrium (10). *GdA* expression is concurrent with pinopode formation in the receptive endometrium (11), indicating that it can potentially be seen as a diagnostic marker of morphological differentiation of human endometrium (12). A lower glycodelin expression in secretory phase was found in eutopic endometrium of endometriosis patients and in uterine flushings from women with unexplained infertility when compared to the healthy controls (13, 14).

Assuming that unexplained infertility can be due to disturbances in the molecular and the cellular biomarkers involved in implantation (15), we hypothesized that continued *ER-α* expression may be detrimental to the development of endometrial receptivity. In present study expression of *GdA*, as a particular marker of endometrial receptivity, was assessed at the time of implantation.

Materials and Methods

This case-control study was approved by the Research Ethics Committee of Shahid Chamran University of Ahvaz, Iran. The study was performed in the Laboratory of Embryology, Department of Biology. Written informed consent was obtained from each participant.

Sample collection

Endometrial biopsy samples were collected using a Novak curette in the mid-luteal phase at day luteinizing hormone (LH)+7 from healthy volunteers women with proven fertility (n=10, age 32.5 ± 3.2 Y) and women with unexplained infertility (n=16, age 31.6 ± 3.0 Y) that showed primary infertility for more than 2 years (30.5 ± 4.7 months). The unfertile females were randomly selected from a population of such females listed in Imam Khomeini hospital medical records. Endometrial samples were divided into two parts. One sample was fixed in 10% formalin and embedded in paraffin. After tissue processing, 5-6 µm sections were stained with haematoxylin-eosin, evaluated histologically to correspond all samples to the assumed time in the cycle according to the Noyes et al. (16) criteria. The other sample was immediately stored in RNA later at -80°C for later use in real-time polymerase chain reaction (RT-PCR). Sample size was determined based on previous studies (17, 18). Sample size was smaller in the fertile group due to the low collaboration. The concentration of LH in morning urine (ACON Laboratories, Inc., USA) was used to determine the day of the surge.

All women included in this study had normal ovarian function and regular menstrual cycles, confirmed based on their menstrual histories, and none of them had used steroid hormones, (for at least 6 months prior to study), and intra-uterine contraceptives. Women with unexplained infertility showed normal ovulatory cycles and mid-luteal serum progesterone levels, normal tubal patency and no recognizable endometriosis based on symptoms and clinical examination in transvaginal ultrasonography or diagnostic laparoscopy. Moreover, unexplained infertile women had partners with normal semen according to WHO criteria. Patients with history of pelvic inflammatory diseases, pelvic surgery including cesarean section, unilateral tubal patency, ovarian hyperstimulation syndrome, diminished ovarian response, endometriosis or multiple female factor were excluded from this study.

Hormone assay

Blood samples were obtained in the fasted state on the same day as endometrial sampling and serum levels of LH, follicle stimulating hormone (FSH), estradiol (E2), and progesterone (P4) were measured using commercially available kits (Abcam plc, UK).

RNA extraction

Total RNA was extracted from the endometrial tissues (approximately 50-100 mg) using Tripure (Roche Diagnostics, Germany), according to the recommended protocol by the manufacturer. RNA integrity was analyzed via electrophoresis and total RNA concentration was obtained using a spectrophotometer at an optical density of 260 nm. The RNA was stored at -70°C for future procedures.

cDNA synthesis

Synthesis of cDNA was carried out using 1 mg of total RNA from each sample with random hexamer primers using prime Script™ RT reagent Kit (Takara Bio Inc., Japan) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction analysis

Real-time PCR was performed for relative quantification of the *ER-α* and *GdA* genes expression using ABI StepOne plus™ System (Applied Biosystems, Germany). Hypoxanthine phosphoribosyltransferase (*HPRT*) gene was used as the housekeeping gene. Forward and reverse primer sequences for each gene are presented in Table 1. The specificity of primers for each gene was analyzed in the BLAST database. The reaction mixture consisted of 10 µl Master mix SYBR Green, 2 µl cDNA, 1 µl of each primer (10 pmol/µl), and 7 µl dH_2O (Qiagen, Germany). The standard cycling protocol used for all genes consisted of DNA denaturation and enzyme activation at 95°C for 10 minutes, denaturation 95°C for 15 seconds, annealing at 62°C for 15 seconds and extension and florescence acquiring at 72°C for 15 seconds. The RT-PCR procedure was carried out 40 cycles. Melting curve analysis was performed by bringing the temperature from 95°C to 60°C for 60 seconds at the transition rate of 1 degree per second. As Livak and Schmittgen (2001) described, for sample analysis the threshold was set based on the exponential phase of products and the $2^{-\Delta\Delta CT}$ method was performed to analyze the data (19).

Table 1: Primer sequences used in real-time polymerase chain reaction

Gene	Primer sequencing (5′→3′)	Accession number
ER-α	F: TGCTTCAGGCTACCATTATGGA	NM-001122742
	R: TGGCTGGACACATATAGTCGTT	
GdA	F: GAGATCGTTCTGCACAGATGG	NM-001018049
	R: CGTTCGCCACCGTATAGTTGAT	
HPRT	F: TGGACAGGACTGAACGTCTTG	NM-000194
	R: CCAGCAGGTCAGCAAAGAATTTA	

ER-α; **Estrogen receptor-alpha,** ***GdA;*** **Glycodelin-A, and** ***HPRT;*** **Hypoxanthine hosphoribosyltransferase.**

Statistical analysis

Data was analyzed by SPSS version 16 software (SPSS Inc., USA). Independent samples t test was performed to compare characteristics and hormonal profile of the fertile and the infertile women. Results are expressed as mean ± SD. Comparison of *ER-α* and *GdA* expression in studied groups was done using Mann-Whitney U-test. Spearman correlation analysis was carried out to investigate the relationship between variables. The level of significance was set at P<0.05.

Results

Of the 54 couples with unexplained infertility, 8 couples were excluded based on their medical records. Among 25 randomly-selected eligible patients with unexplained infertility, 9 couples refused participation. As a result, 16 infertile couples were included in the study. In addition, 10 fertile women (16.1%) out of the 62 eligible couples were included in the study. The mean age, body mass index (BMI), cycle length, duration of menses and hormonal profile in women of both groups are presented in Table 2. There were no differences in age, BMI, cycle length, duration of menses and serum LH, FSH, estradiol and progestrone concentrations between the two groups. Microscopic analysis of the endometrial biopsies showed that all samples corresponded histologically to the mid-luteal phase of endometrial cycle (Fig.1).

Table 2: Characteristics and hormonal profile of the fertile and infertile women in the mid-luteal phase

Parameter	Fertile women n=10	Infertile women n=16	P value
Age (Y)	31.7 ± 5.9	32.2 ± 5.5	NS
BMI (kg/m^2)	23.7 ± 2.8	23.4 ± 2.6	NS
Cycle length (days)	28.2 ± 1.3	28.5 ± 1.5	NS
Menses duration (days)	4.2 ± 0.5	4.5 ± 0.6	NS
LH (mIU/mL)	12.54 ± 6.85	13.27 ± 7.13	NS
FSH (mIU/mL)	5.90 ± 2.62	6.58 ± 2.50	NS
Estradiol (pg/ml)	139.3 ± 55.4	142.9 ± 61.6	NS
Progestrone (ng/mL)	10.93 ± 3.21	11.48 ± 4.86	NS

Independent samples t test was done as the test of significant. Results expressed as mean ± SD. The level of significance was set at P<0.05. BMI; Body mass index, LH; Luteinizing hormone, FSH; Follicle stimulating hormone, and NS; Non significant.

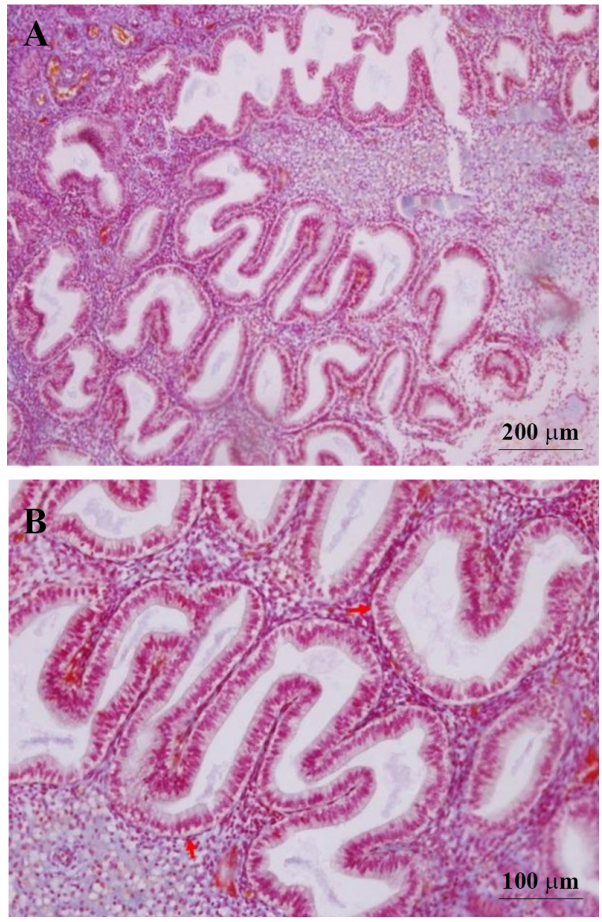

Fig.1: Microscopic structure of endometrium at the mid-luteal phase. **A.** Scale bar=200 μm and **B.** Scale bar=100 μm, H&E. Stromal edema and coiled endometrial glands that contain secretions with sub-nuclear vacuolization (red arrows) in their epithelium exhibit endometrium in the mid-luteal phase.

Relative expressions of *ER-α* and *GdA* in the mid-luteal endometrium of the patients with unexplained infertility and healthy fertile women are shown in Figures 2 and 3. Expression levels of *ER-α* and *GdA* mRNA are given relative to the expression levels of the reference gene, *HPRT*. Levels of *ER-α* mRNA expression in the endometrium of the patients with unexplained infertility were significantly higher than those in the fertile women (P=0.007, Mann-Whitney U-test, Fig.2).

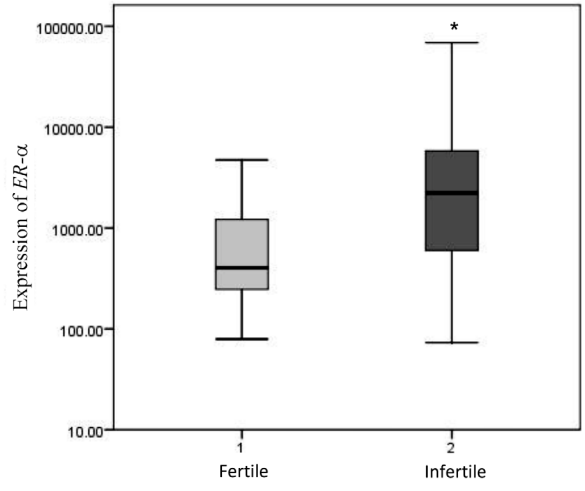

Fig.2: Relative expression of *ER-α* in the mid-luteal endometrium of patients with unexplained infertility (n=16) was significantly higher than those in healthy fertile women (n=10, P=*0.007,* Mann-Whitney U-test). *; P<0.05.

GdA mRNA levels were significantly lower in the infertile women compared to the healthy fertile group (P=0.045, Mann-Whitney U-test, Fig.3).

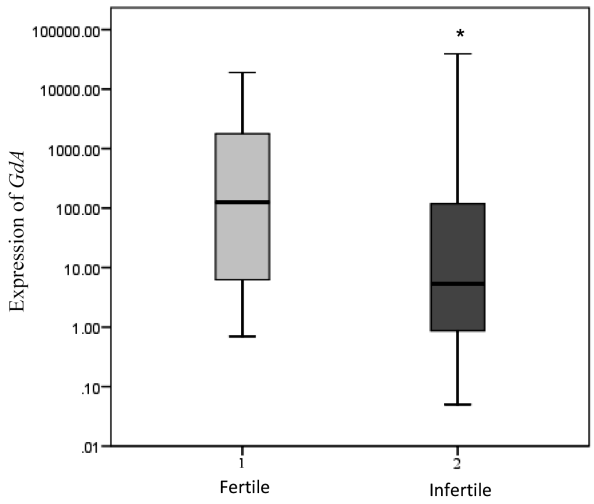

Fig.3: Relative expression of *GdA* in the mid-luteal endometrium of patients with unexplained infertility (n=16) was significantly lower than those in healthy fertile women (n=10, P=0.045, Mann-Whitney U-test). *; P<0.05.

A statistically non-significant negative correlation was observed between *ER-α* and *GdA* mRNA expression levels in the fertile women (r=-0.047, P=0.845) and in the patients with unexplained infertility (r=-0.205, P=0.316, Fig.4).

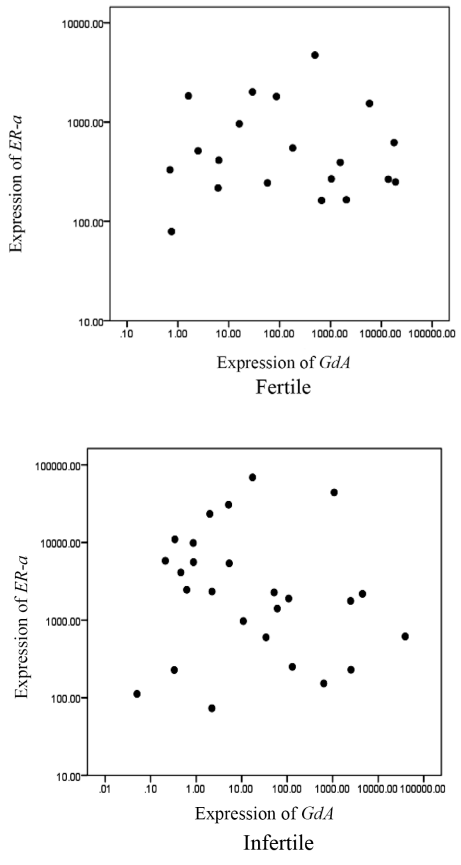

Fig.4: Correlation between *ER-α* and *GdA* mRNA expressions in the mid-luteal endometrium of the healthy fertile women (r=-0.047, P=0.845) and the patients with unexplained infertility (r=-0.205, P=0.316).

Discussion

Implantation failure is believed to be a major cause of infertility (20). Successful embryo implantation depends on the development of an endometrium that is receptive to the embryo (21). Coordinated interactions between estrogen and progesterone resulting in a series of synchronized molecular events during menstrual cycle ultimately lead to the preparation of a receptive endometrium (22).

The present study showed that a lack of appropriate levels of *ER-α* downregulation in the mid-luteal phase in the patients with unexplained infertility relative to the control group. During implantation *ER-α* is being downregulated in response to progesterone. Downregulation of *ER-α* during the mid-secretory phase is one of the primary actions of progesterone. The combination of estrogen withdrawal and progesterone action is required to stimulate the endometrial gene expression in the mid-luteal phase (8). Disappearance of *ER-α* in the mid-luteal phase provides the opportunity for progesterone to act alone specifically on the stroma (6). Paracrine activity of stroma in response to progesterone results in epithelial gene expression (7). Similar findings have been reported in patients with endometriosis and in women with PCOS (9).

Inadequate progesterone levels, defects in the progesterone receptor, hypersensitivity to estrogen, inappropriate expression of aromatase and progesterone resistance are among the reasons that can cause this failure to downregulate *ER-α* in the mid-luteal phase. Insufficient serum level of progesterone in the luteal phase defect (LPD) may delay the timing of *ER-α* downregulation during implantation (23). Resistance to progesterone due to aberrant expression or activity of receptor results in estrogenicity in endometrial tissue (24). The loss of progesterone activity caused by defect in the progesterone receptor (25) and/or an increase in the local estrogen production due to inappropriate expression of aromatase (26) may cause the persistence of *ER-α* in endometriosis patients. A failure in *ER-α* downregulation has been reported in ovarian and peritoneal endometriosis (27). Increased production of estrogen contributes to the pathophysiology of the endometriosis as a mitogen causing aberrant proliferation (28) and inhibition of apoptosis (29). Overexpression of steroid receptor co-activators in PCOS patients which marks the hypersensitivity to estrogen may explain elevated endometrial *ER-α* expression (9).

Moreover, it seems that any change in the balance between estrogen and progesterone could disturb the timing of *ER-α* downregulation in mid-luteal phase. Endocrine disrupting chemicals (EDCs) or xenoestrogens are natural or synthetic chemicals in the diet or the environment that mimic the endogenous estrogens functions or interfere with estrogen signaling pathways (30). Lower levels of progesterone metabolite have been found during the luteal phase with higher concentration of Dichlorodiphenyldichloroethylene (DDE) (31). Impaired implantation has been reported in patients with an increase in serum 17β-estradiol (E2) levels during the pre-implantation pe-

riod, while reducing E2 levels during the pre-implantation period by a step-down protocol increases implantation and pregnancy rates (32). Accordingly, the possibility of manipulating the receptivity window with the use of different doses of E2 has been suggested (33). Aberrant uterine expression of implantation-related genes has been found at high estrogen levels (34), suggesting that in *in vitro* fertilization (IVF) programs estrogen levels regulation is important for improvement of women fertility.

Any inability in the *ER-α* downregulation may lead to failure to express essential proteins associated with uterine receptivity, in turn resulting in either infertility or pregnancy loss (35). The present study showes that *ER-α* overexpression is accompanied by downregulation of *GdA* in the mid-luteal endometrium of the patients with unexplained infertility. *GdA*, a potential diagnostic marker of the endometrial receptivity, is the major progesterone-regulated glycoprotein and has been demonstrated in the pinopodes of receptive-phase human endometrium (11). Lower levels of *GdA* has been reported in the secretory phase of the menstrual cycle in the eutopic tissue of patients with endometriosis (13). In addition, lower levels of *GdA* were detected in the uterine flushings on days LH+10 and LH+12 in women with unexplained infertility (14) and recurrent miscarriage (36). A negative but statistically non-significant correlation was found between *ER-α* and *GdA* in fertile women and in patients with unexplained infertility. Although transcription, synthesis, and secretion of endometrial *GdA* are regulated by progesterone, according to our findings one can assume that the overexpression of endometrial *ER-α* disturbs the expression of special genes during the implantation, which is detrimental to the development of uterine receptivity.

Inadequate uterine receptivity is responsible for approximately two-thirds of implantation failures (37). A range of cellular and molecular endometrial defects has been associated with unexplained infertility (38). Microarray analysis demonstrated that endometrial gene expression at the time of embryo implantation is considerably different in the unexplained infertile patients compared to the fertile women (39).

Therefore, the failure in *ER-α* downregulation and the observed disturbance in *GdA* expression in the patients with unexplained infertility may elucidate the causes of unexplained infertility. Our observations suggest that endometrial *ER-α* expression may participate in the cascade of molecular events leading to successful implantation.

The random inclusion of all cases diagnosed with unexplained infertility is the main strength of this study. Furthermore, real-time PCR based assay of endometrial markers, an extremely sensitive technique that allows the precise measurement of gene expression (40), increases the accuracy and external validity of our results. However, data was collected from a single randomized center and subjects represent only a fraction of the population, thus reducing the population validity. Moreover, unexplained infertile women with secondary infertility were excluded, so its external validity is restricted to women with primary infertility.

Conclusion

The present study shows the prognostic significance of *ER-α* expression in patients with unexplained infertility. Disruption in the endometrial *ER-α* expression, which leads to defects in the uterine receptivity may contribute to unexplained infertility. In addition, our findings demonstrate that reduction in endometrial *GdA* expression was associated with elevated expression of *ER-α* in the mid-luteal phase. However, our study has some limitations including the low number of cases of unexplained infertile women with primary infertility. Studies including more tissue samples and protein-based assays such as immunohistochemistry and western blot analysis are also needed to further determine the role of endometrial *ER-α*.

Understanding of biomarkers involved in the implantation and the mechanisms governing their relationships in endometrial receptivity could provide new therapeutic strategies for unexplained infertility. Whether such defects of uterine receptivity could be treated by the therapeutic blockage of *ER-α* activity or by dealing with the related causes of *ER-α* overexpression, e.g., using progestins or aromatase inhibitors to normalize the expression pattern of endometrial biomarkers associated with implantation, requires further investigation.

Acknowledgements

The authors wish to thank the Vice Chancellor for Research of Shahid Chamran University of Ahvaz for providing the research grants (No. 867615). The authors declare that there is no conflict of interest.

Author's Contributions

M.D.; Designed and directed the project. F.M.; Contributed to sample preparation. H.-o-a.G.; Planned the RT-PCR method. N.K.; Performed the experiments. All authors discussed the results and contributed to the writing and read and approved the final manuscript.

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Original Article

Decreased Expression of *Arginine-Phenylalanine-Amide-Related Peptide-3* Gene in Dorsomedial Hypothalamic Nucleus of Constant Light Exposure Model of Polycystic Ovarian Syndrome

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Abstract

Background: An abnormality in pulse amplitude and frequency of gonadotropin releasing hormone (GnRH) secretion is the most characteristics of polycystic ovarian syndrome (PCOS). On the other hand, arginine-phenylalanine-amide (RFamide)-related peptide-3 (RFRP3) inhibits the secretion of GnRH in mammalian hypothalamus. The current study performed in order to investigate the expression of *RFRP3* mRNA in the dorsomedial hypothalamic nucleus (DMH) after the induction of PCOS in a rat model of constant light exposure, and the possible role of parity on occurrence of PCOS.

Materials and Methods: In the experimental study, female nulliparous (n=12) and primiparous (n=12) rats were randomly subdivided into control and PCOS subgroups (n=6). PCOS were induced by 90 days exposure to constant light. After 90 days, blood, brain, and ovaries were sampled. Serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were evaluated. In addition, six adult female ovariectomized rats as a control of real-time polymerase chain reaction (PCR) tests were prepared and in the DMH of all rats, the relative mRNA expression of *RFRP3* was assessed.

Results: Histological evaluation of ovaries represented the polycystic features. In addition, serum concentrations of testosterone in the PCOS subgroups were more than the controls ($P<0.05$). Furthermore, the relative expression of *RFRP3* mRNA in PCOS subgroups was lower than the controls ($P<0.05$).

Conclusion: Constant light model of the PCOS-induced rats decreased the gene expression of *RFRP3* in the DMH that suggests the decrease of *RFRP3* may reduce its inhibitory effect on GnRH during the PCOS pathogenesis. This effect was stronger in the nulliparous rats than the primiparous.

Keywords: Constant Light, Dorsomedial Hypothalamic Nucleus, Polycystic Ovary Syndrome, Rats, RFamide-Related Peptide-3

Citation: Shaaban Z, Jafarzadeh Shirazi MR, Nooranizadeh MH, Tamadon A, Rahmanifar F, Ahmadloo S, Ramezani A, Zamiri MJ, Razeghian Jahromi I, Sabet Sarvestani F, Koohi Hosseinabadi O. Decreased expression of Arginine-Phenylalanine-Amide-Related Peptide-3 gene in dorsomedial hypothalamic nucleus of constant light exposure model of polycystic ovarian syndrome. Int J Fertil Steril. 2018; 12(1): 43-50. doi: 10.22074/ijfs.2018.5206.

Introduction

Polycystic ovarian syndrome (PCOS) as an complex endocrine disorder in women is accompanied with ovarian dysfunction, metabolic disorders (e.g., obesity), and a myriad of causes, including genetic abnormalities, fetal epigenetic alterations, maternal or postpubertal hormonal imbalances, lifestyle, and environmental factors have been explained (1), have been proposed to explain PCOS. However, and despite the prevalence of PCOS and its effects on health, the causes of this syndrome, especially in hypothalamus, have been not completely understood.

Common neuroendocrine disorder of PCOS, increased frequency and amplitude pulses of gonadotropin releasing hormone (GnRH) (2). Furthermore, elevated secretion and pulse amplitude and frequency of luteinizing hormone (LH) release are the prominent pathophysiological features of PCOS (2, 3). LH secretion increment in 70% of women with PCOS has been accessed; this increase accompanied with in-

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 1, Apr-Jun 2018, Pages: 43-50

creases in LH/follicle stimulating hormone (FSH) ratio (3). Suppression of FSH secretion inhibits proper development of follicles and overproduction of LH led androgen synthesis in follicular theca cells (2). Therefore, measuring of FSH and LH levels in PCOS will reflect a GnRH pulse frequency. Increase of both amount and pulse frequency of GnRH have importance in pathophysiology of PCOS.

On the other hand, some evidences show that PCOS may be originated from dysfunctions in regulating neuronal circuits of negative feedback of steroids to hypothalamus-pituitary-gonads (HPG) axis (4). Furthermore, it is possible that change in GnRH release inhibitors, such as arginine-phenylalanine-amide (RFamide)-related peptide-3 (RFRP3), may control the hormonal irregularities of PCOS. RFRP3 neurons localize in the dorsomedial hypothalamic nucleus (DMH) of rat brain (5). Furthermore, peripheral or intracerebroventricular injection of RFRP3 inhibited LH secretion in sheep (6). Although, there is evidence supporting the existence of the RFRP3 receptor in the pituitary, but the inhibitory effect of RFRP3 neurons on GnRH at the level of hypothalamus were achieved (7). In general, the inhibitory signals of RFRP3 on GnRH neurons allow preovulatory LH surge happen at the right time (8). Also, there are reports that show PCOS is common in nulliparity and multiple gravidity can reduce PCOS (9). In the present study a constant light model was induced in both nulliparous and uniparous rats to evaluate the mRNA expression of RFRP3 in the DMH of PCOS rats.

Materials and Methods

All experimental procedures on rats were performed based on the instructions of Animal Care Committee of Shiraz University. The experimental procedure had been approved by Chancellor of Research Committee of the Shiraz University.

Animal and polycystic ovarian syndrome induction

In the experimental study, 30 female Sprague-Dawley rats were purchased from and kept in the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences. The rats were housed in standard condition of laboratory animal center (22 ± 1˚C temperature) and food and water were available ad libitum. Twelve nulliparous (38 days-old, 177 ± 20 g) and 12 uniparous (80 days-old, 226 ± 20 g) rats were randomly allocated into two PCOS and control normal sub-groups (n=6). PCOS was induced using constant light (10). Briefly, the both the PCOS sub-groups were exposed to 90 days constant 24 hours per day fluorescent light with 350 lux intensity to 1 m^2 on floor. The control normal sub-groups were housed in 12 hours light to 12 hours dark condition. After 90 days, blood, brain and ovaries of the PCOS and control rats were collected.

Six remained nulliparous rats were used as the ovariectomized control group for real-time polymerase chain reaction (PCR). The ovariectomy was performed through a ventral midline incision after anesthetizing with an intraperitoneal injection of xylazine (7 mg/kg, Alfazyne, Netherlands) and ketamine (100 mg/ kg, Netherlands). Brain tissue sampling in this group was done after two weeks.

Sampling and histological evaluation

For sampling, the rats were euthanized with ether and blood was sampled in tubes without anticoagulants by cardiac puncture. Serum was collected by centrifuging 2000 rpm for 15 minutes and then stored at -80°C until analysis.

Brain was dissected out from skull and DMH was sampled (11). In brief, the diencephalon was rapidly separated in cold condition by an anterior coronal section to the optic chiasm and a posterior coronal cut at the mammillary bodies. To separate DMH from anteroventral periventricular nucleus, the third coronal sectioning was performed through the middle of the optic tract and rostral to infundibulum. The samples were covered in aluminum foil and rapidly stored in liquid nitrogen.

Then ovaries were removed through ventral midline incision and kept in 10% buffer formalin solution. Ovaries were dehydrated by graded concentrations ethanol and xylene and then were embedded in paraffin. Serial sectioning was performed at thicknesses of 10 μm. Sections were deparaffinized in 60°C. In room temperature, sections were rehydrated in xylene and graded concentrations of ethanol. Samples were stained with hematoxylin and eosin (H&E) stain. Follicle types in ovarian sections were defined (12) with light microscope (CX21, Olympus, Japan).

Measurements of serum hormone

Serum concentration of testosterone with 0.2 nmol/L sensitivity (catalog# RK-61M, Institut des Isotopes Ltd, Hungary) was measured by radioimmunoassay (RIA) technique. In addition, serum concentrations of follicle stimulating hormone with 0.09 mIU/mL sensitivity (catalog# RF01N, Gyeonggi-do, South Korea) and luteinizing hormone with 0.22 mIU/mL sensitivity (catalog# RF03N, Gyeonggi-do, South Korea) were determined using immunoradiometric assay (IRMA) technique.

RFRP3 expression by real-time polymerase chain reaction

RFRP3 mRNA relative expression in DMH of rat brains was measured (13). Total RNA from DMH was extracted from the frozen brain samples after grounding it in liquid nitrogen and adding extraction buffer by Tripure isolation reagent kit (Roche Life Science, Branford, CT) according to manufacturer's instructions. Brain samples were transferred to the free RNase microtube and after mixing the solution were kept at room temperature for 5 minutes. Then, 0.2 ml chloroform was added to the solution and was held at room temperature for 15 minutes. Afterwards, the supernatant phase of mixture was transferred to another microtube after centrifuging of mixture at 12,000 rpm for 20 minutes. The same volume of isopropanol was added to the microtube. After washing the RNA pellet with 75% ethanol, it was quickly dried. The total purified RNA was measured by spectropho-

tometer (Nano-Drop ND-1000, Nano-Drop Technologies, Wilmington, DE, USA). To ensure the removal of genomic contamination, the DNase treatment was done using a DNase kit (Fermentas, St. Leon-Roth, Germany). The first strand cDNA synthesis using cDNA synthetize kit was performed (Fermentas, St. Leon-Roth, Germany). Primers for *RFRP3* target gene and rat *β-actin* reference gene were designed using Allele ID software version 6.0 (Premier Biosoft International, USA). Relative real-time PCR reactions was performed by 20 µL real time master mix (Yekta Tajhiz Azma, Iran) containing 1 µL cDNA, 4 pmol of primer, and 1X SYBR Green buffer. The cDNA samples were amplified (Table 1) by a StepOne cycler (Applied Biosystems, CA, USA). Amplification condition was 15 minutes at 94°C, 40 cycles of 94°C 10 seconds, 58°C 15 seconds, and 72°C 30 seconds for *RFRP* and 15 minutes at 94°C, 40 cycles of 94°C 15 seconds, 57.8°C 20 seconds, and 72°C 30 seconds for *β-actin*.

Table 1: Designed primers for *arginine phenyl alanine related peptide-3* (*RFRP3*) and *β-actin* genes and their amplification reaction conditions

Gene	Primer sequencing (5´-3´)	Amplicon length (bp)
RFRP3	F: AAGACACTGGCTGGTTTG R: TTGAAGGACTGGCTGGAG	192
β-actin	F: CCACACTTTCTACAATGAGC R: ATACAGGGACAACACAGC	169

For quantitative assessment and evaluation of the relative mRNA expression of *RFRP3* gene the CT values were estimated with real-time PCR Step One software version 2.1 (Applied Biosystems, CA, USA). Accordingly, CT of *RFRP3* and CT of reference gene were entered in the $2^{-\Delta\Delta CT}$ equation. ΔCT is a difference between the internal control gene CT value and the target gene CT value. ΔΔCT was obtained by subtracting the ΔCT of each sample from the average of CT value of calibrators (ovariectomized rats).

Statistical analysis

The normality of data from hormone measurements and the *RFRP3* mRNA relative expression were evaluated by the Kolmogorov-Smirnov test. Then, they were analyzed by one-way ANOVA (SPSS for Windows, version 20, SPSS Inc, Illinois), and mean differences was compared by post hoc LSD test at $P<0.05$. Data are presented as mean and SE.

Results

Histological evaluation of ovaries

The microscopic evaluation of ovaries in the PCOS groups showed formation of cystic follicles (Fig.1B, D) in comparison with controls (Fig.1A, C) and alterations of thickness and structure of follicular wall layers (Fig.2B, D) in comparison with controls (Fig. 2A, C). Active corpora lutea was not observed in the PCOS rats, but in the control group numerous corpora lutea were obvious (Fig.1). The number of the secondary follicles was lower in the PCOS rats compared with control rats (Fig.3). In medulla, stroma cells' cytoplasm of the PCOS rats demonstrated high amount of vesicles in comparison with controls (Fig.4).

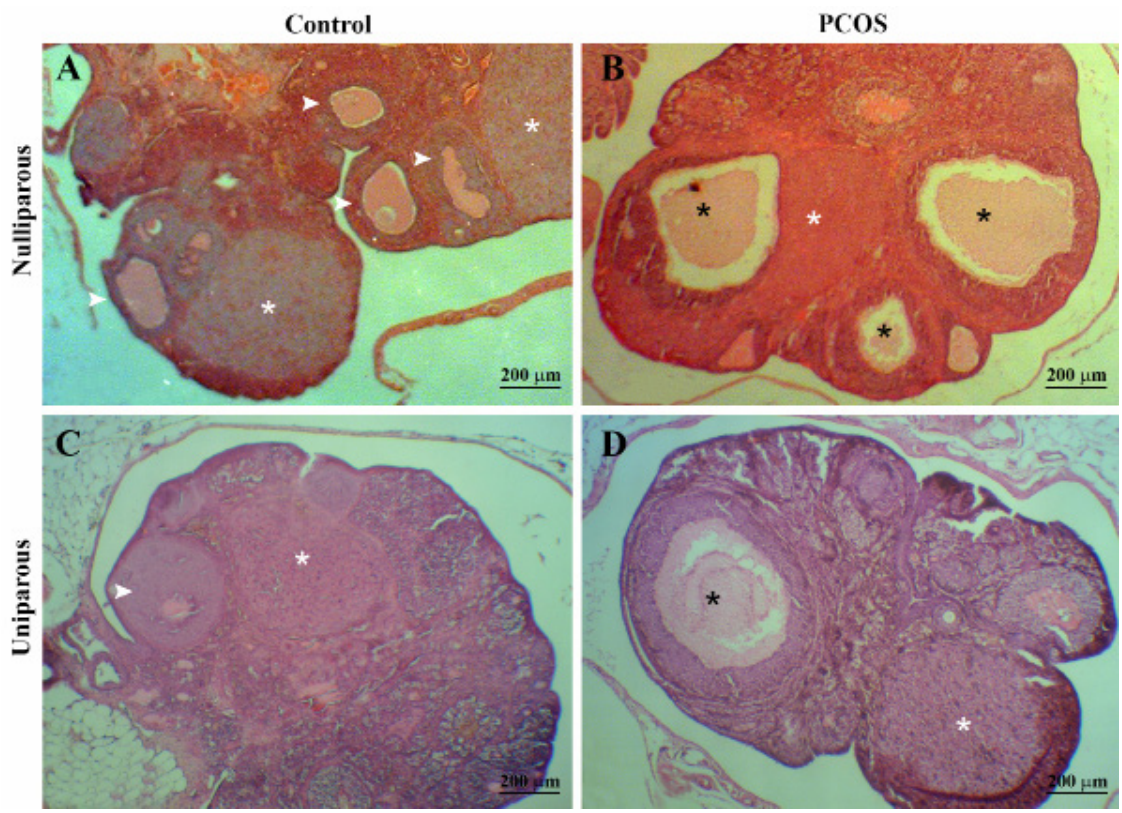


Fig.1: Alterations of histological charecters of ovaries in the female nulliparous and the primiparous rats after the exposition to continuous light during 90 days. The control groups show normal ovarian feature with Several corpus luteum (white stars) and normal tertiarry follicles (arrows). The polycystic ovary syndrome (PCOS)-induced groups showed considerably distended and cystic tertiary follicles [black stars, hematoxilin and eosin staining (H&E)]. **A.** Nulliparous control, **B.** Nulliparous PCOS, **C.** Uniparous control, and **D.** Uniparous PCOS.

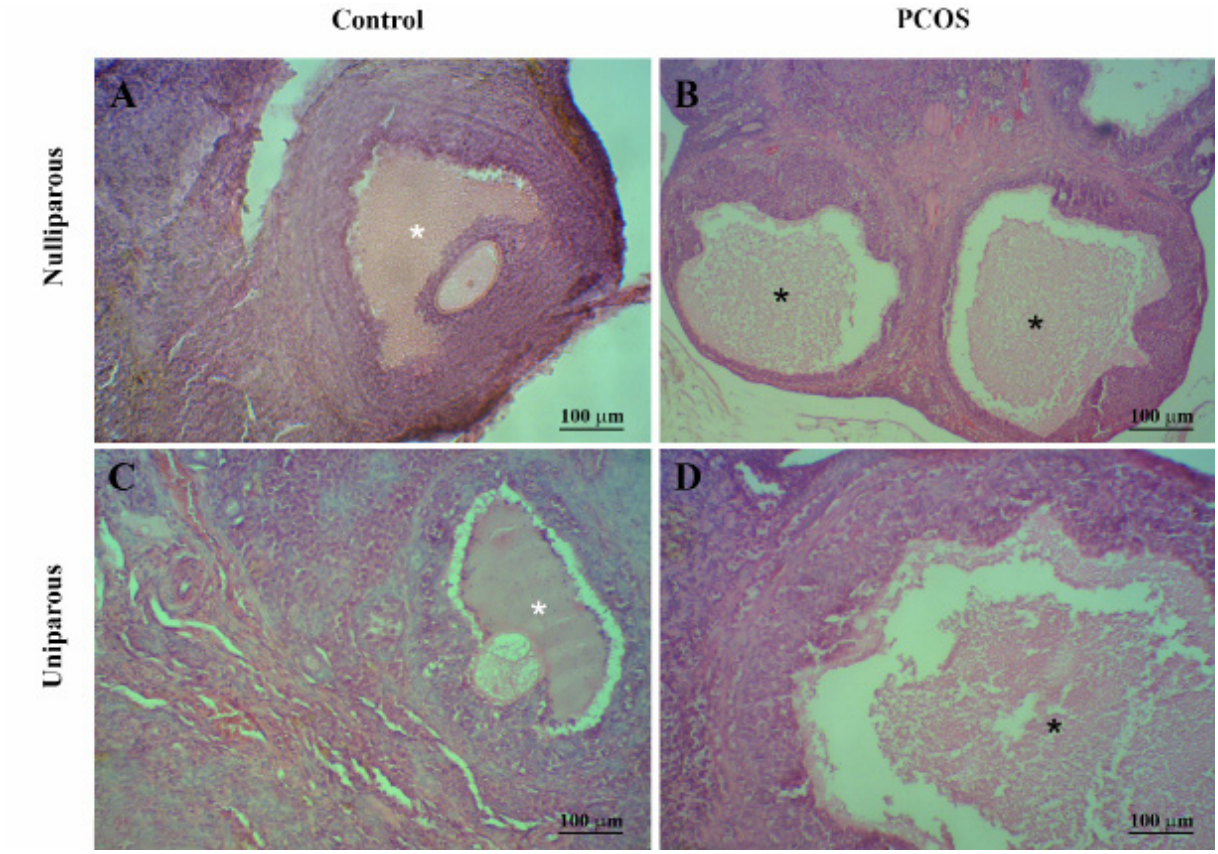


Fig.2: Alterations of tertiarry follicles features in the female nulliparous and the primiparous rats after continuous light exposure during 90 days. Ovary of the control rat with normal tertiary follicles (white stars). Oocytes and corona radiata are absent in the polycystic ovary syndrome (PCOS)-induced groups and atretic follicles (black stars) are more observable [hematoxilin and eosin staining (H&E)]. **A.** Nulliparous control, **B.** Nulliparous PCOS, **C.** Uniparous control, and **D.** Uniparous PCOS. [hematoxilin and eosin staining (H&E)]. PCOS; Polycystic ovary syndrome.

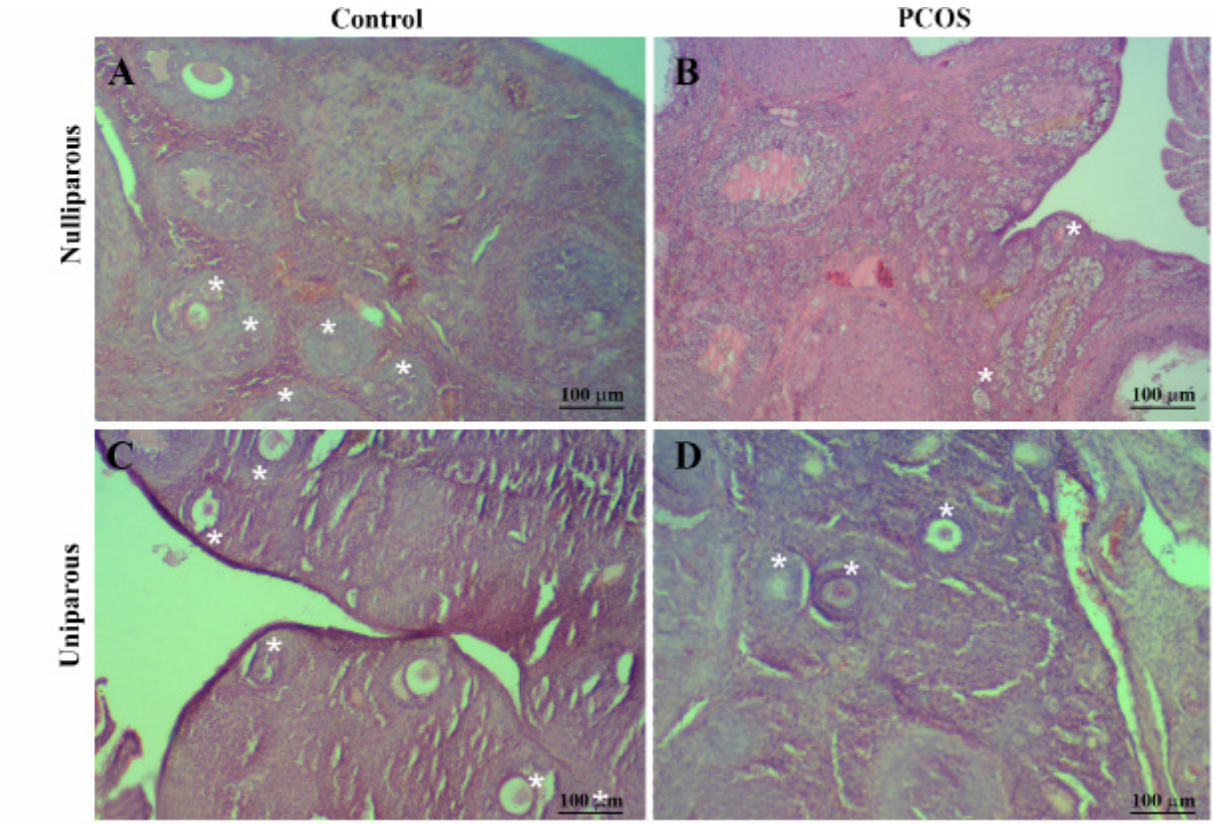


Fig.3: Decrease in the number of secondary follicles (stars) in ovary of the rat model of polycystic ovary syndrome (PCOS) in comparison with the control rat [hematoxilin and eosin staining (H&E)]. **A.** Nulliparous control, **B.** Nulliparous PCOS, **C.** Uniparous control, and **D.** Uniparous PCOS. [hematoxilin and eosin staining (H&E)]. PCOS; Polycystic ovary syndrome.

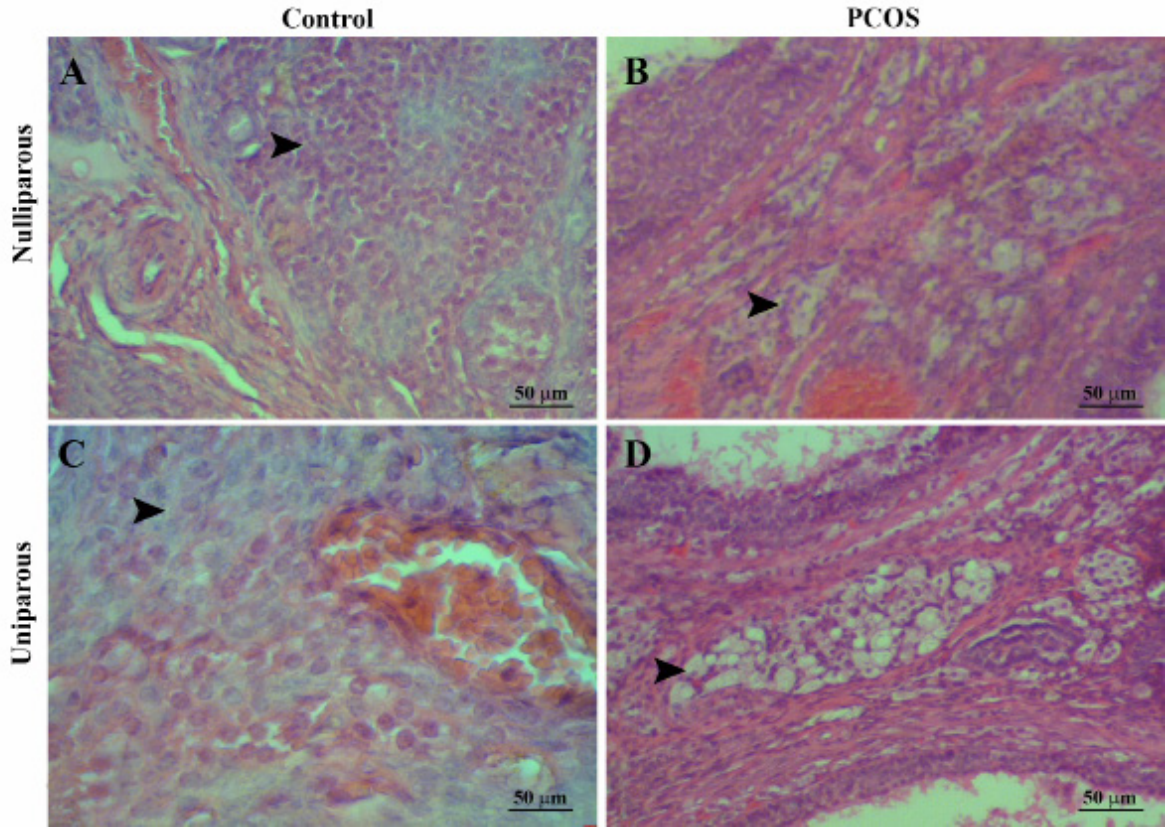


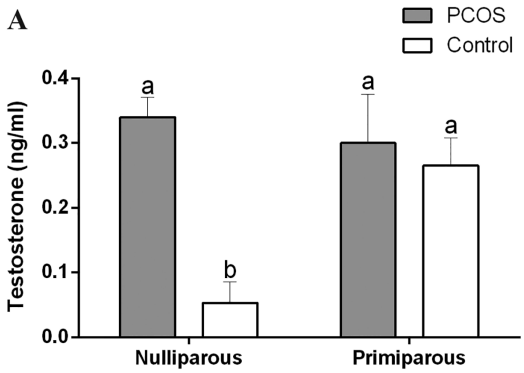
Fig.4: Hypertrophied and hyper-vaculated stromal cells in the ovarian medula of the polycystic ovary syndrome (PCOS) rats in comparison with normal stromal cells in the control rats [hematoxilin and eosin staining (H&E)]. **A.** Nulliparous control, **B.** Nulliparous PCOS, **C.** Uniparous control, and **D.** Uniparous PCOS. [hematoxilin and eosin staining (H&E)]. PCOS; Polycystic ovary syndrome.

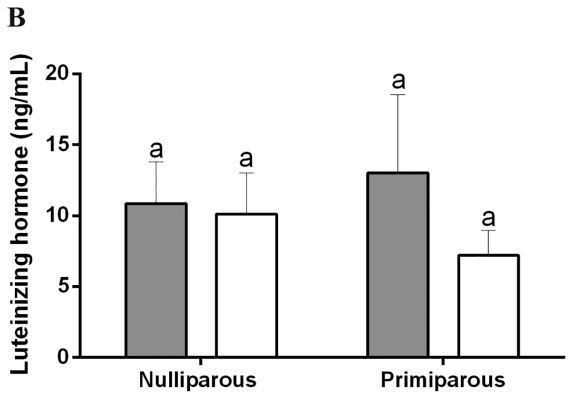
Serum hormone measurements

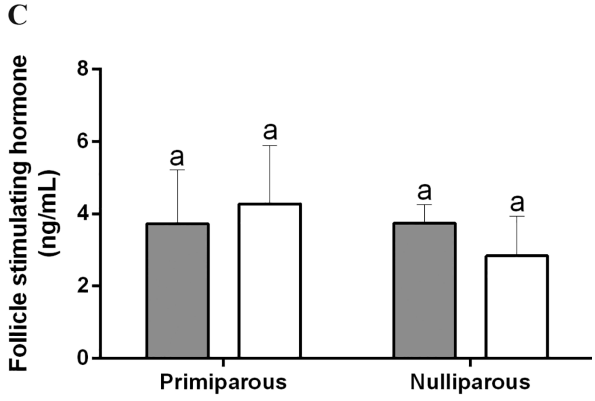
Serum testosterone concentrations of nulliparous the PCOS rats was more than the nulliparous control (Fig.5A, P<0.05), but not significantly different from the uniparous rats (P>0.05). FSH and LH concentrations were not significantly different between the control and the PCOS sub-groups (Fig.5B, C, P>0.05).

The *RFRP3* gene expression in hypothalamus

The real-time PCR analysis showed that the expression of *RFRP3* gene in the PCOS groups reduced (Fig.5D, P<0.05). Expressions of *RFRP3* gene in the uniparous rats of the PCOS and the control sub-groups were not different (P>0.05).







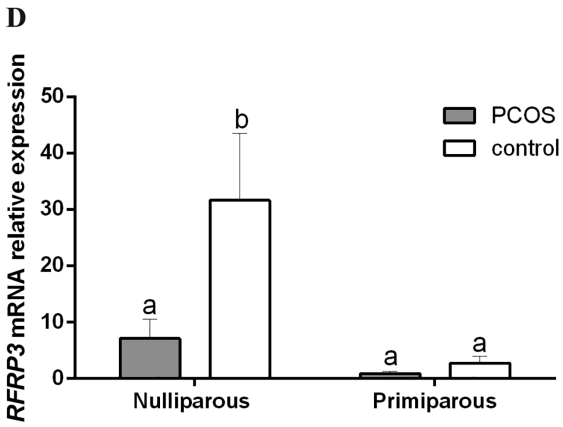


Fig.5: Alterations of the mean (+SE) of serum hormone concentrations in the female nulliparous and the primiparous rats after the exposition to continuous light during 90 days for induction of polycystic ovary syndrome (PCOS). **A.** Testestrone, **B.** Luteinizing hormone (LH), **C.** Follicle stimulating hormone (FSH), and **D.** Decrease in the mean (+SE) expression of arginine phenyl alanine related peptide-3 (*RFRP3*) mRNA in the dorsomedial nucleus of hypothalamus of nulliparous PCOS-induced.
a,b; Different letters show statistically significant differences between groups (P<0.05).

Discussion

The present study for the first time showed that the PCOS induction by constant light decreases *RFRP3* gene expression in the DMH of rats in the nulliparous group, an effect that was not observed in the uniparous group. Consistent with our finding, in a prepubertal rat model of androgen-induced PCOS, reduction in the transcription of RFRP3 inhibitory neuropeptide in whole hypothalamus has been recently reported (14). Furthermore, in letrozole-induced PCOS rats, gene expression of *RFRP3* and increased in RFRP3 receptor in pituitary was observed. Although, exogenous hormones may alter the pathogenesis of PCOS in those models, our findings, similar to (15), shows the role of RFRP signaling in PCOS.

In women suffering from PCOS, the concentrations of LH increases and FSH decreases in comparison with healthy women (16). Interestingly, in the current study, long term constant light (90 days) with intensity of 350 lux increased the mean concentrations of LH and decreased the mean of concentrations FSH in the uniparous rats in comparison with the control group but the alterations were not significant. Consistent with our findings, 100 days constant light exposures with about two times illumination intensity (500-600 lux) in rats could induce higher LH and lower FSH concentrations than the control group (17). While, long term exposure to continuous luminescence much lower than 350 lux (the rats were kept in a room with light) could slightly increase LH hypersecretion in rats (18), short-term continuous exposure to light (3 days) could suppress the synthesis of LH in female rat by reducing the sensitivity of the LH-releasing hormone release centers to estrogen (19). Therefore, increasing the intensity of the light could induce alterations, akin to those observed in PCOS rat models, to in gonadotropins concentrations in human PCOS.

Hyperandrogenism is accepted as an important attribute of PCOS; therefore, in most animal models of PCOS, androgen hormones have been used to stimulate the PCOS (20, 21). Although, these androgen models or other hormone-induced models of PCOS, especially in the prepubertal and pubertal models, have been used for evaluation of hypothalamic functions in PCOS, but there is a concern that these exogenous hormones may directly disturb the neuronal circuits and the observed alternations are not related to the induced PCOS. Therefore, prenatal androgen models or non-hormonal induced models of PCOS, such as constant light model of PCOS (10), may demonstrate closer hypothalamic features of PCOS to the human PCOS than the others.

Increase in serum concentrations of testosterone in the PCOS rats of the nulliparous group compared to the controls showed the efficiency of this model for evaluation of PCOS in hypothalamus without exogenous androgens. Consistent with our findings, increase in serum testosterone levels of rats that were exposed to 112 days constant light exposures with illumination intensity of 600 lux was shown (22).

To explain this phenomenon, it has been shown that the testosterone concentrations are positively correlated with the expression of the androgen receptor in the hypothalamic suprachiasmatic nucleus (SCN). This locus regulates circadian rhythms and light exposure controls it (23). On the other hand, SCN sends direct and indirect projections to DMH (24), which suggests the role of SCN in control of DMH reproductive function, and can also explain the observed relationship between testosterone increase and constant light exposure. Furthermore, it has been shown that SCN projects to RFRP neurons in DMH of hamster (25). Therefore, our findings in combination with previous findings, suggest the relationship of RFRP3 in DMH and testosterone effects on SCN. However, clarifying this pathway in pathogenesis of PCOS needs further investigation.

Histopathologic evaluation of ovaries showed that continuous light exposure increased the number of antral follicles and atretic follicles. Consistent with our results, increase in large antral and atretic follicles and reduction of the number of early growing follicles have been previously reported in rats that were subjected to 13 weeks of continuous exposure (26). Continuous light exposures of rats for 100 days led to atresia of ovarian follicles due to lack of preovulatory LH surge and resulted in cyst formation (17). Although, corpora lutea were present in the uniparous PCOS-induced group, but the absence of corpus luteum is another attribute of PCOS in the current study in the nulliparous rats, in accordance with a previous report (26).

However, the nulliparous group represented a better PCOS model than the primiparous rats, but it is not clear if gravidity can influence the occurrence of PCOS or

not. Consistent with the current finding, in the consistent light model of PCOS, the PCOS criteria in the nulliparous rats were more than the primiparous group. In women, obesity would exacerbate the insulin resistance, a predisposing factor for PCOS. It has been reported that pregnancy can be a risk factor for obesity (27). Therefore, it can be expected prevalence of PCOS increase in the uniparous women. While, evidences showed that nulliparous women are susceptible to PCOS (28). Therefore, based on the current findings and the other published reports it can indirectly conclude that increase in gravidity may be associated with decreased PCOS, although confirmation of it and knowing its mechanism need further investigations.

Conclusion

The constant light model of PCOS induced decrease in the gene expression of *RFRP3* in the DMH that suggests the decrease in *RFRP3* reduces its inhibitory effect on GnRH in the PCOS pathogenesis. The Continuous light exposure model of PCOS in rats could trigger the creation of phenotypic traits of PCOS with similar histopathologic and hormonal phenomenon in human PCOS. Furthermore, by removing exogenous androgen, this model can be applied to hypothalamic-pituitary-ovarian disorders in PCOS studies.

Acknowledgements

The current project has been supported by Shiraz University, Vice Chancellor of Research as a master thesis no. 3074 financially and performed using the instruments and equipment in Stem Cell Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. The authors declared no conflict of interest.

Author's Contributions

A.T., M.R.J.S., M.J.Z., F.R.; Conception and design of the study. Z.S., M.H.N., A.T., F.R., S.A., A.R., I.R.J., F.S.S., O.K.H.; Acquisition and analysis of data including animal modeling by M.H.N., O.K.H., F.S.S.. A.T., O.K.H., M.H.N., Z.S.; Sampling. M.H.N, Z.S.; Hormone analysis. F.R., Z.S.; Histological evaluation. S.A., A.R., I.R.J.; Molecular analysis. A.T., Z.S., M.R.J.S., F.R., M.J.Z.; Drafting the manuscript, finalizing the last version and preparing the figures. All authors read and confirmed the final draft.

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Original Article

Screening for Causative Mutations of Major Prolificacy Genes in Iranian Fat-Tailed Sheep

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Abstract

Background: The presence of different missense mutations in sheep breeds have shown that the bone morphogenetic protein receptor 1B (*BMPR1B*), bone morphogenetic protein 15 (*BMP15*) and growth differentiation factor 9 (*GDF9*) genes play a vital role in ovulation rate and prolificacy in ewes. Therefore, the present study aims to investigate *BMPR1B*, *BMP15* and *GDF9* gene mutations in prolific ewes of Iranian fat-tailed Lori-Bakhtiari sheep.

Materials and Methods: In the present experimental study, genomic DNA was extracted from whole blood of 10 prolific Lori-Bakhtiari ewes with at least two twinning records in the first four parities to identify point mutations of the *BMPR1B*, *BMP15* and *GDF9* genes, using DNA sequencing.

Results: The results obtained from DNA sequencing showed a new synonymous mutation (g.66496G>A) in exon 8 of the *BMPR1B* gene, without any amino acid change. Sequencing of the *BMP15* gene revealed a deletion of 3 bp (g.656_658delTTC) in exon 1, leading to an amino acid deletion (p.Leu19del). Four single nucleotide polymorphisms (G1:g.2118G>A, G2:g.3451T>C, G3:g.3457A>G and G4:g.3701G>A), were detected in exons 1 and 2 of the *GDF9* gene, two of which caused amino acid substitutions (G1: p.87Arg>His and G4: p.241Glu>Lys). These amino acid alterations are proposed to have a benign impact on structure and function of the *GDF9* polypeptide sequence.

Conclusion: Three major prolificacy genes (*BMPR1B*, *BMP15* and *GDF9*) were polymorphic in Lori-Bakhtiari sheep, although none of the major causative mutation was detected in this sheep type. Further studies using high throughput methods such as genome-wide association study (GWAS) and evaluation of other candidate genes are necessary in the future.

Keywords: Fertility, Litter Size, Single Nucleotide Polymorphism, Sheep

Citation: Abdoli R, Mirhoseini SZ, Ghavi Hossein-Zadeh N, Zamani P. Screening for causative mutations of major prolificacy genes in Iranian fat-tailed sheep. Int J Fertil Steril. 2018; 12(1): 51-55. doi: 10.22074/ijfs.2018.5247.

Introduction

Reproductive traits such as ovulation rate and litter size are genetically influenced by several minor genes as well as some major genes, called fecundity (*Fec*) genes (1). Three major genes including bone morphogenetic protein receptor 1B (*BMPR1B*) or *FecB*, bone morphogenetic protein 15 (*BMP15*) or *FecX* and growth differentiation factor 9 (*GDF9*) or *FecG*, belong to the transforming growth factor-beta (TGF-β) superfamily, located on ovine chromosomes 6, X and 5, respectively, and they affect the prolificacy (2).

Different causative mutations in the exon 8 of *BMPR1B* (*FecB*), both exons 1 and 2 of the *BMP15* ($FecX^{G}$, $FecX^{B}$, $FecX^{I}$, $FecX^{H}$, $FecX^{L}$, $FecX^{R}$, $FecX^{O}$, $FecX^{Gr}$) and *GDF9* ($FecG^{H}$, $FecG^{T}$, $FecG^{E}$, $FecGN^{W}$) with major effects on ovulation rate and litter size have thus far been distinguished in various sheep breeds around the world (3). In this regard, the importance of the BMP system as an intra-ovarian regulator of follicular growth and maturation has been described (4, 5).

Lori-Bakhtiari sheep is an important heavyweight indigenous breeds, mainly raised in a wide range of Zagrous Mountains in southwestern part of Iran, with a current census over 1.7 million heads. This breed has the largest fat-tail size among all Iranian sheep breeds. Lori-Bakhtiari sheep is well known for providing a major source of meat with an average litter size of 1.17 ± 0.38 at birth and a conception rate of more than 90 percent (6). Considering that increasing the reproductive ability of the Lori-Bakhtiari sheep has always been an important breeding goal, genetic strategies have currently focused on reproduction traits to improve the profitability of sheep operations.

The aim of this study was to identify the possible pres-

Received: 7 Mar 2017, Accepted: 3 Jun 2017
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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 1, Apr-Jun 2018, Pages: 51-55

ence of known main mutations in *BMPR1B*, *BMP15* and *GDF9* genes affecting reproductive performance in Lori-Bakhtiari sheep.

Materials and Methods

Experimental animals and DNA isolation

In the present experimental study, a total of 10 prolific Lori-Bakhtiari ewes with at least two twinning records in the first four parities were selected from different half-sib families at Sholi Sheep Breeding Station, Charmahal and Bakhtiari province, Iran. From the prolificacy point of view, the average of twinning rate in the studied population was about 18%. Blood samples were collected from jugular vein (5 ml per ewe) by venoject tubes contained ethylene diamine tetra acetic acid (EDTA) and immediately transported to the laboratory with ice before DNA isolation. Genomic DNA was extracted from whole blood by the CinnaGen DNP kit (CinnaGen Co, Iran).

All protocols were adhered in accordance with the ethical standards of the National Research Council's 2011 guideline for the care and use of animals, approved by the research Ethics Committees of University of Guilan (Guilan, Iran) and Bu-Ali Sina University (Hamedan, Iran).

Polymerase chain reaction amplification and sequence analysis

Five pairs of primer (Table 1) were designed to amplify exon 8 of *BMPR1B* (Gene ID: 443454) as well as exons 1 and 2 of both *BMP15* (Gene ID: 100141303) and *GDF9* (Gene ID: 100217402) genes, using Primer3 software online.

Table 1: Primer sequences

Gene	Region	Primer sequence (5′-3′)	Product size (bp)
BMPR1B	Exon 8	F: CCAGAGGACGATAGCAAAGCAA	190
		R: CAAGATGTTTTCATGCCTCATC	
BMP15	Exon 1	F: AAGCGTTATCCTTTGGGCTT	380
		R: CTGAGAGGCCTTGCTACACT	
	Exon 2	F: CGCTTTGCTCTTGTTCCCTC	906
		R: TAGCTGCACCTTTGCCGTC	
GDF9	Exon 1	F: GAAGACTGGTATGGGGAAATG	462
		R: CCAATCTGCTCCTACACACCT	
	Exon 2	F: TGGCATTACTGTTGGATTGTTT	1019
		R: GGTTTTACTTGACAGGAGTCTG	

Polymerase chain reaction (PCR) amplification protocols were similar in designated regions and they were carried out in a 50 µl volume, consisting of 25 µl Taq DNA Polymerase Master Mix 2X (CinnaGen, Iran), 10 pM of each primer (OD:2), 50-100 ng of DNA template and distilled water. PCR reactions were run in Applied Biosystems thermal cycler (Life technologies, USA) under the following thermal condition: Initial denaturation at 95°C for 5 minutes, followed by 35 cycles consisting of denaturation at 95°C for 1 minute, annealing at 60°C for 45 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 5 minutes.

Individual fragments were distinguished by electrophoresis of PCR products in 2% agarose gel (CinnaGen, Iran). The gels were stained with ethidium bromide and photographed under UV light (BTS-20.M model, UVItec Ltd, UK) (Fig.1).

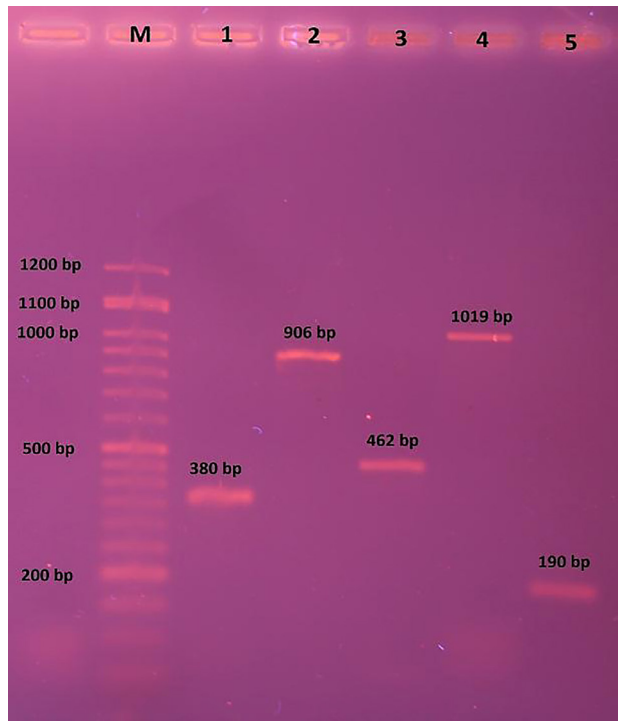

Fig.1: Polymerase chain reaction (PCR) products of *BMPR1B*, *BMP15* and *GDF9* genes bands. M; DNA molecular weight marker (Orange Ruler 50 bp DNA Ladder, CinnaGen, Iran), 1; 380 bp fragment of *BMP15* exon 1, 2; 906 bp fragment of *BMP15* exon 2, 3; 462 bp fragment of *GDF9* exon 1, 4; 1019 bp fragment of *GDF9* exon 2, and 5; 190 bp fragment of *BMPR1B* exon 8.

All 50 samples (five fragments from each animal) were submitted to DNA sequencing. The purified PCR products were sequenced on both strands by Bioneer Co., Korea. The identified single nucleotide polymorphisms (SNPs) were compared to the referring sequences at NCBI database using BLAST (7). Ultimately, any potential effect of the identified mutations, in terms of the structure and function of codified polypeptides, was predicted using PolyPhen-2 online software tool (8).

Results

Sequence analysis of the *BMPR1B* gene exon 8

The sequence of exon 8 for *BMPR1B* gene did not show any *FecB* mutation corresponding with the increase of litter size and ovulation rate (9). However, the sequencing showed a novel synonymous mutation in the location of g.66496G>A (Fig.2).

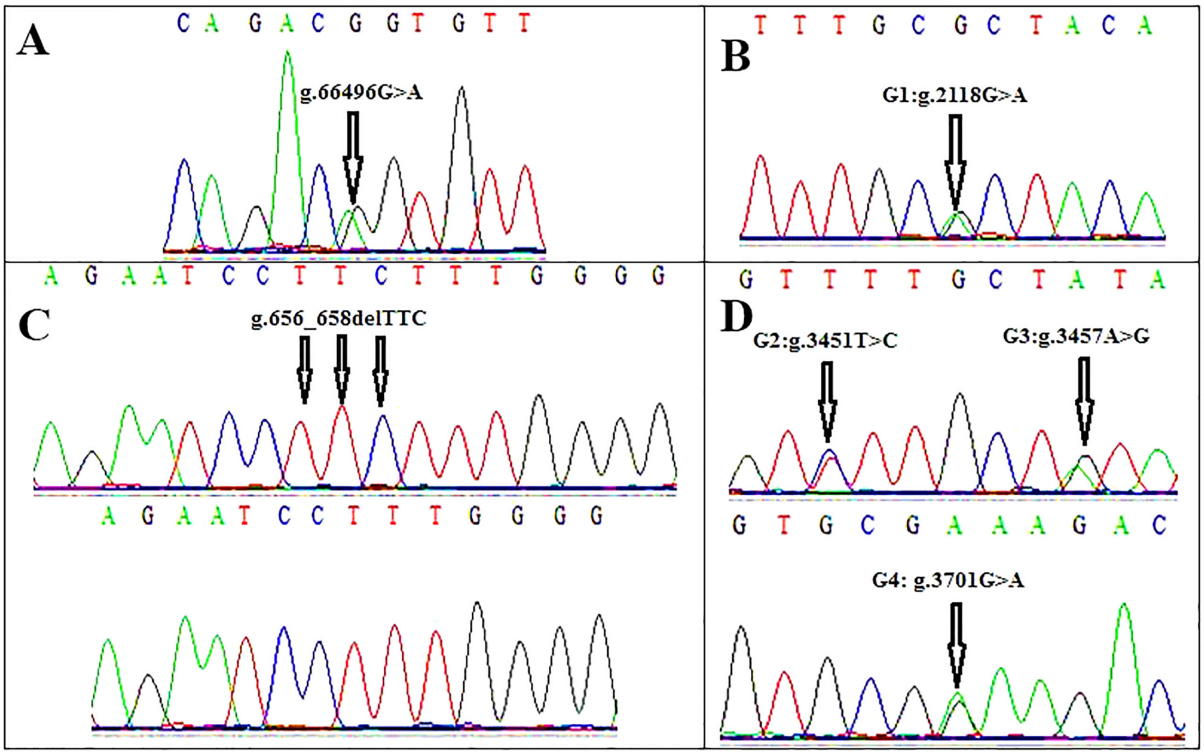

Fig.2: Sequencing chromatograms of the detected mutations of major prolificacy genes in Lori-Bakhtiari sheep. **A.** The identified transition (g.66496G>A) in *BMPR1B* exon 8, **B.** The identified 3bp deletion (g.656_658delTTC) in *BMP15* gene exon 1, **C.** The identified polymorphism in exon 1 of the *GDF9* gene (G1:g.2118G>A), and **D.** The identified polymorphisms in exon 2 of the *GDF9* gene (G2:g.3451T>C, G3:g.3457A>G and G4: g.3701G>A). Positions of the mutations are based on the full sequences of *BMPR1B*, *BMP15* and *GDF9* genes (Gene IDs; 443454, 100141303 and 100217402, respectively).

Table 2: Identified mutations of the major prolificacy genes in Lori-Bakhtiari sheep

Gene	Variation	Amino acid substitution	Frequency	Type of mutation
BMPR1B exon 8	g.66496G>A	-	2 out of 10	Synonymous
BMP15 exon 1	g.656_658delTTC	p.Leu19del	1 out of 10	Non-synonymous
GDF9 exon 1	g.2118G>A	p.87Arg>His	2 out of 10	Non-synonymous
GDF9 exon 2	g.3451T>C	-	2 out of 10	Synonymous
	g.3457A>G	-	2 out of 10	Synonymous
	g.3701G>A	p.241Glu>Lys	2 out of 10	Non-synonymous

Sequence analysis of *BMP15* gene exons 1 and 2

The sequence of exon 1 of *BMP15* showed a 3 bp nucleotide deletion (g.656_658delTTC) just in 1 out of 10 sheep samples (Fig.2). This mutation, leading to an amino acid deletion (p.Leu19de), has been described in Cambridge and Belclare sheep for the first time (10). So that nine sheep had two leucine codons and one showed this mutation with only one codon at this position (Table 2). No polymorphism was found in exon 2 of the *BMP15* gene.

Sequence analysis of *GDF9* gene exons 1 and 2

Four single nucleotide polymorphisms (g.2118G>A, g.3451T>C, g.3457A>G and g.3701G>A), respectively known as G1-G4, were detected in the exons 1 and 2 of *GDF9* gene (Fig.2). Two of these mutations deduced amino acid changes, including G1: p.87Arg>His and G4: p.241Glu>Lys (Table 2). Potential effect(s) of the identified mutations on the structure and function of codified polypeptides were not significant, proposing no phenotypic effect (Fig.3).

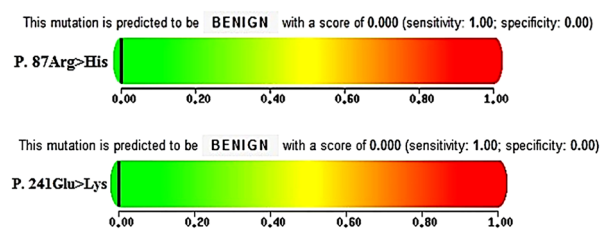

Fig.3: Prediction of the amino acid substitutions impact (G1: P.87Arg>His and G4: P.241Glu>Lys) on structure and function of the *GDF9* codified polypeptide.

Discussion

Considering the hotspot regions involved in prolificacy of

sheep, selection of a limited number of high prolific ewes and sequencing of their *BMPR1B* exon 8, in addition to the both exons 1 and 2 of *BMP15* and *GDF9* genes could be contemplated as an ideal approach to find related causative mutations. This approach would be less expensive than other methods and may lead to find new causative mutations in the indicated fragments. If this approach does not detect any important mutation, genome-wide association study (GWAS) implications would be a more appropriated method to determine causative mutations or candidate genes affecting prolificacy in the other parts of genome.

Evidences show that Booroola Merino ewes have high ovulation rate and litter size, due to the effects of a missense mutation (*FecB*) in the exon 8 of *BMPR1B* gene, located on chromosome 8 (9). This mutation has also been reported in Indian Garole and Kendrapada sheep breeds (11) as well as Chinese sheep breeds, Small Tail Han and Hu (12). The *FecB* was also found in Kalehkoohi sheep, as an Iranian breed (13).

In the present study, analysis of the sequences for *BMPR1B* gene exon 8 did not show the *FecB* alteration, as a mutation coordinating with increase of litter size and ovulation rate in the sheep. However, this analysis showed a new transition of g.66496G>A, compared to sequences reported for Garole (GenBank: GQ863576.1) and Booroola merino sheep, which is very close to the position of *FecB* mutation. This mutation causes a synonymous substitution which does not alter amino acid sequences. Western blot and qRT-PCR techniques could in future be used to verify possible effects of this SNP on translation and transcription aspects respectively. Moreover, linkage disequilibrium of this SNP with variants in other loci should be considered and investigated (14).

Eight different causative mutations in ovine *BMP15* gene with major effects on ovulation rate and litter size have previously been reported in literature (3). Hence, the *BMP15* gene could be considered as the most polymorphic locus among the major genes affecting prolificacy in sheep. As a case, evidences showed that a nonsense mutation, due to deletion of 17 bp nucleotides, altered the amino acid sequence and introduced a premature stop codon in this protein (15).

In the present study, sequencing of the exon 1 of *BMP15* gene showed 3 bp nucleotides deletion (g.656_658delTTC) in one sample, whereby nine sheep had two leucine codons at this position while this sheep showed heterozygote mutation. This mutation has been reported for the first time in Cambridge and Belclare sheep, without any determined significant effect on prolificacy (10). However, because of low frequency of this deletion and missing the infertile or singleton-bearing ewes, investigation of this aberration was not feasible in the present study. Further investigations demonstrated no polymorphism in the exon 2 of *BMP15* gene.

Hanrahan et al. (10) reported eight point mutations (i.e. G1-G8) in the *GDF9* gene of Cambridge and Belclare sheep, five of which deduced amino acid changes (G1: p.87Arg>His; G4: p.241Glu>Lys; G6: p.332Val>Ile; G7: p.371Val>Met and G8: p.395Ser>Phe), while only one of them (G8; also known as $FecG^{H}$) had additive effects on prolificacy. The first mutation (G1: g.2118G>A) was also reported to associate with an increased ovulation rate and litter size in some sheep breeds (16, 17). In the present study, the mutations of G1 (g.2118G>A), G2 (g.3451T>C), G3 (g.3457A>G) and G4 (g.3701G>A) were identified in exons 1 and 2 of the *GDF9* gene. With exception for G1 mutation, which had an additive effect (16, 17), other mutations (G2, G3 and G4) did not carry any significant effect on litter size in some sheep breeds (18). The impacts of amino acid substitutions for two non-synonymous mutations (G1: p.87Arg>His and G4: p.241Glu>Lys) were also benign with scores of 0.00. In fact, the phenotypic expression of an allele, to some extent, depends on other alleles, mainly multiple interacting mutations, and thus, a phenotypic effect of an allele may be observed in one breed while being absent in the other (3, 19). For instance, a GWAS analysis showed a missense mutation in the *GDF9* ($FecG^{NW}$) with strong association with litter size in Norwegian white sheep (20). This mutation, known as G7, has also been identified in Belclare and Cambridge sheep as well as G1, but without any phenotypic effects (10). Hence, more studies are still needed to determine the precise effects of this kind of mutations and epistatic effects evaluation.

Regarding the absence of known main causative mutations of *BMPR1B*, *BMP15* and *GDF9* genes in the studied population, high prolificacy could be attributed to the other genetic factors not studied here. Thus, seeking for other related major genes, such as a regulatory mutation in intron 7 of *B4GALNT2* (Fec^{L}) largely affecting the respective gene expression in prolific ewes and most likely taking responsibility for high prolificacy in Lacaune sheep (1), could be considered as an interesting subject for similar studies in the future. However, regarding the polygenic control of reproductive traits, GWAS and evaluation of linkage disequilibrium with other mutations or transcriptome analysis are also necessary to understand the precise underlying pathway(s) of prolificacy in Lori-Bakhtiari sheep.

Conclusion

We can state that major prolificacy genes (i.e. *BMPR1B*, *BMP15* and *GDF9*) were polymorphic in the studied population of Lori-Bakhtiari sheep, but none of the previously identified mutations contributing to prolificacy was detected in the studies genes. Moreover, no clear statistical association was determined among the observed polymorphisms and prolificacy. More studies on other candidate genes or use of high throughput methods such as GWAS are necessary in the future studies.

Acknowledgements

The authors are grateful to Agricultural Jihad Institutes of Charmahal and Bakhtiari Province for their kindly cooperation. The authors also would like to thank to University of Guilan for financial supporting the costs of this study. The authors declare that there is no conflict of interest for this article.

Author's Contribution

R.A.; Is the first author and participated in study design, data collection and evaluation. S.Z.M.; Is the corresponding author and performed scientific editing and writing. N.G.H.-Z., P.Z.; Co-edited the article. All authors read and approved the final manuscript.

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Original Article

Effects of Crocin on The Pituitary-Gonadal Axis and Hypothalamic *Kiss-1* Gene Expression in Female Wistar Rats

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Abstract

Background: Saffron (*Crocus sativus L.*) has been traditionally used as a spice for coloring and flavoring in some countries cuisine. One of the main components of saffron is Crocin. Recent research have shown that crocin has various pharmacological effects. The aim of this study was to assess the effects of crocin on the Pituitary-Gonadal axis and *Kiss-1* gene expression in hypothalamus and ovarian tissue organization in female Wistar rats.

Materials and Methods: In this experimental study, 18 adult female Wistar rats were randomly divided into three groups. Control group received normal saline and experimental groups received two different doses of crocin (100 and 200 mg/kg) every two days for 30 days. After the treatment period, blood samples were obtained from the heart and centrifuged. Next, the serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), estrogen and progesterone hormones were measured by ELISA assay. The ovarian tissues were removed and fixed for histological investigation. The hypothalamic *Kiss-1* gene expression was measured using real-time polymerase chain reaction (PCR). All data were analyzed using one-way ANOVA.

Results: A significant reduction (P=0.038) in the number of atretic graafian follicles (0.5 ± 0.31) was observed in rats treated with 200 mg/kg crocin. In addition, estrogen concentration in experimental groups (35.04 ± 0.85 and 36.18 ± 0.69 in crocin 100 and 200 mg/kg groups, respectively) compared to control group (38.35 ± 0.64) and progesterone concentration in rats treated with crocin 200 mg/kg (2.06 ± 0.07) compared to control group (2.16 ± 0.04), significantly decreased. Interestingly, relative expressions of Kiss-1 mRNA significantly decreased in experimental groups (0.00053 ± 0.00051 and 0.0011 ± 0.00066 in crocin 100 and 200 mg/kg groups, respectively) (P=0.000) compared to control group (1 ± 0).

Conclusion: Crocin, at hypothalamic level, reduces *Kiss-1* gene expression and it can prevent follicular atresia and reduce serum levels of estrogen and progesterone.

Keywords: Crocin, Folliculogenesis, Gonadal Steroid Hormones, Gonadotropins, *Kiss-1*

Citation: Zohrabi D, Parivar K, Sanati MH, Hayati Roodbari N. Effects of crocin on the pituitary-gonadal axis and hypothalamic Kiss-1 gene expression in female wistar rats. Int J Fertil Steril. 2018; 12(1): 56-60. doi: 10.22074.ijfs.2018.5139.

Introduction

Hypothalamic-pituitary-gonadal axis (HPG axis) has an important role in hormonal regulation of reproductive system. Disruption of this axis can have unpleasant consequences on fertility (1). Kisspeptin, also known as metastin, is a hypothalamic peptide encoded by the *Kiss-1* gene, which was first discovered as a metastasis inhibitor in melanoma cell lines (2). Recent studies have shown that the *Kiss-1* gene is also a key regulator of female gonadotropic axis in mammals (3) and is required for follicular development and ovulation during reproduction (4).

In rodents' central nervous system, Kisspeptin expressing neurons were found in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) of the hypothalamus (5). Kisspeptin neurons send projections to gonadotropin-releasing hormone (GnRH) cell bodies, regulate the secretion of GnRH (6) and thereby control the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (7). Based on its role in sex organ development and the HPG-axis, kisspeptin neurons dysfunction can lead to abnormal fetal development and infertility (8).

Medicinal plants like *Pongamia pinnata*, *Trachyspermum ammi* and *Semecarpus anacardium* have shown to be able to improve fertility and resolve hormonal imbalances (9) while *Trigonella foenum, Carum carvi, Achyranthes aspera*, and *Rivea hypocrateriformis* have contradictory effects (10). So far, four types of phytochemicals including carotenoids, flavonoids, terpenoids and curcumins have been reported to be responsible for phytochemical activities of herbal drugs (11). Crocin is a carotenoid that is found in saffron (*Crocus sativus* L.). Saffron is traditionally has been used as a coloring or flavoring

Received: 9 Dec 2016, Accepted: 5 Feb 2017
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agent, as well as a herbal medicine (12). Moreover, previous studies have introduced crocin as an antioxidant (13), anticancer (14) and tumoricidal (15), anti-inflammatory (16), antinociceptive, antidepressant (17), and antianxiety agent (18). Crocin has been used as an effective treatment against Alzheimer's disease (19), atherosclerosis (20), hyperlipidemia and hypertension (21). Considering various pharmacological properties of crocin, this study aimed to evaluate the effects of this phytochemical on female reproductive functions in Wistar rats.

Materials and Methods

All aspects of animal care complied with the ethical guidelines and technical requirements approved by the Institutional Animal Ethics Committee. In this study, after two-week adaptation period, 18 virgin adult female Wistar rats were maintained under standard laboratory conditions. Rats (160-180 g) were housed under controlled lighting (12 hours light and 12 hours dark) at 20 ± 2°C and had free access to food and water. Synchronization of estrus in rats was performed using estradiol valerate and progesterone. Estrous cycle was monitored by vaginal smears.

In this experimental study, 18 Rats were randomly divided into three groups as follows: control group received 2 ml normal saline, experimental group I received crocin 100 mg/kg body weight (BW) intraperitoneally (Pharmaceutical Research Center, BuAli Research Institute, Mashhad University of Medical Sciences, Mashhad, Iran) and experimental group II received crocin 200mg/kg BW intraperitoneally every two days for 30 days (22).

Hormonal assay

After the treatment period, animals in each group were anesthetized by ketamine/xylazine (k,80-100 mg/kg, X, 10-12.5 mg/kg) and blood samples were collected from their hearts. Blood samples were centrifuged for 10 minutes at 8000 rpm and the serum were separated and stored at -20°C. ELISA technique was used for evaluation of FSH, LH (commercial kits purchased from Pishtaz Teb Co., Iran), estrogen and progesterone hormones (commercial kits purchased from DRG Co., Germany).

Histological investigation

The left ovary of animals were removed and fixed in 10% formalin solution. The specimens were processed through routine paraffin embedding method. Subsequently, 6 µm serial paraffin sections were stained with haematoxylin and eosin (H&E). The total number of sections was counted and the middle section of the ovary was determined. The follicles were counted in 5 sections per ovary which included the middle section and 4 sections from either side of the center. Ovarian follicle counting was performed (23) and atretic graafian follicles were identified (24) and counted using a light microscope (Olympus IX71, Japan).

Evaluation of *Kiss-1* gene expression by quantitative real-time polymerase chain reaction

Brains were removed immediately from the skull. The hypothalami were dissected and frozen at -80°C. These tissues were thoroughly homogenized. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Germany) and RNA concentration was determined by Nano Drop ND-1000. cDNA synthesis was performed using 2 µg of total RNA and QuantiTect Reverse Transcription Kit (Qiagen, Germany). The quality of synthesized cDNA was assessed by polymerase chain reaction (PCR) using *β-actin* gene. PCR products were qualified by electrophoresis on 1% agarose gel. Primers were designed by Primer premier 5 (Premier Bio Soft International, Palo Alto, CA, USA) software for the reference gene, *Kiss-1*. The rat *β-actin* gene was used as the reference gene for data normalization (Table 1).

Table 1: Primers used for real-time quantitative polymerase chain reaction

Primer	Primer sequencing (5´-3´)	Product length (bp)	Annealing temperature (°C)
Kiss-1	F:TGCTGCTTCTCCTCTGTG R:ACGAGTTCCTGGGGTCC	106	59
β-actin	F:CCATCTATGAGGGTTACGC R:TGTAGCCACGCTCGGTC	105	60

Real-time PCR was performed in a thermal cycler Rotor gene 6000 (Corbett, AUS). The PCR mixture for each reaction contained 5 µl SYBR premix Ex Taq II, 0.5 µl of each primer (5 pmol/µl) and 50 ng cDNA adjusted to a final volume of 10 µl using DEPC water. All reactions were carried out in triplicate. The real-time PCR protocol included 5 minutes at 95°C followed by 40 repetitive cycles for 10 seconds at 95°C, 30 seconds at 60°C and 61°C for *Kiss-1* and 30 seconds at 72°C. The expression level of *Kiss-1* mRNA was normalized against *β-actin* expression level as a housekeeping gene. The relative expression of *Kiss-1* gene was assessed using the ΔΔCt method and results were demonstrated as $2^{-\Delta\Delta Ct}$ based on previous reports (25, 26).

Statistical analysis

The results were statistically analyzed using SPSS 19 software. Mean ± SD was calculated for each parameter and differences among means were evaluated by ANOVA followed by the Tukey post-hoc test using the Excel computer-based program. $P<0.05$ were considered statistically significant.

Results

There was no significant differences in FSH hormone levels between experimental groups (0.33 ± 0.042 for crocin 100 mg/kg, P=0.158 and 0.34 ± 0.073 for crocin 200 mg/kg, P=0.302) and control group (0.36 ± 0.008). In addition, administration of crocin 100 and 200 mg/kg doses had no effect on LH levels in female rats ((0.66 ± 0.026, P=0.120)

and (0.67 ± 0.032), P=0.350, respectivly) compared to control groups (0.69 ± 0.019) (Fig.1). However, both doses of crocin significantly decreased serum estrogen levels (35.04 ± 0.85 for crocin 100 mg/kg, P=0.000) and (36.18 ± 0.69 for crocin 200 mg/kg, P=0.000) compared to the control group (38.35 ± 0.64) (Fig.2). Also, progesterone concentrations significantly reduced in rats received 200 mg/kg crocin (2.06 ± 0.07, P=0.009) compared to control group (2.16 ± 0.04) (Fig.1). The expression of Kiss-1 was significantly (P=0.000) reduced following treatment with crocin 100 and 200 mg/kg (Table 2). We also assessed the mean number of primordial, primary, growing, graafian and atretic graffian follicles and corpora lutea among rats treated with crocin 100 and 200 mg/kg compared to the control group. Apart from the number of atretic graafian follicles that was significantly lower in the rats treated with crocin 200 mg/kg (0.5 ± 0.31) compared to the control group (1.33 ± 0.45), we did not find any significant differences in the mean number of other follicles (Table 3). Figure 3 shows images of ovarian tissue in the control group and rats treated with crocin 200 mg/kg.

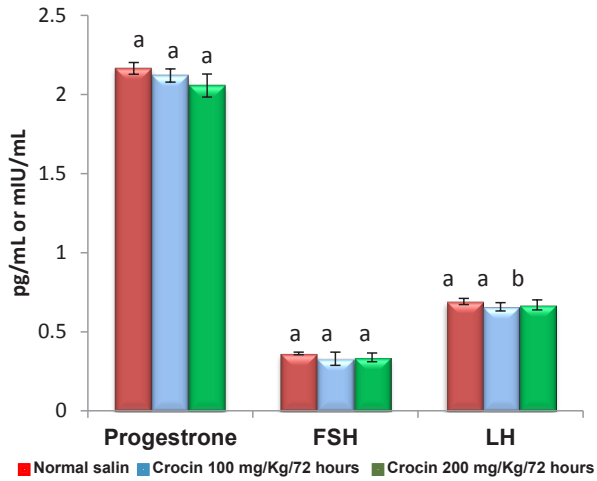

Fig.1: Comparison of serum FSH, LH, and progesterone levels among the experimental and control groups. Bars labeled with different letters are significantly different from each other at P<0.05. FSH; Follicle-stimulating hormone and LH; Luteinizing hormone.

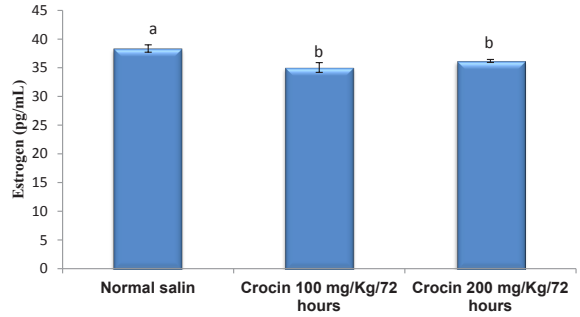

Fig.2: Comparison of serum estrogen level among the experimental and control groups. Significant differences were observed between groups treated with Crocin and control group (P<0.05).

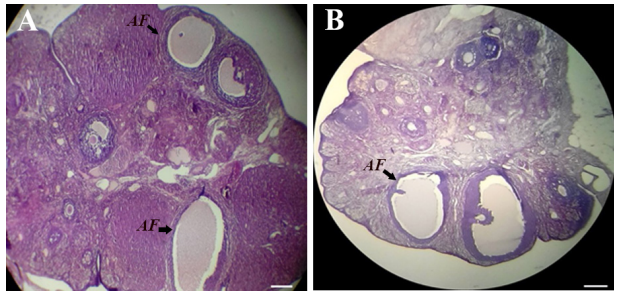

Fig.3: The ovarian tissue. Photomicrographs of ovary tissue (6-µm thick sections were stained with H&E; X100) in **A.** The control animals and **B.** Rats treated with 200 mg/kg crocin. AF; Atretic graafian follicle (scale bar: 100 µm).

Table 2: Mean hypothalamic *Kiss-1* gene expression in the experimental and control groups

Group	RF *Kiss-1* (mean ± SD)	P value compared to control group
Control	1 ± 0	-
Crocin (100 mg/kg)	0.00053 ± 0.0005[a]	0.000
Crocin (200 mg/kg)	0.0011 ± 0.0007[a]	0.000

All data were presented as mean ± SD.
[a]; Indicates a significant difference between experimental groups and control group (P<0.001).

Table 3: Mean number of primordial, primary, growing, atretic graafian follicles, graafian follicles and corpora lutea in the ovaries of rats in the experimental and control groups

Variable	Control	100 mg/kg/72 hours	200 mg/kg/72 hours	P value compared to control group
Primordial follicles	8.5 ± 1.169	5.8 ± 0.98	5.8 ± 1.17	0.052 0.056
Primary follicles	2.17 ± 1.169	3.17 ± 0.408	1.33 ± 0.516	0.097 0.183
Growing follicles	1.67 ± 0.516	1.17 ± 0.408	1.17 ± 0.408	0.163 0.163
Graafian follicles	1.5 ± 0.837	1.17 ± 0.753	1.83 ± 0.753	0.745 0.745
Atretic Graafian follicles	1.33 ± 0.448	1.33 ± 0.448	0.5 ± 0.31[a]	1 0.038
Corpora lutea	2.5 ± 0.548	4 ± 1.549	4 ± 1.265	0.109 0.109

All data were presented as mean ± SD.
[a]; Indicates a significant difference between Crocin 200 mg/kg-treated group and control group (P<0.05).

Along with other hypothalamic factors, the *Kiss-1* gene regulates the release of GnRH and thereby controls the release of FSH and LH from the pituitary gland. FSH and LH control ovarian folliculogenesis .Therefore, an alteration in this axis may affect sex-related endocrine hormones and follicular development. In this study, we evaluated the effect of crocin on these parameters. The results revealed no significant differences in serum concentrations of FSH and LH, but the levels of estrogen following treatment with both doses of crocin and the level of progesterone following administration of crocin 200 mg/kg significantly reduced.

Considering the fact that estrogen is secreted by follicular cells in the ovary, this may explain a role for crocin at the ovarian level. In this regard, previous studies have shown that carotenoids reduce the activity of cytochrome p450, thus inhibiting the transformation of cholesterol to pregnenolone, and consequently reducing the amount of estrogen. This effect of carotenoids is believed to be mediated by reduced expression of the *CYP19* gene which encodes an aromatase belonging to the cytochrome P450 family (27, 28). In addition, it has been shown that crocin reduces plasma levels of total cholesterol in a dose-dependent manner (29). Therefore, reducing estrogen and progesterone levels reported in the present study could be due to a reduction in cholesterol levels.

Considering the reduction in estrogen, an increase in *Kiss-1* gene expression is expected. It is well established that *Kiss-1* gene expression is negatively regulated by circulatory estrogen. In contrast to this expectation, the relative expression of *Kiss-1* gene was significantly down-regulated by crocin; the underlying reason(s) for this observation should be investigated in future studies. Despite reduction in *Kiss-1* gene, we observed no changes in FSH and LH levels. These observations suggest that reduced expression of *Kiss-1* gene is likely counterbalanced by other mechanisms controlling GnRH release and thereby pituitary FSH and LH secretion. Previous study have shown that some parameters such as NPY, GABA, Glutamate, etc. affect GnRH neurons and reproductive axis (30).

Also, differential action of estrogen on kiss-1 neurons in the AVPV and ARC of the hypothalamus, may explain our results. Estrogen increases *Kiss-1* gene expression in the AVPV while it reduces the expression of *Kiss-1* gene in the ARC. Hypothetically, based on this mechanism, one might expect crocin to reduce the expression of *Kiss-1* gene in ARC but not in AVPV nucleus. However, we believe that this is very unlikely and further research is required to clarify mechanisms via which crocin reduces *Kiss-1* gene expression.

Following hormonal evaluation, we assessed histological sections for any alteration in folliculogenesis. The results revealed no significant changes in the mean value of the number of different follicles between the control and treated groups except for a reduction in the number of atretic graafian follicles which were significantly reduced in crocin 200 mg/kg-treated group. This observation may be related to the anti-apoptotic effect of carotenoids, like crocin. Carotenoids up-regulate the expression of *Cx43* (31), which is dominantly expressed in granulosa cells and maintains the integrity of the follicle thus reducing follicular apoptosis (32). In this regard, previous studies indicated that crocin can inhibit apoptosis via increasing *Bcl2/Bax* expression ratio (33, 34).

Another process involved in the generation of atretic follicles is excessive production of reactive oxygen species (ROS). Assimopoulou et al. (13) showed that crocin has a marked radical-scavenging activity. In this regard, Soeda et al. (35) reported that crocin inhibits oxidative stress-induced cell death via a glutathione (GSH)-dependent mechanism. Also, Hosseinzadeh et al. (36) showed that crocin decreases malondialdehyde (MDA) generation. The role of crocin as an anti-apoptotic agent is well established in different systems (37, 38).

As ovarian follicles synthesize estrogen, one may expect that a decrease in the number of atretic follicles may result in an increase in estrogen production, which is contrary to results showing a reduction in estrogen levels. This effect might be due to a reduction in aromatase activity induced by crocin. The reduction in estrogen might be possibly due to crocin effect on the ovary rather than on the hypothalamus, as crocin had no effects on FSH and LH levels.

Based on the literature, crocin can induce the expression of genes like *XBP, BiP, CHOP, F4/80, TNF-α, NOS-2, IFN-α* (39), *Mmp-9, Cox-1, Cox-2, Bcl-2,* and *Bax* (40). In our study, crocin reduced the expression of the *Kiss-1*gene which might be a result of crocin interaction with factors involved in regulation of gene expression.

Conclusion

Our results revealed that at the hypothalamic level, crocin reduces *Kiss-1* gene expression; however, reduced *Kiss-1* gene expression did not affect sex-related hormones, indicating that other mechanisms may have counterbalanced this reduction. At the ovarian level, crocin acts as an anti-apoptotic agent and reduces follicular atresia. Crocin may also indirectly reduce aromatization via regulating genes involved in this process. Overall, these data suggest that crocin may interfere with factors regulating gene expression and this hypothesis needs further investigations.

Acknowledgements

This work was financially supported by Science and Research Branch, Islamic Azad University, Tehran, Iran. There is no conflict of interest in this article.

Author's Contributions

K.P., M.H.S., N.H.R.; Contributed to conception and design. D.Z.; Performed data collection and evaluation, drafting and statistical analysis. All authors performed editing and approving the final version of this paper for

submission, also participated in the finalization of the manuscript and approved the final draft.

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Original Article

Comparison of The Effects of Vitrification on Gene Expression of Mature Mouse Oocytes Using Cryotop and Open Pulled Straw

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Abstract

Background: Oocyte cryopreservation is an essential part of the assisted reproductive technology (ART), which was recently introduced into clinical practice. This study aimed to evaluate the effects of two vitrification systems-Cryotop and Open Pulled Straw (OPS)-on mature oocytes gene expressions.

Materials and Methods: In this experimental study, the survival rate of metaphase II (MII) mouse oocytes were assessed after cryopreservation by vitrification via i. OPS or ii. Cryotop. Then we compared the fertilization rate of oocytes produced via these two methods. In the second experiment, we determined the effects of the two vitrification methods on the expression of *Hspa1a, mn-Sod*, and *ß-actin* genes in vitrified-warmed oocytes. Denuded MII oocytes were vitrified in two concentrations of vitrification solution (VS1 and VS2) by Cryotop and straw. We then compared the results using the two vitrification methods with fresh control oocytes.

Results: *mn-Sod* expression increased in the vitrified-warmed group both in OPS and Cryotop compared with the controls. We only detected *Hspa1a* in VS1 and control groups using Cryotop. The survival rate of the oocytes was 91.2% (VS1) and 89.2% (VS2) in the Cryotop groups (P=0.902) and 85.5% (VS1) and 83.6% (VS2) in the OPS groups (P=0.905). There were no significant differences between the Cryotop and the OPS groups (P=0.927). The survival rate in the Cryotop or the OPS groups was, nevertheless, significantly lower than the control group (P<0.001). The fertilization rates of the oocytes were 39% (VS1) and 34% (VS2) in the Cryotop groups (P=0.902) and 29 %(VS1) and 19.7% (VS2) in the OPS groups (P=0.413). The fertilization rates were achieved without significant differences among the Cryotop and OPS groups (P=0.755).

Conclusion: Our results indicated that Cryotop vitrification increases both cooling and warming rates, but both Cryotop and OPS techniques have the same effect on the mouse oocytes after vitrification.

Keywords: Cryotop, Gene Expression, Oocyte, Vitrification

Citation: Amidi F, Khodabandeh Z, Nori Mogahi MH. Comparison of the effects of vitrification on gene expression of mature mouse oocytes using cryotop and open pulled straw. Int J Fertil Steril. 2018; 12(1): 61-67. doi: 10.22074/ijfs.2018.5112.

Introduction

Oocyte cryopreservation is an essential part of the assisted reproductive technology (ART), which has been recently introduced into clinical practice. Additionally, this method is useful for the preservation of female genetic resources through oocyte banking (1, 2). The cryopreservation of the mammalian oocyte has proven to be more difficult than other cell types because of its sensitivity towards ice crystal formation and the sensitivity of meiotic spindle to changing temperature during the process of freezing and thawing (3). The freezing and thawing cause meiotic spindle destruction; therefore, it is essential to incubate the oocytes for 3-5 hours post-warming. Then, the meiotic spindle can regenerate (4, 5). Vitrification is a practical method that produces a glass-like solidification of the cells by rapid cooling and high concentrations of cryoprotective agents (CPAs). Consequently, this method can decrease the formation of ice-crystals and cell injury (6, 7).

Different types of cryoprotectants are used for vitrification protocols, including ethylene glycol (EG), dimethyl sulfoxide (DMSO), and 1, 2-propanediol (PrOH). EG is a common CPA that is used for oocyte vitrification. DMSO and PrOH are used regularly as permeating CPAs to cryopreserve oocytes and embryos to prevent the intra-cellular ice crystal formation. The combinations of CPAs can decrease the concentration of each CPA, as well as diminishing the toxic effects of CPA on the oocytes (8, 9). Non-penetrating CPAs, such as sucrose, are often used in combination with other

Received: 30 Oct 2016, Accepted: 2 Jul 2017
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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 1, Apr-Jun 2018, Pages: 61-67

permeating CPAs to prevent ice crystal formation and decease the CPA toxicity (10).

There are many vitrification devices that increase the cooling rate, such as cryoloop, solid surface (11), Cryotop (12), and open pulled straw (OPS) (13). The Cryotop consist of a hard plastic and a fine thin film-strip (14). The minimum amount of vitrification solution (~0.1 µl) remaining in Cryotop is in direct contact with liquid nitrogen during cooling. As a result, ice crystal formation is prevented due to dramatically increased cooling rate (12). OPS vitrification is another popular method for human oocyte and embryo vitrification (15-17). OPS has a small effect for reducing the volume of vitrification solution to 0.5 µl and thus increasing the cooling rate (18). In recent studies, the advantages of Cryotop was compared with OPS in different species, including pig (19), human (20), and matured bovine oocytes (15). However, additional information is required to identify the effect of these two devices on mouse oocytes (21).

Other studies have reported that the structural and morphological injures occur in the vitrified-warmed oocytes. These include zona hardening, variation in selective permeability of plasma membrane, aneuploidy, and nuclear fragmentation (8, 6, 22). Vitrification may also result in changes at the molecular level in vitrified oocytes. Heat shock protein *(Hsp) a1a* and the manganese super oxide dismutase *(mn-Sod)* are two critical genes related to stress. Hsps play a protective function against heat, stress response, or both in cellular auto-regulation. The critical role of *Hspa1a*, as a defensive protein resulting from external stress, has been proven. It is confirmed that knock-out Hsp70.1 mice have higher sensitivity to osmotic stress after preconditioning them with heat (23, 24).

Hut et al. (25) showed that *Hspa1a* has a protective effect on the mitotic cell cycle against heat-induced centrosome damage, preventing chromosomal division. *Mn-Sod* is an anti-oxidant enzyme that protects the oocytes and embryos against the oxidative stress damages. It was stated that adding antioxidant enzymes such as catalase or *Sod1 (Cu-Zn-Sod)* to culture media leads to an improved rate of blastocyst formation in rabbit (26), and mouse (21). Sonna et al. (27) reported that cold stress can influence the expression of genes associated with stress (stress-response genes).

In this study, the effect of vitrification protocols on the oocyte's gene expression was investigated using mature mouse oocytes. Hence, the efficiency of the two vitrification methods (OPS vitrification to Cryotop method) was compared on fertilization percentage, morphological survival, and gene expression of *Hspa1a* and *mn-Sod* in the mouse oocytes.

Materials and Methods

The present experimental study was conducted using mouse oocytes and sperm. The study protocol was approved by the Research Ethics Committee of Tehran University of Medical Sciences. All chemicals and media were purchased from Sigma-Aldrich Co (St.Louis, Mo, USA), unless otherwise mentioned.

Experimental design

The fertilization rate of metaphase II (MII) mouse oocytes was assessed after cryopreserving by vitrification using: i. OPS or ii. Cryotop. In the second experiment, we determined the effects of two vitrification methods on the oocytes gene expression.

Experiment 1

Mature oocytes were randomly selected and distributed amongst three experimental groups (OPS, Cryotops, and controls). All vitrification groups were divided into VS1 (10% v/v cryoprotectants) and VS2 (14.5 %v/v cryoprotectants) subgroups and a total of 119 and 114 were OPS-vitrified in VS1 and VS2. Also, 135 and 136 were cryotop-vitrified in VS1 and VS2; finally, 136 oocytes were used as controls. After vitrification and warming, the oocytes in all groups were fertilized and cultured *in vitro*.

Experiment 2

The oocytes were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) to evaluate changes in *Hsp70* and *mn-Sod* expression in all groups.

Oocyte collections

Female NMRI mice aged 8 to 10 weeks were kept under 12 hours of light/dark condition. The female mice were superovulated by intraperitoneal (i.p.) injection of 10 IU pregnant mare's serum gonadotropin (PMSG), followed by i.p. injection of 10 IU human chronic gonadotropin (hCG) 48 hours later. The mice were sacrificed by cervical dislocation 13-15 hours post-hCG administration (6). The cumulus-oocyte complex (COC) were collected from the oviduct and oocytes denudation were performed using 300 µg/ml hyaluronidase in hepes-buffered TCM199 for 30 seconds. The normal mature oocytes were selected with first polar body, intact zona pellucida, and plasma membrane.

Preparation of vitrification and dilution solution

TCM199 supplemented with 20% fetal bovine serum (FBS) were used as a base medium. The first vitrification solution (VS1) consisted of 10% EG, 10% DMSO, and 0.5 M sucrose in the base medium (21). The second vitrification solution (VS2) was contained 14.5% EG+14.5% PrOH and 0.5 M sucrose in the base medium. The equilibration solution included (ES1) 5% EG and 5% DMSO without sucrose in the base medium and the second equilibration solution (ES2) contained 7.25% EG+7.25% PrOH without sucrose in the base medium. Warming solution (WS) contained 1 M sucrose in the base medium, and diluents' solution (DS) contained 0.5 M (DS1) and 0.25 M

(DS2) sucrose, respectively. All vitrification process steps were performed at room temperature (25°C) (13, 15).

Occyte vitrification/warming

The COC were isolated from 32 female mice by simple random sampling. Then, the denuded MII oocytes were vitrified in two concentrations of VS1 and VS2 by Cryotop and OPS (13). Oocytes at VS1, VS2, and control groups were exposed to the first equilibration drop for 3 minutes and then the first drop was merged with adjacent ES drop. Subsequently, the oocytes were incubated in vitrification solution, VS1 and VS2, each one for less than 1 minute. Every five oocytes were quickly loaded on the top of per Cryotop (Kitzato, Ltd, Japan, Cryotop group) or loaded into OPS. Excess media were carefully removed around the oocyte in the Cryotop and then immediately submerged in liquid-nitrogen (LN2). OPS was also sealed and plunged directly into LN2. The oocytes were stored in LN2 for 7 days.

During warming, the Cryotop was immediately inserted into WS at 37°C for 1 minute (Cryotop group) or the straw was taken out and immersed into 37°C water for 30 seconds. The straw end was cut and its contents were transformed into a drop of 1 M sucrose (straw group). Then, the oocytes were placed onto decreasing sucrose concentrations (DS1 and DS2) to remove cryoprotectants, for 3 minutes each. Finally, the warmed oocytes were washed twice in the base medium using WS, each time for 5 minutes (Fig.1). We assessed the survival rates of vitrified-warmed oocytes on the basis of normal appearing zona pellucida and intact polar body (Fig.2). After warming, groups of 15 oocytes were stored at -80°C in Tripure isolation reagent for RNA extraction and groups of 15 oocytes were also incubated in the base medium before *in vitro* fertilization (IVF).

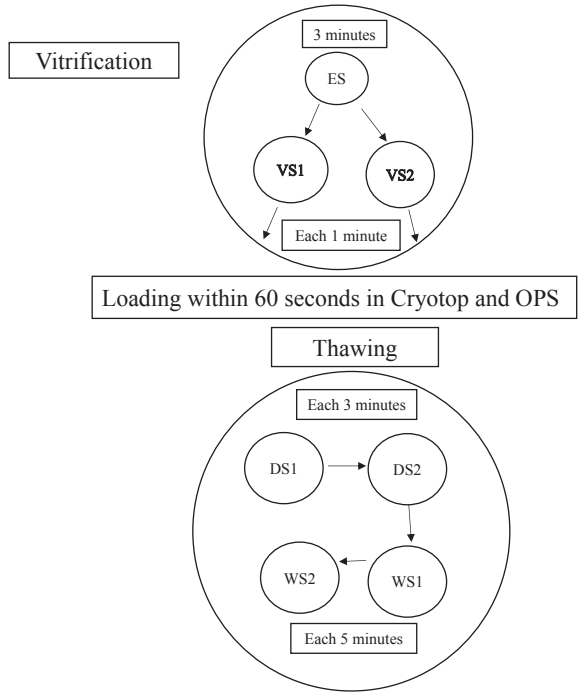

Fig.1: A schematic of vitrification and warming procedure.

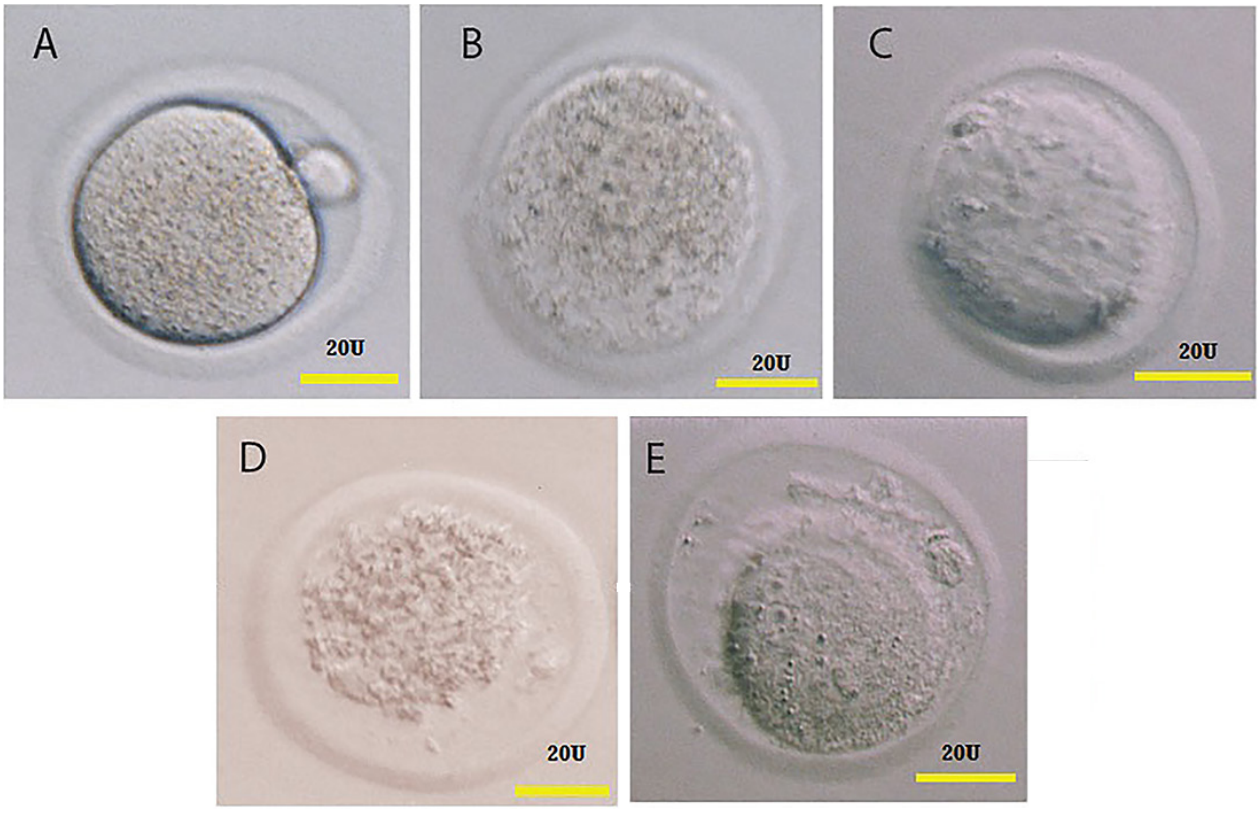

Fig.2: Morphology of vitrified MII oocytes after warming. Oocyte vitrified in two vitrification solution (VSI and VSII) by OPS and Cryotop. **A.** Control, **B.** VSI, Cryoptop, **C.** VSII, Cryotop, **D.** VSI, OPS, and **E.** VSII, OPS 20 U means 20 micron).
MII; Metaphase II, VS; Vitrification solution, and OPS; Open Pulled Straw.

Table 1: The Primer sequences for reverse transcription-polymerase chain reaction

Gene	Gene bankaccession number	Primer sequencing (5´-3´)	Annealing temperature (ºC)	Location	Size bp
ß-actin	NM_001101	F: tcatgaagatcctcaccgag R: ttgccaatggtgatgacctg	60	650-839	190
Sod2	NM_001024466.1	F: ggaagccatcaaacgtgact R: ccttgcagtggatcctgatt	55	237-398	161
Hspa1a	ENST00000375651	F: cgacctgaacaagagcatcaac R: tgaagatctgcgtctgcttggt	59	668-862	194

In vitro fertilization

The vitrified/warmed oocytes with intact zona pellucida, intact plasma membrane plus homogeneous cytoplasm were chosen and placed in 200 µl drops of IVF medium [human tubal fluid (HTF)+15 mg/ml bovine serum albumin (BSA)] layered under mineral oil (Sigma, 8410). The medium was prepared earlier to equilibrate and incubated at 37°C in 5% CO_2 for 2 hours. A suspension of epididymal spermatozoa was prepared and the sperms were capacitated in the medium (HAM's F10+4 mg/ml BSA) at 37°C in 5% CO_2 for 45-60 minutes. A final concentration of 2×10^6 spermatozoa/ml was added to IVF medium containing 15 oocytes and incubated at 37°C in 5% CO_2 for 6 hours. Finally, the oocytes that developed into pronuclear stage were used for fertilization.

RNA isolation and reverse transcription

Total RNA was extracted from the vitrified and non-vitrified oocytes. A number of oocytes were lysed with Tripure isolation reagent (Roche, Germany), according to the manufacturer's instructions. The concentration and purity of the extracted RNA were determined by ND-1000 spectrophotometer (Nanodrop, USA). To synthesize cDNA, we used 300 ng/µl of total RNA and cDNA Synthesis Kit (Bioneer, South Korea) by following the manufacturer's protocols.

Polymerase chain reaction

RT-PCR was performed using Taq polymerase enzyme (Roche). Reactions (25 µl) contained 1 µl of each primer mix, 2 µl dNTP, 2.5 µl 10X buffer with $MgCl_2$, 0.3 µl rTaq polymerase enzyme, 1 µl cDNA, and 18.2 µl DEPC water in every well. The initial denaturation step was 3 minutes at 94°C and then denaturation in each cycle was 30 seconds at 94°C. Then annealing was done for 30 seconds at 55°C for *mn-Sod*, and 59°C for *Hspa1a* and it was extended for 1 minute at 72°C. Expression of *β-actin* housekeeping gene was used as a reference for the level of target gene expression.

PCR primers were designed using primer 3 software based on mouse DNA sequences found in the Gene Bank (NCBI) (Table 1) (28). The primers were placed into BLAST search to examine the aligned sequences for polymorphisms and avoided these regions for primers or probe design. RT-PCR products were electrophoresed on a 2% agarose gel. After stained by ethidium bromide (Cina Gene), the products were then visualized under ultraviolet. The no template control (NTC) includes all the RT-PCR reagents except that the template was considered as a negative control. A run on 2% agarose and no DNA band was also visualized (data was not shown).

Statistical analysis

Oocyte survival and fertilization rates were analyzed by SPSS version 16 software package. All percentages of values were subjected to arcsine transformation prior to analysis. All data were expressed based on mean ± SEM. The level of statistical significance was set at $P<0.05$.

Results

Vitrification and *in vitro* fertilization

The survival of the vitrified/warmed oocytes were assessed according to their morphology in the control, the Cryotop, and the OPS groups (Fig.2). There was no difference in oocyte survival between the VS1 group and the VS2 group when using the Cryotop method. Similarly, there was also no significant difference in oocyte survival between the VS1 and the VS2 group when oocytes were vitrified by the OPS method ($P=0.905$). There were also no significant differences in oocyte survival between oocytes vitrified by the Cryotop and the OPS methods within the same vitrification solution group ($P=0.927$). The survival rate in the Cryotop or the OPS groups was, nevertheless, significantly lower than the control group ($P<0.001$).

The results showed a significant reduction in the fertilization rate of each group in comparison with the control ($P<0.05$). There is also no significant difference in oocyte fertilization between the VS1 and the VS2 group when oocytes were vitrified by Cryotop ($P=0.902$). There is also no significant difference in oocyte fertilization between the VS1 and VS2 the group when oocytes were vitrified by OPS method ($P=0.413$). The fertilization rates were achieved without significant differences among the Cryotop and the OPS groups ($P=0.755$, Table 2).

Table 2: Effects of two different vitrification solutions on the survival and the fertilization rate of the MII oocytes

	Survival Mean ± SEM			Fertilization Mean ± SEM		
Device	**Control**	**Cryotop**	**OPS**	**Control**	**Cryotop**	**OPS**
Control	100 ± 0.001			88.0 ± 2.3 (131/136)		
VS1		91.2 ± 6.7	85.5 ± 1.2		39.0 ± 5.8 (58/135)	29.2 ± 2.4 (57/119)
VS2		89.2 ± 6.1	83.6 ± 1.19		34.0 ± 5.7 (48/133)	19.7 ± 2.3 (49/114)
P value		0.004			0.001	

Tukey's method was used for multiple comparisons. No significant differences were detected amongst the treatment groups (P<0.05). The experiments were replicated 3 times. MII; Metaphase II, VS; Vitrification solution, and OPS; Open Pulled Straw.

Gene expression analysis

Cryotop groups

The expression of all genes in the vitrified-warmed oocytes in Cryotop was compared to the control (Fig.3). RT-PCR was prepared to investigate the alternation in gene expressions. The abundance of mRNA declined in the oocytes as a by-product of the vitrification procedures, but the expression of *mn-Sod* increased in the vitrified-warmed oocytes in comparison with the control group. We also detected *Hspa1a* in the control and VS1 in the Cryotop group.

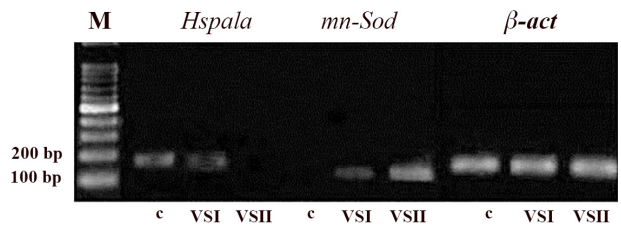

Fig.3: The expression of *Hspa1a* and *mn-Sod* genes was examined by reverse transcriptase- polymerase chain reaction; then, products run on 2 percent agarose gel (Cryotop groups). M; Marker, c; Control, and VS; Vitrification solution.

Open Pulled Straw groups

The expression of *Hspa1a* and *mn-Sod* was assessed in the OPS group and compared to the control group. The results presented in Figure 4 show that *Hspa1a* was expressed in the VS1, the VS2; and the control groups, but *mn-Sod* was expressed only in the VS1 and the VS2 groups.

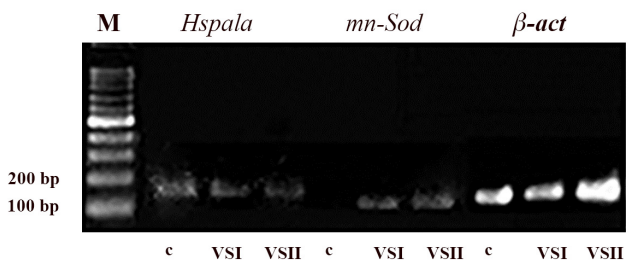

Fig.4: The expression of *Hspa1a* and *mn-Sod* genes was examined by reverse transcriptase- polymerase chain reaction; then, products run on 2% agarose gel [Open Pulled Straw (OPS) groups].
M; Marker, c; Control, and VS; Vitrification solution.

Discussion

In the present study, we observed that the Cryotop or the OPS changed the expression levels of a *Hsp70* family (*Hspa1a*), and an antioxidant enzyme (*mn-Sod*) in the vitrified-warmed MII-oocytes. The results showed that there were no a significant differences between the quality of the Cryotop and the OPS methods in the morphology and the fertilization rates in mouse MII oocytes. Significant decreases in the fertilization rate of the vitrified-warmed oocytes compared to the control in both the VS1 and the VS2 groups were observed regardless of the vitrification methods.

Optimal cryopreservation can be achieved by limiting the two essential factors in various vitrification protocols: chilling injury and ice formation (15). To minimize the chilling injury, the vitrification procedure can use high cooling rate. This can be achieved by minimizing the volume of vitrification solution and direct contact between the sample and liquid nitrogen. Furthermore, in the vitrification protocol, high concentrations of CPAs were used to avoid ice crystal formation, but the cytotoxicity and the osmotic stress were increased. Permeating cryoprotectants were used to prevent intracellular ice crystal formation. Therefore, the use of various CPAs combinations can be efficient in reducing the concentration and the individual-specific toxicity of each CPA (29).

Vitrification process can induce stress. Hence, it is critical to choose an appropriate approach in order to minimize oxidative, osmotic, and heat stress (23). In this study, we attempted to increase the cooling rate by using a minimum volume cooling method (Cryotop) or the OPS, and then compare them with each other. It has been demonstrated that a high cooling rate reduces the toxicity of high CPAs concentrations, thus minimizing the oxidative stress and also improving the efficiency of cryopreservation (18, 30). In this study, we compared the Cryotop and the OPS vitrification, two popular minimum volume vitrification methods that provide high cooling rates, for mouse oocyte cryopreservation. The results demonstrated that the efficacy of both methods to allow mouse oocytes to undergo normal fertilization after warming.

Cryotop vitrification has been a widely used method for oocyte vitrification. Previously, we reported that using the Cryotop vitrification with a mixture of 15% EG and 15%

DMSO is beneficial for vitrifying oocytes (30). Chian et al. (8) and Habibi et al. (31) also obtained a high survival rate of *in vitro* matured bovine oocytes vitrified by the Cryotop method using various combination of CPAs. In this study, oocytes vitrified by the Cryotop method resulted in a higher survival rates compared with those vitrified by OPS method. However, the differences were not significant. These results were in agreement with a previous report that compared the two vitrification methods (the Cryotop and the OPS) using calf and cow oocytes with different combinations of CPAs (15).

In addition to evaluating the effects of the vitrification methods on the oocyte viability, we also assessed the *Hsp70* and *mn-Sod* expression in the oocytes vitrified by the OPS or the Cryotop. Based on the works done on the animal models, reduced fertilization rate and low competency of the oocytes after warming may be associated with alternation in expressions of antioxidant enzymes and also hereditary factors in the oocytes (32, 33), as well as the toxicity of cryoprotectants. The development of the oocytes is dependent on the presence of specific transcripts (34).

The selected genes were involved in response to stress (*mn-Sod*, and *Hspa1a*). Changes in gene expression are considered as an integral part of cellular response to thermal stress. It is widely accepted that *Hsps*, whose expression is affected by heat shocks, are the best candidate. It was recently indicated that thermal stress can induce expression in a number of non-Hsps genes like *mn-Sod* (25, 29).

Hspa1a is a member of the inducible heat-shock family that can protect the oocytes against oxidative stress (35). In the present study, we only detected *Hspa1a* in the control and the VS1 group in the Cryotop groups, but *Hspa1a* was expressed in both the VS1 and the VS2 as well as the controls in the OPS groups. Boonkusol et al. (36) reported a similar result after vitrification with straw. The difference in gene expression observed in present study suggests that different vitrification methods may in affect the oocytes differently at the molecular level.

Oxidative stress may weaken the intracellular function and affect further development of the oocytes. Oxidative stress caused DNA instability in the mouse oocyte (37). Moreover, Bilodeau et al. (38) reported that during cryopreservation, the activity of Sod was reduced by 50% in bovine spermatozoa. Therefore, high expression of *mn-Sod* in the vitrified-warmed oocytes can be a defense mechanism against oxidative stress. In the present study, the expression of *mn-Sod* was increased in both the VS1 and the VS2 in the Cryotop and the OPS groups. We found that the survival rate and the developmental competence of the mouse MII oocytes after being vitrified both in 10% EG+10% DMSO mixture and 14.5% EG+14.5% PrOH in the Cryotop and the OPS groups showed the same effects.

Conclusion

Our findings confirmed that the Cryotop and the OPS both can be a good candidate in mouse oocytes vitrification. It is crucial to perform further studies focusing on the expression patterns of the genes involved in early differentiation stages.

Acknowledgements

This project was financially supported by the research deputy of Tehran University of Medical Sciences and Health services grant No. 88-01-30-8402. The authors would like to thank the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for their invaluable assistance in editing this article. The authors declared no conflict of interest.

Author's Contributions

F.A.; Study conception and design, analysis and interpretation of data, critical revision. Z.Kh.; Acquisition of data, analysis and interpretation of data, drafting of manuscript, critical revision. M.H.N.M.; Analysis and interpretation of data. All authors read and approved the final manuscript.

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Original Article

Combined Effect of Retinoic Acid and Basic Fibroblast Growth Factor on Maturation of Mouse Oocyte and Subsequent Fertilization and Development

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Abstract

Background: Many autocrine and paracrine elements that are produced within follicular niche have been the focus of much *in vitro* maturation (IVM) research. The present study was carried out to compare retinoic acid (RA) and basic fibroblast growth factor (bFGF) efficacy on IVM of mouse oocytes, and their further dual consumption to reach an optimal protocol.

Materials and Methods: In this experimental study, germinal vesicle (GV) oocytes obtained from two-months-old NMRI mice were randomly divided into control, sham and three experimental groups. The basic culture medium was α-MEM supplemented with 10% fetal bovine serum (FBS), 50 mg/l streptomycin, 60 mg/l penicillin and 10 ng/ml epidermal growth factors. Each of the experimental groups received one of the following treatments: RA (2 μM), bFGF (20 ng/ml) or combination of RA and bFGF with the indicated concentrations. After 24 hours, capacitated spermatozoa were added to in vitro matured oocytes. Five hours later, the oocytes were cultured in fresh droplets of M2 medium for 24 hours and assessed for cleavage to the two-cells stage.

Results: As compared with the control group, the rate of maturation was significantly increased in the RA ($P<0.001$) and bFGF+RA ($P<0.02$) groups with 58 ± 10 and 57 ± 3.46, respectively. The rate of maturation was significant in the RA ($P<0.02$) and bFGF+RA ($P<0.03$) groups, in comparison with the bFGF group. The bFGF+RA group had higher rate (83 ± 1.52) of two-cells development, than control (33 ± 1, $P<0.001$).

Conclusion: Our findings showed beneficial effects of 2 μM RA and 20 ng/ml bFGF combination on mouse oocyte IVM.

Keywords: Basic Fibroblast Growth Factor, *In Vitro* Maturation, Oocyte, Retinoic Acid

Citation: Abouzaripour M, Fathi F, Daneshi E, Mortezaee K, Rezaie MJ, Abdi M. Combined ffect of retinoic acid and basic fibroblast growth factor on maturation of mouse oocyte and subsequent fertilization and development. Int J Fertil Steril. 2018; 12(1): 68-71. doi: 10.22074/ijfs.2018.5293.

Introduction

In spite of great scientific breakthrough for *in vitro* maturation (IVM), the number of mature oocytes obtained from these methods and their fertilization rates is still too low. So, researchers are trying to achieve a superior approach for in vivo recapitulation of follicular environment. Thus far, many elements have been surveyed to assess oocyte maturation within follicular niche. Supplementation of maturation medium with various complements is a promising method. The active form of vitamin A, retinoic acid (RA), is an example of these complements involved in very initial events of mammalian reproduction, including follicular growth, oocyte maturation, embryonic growth and its development (1).

Positive effects of RA on IVM of cumulus oocyte complexes have previously been described (2-5). Fibroblast growth factors (FGFs) produced by theca and granulosa cells are involved in diverse biological processes during folliculogenesis, but the role of these factors during the ultimate period of oocyte maturation remained yet unknown (6). The present study was accomplished to survey the combined role of RA and bFGF in IVM of mouse oocytes to reach an optimal protocol. We propose that providing dual supplementation of maturation medium with RA and bFGF during IVM may probably be beneficial for oocyte maturation and the subsequent embryo development.

Materials and Methods

In this experimental study, the animals were kept under controlled conditions (12 hour light: 12 hour dark), fed with water ad libitum. All procedures were performed in accordance with the approval of the Institutional Animal Care and Use Committee at the Kurdistan University of Medical Sciences (MUK, Iran). All reagents were purchased from Sigma-Aldrich Co, USA.

Received: 6 May 2017, Accepted: 26 Jul 2017
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Table 1: Outcome of oocytes IVM in different groups

Group	GV numbersn	Arrested GV Mean ± SD	Degenerated GV Mean ± SD	GVBD Mean ± SD	MII Mean ± SD
Control	110	39.33 ± 2.08	10 ± 4	30.33 ± 1.52	31.66 ± 1.52
sham (ethanol)	120	41.33 ± 1.15	13.66 ± 2.30	34 ± 1.73	32.66 ± 0.57
bFGF	115	18.33 ± 1.52	12.33 ± 1.15	44.66 ± 3.21	40.66 ± 2.30
RA	125	16.33 ± 0.57	4.33 ± 0.57	47 ± 2	58 ± 1[$,@]
bFGF+RA	120	17.33 ± 2.30	6.33 ± 0.57	38.66 ± 1.15	57 ± 3.46[*,#]

*; P<0.03 vs. bFGF, #; P<0.02 vs. control, $; P<0.02 vs. bFGF, @; P<0.001 vs. control and sham, IVM; *In vitro* maturation, GV; Germinal vesicle, GVBD; GV break down, MII; Miosis phase II, bFGF; Basic fibroblast growth factor, and RA; Retinoic acid.

Collection of immature mouse oocytes

Animals were superovulated by an intraperitoneal injection of 10 IU pregnant mare's serum gonadotropin (PMSG). Mice were sacrificed 44 hours later by cervical dislocation and their ovaries were placed in α-MEM culture medium supplemented with 10% fetal bovine serum (FBS). Immature oocytes in the germinal vesicle (GV) stage were mechanically dissected using 26-G needles attached to a 1 ml syringe under a stereo microscope (Olympus, Japan). The collected GV-stage oocytes obtained from 2-months-old NMRI mice were randomly divided into control, sham and three experimental groups (7).

In vitro maturation

The collected GV-stage oocytes of each group were placed in 25 µl drops of maturation medium consisting of α-MEM supplemented with 10% FBS, 50 mg/l streptomycin, 60 mg/l penicillin and 10 ng/ml epidermal growth factors (EGF), and then they were incubated in a humidified atmosphere of 5% CO_2 at 37°C for 24 hours.

In the first experimental group, maturation medium was incubated with 2 µM RA dissolved in pure ethanol (8), and in the second experimental group, it was incubated with 20 ng/ml bFGF (9). In the third experimental group, combined RA and bFGF with the same concentrations was added to the maturation medium. In the sham group, 0.2% (v/v) ethanol was added to the maturation medium. After 24 hours, oocytes were observed under inverted microscope. Nuclear maturation of GV stage was determined by evaluation of morphological changes in the nucleus or appearance of the first polar body (MII). Matured oocytes were collected and used for *in vitro* fertilization (IVF).

In vitro culture and *in vitro* fertilization

Sperms of 12-weeks-old male NMRI mice were collected from the tail of epididymis. Sperm suspension (1×10^6 motile spermatozoa/ml) was capacitated for 1 hour in 500 µl human tubular fluid (HTF) culture medium. *In vitro* matured oocytes from each group were added to 100 µl droplets of HTF to which 0.1 ml of capacitated spermatozoa was added. After 5 hours of incubation, the oocytes were washed with three droplets of HTF medium and checked for appearance of the second polar body and formation of male and female pronuclei indicating fertilization. Then, oocytes were cultured in fresh droplets of M2 medium (25 µl) covered by mineral oil and assessed for cleavage to the two-cells stage after 24 hours (1).

Statistical analysis

Data were analyzed using One-way ANOVA with a post-hoc Tukey and presented as mean ± SD. The differences in the values of maturation, fertilization and developmental rates were considered significant at P<0.05. All computations were carried out using SPSS 16 for Windows.

Results

In vitro maturation of mouse oocytes

Development of oocytes from GV break down (GVBD) to two-cells stage has been shown in in the Figure 1. The maturation rate of cultured GV-stage oocytes was low in both control and sham groups with 31.66 ± 1.52 and 32.66 ± 0.57, respectively. As compared with the control group, the rate of maturation was significantly increased in the RA (P<0.001) and bFGF+RA (P<0.002) groups with 58 ± 1 and 57 ± 3.46, respectively. The rate of maturation was significant in the RA (P<0.02) and bFGF+RA (P<0.03) groups compared to the bFGF group (Table 1).

In vitro fertilization and development of mouse oocytes

Data from Table 2 showed that the bFGF+RA group had a higher rate 83 ± 1.52 (47.7%) of two-cells development, compared to the control 33 ± 1 (34%) (P<0.001). The number was significant in the bFGF+RA group in comparison with the bFGF (P<0.001, Table 2).

Table 2: The number and percentage of oocytes attaining the two-cells stage after 24 hours of culture

Group	Number of MII n	Number of two-cells stage Mean ± SD (%)
Control	95	33 ± 1 (34)
sham (ethanol)	65	20 ± 0.57 (30)
bFGF	122	51 ± 1 (41)[#]
RA	174	58 ± 0.57 (50)[*]
bFGF+RA	116	83 ± 1.52 (47.7)[*]

*; P<0.001 vs. bFGF, sham and control, #; P<0.001 vs. all groups, MII; Miosis phase II, bFGF; Basic fibroblast growth factor, and RA; Retinoic acid.

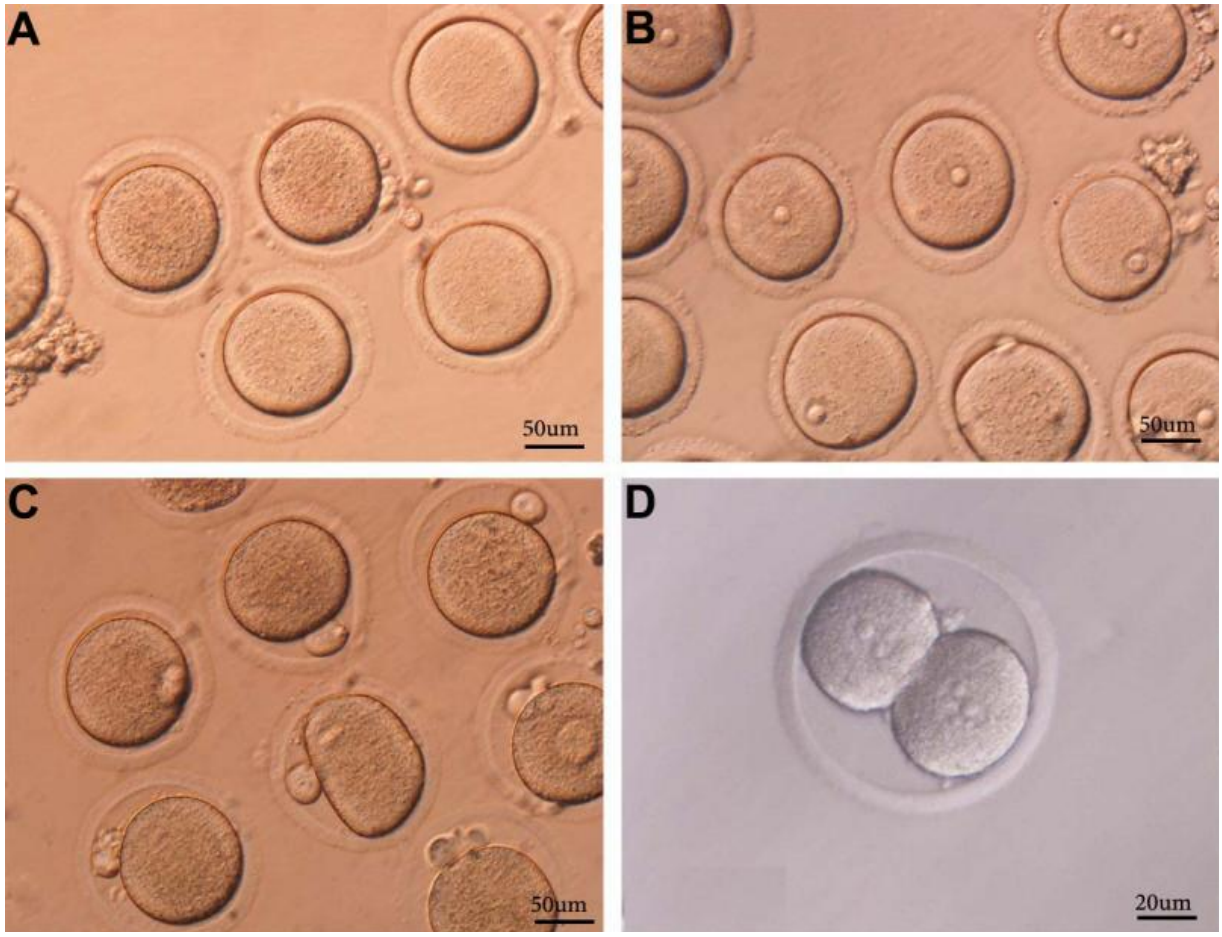

Fig.1: Oocytes in various stages of development. **A.** Germinal vesicle break down (GVBD), **B.** GV, **C.** Mature oocytes with polar bodies, and **D.** Two-cells stage.

Discussion

In the present survey, we compared the effect of RA and bFGF on maturation of mouse oocytes and their further development into two-cells stage. We found that separate usage of either RA or bFGF in basic culture medium could improve outcomes of IVM. Achieving an efficient culture system for IVM is an important criterion in reproductive research. The advantageous roles of retinol metabolites in *in vitro* cytoplasmic maturation and embryonic development have formerly been demonstrated (10, 11). Previous studies reported that RA may stimulate follicle-stimulating hormone (FSH) for induction of luteinizing hormone (LH) receptors RA regulates progesterone generation and reduces cAMP levels (12). It could also protect oocyte against oxidative stress induced by apoptosis (13, 14) through reduction of free oxygen radicals and interaction with other antioxidant compounds (15).

bFGF has been known as an oocyte competency factor due to its formation from theca, granulosa and cumulus cells throughout folliculogenesis (16). Researchers asserted that bFGF is localized in the primordial and early developing follicles, and that this growth factor stimulates primordial follicle development and further cell growth (17). Addition of bFGF to the medium has also been shown to be beneficial in improvement of oocyte development (18, 19). We found an increase in the number of oocytes attaining two-cells stage after addition of bFGF to the medium for 24 hours. This number was considerably lower compared to the RA group. When combination of RA and bFGF was used, there were no significant changes compared to the RA group. Therefore we propose that both RA and bFGF could improve IVM quality, and the role of RA was more noticeable than that of bFGF to develop into two-cells stage.

Conclusion

Our findings showed beneficial effects of 2 µM RA and 20 ng/ml bFGF on mouse oocyte IVM.

Acknowledgements

This work was financially supported by Cellular and Molecular Research Center, Kurdistan University of Medical Sciences, Sanandaj, Iran. All authors declare that there is no conflict of interest.

Author's Contributions

M.A.; Contributed to the conception and design of the study, data collection, statistical analysis and writing the

manuscript. M.A.; Contributed to the conception, design of the study and writing the manuscript. F.F., M.J.R.; Contributed to the conception and design of the study. E.D.; Contributed to the conception, design of the study and provided critical revision of the article. K.M.; Contributed to statistical analysis and provided critical revision of the article. All authors read and approved the final manuscript.

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Original Article

Curcumin and Quercetin Ameliorated Cypermethrin and Deltamethrin-Induced Reproductive System Impairment in Male Wistar Rats by Upregulating The Activity of Pituitary-Gonadal Hormones and Steroidogenic Enzymes

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Abstract

Background: Dietary antioxidants protect tissues and organs against insecticides/xenobiotic-induced damage. In the present study, we evaluated the results of exposure to synthetic pyrethroid insecticides, cypermethrin (Cyp) and deltamethrin (Del) and possible protective effects of curcumin and quercetin on reproductive system in male Wistar rats.

Materials and Methods: In this controlled experimental study, 42 male Wistar rats were randomly divided into 7 groups of 6 animals. Group A served as control, group B was exposed to Cyp (2 mg/kg.bw), group C was exposed to Del (2 mg/kg.bw), group D was exposed to Cyp+Del (2 mg/kg.bw each), group E was exposed to Cyp+Del and treated with curcumin (100 mg/kg.bw), group F was exposed to Cyp+Del and treated with quercetin (100 mg/kg.bw) and group G was exposed to Cyp+Del and treated with quercetin+curcumin for 45 days.

Results: Exposure to Cyp and Del caused decreases in reproductive organs weight, sperm count, sperm motility, level of sex hormones viz. testosterone (T), follicle stimulating hormone (FSH) and luteinizing hormone (LH), steroidogenic enzymes viz. 3β-hydroxyl steroid dehydrogenase (3β-HSD) and 17β-HSD, non-enzymatic antioxidant glutathione (GSH) and enzymatic antioxidants viz. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR) activity and increases in sperm abnormalities and lipid peroxidation (LPO). The exposure also adversely affected the histo-achitecture of testes. Single and combined treatment with curcumin and quercetin significantly ameliorated Cyp and Del-induced damage in reproductive system.

Conclusion: Curcumin and quercetin protected against Cyp and Del-induced reproductive system toxicity and oxidative damage in rats. The increases in activities of 3β-HSD and 17β-HSD with concomitant increases in testosterone were mainly responsible for ameliorating effects of curcumin and quercetin. Curcumin showed slightly better activity as compared to quercetin. The combination of both antioxidants offered more protection compared to each one alone.

Keywords: Curcumin, Cypermethrin, Deltamethrin, Quercetin

Citation: **Sharma P, Khan IA, Singh R. Curcumin and quercetin ameliorated cypermethrin and deltamethrin-induced reproductive system impairment in male wistar rats by upregulating the activity of pituitary-gonadal hormones and steroidogenic enzymes. Int J Fertil Steril. 2018; 12(1): 72-80. doi: 10.22074/ijfs.2018.5160.**

Introduction

Synthetic pyrethroids insecticides are widely used because of their high effectiveness against a large number of insects, rapid biodegradation, low mammalian toxicity and target-oriented mechanism of action (1). Cypermethrin (Cyp) and deltamethrin (Del) are synthetic pyrethroids used in agriculture, veterinary and public health programs for management of insects and pests (2). Although pyrethroids are considered to be safe for humans, indiscriminate uses of these insecticides have induced carcinogenicity, neurotoxicity, genotoxicity and developmental toxicity in domestic animals and humans (3). The reproductive toxicity of Cyp (4) and Del (5) was previously evaluated in our laboratory. Increased oxidative stress and augmented generation of reactive oxygen species (ROS) are among the underlying mechanisms via which these insecticides induced toxicity.

Received: 6 Jan 2017, Accepted: 23 Jun 2017
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Dietary antioxidants, chiefly plant phenolics, flavonoids and carotenoids that have ROS scavenging activity, are considered important for a healthy life. Curcumin, a polyphenolic compound obtained from turmeric is an excellent antioxidant and possesses a number of pharmacological activities (6). Quercetin, the flavonoid present in several vegetables and fruits also possesses antioxidant and other biological activities (7). The ameliorative effects of curcumin (8) and quercetin (9) on xenobiotic-induced reproductive toxicity have largely been attributed to their ability in decreasing oxidative stress in testicular tissue, in laboratory animals.

The present study was planned to investigate the role of curcumin and quercetin in Del and Cyp-induced reproductive toxicity in male Wistar rats. Apart from evaluating their antioxidant potential, we explored the effect of these phytochemicals on sperm parameters, hormones of the pituitary-gonadal axis and enzymes involved in testosterone biosynthesis.

Materials and Methods

Male Wistar rats, weighing about 200-250 g, were used in this controlled experimental study. Animals were kept in the animal house at 22 ± 3°C, with relative humidity of 45-55%, and 12 hours/12 hours dark/light cycles. The animals were fed with pelleted diet and water ad-libitum. All animal experiments were performed as per approval of the Institutional Animal Ethics Committee (BU/Pharma/IAEC/12/032).

Chemicals

Technical grade Cyp (99.2%) and Del (98.5%) were obtained from Gharda chemicals (Mumbai, India). Curcumin (95%) was purchased from Sigma-Aldrich (St. Louis, MO. USA) and quercetin dihydrate (98%) from Himedia (Mumbai, India). All other chemicals used in this study were of high purity and purchased from standard firms.

Treatment schedule

Forty two male Wistar rats were randomly divided into 7 groups of 6 animals. Cyp, Del, curcumin and quercetin were dissolved in polyethylene glycol (10) and administered orally for 45 days. The doses of curcumin (11), quercetin (12) and Del (13) were selected based on previous studies. For effective comparison, equivalent doses of Cyp and Del were used. Group A served as control and each animal in the group received 1 ml of polyethylene glycol. Group B was exposed to Cyp (2 mg/kg.bw), group C was exposed to Del (2 mg/kg.bw), group D was exposed to Cyp+Del (2 mg/kg.bw each), group E was exposed to Cyp+Del and treated with curcumin (100 mg/kg.bw), group F was exposed to Cyp+Del and treated with quercetin (100 mg/kg.bw) and group G was exposed to Cyp+Del and treated with quercetin (100 mg/ kg.bw)+curcumin (100 mg/kg.bw) for 45 days.

At the end of the experiment, rats were sacrificed by cervical dislocation, under ketamine-induced anesthesia. The testes and epididymis were removed and weighted. The epididymis was used for sperm motility and sperm morphology studies. One testis was used for sperm head counts and the other was used for estimation of lipid peroxidation, enzymatic and non-enzymatic antioxidants and steroidogenic enzymes. A part of testis was kept in 10% formaldehyde for histological studies. Blood was taken from the heart and used for estimation of various reproductive hormones.

Estimation of sperm parameters

Sperm head counts

Sperm head counts was performed using a hemocytometer as described by Choi et al. (14). The testis was dissected and tunica albuginea (outer covering) was removed. The testis was minced in a solution consisting of 0.9% NaCl and 0.05 triton X and homogenized for 2 minutes at highest speed using a tissue homogenizer. Testis homogenate (10-15 µl) was placed on hemocytometer and after 5 minutes, sperm heads were counted in red blood corpuscles (RBC) chamber at ×40 magnification.

Sperm motility

A segment of distal cauda epididymis was removed and kept in 2 ml of Dulbecco's phosphate-buffered saline (PBS), maintained at 36-38°C on a water bath. Cauda was minced sufficiently to disperse the sperm for 1-5 minutes and gently mixed using pasture pipette. The test sample (5-10 µl) was loaded into the hemocytometer chamber and the motile sperms were counted in white blood cells (WBC) counting area. Sperms were counted as motile if they exhibited any type of movement/motion. Hemocytometer was placed on ice for 10-20 seconds to render all the sperms immotile for counting the total sperms (15).

Sperm morphology

Cauda epididymis was minced with the help of a razor, in 1 ml of 0.9% saline and 1 ml of 10% neutral buffered formaldehyde was added. The suspension was diluted with water to a volume suitable for performing the assay. Next, 1-2 ml of 1% Eosin was added to 20 ml of the above-mentioned mixture and incubated at room temperature for 45 to 60 minutes. One drop of this suspension was taken on slide and a smear was prepared for studying sperm morphology. The head and tail abnormalities were expressed as percentage.

Biochemical estimations

Estimation of testosterone, follicle stimulating hormone and luteinizing hormone

At the end of the experiment, blood was taken from the heart and centrifuged and serum was separated for the estimation of reproductive hormones. Testos-

terone (T), follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels were estimated using rat specific ELISA kits (Qayee-Bio Life Science, China).

Estimation of steroidogenic enzymes

Testis tissue (100 mg) was rinsed and homogenized in 1 ml of 1X PBS and stored at -20°C, overnight. After two freeze-tha wcycles to break the cell membranes, the homogenate was centrifuged at 5000 g for five minutes in a refrigerated centrifuge. The supernatant was removed immediately and 3-β hydroxyl steroid dehydrogenase (3β-HSD) and 17β-HSD were assayed using rat specific ELISA kits (Cusabio, USA)

Estimation of lipid peroxidation, non-enzymatic and enzymatic antioxidants

A part of the testis was homogenized using homogenizing buffer (10 times, w/v, 0.1 M phosphate buffer (pH=7.4)+150 mM KCl) to prepare 10% homogenate. A part of the homogenate was used for lipid peroxidation (LPO) and glutathione (GSH) estimations. The remaining part was centrifuged at 8500 g for 20 minutes in a refrigerated centrifuge to get supernatant (S) fraction. The 'S' fraction was used for measurement of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR) activities (5).

Briefly, LPO and GSH were estimated by the methods of Ohkawa et al. (16) and Elman (17), respectively. The activity of SOD was estimated by the method described by Kakkar et al. (18). CAT activity was estimated by the method described by Sinha (19). GPx, GST and GR activities were assayed by the methods of Rotruck et al. (20), Habig et al. (21) and Carlberg and Mannervik (22), respectively. Protein content in tissue homogenate was estimated by the method of Lowry et al. (23).

Histological studies

The testicular tissues, previously kept in 10% formaldehyde were used for histological studies. The tissues were washed overnight in running water to remove remaining fixative. Dehydration was carried out to remove water using a series of gradually increasing concentrations of alcohol. These tissues were then cleared in xylol, embedded in wax and cut in sections of 5-µm thickness. The sections were recovered from wax blocks, stained with haematoxylin and eosin and analyzed by trinocular microscope with camera (24, 25).

Statistical analysis

The results were expressed as mean ± SEM. Intergroup variations were evaluated by one way analysis of variance (ANOVA) followed by Dunnett's test. Statistical significance was considered at P<0.05. The statistical analysis was performed using Graph Pad In Stat Software Inc., V. 3.06, San Diego, USA.

Results

Effects on weight of testes and epididymis

Non-significant decreases in weight of testis and epididymis were observed between exposure (B, C, and D) and treatment groups (E, F, and G) and control group A. The treatment groups E, F and G showed non-significant (P>0.05) increases in the weight of testes and epididymis as compared to exposure group D (Fig.1).

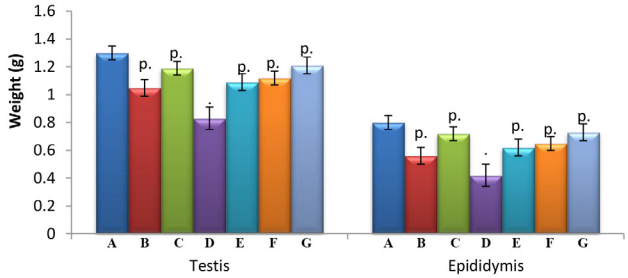

Fig.1: Effect of curcumin and quercetin in Cyp and Del-induced changes in sex organs weight. Each bar represents mean ± SEM of 6 rats.
Cyp; Cypermethrin, Del; Deltamethrin, ▪; P>0.05, compared to group A, p▪; Compared with group A and D both, A; Control, B; Cyp, C; Del, D; Cyp+Del, E; Cyp+Del+curcumin, F; Cyp+Del+quercetin, and G; Cyp+Del+curcumin+quercetin.

Effects on sperm parameters

Sperm head counts were significantly decreased in groups B, C, D, E, and F (29.38, 15.99, 40.46, 15.53 and 17.38%, respectively, P<0.01) and group G (5.15%, P>0.05) as compared to group A. Sperm motility was decreased significantly in groups B and (28.09 and 46.20%, respectively, P<0.01) and groups E and F (17.70 and 14.86%, respectively, P<0.05) whereas it decreased non-significantly (P>0.05) in groups C (14.69%) and G (2.09%) as compared to group A. On the other hand, in group E, F and G, we observed significant (P<0.01) increase in sperm head counts (41, 38.75 and 59.30%, respectively) and sperm motility (52.98, 58.26 and 82%, respectively) as compared to group D. Significant (P<0.01) increases in sperm abnormality were observed in groups B (61.37%), C (52.85%) and D (102.28%) as compared to control group A. Groups E, F and G showed significant increases in sperm abnormality (27.27% for group E (P<0.05), 22.73% for group F (P>0.05) and 8.43% for group G (P>0.05) as compared to group A. On the contrary, a significant (P<0.01) reduction in sperm abnormality was found in group E (37.07%), F (39.32%), and G (46.39%) as compared to group D (Table 1, Fig.2).

Effects on testosterone, follicle stimulating hormone and luteinizing hormone

Testosterone levels decreased significantly in groups B, C, and D (73.37, 65.47, and 90.70%, respectively, P<0.01 for all groups) and groups E and F (54.99 and 56.47%, respectively, P<0.05 for all groups) and non-significantly (P>0.05) in group G (18.84%) as compared to group A. Significant (P<0.05) increases in testoster-

one was observed in groups E, F and G (P<0.01) when compared to group D. A decrease in FSH level was observed in groups B, C, E, F, and G (57.55, 47.08, 27.95, 29.06, and 16.77%, respectively, P>0.05 for all groups) and D (64.55%) as compared to group A. Groups E, F (P<0.05) and G (P<0.01) showed significant increases in FSH level as compared to group D. A significant decrease in LH level was observed in groups B, C, D, E, and F (56.76, 52.52,72.97,49.69 and 57.92%, respectively, P<0.01 or all groups) and G (37.37%, P<0.05) as compared to group A. Groups E, F (P<0.05) and G (P<0.01) showed significant increases in LH level as compared to group D (Table 2).

Effects on steroidogenic enzymes

Non-significant (P>0.05) decreases 3β-HSD was observed in groups B (67.39%), C (49.34%), D (76.84%), E (17.28%), F (31.95%) and G (3.26%) as compared to group A, whereas significant (P<0.05) increases were found in groups E, F and G (P<0.01) as compared to group D. 17β-HSD activity was significantly (P<0.01) decreased in groups B (58.65%), C (52.88%), D (80.28%) and F (52.88%) and also in groups E (44.23%, P<0.05) and G (5.76%, P>0.05) as compared to group A. Significant (P>0.05) increases in 17β-HSD activity were observed in groups E, F and G (P<0.01) as compared to group D (Table 3).

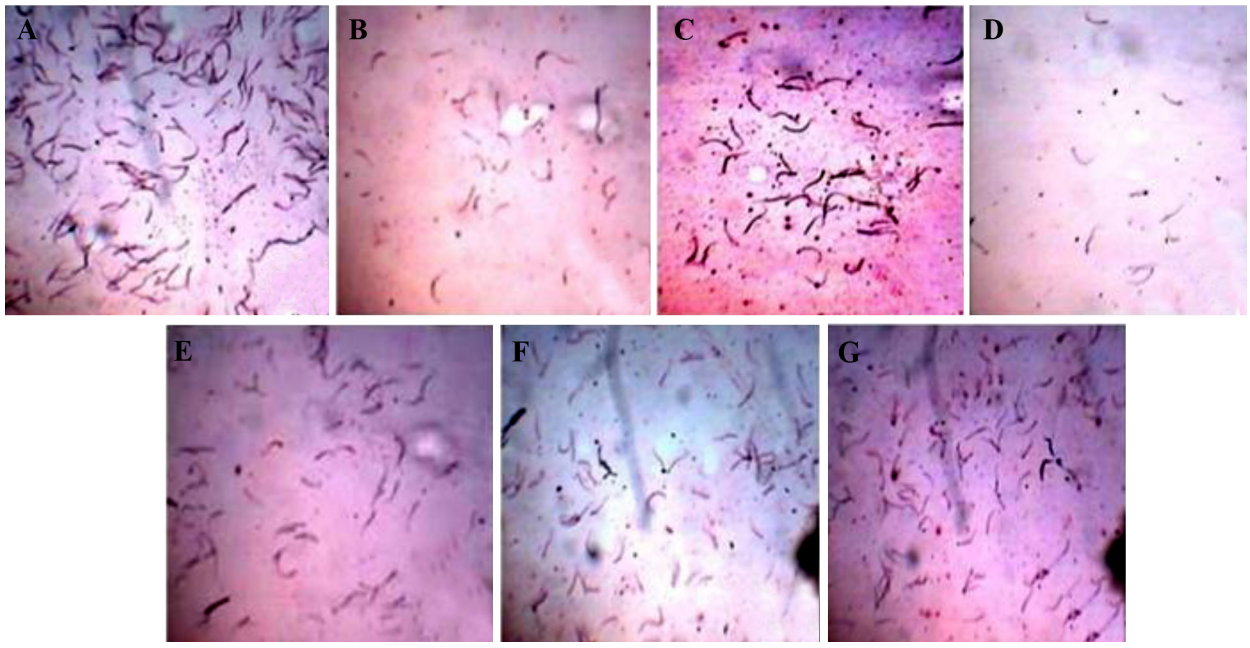

Fig.2: Effect of curcumin and quercetin on Cyp and Del-induced changes in testicular sperm counts. **A.** Control, **B.** Cyp, **C.** Del, **D.** Cyp+Del, **E.** Cyp+Del+curcumin, **F.** Cyp+Del+quercetin, and **G.** Cyp+Del+curcumin+quercetin. Cyp; Cypermethrin, Del; Deltamethrin.

Table 1: Effect of curcumin and quercetin on Cyp and Del-induced changes in sperm parameters

Parameter	Group A	Group B	Group C	Group D	Group E	Group F	Group G
Sperm count×10^6/g tissue	72.22 ± 1.176	51.00 ± 0.730q**	60.666 ± 2.028r**	43.00 ± 1.751**	61 ± 2.129r**	59.666 ± 2.275r**	68.5 ± 2.487r$^{\bullet}$
Sperm motility (%)	70.475 ± 3.367	50.675 ± 2.54q**	60.12 ± 3.245r$^{\bullet}$	37.911 ± 2.909**	60.00 ± 2.745r*	58.00 ± 2.160r*	69.00 ± 1.506r$^{\bullet}$
Sperm abnormality (%)	43.998 ± 3.246	71.00 ± 1.807r**	67.251 ± 2.627r**	89.00 + 3.183**	56.00 ± 3.724r*	54.00 ± 2.769r$^{\bullet}$	47.71 ± 2.911r$^{\bullet}$

Data are presented as mean ± SEM of 6 rats in each group.
Cyp; Cypermethrin, Del; Deltamethrin, $^{\bullet}$; P>0.05, *; P<0.05, **; P<0.01 compared with group A, p; P>0.05, q; P<0.05, r; P<0.01 compared with group D, Group A; Control, B; Cyp, C; Del, D; Cyp+Del, E; Cyp+Del+curcumin, F; Cyp+Del+quercetin, and G; Cyp+Del+curcumin+quercetin.

Table 2: Effect of curcumin and quercetin on Cyp and Del-induced changes in sex hormone level

Parameter	Group A	Group B	Group C	Group D	Group E	Group F	Group G
Testosterone (ng/ml)	3.635 ± 0.745	0.968 ± 0.302p**	1.255 ± 0.170p**	0.338 ± 0.137**	1.636 ± 0.492q*	1.585 ± 0.495q*	2.95 ± 0.556r$^{\bullet}$
FSH (mIU/ml)	2.415 ± 0.446	1.025 ± 0.112p$^{\bullet}$	1.278 ± 0.169p$^{\bullet}$	0.856 ± 0.213*	1.74 ± 0.494q$^{\bullet}$	1.713 ± 0.492q$^{\bullet}$	2.01 ± 0.454r$^{\bullet}$
LH (mIU/ml)	1.98 ± 0.325	0.856 ± 0.213p**	0.94 ± 0.220p**	0.535 ± 0.126**	0.996 ± 0.118q**	0.833 ± 0.113q**	1.24 ± 0.112r*

Data are presented as mean ± SEM of 6 rats in each group.
Cyp; Cypermethrin, Del; Deltamethrin, $^{\bullet}$; P>0.05, *; P<0.05, **; P<0.01 compared with group A, p; P>0.05, q; P<0.05, r; P<0.01 compared with group D, Group A; Control, B; Cyp, C; Del, D; Cyp+Del, E; Cyp+Del+curcumin, F; Cyp+Del+quercetin, and G; Cyp+Del+curcumin+quercetin.

Table 3: Effect of quercetin and curcumin on Cyp and Del-induced changes in steroidogenic enzymes

Parameter	Group A	Group B	Group C	Group D	Group E	Group F	Group G
3-β HSD (pg/ml)	0.92 ± 0.283	0.30 ± 0.125p•	0.466 ± 0.105p•	0.213 ± 0.106•	0.761 ± 0.213q•	0.626 ± 0.175q•	0.89 ± 0.274r•
17-β HSD (ng/ml)	1.04 ± 0.112	0.43 ± 0.104p**	0.49 ± 0.105p**	0.205 ± 0.095**	0.58 ± 0.124q*	0.49 ± 0.100q**	0.98 ± 0.113r•

Data are presented as mean ± SEM of 6 rats in each group.
Cyp; Cypermethrin, Del; Deltamethrin, •; P>0.05, *; P<0.05, **; P<0.01 compared with group A, p; P>0.05, q; P<0.05, r; P<0.01 compared with group D, Group A; Control, B; Cyp, C; Del, D; Cyp+Del, E; Cyp+Del+curcumin, F; Cyp+Del+quercetin, and G; Cyp+Del+curcumin+quercetin.

Effects on lipid peroxidation, and non-enzymatic and enzymatic antioxidants

Significant (P<0.01) increases in LPO level were observed in groups B (142.65%), C (80%), D (188.94%), E (144.70%), F (127.48%) and G (47.70%) as compared to group A. Significant (P<0.05) decreases in LPO level were observed in groups E (15.52), F (86.87%) and also in group G (48.87%, P<0.01) as compared to group D. GSH level was significantly (P<0.01) decreased in group B (40%), C (31.98%), D (75.82%), E (54.69%) and F (46.36%) and non-significantly (P>0.05) in group G (6.67%) as compared to group A. Groups E (87.39%, P<0.05), F(121.81%, P<0.01) and G (285.97%, P<0.01) showed significant increases in GSH level compared to group D (Table 4). The results of SOD, CAT, GPx, GR and GST activities are summarized in Table 4. Rats in groups B, C, D, E, and F showed significant (P<0.01) decreases (44.69, 28.36, 66.68, 39.03 and 31.91%, respectively) in SOD activity, compared to group A, whereas a non-significant (9.51%, P>0.05) decrease was observed in group G. On the other hand, significant (P<0.01) increases in SOD level were observed in groups E (82.98%), F (104.36%) and G (171.60%) when compared to group D. CAT activity was significantly decreased in groups B, C and D (35.77, 18.26 and 50.71%, respectively, P<0.01 for all groups), E and F (6.23 and 6.76%, respectively, P<0.05 for both groups) and non significant in group G (2.88%, P>0.05) as compared to group A.

However, significant (P<0.05) increases were observed in groups E (89.18%), F (90.27%), and G (97%) when compared to group D. Significant (P<0.05) decreases in GPx activity were observed in groups B (36.32%), D (55.59%, P<0.01) and non-significant (P>0.05) decreases were found in groups C (21.23%), F (22.21%), E (27.54%) and G (3.84%) as compared to group A. Significant (P<0.05) increases in GPx activity were observed in groups E (63.17%), F (75.18%) and G (116.55%, P<0.01) as compared to group D. GR activity was significantly (P<0.05) reduced in groups B (31.94%), E (27.77%), F (25.83%) and D (61.55%, P<0.01) and non-significantly (P>0.05) in groups C (20.96%) and G (6.38%) as compared to group A.

On the other hand, significant (P<0.01) increases were observed in groups E (87.84%), F (92.89%) and G (143.48%) as compared to group D. The GST activity was significantly (P<0.01) decreased in groups B (7.19%), D (17.62%), E (14.13%), F (14.40%), G (8.76%) and C (4.60%, P<0.05) as compared to group A. Significant increases in GST were recorded in groups E and F (4.24, 3.91%, respectively, P<0.05 for both groups) and group G 10.75%, P<0.01, as compared to group D (Table 4).

Table 4: Effect of curcumin and quercetin on Cyp and Del-induced changes in lipid peroxidation, non- enzymatic and enzymatic antioxidants

Parameter	Group A	Group B	Group C	Group D	Group E	Group F	Group G
LPO (nmoles MDA/hours/g tissue)	3.165 ± 0.2749	7.68 ± 0.166q**	5.726 ± 0.148r**	9.145 ± 0.489**	7.725 ± 0.319q**	7.2 ± 0.402r**	4.675 ± 0.309r*
GSH (µmole/g tissue)	2.920 ± 0.288	1.735 ± 1.145r**	1.986 ± 0.224r**	0.706 ± 0.071**	1.566 ± 0.03827r**	1.323 ± 0.0388q**	2.725 ± 0.1385r•
SOD (nmole/minutes/mg protein)	31.911 ± 1.262	17.648 ± 1.631r**	22.858 ± 1.474r**	10.631 ± 0.5518**	21.726 ± 0.9145r**	19.453 ± 1.170r**	28.874 ± 0.7624r•
CAT (µ mole/minutes/mg protein)	74.14 ± 1.193	47.62 ± 1.335r**	60.596 ± 1.295r**	36.538 ± 1.331**	69.521 ± 0.659r*	69.123 ± 0.620r*	72.00 ± 0.7857r•
GPx (nmole/minutes/mg protein)	8.396 ± 1.005	5.346 ± 0.715q*	6.613 ± 0.774q•	3.728 ± 0.760**	6.531 ± 0.771q•	6.083 ± 0.736q•	8.073 ± 0.719r•
GR (nmole/minutes/mg	3.553 ± 0.339	2.418 ± 0.250q*	2.808 ± 0.2778r•	1.366 ± 0.1686**	2.635 ± 0.185r*	2.566 ± 0.2008r*	3.326 ± 0.1578r•
GST (µmole/minutes/mg protein)	91.015 ± 0.681	84.471 ± 1.141r**	86.823 ± 0.775r*	74.973 ± 1.284**	78.153 ± 0.673p**	77.906 ± 0.741p**	83.038 ± 0.791r**

Data are presented as mean ± SEM of 6 rats in each group.
Cyp; Cypermethrin, Del; Deltamethrin, •; P>0.05, *; P<0.05, **; P<0.01 compared with group A, p; P>0.05, q; P<0.05, r; P<0.01 compared with group D, Group A; Control, B; Cyp, C; Del, D; Cyp+Del, E; Cyp+Del+curcumin, F; Cyp+Del+quercetin, and G; Cyp+Del+curcumin+quercetin.

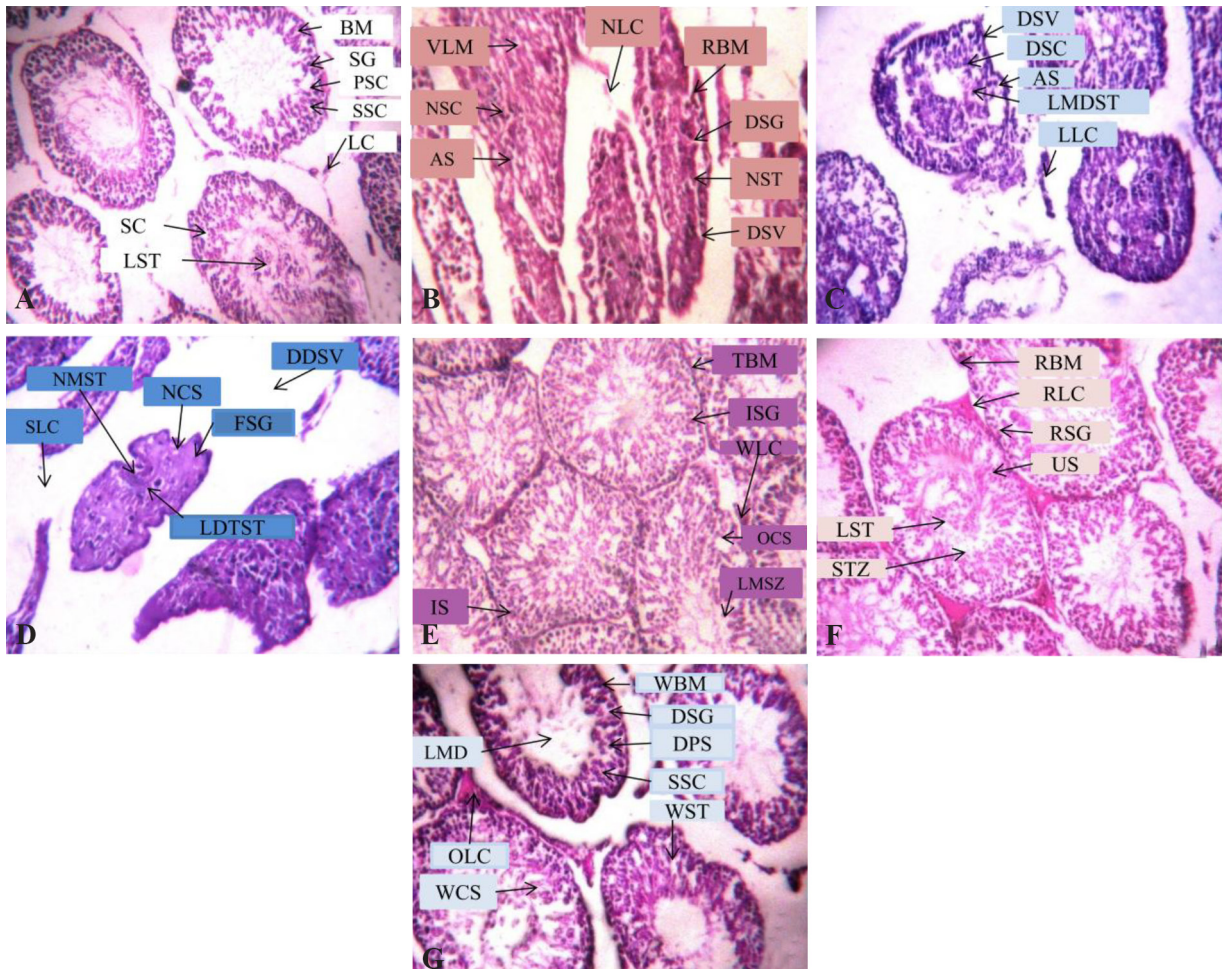

Fig.3: Effect of curcumin and quercetin on Cyp and Del-induced changes in testicular sperm counts. **A.** Control, **B.** Cyp, **C.** Del, **D.** Cyp+Del, **E.** Cyp+Del+curcumin, **F.** Cyp+Del+quercetin, and **G.** Cyp+Del+curcumin+quercetin.
Cyp; Cypermethrin, Del; Deltamethrin, LC; Leydig cells, BM; Basement membrane, SG; Spermatogonia, PSC; Primary spermatocyte, SSC; Secondary spermatocyte, LST; Lumen filled with spermatids, SC; Sertoli cells, RBM; Ruptured basement membrane, DSV; Disorganized structure of seminal vesicles, NSC; Necrosis of sertoli cells, VLM; Vacuolation of lumen, NLC; Necrosis of Leydig cells, AS; Arrested stages of spermatogenesis, DSG; Disorganized spermatogonia, NST, Necrosis of spermatids, DSV; Disorganization of seminal vesicles, LLC; Loosed Leydig cells, DSC; Degeneration of sertoli cells, AS; Arrested spermatogenesis, LMDST; Lumen filled with dead spermatids, DDSV; Disorganization and disappearance of seminal vesicles, NSC; Necrosis of sertoli cell, FSG; Fading of spermatogonia, NMST non-motile spermatids, SLC; Scattering of Leydig cells, LDTST; Lumen filled with dead and tailless spermatids, TBM; Thick basement membrane, ISG; Increased spermatogonia, WLC; Well-developed Leydig cells, IS; Increased spermatogenesis, OCS; Organized sertoli cells, LMSZ; Lumen filled with spermatozoa, RBM; Recovery of basement membrane, RLC; Recovery of Leydig cell, RSG; Recovery of spermatgonia, US; Unaffected spermatogenesis, LST; Lumen filled with spermatids, SZT; Spermatozoa with tail, WBM; Well-shaped basement membrane, DSG; Dense structured spermatogonia, DPS; Densely packed primary spermatocyte, SSC; Secondary spermatocyte, WST; Well-developed spermatids with tails, LMD; Lumen filled with dense materials, OLC; Organized Leydig cells, and WCS; Well-shaped sertoli cells.

Histology of testes

The histology of testes of control rats showed seminiferous tubules separated by basement membrane containing Leydig cells. The germinal epithelium consisted of concentric layers of germs cells viz. spermatogonia, primary and secondary spermatocytes, lumen filled with spermatids and spermatozoa with tail and sertoli cells (Fig.3A). The testes of rats treated with single and combined exposure to Cyp and Del, showed ruptured basement membrane, disorganized seminal vesicles, necrosis of Leydig cells and sertoli cells, vacuolation of lumen, arrested stages of spermatogenesis including disorganized spermatogonia, increased intertubular space and lumen with cellular debris (Fig.3B-D). Treatment with curcumin, quercetin and combination of both recovered the histological damage induced by the insecticides (Fig.3E-G).

Discussion

Cyp and Del showed toxic effects in reproductive system of male Wistar rats. The reproductive toxicity caused by these insecticides was ameliorated by curcumin and quercetin. The weight of the testes and epididymis decreased following single as well as combined exposure to Cyp and Del, as compared to the control. The male reproductive toxicity of Cyp (25) and Del (26) has been previously reported in laboratory animals. The decrease in testes and epididymis weight observed in the present study may be due to the direct cytotoxic action of these insecticides on testicular tissue. Also, a significant decrease in testicular sperm head counts was observed following single and combined exposure to Cyp and Del as compared to the control. Probably, accumulation of the insecticides in the testicular tissue may have adversely affected the sertoli cell population leading to compromised spermato-

genesis and reduction in sperm head counts. Decreases in serum testosterone which were observed in the present experiment and reported by previous studies, may also be responsible for the reduction in sperm head counts (27). Also, an increase in ROS has been reported to decrease sperm counts (28). In the present study, we observed increases in LPO and decreases in enzymatic and non-enzymatic antioxidants following exposure to Cyp and Del. So, decreased sperm counts may also be due to increases in lipid peroxidation and excessive generation of ROS. Dichlorvos (29) has been reported to increase sperm abnormality and reduce sperm motility. We also observed increases in sperm abnormality and reductions in sperm motility after Cyp and Del exposure in our study, which were possibly due to excessive ROS production and decreases in testosterone level.

The endocrine disruptive action of Del (30) and Cyp (31) has been previously reported, though the mechanism is largely unknown. The decrease in testosterone level following Cyp and Del exposure observed in this study, may be due to the direct effect of these pyrethroids on the androgen biosynthesis pathways in the testes or alterations in gonadotropins levels. We observed reduction in steroidogenic enzymes (i.e. 3β-HSD and 17-βHSD) after exposure to Cyp and Del. As there was marked increases in oxidative stress in the testicular tissue, this reduction in 3β-HSD and 17β-HSD may either be due to Leydig cell damage or direct action of these insecticides on gene expression of 3β-HSD and 17β-HSD.

StAR protein transports cholesterol from the cytoplasm to the mitochondrial matrix. The transport of cholesterol is a rate-limiting step in testosterone biosynthesis. There are reports that pyrethroids reduce StAR protein expression (32). Hence, decreases in testosterone observed in the present study following exposure to Cyp and Del, may be possibly induced by inhibition of StAR protein expression. Spermatogenesis is also controlled by the gonadotropins and any alteration in level of gonadotropins may impair spermatogenic activity. Hu et al. (33) reported decreased testosterone after Cyp exposure with concomitant increases in level of FSH and LH, possibly due to negative feedback inhibition. On the other hand, Issam et al. (34) reported decreases in FSH, LH and testosterone after Del exposure in 45 and 60-day experiments. In our study, we observed decreased in testosterone, FSH and LH levels following Cyp and Del exposure. It is possible that the effect of these pyrethroids on pituitary gonadotropin hormones and testicular hormones, is dependent on time of exposure and testicular tissue being their primary target. During short-term exposure, due to direct cytotoxic effect of Cyp and Del on testicular tissue, a decrease in testosterone is observed, which in turn increases the level of FSH and LH due to negative feedback inhibition. However, during long-term exposure, it is possible that the anterior pituitary is also affected by Cyp and Del along with testes, which may result in decreases in testosterone, FSH and LH levels, as observed in present study.

Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors. Lipid peroxidation products (lipid radical and lipid peroxide) are harmful to the cells and are associated with a number of pathological conditions. In the present study, significant increases in the level of LPO was observed following single and combined exposure to Cyp and Del as compared to control group A. Increased LPO has been reported after Cyp exposure in rats brain and liver (35). We observed significant decreases in GSH level after single and combined exposure of Cyp and Del as compared to the control. The decrease in GSH may be due to increased utilization of GSH for detoxification of excessive free radicals generated after pesticide exposure.

Superoxide dismutase is the first line of defense against deleterious effects of oxygen radicals in the cell. It acts by catalyzing the dis-mutation of superoxide radicals to hydrogen peroxide and molecular oxygen. Significant decreases in the SOD activity were observed in the present study, which may be due to the decrease in the ability of the tissues to handle extra free radical. These extra free radicals may attack the thiol group of cysteine residues of proteins and polysaturated fatty acids of biological membranes. Catalase is present ubiquitously in nearly all living organisms exposed to oxygen and catalyzes the decomposition of hydrogen peroxide to water and oxygen (36). In our study, a significant decrease in CAT activity was observed, possibly because of inactivation of the enzyme by excessive ROS production. Also, decreased CAT activity was observed by Latchoumycandane and Mathur following methoxychlorexposure in rats (37).

The present study showed significant decreases in GPx activity which may be due to reduced level of GSH, a substrate of GPx. GR is a member of the pyridine-nucleotide disulfide oxidoreductase family of flavo enzymes which catalyzes the reduction of glutathione disulfide (GSSG) to its reduced form GSH, in the presence of NADPH. The level of GR was decreased in this study, possibly due to the damage caused by Cyp and Del to the tertiary structure of the enzyme. GST catalyzes the conjugation of GSH to electrophiles and protects cellular components from oxidative damage (38). In the present study, significant decreases in the GST activity were observed. Decreased GST may be due to the affinity of this enzyme to the hydrophobic compounds like pyrethroid insecticides. Decreased GST has been previously reported following exposure to phosphorothionate (39) in male rats.

Additionally, exposure to Cyp and Del resulted in marked histo-architectural disturbances in testis. These changes were possibly caused by ROS-induced cell damage. The damage in sperm mother cells as well as supporting sertoli cells resulted in gross changes in sperm parameters and decrease in testosterone.

Curcumin and quercetin are phytochemicals with proven antioxidant and cyto-protective activities. Treatment of

Cyp and Del-exposed rats with curcumin and quercetin in the present study, increased sex organs weights, sperm count, sperm motility, sex hormones (testosterone, LH and FSH) levels, and steroidogenic enzymes (3β-HSD and 17β-HSD) and decreased sperm abnormalities. Treatment with curcumin and quercetin also restored Cyp and Del-induced histo-architectural disturbances by their antioxidant and cyto-protective activities. Since we observed direct cytoprotective effect of these antioxidants, particularly the restoration of testicular histo-architecture, it is possible that curcumin and quercetin have crossed the blood-testes-barrier. The increase in antioxidant defense of the testis, as reflected by increased GSH, CAT, SOD, GPx, GST and GR activities and decreased LPO levels, indicated curcumin and quercetin-mediated scavenging of the hydroxyl, peroxy, and superoxide radicals.

Moreover, increased antioxidant defense protected sertoli and Leydigcells with concomitant increases in the level of sex hormones viz. testosterone, FSH and LH. There are reports of feedback regulation of testosterone biosynthesis by FSH and LH. Hu et al. (33) reported decreases in testosterone and increases in FSH and LH level following Cyp exposure. In our study, we observed increases in all sex hormones. Enhanced testosterone level may by cyto-protective effect of curcumin and quercitin on testicular tissue. Since the animals were orally fed with curcumin and quercitin, the effect of these phytochemicals on other systems can not be ruled out. We propose that these phytochemicals may also protect the pituitary gland and enhance the level of FSH and LH. We also observed increases in steroidogenic enzymes 3β-HSD and 17β-HSD, responsible for enhanced biosynthesis of testosterone. Although, no data is available on the mechanism of induction of steroidogenic enzymes by natural antioxidants, it is possible that curcumin and quercetin might have up-regulatedthe gene expression of these enzymes.

Conclusion

This study indicated that the combined exposure to Cyp and Del was more toxic than exposure to each of the insecticide alone. The 45-day exposure to Cyp and Del showed marked decreases in sperm motility and sperm head counts, increases in sperm abnormality and decreases in testosterone, FSH, LH, 3β-HSD and 17β-HSD in serum. Enhanced activities of steroidogenic enzymes (3β-HSD and 17β-HSD) and concomitant increased levels of testosterone were mainly responsible for ameliorating effect of curcumin and quercetin. We also observed decreases in enzymatic and non-enzymatic antioxidants and disturbance in testicular histo-architecture in the exposed rats. Treatment with curcumin and quercetin ameliorated Cyp and Del-induced toxicity by improving the reproductive system. Curcumin showed slightly better activity as compared to quercetin. Our study further showed that combined treatment of curcumin and quercetin possesses higher activity as compared to treatment with each one alone.

Acknowledgements

The authors are thankful to Innovation Centre, Bundelkhand University, Jhansi, India, for extending equipment and other facilities. No financial support was provided by any external funding agency. There are no conflicts of interest in this study.

Author's Contributions

P.S.; Designed the study, performed histology and analyzed the data. I.A.K.; Performed the animal experiments. R.S.; Conceived the study, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Original Article

Comparison of The Efficacy and Safety of Palomo, Ivanissevich and Laparoscopic Varicocelectomy in Iranian Infertile Men with Palpable Varicocele

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Abstract

Background: This study aimed to compare the effects of three commonly used varicocelectomy techniques namely, open retroperitoneal ligation (Palomo), open inguinal ligation (Ivanissevich) and laparoscopy, in Iranian infertile men.

Materials and Methods: This retrospective study was conducted on 70 infertile men with palpable varicocele who underwent one of the varicocelectomy techniques namely, Palomo, Ivanissevich, or laparoscopy. Basic information about semen parameters were collected and registered prior to the surgery. Three months after the surgery, semen parameters and surgical complications were investigated in all patients.

Results: The Palomo technique was significantly associated with fewer complications compared to other techniques (P=0.006). The means of sperm concentration, normal motility and normal morphology were significantly different among the three groups after surgery (P=0.025, 0.023 and 0.047, respectively); however, after adjustment for poten-tial confounders, in addition to the baseline values of semen parameters, significant differences were observed only in sperm concentration among the groups (P=0.040).

Conclusion: Varicocelectomy improved sperm parameters. The Ivanissevich technique was more effective in improving sperm concentration compared to the laparoscopic method. The lowest rates of complications were related to the Palomo technique.

Keywords: Infertility, Male, Semen Analysis, Varicocele

Citation: Hosseini K, Nejatifar M, Kabir A. Comparison of the efficacy and safety of palomo, ivanissevich and laparoscopic varicocelectomy in Iranian infertile men with palpable varicocele. Int J Fertil Steril. 2018; 12(1): 81-87. doi: 10.22074/ijfs.2018.5158.

Introduction

Reproductive health is a global health priority and infertility is one of its critical components regarded as a global health concern (1). The prevalence of infertility in developed and developing countries is 3.5-16.7% and 7-9%, respectively (2). Although in many communities particularly in developing ones, women are usually held responsible for infertility and male infertility is widely ignored (3). Male factors account for half of the infertility cases (4). In Iran, the prevalence of infertility is 10.9% (10.6 and 2.7% for primary and secondary infertility, respectively). Male factor conditions (with a prevalence of 34%) are the most prevalent causes of infertility in Iranian couples (5). Varicocele is the most well-known reversible cause of male infertility (6).

About one-sixth, two-fifths, and four-fifths of general male population and in men with primary and secondary infertility are diagnosed with varicocele, respectively (7, 8). A majority of varicoceles are unilateral left-sided (90%) (9). Nowadays, radiologic techniques (embolization or sclerotherapy), open surgical techniques for ligation of the spermatic vein (using inguinal, subinguinal, and retroperitoneal methods), microsurgery (using inguinal and subinguinal methods), and laparoscopic varicocelectomy are used for the treatment of varicocele (8). Recurrence and hydrocele are complications which are commonly reported after varicocele surgery (10). Different studies have reported controversial results regarding the effect of various varicocelectomy techniques on male infertility. Therefore, no agreement has been reached yet about the "gold standard" of varicocele treatment (6).

In many developing countries, including Iran, it is not possible for young couples to use the assisted reproductive technology (ART) due to its economic burden; therefore, in these countries, it is required to seek more affordable effective approaches that are associated with fewer side effects (11). This study aimed to compare the effects of three commonly used varicocelectomy techniques namely, open retroperitoneal ligation (Palomo), open inguinal ligation (Ivanissevich) and laparoscopy, in Iranian infertile men.

Received: 24 Dec 2016, Accepted: 24 Apr 2017
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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 1, Apr-Jun 2018, Pages: 81-87

Materials and Methods

This retrospective study was conducted on infertile men with confirmed varicocele. These men had multiple abnormal semen analysis results and a 3-5 year history of primary infertility even after different medical treatments. The subjects were selected from individuals who referred to Hazrat Rasoul-e-Akram medical center in Tehran, Iran between 2009 and 2015. Cases with a history of previous scrotal or inguinal operation were excluded from our study. Using G*Power 3.1.9.2 and considering equal number in each group and 0.386 for effect size from means of postoperative sperm concentration (million/mL), 0.05 for alpha, 0.80 for power, and 3 for number of groups, a total sample size of 70 patients was calculated (12).

Convenience sampling was done by an expert clinician. Then, patients' medical history was recorded and physical examination (mode of presentation laterality and varicocele grade) and semen analysis were done for each subject. The results of the last semen analysis before surgery, were considered as the baseline. The patients were classified into three clinical groups: grade 1 (palpable only with a Valsalva maneuver), grade 2 (non-visible but palpable without a Valsalva maneuver), and grade 3 (palpable and visible).

The status of atrophy in patients before and after surgery was examined using scrotal Doppler ultrasonography. Atrophy was defined as a testicular volume of <16 ml. According to WHO definition, normal semen samples have a volume of ≥1.5 mL, a sperm concentration of ≥15 million per mL, motility (movement of the sperm) value of ≥32% with forward progression (sum of type A and type B), total motility (sum of type A, B and C) of ≥40% and ≥4% normal morphology (13).

The sperm concentration was measured using a haemocytometer utilizing a neubauer sperm counting chamber after immobilization of spermatozoa by neutral formalin. The sperm motility was assessed by scanning a few fields under high-dry objective, until a total of ≥200 spermatozoa was enrolled and the sperm morphology was assessed on the basis of differential counts of morphologically normal and abnormal spermatozoa sorts on Pap-stained slides. Different types of sperm motility were classified into four groups: Quick dynamic (type A), Slow dynamic (type B), Non-dynamic (type C), and Immotile (type D). Based on a previous study, motility was defined as the average percentage of forward progression (sum of type A and B) divided by all four types of motility (14).

Information about time of surgery (since anesthesia time), type of anesthesia, and level of pain after surgery were collected for all the patients. Pain was measured using a visual analogue scale (VAS) ranging from zero (no pain) to 10 (severe pain). Three months after the surgery, semen parameters and surgical complications (atrophy, hematoma, recurrence, hydrocele, pneumoscrotum, significant nausea and vomiting, infection, ileus, next organ damages as well as need for blood transfusion, re-operation, changing the laparoscopic surgery to open, and other conditions) were investigated in all patients via clinical examinations, ultrasound test, and semen analysis. An urologist who was completely blind to the medical history and semen analysis, carried out all physical examinations. Taking into consideration the clinical indication and patients' preference, they were allocated to different groups of varicocelectomy. All surgeries were done by a single urologist. To compare the effects of surgery in each group, we used Mann-Whitney, Wilcoxon, and McNemar's tests. To compare the effects of the type of surgery on semen parameters and to compare their side effects, we used chi-square or Kruskal-Wallis test, as appropriate.

Also, univariate general linear model was utilized to compare the effects of the type of surgery on semen parameters, by considering the baseline values of semen parameters as covariates and controlling the effect of other potential confounders. For sensitivity analysis, an additional univariate general linear model was used by controlling other residual (or potential) confounders that were not different among the three groups.

In order to predict the effect of varicocelectomy on semen parameters, we used linear regression in a stepwise manner. In this study, the level of statistical significance was set as a P value less than 0.05. All analyses were performed using SPSS 20 software (SPSS Inc., Chicago, Illinois, USA). All surgeries performed in this study were in accordance with the institutional ethical standards and the study protocol was confirmed by Ethics Committee of Iran University of Medical Sciences, Tehran, Iran. Written informed consent was signed by all participants.

Results

There were 25, 23 and 22 cases in Palomo, Ivanissevich and laparoscopic groups with the mean age of 25.97 ± 5.7 years old which was not significantly different among the groups (P=0.352). In 76% of subjects, varicocele was in left side with no statistically significant differences among the three groups (P=0.513). Grade 3, 2 and 1 of varicocele were observed in 67, 30 and 3%, respectively. The rate of varicocele grade 3 in the Palomo group was significantly higher than that of laparoscopic (P=0.005) and Ivanissevich (P=0.047) groups. Sperm concentration was abnormal in 30 subjects accounting for 42.85% of patients population; there were no significant differences in this parameter among the three groups (P=0.138).

Moreover, there were no statistically significant differences in other parameters of semen analyzed before the surgery, among the three groups. There was no atrophy before the surgery in 56% of the patients (n=39). Presence of atrophy significantly varied among different groups (64, 35 and 32% of cases in Palomo, Ivanissevich and laparoscopic groups, respectively; P=0.046). There was a significant difference among the three groups in terms of the mean duration of surgery (longer in laparoscopic type than two others) and type of anesthesia (general anesthesia in most cases of laparoscopic type and spinal anesthesia in the other

methods) (P<0.001 for both comparisons) (Table1).

The results showed that after surgery, the Palomo technique was significantly associated with fewer complications compared to other techniques (12, 55 and 44% for the Palomo, laparoscopic and Ivanissevich groups, respectively, P=0.006). In all group, no one had significant nausea and vomiting, infection, ileus, and next organ damages specifically intestinal damage as well as need for blood transfusion, re-operation, and changing the laparoscopic surgery to open method. In general, the most common complications were hydrocele in 21.4% (n=15), recurrence in 10% (n=7), and hematoma and pneumoscrotum each in 8.60% (n=6) of the patients. Pain after surgery was similar among all groups (Table 2).

Post-surgery semen analysis of all 70 subjects showed decreases in sperm concentration, normal motility and normal morphology in 7 (10%), 5 (7.4%) and 4 patients (5.9%), respectively. In these patients with semen parameters worsened after the surgery (n=13), from 5 patients with atrophy at baseline, only one had atrophy after the surgery and recurrence was observed in three of them. Moreover, 30 patients had abnormal sperm concentration and 67 patients had abnormal sperm motility at baseline; following the surgery, sperm concentration and sperm motility were within the normal range in 73.3% (n=22) and 40.2% (n=27) of these individuals, respectively (P<0.001 for both).

Table 1: Basic characteristics of the patients in different groups of surgical treatment

Item	Group			P value	Power (%)
	Laparoscopy n=22	Ivanissevich n=23	Palomo n=25		
Age (Y)[**, ***]	26.59 ± 6.05	26.78 ± 6.01	24.68 ± 5.23	0.352	64.01
Sperm concentration (million/mL)[**, ***]	13.09 ± 9.88	18.26 ± 13.38	14.96 ± 12.89	0.262	67.31
Sperm normal motility (%)[**, ***, #]	16.86 ± 6.77	19.56 ± 8.51	18.72 ± 9.90	0.639	59.32
Sperm normal morphology (%)[**, ***]	46.59 ± 14	48.04 ± 12.13	47.17 ± 15.87	0.871	51.04
Atrophy, n(%)[&]					
Positive	7(32)	8(35)	16 (64)	0.046	
Negative	15(68)	15(65)	9(36)		
Mode of presentation laterality, n(%)[&]					
Left unilateral	18(82)	18(78)	17(68)	0.513	16.31
Bilateral	4(18)	5(22)	8(32)		
Varicocele grade, n(%)[***]					
I	1(5)[a*]	1(4)[a]	0(0.00)	0.020	
II	11(50)[a]	7(31)[a]	3(12)		
III	10(45)[a]	15(65)[a]	22(88)		
Duration of surgery[**, ***]	61.59 ± 9.43	49.35 ± 6.08[b]	51.60 ± 8.50[b]	<0.001	
Type of anesthesia, n(%)[&]					
General	20(91)	5(22)	8(32)	<0.001	
Spinal	2(9)	18(78)	17(68)		

All variables refer to the condition of patients before surgery, [**]; Values are presented as mean ± SD, [***]; The comparisons were made by Kruskal-Wallis test, [#]; Normal motility is sum of A+B motility type, and [&]; The comparisons were made by Chi-Square test. The same lowercases showed no significant differences in the post-hoc Mann-Whitney tests.

Table 2: The occurrence of postoperative complications in different groups

Item	Group			Total	P value	Power (%)
	Laparoscopy n=22	Ivanissevich n=23	Palomo n=25			
Complications[*]	12(55)	10(44)	3(12)	25	0.006	
Hematoma[**]	1(4.5)	4(17)	1(4)	6(8.60)	0.657	51.23
Recurrence[**]	5(23)	2(9)	0(0.00)	7(10)	0.028	
Hydrocele[**]	8(36)	5(22)	2(8)	15(21.40)	0.059	48.49
Pneumoscrotum[**]	6(27)	0(0.00)	0(0.00)	6(8.60)	0.001	
Pain[***]	2.19 ± 1.40	2.09 ± 1.64	2.71 ± 1.87		0.430	59.79

Data are presented as mean ± SD or n (%). [*]; The comparisons were made by Chi-Square test. Complication: refers to any adverse effect observed in everybody, [**]; The comparisons were made by Fisher's exact test between the laparoscopic and open surgical techniques, and [***]; The comparisons were made by Kruskal-Wallis test.

Mean values of semen parameters after surgery indicated significant improvements in all groups of varicocelectomy. The results of the univariate general linear model (by considering the preoperative values of semen parameters as covariates) revealed that the means of sperm concentration, normal motility and normal morphology were significantly different among the three groups after the surgery (P=0.025, 0.023 and 0.047, respectively). Mean values of sperm concentration and normal motility in the patients in Ivanissevich and Palomo groups were better than those of patients in laparoscopic group; however, Palomo technique had significantly better effect on normal morphology only compared to the laparoscopic technique (Table 3).

Comparing the mean differences of semen parameters among the three groups of varicocelectomy confirmed the results of univariate general linear model. We also used a univariate general linear model for controlling other factors (i.e. duration of surgery, atrophy before surgery, type of anesthesia and grade of varicocele) which were different among the three groups. The results of this analysis showed a significant difference among the groups just in terms of sperm concentration (P=0.040). Post-hoc analysis revealed that this difference was statistically significant only when comparing Ivanissevich (15.13 ± 8.69 million/mL) and laparoscopic (8.77 ± 8.94 million/mL) groups (P=0.008) (Table 4).

Age distribution and mode of presentation laterality was not significantly different in the three groups. Nonetheless, the power of this study to detect differences was low. Thus, we can consider the effect of these variables as residual confounders. Controlling these variables in an additional univariate general linear model showed that there was no significant differences among the three groups in terms of improving all semen parameters.

Varicocelectomy helps to improve atrophy (P<0.001). So, at all ages and all surgery groups, among 31 patients who had atrophy at baseline, improvement in this respect was seen in nearly all of them (n=26, 83.9%), except for 2 patients at the age of 24 and 36. Atrophy was unknown for 3 patients. Moreover, except for the patients in Ivanissevich group, this positive effect was confirmed in patients of the other groups (P=0.016 for laparoscopy and P<0.001 for Palomo).

Table 3: Comparison of the results of surgery before and after utilization of three varicocelectomy techniques

Item	Group			P value	Power (%)
	Laparoscopy n=22	Ivanissevich n=23	Palomo n=25		
Sperm concentration, million/mL[*]					
BS[**]	13.09 ± 9.88	18.26 ± 13.38	14.96 ± 12.89		
AS[***]	21.86 ± 10.28	33.39 ± 14.66[a]	29 ± 13.69[a]	0.0255[&]	
P value[#]	0.001	<0.001	<0.001		
Sperm normal motility (%)[*, ^]					
BS	16.86 ± 6.77	19.56 ± 8.51	18.72 ± 9.90		
AS	23.81 ± 9.55	31.95 ± 13.12[b]	32.80 ± 12.99[b]	0.0235[&]	
P value[#]	0.004	<0.001	<0.001		
Sperm normal morphology (%)[*]					
BS	46.59 ± 14	48.04 ± 12.13	43.40 ± 20.03		
AS	54.55 ± 12.71[c]	57.39 ± 10.32[cd]	58.40 ± 15.72[d]	0.0475[&]	
P value[#]	<0.001	0.001	<0.001		
Atrophy, n(%)[£]	0(0.00)	3(14.29)	1(4.55)	0.294	4.26

BS; Before surgery, AS; After surgery, [*]; Values are presented as mean ± SD, [**]; BS refer to values before surgery, [***]; AS refer to values after surgery, [#]; The comparisons were made by Wilcoxon test, [&]; The Univariate general linear model was used for comparisons among the three groups, by considering the preoperative values of semen parameters as covariate, [^]; Normal motility is sum of (A+B) motility type, £; The comparisons were made by Fisher's exact test between the Laparoscopic and the open surgical techniques. The same lowercases showed no significant differences in post-hoc tests.

Table 4: Comparing the mean differences of indices before and after surgery among patients undergoing three different surgical techniques

Item	Group			P value
	Laparoscopy n=22	Ivanissevich n=23	Palomo n=25	
Sperm concentration (million/mL)[***]	8.77 ± 8.94[a]	15.13 ± 8.69[b]	14.04 ± 11.51[ab]	0.023
Sperm normal motility (%)[#, &]	6.95 ± 9.11	12.39 ± 9.87[c]	14.08 ± 8[c]	0.014
Sperm normal morphology (%)[^]	7.95 ± 4.27[d]	9.34 ± 10.47[de]	15 ± 12.4[e]	0.019

[*]; All values are presented as mean ± SD. All comparisons were made by Kruskal-Wallis test. [**]; Mean count after surgery-mean count before surgery, [#]; Mean normal motility after surgery-mean normal motility before surgery, and normal motility is sum of (A+B) motility type, [^]; Mean normal morphology after surgery-mean normal morphology before surgery. The same lowercases showed no significant differences in post-hoc tests.

Table 5: Stepwise linear regression model for indices of semen analysis after the surgery

Dependent variable	Independent variable	Unstandardized coefficients		Standardized beta	P value	Model	
		Beta	SE of beta			R square	P value
Sperm concentration after surgery	Sperm concentration before surgery	0.761	0.105	0.678	<0.001	0.531	<0.001
	Laparoscopic surgical treatment	-7.587	2.917	-0.243	0.012		
	Atrophy before surgery	-5.449	2.614	-0.196	0.042		
Normal motility of sperm after surgery	Sperm normal motility before surgery	0.992	0.146	0.649	<0.001	0.505	<0.001
	Laparoscopic surgical treatment	-6.334	2.637	-0.229	0.020		
Normal morphology of sperm after surgery	Sperm normal morphology before surgery	0.535	0.070	0.629	<0.001	0.608	<0.001
	Sperm normal motility before surgery	0.354	0.123	0.241	0.005		
	Palomo surgical treatment	5.375	1.953	0.217	0.008		

The results of stepwise linear regression showed that sperm concentration prior to the surgery, laparoscopic varicocelectomy, and atrophy prior to the surgery were the prognostic factors that could significantly predict the sperm concentration after the surgery. Laparoscopic varicocelectomy and presence of atrophy before the surgery have a negative impact on sperm concentration after the surgery. The values of normal motility before surgery and laparoscopic varicocelectomy were independent factors for predicting the normal motility after surgery. In addition, the number of sperms with normal morphology after the surgery, depends on the values of normal morphology before surgery and normal motility, as well as the utilization of Palomo technique. The adjusted R-square of the models (0.531, 0.505, and 0.608) indicates the higher accuracy of regression models in predicting the morphology and concentration, regardless of the number of independent variables entered the model. In each of the models, higher standardized beta indicates higher values of a variable in predicting the dependent variable. The values of each semen parameter prior to the surgery (e.g. sperm concentration) had the highest values in prediction of these parameters (e.g. sperm concentration) after the surgery (Table 5).

Discussion

In this study, following the surgery, sperm concentration, normal motility, and normal morphology worsened in 10, 7.4, and 5.9% of patients, respectively. Based on univariate analysis, sperm concentration, normal motility and normal morphology after surgery using Ivanissevich and Palomo techniques, were better than those of laparoscopic group; but after controlling for confounders, a significant difference was seen only between Ivanissevich and laparoscopic techniques.

A similar study on 100 infertile patients who underwent varicocelectomy, showed a significant difference between open inguinal or laparoscopy methods in terms of sperm concentration and motility (15). A quasi-experimental study comparing open inguinal and laparoscopic Palomo in 50 patients, reported no significant differences between them in terms of sperm concentration and morphology, three and six months after the surgery (10).

According to other studies that had no controlling for confounding factors and did not consider the type of varicocelectomy, varicocelectomy could lead to significant improvements in sperm concentration, motility, and morphology. The results of our study are in line with the mentioned studies and confirm their findings (16, 17). Obviously, good quality and quantity of sperm before the surgery lead to better surgical outcome for the majority of patients. However, in this study we sought to find out which varicocelectomy technique has an additive effect in improving semen parameters. As regression models showed, laparoscopy had an inverse relationship with sperm concentration and motility after the surgery. In addition, these models showed that Palomo varicocelectomy was able to improve the mean normal morphology after the surgery.

The results of this study showed that laparoscopic and Palomo surgery had a positive effect on the improvement of the atrophy. Regarding Ivanissevich technique, at least in short-term follow-up in this study, no improvement in atrophy was seen; however, at least in terms of sperm concentration, changes were in direction to improve.

Nevertheless, to check the efficacy of varicocelectomy, we need to know post-surgery fertility status which was not assessed in this study. The best modality for treatment of varicocele in infertile men is a modality which highly improves semen and increases pregnancy rates with minimum complication rates (recurrence, hydrocele, and atrophy). Thus, an ideal technique not only preserves the lymph nodes and spermatic vessels, but also closes all external and internal spermatic veins. Although so far, no treatment modality has been introduced as a "gold standard" of varicocele treatment. According to the literature, compared to other varicocelectomy techniques, microscopic varicocelectomy (MV), despite its need for more operative time, surgical skills and experiences, was accepted as a standard treatment which had the lowest postoperative recurrence and complication rates (4). The findings of our study showed no significant differences among the three types of varicocelectomy in terms of complications after the surgery, which can be attributed to the method of our study (which consisted of non-random

sampling, small sample size, and short follow-up period). It might be also due to the real low incidence of these complications in similar patients.

Overall rate of complications in open varicocelectomy has been reported to be slightly higher than laparoscopic varicocelectomy (8 vs. 6%, respectively) but this difference is not significant. Recurrent symptoms of varicocele were observed only in five and two patients in laparoscopic and Ivanissevich group, respectively. Other studies have also shown that higher grades of preoperative varicocele lead to increased risk of recurrence that can be secondary to multiple collateral venous channels (15). This can be applicable to our study as well because the majority of the patients in both groups were patients with varicocele grade 3. Another study has also reported the high rate of recurrence in laparoscopic surgery (10, 18-20).

Hydrocele after the surgery has an incidence rate of up to 10% of cases regardless of type of varicocelectomy (21). In the present study, hydrocele was observed in eight, five and two patients in laparoscopy, Ivanissevich, and Palomo group, respectively. Some studies have reported lower incidence of hydrocele in open inguinal group than laparoscopy group (10, 18, 19); but, some others have reported completely opposite results (15, 21, 22). A meta-analysis showed that the incidence of hydrocele is 8.24% after Palomo surgery, 2.84% after laparoscopic surgery, and 7.3% after macroscopic inguinal (Ivanissevich) or subinguinal varicocelectomy (21). Another study indicated that the recurrence rate and incidence of hydrocele were higher in patients undergoing Palomo surgery than those undergoing inguinal microsurgical procedure (22). This controversy could be due to an inadequate follow-up period in this study because most cases of hydrocele occur nine months after varicocelectomy (15).

There are some limitations in our study such as the retrospective nature of study, non-random sampling, small sample size, short follow-up period and no hormonal and fertility assessment. Moreover, there is a risk of selection bias. A higher proportion of men who underwent a Palomo repair had bilateral disease. Similarly, those who underwent this procedure had a higher grade of varicocele and higher incidence of atrophy, as defined in the study. This might have an impact on the results. However, we used a general linear model in our analyses to overcome this problem and fix this bias. Performing all surgeries by a single surgeon may cause dependency of the results to the surgeon; although, it removed inter-observer bias. In real scenario, most cases are like our cases with high grade of varicocele. So, our results are relatively generalizable.

Conclusion

Varicocelectomy improves sperm parameters. Palomo, Ivanissevich, and laparoscopy methods were similar in terms of sperm normal motility and morphology. However, Ivanissevich was more effective in improving sperm concentration. Regarding complications, Palomo technique caused the lowest rate of post-surgery complications. It seems necessary to conduct further studies with longer follow-up periods to clarify the effect of different types of varicocelectomy on semen parameters and pregnancy rate in Iran.

Acknowledgements

We would like to express our special thanks to all patients who participated in this study. This study did not receive any financial support. None of the authors are directly funded or employed by the government. There is no conflict of interest.

Author's Contributions

K.H.; Participated in conception and design of the study, doing all surgeries, interpretation of data and revised the manuscript. A.K.; Designed the study, did statistical analysis, and drafted the article. M.N.; Prepared data and did statistical analysis, drafted the manuscript. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work.

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Case Report

Conservative Management of Ovarian Fibroma in A Case of Gorlin-Goltz Syndrome Comorbid with Endometriosis

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Abstract

Ovarian fibromas are the most common benign solid ovarian tumors, which are often difficult to diagnose preoperatively. Ovarian fibromas, especially in bilateral cases, may be cases of Gorlin-Goltz syndrome (GGS), a rare autosomal dominant disorder with predisposition to basal cell carcinomas (BCCs) and other various benign and malignant tumors. This case report describes a 25 year-old female with GGS, bilateral ovarian fibroma, endometriosis and septated uterus, which was referred to the Gynecology Clinic of Rasoul-e-Akram Hospital in October 2016. This patient had facial asymmetry due to recurrent odontogenic keratocysts. In young cases of ovarian fibromas as reported here, conservative surgical management can preserve ovarian function and fertility. These patients must be followed up by a multidisciplinary team and submitted to periodic tests.

Keywords: Endometriosis, Gorlin-Goltz Syndrome, Odontogenic Keratocysts, Ovarian Fibroma

Citation: **Khodaverdi S, Nazari L, Mehdizadeh-Kashi A, Vahdat M, Rokhgireh S, Farbod A, Tajbakhsh B. Conservative management of ovarian fibroma in a case of Gorlin-Goltz syndrome comorbid with endometriosis. Int J Fertil Steril. 2018; 12(1): 88-90. doi: 10.22074/ijfs.2018.5240.**

Introduction

Gorlin-Goltz syndrome (GGS), also known as the nevoid basal cell carcinoma syndrome (NBCCS), is an autosomal dominant inherited disorder (1). The incidence of this disorder is estimated to be 1 in 50,000 to 150,000 in the general population, varying by geographic region (2). Although it occurs in all ethnic groups, it mostly affects whites, with males and females equally affected (3). Pathogenesis of NBCCS is due to mutations in the patched tumor suppressor gene 9q22.32; PTCH1 causing abnormality in the Hedgehog (Hh) signaling pathway, thus resulting in neoplasm formation (4).

GGS is characterized mainly by the presence of multiple basal cell carcinomas (BCC), odontogenic keratocysts (OKCs) of the jaw, palmar pits and ectopic calcifications of the cerebral falx. More than a 100 minor criteria have also been described. The presence of two major and one minor criteria or one major and three minor criteria are necessary to establish a diagnosis (5). Recent consensus statement from the first international colloquium on basal cell nevus syndrome(BCNS) proposed less stringent criteria for diagnosis where one major criterion and molecular confirmation, two major criteria or one major and two minor criteria are sufficient (Table 1) (3).

Table 1: Criteria for diagnosis Gorlin-Goltz syndrome

The major criteria are:	The minor criteria are:
Multiple BCC or one occurring under the age of 20 years	Macrocephaly (adjusted for height)
Histologically proven OKCs of the jaws	Congenital malformation: cleft lip/palate, frontal bossing, coarse face, moderate or severe hypertelorism
Palmar or plantar pits (three or more)	Other skeletal abnormalities: sprengel deformity, marked pectus deformity, marked syndactyly of the digits
Bilamellar calcification of the falx cerebri	Radiological abnormalities: bridging of the sella turcica, vertebral anomalies such as hemivertebrae, fusion or elongation of the vertebral bodies, modeling defects of the hands and feet or flame shaped hands or feet
Bifid, fused or markedly splayed ribs	Ovarian fibroma
A first-degree relative with NBCCS	Medulloblastoma

BCC; Basal cell carcinoma, OKCs; Odontogenic keratocysts and NBCCS; Nevoid basal cell carcinoma syndrome.

Received: 28 Feb 2017, Accepted: 13 Jun 2017
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Early diagnosis of the syndrome is of great clinical importance since the severity of complications, such as maxillofacial deformities related to the jaw cyst, can be avoided and long-term prognosis of malignant skin lesion and brain tumor is better when early diagnosis and treatment is initiated (6).

Diagnosis of NBCCS may be difficult because of variable expressivity and different age-onsets for different traits of this disorder. The average age for diagnosis of NBCCS is 13 years while the average age for detection of basal cell carcinoma is 20 years. The clinical expression of the syndrome varies among individuals within the same family and to a greater extent among families (7). This case report describes a patient with typical features of GS, diagnosed for the first time in our Department.

Case report

A 25 year-old female was referred to the Gynecology Clinic of Rasoul-e-Akram Hospital because of chronic abdominal pain, myomatous uterus and a 6×8 cm^2 right adnexal mass suspicious to be a dermoid cyst in sonography and magnetic resonance imaging (MRI) reports due to the presence of dense calcification in the tumor. Tumor markers were all normal. She had been born by uncomplicated normal vaginal delivery. She spoke and walked at 19 months of age and her neurodevelopment was normal. At 21 years of age, she was diagnosed with OKCs in the mandibular and maxillary regions, and submitted to surgery for the removal of her dental cysts. In less than a year, the surgery was repeated due to recurrent OKCs.

One year later, she complained of pain in her lower abdomen and underwent trans-abdominal ultrasonography and pelvic MRI, which revealed the right ovarian mass, suspicious of being a dermoid cyst, and a myomatosis septated uterus. Physical examination revealed hirsutism with harsh face and multiple nevi on face and upper trunk. She underwent laparoscopic surgery but after abdominal entry, we encountered unusual round solid ovarian masses, which could not be excluded as malignant. Frozen sections, however, showed they were benign. There was a 6 cm endometrioma in the right ovary and multiple bilateral ovarian fibromas (Figs.1, 2). There were also endometriotic patches in a posterior cul-de-sac. We excised 7 fibromas from the left ovary and 5 fibromas from the right ovary in different sizes ranging from 0.3 to 5 cm in diameter. Both ovaries were preserved. The definitive histologic diagnosis confirmed stromal proliferation and no atypia in the ovarian tissue with areas of necrosis, corresponding to an ovarian fibroma and endometriosis.

According to the laparoscopy outcome, pathologic findings and history of recurrent OKCs, Gorlin syndrome was the top differential diagnosis. Investigation for other signs and symptoms of this syndrome confirmed the diagnosis. Chest radiography, posterior-anterior skull view and spine x-rays were normal. We referred the patient to a dermatologist and excisional biopsy of nevi was undertaken. Fortunately, the pathologic examination was benign. A written consent was taken from the patient for publication of this report.

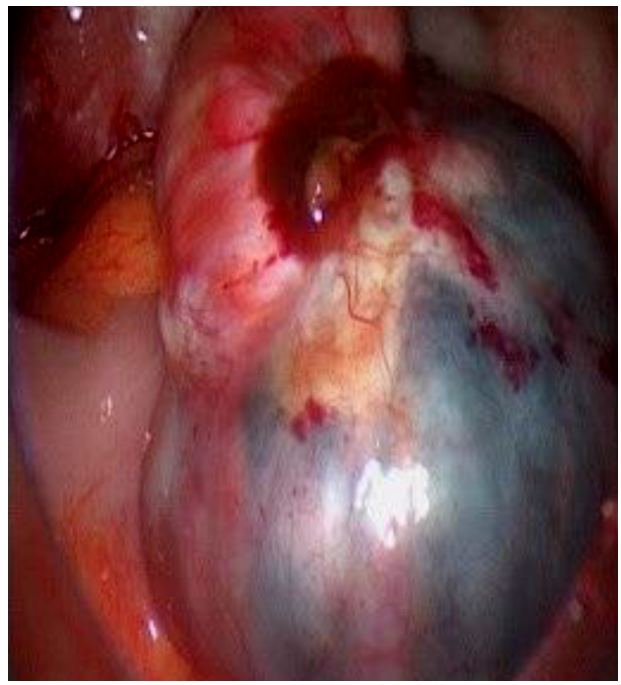

Fig.1: The right ovary with endometrioma and fibroma.

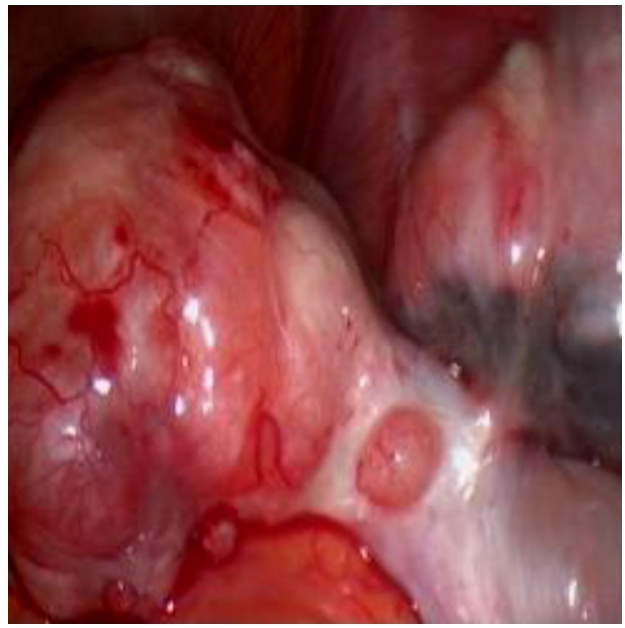

Fig.2: The left ovary with endometrioma and fibroma.

Discussion

GS is an autosomal dominant disorder with near complete penetrance and variable expressivity, and with an estimated birth incidence of 1 in 19,000 individuals (2).

Our patient had one major criterion (i.e. multiple OKCs in the jaw) and 2 minor features (i.e. multiple bilateral ovarian fibromas and a coarse face), thus suggesting it to be a case of GGS.

Conclusion

Three quarters of female patients with GGS are affected with ovarian fibroma that could be bilateral and recurrent,

and thus requires repeated surgery. Fertility of the patient may be influenced by these repeated surgeries and one of the important consultations with these patients has to be about fertility preservation plans. Ovarian fibromas can be excised with minimally invasive methods and the function of the ovary can be preserved at a healthier state.

Acknowledgements

This study was financially supported by Iran University of Medical Sciences and the Endometriosis and Gynecological Disorder Research Center. The authors declare that they have no conflict of interests.

Author's Contributions

S.Kh., L.N., A.M.K.; Contributed to conception and design. M.V.; Were responsible for overall supervision. S.R., A.F. B.T.; Drafted the manuscript, which was revised by L.N. All authors read and approved the final manuscript.

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Correction

Quality of Life and Its Influencing Factors of Couples Referred to An Infertility Center in Shiraz, Iran

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In this article which was published in Int J Fertil Steril, Vol 11, No 4, Jan-Mar 2018, on pages 293-297, the "Duration of tducation (male)" was misspelled in Table 1. The corrected one is "Duration of education (male)".

Table 1: Educational and fertility characteristics of the participants

Variable	n (%)
Cause of infertility	
Male	147 (29.5)
Female	132 (26.5)
Both	45 (9.0)
Unexplained	130 (26.1)
Duration of infertility	
<5 Y	302 (60.6)
5-10 Y	101 (20.3)
>10 Y	63 (12.7)
Duration of education (male)	
<9 Y	89 (18.5)
9-11 Y	221 (46.0)
>11 Y	170 (35.4)
Duration of education (female)	
<9 Y	67 (13.9)
9-11 Y	217 (45.2)
>11 Y	196 (40.8)

In the sentence "These differences might be related to the use of a fertility-specific instrument (FertiQoL) in the study by Huppelschoten et al. (23) and the current study compared to the general QoL assessment instrument by Chachamovich et al. (24) and Rashidi et al. (15)." Which was at the page of 296 in the discussion section, the word "generic" was corrected in to "general".

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 1, Apr-Jun 2018, Page: 91

International Journal of Fertility and Sterility (Int J Fertil Steril)
Guide for Authors

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