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Editorial

Molecular Targets for Endometriosis Therapy: Where We Are and Where We Are Going?

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Endometriosis is a chronic hormone-dependent disease, characterized by the presence of endometrial-like glands and stroma outside the uterine cavity. It could occur in distinct phenotypes: peritoneal superficial lesions, ovarian endometriomas and deep infiltrating endometriosis (DIE) (1), which includes various locations penetrating >5 mm under the peritoneal surface. Prevalence of this disease in women of child-bearing age ranges between 6 and 10% (2), but it may also be diagnosed sometimes in menopause. Endometriosis may be responsible for pain symptoms and infertility, which can severely impact the patient's quality of life (3). Transvaginal ultrasonography is the non-invasive gold standard technique for diagnosing DIE and ovarian endometriomas (4). Magnetic resonance imaging (MRI) can be helpful when the gynecologist is not experienced about ultrasonographic diagnosis of endometriosis or when the findings of ultrasonography are ambiguous (5). Anyway, confirmation of endometriosis diagnosis is only achieved by histological analysis of endometrial stroma and glands.

Medical therapy is usually the first-line option to treat women affected by endometriosis, aiming to improve patient's pain symptoms and to prevent disease recurrence after surgery. Indeed, progestins and combined oral contraceptives (COCs) are usually started in patients with suspicion of endometriosis without any surgical diagnosis (6). Currently, the most appropriate therapy is chosen taking into account several factors, such as patients' age, preference, desire to conceive, intensity and features of pain. Anyway, a long-term regimen is necessary for patients affected by this benign chronic disease, in which, efficacy in improving symptoms has to be balanced with a good tolerability.

Currently available options are not definitely curative for endometriosis, even if women have temporary relief of symptoms. Nevertheless, once the therapy is discontinued, their recurrence happens. Moreover, treatments employed in the clinical practice, with the exception of non-steroidal anti-inflammatory drugs (NSAIDs), are contraceptive, representing a challenge for patients whose want to become pregnant (7). For these reasons, the research of novel alternative active drugs is mandatory.

In this regard, the increasing knowledge of several molecular pathways involved in the genesis of this chronic and progressive disease has pushed forward the investigation of new interesting targets. It is known that implantation, growth and progression of endometriosis are caused by a number of disturbed biological mechanisms including invasion capacity, cell proliferation, apoptosis (8), immune function (9-11) as well as angiogenesis (12).

Research is focalized on finding drugs that specifically target the hormonal and immunological microenvironment of implants, down-regulating endometriotic cells proliferation, enhancing their apoptosis as well as renormalizing their up-regulated mechanisms of invasion and angiogenesis. Over the last 20 years, a wide variety of medical options has been tested: in particular, among experimental hormonal compounds, aromatase inhibitors and gonadotropin realizing hormone (GnRH)-antagonists have been the most studied drug classes in late clinical trials.

Investigation of aromatase inhibitors has greatly been increased over the last decade, considering that important role of aromatase enzyme has been demonstrated in the endometriotic implants. However, majority of data concerning the use of aromatase inhibitors includes a low number of patients receiving them for a limited period of time (maximum 6 months). In addition, frequent presence of drug-related adverse events (such as vasomotor symptoms and musculoskeletal pain) represents an important limitation for their clinical long-term use. Furthermore,

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it has been reported that increasing serum follicle-stimulating hormone (FSH) levels, by these drugs, may cause development of ovarian cysts (13). Nevertheless, in our opinion, research of alternative formulation of aromatase inhibitors is still appealing. For example, a combination of anastrozole and levonorgestrel in a vaginal ring is under evaluation in a randomized, double-blind phase II trial (NCT02203331). In particular, we deem that this vaginal combination may be advantageous for patients with rectovaginal nodules of endometriosis, considering a local action of this drug in high concentration. Anyway, at the moment, administration of aromatase inhibitors should be reserved only in patients not responding to the conventional therapies in the setting of scientific investigations (14).

Contrary to the form of GnRH-analogs, GnRH-antagonists maintain sufficient circulating levels of estrogens, contributing to avoid vasomotor symptoms as well as loss of bone mineral density (15). After promising findings in the multicenter, randomized, double-blinded Elaris Endometriosis I-II studies (16), long-term oral elagolix was effective in improving dysmenorrhea (overall 46-76% of patients) and chronic pelvic pain (50-76%) due to endometriosis with a good safety-profile. These promising data demonstrate that elagolix (in particular at 150 mg, once daily) might be a potential candidate for the management of patients with endometriosis-associated pain who are not responsive to COCs or progestins without the necessity for add-back therapy (differently from the GnRH-analogs). Anyway, new ongoing multicenter double-blinded phase III studies should confirm these preliminary results on larger populations (NCT03343067, elagolix alone; NCT03213457, elagolix plus NETA and estradiol). Moreover, relugolix (TAK-385), as another GnRH-antagonist (17), is currently under investigation in an international phase III study in comparison with placebo (NCT03204318). In general, we think that for this group of innovative hormonal drugs, randomized trials should be necessary in order yet also to compare GnRH-antagonists with COCs or progestins.

Selective hormonal receptor modulators have variable effects on estrogen and progesterone receptors of different tissues, as their pharmacodynamics activity ranges from pure agonism to a pure antagonism. Anyway, the use of selective estrogen receptor modulators (SERMs) and selective progesterone receptor modulators (SPRMs) is improbable to become a first-line strategy to treat endometriosis due to the unproductive results observed in laboratory and animal studies (18). Firstly, these drugs target the same receptors and have the same therapeutic mechanisms with the available hormonal compounds, including the potential contraceptive effect. More importantly, a randomized controlled phase II trial that evaluated a 6-months therapy with raloxifene was prematurely interrupted, because the women allocated to the SERM group showed worsening pain (19). In fact, it has been proposed that raloxifene, differently from rodents that have an estrous cycle, in human may not be able to prevent ovulation. The subsequent production of ovarian estrogens may be continued and, in some cases, even increased following the action on receptors of this drug, causing a worsening of the symptoms. Regarding SPRMs, the studies on anoprisnil have been stopped for the presence of some cases of endometrial hyperplasia (18). Ulipristal, largely used for preoperatively treating myomas, has been investigated by the Pharmacovigilance Risk Assessment Committee of the European Medicines Agency (EMA) for four recent cases of serious liver injury, and thus, its future testing for endometriosis appears improbable.

Currently, new hormonal drugs acting on steroid sulfatase and 17β-hydroxysteroid dehydrogenase are under early pre-clinical investigation (20). Interestingly, a first dual inhibitor of these two pathways has been developed, but no *in vitro* experiment or investigation on animals with endometriosis has been reported yet (21). Generally, more information on efficacy and safety in animals is needed (in particular for 17β-hydroxysteroid dehydrogenase inhibitors) before being able to translate these experimental compounds to women affected by endometriosis. Other non-hormonal targets have been preliminary tested for endometriosis. Anyway, few studies on human have been organized for these compounds.

Angiogenesis is an essential process for establishment and development of implants of endometriosis. Several anti-angiogenic agents (anti-angiogenic antibodies or multi-tyrosine kinases and mTor inhibitors) have been tested in rodents, showing good efficacy in reducing growth of endometriotic lesions (22). However, we deem that the pharmacological activity against angiogenesis in nodules of DIE, which tends to have a high content of fibrosis, remains unclear. Furthermore, due to the important drug-related adverse events (associated to the interference with physiologic angiogenesis) their potential translation into human research should be taken with caution. At the moment, almost none of them have been investigated in clinical trials.

A small pilot study reported encouraging findings in terms of efficacy (70% reduction of endometriotic implants size) and safety by administering quinagolide, a dopamine agonist, for treatment of hyperprolactinemic patients with peritoneal endometriosis. It has been reported by the authors that the mechanism of action of this drug may include an inhibitory effect on the angiogenic pathway (showed in their study by the down-regulation of VEGF/VEGFR2 expression). However, no data regarding patients' symptoms have been reported in this trial (23). Thus, larger, better-designed studies, focalized on clinical efficacy should be organized to confirm its efficacy for endometriosis.

Targeting inflammatory-related pathways in endometriosis appears rational, as it is widely known that the overproduction of prostaglandins, cytokines and other pro-inflammatory mediators characterize endometriotic tissue. Moreover, the action mechanism of non-selective NSAIDs, is largely employed and it is effective for the

treatment of pain associated to endometriosis, including inhibition of the pro-inflammatory prostaglandins synthesis at both the COX-1 and COX-2 sites. For this reason, tumor necrosis factor-α (TNF-α) inhibitors, such as etanercept or infliximab commonly administered in clinical practice for treatment of chronic inflammatory diseases, have been investigated for this chronic hormonal disease (18). Contrary to expectations after promising results in animal models, no clinical trials have been carried out in this setting with the exception of infliximab, which did not demonstrate to be enough active in a small clinical study on women (24).

Oxidative stress displays an important role in the development and progression of endometriotic implants (25). The efficacy of several antioxidants for improving endometriosis-associated pain and reducing size of implants has been successfully assessed in animals. In future, it would be interesting to investigate other potential drugs, like statins, metformin and tiazolinediones, which are not expensive and they are largely available. Moreover, according to latest evidences, they may exert both antioxidant and anti-inflammatory proprieties (26). In this regard, a well-designed in vitro study of human endometrial biopsies and 3-D culture in fibrin matrix investigated the effects of lovastatin on proliferation of stromal cells and invasion of the fibrin matrix (27). Interestingly, a concentration-dependent effect of lovostatin was seen on cell growth and angiogenesis in the experimental groups. Particularly, in the presence of 5 and 10 μM of statin, angiogenesis was abolished, and cell proliferation was inhibited. In the presence of 1 μM of lovastatin, angiogenesis was reduced, but cell proliferation was not affected.

Anyway, a small clinical trial has only been organized to test statins in women after conservative surgery in order to prevent recurrence of endometriosis (28). However, further large studies in vivo are required to draw more accurate conclusions on this topic.

Lastly, methylation of progesterone receptor gene may be part of aberrant gene silencing described in endometriosis (29). Thus, demethylation agents as well as histone deacetylase inhibitors were proposed as potential therapeutic options. At the moment, safety profile of these drugs should be deeply weighed before considering eventual clinical application. In particular, among the others, valproic acid seems to be the most promising option, considering the interesting results obtained from a series of patient case with adenomyosis (30). Anyway, no trial on human has been performed for endometriosis and therefore their beneficial effect should still be confirmed.

In conclusion, a high number of new investigational drugs targeting specific biological mechanisms have recently been proposed for treatment of endometriosis. Although the use of new hormonal treatments, such as GnRH-antagonists, is being deeply tested in the last clinical studies appearing relatively near to introduce into clinical practice, the majority of other innovative agents and targets have been tested only *in vitro* and in the animal model. Thus, more extensive research is mandatory to assess their efficacy and tolerability. In particular, only a minority of these drugs seems suitable for future investigations, considering the cornerstones of the endometriosis management, as well as possible side-effects and impact on quality of life.

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Authors' Contributions

L.F.D., C.S.; Contributed to conception and design and collected, screened and selected relevant literature data. S.F., F.G.; Were responsible for overall supervision. F.B., A.S.L.; Drafted the manuscript, which was revised by J.C., F.G. All authors performed editing and approving the final version of this manuscript for submission, also participated in the finalization of the manuscript and approved the final draft.

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Original Article

Comparison of First Trimester Screening for Down's Syndrome Using Free Beta-Human Chorionic Gonadotropin and Pregnancy-Associated Plasma Protein-A Levels between Spontaneous and IVF Pregnancies at 12 Weeks of Gestation

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Abstract

Background: In some previous studies, it was shown that first trimester screening tests produce equivocal results in *in vitro* fertilization (IVF) pregnancies. The purpose of this study was to compare free beta-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein-A (PAPPA) levels between single normal and IVF pregnancies during 11 to 13 week (+ 6 day) of gestational age.

Materials and Methods: In this observational cohort study, 300 consecutive single IVF pregnancies and 700 single normal pregnancies were enrolled at about 11-13 week + 6 day gestational age and levels of free β-hCG and PAPPA were compared between the groups.

Results: The results demonstrated that PAPPA (P=0.026) was significantly lower and β-hCG (P=0.030) was significantly higher in IVF pregnancies. The other factors including nuchal translucency (NT) and crown-rump length (CRL) and demographic characteristics did not significantly differ between the groups (P>0.05).

Conclusion: This study showed that PAPPA levels are lower but free β-hCG levels are higher in single IVF versus normal pregnancies. This finding could be related to different placentation in intracytoplasmic sperm injection (ICSI) technique because of alterations in oocyte cytoplasm. Therefore, these markers may need to be adjusted in assisted reproductive technology (ART) conceptions. Further research should be done to obtain optimal cut-off for these markers in first trimester screening for detection of Down syndrome in ART pregnancies.

Keywords: Chorionic Gonadotropin, Down Syndrome, *In Vitro* Fertilization, Pregnancy, Pregnancy-Associated Plasma Protein-A

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Introduction

During gestation, pregnancy-associated plasma protein-A (PAPPA) is derivate from the placental trophoblasts and stromal cells at the placental-endometrial surface (1). The pregnancy-associated hormones such as human chorionic gonadotropin (hCG), progesterone and estradiol (E2) are present at high levels at the maternal-fetal surface during the remodeling period and thus could regulate trophoblast invasion (2). High levels of E2 in the placenta may lead to down regulation of the E2 receptors (3, 4). We hypothesized that a higher E2 levels on hCG injection days could result in a suboptimal placental-endometrial interface, and lead to reduced pregnancy-associated hormones concentrations.

Second trimester screening in pregnancy such as triple and quadruple tests are popular examinations used to evaluate for this hormonal imbalances; these examinations first one measure alpha-fetoprotein (AFP), unconjugated estriol, and β-hCG and then, inhibin-A. In case of abnormal second screening test results, complementary tests such as chorionic villus sampling and karyotyping should be performed (5, 6). Since the results of these tests

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may have negative psychological and emotional effects in pregnant women, the use of different methods to decrease false positive results was suggested (7).

In vitro fertilization (IVF) is an important treatment method for infertility. Some studies reported inaccuracy of screening tests in pregnancies developed by this method (8). The purpose of this study was to compare free β-hCG and PAPPA between single normal and IVF pregnancies at 12 gestational weeks.

Materials and Methods

In this observational cohort study, 310 consecutive single IVF pregnancies and 720 single normal pregnancies attending for screenings of the first trimester, to tertiary health care centers, were enrolled at 11-13 week + 6 day gestational age. These women were recruited by random sampling. The study was approved by the Research Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (013648749312). All eligible patients who were enrolled, signed an informed consent.

Inclusion criteria were single pregnancy and lack of complications (abortion, or ectopic or molar pregnancy). Also, subjects who did not have complete medical records, were excluded. Free β-hCG and PAPPA levels measured by kit (the BRAHMS free β-hCG and the PAPPA KRIPTOR CAL). This two automatic system based on immunofluorescence (mIU/mL) and were converted to multiple of the median (MOM) units, respectively and then compared between the groups (9). Also, other variables including maternal and paternal age, body mass index (BMI), gestational history, crown-rump length (CRL), and nuchal translucency (NT) on ultrasonography, were recorded.

t test was used to compare the results of the two groups. Chi-squared test was used for the analysis of differences in proportions among the groups. Data analysis was performed using SPSS software (SPSS, Chicago, Illinois, USA) version 24.0. $P<0.05$ was considered statistically significant.

Results

A total of 1030 women were recruited in the present study; 30 women were excluded from the study due to various reasons, including absence of consent, or loss to follow up (data not shown). The mean age ± SD of the women in the IVF group and normal pregnancy group was 34.05 ± 4.728 and 33.44 ± 4.368 years, respectively (P=0.235).

Demographic characteristics such as BMI and reproductive histories including gravidity and parity and previous abortion did not vary significantly between the groups (P>0.05, Table 1). But, other confounders like fetus sex and maternal behaviors like smoking and alcoholism were not assessed.

Table 1: Demographic characteristics of enrolled patients

Group	IVF pregnancy n=300	Normal pregnancy n=700	P value
Maternal age (Y)	34.05 ± 4.728	33.44 ± 4.368	0.235
Paternal age (Y)	36.70 ± 4.739	36.15 ± 4.903	0.709
Gravidity (n)	1.23 ± 0.701	1.42 ± 0.744	0.101
Parity (n)	1.32 ± 0.478	1.10 ± 0.387	0.116
Living childs (n)	1.05 ± 0.229	1.09 ± 0.422	0.305
Prior abortions (n)	1.38 ± 0.976	1.32 ± 0.637	0.818
BMI (Kg/m^2)	24.95 ± 3.169	24.54 ± 2.615	0.417

Data is shown as mean ± SD. Independent t test was used to compare different variables between the two groups. IVF; *In vitro* fertilization and BMI; Body mass index.

There was no significant difference in NT (1.54 ± 4.643 mm vs. 1.30 ± 1.004 mm respectively, P=0.235) and CRL (56.93 mm ± 7.552 vs. 57.55 mm ± 7.142 for IVF pregnancy and normal pregnant women, respectively, P=0.417) (Table 2).

Table 2: Laboratory screening and ultrasonography findings in two groups

Group	IVF pregnancy n=300	Normal pregnancy n=700	P value
PAPPA levels (mIU/mL)	3.75 ± 0.090	4.10 ± 2.251	0.026
β-hCG titrate (mIU/mL)	44.07 ± 31.366	37.28 ± 23.787	0.030
PAPPA (MOM)	1.19 ± 0.772	1.63 ± 4.375	0.167
β-hCG (MOM)	2.23 ± 2.367	1.67 ± 2.097	0.003
NT (mm)	1.54 ± 4.643	1.30 ± 1.004	0.235[a]
CRL (mm)	56.93 ± 7.552	57.55 ± 7.142	0.417[a]

Values are given as mean ± SD. [a]; Independent t test was used to compare different variables between the two groups, IVF; *In vitro* fertilization, PAPPA; Pregnancy-associated plasma protein-A, β-hCG; Beta-human chorionic gonadotropin, NT; Nuchal translucency, and CRL; Crown-rump length.

Mean PAPPA levels was significantly lower (3.75 ± 0.090 vs. 4.10 ± 2.251 mIU/mL for IVF pregnancy and normal pregnant women, respectively, P=0.026) but β-hCG levels were significantly higher (44.07 ± 31.366 vs. 37.28 ± 23.787 mIU/mL for IVF pregnancy and normal pregnant women, respectively, P=0.030) in IVF pregnancies compared to normal pregnancies. After conversion of the data into MOM unit, no significant difference was found in PAPPA between the two groups (1.19 ± 0.772 vs. 1.63 ± 4.375 MOM for IVF pregnancy and normal pregnant women, respectively, P=0.167), however, a statistically significant difference was observed between the two groups in terms of β-hCG (1.54 ± 4.643 vs. 1.67 ± 2.097 MOM for IVF pregnancy and normal pregnant women, respectively, P=0.003) (Table 2).

Discussion

Results of screening tests obtained for IVF pregnancies are equivocal and accurate risk assessment calculations for better interpretation is crucial. The purpose of this study was to compare free β-hCG and PAPPA levels between single normal and IVF pregnancies at about 12 gestational weeks. It was seen that PAPPA levels (as re-

ported in mIU/mL) was lower but free β-hCG (as reported in both mIU/mL and MOM) was higher in IVF compared to normal pregnancies. In PAPPA levels reported in MOM, there was no significant difference between the two groups. The MOM values reported in the present research were adjusted for smoking status, maternal weight and ethnicity by laboratory software which could explain the differences in PAPPA results (9). Other factors like previous reproductive history, did not differ between the two groups.

This observational cohort study established significantly higher first-trimester β-hCG levels in intracytoplasmic sperm injection (ICSI) pregnancies compared to naturally conceived women (10). In contrast, free β-hCG titrate and NT thickness were similar between ART and normal pregnancies. Nevertheless, the majority of IVF/ICSI pregnant women had an elevated first-trimester combined screening risk estimation. Combined first-trimester screening (cFTS) combines the maternal age-related risk with the levels of maternal serum biomarkers such as free β-hCG, and PAPP-A, and fetal NT to determine the risk of trisomy 21, 18 and 13 (9).

Kagan et al. (8) found that IVF patients had up to 10% lower levels of PAPPA (MOM). Giorgetti et al. (10) reported that PAPPA levels were lower in IVF cases compared to normal pregnancy group but β-hCG levels were same between the groups. Also, they found no relationship between PAPPA levels and the etiology of infertility. However, in our study, significant differences were observed between the two groups for both PAPPA and β-hCG levels measured by ELISA (miL/ml). Giorgetti et al. (10) found that the PAPPA levels following intra uterine insemination (IUI) did not differ from natural conceptions, but in our research, the IUI-conceived women were not included.

Bellver et al. (11) reported lower PAPPA but higher β-hCG levels in IVF cases but no significant differences were found between the groups. However, both PAPPA and β-hCG levels were significantly different between the two groups in our study. Köşüş et al. (12) reported that the etiology of infertility affect β-hCG and PAPPA levels in IVF pregnancies and higher PAPPA level was seen in polycystic ovary (PCO) cases compared to male-factor infertile patients. However, infertility causes were not investigated in our research.

Orlandi et al. (13) reported that PAPPA level was significantly higher (up to 21 %) in IVF cases which was consistent with our data. Nevertheless, they evaluated singleton and twin pregnancy in a small population which was different from the population enrolled in the current study. Engels et al. (14) demonstrated that β-hCG after correction, was higher in IVF group compared to normal pregnancy group which was consistent with our results; however, PAPPA levels were lower in IVF/ICSI groups, they found that false positive rates were higher in IVF pregnant women and needed to be adjusted for better interpretation. However, unlike our study, their study was a retrospective study.

Gjerris et al. (15) reported similar β-hCG, but lower PAPPA levels and NT in ART groups compared to control; the inconsistency between their results and our findings may be due to the larger difference existed between the two groups included in their study compared to our study.

Cavoretto et al. (16) in a systemic review in 2017 reported that β-hCG was slightly higher in ICSI groups, but it did not vary significantly between IVF and normal pregnancy; however, PAPPA level was significantly lower in IVF group in which was consistent with our study. These authors suggested that such differences could be due to changes in the placentation of ART conceptions and recommended to define subgroups of ART conception to explore this discrepancy and better predict obstetrics outcomes. Cavoretto et al. (17) in a retrospective case-control study, found that PAPPA and CRL did not differ between IVF and normal pregnancy; however, β-hCG and NT were significantly higher in IVF group. They studied fresh and freeze single blastocyst transfer and found that pregnancy outcomes were similar when comparing the groups. These authors showed that this difference was not correlated with the pregnancy outcome but it could be due to changes or delay in embryogenesis or placentation and may depend on screening test performance. However, we did not perform blastocyst transfer and did not evaluate pregnancy outcomes. Our finding is in-line with the study as reported by Savasi et al. (18). They found that, free β-hCG levels are considerably greater in IVF/ICSI pregnancies. But, they studied another group of patients as oocyte donor IVF/ICSI cycles that could be different from our research that was done in autologous IVF cycles with their own eggs. They pointed that such variations could be due to the ART techniques.

A limitation of our study was lack of information on the causes of infertility, number and kind of embryos, freeze or fresh embryo transfer; also, we did not evaluate its correlation with biomarker levels such as PAPPA and free β-hCG. Moreover, we did not evaluate pregnancy outcomes in ART patients and controls.

One of the strengths of this cohort research is the large number of the participants. In addition, to avoid misinterpretation, we excluded the patients who received their ART treatment in another place.

PAPPA is an important indicator of the early growth and later development of the placenta (19). A prominent delay in fetal and placental growth leading to numerous metabolic disorders and greater risk of obstetrical complications (such as fetal growth restriction and preeclampsia). It is associated with assisted reproduction techniques and may also cause changes in serum markers levels (20, 21). Since just one corpus luteum is generally observed in pregnancies after freeze embryo transfer, which could explain the normal levels of PAPPA in these patients.

Finally, according to the results obtained in this study, it may be concluded that PAPPA levels are lower and free

β-hCG levels are higher in single IVF compared to normal pregnancies. However, further studies are required to attain more definite results and assess other factors affecting first and second screening tests results.

Conclusion

We found lower PAPPA but higher free β-hCG levels in single IVF compared to normal pregnancies. It could be related to different placentation in ICSI technique because of alteration in oocyte cytoplasm. Therefore, these markers may need to be adjusted in ART conceptions. Further research should be done to obtain optimal cut-off and more definite results for these biomarkers in first-trimester screening of Down syndrome in ART pregnancies.

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Authors' Contributions

R.T., M.T., M.Z., D.Kh., A.T.; Participated in study design, data collection, evaluation, drafting and statistical analysis. A.T.; Conducted biochemical analysis. R.T., M.T., M.Z.; Extensively Contributed to interpretation of the data and drawing conclusion. M.Z.; Was corresponded. All authors performed editing and approved the final version of this manuscript for submission.

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Original Article

Correlation of Copper and Zinc in Spontaneous Abortion

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Abstract

Background: Humans require minute amounts of trace metals to maintain body's normal growth and physiological functions; such elements may also play a vital role in pregnancy and pregnancy outcome. The present study was conducted to assess the role of two trace metals, zinc (Zn) and copper (Cu) in women with history of spontaneous abortion (SAb cases) in comparison to women without such history (controls).

Materials and Methods: In this retrospective study, a total of 277 subjects were enrolled from the Obstetrics and Gynecology Department, Civil Hospital, Ahmedabad, India. Personal demographic information, medical history, reproductive history especially details of number of SAb, duration of last SAb, number of children, etc. were recorded using predesigned and pre-tested proforma. Serum Zn and Cu levels were measured by an atomic absorption spectrophotometer.

Results: The data indicated that the serum level of Cu ($P<0.01$) and Zn was lower in SAb cases as compared to controls. Correlation between the number of SAbs and trace metals levels showed a significant negative correlation between Cu and Cu/Zn and the number of SAbs. Cu/Zn was higher in controls and women having at least one child as compared cases and women without child, respectively. Pregnant women had higher levels of trace elements as compared to non-pregnant women at the time of enrollment.

Conclusion: The data revealed that trace metals such as Zn and Cu have a positive role in pregnancy outcome and optimum levels of Zn and Cu might be able to decline the chances of SAb occurrence in addition to other factors. The ratio of Cu/Zn has a positive role in reproductive outcomes.

Keywords: Copper, Pregnancy Outcome, Spontaneous Abortion, Trace Elements, Zinc

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Introduction

In the advent of industrialization and increasing need for foodstuffs and other requirements for ever growing population, the levels of toxic substances in the environment have increased considerably. Some heavy metals are toxic to humans especially to the pregnant women and developing fetus, even at very low doses, also. However, some trace metals are necessary for normal growth, development and various other physiological functions. Trace elements include more than 60 substances that are generally present at low concentrations in the environment and mammalian tissues. They are present in tissues and serum at a very low concentration (i.e. within picogram or microgram levels), and their absorption, distribution, storage and excretion are firmly controlled. At least a dozen of trace elements are considered essential minerals for human (1). Earlier, it is reported that trace metals like zinc (Zn), copper (Cu), selenium, chromium, cobalt, iodine, manganese, and molybdenum are indispensable for human body and these trace elements accounts for only 0.02 % of the total human weight and play noteworthy role in physiological functions of the body (2). Their deficiencies can lead to reduced activities of associated enzymes and cellular function.

Recently Prashanth et al. (3) reported that trace elements facilitate various vital biochemical reactions by acting as cofactors for many enzymes, and stabilizing structures of enzymes and proteins and they are significant for cell function at biological, chemical and molecular levels. Some of the trace elements govern vital biological processes by binding molecules on the receptor site on cell membrane or by altering the structure of membrane to avert the entry of specific molecules into the cell. At optimum concentrations, they are significant for maintenance of cellular structures, but at insufficient levels, they may adopt different pathways and cause diseases. Earlier, Savory and Wills (4) reported that trace metals play a vital role in biological processes, either as indispensable components or toxins. Pathak and Kapil (5) also reported that inadequacies of trace metals such as Cu, Zn and magnesium were implicated in various adverse reproductive events such as infertility, congenital anomalies, pregnancy wastage, pregnancy-induced hypertension, premature rupture of membranes, placental abruption, still births, low birth weight, etc.

Several reports indicated that optimum concentrations of trace metals are also essential for favorable pregnancy and pregnancy outcome. One such pregnancy outcome

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focused here is spontaneous abortion (SAb). SAb, or miscarriage, is a clinically acknowledged pregnancy loss before the 20th week of gestation (6, 7). The World Health Organization defines SAb as expulsion or withdrawal of an embryo or fetus weighing 500 g or less. Whereas recurrent pregnancy loss is generally defined as 3 consecutive pregnancy losses prior to 20 weeks from the last menstrual period (8). Ajayi et al. (9) reported that decline in vital micronutrients such as Zn, Cu and vitamin E may be associated with recurrent SAb. Both Zn and Cu are essentials to the body but Cu to Zn ratio is clinically more important as compared to the concentration of either elements alone (10). Recently Shen et al. (11) reported that trace elements are closely linked with fetal growth and development throughout pregnancy and their shortage can lead to adverse pregnancy outcomes. Jariwala et al. (12) found that Zn and selenium levels were lowered in pregnant mothers supporting the idea of the need of Zn and selenium supplementation along with iron during pregnancy. Thus, the present study was conducted to understand the role of Zn and Cu with respect to SAb.

Materials and Methods

In this retrospective study, a total of 277 subjects (118 control-subjects without history of SAb and bearing at least one child, and 159 case-subjects with history of SAb) were enrolled from the Out-Patient Department (OPD), Obstetrics and Gynecology, Civil Hospital, Ahmedabad, India. The control subjects (n=118) included 74 pregnant women with children enrolled from OPD and 44 non-pregnant with children enrolled from ward. While SAb cases (n=159) included 86 pregnant women with history of SAb enrolled from OPD and 73 non-pregnant (at the time of enrolment) recruited from ward with history of SAb/current SAb faced. An informed consent was obtained from each participant after explaining the aims and objective of the study as well as benefit of the study in general to the society. The ethical approval of the study was attained from the Institutional Human Ethical Committee of National Institute of Occupational Health (NIOH), Ahmedabad. The personal demographic information, habits, medical history, reproductive history especially details of number of SAb, duration of last SAb, number of children born, etc. were collected and recorded on pre-designed and pre-tested proforma through questionnaire interviews.

Blood samples (about 2 ml) were collected from each subjects and serum was separated using centrifugation (REMI R-8C, India) at 3000 rpm and kept in different aliquots in deep freeze till analysis. The serum levels of Zn and Cu were measured at 213.9 and 324.8 nm, respectively using an atomic absorption spectrophotometer (model no: AAnalyst-800, Perkin Elmer, USA) after preparing proper dilutions. The data were computerized using Microsoft Excel and presented as mean ± SE. Independent student's t test and one-way ANOVA were applied with a significance level of $P<0.05$ to analyze the data using SPSS 16 (SPSS Inc., Chicago, USA). Also, Cu/Zn ratio with respect to different categories/variables was also determined.

Results

The characteristics of both SAb (cases) and control subjects, is depicted in Table 1. The mean age of the SAb group was more than a year higher than that of the control group. Most of the cases of both SAb and control groups were residing in residential area; however, almost about 15-16% of subjects were living in agricultural or industrial areas. Further, about 92 and 88% subjects were literate in control and SAb groups, respectively and about 22 and ~10 % of SAb and control subjects were employed, respectively. Further, about 75% of SAb subjects had a history of one SAb and 20 and 5% of subjects had a history of two and more than 2 SAb, respectively. Further, all the control subjects were having children while about 34.6% SAb group subjects did not have children and the rest of them had children and a history of SAb (Table 1).

Table 1: Characteristics of study population (control and SAb cases)

Characteristic	Cases n=159	Controls n=118
Mean age at the time of SAb/child birth*	24.85 ± 0.32	23.65 ± 0.33
Area of residence		
Agricultural area	10 (6.29)	5 (4.24)
Industrial area	16 (10.06)	13 (11.02)
Residential area	133 (83.65)	100 (84.75)
Educational status		
Illiterate	19 (11.95)	9 (7.63)
Literate	140 (88.05)	109 (92.37)
Employment status		
Employed	36 (22.64)	12 (10.17)
Unemployed	123 (77.36)	106 (89.83)
Pregnancy status		
Pregnant (at the time of interview and sample collection)	86 (54.09)	74 (62.71)
Non-pregnant (at the time of interview and sample collection)	73 (45.91)	44 (37.29)
Number of SAb		
One SAb	119 (74.84)	-
Two SAb	32 (20.13)	-
More than two SAb	8 (5.03)	-
Mean gestational age at the time of SAb (in weeks)	10.37 ± 0.32	-
Pregnancy loss in trimester		
First trimester	117 (73.58)	
Second trimester	27 (16.98)	
PL in 1st or 2nd trimester (subjects more than one SAb, one SAb in 1st trimester and another SAb in 2nd trimester)	15 (9.43)	
Number of children		
0 child	55 (34.59)	-
1 child	68 (42.77)	63 (53.39)
2 children	23 (14.47)	41 (34.75)
3-4 children	13 (8.18)	14 (11.86)

Data are mean ± SE or n (%). SAb; Spontaneous abortion, PL; Pregnancy loss, and *; Calculated as: age at interview-duration of last SAb (cases)/child birth (control).

The data revealed that the mean serum levels of Cu and Zn were higher in control subjects as compared to SAb subjects. There was a significant difference between case and control groups with respect to Cu levels. Further, it was observed that the levels of these trace metals in the pregnant women were higher than non-pregnant women at the time of enrollment. Serum Cu level was higher and serum Zn level was lower in women bearing child in comparison to women bearing no child; however, these differences were statistically non-significant (Table 2). Moreover, Cu/Zn ratio was higher statistically non-significant in controls compared to SAb cases.

Table 2: Level of Serum Zn and Cu with respect to reproductive history

Group	Serum Cu (mg/L)	Serum Zn (mg/L)	Cu/Zn
Case n=159	1.59 ± 0.05	1.430 ± 0.03	1.28 ± 0.066
Control n=118	1.81 ± 0.06**	1.463 ± 0.05	1.46 ± 0.091
P value	0.008	0.594	0.107
Pregnant n=160#	1.75 ± 0.05	1.491 ± 0.03	1.34 ± 0.067
Non-pregnant n=117##	1.59 ± 0.06	1.380 ± 0.04	1.38 ± 0.091
P value	0.103	0.071	0.728
With child n=222	1.714 ± 0.04	1.417 ± 0.03	1.40 ± 0.063
Without child n=55	1.584 ± 0.09	1.553 ± 0.06	1.17 ± 0.104
P value	0.231	0.075	0.061

The data are presented as mean ± SE. SAb; Spontaneous abortion, Cu; Copper, Zn; Zinc, **; P<0.01 show significant differences between the two groups based on independent student's t test, #; Pregnant 160 includes 74 pregnant women having previous child birth and 86 case women with history of SAb, and ##; Non-pregnant 117 includes 44 non-pregnant with previous child birth and 73 non-pregnant with present and past history of SAb, at the time of enrolment.

Besides, the levels of trace metals were analyzed with respect to lifestyle habits of the study population. The data revealed that women having vegetarian or mixed diet had almost similar levels of Cu. While the Zn level was slightly higher in women who adopted mixed diet as compared to those who adopted vegetarian diet. Also, women having habit of tobacco chewing had a lower level of serum Cu and a slightly higher level of Zn as compared to women with no such habits. In terms of caffeine consumption, it was observed that the caffeine consumers (in the form of tea or coffee) had significantly higher level of serum Cu and lower level of serum Zn compared to those who were not consuming caffeine (Table 3). Cu/Zn ratio was significantly higher in caffeine consumers.

Serum levels of Cu and Zn were correlated with the number of SAbs; it was observed that the serum level of Cu was significantly negatively correlated (r=-0.175, P=0.003) with the number of SAbs. As the number of SAb increased, the level of serum Cu decreased. No such correlation was observed between serum Zn and the number of SAbs (Table 4). It was also found that the number of SAb was significantly negatively correlated with Cu/Zn.

Table 3: Serum levels of Zn and Cu and their correlation with lifestyle habits

Variable	Serum Cu (mg/L)	Serum Zn (mg/L)	Cu/Zn
Vegetarian diet n=170	1.690 ± 0.05	1.403 ± 0.03	1.41 ± 0.070
Mixed diet n=107	1.672 ± 0.06	1.509 ± 0.04	1.28 ± 0.088
P value	0.777	0.091	0.250
Chewing habit n=15	1.591 ± 0.10	1.477 ± 0.10	1.12 ± 0.15
No chewing habit n=262	1.693 ± 0.04	1.442 ± 0.03	1.37 ± 0.057
P value	0.591	0.792	0.145
Caffeine consumption n=237	1.730 ± 0.04	1.471 ± 0.03	1.41 ± 0.060
No caffeine consumption n=40	1.439 ± 0.08*	1.641 ± 0.09**	1.06 ± 0.117*
P value	0.017	0.008	0.010

The data are presented as mean ± SE. *; P<0.05, **; P<0.01 show significant differences between two variables groups based on independent student's t test, Cu; Copper, and Zn; Zinc.

Table 4: Correlation between trace metals levels and the number of SAb

Variable	Cu	Zn	Cu/Zn
Number of SAb			
Pearson correlation	-0.175**	-0.006	-0.120*
Sig. (2-tailed)	0.003	0.915	0.046
Cu			
Pearson correlation		-0.040	0.698**
Sig. (2-tailed)		0.511	0.000
Zn			
Pearson correlation			-0.601**
Sig. (2-tailed)			0.000

****; Correlation is significant at the 0.01 level (2-tailed), *; Correlation is significant at the 0.05 level (2-tailed), SAb; Spontaneous abortion, Cu; Copper, Zn; Zinc, and Sig.; Significance.**

The data of trace metals was also analyzed with respect to the duration of the last SAb. The level of Cu was least significantly lower in women who had a recent SAb but highest in controls. No such pattern was observed in Zn serum level. It was also observed that Cu/Zn ratio was increasing as the duration of the last SAb decreased. Controls had the highest Cu/Zn ratio (Fig.1).

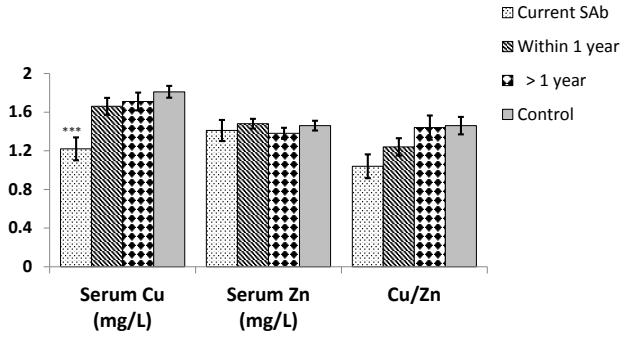

Fig.1: Level and ratios of trace metals (Cu and Zn) with respect to duration of last SAb faced. ***; P<0.001 shows significant differences compared to control based on independent student's t test, Cu; Copper, Zn; Zinc, and SAb; Spontaneous abortion.

Discussion

It is well known that both Cu and Zn are indispensable trace metals that are involved in important physiological functions of the human body. Their deficiencies lead to various diseases and disorders. However, excess levels of these elements can also lead to various pathological conditions. In the present study, both Cu and Zn level were lower in SAb group as compared to control, but differences were significant only in terms of Cu level. There are several reports which indicated that Zn has positive role in female reproduction as well as pregnancy outcomes (13-16). Further, Ajayi et al. (9) also found substantial decline in serum levels of Cu, Zn and vitamin E while a noteworthy elevation in serum levels of lead, selenium and cadmium in recurrent spontaneous abortion (RSA) cases compared to controls. Earlier, Jameson also reported that women who delivered pre-term (37th week or earlier) or post-term (43rd week or later), exhibited significantly lower serum levels of Zn during early pregnancy compared to women delivered a full-term (40th week) pregnancy. Mothers with normal deliveries with normal infants exhibited significantly higher serum Zn but significantly lower serum Cu during early pregnancy compared to women with abnormal labors and immature infants (17). Later, Kiilholma et al. (18) reported that maternal serum Zn and calcium were lower in preterm subjects than in full-term groups and the cord Cu concentration and ceruloplasmin and their fetal/maternal ratios were significantly lower in women with preterm premature rupture of membranes (PPROM) compared to other groups. This indicates a role for Cu in PPROM and Zn in initiation of preterm labor, while calcium and iron may not be associated in the causation of prematurity or PPROM. Very recently, it was also found that Zn levels were lower in the sera of mothers with preterm deliveries with PPROM compared to those without PPROM; but Cu level did not differ between the groups either for maternal or umbilical cord serum or placental tissue (19). The mean serum levels of Cu and Zn were higher in pregnant women as compared to non-pregnant women at the time of subjects' enrollment (cases-history of SAb and control subjects with children). Higher levels of Zn in pregnant women might be due to the presence of 73 non-pregnant subjects with a history of SAb out of a total 107 subjects in this group. Earlier, Izquierdo Alvares et al. (20) reported that serum Zn and Se levels decreased as gestation progresses, while serum Cu concentrations increased, and all the variation occurred mostly in the first 3 or 4 months.

Further, Zare et al. (21) reported that Zn deficiency may be one of the significant causes of adverse outcomes for lymphocyte immunotherapy (LIT) in RSA patients. Hence, compensation for Zn deficiency before LIT can be a promising approach to improve the immune response in patients with RSA. Earlier, Buamah et al. (22) found lower levels of maternal Cu in the abnormal pregnancies compared to the normal ones and these levels did not differ with increasing gestational age. Cu deficiency during pregnancy and post-natal development may have adverse effects on pregnancy and the developing fetus (23-26).

We found that Cu/Zn ratio was lower in SAb subjects than controls. Malavolta et al. (27) reported that the concentrations of Cu and Zn in serum are strictly regulated. There are several mechanisms that are responsible to decline serum concentration of Zn and raise serum concentration of Cu under inflammatory conditions. Recently, Shen et al. (11) observed statistically significant lower serum levels of Zn and iron in subjects with preterm delivery or miscarriage compared to control. Serum Zn levels were significantly lower in subjects with premature rupture of membrane while serum Cu, Zn, calcium and iron were significantly lower in subjects with intrauterine growth restriction.

Conclusion

The present study together with the available information on this issue, suggests that Cu and Zn deficiency as well as Cu/Zn ratio might be associated with occurrence of SAb. Thus, further studies should be warranted on supplementation of these trace elements in clinical practices for the well being of pregnancy and pregnancy outcome.

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Authors' Contributions

S.K.; Served as the principal investigator of the project, involved in conception and design of the study, obtained ethics approval, and prepared the manuscript. R.T.; Was involved in the collection and analysis of the data, and laboratory work, involved in the writing and revision of the manuscript. H.O.; Provided clinical support and expertise in the enrollment of subjects and analysis. I.S.; Was involved in metals analysis and data interpretation. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Original Article

Comparison of Oocyte Maturation Trigger Using Follicle Stimulating Hormone Plus Human Chorionic Gonadotropin versus hCG Alone in Assisted Reproduction Technology Cycles

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Abstract

Background: The goal of this study was to investigate oocyte maturation, fertilization and pregnancy rates among infertile women, by concomitant follicle stimulating hormone (FSH) administration at the time of human chorionic gonadotropin (hCG) trigger, compared to hCG trigger alone.

Materials and Methods: In this prospective randomized controlled trial, 109 infertile women between the ages of 20 and 40 years, received gonadotropin-releasing hormone (GnRH) antagonist and fresh embryo transfer. Following the procedure, the subjects were randomly divided into two groups on the oocyte-triggering day. In the experimental group, final oocyte maturation was achieved by 5000 IU hCG plus 450 IU FSH. In the control group, however, oocyte triggering was performed by 5000 IU hCG, only. The primary outcome was clinical pregnancy and the secondary outcomes included oocyte recovery rate, oocyte maturity rate, fertilization proportion rate, fertilization rate, implantation rate and chemical pregnancy rate.

Results: Fifty-four women were appointed to the group with the FSH bolus injection at the time of hCG trigger and 55 women were assigned to the hCG alone group. Women in the FSH group had a significantly higher metaphase II (MII) oocyte (7.17 ± 3.50 vs. 5.87 ± 3.19), 2 pronuclear embryos (2PNs) (5.44 ± 3.20 vs. 3.74 ± 2.30) and total embryos (4.57 ± 2.82 vs. 3.29 ± 2.13) compared to hCG alone group, respectively. Furthermore, fertilization rate (0.75 ± 0.19 vs. 0.68 ± 0.25), implantation rate (14.2 vs. 8.5%) as well as clinical (27.9 vs. 15.9%) and chemical (32.6 vs. 20.5%) pregnancy rates were higher in the FSH group, but no statistically significant difference was found (P>0.05).

Conclusion: Combination of FSH and hCG for oocyte triggering improves oocyte maturity and fertilization proportion rates without increasing the chance of implantation, chemical and clinical pregnancy rates (Registration number: IRCT2017082724512N5).

Keywords: Follicle Stimulating Hormone Co-Trigger, Human Chorionic Gonadotropin Trigger, Pregnancy Rate

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Introduction

The success rates of assisted reproduction technology (ART) have extremely improved in the recent years. This improvement is due to the new developments in laboratory techniques along with the enhancement of ovarian stimulation protocols. Several studies have investigated the efficacy of different ovarian stimulation protocols. However, the development of final oocyte maturation has not been fully evaluated (1).

The natural ovulation cycles in many mammalian species involve a surge of follicle stimulating hormone (FSH) together with luteinizing hormone (LH) during the mid-cycle phase (2). Several studies have suggested a possible biological role for FSH rising at the time of final oocyte maturation. FSH motivates LH receptor activation in luteinizing granulosa cells and stimulates oocyte nuclear maturation through meiosis resumption and oocyte cumulus expansion (3). In a regular *in vitro* fertilization (IVF) cycle, human chorionic gonadotropin (hCG) is a gold standard for triggering final oocyte maturation, mimicking the effects of the natural mid-cycle LH surge (4). However, the risk of ovarian hyperstimulation syndrome (OHSS) has been increased with hCG treatment for oocyte triggering (5). Moreover, several reports have shown that induction of final oocyte maturation using gonadotropin releasing hormone agonists (GnRHa) is more advantageous than hCG triggering; as it provides a surge of gonadotropins similar to the natural mid-cycle occurrence, and thus prevention of OHSS (4, 6, 7). In addition, it is demonstrated that FSH surge before ovulation could improve oocyte maturation and recovery in ART cycles (8). Regardless of the currently available animal studies (2, 9, 10), there are limited reports evaluating the role of

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FSH in final oocyte triggering. It has been shown that oocyte triggering using 1500 IU hCG plus 450 IU FSH may reduce OHSS compared to the routine trigger methods such as (5000 IU hCG alone), without any positive effects on the outcomes of IVF and pregnancy (11).

The goal of this study was to compare oocyte maturation as well as fertilization and pregnancy rates among women receiving high-dose concomitant FSH administration at the time of hCG trigger to those with hCG trigger alone. We also aimed to compare OHSS development between these two groups.

Materials and Methods

This prospective randomized controlled trial was performed at Yazd Reproductive Sciences Institute between August and November of 2017. The study was approved by the Ethics Committee of Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran (IR.SSU.RSI.Rec.1396.7). All couples signed a written informed consent for participation. The study was registered in Iranian Registry of Clinical Trials (IRCT2017082724512N5) and was indicated according to the CONSORT statement.

Subjects

For this study, 109 infertile women at the ages of 20 to 40 years underwent GnRH antagonist protocol for controlled ovarian hyperstimulation and fresh embryo transfer in the same cycle. Exclusion criteria were severe male factor infertility, cycle cancellation or changing to intrauterine insemination in the subjects, and a cycle containing preimplantation genetic diagnosis (PGD) and estradiol level of more than 2500 pg/mL on the day of hCG injection. Women who did not undergo embryo transfer due to the freeze-all policy, donor, or surrogate cycle, were also excluded.

Stimulation protocol and randomization

All patients who participated in this trial were stimulated using gonadotropins Cinnal-f (CinnaGen, Iran), which started on day 2 of the menstrual cycle. The initial dose of gonadotropin was individualized for each patient based on the age, antral follicle count (AFC), and anti-mulerian hormone (AMH) level. Gonadotropin dose adjustment was done based on ovarian response by follicular diameter measurement with transvaginal ultrasound, which was done every 2 to 3 days from the 7th day of stimulation. The GnRH antagonist (Cetrorelix, Merck Serono Laboratories, Aubonne, Switzerland) was administered when the mean diameter of dominant follicles reached 13-14 mm. In all patients, oocyte triggering was performed when at least three follicles with a diameter of 18 mm or greater were found in the ultrasound examination. The participants were randomly divided into two groups on the day of trigger. Randomization was performed using computer-created random numbers in covered, unlabeled envelope each holding a single number. The patients, nurses, and physicians were not blinded to the allocated treatment groups. In the first group, final oocyte maturation was done by 5000 IU hCG (Pregnyl, Organon, Netherlands) plus 450 IU FSH (Cinnal-f Cinnagen, Iran). In the control group, oocyte triggering was performed by 5000 IU hCG alone. The dose of 450 IU FSH was chosen as it seems to be the maximum practical dose with the goal of making a FSH surge with a natural cycle (1:4-5 FSH:LH ratio) (1).

Transvaginal oocyte retrieval was done 36 hours after triggering for all subjects. Routine IVF/intracytoplasmic sperm injection (ICSI) was performed according to standard protocols (72.7% ICSI and 27.3% IVF). Oocyte maturity was assessed after cumulus cell denudation, and fertilization was evaluated 18 hours after insemination or sperm injection. The best embryos with at least 7 blastomeres (7-9 blastomeres) and a maximum of 20% cytoplasmic fragmentation were considered as grade A. Grade B embryos had 7-9 cells with over 20% fragmentation. Grade C embryos had 4-6 cells with a maximum of 20% fragmentation.

Two or three good quality embryos were transferred 48-72 hours after oocyte retrieval, using an embryo transfer Labotect catheter (Labor-TechnikGöttingen GmbH, Gottingen, Germany) or a Cook (Sydney, Australia) catheter.

Outcome parameters

The primary outcome was clinical pregnancy, defined as the observation of fetal heart activity by transvaginal ultrasound 2-3 weeks after positive β-hCG test. Secondary outcomes included oocyte recovery rate, oocyte maturity rate, fertilization proportion rate, fertilization rate, implantation rate and chemical pregnancy, which were defined as follows: oocyte recovery rate was the number of retrieved oocytes divided by the number of follicles >10 mm in size counted on the day of trigger; oocyte maturity rate was the number of metaphase II (MII) oocytes divided by the number of oocytes retrieved; fertilization proportion was the number of 2 pronuclear (2PNs) divided by the number of oocytes retrieved; fertilization rate was the number of 2PNs divided by the total number of MII oocytes; implantation rate was the number of intrauterine gestational sacs observed by transvaginal ultrasonography divided by the total number of transferred embryos; and chemical pregnancy rate was positive β-hCG test 14 days after embryo transfer. OHSS development was also considered as a secondary outcome, so women with signs of OHSS were separated into three categories according to the signs and symptoms. Mild OHSS was considered as ovarian enlargement, lower abdominal discomfort, mild nausea, vomiting, and abdominal distention. Getting worse of symptoms, ascites, and ovarian enlargement up to 12 cm were characterized as moderate OHSS. Lastly, severe OHSS was defined by severe pain, rapid weight gain, tense ascites, hemodynamic instability, difficulty of respiration, progressive oliguria, and laboratory abnormalities (12).

Statistical analysis

We assumed that at least a total of 100 cases are needed (50 in each group) to achieve a 15% difference in the clinical pregnancy rate as our primary outcome between the two groups. A power of 80% and P<0.05 level of significance were set for this study. The Statistical Package for the Social Science version 20 for Windows (SPSS Inc, Chicago. IL, USA) was applied for data analysis. Differences between continuous variables without normal distribution were measured by Mann-Whitney U test. The Chi-square test was used to compare categorical variables.

Results

Initially one hundred and fifty-two infertile women enrolled in the study. Of those, 109 participants met the inclusion criteria on the day of oocyte trigger and were randomized into two groups. Fifty-four women were appointed to the group with the FSH bolus injection at the time of hCG administration and the remaining 55 women were assigned to the hCG alone administration group. A total of 43 women who were excluded before randomization were as follows: 9 cycles were canceled or converted to intrauterine insemination, 13 women had estradiol levels more than 2500 pg/mL at the time of triggering, 2 women were planned to have PGD, 19 couples were diagnosed with severe male factor infertility, 2 women in the FSH group forgot to take the ordered medicine, and 1 patient in the control group withdrew from the treatment prior to oocyte retrieval due to personal reasons. All other participants were available throughout the study for follow-up in both groups (Fig.1). Demographics characteristics of both groups are listed in Table 1. The demographic features did not differ significantly between the two groups.

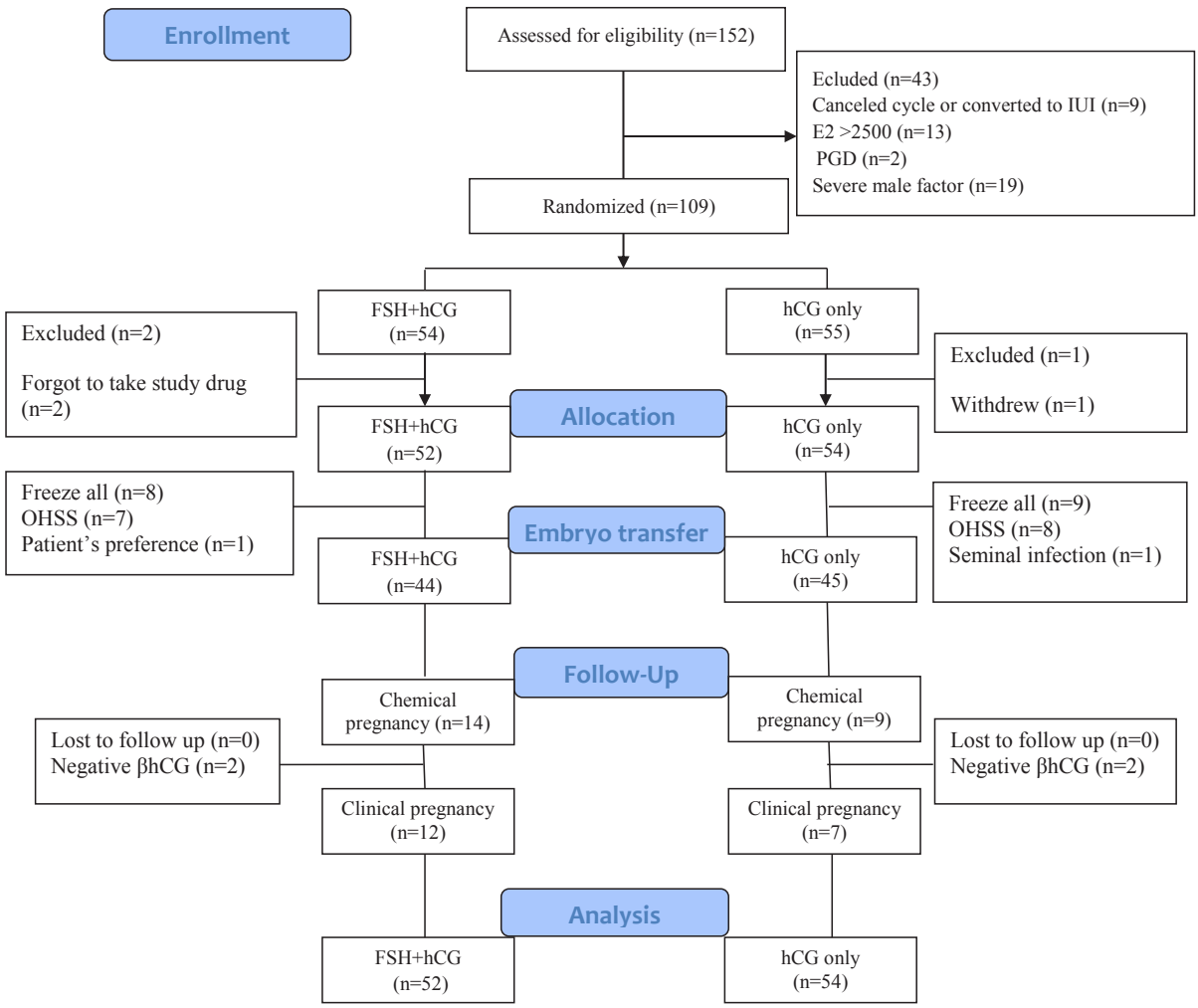

Fig.1: Flowchart of participants' allocation, treatment, follow-up, and analysis. IUI; Intrauterine insemination, E2; Estrogen, PGD; Preimplantation genetic diagnosis, OHSS; Ovarian hyperstimulation syndrom, FSH; Follicle stimulating hormone, and hCG; Human chorionic gonadotropin.

Table 1: Baseline characteristics of "FSH+hCG" group versus "hCG only" group

Variables	FSH+hCG n=52	hCG only n=54	P value
Age (Y)	29.84 ± 4.24	30.83 ± 4.66	0.258*
Duration of infertility (Y)	6.18 ± 3.69	6.05 ± 4.14	0.611*
Type of infertility			
Primary	42 (80.8)	36 (66.7)	0.125**
Secondary	10 (19.2)	18 (33.3)	
AMH (ng/ml)	3.65 ± 2.56	4.00 ± 2.06	0.158*
Endometrial thickness (mm)	9.55 ± 1.72	11.30 ± 13.64	0.952*
Cause of infertility			0.706**
Male factor	20 (38.5)	15 (27.8)	
PCOS	4 (7.7)	7 (13)	
TF	3 (5.8)	3 (5.6)	
MIX	15 (28.8)	15 (27.8)	
Unexplained	10 (19.2)	14 (25.9)	

Data are presented as mean ± SD and number (%). "FSH+hCG" group versus "hCG only" group using *; Mann-Whitney U test, **; Chi-squared test, FSH; Follicle stimulating hormone, hCG; Human chorionic gonadotropin, AMH; Anti mullerian hormone, PCOS; Polycystic ovary syndrome, and TF; Tubal infertility.

Furthermore, total gonadotropin dose, serum estradiol on the day of trigger, number of total follicles on the day of trigger and number of retrieved oocytes were similar in both groups. Moreover, number and quality of the transferred embryos were comparable between the two groups. Nevertheless, women in the FSH group had a significantly higher MII oocyte, 2PNs and total embryos compared to the hCG alone group (Table 2).

Table 2: ART cycle characteristics of "FSH+hCG" group versus "hCG only" groups

Variables	FSH+hCG n=52	hCG only n=54	P value
Total gonadotropin dose (IU)	1537 ± 422	1567 ± 500	0.831*
Number of days of stimulation	10.08 ± 2.04	9.7 ± 1.69	0.641*
Serum estradiol on the day of trigger (pg/ml)	1246 ± 462	1244 ± 460	0.982*
Number of total follicles on the day of trigger	9.71 ± 3.21	10.16 ± 3.73	0.503*
Number of oocyte retrieved	8.32 ± 3.88	7.62 ± 3.93	0.322*
Number of MII oocytes	7.17 ± 3.50	5.87 ± 3.19	0.049*
Number of 2PNs	5.44±3.20	3.74 ± 2.30	0.002*
Number of total embryos	4.57 ± 2.82	3.29 ± 2.13	0.012*
Number of embryos transferred	n=44 1.90 ± 0.29	n=45 1.84 ± 0.52	0.400*
Quality of embryo transferred	n=44	n=45	0.231**
A	13 (29.5)	13 (28.8)	
B	26 (59.1)	27 (60)	
C	5 (11.4)	5 (11.1)	

Data are presented as mean ± SD and number (%). "FSH+hCG" group versus "hCG only" group using *; Mann-Whitney U test, **; Chi-squared test, ART; Assisted reproductive technology, FSH; Follicle stimulating hormone, hCG; Human chorionic gonadotropin, MII; Metaphase II, 2PN; 2 pronuclear, quality of embryos A-C as described in materials and methods.

Eight women in the FSH group and 9 women in the control group decided to use the freeze-all porcedure for future embryo transfer. Therefore, fresh embryo transfer was performed in 44 and 45 cycles in the FSH and control groups, respectively. For comparing the two treatment groups (FSH plus hCG vs. hCG only), we analyzed the reproductive outcomes such as fertilization rate (0.75 ± 0.19 vs. 0.68 ± 0.25), implantation rate (14.2 vs. 8.5%), as well as clinical (27.9 vs. 15.9%) and chemical (32.6 vs. 20.5%) pregnancy rates, which were all higher in the FSH group, but not at a statistically significant level (P>0.05). Also, we found that oocyte recovery rate showed no statistically significant difference between the two groups, however, there is a general trend towards greater oocyte recovery rate in the FSH group. In addition, women who received FSH showed significantly higher oocyte maturity rate and fertilization proportion than the other group (Table 3). Proportion of mild and moderate OHSS development was similar between the two groups (P>0.05). There was no case of severe OHSS in either group (Table 4).

Table 3: ART outcome of "FSH+hCG" group versus "hCG only" groups

Cariables	FSH+hCG n=44	hCG only n=45	P value
Oocyte recovery rate	0.84 ± 0.19	0.75 ± 0.26	0.066*
Oocyte maturity rate	0.87 ± 0.16	0.77 ± 0.19	0.004*
Fertilization proportion	0.65 ± 0.20	0.51 ± 0.22	0.001*
Fertilization rate	0.75 ± 0.19	0.68 ± 0.25	0.124*
Implantation rate	12/84 (14.2)	7/82 (8.5)	0.244**
Chemical pregnancy rate	14 (32.6)	9 (20.5)	0.231**
Clinical pregnancy rate	12 (27.9)	7 (15.9)	0.203**

Data are presented as mean ± SD and number (%). "FSH+hCG" group versus "hCG only" group using *; Mann-Whitney U test, **; Chi-squared test, ART; Assisted reproductive technology, FSH; Follicle stimulating hormone, and hCG; Human chorionic gonadotropin.

Table 4: OHSS occurrence in "FSH+hCG" group versus "hCG only" groups

OHSS occurrence	FSH+hCG n=52	hCG only n=54	P value
No OHSS	45 (86.5)	46 (85.2)	0.968
Mild	5 (9.6)	6 (11.1)	
Moderate	2 (3.8)	2 (3.7)	
Severe	--	--	

Data are presented as number (%). "FSH+hCG" group versus "hCG only" group using Chi-squared test. FSH; Follicle stimulating hormone, hCG; Human chorionic gonadotropin, and OHSS; Ovarian hyper stimulation syndrome.

Discussion

Our results showed that co-administration of a bolus dose of FSH and hCG for oocyte triggering improves the number of MII oocytes, 2 PNs, embryos, as well as oocyte maturity rate and fertilization proportion, compared to hCG injection only.

It is well-documented that mid-cycle LH surge is necessary for oocyte maturation, but the possible alternative is poorly investigated. Animal studies have indicated that FSH is capable of inducing ovulation. In hypophysectomized rats ovulation was induced using LH-free recombinant FSH, and as a result a 100% dose-dependent ovulation rate was obtained (13). Similarly, another study found comparable ovulation rates in both FSH-stimulated and hCG-induced mice (14). Moreover, Zelinski-Wooten et al. (15) showed that a single bolus of 2500 IU recom-

binant FSH was equivalent to 1000 IU hCG for induction of mid-cycle FSH surge, meiosis resumption and fertilization in rhesus monkeys. The first human report of FSH injection at the time of oocyte trigger was a case study, which administered 1050 IU FSH instead of standard 10000 IU hCG before oocyte retrieval. This case report indicated that administration of recombinant human FSH results in production of good quality oocyte with the maturity rate of 90% and consequent high-graded embryos. Although no pregnancy was achieved after transfer of fresh or frozen-thawed embryos in this study (2).

Similar to our findings, in another study the authors triggered oocytes by adding either 450 IU FSH injection or normal saline as placebo at the time of hCG administration. The results showed a significant rise of oocyte recovery rate and fertilization proportion in FSH group compared to the placebo-treated group. Furthermore, we found higher fertilization and implantation rates along with chemical and clinical pregnancies, but the difference did not reach significance. In the same way, Lamb and colleagues, reported insignificant increase in implantation rate, clinical and ongoing pregnancy rates. Although they did not assess oocyte maturity rate, but their IVF fertilization rate was reported significantly higher in the FSH group (1).

Mid-cycle FSH surge promotes oocyte cumulus expansion and oocyte nuclear maturation through constitution of signaling pathways and bilateral communications between oocytes and cumulus cells by opening the gap junctions (16). On the other hand, FSH induces plasminogen activator gene expression (13) as well as elevation of plasminogen activator (17, 18). In primates, FSH facilitates detachment of oocytes from follicular wall and provokes follicular rupture (19). In rodents, this possibility is confirmed by an increase in oocyte recovery following a bolus injection of recombinant FSH (20), which is similar to that in humans (1, 21). In agreement with aforementioned studies, we found higher oocyte recovery rate in the FSH group compared to the control women.

Several studies have shown indirect beneficial effects of FSH on the occurrence of OHSS. For instance, using GnRHa for final oocyte maturation minimizes the chance of OHSS, with comparable results than conventional oocyte triggering (6, 22-24). Nonetheless, an important disadvantage of GnRHa triggering is failure to induce LH surge in downregulated stimulation cycles in patients with hypothalamic dysfunction. Oocyte triggering by low dose hCG plus FSH, however, could be beneficial in all types of stimulation protocols. We also compared OHSS development in the two study groups and found similar rates between FSH plus hCG and hCG alone groups. We know of only one study, in which the authors assessed the effects of FSH for oocyte triggering in high responder women. They applied 1500 IU hCG plus 450 IU FSH for OHSS prevention and found that OHSS associated symptoms were significantly lower among women receiving FSH and hCG compared to those treated with hCG alone. Moreover, the authors claimed that this strategy induced meiosis resumption and cytoplasmic maturation, which resulted in high quality oocytes with competence to generate normal embryos leading to live births (11).

Conclusion

Our results showed that adding 450 IU FSH to 5000 IU hCG for oocyte triggering improves oocyte maturity and fertilization proportion rates. However, it does not increase the chance of successful implantation or chemical and clinical pregnancy rates. Prominently, further studies are required to optimize this novel triggering strategy with regards to concentration, sample size, etc. to provide significantly higher pregnancy percentages along with reduced OHSS rate.

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Authors' Contributions

S.D., A.A.; Original idea and design. S.D.; Data acquisition and interpretation. A.A.; Data interpretation, and supervision. S.D., N.T.; Drafting of the manuscript. N.T.; Data analysis and interpretation. A.A., N.T.; Revising the manuscript for critically important intellectual content. All authors read and approved the final manuscript.

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Original Article

Outcomes after Hysteroscopic Treatment of Symptomatic Isthmoceles in Patients with Abnormal Uterine Bleeding and Pelvic Pain: A Prospective Case Series

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Abstract

Background: Isthmoceles are described as complications associated with caesarean section (CS). Only symptomatic isthmoceles should be treated. The main symptoms are abnormal uterine bleeding (AUB) in the absence of any other causes, pelvic pain and secondary infertility. There are several techniques described for the correction of isthmoceles. Isthmoplasty can be performed by hysteroscopy, laparoscopy or vaginal surgery. The aim of this study was to assess the effectiveness of hysteroscopic surgical treatment of isthmoceles in women with associated symptoms such as pelvic pain and AUB.

Materials and Methods: A prospective case series study was performed; this study included all women with AUB, pelvic pain and ultrasonographic (US) diagnosis of isthmocele, who had undergone hysteroscopic correction between June 2014 and December 2017 in our Hospital.

Results: Thirty eight women underwent surgical hysteroscopy for correction of symptomatic isthmoceles. All patients presented AUB, 42.1% experienced pelvic pain and 28.9% had secondary infertility. US evaluation of isthmoceles was performed using 2D ultrasound. The residual myometrial thickness (RMT) above the isthmocele was measured in women who expected future pregnancy; if it was <2.5 mm the patient was not included in the study because the correction was performed laparoscopically. Follow-up was performed one and two months after the surgery. In all cases, pelvic pain was resolved one month after the surgery. AUB disappeared within the first month in 87.5% of patients and in the second month in 96.8% of subjects; however, one patient needed further surgery to alleviate her symptoms. Secondary infertility was assessed one year after surgical isthmoplasty. Seven women completed the first year of follow up, and three of them (42.8%) reported pregnancy after treatment between six and eight months after the surgery.

Conclusion: Hysteroscopic correction of symptomatic isthmoceles may constitute a safe and effective technique for patients who present AUB and pelvic pain.

Keywords: Caesarean Section, Hysteroscopy, Infertility, Metrorrhagia, Pelvic Pain

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Introduction

The number of deliveries by caesarean section (CS) has increased during the last 15 years (1). In 2016, the global rate of CS in Spain was about 22%, even higher in private hospitals (2). This rise probably leads to a greater incidence of complications. Uterine scars defects, also known as isthmoceles, are described as complications associated with CS. An isthmocele is an anatomical uterine defect, defined as a reservoir-like pouch in the isthmus of the anterior uterine wall, at the site of the CS scar (3-5). This complication is more frequently observed in women with retroverted uterus and those with multiple CS (6).

It is thought that the scar defect appears due to tissue healing impairment, probably secondary to reduced vascular perfusion in this area (6, 7). Although the mechanisms are unknown, several factors such as differences in the thickness between the superior and inferior edges of the hysterotomy (8), the stage of labour at the moment of the CS (9, 10) or the suturing technique (8, 11-14) may contribute to the formation of the defect. Isthmoceles are usually asymptomatic, and may be incidentally diagnosed by ultrasonography; in such cases, treatment is not required. The niche (anechoic area) may vary in sizes and symptoms may be related to the

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size of the defect (6, 15, 16). The main symptoms are abnormal uterine bleeding (AUB) in the absence of any other causes, pelvic pain and secondary infertility (3, 6, 8, 9, 17). The typical pattern of bleeding is postmenstrual dark spotting.

The presence of an isthmocele may also cause complications during some gynaecological procedures such as curettages, hysteroscopy, intrauterine device insertion or in embryo transfers, because of alteration of uterine anatomy (18). Diagnosis is based on the symptoms and complementary exams. Transvaginal ultrasound (TVUS) and hysterosonography measure not only symptomatic defects but also isthmoceles in asymptomatic patients (14, 16, 18-20). Hysteroscopy is also a very effective technique that ensures diagnostic confirmation by direct visualization of the pouch enabling direct correction of the defect (21).

The aim of this study was to assess the effectiveness of hysteroscopic surgical treatment in patients with pelvic pain, AUB and TVUS diagnosis of isthmocele, in the absence of other causes.

Materials and Methods

This prospective case series study included all women with AUB, pelvic pain and US diagnosis of isthmocele, in the absence of other causes, who had undergone hysteroscopic correction between June 2014 and December 2017 in our hospital. The study was approved by the Ethical Committee of the Hospital with code 18.07.1270-GHM.

The diagnosis was based on the symptoms, patients' background once other possible causes of AUB had been excluded. As complementary test at office we used TVUS. TVUS was performed in early proliferative phase. The isthmocele is identified as an anechoic triangular-shaped area, with the vertex pointing to the bladder, in the isthmus of the anterior uterine wall. Both depth and width of the defect were measured (Fig.1).

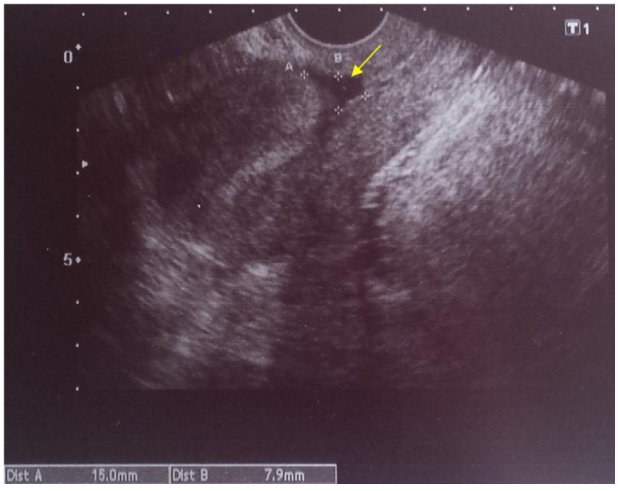

Fig.1: Ultrasound image of an isthmocele. Yellow arrow points towards the isthmocele.

Scar defects were classified based on their size according to the classification proposed by Gubbini et al. (4) using the triangle area formula: base x height/2. Gubbini et al. (5) established 3 grades as follows: grade I: <15 mm^2; grade II: 16-25 mm^2; and grade III: >25 mm^2 (4, 5). The residual myometrial thickness (RMT) above the vertex of the isthmocele, was also measured in patients who expected future pregnancy. When the RMT was <2.5 mm, the correction of the defect was performed by laparoscopic technique and these patients were not included in this case series. All women were assessed by the anaesthesiology team and provided with informed consent.

Hysteroscopy was performed under general anaesthesia in the operating room, using saline solution as distending media. All hysteroscopies were done by two experienced surgeons who followed the same protocol. Initially, a diagnostic hysteroscopy using 5-mm, 30º angle lens, rigid hysteroscope (Karl Storz GmbH and Co, Tuttlingen, Germany), without cervical dilatation, was done in order to achieve direct view of the scar defect and to exclude other intrauterine anomalies. Afterwards, hysteroscopic niche resection was performed using a 9-mm bipolar loop resectoscope (Ethicon Gynecare Inc., Johnson and Johnson). Small defects were resected by a 5-mm hysteroscope and a 5-Fr bipolar electrode. Anterior and posterior fibrotic arch of the isthmocele were identified. The anterior arch was resected by the bipolar loop resectoscope or the 5-Fr bipolar electrode in cases of small defects, until the bottom of the isthmocele reached the level of the cervical canal. The bottom of the sacculation was coagulated (Fig.2).

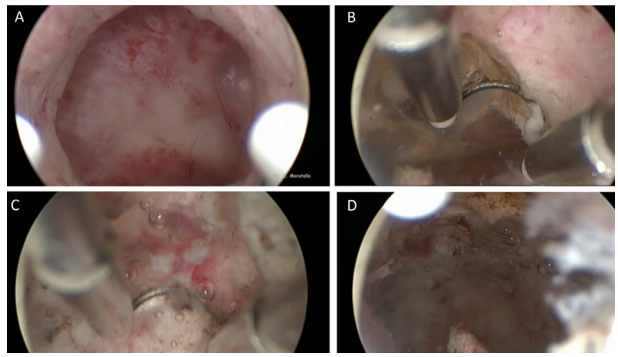

Fig.2: Hysteroscopic isthmoplasty. **A.** Isthmocele, **B.** Resection of the anterior arch, **C.** Coagulation of the bottom of the niche, and **D.** Image after resection.

Results

Between June 2014 and December 2017, 38 patients underwent surgical hysteroscopy for correction of symptomatic isthmoceles. Mean age of the patients at the intervention was 40 [31-47] years. All women presented postmenstrual AUB (PAUB). Among them, 16 patients (42%) had pelvic pain and 11 (29%) had secondary infertility. All women had at least one previous CS (63.1%), nine women (23.6%) had 2 CS, and five women (13.1%) had 3 previous CS. Regarding the

anatomical position of the uterus, 65.7 and 34.3% of patients presented anteverted and retroverted uterus, respectively. Isthmoceles were classified according the US based classification described above. Nine out of the 38 women (23.6%) presented grade 1 isthmocele, eight women (21%) presented grade 2 defects and 21 women (55.2%) had grade 3.

In 81% of cases (31 patients) the procedure was performed using a bipolar loop resectoscope as the diagnosis had already been established on a previous diagnostic hysteroscopy. For the rest of the patients (19%) who had smaller defects, correction of the isthmocele was carried out using a 5-Fr bipolar electrode. All patients were discharged on the day of the surgery. No complications or adverse effects were reported after hysteroscopic resection. The criterion for selecting a specific hysteroscopic resection technique was the size of the niche. The RMT was also taken into consideration in patients with secondary infertility who expected future pregnancy, those women who presented an RMT <2.5 mm were excluded from hysteroscopic correction and underwent laparoscopic correction of the isthmocele. Follow-up was performed 1 and 2 months after the surgery. PAUB was the most frequently reported complaint, which was resolved within 2 months in almost all women; however, 1 woman needed a second surgery to eliminate the spotting. In 79.5% of patients, PAUB disappeared within the first month, and after two months of follow-up, 97.4% of women did not present with AUB. Pelvic pain was resolved in 100% of the patients 1 month after surgery.

Ultrasonographic (US) follow-up showed that after the surgery, 100% of grade I and II isthmoceles were completely resected. On the other hand, in three of the twenty one grade III isthmoceles, despite the resolution of the symptoms, small defects could still be observed on US, two months after the surgery (Table 1).

Table 1: Results of ultrasonographic follow up

Ultrasonographic image	Before surgery	1 month after surgery	2 months after surgery
Grade I	23.6%, n=9	0	0
Grade II	21%, n=8	0	0
Grade III	55.2%, n=21	4 grade I	3 grade I

Secondary infertility was assessed one year after surgical isthmoplasty. Eleven patients showed infertility, seven completed the first year of follow up, and three of them reported pregnancy after treatment (42.8%) between six and eight months after the surgery. One patient was lost to follow-up, and the remaining three women have not yet completed one year of follow-up. Among the patients who reported pregnancy, one presented a miscarriage after 7 weeks of pregnancy and in the two other cases, pregnancy evolved without incidents undergoing CS after 38 weeks of pregnancy (Fig.3).

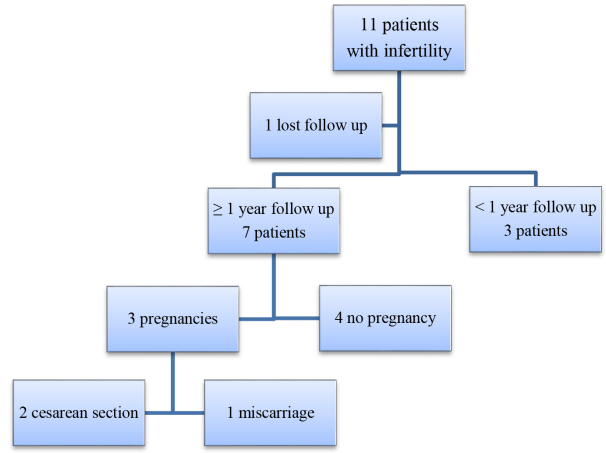

Fig.3: Follow up and results in patients with secondary infertility after hysteroscopic isthmoplasty.

Discussion

Postmenstrual AUB (PAUB) is the most frequent complaint among patients with symptomatic isthmoceles. In 1995, Morris (3) was the first to describe the caesarean scar defect. He examined the uterus of women who had undergone hysterectomy due to AUB symptoms in the absence of any identifiable cause and did not respond to hormonal therapy. All women had at least one previous CS. He found that most of these women presented distortion and widening of the lower uterine segment as well as inflammatory changes in this site. It was proposed that menstrual blood accumulates in the isthmocele and delay menstrual bleeding, causing PAUB (18). Not only the anatomical defect is responsible for the spotting, but also other mechanisms such as, in situ production of blood (3) and decreased contractility of the myometrium in this area (22) were suggested to contribute to blood accumulation.

Surgical hysteroscopy enables correction of the anatomical defect by removing the edges of the niche, avoiding, in this way, the accumulation of the menstrual blood. In addition, the cauterization of the pouch of the isthmocele reduces the in situ production of blood and release of inflammatory factors, and produces a scar retraction of the pouch. Several authors, in non-controlled reports, suggested that these hysteroscopic procedures seem to be effective in improving isthmocele symptoms, even achieving the resolution of the AUB in the majority of the patients (3, 5, 14, 23-26). So far, only one controlled study was conducted to compare the resectoscopic treatment of symptomatic isthmoceles to the expectant management, reporting the complete resolution of symptoms in 87% of the treated patients, with a significant difference compared to untreated women (27). So, as we can see, our results are consistent with previous studies. In relation to secondary infertility, it is thought that the isthmocele produces a toxic environment due to the accumulation of blood and the release of inflammatory factors, obstructing the passage of sperms and preventing embryo implantation (5, 8, 17). Hysteroscopic correction of the isthmocele may also improve pregnancy outcomes (5, 23, 26).

Although no complications were reported after hysteroscopic isthmoplasty, it is important to consider that the surgical technique is not exempt from complications. Besides the general risks of hysteroscopy, in this case, it should be noted that the myometrium above the isthmocele is thinned, which implies a greater risk of perforation and therefore, vascular, bladder or bowel injury (28).

To prevent the risk of uterine perforation and bladder injury, it is recommended to measure the RMT above the isthmocele. In our series, patients with an RMT <2.5 mm were not included, because in such cases, the correction was performed laparoscopically, as suggested by Tanimura et al. (17). At the moment we began our study, there was controversy over the value of the RMT that was safe and recommended for the hysteroscopic correction of the isthmocele. They established the cut-off point of 2.5 mm for RMT, and Marotta et al. (28) and Donnez et al. (29) proposed the laparoscopic correction of isthmocele when the RMT above the isthmocele is <3 mm; on the other hand, Raimondo et al. (24) suggested to avoid hysteroscopic correction in patients with an RMT <4 mm. In 2018, the Global Congress on Hysteroscopy Scientific Committee (30) published a consensus statement for the management of symptomatic isthmoceles, establishing that when myometrial thickness is <3 mm, the laparoscopic approach is preferred to reduce the risk of perforation. This is the limit (cut-off) we are currently using. Moreover, in patients with secondary infertility, who expect future pregnancy and undergo isthmoplasty, it seems especially important to avoid excessive myometrial resection. In these cases, the goal will be to achieve a pregnancy, and extremely thin residual myometrium increases the risk of uterine rupture (17). Therefore, for patients who are looking for pregnancy and have a RMT < 3 mm, laparoscopic correction is the recommended option, since it also favours the restoration of the myometrial thickness (17, 28, 29, 31).

Being conscious of the limitations of our study, a case series study with a limited number of patients, and knowing that more randomized control trial are needed to demonstrate the efficacy of the hysteroscopic treatment of symptomatic isthmoceles, it seems that this technique can be effective to resolve PAUB and pelvic pain in women with symptomatic isthmoceles. Another limitation of our study was the assessment of the fertility outcome as one year follow-up of patients who presented secondary infertility was difficult and some of them were lost follow-up.

Conclusion

Isthmoceles constitute a frequent cause of AUB and pelvic pain in patients with CS. Therefore, isthmoceles should be included in the differential diagnosis of AUB and pelvic pain in premenopausal women with history of previous CS. Symptomatic isthmoceles should be treated. In patients with AUB or pelvic pain who do not expect future pregnancy, hysteroscopic correction of the isthmocele may constitute the first choice of treatment being a minimally invasive technique that improves the symptoms. On the other hand, in women who expect future pregnancy, it seems to be important to consider the RMT above the vertex of the isthmocele to select the best surgical technique for correction of the defect. Hysteroscopic isthmoplasty also seems to be a safe and effective technique in patients who present an RMT of >3 mm. Nevertheless, further studies are needed to determine the surgical technique and type of treatment which would be better for each patient.

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Authors' Contributions

A.V., C.M., M.M.; Participated in study design, and data collection, evaluation, and analysis. E.M., I.L., N.M.; Performed the ultrasonographies and hysteroscopies. E.M.; Was responsible for overall supervision. A.V., I.L.; Drafted the manuscript. E.M, N.M., L.A.; Revised the manuscript. L.A.; Been scientific advisor and surgical reviewer. All authors read and approved the final manuscript.

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Original Article

Practical Difficulties in Estimating The Prevalence of Primary Infertility in Iran

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Abstract

Background: According to the World Health Organization (WHO)'s clinical, epidemiological and demographic definitions, infertility is an inability to become pregnant within one, two or five years of exposure to pregnancy, respectively. Inconsistent infertility-related definitions and various methodological approaches make it difficult to compare quantitative data in this regard and consequently, have negatively influenced estimating the prevalence of infertility. The present study reviewed the results of a large population-based survey on how the clinical, epidemiological and demographic definitions of infertility produce different results in terms of infertility prevalence in Iran and subsequently, compared the findings in order to find the right time of treatment-seeking by couples.

Materials and Methods: This community-based, cross-sectional study was carried out by Avicenna Research Institute in the urban and rural parts of Iran between 2010 and 2011. Using cluster sampling, the reproductive history of 17,187 married women aged 20-40 years, was recorded. Totally, 1011 clusters were randomly selected according to post office codes, proportional to the population of the province. Descriptive and inferential statistical analysis of the data was carried out by SPSS statistical software.

Results: The prevalence of primary infertility based on the WHO's clinical, epidemiological and demographic definitions were 20.2, 12.8 and 9.2%, respectively. In addition, secondary infertility rate was 4.9%.

Conclusion: Infertility estimates over a two-year exposure period made a 50% decrease in infertility rate; however, increasing exposure period to five years made no significant difference in infertility rate. The findings showed that most of the couples will get pregnant within two years of unprotected sexual intercourse and thus, need no treatment. Due to practical difficulties in estimating the prevalence of primary infertility, the reference limit for time to pregnancy, should be reconsidered and giving more time to younger women to become pregnant, seems reasonable.

Keywords: Epidemiology, Infertility, Iran, Prevalence, Reproduction

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Introduction

According to the World Health Organization (WHO), about 60 to 80 million couples in the world have difficulties in getting pregnant and suffer from infertility as a universally common problem. Obesity, increasing rate of sexually transmitted diseases (STDs) and life style changes increased the prevalence of infertility (1). In the most recent years, the factor of life style was shown to play an important role in decrement of fertility and increment of the use of assisted reproductive techniques (ART) (2). Since infertility may change demographic patterns and lead to economic, social and health complications, different groups of sociologists, epidemiologists and researchers in medical sciences focused on it. In order to understand the magnitude and scope of infertility, it is necessary to consider the infertility definition, socio-demographic context and the study population (3).

Inconsistent definitions of infertility and various methodological approaches make it difficult to compare the quantitative data and have negatively influenced estimating the prevalence of infertility (3, 4). In demography, infertility refers to women who are sexually active and do not use any contraceptive methods but unable to have a live birth. Demographers focus on the end-point of the fertility process because demographic analysis of infertility is often based on second-

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ary data such as the Demographic and Health Surveys (DHS). Although in sociological studies, the most prominent issue is giving birth to a live baby which is a key problem for couples who suffer from infertility, it is clinically important to know whether the woman has difficulties in conceiving or in carrying a pregnancy to term. This different attitude relatively explains the diversity in infertility-related definitions in research and practice (5, 6). Other controversial issue is the time of trying to get pregnant. Based on the clinical definitions, infertility is failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (7, 8). According to the WHO's epidemiological and demographic definitions, infertility is an inability to become pregnant within two or five years of exposure to pregnancy, respectively (9). It seems that the exposure time of five years reduces biases and consequently, the fertile population is not classified as infertile (4). Secondary infertility is the inability to bear a child, either due to the inability to become pregnant or the inability to carry a pregnancy to a live birth following either a previous pregnancy or a previous ability to carry a baby to term (9, 10).

In order to avoid over- or under-treatment, the right time of treatment-seeking by couples should be investigated. Not considering this issue may result in unnecessary costs and iatrogenic complication of assisted reproduction such as ovarian hyper-stimulation syndrome (OHSS), multiple pregnancies in the short-term, and shortage of required resources in the long-term. The present study reviewed the results of a large population-based survey on how the definition of infertility affect the infertility prevalence in Iran. The present study also provided the prevalence of primary infertility (in all provinces) and secondary infertility in Iran.

Materials and Methods

The community-based, cross-sectional study is part of a population-based cross-sectional survey on the reproductive history of Iranian women conducted by the Avicenna Research Institute in 2010 and 2011 in the urban and rural parts of Iran. The study and its written consent form were approved by the Research Ethics Committee of Avicenna Research Institute (No: 29/51/7509). Date of pregnancy, child birth, method of contraception, contraceptive use, stopping and switching contraceptive methods, desire to become pregnant, previous history of abortion or miscarriage, beginning or stopping infertility treatments and divorce were recorded. These data were required for completing the reproductive history of each woman and providing an accurate estimate of the infertility prevalence because measuring continuous exposure to the risk of pregnancy over a period of one year is complicated and detailed information about reproductive history is necessary (11). Before distributing the questionnaire to the participants, it was piloted in three phases. The study sample consisted of Iranian married women aged 20 to 40 years old. We only recruited women aged 20-40 years old to reduce recall bias in taking reproductive history. Overall, the reproductive history of 17,187 women aged 20-40 years was recorded.

We used randomized cluster sampling, in which 1000 clusters were determined based on the proportion of the population in every province. In provinces such as Ilam, Kohgiluyeh and Boyer-Ahmad and South Khorasan, the number of clusters reached 12. Finally, 1011 clusters were determined according to the postal codes and 17 questionnaires were completed in every cluster. Data collection was carried out by 280 trained and qualified interviewers. The interviewers selected households in the field according to postal codes and regional map and recorded the subjects' demographic characteristics as well as their reproductive history. Written informed consent was obtained from all the participants in this study.

Statistical analysis

Primary infertility was estimated using a quantitative method based on the reproductive history of participants. Primary infertility was defined as inability to have live birth in women who are sexually active and do not use any contraception after 12 months. To assess the primary infertility rate, the reproductive history of the participants was used as discussed in more detail in previous papers (11, 12). The study data was statistically analyzed using SPSS software (SPSS Inc. Chicago, USA Version 11.5), including descriptive statistics (mean, range, frequency and distribution) and analytic statistics (Chi-square and t test). It should be noted that a $P \leq 0.05$ was considered significant.

Results

The present study included 2216 rural women and 14971 urban women. According to the findings, among 17178 women aged 20 to 40 years old who participated in the study, a total of 456 participants (16.3% of subjects with primary infertility and 3.3% of total number of participants) were infertile until the completion of the interview. Primary infertility rate, based on the definition of infertility in clinical practice, was 20.2% (2783 individuals) and secondary infertility rate was 4.9% (36 subjects). Table 1 shows the prevalence of primary infertility in every province based on the definition of infertility in clinical practice. The prevalence of secondary infertility could not be estimated in each province.

Figure 1 depicts the prevalence of infertility based on the period length of exposure to unprotected sexual intercourse that varied from 12 months to 5 years. All seeking-treatment women were considered infertile. We only considered part of this group who got pregnant in the first year after marriage or contraceptive discontinuation, fertile women. With increasing the exposure period to five years, the prevalence of infertility decreased to 9.2% (Fig.1).

Table 1: Prevalence of primary infertility in different provinces of Iran based on clinical definition

Name of province	n (%)	Prevalence (%)	2SE
East Azarbayejan	765	21.05	0.21
West Azarbayejan	680	26.24	0.26
Ilam	323	12.04	0.12
Ardebil	1020	33.1	0.33
Isfahan	306	18.05	0.18
Alborz	204	16.81	0.17
Bushehr	221	21.31	0.21
Tehran	3230	18.85	0.19
Cha-harmahal & Bakhtiyari	204	25.17	0.25
South Khorasan	204	36.21	0.3621
Khorasan-e- Razavi	1054	18.96	0.19
North Khorasan	204	18.67	0.19
Khusestan	901	23.89	0.24
Zanjan	238	21.61	0.22
Semnan	204	20.08	0.20
Sistan & Baluchestan	578	11.56	0.12
Fars	884	20.77	0.21
Qazvin	323	17.11	0.17
Qom	340	27.21	0.27
Kordestan	456	14.33	0.14
Kerman	561	18.45	0.18
Kermanshah	425	22.48	0.22
Kohgiluyeh & Boy-erahmad	204	19.77	0.20
Golestan	425	9.52	0.10
Gilan	680	23.81	0.24
Lorestan	408	12.53	0.13
Mazandaran	850	21.23	0.21
Markazi	340	19.05	0.19
Hor-mozgan	340	23.21	0.23
Hamedan	357	23.13	0.23
Yazd	255	21.39	0.21
Total	17187	20.17	0.20

SE; Standard error.

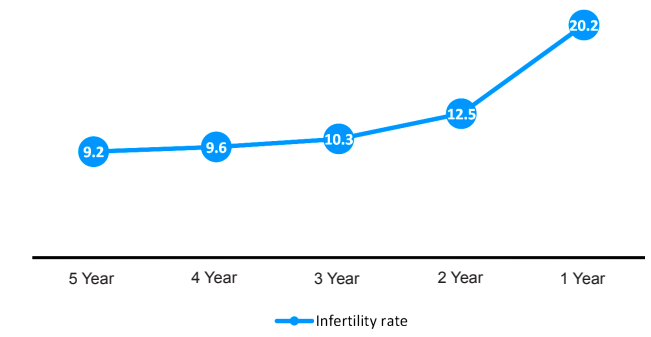

Fig.1: Prevalence of primary infertility according to exposure period to the risk of conception.

There was a significant difference in age during the first conception attempt between the infertile and fertile groups (P≤0.001). The findings related to the distribution of fertile and infertile couples according to women's age at time of attempt to get pregnant (no contraception after marriage or contraceptive discontinuation after marriage) are presented in Table 2. It is worth mentioning that 45.0% of the participants were <19 years old on their first attempt to get pregnant. Only 45% of participants with primary infertility (1255) received medical treatments. Among all the study participants (n=1255), the majority (82.6%) were presented first to gynecologists because of infertility problems. It should be noted that referral to specialized centers such as hospitals and infertility clinics was lower than that observed for private gynecology clinic.

Table 2: Age distribution of women at the first time of attempt to get pregnant

Women's age (Y)	Fertile n (%)	Infertile n (%)	Total n (%)
≤14	526 (4.8)	273 (9.8)	799 (5.7)
15-19	4241 (38.5)	1178 (42.3)	5419 (39.3)
20-24	4411 (40.0)	944 (33.9)	5355 (38.8)
25-29	1559 (14.1)	304 (10.9)	1863 (17.2)
30-34	259 (2.3)	72 (2.6)	331 (2.4)
≥35	22 (0.2)	12 (0.4)	34 (0.2)
Total	11018 (100)	2783 (100)	13801 (100)

Discussion

Generally, infertility and sub-fertility definition is important to manage infertility appropriately (13). Health care providers should be aware of the prevalence of infertility to estimate the likelihood of seeking and undergoing infertility evaluation and treatments. Therefore, there is a need to have a consistent definition for infertility to be used in clinical practice and epidemiological researches (6). In addition to the importance of data collection and analysis to estimate infertility prevalence (11), inconsistency among definitions of infertility across research and clinical practice, can lead to different estimates which makes it difficult to manage infertility.

Also, it should be noted that the infertility prevalence rate in Iran is higher than the global level (12), and infertility has become a national public health problem. Hence, the present study examined the prevalence of infertility in Iran based on different definitions and compared the results subsequently. The prevalence of primary infertility in Iran was 20.2% based on the classic definition used in the clinical practice. Accordingly, one fifth of Iranian couples experienced primary infertility. The results of the present study were similar to other national surveys with regard to the prevalence of primary infertility (based on the classic definition) (14-16). Infertility estimates over a two-year exposure period showed a 50% decrease in infertility rate; however, increasing the period length to five years made no remarkable difference in infertility rate. Infertility estimates over a two-year exposure period in the present study were similar to those reported by Safarinejad (17) in Iran for the same exposure period. Consistently, Gnoth et al. (13) reported that the duration of unwanted

non-conception is the main factor in spontaneous fertility.

Mascarenhas et al. (4) suggested considering five-year exposure period for an accurate infertility measurement because longer exposure periods decrease the possibility of the recall bias and are less likely to categorize fertile people as infertile. In another study conducted on infertility, Larsen concluded that the WHO's epidemiological definition which considers an exposure period of 24 months is more applicable in both research and clinical practice. They divided subjects into infertility and subfertility categories and considered those who get pregnant after 12 months and before 24 months as sub-fertile individuals (6). The results of the present study are in line with the results of the Larsen's study according to which, it seems that considering two-year exposure period produce more accurate results in identifying infertile individuals and is helpful to be used both in clinical practice and epidemiological research. It can be concluded that clinical definition of infertility suggested by the WHO may lead to over-treatment and increase in potentially life-threatening OHSS and multiple pregnancies. Bushnik et al. (18) found that considering questions about the "use of birth control in the previous 12 months", "the regular sexual activity in the previous 12 months", and "trying for pregnancy" or "pregnancy intent" in the clinical definition of infertility, results in a lower rate of infertility prevalence.

Unlike the study done by Mascarenhas et al. (4), extending the exposure period to five years made no remarkable difference in infertility rate in the present study. As a result, five-year exposure period cannot be considered a good criterion in the clinical practice or national policy makings. However, delayed fertility in couples trying to conceive for two years is seriously important, and needs thorough examination to understand the causes of infertility.

In addition, women's age is also an effective issue during the first conception attempt and should be considered in studies estimating the infertility prevalence. There was a significant difference between fertile and infertile couples in terms of women's age in the first conception attempt. The first conception attempt was defined as not using contraception after marriage or contraceptive discontinuation after marriage. Unlike expectations, the infertile participants were so young (≤19 years old). Over half of the infertile participants were less than 20 years old in their first pregnancy attempt and the number of participants over 35 years old was very few. Although the sample size was too small to draw any relevant conclusions on the effect of higher age on infertility, the effect of women's age as reflected by decreased pregnancy chance among women over 35, cannot be ignored in presenting an appropriate definition for infertility rate. It was suggested that the age range of 19 to 30 years is the appropriate age for Iranian women to conceive and in fact, teenage pregnancy is not suggested due to its risk for mother and baby (12). Gurunath et al. (19) suggested that an appropriate clinical definition should consider both exposure period and the female age. Moreover, Gnoth et al. (13) recommended that a basic infertility evaluation following failure to achieve a pregnancy after 6 cycles, identifies couples with serious infertility problems and may decrease over- or under-treatment despite the age factor. According to this study, couples with good prognosis such as unexplained infertility may be encouraged to wait longer because there is no chance for fertility even through treatment, though others may take advantage of undergoing early ART. Considering the above-mentioned studies, for women of <35 years old, it is suggested to perform a basic infertility evaluation after at least one year and at most two years of unsuccessful conception attempts. For women of <35 with no definitive cause of infertility, it is suggested to continue attempting to conceive and seek medical treatments after two years of trying, whereas for women of >35 years old, it applies after six months of attempting to conceive. Since inability to have a child after two years is a serious issue, it is not reasonable to delay the treatment. Unfortunately, a high percentage of girls in low- and middle-income countries marry before the age of 18 years old. If there is a fertility problem in women aged under 18 years, it will be better to delay fertility treatments.

Based on the present study, current infertility rate (i.e. 3.3%) indicates that about 3 percent of all women of reproductive age have infertility problems and current national facilities, including 70 fertility centers, of the country should support them. Similarly, current infertility rate was reported 3.3, 6.4 and 4.3% in studies conducted by Rostami Dovom et al. (14), and Vahidi et al. (15), respectively. Recent studies reported a secondary infertility rate of about 3 to 8%, which is consistent with the present study (14, 20).

It was reported that just less than half of participants with delayed fertility referred to the specialists for infertility treatments. According to Rostami Dovom et al. (21), about 56% of women with delayed first pregnancy sought to undergo treatment. Boivin et al. (22) reported that 56% of couples with infertility problems were seeking treatment and stated that lack of access or limited access to fertility services is the probable reason for unwillingness to undergo infertility treatment. However, the researchers acknowledged that the demand for infertility treatment is approximately similar in different countries (including developed and developing countries). It is noteworthy that seeking infertility treatment is completely different from giving suitable services. It seems that limited access to appropriate fertility services, high cost of related services and no insurance coverage are among the most important reasons why infertile couples do not refer for the treatment. Out of 70 fertility centers in Iran, over 25 ones are in the capital and other provinces suffer a serious limitation in this regard (23). However, more quantitative and qualitative studies are needed to examine the treatment-seeking behavior among infertile couples in Iran due to cultural and social aspect of infertility, especially considering the stigma associated with infertility in Iran (24). The limitation of present study was including only women of 20-40 years old to prevent recall bias; so,

the results might not be extended to all of the infertile women's population.

Conclusion

In the present study, we found that most of the participants got pregnant with no infertility treatment over a two-year exposure period and women's age (≤19 years old) is one of the most important reasons of delayed pregnancy. Due to practical difficulties in estimating the prevalence of primary infertility, it seems that the reference limit for time to pregnancy should be reconsidered in future studies and giving more time to younger women seems reasonable. As the resources are limited, following this policy can greatly reduce costly diagnostic procedures and additional treatments. Since infertility is a serious issue after two years of unsuccessful attempt, it is not reasonable to delay the diagnostic and therapeutic approaches. Caution must be taken in applying these findings to the clinical practice and more studies are required to choose an accurate criterion for both clinical practice and national policy-making.

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Authors' Contributions

M.M.A., K.K., K.M., Z.B.A.; Participated in designing the study and managing the project. K.M, M.Sh., F.R., K.K.; Extensively contributed to data analysis, interpretation of the data and drawing conclusion. F.R.; Was involved in collecting the data, reviewing the literature and drafting the manuscript. All authors edited and approved the final version of this manuscript for submission. In addition, they all participated in finalizing the manuscript and approved the final draft.

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Original Article

The Need for A Training Software among Iranian Infertile Couples: A Qualitative Study

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Abstract

Background: Training needs are multidimensional requirements affected by social and cultural background, level of knowledge and personal and health conditions. This study was conducted to explain the needs for a training software among Iranian infertile couples.

Materials and Methods: In this qualitative study, we used content analysis to examine the need among ten infertile participants (four men and six women) and six health care professionals (including two gynecologists, two reproductive health specialists and two midwives). The present research was carried out from January 2017 to July 2018 at Rouyesh and Ibn Sina infertility treatment centers in Karaj, Iran. The participants were selected through purposive sampling with maximum variation. Four focus group discussions with the health care professionals and twelve semi-structured, in-depth interviews with the infertile participants were held for data collection. Data were analyzed using conventional content analysis in MAXQDA-10.

Results: Data analysis led to the extraction of a central theme of "a multidimensional training application" and its four main categories, including "pre-treatment training", "diagnostic training", "mid- and post-treatment training" and "continuous psychological training". These main categories also had 20 subcategories.

Conclusion: Based on the results of this study, infertile women and men have multidimensional training needs before and after treatment and during the process of diagnosis; psychological aspects should also be considered.The interviewed health care professionals helped to explain these training needs. A training software thus needs to be designed based on the real needs of the infertile community.

Keywords: Infertility, Knowledge, Qualitative Research, Training Programs

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Introduction

Adult training programs are developed based on the priorities of the learners' needs (1-3). Meeting the population's diverse and changing health needs is the most important mission of health education organizations. If the training programs developed by these organizations are based on the needs of different target groups of different social, economic, cultural and psychological backgrounds within the community, they can effectively improve the community's health (4). The infertile population is a target group that requires an effective training program for their condition. Having children is one of the most basic needs in married people's lives and forms the basis of all human life (5-7). Infertility is "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse". The condition is divided into primary and secondary types. There is no pregnancy in primary infertility, while one or more previous pregnancies existed in those with secondary infertility. Couples with secondary infertility were able to conceive at least once, but are now unable to conceive again (4).

The current rate of primary infertility is estimated

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2.8% by the National Health Survey (NHS) and 3.4% by the National Infertility Survey (NIS). NIS conducted a survey of 12,000 women aged 19 to 49 years in all 28 provinces of Iran in 2004-2005. A study in Tehran and also the NIS report estimated the prevalence of lifetime primary infertility to be 21.9 and 24.9%, respectively. The minimum prevalence of lifetime primary infertility was found to be 15.8% for the marriage age of 19-27 years in the study conducted in Tehran. Some studies in Iran reported that an average of 21-22% of women experience primary infertility during their marital life (3).

As a significant multidimensional issue, infertility can affect couples' physical, emotional, sexual and social health (5). One of the issues which infertile couples would face is lack of awareness about multiple dimensions of dealing with infertility. Some of these dimensions include diagnosis and treatment. The lack of awareness and training causes more confusion and psychological stress for infertile couples and leads to more complex medical treatments, higher financial costs and prolongation of treatment process (2, 3).

The first step in educational interventions is understanding the training needs of the target population. The final process of training pertains to the correct implementation of the needs assessment before designing the training program. Needs assessment is the process of collecting and purposefully analyzing data for identifying the needs of individuals, groups, organizations and communities; before presenting or prioritizing the obtained information, it is essential to get the patients' actual needs (i.e. the gap between the status quo and the desired situation) as assessed by health care professionals (8).

Using qualitative methods is a way to understand the needs of infertile couples. Quantitative approaches fail to process complex multidimensional concepts. Quantitative research often involves limited variables and does not reveal the depth of the existing reality. Therefore, conducting qualitative research would result in a better understanding of the needs of infertile women and men (8-10). Content analysis is a qualitative research technique used to make valid replicable inferences by interpreting and encoding text. Qualitative content analysis is suitable for obtaining valid results as text data for generating knowledge, new ideas, facts and guidelines for proper performance (11). This method is used to interpret and classify textual data by considering the cultural and environmental aspects affecting the studied phenomenon (12, 13). In Iran, Karaj is considered the "tiny Iran", since it is composed of several demographic groups of different cultures. This city offers limited resources to infertile couples. It is therefore beneficial to develop an application based on patients' educational/ training needs.

Needs comprise a multidimensional phenomenon (5) affected by the socio-cultural context in place. Very few studies were done on the training needs of the infertile population in the country, and none them examined the training needs of infertile couples with respect to development of an application. Although some qualitative studies on infertility explained the needs of infertile couples, the present study, for the first time, explored their needs in terms of having access to a software application. The main question of the present study is 'What types of training do you expect from an application to provide? In other words, what should such an application include to be able to increase your knowledge and awareness about your areas of need?' This study was thus conducted to discover the training needs of infertile couples to be considered when designing a software application based on the explored real needs of infertile couples and health care professionals.

Materials and Methods

Participants and data collection

Four focus group discussions (done by six health care professionals) and 12 semi-structured interviews (held with ten infertile participants) were completed in this study on a total of 16 participants. The inclusion criteria included: i. Being an infertile man or woman or an infertility healthcare provider, ii. Having Iranian nationality, iii. Having at least reading and writing nationality, iv. No mental disorders based on the self-report, and v. The healthcare providers and clinicians with at least two years of work experience were taken as the group of health care professionals.

This qualitative study used a content analysis approach to examine ten infertile participants (four men and six women) and six health care professionals (two gynecologists, two reproductive health specialists and two midwives), from January 2017 to July 2018 at Rouyesh and Ibn Sina infertility treatment centers, Karaj, Iran.

First, based on the health records available at these two infertility treatment centers, 42 infertile couples were contacted by text message and invited to attend a briefing session at a specified time and place. A total of 25 people showed up. Twenty of the attendees volunteered to take part in the study. Then, based on the inclusion criteria, ten infertile couples entered the study. Six health care professionals including two gynecologists, two reproductive health specialists and two midwives, with at least two years of work experience in infertility treatment, were also recruited. The participants were selected through purposive sampling with maximum variation. Four focus group discussions were held with the health care professionals and 12 in-depth semi-structured interviews were held with the infertile participants for data collection. It should be noted that two out of ten infertile couples were interviewed twice due to their higher knowledge and experience about infertility; therefore, a total of 12 interviews were ultimately held with the couples.

This study examined the training need of Iranian infer-

tile couples and health care professionals with respect to development of a training software or application. The main questions of the study were "What are the training needs of infertile women and men regarding their infertility?" and "What are the training needs of infertile women and men that should be considered when developing an application?" The interviews were tape-recorded with participants' permission. The interviewer took field notes immediately after each interview. All interviews were held by corresponding author (M.Y) after approval of all research team members. The interviews lasted from 40 to 60 minutes, with an average duration of 50 minutes. The average time for the four focus group discussions was 75 minutes.

The infertile participants were selected through purposeful sampling. One type of purposeful sampling is variation sampling method. In this study, variation sampling was considered in terms of age, gender, education, occupation, socioeconomic status, duration of marriage, duration of infertility and cause of infertility (Table 1). The health care professionals were selected through the same method, with variation in terms of gender, education and work experience (Table 2).

Table 1: Demographic characteristics of the infertile couples

Variable	n	Variable	n
Age (Y)		Socioeconomic status	
25-30	5	Good	4
31-35	2	Moderate	3
36-40	3	Poor	3
Sex		Duration of marriage (Y)	
Female	6	1-5	6
Male	4	6-10	2
		11-15	2
Education level		Time of infertility (Y)	
Diploma	4	1-5	7
Bachelor	3	6-10	2
Masters	3	11-15	1
Job		Cause of infertility	
House keeper	5	Female factor	5
Employee	3	Male factor	2
Informal Job	2	Unexplained	3

Table 2: Characteristics of the health care professionals participated in the present study

Variable	n
Sex	
Female	5
Male	1
Education level	
Specialty	2
PhD	2
Masters	1
Bachelor	1
Work experience (Y)	
1-10	3
11-20	3

The individual interviews and focus group discussions were held at the time and place of participants' choosing. Table 3 lists some of the interview's questions. Probing questions were also asked, such as "Can you elaborate on that/provide an example?"

Table 3: A sample of interview questions

1. What are your training needs with regard to your infertility? (infertile couples)
2. Based on your experience, what features should a well-designed educational computer application have to have in order to meet the needs of infertile people? (infertile participants)
3. How do you suit your training needs regarding infertility? (infertile couples)
4. What are your training needs regarding infertility? (infertile couples)
5. What questions do you tend to ask infertility health care professionals to meet your training needs? (infertile couples)
6. What questions are more frequently asked by your patients about infertility diagnostic-therapeutic steps? (health care professionals)
7. Based on your experience, what kinds of training needs do couples have regarding infertility? (health care professionals)

Data analysis

Data were analyzed using the method of data analysis provided by Graneheim and Lundman (14); the following steps were taken to analyze the collected data: i. Transcribing the interviews verbatim and reading through several times to get a general sense of the material, ii. Dividing the text into meaning units, which are key phrases in the text, iii. Abstracting the condensed meaning units and outlining using codes, iv. Grouping codes into subcategories and categories based on comparisons made based on similarities and differences, v. Re-organizing and merging into sub-themes and overarching themes as the expression of the latent content of the text participant.

MAXQDA-10.0 was used to facilitate data coding, categorizing and retrieval. This tool allows to analyze the combination of activated codes in different ways, taking into account the different groups and positions of coded segments. It helps to retrieve and arrange codes in a category.

Trustworthiness of the data

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

Ethical considerations

This project was approved by the Ethics Committee of Alborz University of Medical Sciences (IR-ABZUMS.REC.1397.032). All the participants were briefed on the objectives and methods of the study and they signed informed consents. Before the interviews, the participants were ensured that they have the right to refuse to participate in the interviews at any time without any negative consequences for the services they receive. The authors

have fully observed the ethics of research, including submitting informed consent and avoiding plagiarism, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.

Results

Tables 1 and 2 present participants' characteristics. After analyzing the data, the findings were explored in one theme, entitled "A multidimensional training application". The theme includes four main categories (Table 4) namely, "pre-treatment training" (with ten subcategories), "diagnostic training" (two subcategories) "mid- and post-treatment training" (five subcategories) and "continuous psychological training" (three subcategories), and 130 codes (from originally 658 primary codes). Figure 1 presents the relationship between the theme and the main categories.

Table 4: The theme, main categories and subcategories

Theme	Main category	Subcategory
A multidimensional training application	Pre-treatment training	The fertility process
		The causes of infertility
		Assisted fertility techniques
		The process of transfer in assisted fertility techniques
		Pre-treatment nutrition
		The necessity of scientifically-filtered training
		Understanding infertility by visiting the web
		Access to valid infertility centers
		Pre-treatment medications
		Ethics
	Diagnostic training	The step-by-step of diagnostic measures
		Learning effective measures for improving the quality of spermogram
	Mid- and post-treatment training	Mid- and post-treatment medications
		Mid- and post-treatment self-care
		Mid- and post-treatment complications
		Mid- and post-treatment nutrition
		Mid- and post-treatment feedback
	Continuous psychological training	Stress management strategies in diagnostic and treatment procedures
		Mental relaxation
		Strategies for improving self-confidence or self-esteem

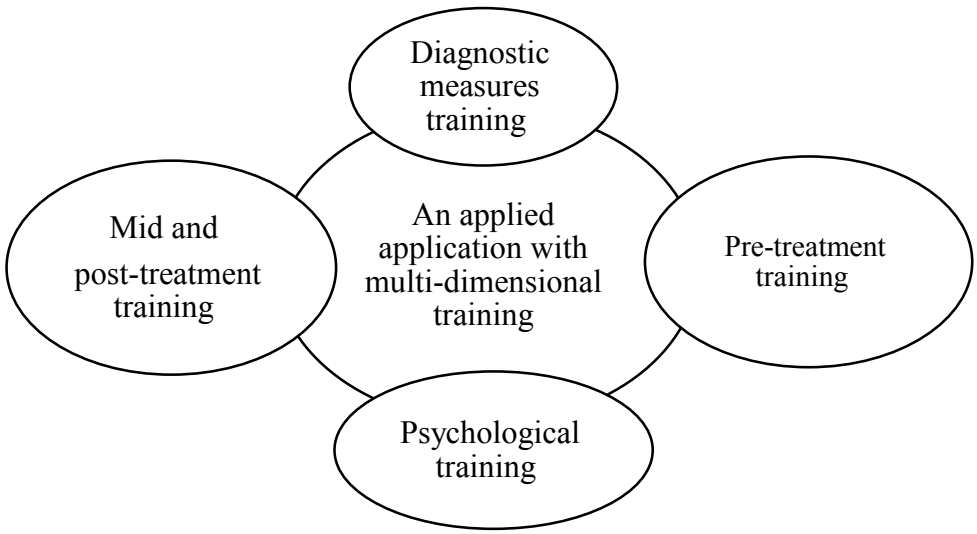

Fig.1: The relationship between the theme and the main categories.

Pre-treatment training

The first step in maintaining future fertility is awareness about the risk factors of infertility. The majority of the participants of this study prioritized the design of a training program that covers infertility issues and trains couples about their fertility health, although they receive some sort of training on these subjects in different contexts and at various times, they were seeking unlimited access to more comprehensive written information. Further access to this information would make them examine their fertility health and infertility issues sooner, and help them begin treatments on time. Participant 3, who was infertile, made the following comment:

“When we are informed about the natural process of fertility and the things needed for its success, we start looking for treatment sooner”.

Considering the causes of infertility, the participants believed that couples need to know the exact causes of their infertility to better deal with their particular case of infertility. Participant 4, also infertile, commented:

"I think that the causes of infertility should be fully explained in a simple and accurate manner, so they can be easily understood; for example, your infertility is caused by these four reasons and you are lacking in these four areas. I get a lot of my information on the web, but I like to get information from a valid source as well".

A training tailored to the cultural context of the society in question and offered in a simple and easily-comprehensible language helps to improve couples’ process of infertility treatment. According to the participants, awareness about the available methods of treatment can help to choose the correct infertility treatment and reduce the errors. Participant 6 stated:

“Before beginning any type of treatment, we gather a lot of accurate information to be able to choose the correct route faster. A software application is way better, since it is always available on your cellphone, offers you precise information and reduces the probability of error".

The other participants discussed their need for knowledge about the reasons for their treatment failure. Participant 2, also infertile, said:

"In case of a potential treatment failure, for example, there should be a brief explanation of the issue to satiate your curiosity and prepare you for upcoming events."

The need for proper nutrition training was another significant component of infertility pre-treatment that was considered a necessary part of the training software by the participants. Participant 4, also infertile, said:

“It is better that the software include some sort of diet that help to improve your treatment outcome and follicle growth during the treatment period, since our information is limited".

One of the health care professionals (a gynecologist) said:

"A poor long-term diet disrupts the body’s physiology. For the patients who do not have access to a nutritionist, you can suggest a brief simple diet to follow. For example, the number of calories needed in the main meals can be stated".

According to the participants, advanced technologies are a step toward training services with a higher quality, greater validity and less damage to the environment. Participant 8, also infertile, said:

"I always wonder whether these websites are valid or not. This way, the need for paper is reduced, and it is good in terms of environmental protection. I think the software application can provide better training with better quality".

The participants said that the training application is a way to enhance their awareness and contributes to their readiness to move along with their infertility treatment procedures. Participant 3, who was infertile, said:

"Awareness empowers couples in terms of knowledge and leads to a better understanding of the issue. A well-informed and aware person is always successful".

The participants also needed pre-treatment training on ovum donation concerning family and religious issues. For example, some couples were worried about the similarity of their future child’s appearance to themselves and the religious issues concerning the donor family. In this respect, participant 3 (infertile) stated:

"For example, when they say that they give you another woman’s ovum, I want to know more about it. How exactly does it work? Is the donor a good person? I do not know her. Can I get involved in this procedure? What is the Islam’s point of view on this subject?"

Diagnostic training

Based on the experiences of the participants, a step-by-step training process can help with diagnoses. Participant 6 (infertile) said about diagnostic training:

"Why does each diagnostic process have to be carried out? For example, what is a Doppler sonography? How does it work? How does it help to make a diagnosis? I assume it would be better if these were explained one by one and step by step."

Explaining the prerequisite conditions of diagnostic measures was also considered necessary for reducing the frequency of test repeats. One gynecologist stated:

“They mostly ask if it is OK to provide the samples at home or not. Well, if the samples are left in the open air for more than 20 minutes or half an hour, or if they are not kept under appropriate storage conditions, the test results would be affected. If the patients understand the significance of speed in these processes, they can cooperate more easily".

Mid- and post-treatment training

Many infertile couples experienced the need for mid-

and post-treatment training in many aspects. One of these needs is the timing of the prescribed medications. Participant 5 (infertile) said:

"I even set the alarm, but I am still worried about not waking up. Now, it would be great if something like a medication alarm could remind us of the time I should take my medicines".

Training on self-care behaviors is crucial for couples who have various diseases (15, 16). Mid- and post-treatment self-care was another need discussed by the participants in this study. Participant 4 (infertile) said:

“Couples are often stressed, but if they knew what to do and how to take care of themselves, it would be better. It would be quite useful if your training program, in a simple language, explains what self-care measures are necessary to take after infertility treatment".

Continuous psychological training

The experience of infertility imposes considerable psychological pressure on infertile couples in the sociocultural context of the society. Based on participants’ experiences, the need for psychological training is great in infertile couples. The need for training on how to achieve mental relaxation, increase self-esteem and cope with stress was a part of participants’ psychological needs. Participant 5 (infertile) said:

"Well, couples tend to emotionally collapse in such situations. It is more helpful if this app also incorporates training on how to calm oneself down. This way, better results can be expected for infertility treatment".

One of the infertile male participants said:

"It would be really good if there was training about how to repel or reduce negative pressures somewhere in the app".

Another infertile male participant said:

“My wife has no fertility problems; sometimes, I blame myself, and wish we had not got married. Negative thoughts put a lot of pressure on me and it would be helpful if there were strategies to get me more relaxed. For example, I try to calm myself down with praying and saying prayers. I need to learn some other strategies too".

In this regard, a gynecologist at the infertility center said:

"Many cannot attend the classes in person. Now, if these items are included in the app, they can have a positive effect on all patients and keep the negative thoughts away".

Supporting the infertile spouse in the process of dealing with infertility was another need discussed by the participants. Participant 7 stated:

"I did not receive emotional and mental support from my husband’s family; however, my husband himself is always besides me, gives me positive energy and supports me when I get tired of taking my medications. His behavior is so inspiring for me to keep up with this whole thing and not get disappointed".

Discussion

Multidimensional training is a fundamental need for promoting health and improving the quality of life. The training needs of infertile people are derived from their cultural, social and religious background (9, 10).

This qualitative study explains the training needs of Iranian infertile women and men and health care professionals working in the field, for development of a training software program. Based on previous studies, infertility training needs require more efficient and up-to-date methods. In today’s world, e-learning has addressed many of these training challenges, such as the challenges of formal training, which requires a physical location, training facilities and instructors (17). During the period of infertility treatment, there is a considerable lack of awareness about the pre-, mid- and post-treatment measures as well as the medical and therapeutic measures that should be taken, which doubles the difficulty of this period. Easy access to information and training materials through the internet facilitates reaching a larger number of audiences in different environments. Providing information about infertility through software programs, enables couples to have easier and more extensive access to the information (7). Poor knowledge and inadequate awareness about fertility health reveals the need for further training. The participants, in the present study, noted the need for more training about fertility health and the reasons for infertility as parts of the reason for delaying their infertility treatment. In a study conducted by Kudesia et al. (18), the need to train women and men was emphasized due to the inadequate knowledge about fertility health. During their reproductive years, 55.9% of men and women lack adequate knowledge about fertility. Such knowledge gap has a direct relationship with the delay of infertility treatment and the delay in its start. Awareness about the problem was discussed by 40% of the participants as the first step in seeking infertility treatment. The participants emphasized the need for being trained about fertility health and the reasons for infertility through training programs. In another study, Swift and Liu (19) reported a level of knowledge of 49.9% about fertility health in infertile women in Canada. This research demonstrated women’s need for more training on fertility health issues.

The participants of the present study needed to be trained on a variety of diagnostic measures and infertility treatments. They considered using the software application as a reliable and valid method for getting answers to their questions. A total of 92.2% of the subjects in the study done by Hampton and Mazza (20) stated a need for more infertility training programs to learn the causes of their infertility and various diagnostic and therapeutic measures they had to undertake in a simple and comprehensible written form for better dealing with the issue of infertility.

The participants in the present study were also concerned about collecting information and becoming aware about fertility or infertility issues as they sometimes received contradictory or invalid information through invalid resources. They needed scientific information in the form of a software application. Conceição et al. (21) conducted a study and found that infertile men and women suffer from poor knowledge on their health issues and have contradictory infertility information. The study concluded that these groups needs more valid information on fertility and infertility health issues. Valid e-learning training was therefore used due to its ease of access and scientifically-filtered information. After this e-learning intervention, the subjects were reported to have higher knowledge about fertility health and infertility, and a statistically significant increase was reported in their knowledge and awareness.

Many questions were posed by the participants about diagnostic and treatment measures, which increased the personnel's workload in infertility treatment centers, and the software application was noted by them as a better way to answer such questions. In the study done by Hampton et al. (22) in Australia, time limits were reported by the subjects as the greatest barrier to training the staff and physicians in infertility centers. Inappropriate training materials were also noted to cause a significant gap in providing primary care to infertile women. The participants considered the need for training on fertility and infertility as one of the main steps in infertility pre-treatment that reduces their mental stress and anxiety (22-24). In this regard, Dawdy et al. (23) reported multimedia information as a method for minimizing the waiting time for the patients and improving their awareness. They also reported that using appropriate training tools can improve treatment outcomes and reduce the costs of hospitalization. In other words, in their study, the readiness of the patients, obtained through multimedia training, reduced stress and anxiety.

In the present research, the participants stated that a training software program is an appropriate way for increasing awareness and empowerment. In the study entitled "The theoretical framework of women's health in Iran: The Farmehr model", security in the family, the family dimension and composition, accessing and utilizing material and spiritual resources, and social capital, social support, social networks, public trust, social norms and social actions contributed to women's health and formed a sense of power/empowerment or powerlessness in them (25). In another study conducted in Iran, Mohamadirizi et al. (26) trained health care providers and reported that pamphlets or training materials alone cannot always meet the different levels of learners' expectations. In their study, using e-learning led to an increase in satisfaction and improved the level of learners' knowledge.

The other needs raised by the participants included self-care and infertility treatment training, which should be included in the training software application in a simple comprehensible language. The module contained authentic and scientific information with a simple and comprehensible language to help patients pursue self-care (27). The results showed that e-learning increases the level of knowledge and improves self-care and self-efficacy in patients (28).

Infertility was described by the participants as a reason for their mental and psychological pressures that exposed them to contempt in the society and made them ask for psychological training and its inclusion in the training software application. In the Iranian culture, men's conception of a woman is a significant construct in the image she has of herself, i.e. her self-concept (29). The childbearing and motherhood role is the centerpiece of the role of women in traditional societies. With this social perspective dominating societies, the threat to femininity is more manifested in infertile women and causes more damage to women's identities and leads to their humiliation and lower self-esteem (9, 29, 30). The participants who experienced more support and attention from their spouse were better able to calm down and face their infertility. In their mixed study, van Empel et al. (31) reported the lack of emotional support as a considerable weakness in infertility treatment. They considered the involvement of the spouse in the other partner's care a significant source of positive energy that helps to improve the treatment outcomes by reducing psychological stress.

In recent years, medical sciences have emphasized the relationship between infertility and psychological factors (29). Rahmani Fard et al. (6) argued that by eliminating or reducing the feelings caused by infertility, mindfulness exercises can make a significant contribution to the quality of life of this group of women. They concluded that training infertile women with mindfulness techniques can help them to improve the physical and mental health.

Resorting to worship, saying prayers and praying were measures that had helped the participants to achieve mental relaxation. Spiritual measures pushed away any negative thoughts and they emphasized the importance of including spiritual stress management techniques in the training software application to be able to go through the complex and sometimes annoying stages of coping with infertility. The interconnection between the body and the mind is irrefutable, such that the body-mind relationship can associate any natural or psychological change in life to psychological symptoms and cause adverse health effects (32). Praying gives the individual spiritual agency, and alongside material factors, plays an effective role in controlling, managing and adapting to the complex issues of life and the treatment of diseases. Prayer is a type of meditation in which the person joins the Divine through connectivity to the extreme energy. The belief in a superior strength pushes away person's sense of loneliness and helplessness. Praying also helps the person to find a partner for sadness, talk about their uncomfortable thoughts and think deeper about the issue. In other words, prayer acts as a source of inspiration for the person to find power and ability to continue his efforts. In a qualitative study

done by Soleimani et al. (32), spiritual care was proposed as a concept experienced by patients with Parkinson's disease. Religious beliefs and practices such as praying and saying prayers were among the coping strategies that most Parkinson's patients used to achieve psychological peace of mind and stability and resistance against illness. Spiritual health therefore plays a significant role in mental health and can facilitate adaptation to health crises and health-threatening conditions. There is also a relationship between spiritual health and the symptoms of infertility-induced depression and stress (33). The results of a study done by Mehrabi et al. (33) showed that there is a direct and significant relationship between the score of spiritual health and the quality of life. To justify this finding, they stated that couples with chronic illness are likely to suffer from stressful social and psychosocial stresses, such as existential conflicts related to meaning and purpose, and suffering from illness often challenges the meaning and purpose of life too. Adopting religious practices can thus help to manage thoughts and feelings and improve the quality of life.

A limitation of this study was its small sample size. Also, similar to all qualitative studies, its results are not intended to be generalized, although maximum variation sampling was used.

Conclusion

Based on the results of this study, infertile women and men have multidimensional training needs before and after treatment, and during the process of diagnosis; also, they need psychological supports. These concepts were extracted based on the real needs of these couples. The health care professionals also elaborated on these needs. Designing a training software based on the real needs of the infertile community thus seems essential and can partly cover the shortage of training staff employed in infertility centers. Based on the findings of this study and the themes extracted from them, the researchers found that the design of this application must incorporate main themes such as fertility (proper explanation of the inner and outer genital system), diagnostic measures (proper explanation of tests such as blood tests, proper sampling time and requirements of the spermogram), infertility treatment [proper explanation of intrauterine insemination (IUI), *in vitro* fertilization (IVF) intracytoplasmic sperm injection (ICSI) and their complications] and psychological and mental health issues experienced by couples using appropriate skills, such spiritual care and mind control. The researchers recommend more qualitative studies to be conducted on the training needs of particular infertile groups, such as infertile patients with multiple sclerosis, spinal cord injury or recurrent abortion, to design better educational content on infertility.

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Authors' Contributions

Z.H.N.A.H.; Participated in data collection, the analysis and interpretation of data. B.B., B.P.; Design of the study, contributed substantially to the conception. R.L.; Evaluation and interpretation of data and provided initial the manuscript. M.Y.; Provided final approval of the version to publish and supervised the research. Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors performed editing and approving the final version of this manuscript for submission, also participated in the finalization of the manuscript and approved the final draft.

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Original Article

The Effect of Imbalanced *Progesterone Receptor-A/-B* Ratio on Gelatinase Expressions in Endometriosis

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Abstract

Background: Gelatinases degrade extracellular matrix (ECM) components to allow for physiological remodeling and contribute to pathological tissue destruction in endometriosis. It is known that the function of gelatinases is resistant to suppression by progesterone in endometriosis. The ability of progesterone to impact gene expression depends on the *progesterone receptor-A/-B (PR-A/PR-B)* ratio. An imbalanced *PR-A/PR-B* ratio in endometriotic tissue may be the result of the differential expression of *MMP-2* and *MMP-9*, which could be important in the etiology and pathogenesis of the disease. Hence, we decided to study the association of *PR-A/PR-B* ratio and gelatinases expression in endometriosis.

Materials and Methods: In this prospective case-control study, we enrolled 40 women, 20 in the case group who were diagnosed with stage III/IV endometriosis and 20 normal subjects without endometriosis (controls) who referred to Royan Institute, Tehran, Iran during 2013-2014. We obtained 60 tissue samples [ectopic (n=20), eutopic (n=20), and normal endometrium (n=20)]. RNA was extracted from the tissue samples in order to analyze *PR-A, PR-B, MMP-2,* and *MMP-9* mRNA levels through real-time polymerase chain reaction (PCR).

Results: There was significantly lower expression of the *PR-B* isoform in ectopic tissues compared to the control (P=0.002) and eutopic endometrium (P=0.006) tissues. *PR-A* expression was higher, but not significantly so, in the same ectopic and eutopic endometrium tissues compared to the control tissues (P=0.643). There was significant overexpression of *MMP-9* in ectopic samples compared to control (P=0.014) and eutopic endometrium (P=0.012) samples. The *PR-A/PR-B* ratio was not significantly higher in either eutopic or ectopic samples compared to the control samples (P=0.305).

Conclusion: Our findings support an altered *PR-B* expression in endometriosis, which may be associated with *MMP-9* overexpression. This finding can be important for disease pathogenesis.

Keywords: Endometriosis, Gelatinases, Progesterone, Progesterone Receptor

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Introduction

Infertility is a persistent and frustrating problem in women with endometriosis (1). The frequency of endometriosis in females with complaints of pain, infertility, or both symptoms is between 35 and 60% (2). It is suggested that endometriosis affects the follicular microenvironment, oocyte maturity and embryo development (1, 3). Extensive remodeling in the endometrial layer and its extracellular matrix (ECM) is one of the reasons for infertility in endometriosis (1, 4, 5). This remodeling of the ECM is required for the activation of matrix metalloproteinases (MMPs) and their inhibitors (6). The decreased potential for embryo implantation is thought to be one of the critical reason for infertility in women with this disease (1). High concentrations of activated macrophages, prostaglandins, IL-1, TNF, and proteases have been reported in peritoneal fluid of women with endometriosis. These abnormalities may adversely impact oocyte function, embryo development, and implantation (4).

MMPs or Matrixins are calcium/zinc-dependent endoproteinases encoded by 24 distinct genes and expressed as 26 distinct proteins in humans (7). They are secreted in a latent form (pro-MMPs) that require proteolytic activation (8). The biological roles of MMPs

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are associated with degradation of the ECM to provide normal endometrial remodeling that accompanies menstruation (9), proliferation, angiogenesis, and apoptosis (7). Endogenous tissue inhibitors of MMPs (TIMPs) regulate MMPs under physiological conditions such as tissue repair and menstruation (10-12). Numerous studies have discussed the role of endogenous proteolytic MMPs in the pathogenesis of endometriosis (13) and have reported a significantly different pattern of MMP expression in endometriosis patients compared to healthy women (14, 15). Over-expressions of MMPs alter the MMPs/TIMPs ratio that may underlie the pathogenesis of diseases including tumor invasion, fibrosis, and endometriosis (8, 16-18). MMPs are involved in all steps of endometriotic tissue migration such as degradation, invasion, and implantation to the ECM outside of the uterine cavity (19). Proteolytic enzymes, like gelatinases (MMP-2 and MMP-9), play an important role in the initial development of endometriosis through ECM degradation (20). The role of gelatinases in the development of diseases has been shown through the participation of MMP-2 and MMP-9 in tumor invasion and progression (1, 13).

Under normal conditions progesterone prevents endometrial breakdown by inhibiting MMPs (21) via its nuclear receptors (21, 22). However, in subjects with endometriosis there is a certain degree of resistance to the action of progesterone (23). In women with this condition, the eutopic endometrium is purportedly resistant to the action of progesterone and inhospitable for embryonic implantation (5). The effects of progesterone are controlled by the two progesterone receptor (PR) isoforms, namely PR-A (94 kDa) and PR-B (114 kDa). These isoforms are functionally different. The PR-B isoform is an activator of progesterone target genes, whereas PR-A is an inhibitor of the PR-B isoform (23). In addition, they are members of the superfamily of ligand-activated transcription factors that bind to sequence-specific sites in the promoters of target genes (22).

On the other hand, progesterone represses *MMP-2* transcription in cells from the jar choriocarcinoma cell line by reducing PR and specificity protein 4 (SP4) through binding to the *MMP-2* promotor (24). Both overexpression and elevated activity of MMP-9 in endometriosis are believed to be regulated by nuclear factor kappa-B (NF-κB) (25). PR can directly interact with one of the subunits of NF-κB, RelA (p65) (26), which is necessary for NF-κB activation. Progesterone efficacy in gene expression depends on the ratio of *PR-A* to *PR-B* (27). An altered ratio in ectopic tissue might play an important role in the mechanism that causes progesterone resistance and modifies progesterone activity related to differential regulation of specific progesterone response genes, such as MMPs, which promote endometriosis. Greater understanding of the abnormal genetic mechanisms involved in the etiology and pathogenesis of endometriosis should lead to better diagnostic methods and targeted treatments that counter endometriosis and its symptoms.

Materials and Methods

We conducted this prospective, case-control study in the Department of Genetics at Royan Institute, Tehran, Iran. Approval was achieved from the Institutional Research Ethics Board. The Ethics Committee of Royan Institute approved this study (No: EC/93/1047). All members signed an informed consent form prior to participation.

Subject selection

This study was conducted from 2013 to 2014 at Royan Institute. We obtained 60 tissue samples (ectopic, eutopic, and normal endometrium) from 40 women. The case group comprised 20 patients with stages III and IV endometriosis. The control group consisted of 20 normal healthy women without endometriosis. Endometriotic (ectopic) tissues were collected during laparoscopy from all patients with ovarian endometriosis. The eutopic samples were obtained by pipelle sampling of endometrial tissues obtained from all patients. Endometrial samples from the control women were also obtained by pipelle sampling. The presence or absence of endometriosis was confirmed by laparoscopy and postoperative histology analyses in endometrial tissue samples from all study participants. Patients with confirmed diagnosis of endometriosis were placed in the patient group. Participants without endometriosis (normal tissue results) were assigned to the control group. None of the patients received hormonal treatments for at least 3 months prior to surgery and all reported regular menstrual cycles. Control group participants did not have any visible endometrial hyperplasia or neoplasia, inflammatory or autoimmune diseases, or endometriosis at the time of the clinical examinations. We also confirmed that women in the control group had given birth to at least one child conceived through natural conception. The menstrual cycle phase at the time of surgery and biopsy was either during the proliferative phase (days 8-14) (80%) or secretory phase (20%) for both patients and controls.

RNA extraction and cDNA preparation

RNA was extracted from snap-frozen tissue samples using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. Genomic DNA contamination was removed by RNase-free DNase I (#EN0521, Fermentas, Thermo Fisher Scientific, USA) and incubation at 37°C for 30 minutes. DNase I enzyme was inactivated by EDTA (50 mM, Fermentas, Thermo Fisher Scientific, USA) and incubation at 65°C for 7 minutes. cDNA samples were prepared from total RNA for each sample by one-step reverse transcriptase-polymerase chain reaction (RT-PCR) and a First-strand cDNA Synthesis Kit (K1632, Fermentas, Thermo Fisher Scientific, USA). Synthesized cDNA was stored at -20°C until later use.

Quantitative real-time polymerase chain reaction

mRNA expression analysis was performed using SYBR® Pre mix Ex Taq II (Applied Biosystems, USA) on a Lightcycler System, 7500 software version 2.0.1 (Ap-

plied Biosystems, USA) as recommended by the manufacturer. We used Primer 3 (version 4.0; http://primer3.ut.ee/), Gene Runner (version 3.05; www.generunner.net), and Perl Primer software (version v1.1.20; perlprimer.sourceforge.net) to design the specific primers used for amplification of *MMP-2, MMP-9, PR-A, PR-B,* and *β-actin* (internal control gene). These sequences were analyzed by Nucleotide Blast and Primer Blast in the NCBI database (http://blast.ncbi.nlm.nih.gov/). Table 1 lists the primers used in this current study and their expected product-sizes. Primers were purchased from Pishgam Co., Iran.

Table 1: Sequences of *β-actin*, *MMP-2*, *MMP-9*, *PR-A*, and *PR-B* primers

Name	Primer sequence (5´-3´)	PCR product (bp)
β-actin	F: CAAGATCATTGCTCCTCCTG R: ATCCACATCTGCTGGAAGG	90
MMP-2	F: GCAACCTGTTTGTGCTGAAG R: GTAGCCAATGATCCTGTATGTG	198
MMP-9	F: TCCAGTACCGAGAGAAAGCCTA R: GCAGGATGTCATAGGTCACG	114
PR-A	F: AATGGAAGGGCAGCACAACT R: TGTGGGAGAGCAACAGCATC	192
PR-B	F: AAGGGGAGTCCAGTCGTCAT R: CGAAACTTCAGGCAAGGTGT	165

MMP; Matrix metalloproteinase, *PR; Progesterone receptor*, **and PCR; Polymerase chain reaction.**

Each reaction contained 10 µl SYBR® Premix Ex Taq II that consisted of Taq DNA polymerase reaction buffer, dNTP mix, SYBR Green II, $MgCl_2$ and Taq DNA polymerase; 5 pmol of either *MMP-9, PR-A,* or *PR-B* primers, or 3 pmol of *MMP-2* primer; 25 ng/µl of synthesized cDNA; and water to reach 20 µl. The target gene levels were compared to that of a housekeeping gene, *β-actin*, from the same cDNA. Each real-time quantitative PCR assay was done in duplicate for each sample to confirm the reproducibility of the results. In this study, both housekeeping genes *GAPDH* and *β-actin* were optimized; however, the expression of *β-actin* appeared to be more stable in our samples. The amplification program contained the following 3 steps. Step 1: a primary heating for 10 minutes at 95°C to denature the cDNA and activate the Taq DNA polymerase. Step 2: DNA amplification for 40 cycles of 15 seconds at 95°C (denaturation) and one minute at 60°C (annealing) for *β-actin, MMP-2, MMP-9, PR-A*, and *PR-B*. Step 3: increasing temperature gradually from 60°C to 95°C for 15 seconds and one minute at 60°C for melting curve analysis. After each run, a melting curve analysis was done to confirm the specificity of the PCR reaction. All samples were retested with a cycle threshold coefficient of variation value higher than one degree. To confirm the melting curve results, we assayed representative samples of the real-time PCR products on 2% ultra-pure agarose (Invitrogen, USA) gel electrophoresis (Paya Pazhoh Pars, Iran), and stained them with ethidium bromide (Sigma Aldrich, USA) prior to visualization on a Molecular Imager® Gel Doc™ XR+ (BioRad, USA).

Statistical analysis

We compared the participants' clinical information between groups (endometriosis and control) using the independent t test. The expression levels of *MMP-2, MMP-9, PR-A*, and *PR-B* were compared between tissue extracts of endometriotic or ectopic lesions and eutopic endometrium samples (patient group) to endometrial samples (control group) using one-way analysis of variance (ANOVA) followed by Tukey's test to conclude significant differences between our groups and pair-wise comparisons. In cases where the data were not distributed normally, we conducted natural logarithmic (Ln) transformation for *MMP-2, MMP-9, PR-A, PR-B,* and *PRA/PR-B* before analysis. The relationships between the Ln-transformed expressions of *PR-A* and *PR-B*, as well as the *PR-A/PR-B* ratio with *MMP-2* and *MMP-9* were assessed by Pearson's correlation. Statistical analysis was done using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). All statistical tests were two-tailed and a $P<0.05$ was considered statistically significant.

Results

Table 2 shows the main clinical characteristics of the 40 participants who provided tissue samples. All 20 women with endometriosis were infertile. There were no statistically significant differences in the sample distributions according to the phases of the menstrual cycles, mean age, or body mass index (BMI) in patients with endometriosis compared to the control group.

Expression of *MMP-2* and *MMP-9* in endometriosis

We assessed the differences between the means of mRNA levels in patients and controls with one-way ANOVA. We observed no significant difference in the expression levels of *MMP-2* among these groups ($P>0.05$, Table 3, Fig.1A). Our results showed a significant increase in the expression of *MMP-9* in endometriotic tissues compared to eutopic endometrium samples ($P=0.012$) and the control group ($P=0.014$, Table 3, Fig.1B).

Table 2: Clinical characteristics of participants in expression assays

Groups	Menstrual cycle phase (%)	Disease stage (%)	BMI (kg/m²)	Age (Y)
Endometriosis n=20	Proliferative (80) Secretory (20)	IV (60) III (40)	25.82 ± 4.91	30.03 ± 8.31
Controls n=20	Proliferative (80) Secretory (20)	-	24.35 ± 4.32	29.21 ± 8.72
P value	NS	-	NS	NS

Data are expressed as mean ± SEM and values in parentheses are percentages. BMI; Body mass index and NS; Not significant.

Table 3: mRNA expression levels of *MMP-2, MMP-9, PR-A,* and *PR-B* in ovarian endometriosis and endometrial tissues obtained from women with and without endometriosis

Different object	Endometriotic lesions (ectopic)	Eutopic endometrium (endometriosis group)	Endometrium (control group)	P value
MMP-2	0.16 (0.05, 1.45)	0.15 (0.05, 0.87)	0.12 (0.06, 0.29)	0.512
MMP-9	0.02E-1 (0.03E-2, .021)*Δ	0.03E-2 (0.01E-2, 0.06E-2)	0.03E-2 (0.09E-3, 0.07E-2)	0.005
PR-A	27.20 (9.99, 206.33)	76.98 (9.00, 268.03)	67.05 (16.60, 231.78)	0.643
PR-B	0.04E-2 (0.03E-3, .01E-1)*Δ	0.03E-1 (0.01E-1, 0.07E-1)	0.04E-1 (0.01E-1, 0.09E-1)	0.001
Ln *(PR-A/PR-B)*	11.79 ± 4.82	10.28 ± 4.64	9.81 ± 2.93	0.305

Data are expressed as mean ± standard deviation or median (inter-quartile range) when appropriate. ANOVA was performed on the natural-log-transformed values when appropriate. *MMP; Matrix metalloproteinase, PR; Progesterone receptor,* *; P<0.05 versus endometriotic lesions compared to the controls, and Δ; P<0.05 versus endometriotic lesions compared to the eutopic endometrium.

Progesterone receptor isoforms *PR-A* and *PR-B* expression in endometriosis

Extracts of endometriotic lesions from women with endometriosis presented a slight decrease in mRNA level of *PR-A* in comparison to the eutopic endometrium (Table 3, Fig.1C), while the mRNA levels of this isoform were slightly higher in eutopic endometrium samples compared to the control group (Table 3, Fig.1C). However, our data presented no significant differences between these groups (P=0.44). The results generally confirmed that the expression level of *PR-B* significantly differed between groups (P<0.001, Table 3). As shown in Figure 1D, we found significantly lower expression levels of *PR-B* in endometriotic tissues compared to the controls (P=0.002) and eutopic endometrium tissues (P=0.006, Table 3). Although eutopic endometrium tissues showed low levels of *PR-B* expression compared with the control samples, there were no significant differences observed among these two groups (P=0.95).

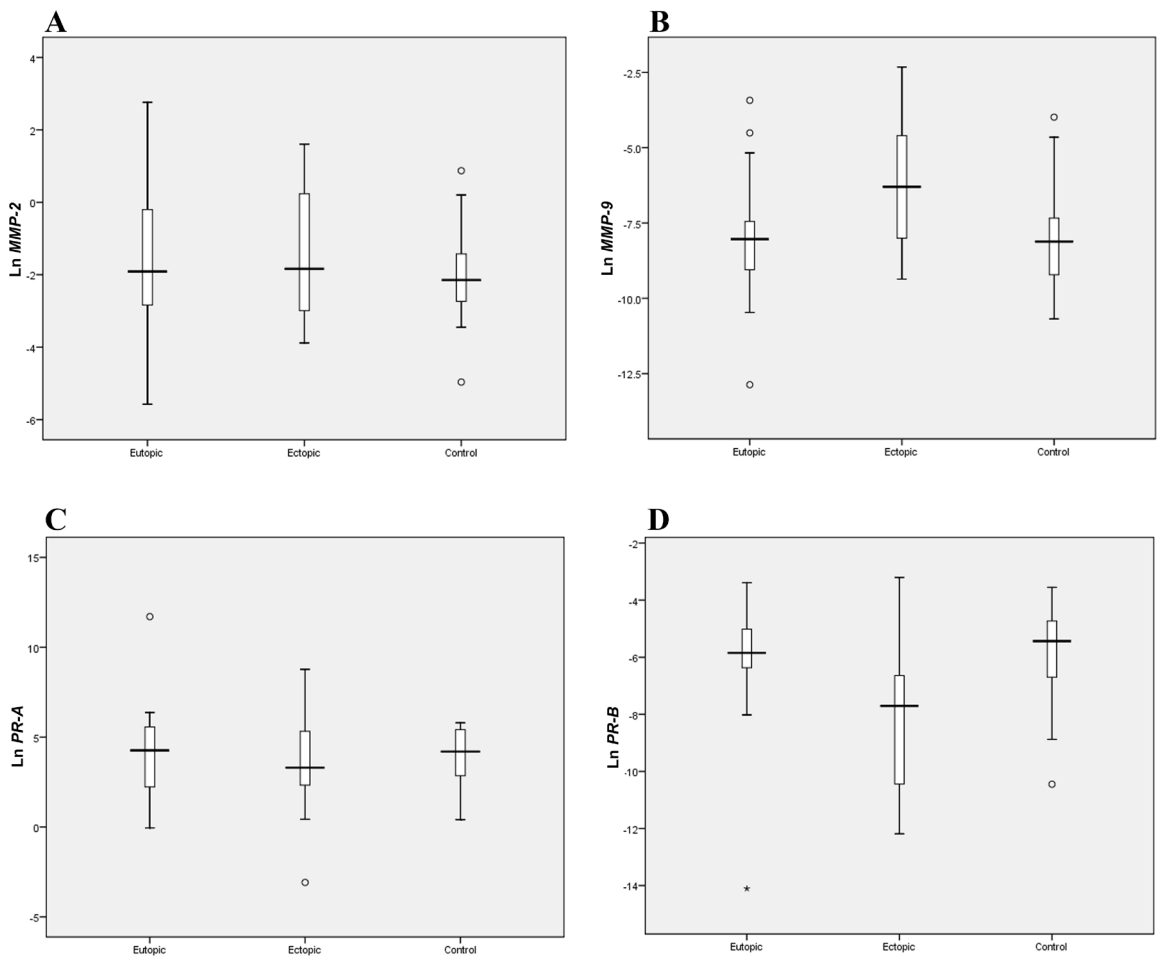

Fig.1: Expression levels of matrix *metalloProteinases (MMPs)* and *progesterone receptors (PRs)*.
A. *MMP-2*, **B.** *MMP-9*, **C.** *PR-A*, and **D.** *PR-B* in ovarian endometrioma (ectopic) and endometrial tissues from women with (eutopic) and without endometriosis (control). Ln: Logarithmic.

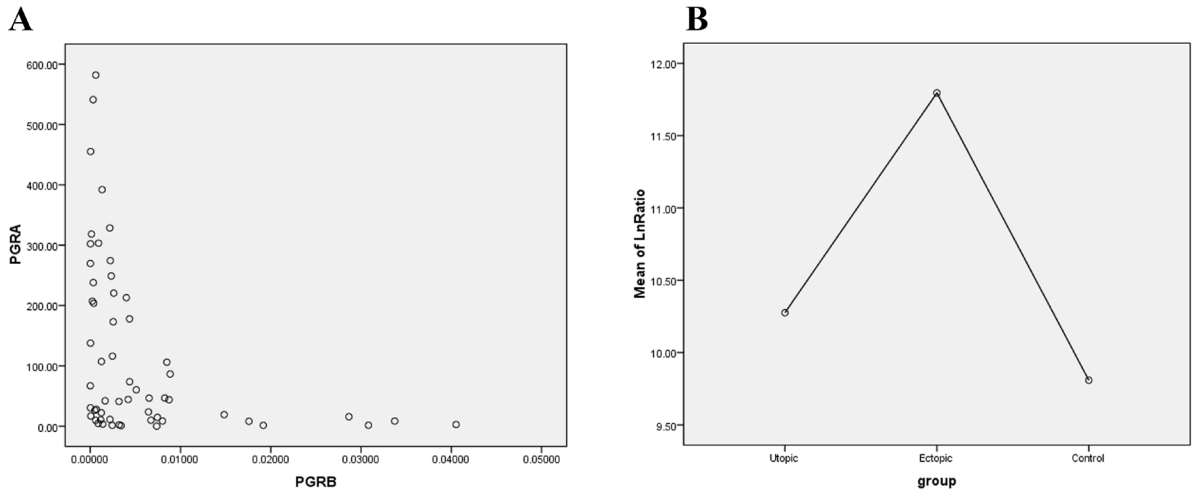

Fig.2: Association of progesterone receptor *(PR)-A* and *PR-B* gene expressions in ovarian endometrioma and endometrium tissues from women with and without endometriosis. **A.** Overexpression of *PR-A* was associated with a low expression levels of the *PR-B* isoform in the three different groups and **B.** There was a higher *PR-A/PR-B* ratio in endometriutic tissue and eutopic endometrium compared with the control group. Ln; Logarithmic.

Table 4: Correlation between mRNA levels of *MMP-2, MMP-9*, and *PR-A/PR-B* ratio in ovarian endometrioma and endometrial tissues from women with and without endometriosis

Different object	Ln*PR-A/PR-B*		
	Endometriotic lesions	**Eutopic endometrium**	**Control endometrium**
Ln*MMP-2*	r=0.09	r=0.09	r=-0.19
	P=0.701	P=0.701	P=0.413
Ln*MMP-9*	r=-0.21	r=-0.62*	r=0.14
	P=0.365	P=0.003	P=0.542

Pearson test: analysis of correlation between different groups. P values are calculated on logarithmic (Ln)-transformed data. *MMP; Matrix metalloproteinase*, and *PR; Progesterone receptor*, and r; Spearman's rho test.

Association between expression levels of progesterone receptor isoforms *PR-A* and *PR-B* in endometriosis

There was a strong negative correlation between *PR-A* and *PR-B* isoforms in endometriotic lesions. When the PR-A isoform had increased mRNA levels, we found significantly lower levels of *PR-B* isoform expression and vice versa (r=-0.789, P<0.001, Fig.2A). Similar results were observed in endometrial tissue from women with (r=-0.844, P<0.001) and without endometriosis (r=-0.579, P=0.008).

PR-A/PR-B ratio and its association with *MMP-2* and *MMP-9* expressions in endometriosis

We observed higher *PR-A/PR-B* ratios in both eutopic and endometriutic tissues related to the control group, but this finding was not significant (P>0.05, Table 3, Fig.2B). We were interested to assess the correlation between the mRNA levels of *MMP-2* and the *PR-A/PR-B* ratio in each group. However, our data did not show any significant correlation between overexpression of *MMP-2* and an altered *PR-A/PR-B* ratio in any of the groups (Table 4). We found no significant correlation between the expressions of *MMP-2* and *PR-A* or *PR-B* (P>0.05, data not shown).

Our results indicated that the expression level of *MMP-9* only had a significant relationship to the mRNA levels of the progesterone receptor ratio (*PR-A/PR-B*) in eutopic endometrial tissue (P=0.003, Table 4). On the other hand, we found a significant association among the expression level of *MMP-9* and the *PR-A* isoform in eutopic endometrial tissue (P=0.03). There was no significant relation between the expression levels of *MMP-9* and the *PR-B* isoform in the study groups (P>0.05, data not shown).

Discussion

Endometriosis develops as a consequence of ectopic implantation of retrograded menstrual tissue, although the mechanisms that underlie this process are unknown (21). Several studies have underlined a correlation between MMPs and the invasive behavior of endometriotic tissues for establishment of endometrial glands and stromal cells at ectopic sites (7). MMPs coordinate general endometrial remodeling through menstrual cycles, which mediates ECM turnover (21). Upregulation and activation of MMPs related to tumor progression have been found in metastatic activity of tumors dependent on MMP synthesis (28). Hence, the expression of MMP enzymes is tightly regulated in normal tissues, because the delicate balance between MMPs and their inhibitors is crucial to preventing excessive matrix destruction (21).

Follicular fluid surrounds the microenvironment of maturing oocytes and has an important role in this process, affecting fertilization and consequent of embryo development (1). The opposed effect of endometriosis on fertilization has been attributed to its impact on the follicular microenvironment, poor oocyte development, and poor embryo formation (4). Studies indirectly suggest that MMP-2 and MMP-9 in follicular fluids have a direct effect on follicular development and rift of the follicular wall (29). A high level of *MMP* expression by the endometriotic tissues can be initiated in the pathogenesis of endometriosis (7). It might be responsible for intrafollicular modifications that result in infertility.

Overexpression of different *MMPs* have been reported in endometriosis and include MMP-1 (30), MMP-2 (18), MMP-9 (20), and MMP-7 (31). The degradation of vascular and epithelial basement membrane components and ECM proteins are mediated by gelatinases (MMP-2 and MMP-9). Gelatinases have been associated with the malignant potential of tumors by increasing tumor invasion and metastasis (32). The role of MMP-2 in endometriosis is debatable. In the current study, we have detected elevated *MMP-2* expression in both ectopic and eutopic tissues of endometriosis patients compared to the normal control group. However, no significant difference in *MMP-2* expression was observed in our groups. Previous studies have reported higher levels of MMP-2 expression and lower mRNA levels for *TIMP-2* in eutopic tissues of endometriosis patients relative to the endometrium from control groups (33, 34).

This highlights potential changes in MMP activity in endometriotic tissues and suggested improved proteolysis activity, which could play an important role in implantation of this tissue in ectopic sites. In addition, our data showed significantly higher expression levels of *MMP-9* in the ectopic versus the eutopic and control endometrial tissues. Several researchers have focused on the role of *MMP-9* in tumor invasion and metastasis (35, 36). The involvement of this proteolytic enzyme in vascular growth and angiogenesis has been previously reported (20). A higher gelatinase activity was found in endometriotic tissues compared to eutopic endometrium in endometriosis (37). Previous investigations have demonstrated higher expression of *MMP-9* in ectopic versus the eutopic endometrium (38). In patients with endometriosis, elevated levels of *MMP-9* mRNA in ectopic tissues might play an essential role in endometrial tissue invasion and its ability to be implanted in ectopic sites. High levels of *MMP-2* and *MMP-9* and low levels of *TIMP-1* were related with low production of mature oocytes and subsequent decreased quality of embryos in endometriosis patients who underwent *in vitro* fertilization (IVF) (1). As a result, *MMP-2* and *MMP-9* overexpression have adverse effects on the function of the follicular microenvironment, as well as oocyte and embryo quality. These changes might be the cause of infertility due to endometriosis.

Endometriosis is known as a progesterone resistant disease (23). The ability of progesterone to affect gene expression is reliant on the *PR-A/PR-B* ratio (27). An altered *PR-A/PR-B* ratio modifies progesterone activity due to differential regulation of specific progesterone response target genes that may lead to the progression of endometriosis. Progesterone reduces the expression of pro-inflammatory genes when the *PR-A/PR-B* ratio favors *PR-B* and increases their expression when the ratio tilts towards the *PR-A* isoform (39, 40). The present study has shown a slightly increased level of *PR-A* expression in eutopic tissues compared to controls. This increased expression was slightly higher in controls compared to ectopic tissues. On the other hand, *PR-B* showed a significantly differential expression pattern between the groups. The results clearly showed a decreased expression level for *PR-B* in endometriotic tissues compared to control and eutopic groups, which can disrupt the *PR-A/PR-B* ratio in ectopic samples. Eutopic tissues also had decreased *PR-B* expression. Progesterone resistance might account for the existence of the inhibitory PR isoform, *PR-A*, and the lack of the stimulatory isoform, *PR-B*, in endometriotic tissues (23). These results suggested that a decrease in the expression level of *PR-B* and overexpression of *PR-A* could alter this ratio in endometriotic tissues. Following this, the imbalanced ratio could alter progesterone activity related to differential regulation of specific progesterone target genes and improve endometriosis. On the other hand, we have demonstrated an association between overexpression of *PR-A* with low expression of the *PR-B* isoform, particularly in ectopic tissue and the endometria of women with and without endometriosis.

It has been shown that transcriptional regulation of *MMP-2* in the JAr choriocarcinoma cell line is mediated by progesterone treatment with progesterone inhibiting the expression of *MMP-2*. *MMP-2* expression is mediated through the binding of the primary transcription factor SP4 to the *MMP-2* proximal promoter. Progesterone inhibits *MMP-2* expression by decreasing PR and SP4 binding to the *MMP-2* promoter (24). Progesterone also suppresses TGFβ1-induced stimulation of *MMP-2* through its nuclear hormone receptors in human endometrial stromal cells (22). Therefore, our data imply that observed alteration in *PR-A/PR-B* expression ratio may cause overexpression of *MMP-2* in endometriotic tissues. However, our analysis did not show any significant correlation between the high level of *MMP-2* expression and imbalance in *PR-A/PR-B* ratio expression in endometriotic tissues.

In contrast, we have shown, for the first time, a significant association between the expression of *MMP-9* and altered an *PR-A/PR-B* ratio in endometrium (eutopic) tissues of women with endometriosis compared to a normal control group. *MMP-9* activity in the human endometrium is controlled by estradiol and progesterone (26). This hypothesis can be supported by the fact that progesterone increases the expression level of inhibitor-κBα, a repressor of the NF-κB transcription factor, and inhibits basal and lipopolysaccharide-induced proinflammatory gene expressions via *PR-B*, which are inhibited by *PR-A*

(27). NF-κB is involved in the regulation of cytokines and MMP transcription (including *MMP-9*) in the human endometrium. PRs can directly interact with the RelA (p65) subunit of NF-κB, which is necessary to activate NF-κB (26). Thus, an altered *PR-A/PR-B* ratio may impact the expression level of *MMP-9* through the regulation of NF-κB activity, which could be important in the pathogenesis of endometriosis. However, we have not observed any significant correlation between this altered ratio and *MMP-9* expression in ectopic tissues in comparison to the control endometrium samples.

Conclusion

We sought to assess the correlation between the expression of *MMP-2* and *MMP-9* and the *PR-A/PR-B* ratio in endometriosis. Our data showed a significant negative association between expression levels of *MMP-9* and an altered *PR-A/PR-B* ratio in the eutopic endometrium group compared with the control samples. To our knowledge, there have been few attempts to report these correlations between the *MMPs* and *PR* isoforms in endometriosis. It is known that endometriosis affects the follicular microenvironment, oocytes maturity and consequent embryo development. This hypothesis may be correlated further by our observations since overexpression of *MMP-9*, as a consequence of an imbalanced *PR-A/PR-B* ratio in endometriosis, may affect the function of the follicular microenvironment, as well as oocyte and embryo quality, which cause infertility in endometriosis.

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Authors' Contribution

S.M.; Participated in study design, data collection and evaluation, drafting and statistical analysis. A.Gh.; Contributed to all data and statistical analysis and interpretation of data. M.Sh., R.A.; Contributed to conception and design. P.A.; Managed substantially of the design, all experimental work, data and statistical analysis of the study and provided critical revision of the manuscript. All authors performed editing and approving the final version of this manuscript for submission, also participated in the finalization of the manuscript and approved the final draft.

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Original Article

A Novel Mutation in *NLRP7* Related to Recurrent Hydatidiform Mole and Reproductive Failure

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Abstract

Background: Hydatidiform mole (HM) is an abnormal human pregnancy with excessive trophoblastic proliferation and abnormal embryonic development, dividing into two complete HM (CHM) and partial HM (PHM) groups. One subcategory of the CHMs is recurrent and familial, which is known as biparental HM (BiHMs) or recurrent HM (RHM). *NLRP7*, *KHDC3L* and *PADI6* are maternal-effect genes involved in RHMs. *NLRP7* is a major gene responsible for RHMs. This study was performed on patients with molar pregnancies and miscarriage. The aim of this study was to genetic screen for mutations in *NLRP7* and *KHDC3L* genes in an affected woman with previous history of 5RHM and the sibling with history of miscarriage.

Materials and Methods: In this experimental study, DNA was extracted from blood samples. *KHDC3L* and *NLRP7* were polymerase chain reaction (PCR) amplified. The PCR products were purified and Sanger sequenced.

Results: In this study, there is no mutation in *KHDC3L* gene but a novel mutation was identified in the NACHT domain of *NLRP7* gene. Patient with five recurrent moles had this mutation in the homozygous state while her sister with one miscarriage and one normal child showed this mutation in the heterozygous state.

Conclusion: In this study, we identified a new mutation in *NLRP7* gene of a patient with recurrent HM. Following egg donation, this patient has a normal boy. The sister of this patient with heterozygous mutation has a spontaneous abortion and one normal child that confirm the impact of a defective allele of *NLRP7* on reproductive wastage in a recent finding.

Keywords: Hydatidiform Mole, *KHDC3L*, *NLRP7*

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Introduction

Hydatidiform mole (HM) is an abnormal human conception with a defect in fetal development and growth (1). HM is divided into two categories, complete HM (CHM) and partial HM (PHM). CHMs are commonly androgenetic diploid conceptions (2) and PHMs are mostly dispermic triploid conceptions (3). Both CHM and PHM have an extra set of the paternal genome, therefore, paternal genes are more expressed and consequently show excessive trophoblastic proliferation (4). In most of the cases, HM is sporadic, however, in a subgroup of CHM, it is recurrent and familial condition which is known as biparental HM (BiHMs) or recurrent HM (RHM) (OMIM 231090). Occurrence of at least two moles in the same woman is referred to recurrent type and this form is inherited in an autosomal recessive fashion. Frequency of RHMs in the Middle and Far East is reported about 2.5% up to 9.4% of all HMs, which is twice or more compared to Western countries (5-9).

So far, three maternal-effect genes, *NLRP7*, *KHDC3L* and recently *PADI6*, have been identified to be responsible for RHMs (10-12). It is suggested that these three genes function in setting genomic imprinting process (13). *NLRP7* mutations have been reported in 48-80% of RHMs cases (14-19), while mutations in *KHDC3L* was only reported in 10-14% of these patients with no *NLRP7* mutations (10, 20, 21). Homozygote or compound heterozygote mutations of these three genes have been observed in most of the affected women (22). There is still a few fractions of RHM patients with the unidentified responsible gene. *NLRP7* is the principal gene responsible for RHMs, identified by Murdoch and colleagues in

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2006. *NLRP7* as the candidate of maternal-effect gene is responsible for RHMs and reproductive disorders such as spontaneous abortions and stillbirths (11).

NLRP7, which encodes a protein with 1037 amino acids, is a member of the CATERPILLER protein family with four conserved and functional pyrine, 9-10 leucine-rich repeats, NACHT-associated domain (NAD) and a NACHT domain (Fig.1A) (23, 24). About 48% of intronic sequences of *NLRP7* gene contain Alu repetitive elements. It is believed that Alu repeats act as a hot spot for INDEL mutations (20). To date, 60 pathogenic point and INDEL mutations have been reported in *NLRP7* (20, 25). In this study, a new mutation was identified in *NLRP7* gene in a patient with recurrent HM. This patient has a normal boy using egg donation. Also, the sister of this patient with heterozygous mutation has a spontaneous abortion and one normal child.

Materials and Methods

In this experimental study, two sisters with molar pregnancies and miscarriage referred to the Infertility Center in Shiraz University of Medical Sciences, Shiraz, Iran. In the patient, as proband, five moles were reported without any normal child. Patient's sister represented one normal child and one miscarriage. Proband was diagnosed as Bi-HMs because she has more than two moles and genetic studies were performed on *NLRP7* and *KHDC3L* genes. Genomic DNA was isolated from whole blood cells using DNA Kit (Cinnaclon, Iran). Three exons and intron boundaries of *KHDC3L* and 11 exons and intron boundaries of *NLRP7* were polymerase chain reaction (PCR) amplified using our previously designed primers and conditions (20, 26). PCR products were purified and Sanger sequenced (Eurofins, Germany). The Ethics Committee of Shiraz University of Medical Sciences approved the study protocol and patients gave written consent to participate in the study (code: IR.SUMS.REC.1396.540).

Results

The sequence of *NLRP7* and *KHDC3L* were analysed by Chromas software (Technelysium Pty Ltd, Australia). BLAST of sequences was performed for two genes based on the reference sequences in the NCBI database (*NLRP7*, NG_008056.1, and *KHDC3L*, NG_031942.1). Sequencing analysis of *NLRP7* in the patient revealed a new three nucleotides deletion in exon 4 in a homozygous state (Fig.1B). Sequence analysis of the patient's sister with one spontaneous abortion and one normal child showed a heterozygous deletion status for these three nucleotides (Fig.1C). Normal sequence is provided in Figure 1D. This deletion is expected to remove amino acid Threonine in codon 185 (*c.555_557delCAC, p.Thr185del*) . The mutation was evaluated by parameters of Mutation Taster (www.mutationtaster.org) and it was regarded as disease-causing alteration. In addition, the mutation was analysed by PROVEAN parameter (http://provean.jcvi.org). Variants with a score equal to or below -2.5 are considered "deleterious," and variants with a score above -2.5 are considered "neutral." PROVEAN score was estimated -13.000 for this mutation. This means that the mutation is deleterious.

In addition, Threonine in codon 185 is conserved in various species using multiple sequence alignment by Clustal Omegam (www.ebi.ac.uk/Tools/msa/clustalo/) (Fig.1E). Histopathology of the molar tissue for the patient is provided in Figure 2. Excessive proliferation of trophoblastic tissue has been observed around chorionic villi, while fetal tissues were clearly absent.

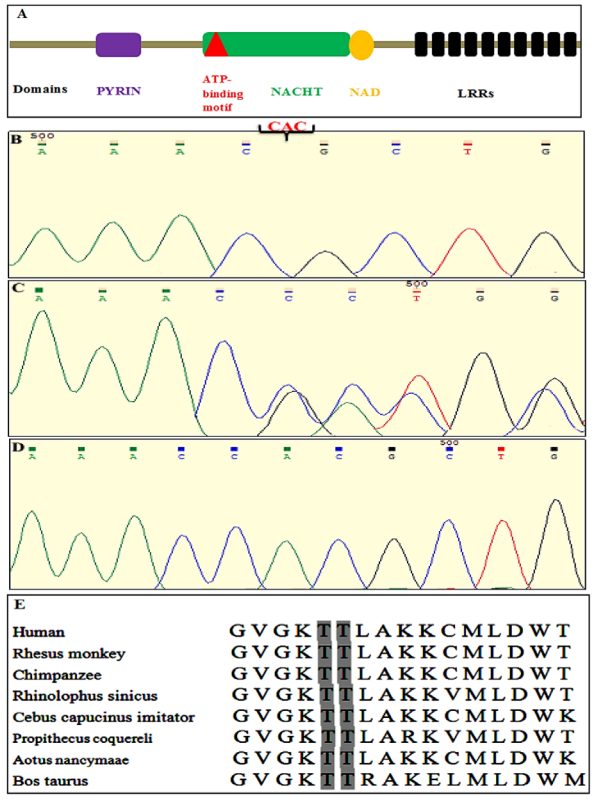

Fig.1: Deletion of the highly conserved Threonine amino acid from NACHT domain. **A.** NLRP7 protein domains including PYRIN, NACHT, NAD, leucine-rich repeats and ATP binding motif in the NACHT domain is depicted, **B.** Sequence chromatogram show deletion of CAC nucleotides in homozygote state in the patient's *NLRP7* gene, **C.** Heterozygote deletion of CAC nucleotides in her sister, **D.** Normal allele in the wild type individual, and **E.** Threonine 185 residue is highly conserved during evolution.

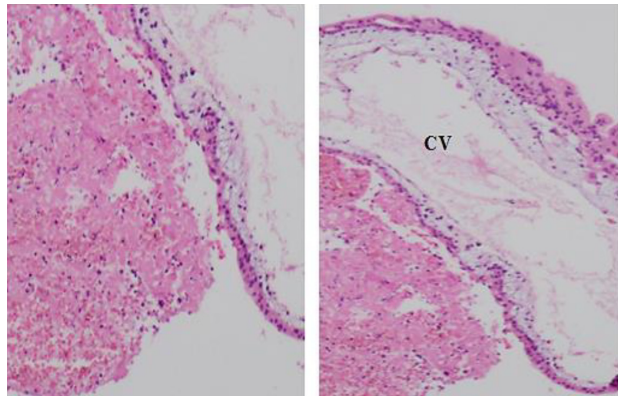

Fig.2: Photomicrograph of molar tissue from the patient. Excessive proliferation of trophoblastic tissue is seen around chorionic villi (CV) by hematoxilin/eosin staining histopathology analysis (magnification: left: × 100, right × 40).

Discussion

In this study, a new mutation in the homozygous state has been identified within the NACHT domain of NLRP7 protein, suggesting the importance of this domain in normal function. This study on a patient with a homozygous mutation in *NLRP7*, while she has a healthy boy via ovum donation, add further evidence that pathology of RHM is restricted to the oocyte and normal ovum is able to rescue defects of these patients for normal pregnancies. To date, four cases of ovum donation in patients with a mild missense mutations in *NLRP7* have been reported (27, 28). Investigations on healthy reproductive male individual with a homozygous mutation in *NLRP7* show that function of this gene is not necessary for normal sperm, in contrast to ovum (14, 15). The sister of indicated patient with heterozygous mutation has a spontaneous abortion and one normal child, confirming the impact of the defective allele of *NLRP7* on reproductive wastage, reported in recent finding (25).

Conclusion

We report a new mutation in *NLRP7* gene, related to RHM and spontaneous abortion in homozygous and heterozygous states, respectively. Regarding this study and four previous reports, patients with homozygous mutation in *NLRP7* are able to have live birth with egg donation. In contrast to four previously reported cases with a mild missense mutations, investigation on this new patient shows that more deleterious mutations with severe functional effect are also good candidate for egg donation.

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Authors' Contribution

M.F., Z.A.; Contributed to conception and design. J.F.; Contributed to all experimental work. M.F., J.F., V.R.; Contributed to interpretation of data and drafted the manuscript. M.A.-J.; Contributed to interpretation of the molar tissue. M.M., B.N.-J.; Contributed to consulting and detection of patients through clinical specification also performed editing and approving the final version of this manuscript for submission. All authors read and approved the final manuscript.

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Original Article

Comparison of Germ Cell Gene Expressions in Spontaneous Monolayer versus Embryoid Body Differentiation of Mouse Embryonic Stem Cells toward Germ Cells

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Abstract

Background: Genetic and morphologic similarities between mouse embryonic stem cells (ESCs) and primordial germ cells (PGCs) make it difficult to distinguish differentiation of these two cell types *in vitro*. Using specific GC markers expressed in low level or even not expressed in ESCs- can help recognize differentiated cells *in vitro*. We attempted to differentiate the mouse ESCs into GC-like cells spontaneously in monolayer and EB culture method.

Materials and Methods: In this experimental study, we attempted to differentiate ESCs, Oct4-GFP OG2, into GC-like cells (GCLCs) spontaneously in two different ways, including: i. Spontaneous differentiation of ESCs in monolayer culture as (SP) and ii. Spontaneous differentiation of ESCs using embryoid body (EB) culture method as (EB+SP). During culture, expression level of four GC specific genes (*Fkbp6, Mov10l1, Riken* and *Tex13*) and *Mvh*, *Scp3*, *Stra8*, *Oct4* were evaluated.

Results: In both groups, *Mov10l1* was down-regulated (P=0.3), while *Tex13* and *Riken* were up-regulated (P=0.3 and P=0.04, respectively). *Fkbp6* and *Stra8* were decreased in EB+SP and they were increased in SP group, while no significant difference was determined between them (P=0.1, P=0.07). Additionally, in SP group, gene expression of *Mvh* and *Scp3* were up-regulated and they had significant differences compared to EB+SP group (P=0.00 and P=0.01, respectively). *Oct4* was down-regulated in the both groups. Flow-cytometry analysis showed that mean number of Mvh-positive cells in the SP group was significantly greater compared to ESCs, EB+SP and EB7 groups (P=0.00, P=0.01, and P=0.3, respectively).

Conclusion: These findings showed that ESCs were differentiated into GCLCs in both group. But spontaneous differentiation of ESCs into GCLCs in SP group (monolayer culture) compared to EB+SP (EB culture methods) has more ability to express GCs markers.

Keywords: Differentiation, Embryoid Body, Embryonic Stem Cells, Monolayer

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Introduction

Embryonic stem cells (ESCs) can proliferate unlimitedly *in vitro* and they are unique in their ability by growing as immortal cells, expressing high telomerase (1) and preserving a normal karyotype during multiple passages (2). The medium supplemented by myeloid leukemia inhibitory factor (LIF) causes the ESCs to remain in an undifferentiating state (3). Spontaneous differentiation of ESCs can be easily triggered by the withdrawal of LIF from the medium culture of embryoid body (EB) *in vitro* (4, 5) and monolayer cells (6). EBs can be developed by aggregation of ESCs in suspension or hanging drops. They are rounded and three-dimensional structures which can generate populations of cells expressing genes indicative of lineages from all three germ layers (7). Under certain conditions, ESCs can also differentiate into different cell types such as neural progenitors (8), primordial germ cells (PGCs) (9), pancreatic linage (5) and blood cells (6). Over the past several decades, researchers have attained significant results in designing an appropriate *in vitro* model for the differentiation of ESCs into GCs (10, 11). It seems that these ESC-derived PGCs have the ability to enter meiosis as male and female gametes. However, compared to endogenous GCs, they do not undergo normal meiosis or become a functional gamete (12). Defects in natural and complete meiosis are one of the obstacles in achieving functional gametes.

In mice, over 53 genes are involved in the regulation of cell cycle (13). In a spontaneous differentiation protocol, expression of the GC markers was demonstrated (14). With regard to the literature, it can be suggested that continuing ESC culture in monolayer system for more than 10 days would lead to an increase in the GC marker expressions (15). Induced pluripotent stem cells express male GC genes during their spontaneous differentiation through EB formation (16).

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Genetic and morphologic similarities between ESCs and PGCs make it difficult to diagnose these two cell type differentiations *in vitro*. Some specific GC markers, such as *4930432K21Rik*, *Mov10l1*, *Fkbp6* and *Tex13*, are expressed in reproductive system. Despite the low expression level of these genes in ESCs, they are highly expressed in PGCs (17). This will facilitate tracing differentiated cells *in vitro*. The Moloney leukemia virus 10-like 1 (*Mov10l1*) is a GC-specific autosomal gene in the mouse spermatogonia cells (18). *4930432K21Rik* is a new gene expressed in PGCs and gametes (17). *Fkbp6* is expressed in mouse testis (19). In human, mutations of this gene have been associated with male infertility (20). In mouse, *Tex13* is also an X-linked gene, expressed in a GC-specific manner beginning at the spermatogonia stage (21, 22). In the present study, we attempted to differentiate the mouse ESCs, Oct4-GFP, into GC-like cells (GCLCs) spontaneously in two different ways: i. Spontaneous differentiation of ESCs in monolayer culture (SP) group and ii. Spontaneous differentiation of ESCs in EB culture method as (EB+SP) group. We tried to evaluate and compare expression level of GC specific genes in both groups, during culture *in vitro*.

Materials and Methods

Animals

Eight healthy adult NMRI mice, weighting more than 30 g, were usually housed in a light cycle of 12 hours light (6:00 AM to 6:00 PM) and 12 hours dark. Mice were obtained from the Animal Research Unit, Babol Medical University, Babol. Animal care and handling was done based on Animal Research Unit following the approval of Ethics Committee (Babol Medical University, Iran; MUBABOL.REC.1393.7).

Study design

In this experimental study, samples were classified to two groups: i. Spontaneous differentiation of ESCs without LIF in its feeder cells (MEF) for 14 days as a monolayer culture (SP) group and ii. Spontaneous differentiation of ESCs using EB method. After 3 days culture for hanging drop and 4 days in bacterial plate, totally 7 days known as (EB7), single EB cells were cultured for 7 more days without LIF, totally 14 days; the latter group was named as EB culture methods (EB+SP).

Cell culture

The utilized medium for MEF culture was a knock out-Dulbecco's modified Eagle's medium (DMEM, Gibco, UK) containing 15% fetal bovine serum (FBS, Bio west, USA), Pen/Str/Glu 100 U/ml , 100 mM non-essential amino acids and 0.1 mM 2-Mercaptoethanol (all from Gibco, UK). The applied medium for ESCs culture contain knock out-DMEM, 15% knock out-SR (Gibco, UK), Pen/Str/Glu 100 U/ml, 100 mM non-essential amino acids, 0.1 mM 2-Mercaptoethanol and 1000 IU/ml LIF (Chemicon, UK). ESCs differentiation and EB medium was similar to ESC medium, without LIF.

Culture of mouse embryonic fibroblast

ESCs need feeder layer for growth. We cultured E13.5 mice in our study (23). Briefly, two female and one male mouse were put together in the same cage to mate. The morning after mating, vaginal plugs were checked and pregnant mice were identified (24) and scored as E0.5. After 13 days, pregnant mice were sacrificed to extract embryos. Embryos were isolated and washed, and then head and liver were separated from embryo and crushed using an 18-gauge needle, followed by culturing in MEF media. After two passages, MEF cells were ready for inactivation using incubation with 10 µg/ml mitomycin C (Sigma, Germany) for 1.5-2 hours. We used the cultured cells with passages 2-4, in this work.

Culture and passage of mouse embryonic stem cells

The OG2 (ΔPE-GFP) ESC line (a kind gift from Dr. Sabour, Max Planck institute, Germany) was used in this study. Briefly, mouse ESC line was cultured on mitomycin C-treated MEFs with 0.1% gelatin-coated 25-cm^2 flasks in ESC medium. Undifferentiated ESCs were cultured at 37˚C, 5% CO_2 and 95% humidity. The medium was renewed daily. Seven two hours after primary culture, when the colony size was increased, cultured cells were trypsinized and expanded at a ratio of 1:3 on fresh feeder cells. The medium was changed every day.

Embryonic stem cells differentiation *in vitro*

ESC colonies were dissociated with 0.25% trypsin-EDTA (Invitrogen, USA) (23). In order to remove MEFs from ESCs, we used the MEF faster reattachment potential, compared to ESC. After two rounds of reseeding (about 30-40 minutes), EB formation was induced with hanging drop prepared with a cell suspension containing 150-200 ESCs per 25 µl of mouse EB differentiation media for 3 days. EBs were next collected and transferred into non-attachment 10-cm^2 bacterial dish for 4 additional days. EBs on seventh day were dissociated and digested by treatment with collagenase IV (0.01%), in order to obtain cell suspensions. The cells were then filtered and seeded in a gelatinized dish at about 20,000 cells per cm^2. EB+SP groups were fed daily with EB medium for additional 7 days. On the other hand, single ESCs were seeded in a gelatinized dish at about 20,000 cells per cm^2, for SP group. The cells were fed daily with ESC cell medium without LIF, for 14 days.

Morphological evaluation

Morphological changes of differentiated cells were assessed by invert microscope (Nikon, Japan) during and after 14 days differentiation *in vitro*.

RNA isolation and quantitative reverse-transcription polymerase chain reaction

Total RNA was isolated from ESCs, MEF cells, spontaneous differentiation of GCLCs (SP), 7 day EBs (EB7), spontaneous differentiation after EB formation (EB+SP) of GCLCs and somatic tissues of the testis and brain using RNA Isolation Kit (Roche, Germany). DNase I was used to eliminate genomic DNA contamination. RNA quality was determined using a Nano drop 2000c (Thermo Scien-

tific, USA). cDNA was prepared in a total volume of 10 µl using a cDNA synthesis kit (TaKaRa, Japan) according to the manufacturer's protocol. Target gene expressions were normalized based on the mouse housekeeping gene, *Hprt*. Gene transcripts were determined using SYBR Green I PCR Master Mix (Applied Biosystems, USA) containing 150 nmol of each forward (F) and reverse (R) primers (Table 1). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis was performed using the ABI 7300 (Applied Biosystems). Relative quantification of gene expression was calculated using $2^{-\Delta\Delta Ct}$ method. Three technical replicates were used for each qRT-PCR reaction. No template control (blank test) was served as a negative control. In all reactions, mouse testis and brain were respectively used as positive and negative controls.

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

Gene	Primer sequence (5´→3´)	Length (bp)
Oct4	F: TGTTCCCGTCACTGCTCTGG R: TTGCCTTGGCTCACAGCATC	82
Sycp3	F: GCAGTCTAGAATTGTTCAGAGCCAGA R: TCCAAACTCTTTATGAACTGCTCGTG	75
Vasa	F: GGAGAGAGAGCAAGCTCTTGGAGA R: TGGCAGCCACTGAAGTAGCAA	74
Stra8	F: GACGTGGCAAGTTTCCTGGAC R: TTCTGAGTTGCAGGTGGCAAA	81
Tex13	F: GCCACAGGAAGACCGAATGAG R: TCTCTGCCTTTTCAGGGGATA	156
Fkbp6	F: CCCCTCATCCCGCCAAATG R: TGCCAAACTCCCTCTCAGTTG	163
Mov10l1	F: CGCTGTGACGAGTACAGTG R: CTGACAACCCTTTGCTAGAGTTT	155
4930432K21Rik	F: AGAGAGTCGGAAGACAGCTCA R: CAGGGGGACCAGCTCTTTG	144
Hprt	F: GTTAAGCAGTACAGCCCCAAA R: AGGGCATATCCAACAACAAACTT	140

Immunofluorescent staining

ESCs were cultured in two wells chamber slides at the end of previous culture step. They were fixed in 100% methanol (chilled at -20°C) at room temperature for 5 minutes. The cells were then heated in antigen retrieval buffer (100 mM Tris, 5% (w/v) urea, pH=9.5) at 95°C for 10 minutes, in order to obtaining optimal performance of certain antibodies. The cells were incubated for 10 minutes in phosphate buffer saline (PBS, Merk, USA) containing 0.1-0.25% TritonX-100 (ICN) for permeabilization. Subsequently, the cells were incubated with 1% bovine serum albumin (BSA, Bio west, USA), 22.52 mg/ml glycine in PBST (PBS+0.1% Tween 20) for 30 minutes to block non-specific binding of the antibodies. The cells were then overnight incubated with diluted Mvh primary antibody (1/100, Abcam13840, UK) in 1% BSA in PBST, using a humidified chamber at 4°. The cells were incubated with the secondary antibody (goat anti-rabbit IgG-PE: sc-3739,1/100, Santacruze, USA) in 1% BSA for 1 hour at room temperature in dark place, followed by incubation with 0.1-1 µg/ml DAPI (DNA stain, Sigma, USA) for 1 minute. Cover slips were mounted with a drop of mounting medium. Finally, the cells were evaluated under an inverted fluorescence microscope (Canada smart, Canada).

Immunohistological examination

After specimen preparation of testis on the slides, they were preserved at room temperature. Slides were washed three times in TPBS (tween PBS) for 5 minutes. It was each time followed by immersing Triton X-100 (0.2% for cytoplasmic antigen) for 20 minutes. Blocking was performed using 10% normal serum with 1% BSA in PBST for 2 hours at room temperature, followed by adding 1% BSA and Mvh primary antibody (1/100) diluted in PBST and overnight incubation at 4°C in the dark. Fluorochrome-labeled secondary antibody (goat anti-rabbit IgG-PE: sc-3739;1/100, Santacruze, USA), diluted in TPBS containing 1% BSA, was applied to the slide and incubated for 1 hour at room temperature in dark. The coverslip was mounted using a compatible mounting medium.

Flow-cytometry analysis

Following differentiation, the cells were fixed before intracellular staining. Fixation was occurred by placing the cells in 0.01% formaldehyde for 10-15 minutes. One hundred microliter of detergent-based permeabilizing agent Triton x100 (0.1-1% in PBS) was added and the cells were incubated in dark at room temperature for 15 minutes. Mvh primary antibody (0.1-10 µg/ml) was added and the cells were incubated for at least 30 minutes at 4°C in dark. The fluorochrome-labeled secondary antibody was diluted in 3% BSA/PBS at the optimal dilution (1:100-1:400) and added to the cells. This was followed by incubation for at least 20-30 minutes at 4°C in the dark. The cells were suspended in ice cold PBS, 3% BSA and 1% sodium azide. Secondary antibody IgG-PE was detected using the FL1 channel of the FACS Calibur TM flow-cytometer (BD Biosciences, USA) and the percentage of positive cells was measured by the FlowJo software.

Statistics

All experiments were independently repeated at least three times. Data are presented as mean ± SD. Statistical analysis was determined using ANOVA, independent t test. All statistical tests were performed using SPSS (Statistical Package for the Social Sciences, version 20, SPSS Inc., USA) software. Flow cytometry data were analyzed using FlowJo 7.6 software. A $P<0.05$ was considered significant.

Results

Morphological evaluation

Figure 1I.A-C shows ESC colonies after 48 hours culture *in vitro*. Colonies were dense with distinct and tight borders, while individual cells were not visible. Colonies did not touch each other. Figure 1.I.D-F shows EB aggregation with consistently round-shapes. Morphological evaluation of these cells during culture in EB+SP group shows that those small and single EB cells were changed into round-shapes.

Single cells in tissue culture plate attached and formed an integrated and fabric building tightly stuck to the bottom of dish. This made trypsinization process very hard (Fig.1II.A´-C´). We also cultured and differentiated EB aggregation on day seven, without dissociating to the single cell (aggregated EB), in tissue culture plate just to look up and compare. After culture of aggregated EB for 7 days in ESC differentiation medium, we saw loose cell-cell adhesions in the colonies and the cells shape was changed to round-shape as well as single EB cells (Fig.1II.D´, E´). In SP group, ESCs colonies were also changed to form round-shaped cells. They were slowly separated from the colony, while clinging tightly to their feeder layer (Fig.1II.F´). Differentiated mESCs on MEFs showed that colonies were merged and lost border integrity (Fig.1II.G´-I´).

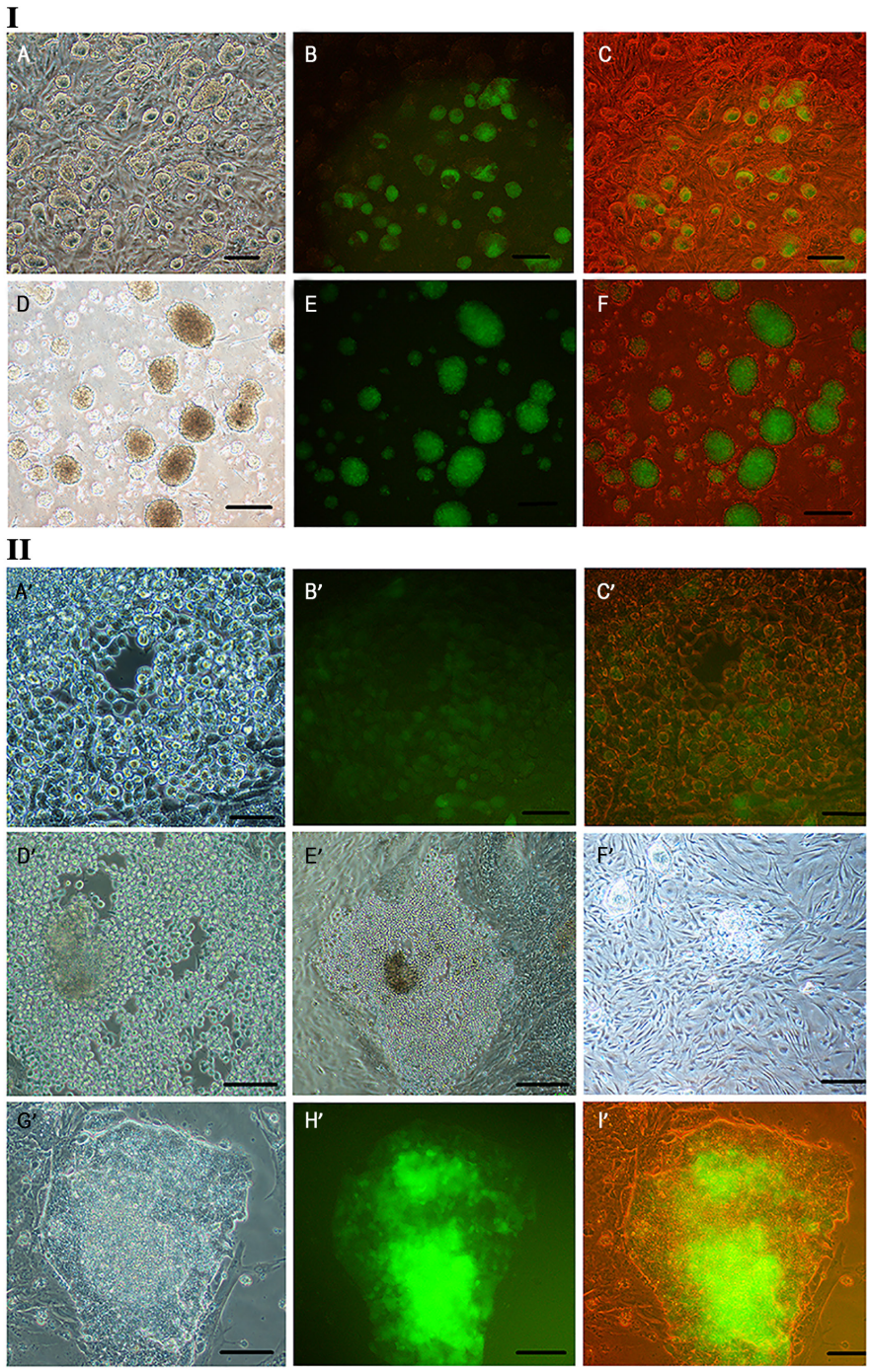

Fig.1: Morphological assessment of Oct4-GFP embryonic stems cell (ESC) colonies, at day 7th of embryoid body (EB) culture (EB 7). **I: A.** Bright-field image of Oct4-GFP ESC colonies growing on an embryonic fibroblast feeder layer, **B.** Fluorescent image of Oct4-GFP ESC colonies show Oct4 expression with green color, **C.** Merged fluorescent and bright-field images of Oct4-GFP ESCs, **D.** Bright-field image of EB colonies after 7 days suspension culture in bacterial plate, **E.** Fluorescent image of EB aggregates show Oct4 expression in green, and **F.** Merged fluorescent and bright-field images of EB aggregates (A-C: ×10), (D-F: ×4) (scale bar: 100 µm). **II. A'.** Bright-field image of germ cell like cells (GCLCs) after 7 days, without feeder cells in (EB+SP) group, **B'.** Fluorescent image of Oct4-GFP GCLCs show Oct4 expression with green color, **C'.** Merged fluorescent and bright-field image, **D', E'.** Bright-field image of differentiated cells after culture of EB aggregates for 7 days, without feeder cells in ESC differentiation medium, **F'.** Bright-field image of ESCs colony during differentiation in SP group, **G'.** Bright-field image of differentiated cells after 14 days culture of singled ESCs in SP group, **H'.** Fluorescent image of section G shows Oct4 expression in green, and **I'.** Merged fluorescent and bright-field images of section G (A´-C´, F´-H´, I´: ×20), (D´: ×10 and E´: ×4) (scale bar: A´-D´, F´: 10 µm, E´, G´-I´: 100 µm).

Expression of germ cell-specific genes

The expression levels of four GC-related genes (*Fkbp6, Mov10l1, 4930432K21Rik* and *Tex13*) as well as *Oct4, Mvh, scp3, stra8* and *HPRT* was determined by qRT-PCR. These findings were confirmed by determining their expression in mouse brain (as a negative control) and testis (as a positive control) somatic tissues. The expression levels of above GC markers were compared in the two study groups: i. SP and ii. EB+SP. Gene expression levels between different groups indicated some variations. qRT-PCR showed that in the both groups, expression of *Mov10l1* was down-regulated and there was no significant difference between them (P=0.3). *Tex13* was up-regulated in both groups, but there was no significant difference between them (P=0.3). *Riken* was up-regulated in both groups and this elevation was significantly higher in SP group compared to EB+SP (P=0.04). *Fkbp6* was down-regulated in EB+SP and up-regulated in SP groups with no significant difference between them (P=0.1, Fig.2).

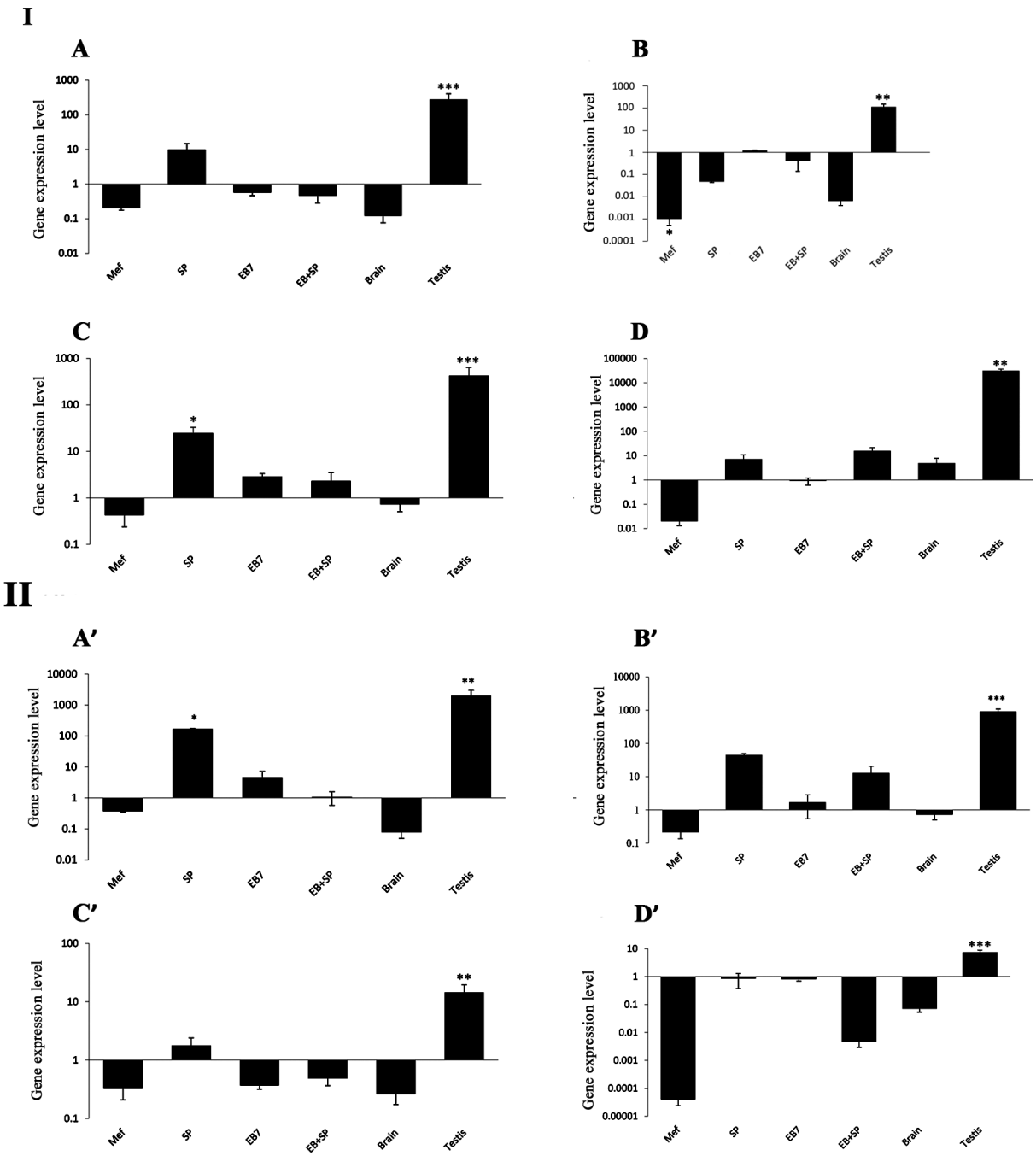

Fig.2: Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) in embryonic stems cell (ESC)-derived cells of study groups. **I:** Gene expression level of specific germ cell markers (**A.** *Fkbp6*, **B.** *Mov10l1*, **C.** *4930432K21Rik*, and **D.** *Tex13*) in ESC-derived cells of mouse embryonic fibroblast (MEF), SP, day 7 of embryoid body (EB) culture (EB7), spontaneous differentiation after EB formation (EB+SP), brain as negative control and testis as positive control compared to ESCs. **II:** Gene expression level of **A'.** *Mvh*, **B'.** *Scp3*, **C'.** *Stra8*, and **D'.** *Oct4* in ESC-derived cells of MEF, SP, day 7 of EB culture (EB7), spontaneous differentiation after EB formation (EB+SP), brain as negative control and testis as positive control compared to ESCs. Values are mean ± SD. *; P<0.05, **; P<0.01, ***; P<0.001. The amount of the undifferentiated mESC is normalized to 1.

Vasa and *Scp3* were up-regulated in both groups, while it was increased with significant difference in SP group, compared to EB+SP (P=0.00 and P=0.01, respectively). Additionally *Oct4* in both groups and *Stra8* in EB+SP group were decreased, while no significant difference was observed between them (P=0.1 and P=0.1, respectively). *Oct4* level was down-regulated in all study groups, compared to ESCs (P<0.05, Fig.3A).

Four GC-specific genes *(Fkbp6, Mov10l1, 4930432K21Rik,* and *Tex13*) were analyzed in differentiated cells. All GC-specific genes (except *Mov10l1*) were expressed at moderate levels in SP group and they had no or low expression level in EB+SP groups. *Fkbp6, Mov10l1, Tex13* and *4930432K21Rik* were expressed at moderate-to-high levels in adult testis. In addition, *Fkbp6, Mov10l1, 4930432K21Rik* and *Tex13* exhibited very low or no expression in brain tissues (Fig.3B).

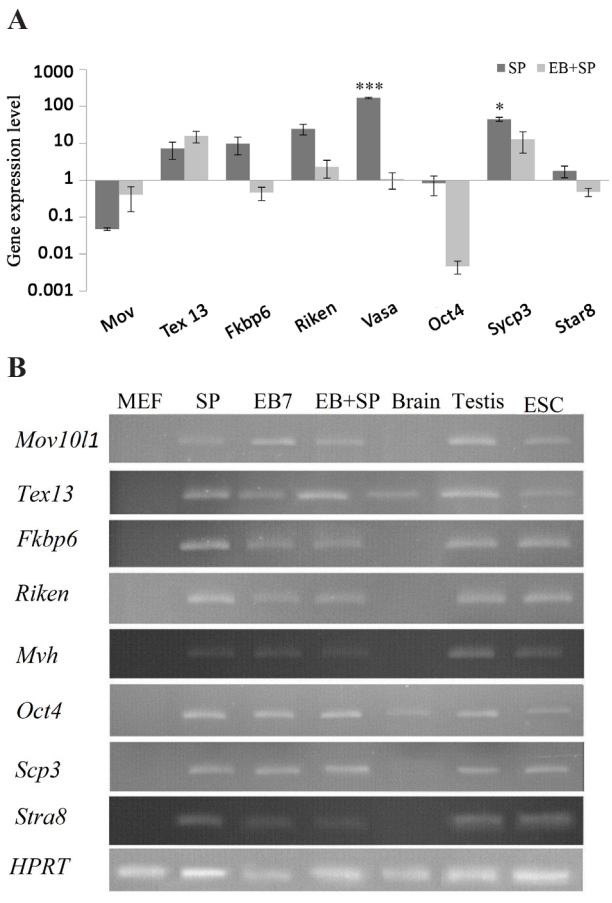

Fig.3: Comparison of meiotic marker gene expression levels. **A.** Graph shows expression level of *Fkbp6, Mov10l1, 4930432K21Rik, Tex13, Mvh, Scp3,* and *Stra8* in SP and embryoid body (EB) EB+SP groups. The amount of the undifferentiated mouse embryonic stems cell (ESC) mESC is normalized to1 and **B.** Graph shows expression of germ-cell genes during ESCs differentiation. RNA was isolated from mouse embryonic fibroblast (MEF), SP, EB7, EB+SP cells, brain and adult testis tissues as well as ESCs, for Quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Values are mean ± SD. *; P<0.05 and ***; P<0.001.

It was found that *4930432K21Rik* was expressed in higher level than other genes in SP group. It was approximately 617-fold higher than that of *Mov10L1*, 3.4- fold higher than that of *Tex13* and 2.4-fold higher than that of *Fkbp6* in SP group. However, in EB+SP group, *Tex13* was expressed in higher level than other genes. It was approximately 39.2-fold higher than that of *Mov10L1*, 6.8- fold higher than that of *4930432K21Rik*, and 39.2-fold higher than that of *Fkbp6*.

Immunostaning

To show the expression of Mvh (Vasa, Ddx4) protein, as a GC marker, in differentiated cells and testis tissue (positive control), primary and secondary antibodies staining was performed. Fluorescent microscope analysis showed positive red color due to the expression of Mvh protein. The nucleus of defined GCLC round cells were counterstained with DAPI. We observed these round-shaped cells were red, indicating expression of Vasa protein. This coloration in the cells of SP group was greater than that of EB+SP group (Fig.4).

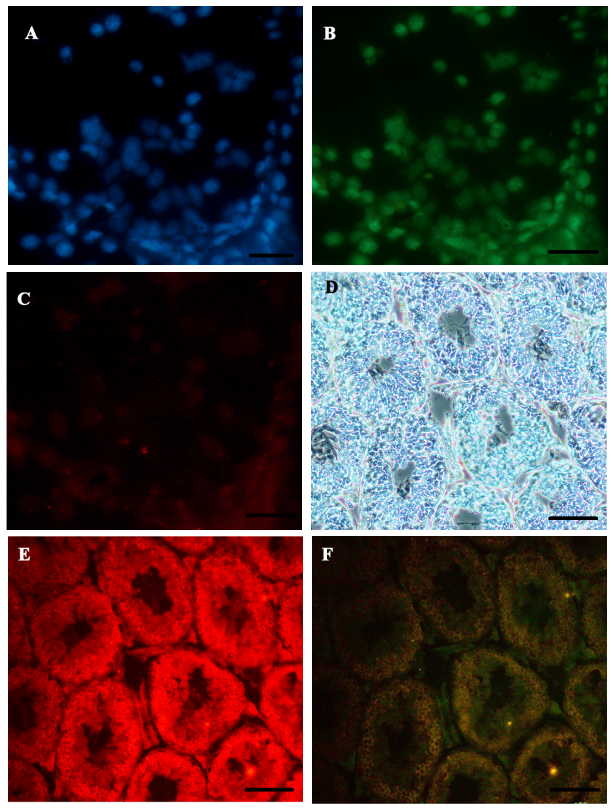

Fig.4: Cytoplasmic protein expression analysis of Mvh in embryonic stems cell (ESC)-derived differentiated cells and mouse adult testis, using immunofluorescent staining. **A.** Image shows that nuclei were stained with DAPI (in blue), **B.** Oct4-GFP expression (in green), **C.** Anti-Mvh antibody as a germ cell (GC) marker (in red) (scale bar: 10 µm), **D.** Image shows section of mouse adult testis in bright-field, **E.** Anti-Mvh antibody as a GC marker (in red), and **F.** When the light of the microscope is off and the fluorescent light is on (in dark) (scale bar: 100 µm).

Flowcytometry

Since qRT-PCR showed that the expression of GC markers were enhanced in differentiated cells, we investigated the protein expression of Mvh by flow-cytometer. Mean fluorescent intensity (MFI) of the cells showed significantly higher Mvh positive cells in the SP group (87.2 ± 2.61) compared to undifferentiated ESC (71 ± 3.02) and EB+SP (75.74 ± 3.90) groups (P=0.00 and P=0.01, respectively). However, compared to EB7 group (82 ± 2.61), Mvh increased MFI of the cells was not significantly different (P=0.3, Fig.5).

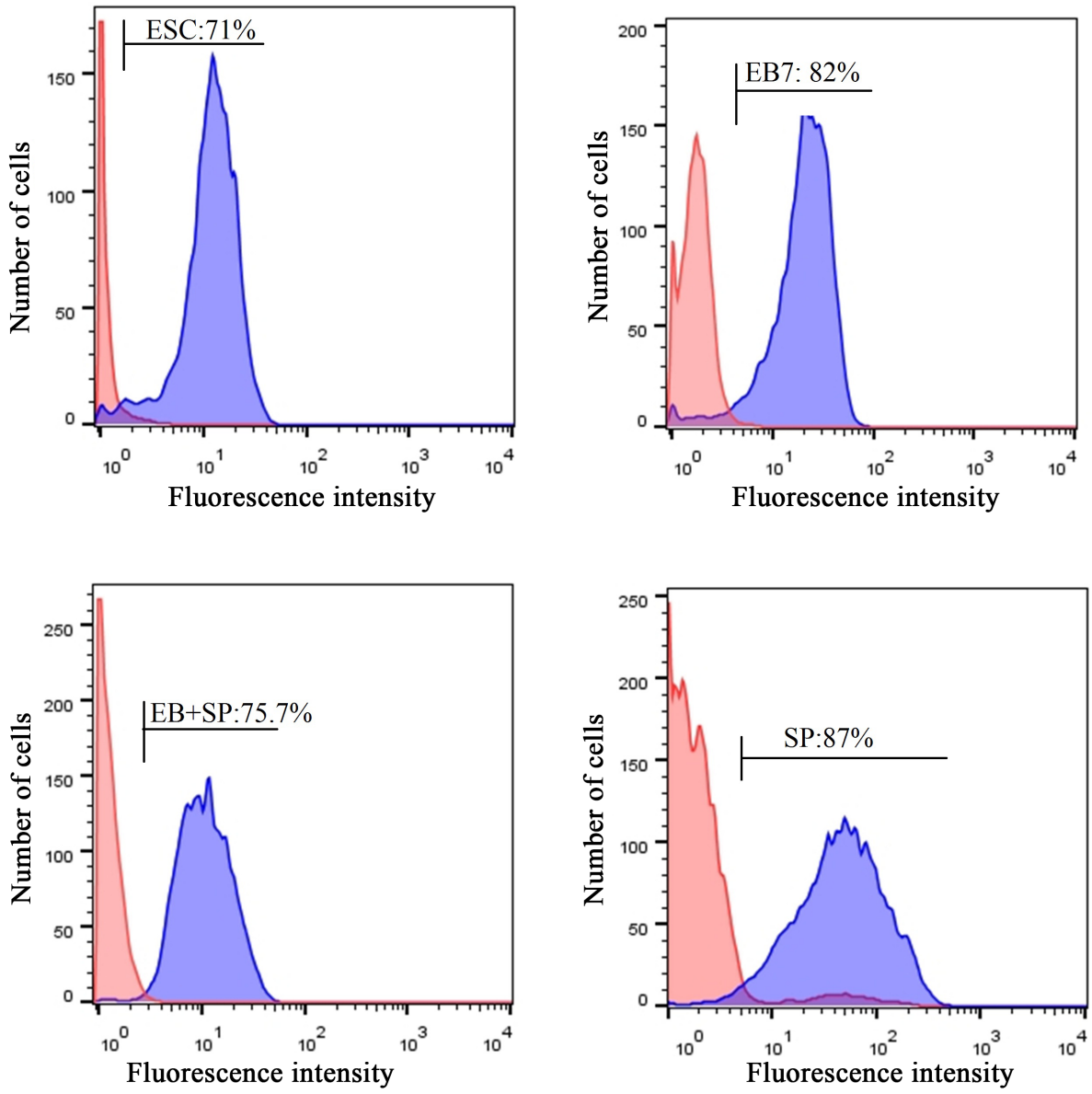

Fig.5: Flow-cytometer analysis for the expression level of Mvh in study groups. Expression level in undifferentiated embryonic stems cell (ESC), spontaneous differentiation of ESCs in monolayer culture (SP), day 7 of embryoid body (EB) culture (EB7), spontaneous differentiation of ESCs in EB culture method (EB+SP).

Discussion

In this study, morphological evaluation showed that stem cell colony formed round-shaped cells, tightly sticking to the bottom of dish, as with Nagai et al. (25) report indicating that PGCs have round-oval shapes with large size and large nucleus. In this study, GC specific genes (*Fkbp6*, *Mov10l1*, *4930432K21Rik* and *Tex13*) displayed different levels of gene expression in GCLCs (both groups), somatic tissues and ESCs. Expression of specific GC markers, with no or low level in ESCs, can appropriately facilitate recognition of differentiated cells *in vitro*. This study showed that expression of the GC genes in SP group (monolayer) were more than EB+SP (EB system). In terms of gene expression patterns, monolayer culture condition was suggested to be superior to the EB culture system (26). Monolayer culture system was also suggested as a more appropriate protocol to promote female GC (23) and neural differentiations (8). On the other hand, Talaei-Khozani et al. (27) showed that in spontaneous differentiation, when no growth factors were used, the expression of meiosis markers in EB method were greater than monolayer culture system. The expression of *Mov10l1* gene was decreased in SP and EB+SP groups. There was no significant difference between expression levels of these two groups. Expression of this gene was increased in the leptotene and zygotene stages of meiosis and decreased at the end of meiosis (28). After 21 postnatal days (dpp), this gene transcription frequency was continuously reduced, coinciding with the emergence of the first generation of rounded spermatozoa (29).

Results of this study showed that *Tex13* was expressed in GCLCs of SP and EB+SP groups. This gene was expressed in the embryo of 12.5 dpp male mice, as well as the testicular tissue and sperm cells (17). *Tex13* is an X-

link gene expressed in the early stages of spermatocyte, during the leptotene and zygotene stages of meiosis (28). However, it appears to undergo translational suppression before late meiosis. Our results showed that *Fkbp6* was expressed in GCLCs of SP, but not EB+SP group. Mouse *Fkbp6* is not involved in the initiation of synapsis. It plays role in monitoring progression and/or maintaining synapsis between homologous pairs (30). However, deficient Fkbp6 male mice were completely sterile and they had abnormal pachytene spermatocytes which failed to proceed beyond the pachytene stage (31).

In this study, *4930432K21Rik* expression level was elevated in both groups, while the level of expression was not significantly different compared to EB+SP group. It is worthy to note that expression of this gene was higher than the other three above genes. *4930432K21Rik* is a new gene with unknown function expressed in PGCs and gametes of mouse embryos (17). Expression level of *Mvh*, as a sex-linked gene, in SP cells was significantly increased in comparison with EB+SP cells. The expression of this gene was increased with the onset of meiosis and remained high up to the end of spermatogenesis (28). Absence of *Mvh* results in the arrest of zygotene stage in male gametes (32). Analysis of Mvh expression at the protein level, using flow-cytometer, showed a greater increase in the SP group rather than other groups, confirming the findings obtained from RT-PCR. Mvh is a cytoplasmic protein and product of the Vasa homolog gene, induced by somatic cell of the genital crest, and it remains until formation of the post meiotic GCs. Hence, mutation in this gene leads to defect in proliferation and differentiation of PGCs (33).

The current study demonstrated down-regulation of *Oct4* in all of the studied groups, compared to ESCs. *Oct4* is a well-known factor, which plays major role in pluripotency maintenance of ESCs. *Oct4* begin to show high expression level in the inner cell mass, but its level is decreased as the cells enter to epiblast stag. Oct4 expression level of GCs *in vivo* is high until E13.5. Then, it is decreased in the zygote/pachytene stages of first meiosis around E16.5 (34). In this study, expression level of Sycp3 was obviously higher in the SP, rather than other groups. This gene is essential for synaptoneal complexes, chromosomal synapse and male fertility (35). The chromosomes lacking this gene are not able to form synaptic complexes (34). This event caused Sycp3$^{-/-}$ mice to stop development at zygote stage (36). Our findings showed that expression of *Stra8* gene was slightly increased in the SP group and decreased in the EB+SP group. Male and female mice that do not express *Stra8* are infertile (37). Expression of this gene is initially determined in immature testis as well as GCs with mitotic activity after birth, and then its expression is increased in undifferentiated GCs of adult testis (38).

Our result showed that after terminating culture, mESCs were differentiated and changed into the round-shape cells with relatively large and distinct nuclei. In this study, some GC gene expression and meiosis marker were increased in both groups. The level of GCs-specific gene expressions in SP group was higher than EB+SP group. *Oct4* and *Mov10l1* were down-regulated and *Tex13, Riken*, *Fkbp6*, *Vasa*, *Stra8* and *Scp3* were up-regulated in the SP group after differentiation. Furthermore, expression levels of Mov10l1, *Fkbp6*, *Oct4* and *Stra8* were down-regulated in EB+SP group, while *Tex13*, *Riken*, *Vasa* and *Sycp3* were up-regulated. On the other hand, findings obtained from flow-cytometry indicated that SP group cells had more MFI in Vasa protein than EB+SP group cells.

Conclusion

Oct4 down-regulation, as a pluripotency factor, and expression of meiosis markers indicated that ESCs were successfully differentiated into GCLCs in both groups. Evaluation of gene expression patterns in both groups demonstrated that monolayer culture was more efficient to produce GCLCs, in compression with EB methods. Further recruitment of culture conditions and optimization will still be needed for successful and high quality GCs differentiation *in vitro*.

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Authors' Contributions

M.Gh.T.; Designed and performed the experiments, analyzed data and contribute to writing the manuscript. S.Gh.A.J.; Supervised the research, designed experiments and coordinate in writing the manuscript. M.Gh.-H.; Designed the experiments and performed transporter experiments. A.A.A., M.Gh.; Helped in the implementation of the experiments, cell culture and the process of genes expression. All authors read and approved the final manuscript.

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Original Article

Impact of Metformin and Pioglitazone on Serum Level of Tumor Necrosis Factor-Alpha and Lipid Profiles during Implantation Window in Diabetic Rats

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Abstract

Background: The present study was designed to evaluate serum lipid profile and tumor necrosis factor-alpha (TNF-α) level in diabetic rats at implantation time. Type 2 diabetes mellitus (T2DM) could affect various systems, including innate immune system and it causes chronic low-grade inflammation, increasing level of TNF-α. Furthermore, T2DM is often accompanied by impaired lipid profile. Metformin and pioglitazone are used as the first and second lines of treatment for T2DM.

Materials and Methods: In this experimental study, 35 adult virgin female wistar rats, weighting 175-225 g, were randomly categorized into five groups: i. Control, ii. Sham, iii. Nicotinamide (NA)+streptozotocin (STZ) induced T2DM, iv. Diabetic+pioglitazone (20 mg/kg/day for 28 days oral administration), and v. Diabetic+metformin (100 mg/kg/day for 28 days oral administration). At the time of implantation, TNF-α level in serum of rats was measured by ELISA kit. Glucose was measured using photometric method and lipid profiles were calculated by enzymatic methods.

Results: Level of TNF-α in the diabetic group was significantly higher than other groups ($P<0.001$). In metformin treated group, TNF-α serum level was also significantly higher than pioglitazone treated group ($P<0.001$). Fasting blood sugar (FBS) and lipid profiles were significantly higher in diabetic group.

Conclusion: Metformin and pioglitazone have similar effects on glucose, lipid profiles and TNF-α serum levels. Among these drugs, pioglitazone has more efficient influence on TNF-α serum level, in comparison with metformin.

Keywords: Diabetes Mellitus, Embryo Implantation, Metformin, Pioglitazone, Tumor Necrosis Factor-Alpha

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Introduction

Type 2 diabetes mellitus (T2DM), especially while it is not well controlled, can affect various systems including the innate immune system, and cause chronic low-grade inflammation in the body (1, 2). In addition, diabetes also affects the functions of female reproductive system and occurrence of subfertility (3) or fetal loss after implantation in diabetic women is more than healthy individuals (4). Different functions of female reproductive system, such as the menstruation, pregnancy, ovulation and implantation, are affected by several hormones and various inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and IL-1 (5).

Studies suggest that hormone-based disorders and high levels of inflammatory cytokines can lead to interruptions in the immune-endocrine cross talk within endometrium, myometrium and blastocyst that could interferes trophoblast and decidua interaction during pregnancy (6, 7). Furthermore, increased TNF-α production is related to infertility and recurrent spontaneous abortion, but the issue is open to further discuss (8). Localized inflammation improves the implantation outcomes and it has positive relationship with cytokine expressions, such as TNF-α, in endometrial biopsies (9, 10). Therefore, optimal expression of TNF-α could be useful during pre-implantation and implantation periods (11).

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Up to now, several drugs are available for the treatment of T2DM, through which, biguanides (metformin) and thiazolidinedione (pioglitazone) were used in this study. Metformin is used as the first line treatment to reduce serum level of glucose in diabetic patients (12). It has been reported that the aforementioned drug plays a crucial role in modulating inflammatory cytokine levels and lipid profile (13, 14).

Pioglitazone is a member of the thiazolidinedione family which binds to the peroxisome proliferator-activated receptor gamma (PPAR-γ), increasing insulin sensitivity, improving lipid profile in serum and regulating blood pressure (15). Pioglitazone also reduces TNF-α level in serum (16).

Therefore, the aim of this study was to determine TNF-α levels and lipid profile in serum of diabetic rat models after treatment with metformin and pioglitazone during embryo implantation window period.

Materials and Methods

Animal maintenance

This study was an experimental study on diabetic rat models, conducted at the central laboratory of Isfahan University of Medical Sciences (Isfahan, Iran) in 2018. All experimental procedures were approved by Isfahan University of Medical Sciences Animal Ethical Committee (code number IR.MUI. REC.1394.1.184.). Adult virgin female Wistar rats, weighting 175-225 g and aged 6-8 weeks, were purchased from Pasteur Institute of Iran (Tehran, Iran), maintained in conventional wire mesh cages at room temperature regulated at 21 ± 1°C, humidity 45-50%, and 12 hours light/dark cycle, while they were accessed to standard dry pellets and water.

Induction of diabetes

Nicotinamide (NA) and streptozotocin (STZ, both from Sigma-Aldrich, Germany) were used to induce T2DM in animals. First, NA with 200-230 mg/kg dose was injected intraperitoneally (IP). After 15 minutes, STZ was IP injected with dose of 60 mg/kg (17). Three days after T2DM induction, blood samples were taken to measure blood glucose in animals using a glucometer (HemoCue Glucose 201+, Sweden). If fasting blood sugar (FBS) level was higher than 250 mg/dl, it was considered as diabetic rat (18).

Study design and serum collection

Thirty-five rats were randomly divided into five groups as follows, existing seven rats in each group. Control group, sham group that received just normal saline using IP injection, STZ+NA-induced diabetic group without any treatment (FBS ≥250 mg/dl), diabetic groups which received pioglitazone 20 mg/kg/day for 28 days by orogastric gavage (19) and the last group was diabetic rats which received metformin 100 mg/kg/day for 28 days by orogastric gavage (20).

Animals were maintained in diabetic condition for four weeks and then drug therapy was started for the next 4 weeks as shown in Figure 1. FBS levels were measured every 4 days by glucometer (HemoCue Glucose 201+, Sweden) and droplet samples were collected from dorsal vein of tail.

In the 4th week, twenty-four days after administration of metformin or pioglitazone, two females and one male rat, in all groups, were placed in the separate cages for mating. The next day, the female rats were checked for the presence or absence of vaginal plugs. Presence of the vaginal plug revealed the first day of pregnancy and the time of implantation window was considered 4 days after that (21), which means 28th day after beginning of the treatment. Four weeks after treatment with metformin or pioglitazone, at the time of blastocyst implantation in rats, animals were fasted overnight and sacrificed by injection of ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (7 mg/ kg) (IP). Blood samples were taken by cardiac puncture then placed in tubes at room temperature for 30 minutes and allowed to get clotted. Aforementioned samples were centrifuged at 3000 rpm for 10 minutes. Serum was removed and stored at -20 ºC until biochemical analysis.

Determination of TNF-α, lipid profile and glucose

Serum level of TNF-α was measured by enzyme-linked immunosorbent assay (ELISA) using readymade kit reagents supplied by Eastbiopharm, China (22). Serum glucose level was measured using photometric method and lipid profiles, including triglyceride (TG), cholesterol (Chol), low density lipoprotein (LDL) and high density lipoprotein (HDL) were calculated by enzymatic methods. Measurements were performed by 14000 auto-analyzer (Toshiba, Japan) using manual colorimetric method (23).

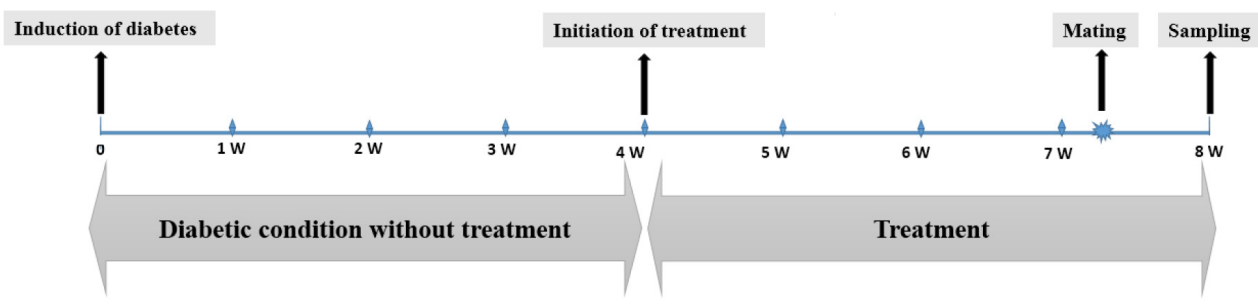

Fig.1: The study was designed for 8 weeks, at the end of week 4, after induction of diabetes, administration of drugs were started. Mating was occurred at the 8th week and sampling was performed 4 days later.

Statistical analysis

Statistical analyses were performed using SPSS version 20.0 (SPSS Inc., USA). The differences were compared using one-way analysis of variance (ANOVA), following Tukey post hoc test for TNF-α and lipid profiles in all groups. $P<0.05$ was statistically considered significant difference.

Results

Pioglitazone and metformin decreased glucose level in the treated rats

Glucose levels were measured every 4 days, in all groups, until 28th day. As Figure 2 illustrates, both of pioglitazone and metformin regulated blood glucose level after 8 days administration. On the twelfth day, blood glucose reached to normal level and there was no significant difference between normal control group compared to pioglitazone treated group (P=0.103) and metformin treated group (P=0.105).

Serum glucose levels were significantly higher (P=0.000) in the diabetic group than others control and treatment groups (Fig.3). As shown in Figure 3, metformin and pioglitazone reduced blood glucose levels; so that, there was no significant difference in blood glucose levels between the treated groups compared to normal control group (P=0.363 and P=0.410, respectively). Furthermore, there was no significant difference in blood glucose levels between these two treated groups (P=0.910).

Pioglitazone and metformin decreased lipid profiles

Based on our statistical analysis performed on lipid profiles at the end of study, the diabetic group had higher serum TG (P=0.000), Chol (P=0.000), HDL (P=0.000) and LDL (P=0.000) levels than the control, sham and treated groups (Fig.4). There was no considerable difference in lipid profiles, including TG (P=0.643), Chol (P=0.597), HDL (P=0.571), LDL (P=0.281), between two treated groups.

Increased TNF-α in the diabetic group and decreased TNF-α after treatment

Measurement of TNF-α showed that there was no significant difference between sham and control groups (P=0.335). In addition, there is no significant difference between pioglitazone and sham treated groups (P=0.075). However, our results revealed a meaningful difference between pioglitazone and the control group (P=0.008), because TNF-α level in sham group is higher than control, despite not being statistically significant (P=0.335). Based on the ELISA outcomes of TNF-α levels, there was a significant difference between the diabetic group and all of the other studied groups (Fig.5). Moreover, there was a significant difference between the two treated groups with pioglitazone and metformin (P=0.000), and the level of TNF-α in the pioglitazone treated group was significantly lower than the metformin treated group.

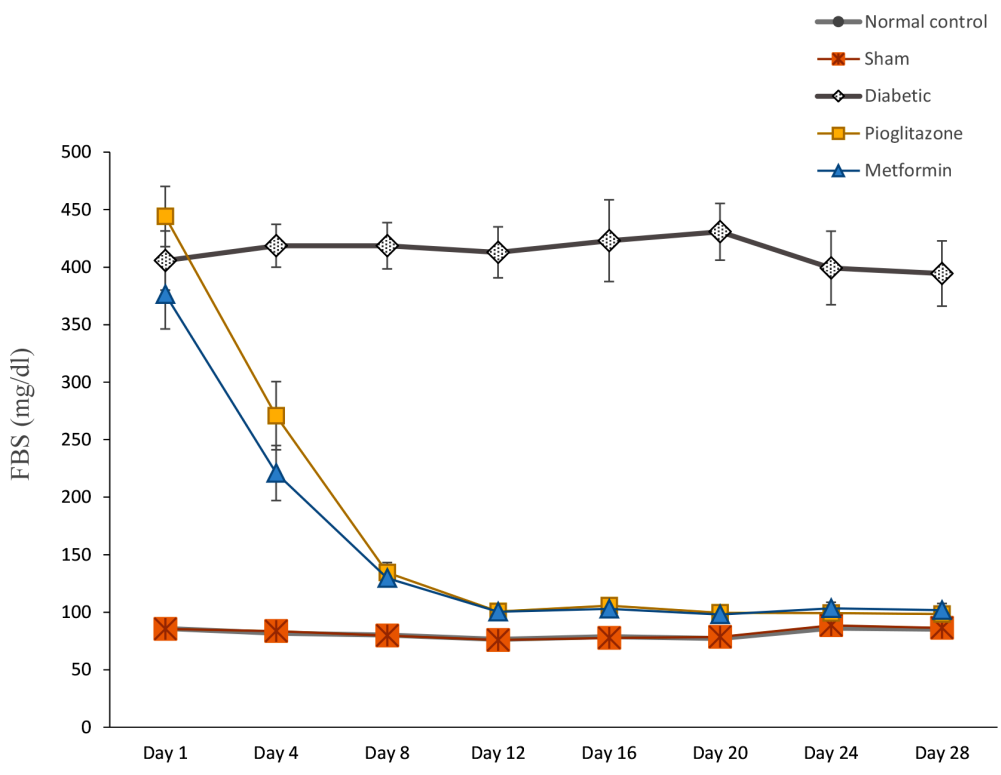

Fig.2: Effects of pioglitazone and metformin on blood glucose level compared to STZ+NA induced diabetic rat models during 4 weeks treatment. FBS was measured every 4 days, during 4 weeks treatment. All values were presented as mean ± standard error mean (mean ± SEM) and there are seven rats in each group. STZ; Streptozotocin, NA; Nicotinamide, and FBS; Fasting blood sugar.

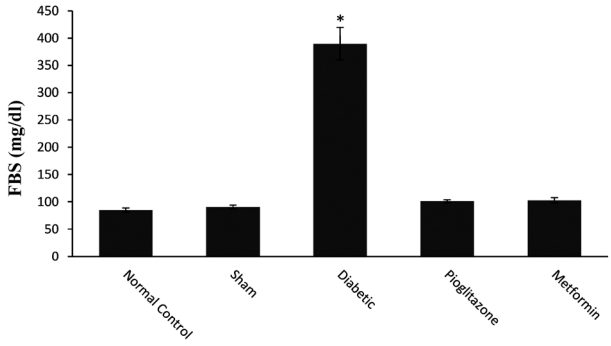

Fig.3: Effects of pioglitazone and metformin, at the time of blastocyst implantation on serum glucose level of rats, compared to STZ+NA induced diabetic rat models at the 28th day of treatment. All values are presented as mean ± SEM. Significant differences in FBS level between diabetic group and all of the other groups were observed (P<0.001). *; Shows significant difference, STZ; Streptozotocin, NA; Nicotinamide, and FBS; Fasting blood sugar.

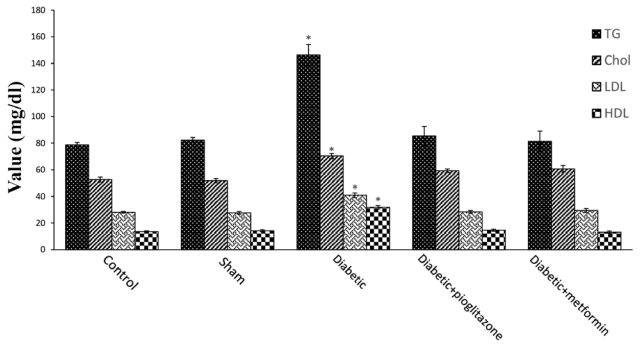

Fig.4: Effect of pioglitazone and metformin, at the time of blastocyst implantation, on lipid profiles in serum of diabetic rats in all five groups. Data are represented as mean ± SEM. *; P≤0.05 vs. control. Lipid profile levels include: TG, Chol, LDL and HDL, measured in 28th day after initiation of treatment. *; Shows significant difference in lipid profile level between diabetic group and all of the other groups (P<0.001), TG; Triglyceride, Chol; Cholesterol, HDL; High density lipoprotein, and LDL; Low density lipoprotein.

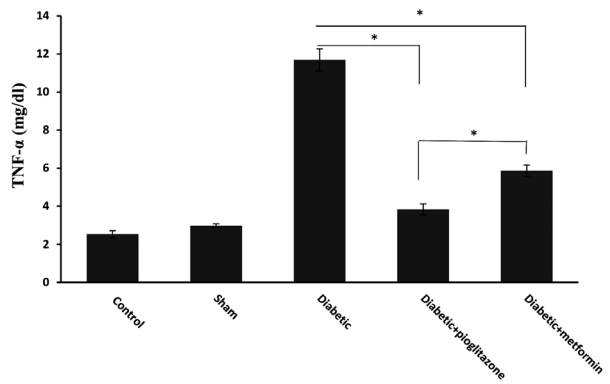

Fig.5: TNF-α serum concentration of T2DM rats treated with metformin and pioglitazone, 4 weeks after treatment. Pioglitazone and metformin significantly decreased TNF-α level compared to diabetic group (P<0.001). A significant difference between treated pioglitazone and metformin was observed (P<0.001). *; Shows significant difference in TNF-α serum levels, TNF-α; Tumor necrosis factor-alpha, and T2DM; Type 2 diabetes mellitus.

Discussion

Diabetes mellitus, as a long term metabolic perturbation, could lead to reduction of life quality in the affected population as well as increases morbidity, mortality and complications in patients (24). Statistics indicated that global outbreak of the diabetes mellitus is increasing and is going to be a serious problem in the health care debates around the world (25).

According to our study, at the time of rat blastocyst implantation, day 4 of post-coitum, serum glucose level in diabetic group was higher and as expected, significantly different from all other groups. In addition, significant differences were again observed in lipid profile between diabetic group and the other four groups. After 28 days treatment by metformin and pioglitazone, serum glucose level was normalized in the treated groups. Along with the improvement of blood glucose levels, both drugs had an appropriate impression on lipid profiles (i.e. the levels of TG, Chol, LDL and HDL). Our study revealed that TG, Chol, LDL and HDL levels were significantly raised in the diabetic group. Regarding the literature reviews, HDL should be reduced in T2DM (26-28).

Considering the impression of changes in female hormones, especially estrogen, through pregnancy -from implantation to child birth- HDL level was increased (29-31). On the other hand, in early stage of T2DM, HDL serum level is significantly higher than control group (32, 33). Lawrence et al. (26) performed a study on diabetic patients and compared the effect of metformin, pioglitazone and gliclazide on lipid profile. They concluded that there is no significant difference in lipid profile before and after administration of these drugs. With regards to administration of pioglitazone in diabetic patients, Aghamohammadzadeh et al. (28) mentioned that pioglitazone down-regulates FBS, hemoglobin A1C (HbA1c) and TG levels significantly, but no significant reduction was observed in cholesterol, LDL and HDL levels. These results were not in accordance with our findings. It seems that performing these human studies, without considering life styles, were probably the cause of controversy in variation of lipid profile outcomes. Exercise, individual diet and BMI in each patient could lead to bias in the study. On the other hand in animal experiments, the confounding factors, such as age, sex, exercise and dietary programs, weight, circadian cycle and etc., were taken under precise controlled condition (34).

The other objective, in our study, was to compare the effects of metformin and pioglitazone on the serum level of TNF-α, as a member of pre-inflammatory cytokine family. T2DM, as an inflammatory condition, could elevate various inflammatory serum cytokines, such as TNF-α, which increase the subsequent complications of this disease (7). The pathogenesis of 10-20% of infertile cases is related to higher level of serum immunological factors, compared to fertile individuals (35). IL-2 and TNF-α cytokine increases could have negative impression on successful pregnancy. When these two cytokines were injected into pregnant mice, the carriages were terminated (36). However, cytokines also exert beneficial effects on pregnancy, including resistance to intrauterine infections and important roles in angiogenesis and tissue regeneration (37). Therefore, to achieve normal and healthy pregnancy, appropriate and adequate presence of pre-inflammatory

cytokines in the endometrium has high necessity.

In our present study, level of TNF-α was raised in the serum of diabetic rat model at the time of implantation window, while this level was reduced in rats treated with any of both drugs. Interestingly, our study revealed that the influence of pioglitazone on TNF-α level was significantly more efficient than metformin.

Pioglitazone can reduce serum TNF-α level by several mechanisms, including inhibition of TNF-α production from macrophages (38), suppression of TNF-α mRNA expression from subcutaneous adipose tissue (16), reduction of the number of CD3+ T lymphocytes in diabetic rats, producing higher levels of TNF-α and IL-1β (39).

Embryo implantation is a multifactorial phenomenon which involves precise molecular programming. Accurate duration of existence and efficient levels of the molecular elements depends on healthy and normal metabolism. Diabetes mellitus, as one of the most common metabolic disorders, makes deep disturbances in the levels of molecular production and the period of their presence (40). Therefore, there is an important necessity to pay attention to underlying metabolic diseases and their effects on the fate of natural and assisted pregnancies.

Conclusion

In this study, at the time of implantation window (i.e. blastocyst-endometrium dialog period), T2DM increases glucose and lipid profile as well as the serum level of TNF-α. Regulation of these parameters was observed after administration of each of metformin and pioglitazone. Additionally, our study reveals that pioglitazone has significantly more efficient influence on TNF-α serum level in comparison with metformin.

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Authors' Contributions

R.A., P.N. A.B.; Participated in study design, data collection and evaluation, drafting and statistical analysis. N.E.; Performed ELISA method. S.S.A; Helped in sample collection. F.S.M., M.M.; Contributed in interpretation of data and conclusion. All authors performed editing and approving the final version of this manuscript for submission, in addition to participation in preparation of the final draft.

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Original Article

The Effect of Diazinon on Cell Proliferation and Apoptosis in Testicular Tissue of Rats and The Protective Effect of Vitamin E

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Abstract

Background: Diazinon (DZN) is an organophosphate pesticide, and nowadays this pesticide is mostly used in agriculture. In this study, we analyzed the effects of DZN and vitamin E (Vit E) on apoptosis and the proliferation of germ cells in rat testis.

Materials and Methods: In this experimental study, 30 male Wistar rats were divided into five groups (n=6 per group) consisting of control, sham (received olive oil), experimental group i (60 mg/kg DZN), experimental group ii (60 mg/kg DZN and 200 mg/kg Vit E), and experimental group iii (200 mg/kg Vit E). After six weeks, left testis of rats was removed for the detection of proliferative cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase end-labeling (TUNEL).

Results: Compared with the control group, DZN in the experimental group i decreased the number of PCNA-positive cells and increased the number of TUNEL-positive cells ($P<0.001$). Vit E improved detrimental changes by the decrease in the rate of apoptosis and the increase in the proliferation of testicular germ cells ($P<0.001$).

Conclusion: Vit E can decrease the number of TUNEL-positive cells and increase the number of PCNA-positive cells by the neutralization of the toxicity caused by DZN in the testicular tissue.

Keywords: Apoptosis, Diazinon, Proliferation, Testis, Vitamin E

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Introduction

Pesticides are widely used in agricultural production to prevent or control pests, diseases, weeds, and other plant pathogens in an effort to reduce or eliminate yield losses and maintain high product quality. Despite their popularity and extensive use, pesticides have raised serious concerns about human health arising from the exposure of farmers when mixing and applying pesticides or working in treated fields and from residues on food and in drinking water for the general population. The wide usage of pesticides in agriculture and general hygiene causes serious problems in ecosystem and hygiene risks including acute, sub-acute, and chronic human and animal poisoning and therefore it is an important concern (1, 2).

Because of the non-polar and lipophilic structure of organophosphates, they are absorbed quickly after eating or breathing, and after the absorption, they aggregate in adipose tissues, kidney, liver, and salivary glands. The long-term exposure of diazinon (DZN) to the skin can cause severe poisoning. Organophosphates undergo different metabolic reactions, and finally, their metabolites are excreted by urine, faces, and expiration. Some of the poisonous metabolites are accumulated in adipose tissue requiring long time periods to be excreted from the body (3).

DZN is one of the most well-known organophosphate pesticides which is synthetic and not naturally found in the environment. It is more common to be used for paddies, fruit trees, and ectoparasites of the livestock. DZN activity is similar to other organophosphates which are able to block acetylcholinesterase enzyme. These compounds can bind to some enzymes in the human body; however, its effects on acetylcholinesterase blockade has been clinically important (4). DZN causes pathological changes in the human body such as hematological and reproductive disorders, as well as kidney, liver, cardiovascular, and the central nervous system (CNS) toxicity through an elevation in oxidative stress and free radicals (5, 6). The toxicity of organophosphate compounds are

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not confined to inhibit acetylcholine enzyme; rather, they are capable of inducing programmed cell death via internal and external apoptosis pathway (7). Moreover, the induction of apoptosis in various tissues of the body is mediated by the activation of caspase-dependent pathways leading to cell death (8). Spermatogenesis is a process in which immature germ cells are matured to mature cells in testicular tubes (9). According to Dadhich et al. (10) apoptosis and cell proliferation play important roles in controlling the number of testicular cells thereby a hormonally controlled process that precisely regulates the balance between the generation of Sertoli and germ cells. Apoptosis has two main roles in the normal spermatogenesis namely, decreasing the number of germ cells that can be supported by Sertoli cells and the removal of abnormal sperms by which ineffective cells such as old, immature, and damaged cells are omitted (10, 11).

DZN can alter the diameter of seminiferous tubules by loosening the connective tissue and the muscles around them. Moreover, the size of the germinal cells in response to intoxication with DZN changes and becomes smaller than their normal counterparts (12). Vitamin E (Vit E) as a lipid-soluble antioxidant inhibits lipid peroxidation thereby maintaining the integrity of the cell membrane. Regarding a study conducted by John et al. (13) Vit E ameliorated (not blocked) organophosphate-induced oxidative stress by decreasing lipid peroxidation and altering antioxidant defense system in erythrocytes. Although the antioxidant and anti-apoptotic effects of Vit E have been shown in a vast number of studies, no study has been so far performed to study the effect of this vitamin on testicle toxicity caused by DZN. Hence, in this study, the apoptosis and proliferation rate of germ cells in testicles of male rats intoxicated with DZN were examined, and the protective impacts of Vit E administration were assessed.

Materials and Methods

Animals

In this experimental study, thirty adult male Wistar rats weighing 200-250 g were procured from the Animal House of the Medical University of Mashhad. They were housed in the standard situation (six animals per cage) at a temperature of 22 ± 2°C and 12:12 hour light dark cycle. The animals had free access to food and tap water during the experiment. Rats were randomly divided into five groups as follows: i. Control group that received no therapy or drug solvent, ii. Experimental group i that received 60 mg/kg DZN, dissolved in olive oil and intraperitoneally administered, iii. Experimental group ii that received 60 mg/kg DZN along with 200 mg/kg Vit E by intraperitoneal injection and daily gavage, respectively, iv. Experimental group iii that received 200 mg/kg Vit E by daily gavage, and v. Sham group that received pure olive oil as an intraperitoneal injection (IP).

After six weeks, rats were anesthetized, and their testicles were removed for further study. This study was carried out in the Histology Laboratory of the Medical University of Mashhad and approved by the Ethics Committee of Mashhad University of Medical Sciences (no.911096).

Chemicals

DZN 98% was purchased from Ariashimi Company. TUNEL assay kit was procured from Roche, Germany. PCNA kit was purchased from Zymed Company. Vit E (α-tocopherol acetate) was procured from Sigma.

Testicular perfusion protocol

Tissue perfusion is necessary for the drainage the blood from tissues, and it enhances the background and the quality of staining. To do perfusion, first, the animals were anesthetized by ether and their thorax was opened through the surgical procedure. Perfusion was carried out by the injection of rinse and fixative solutions (by 10 or 20 ml syringes) into the left ventricle. During the opening of the thorax, an incision was made in the midline to minimize the damage to the vascular walls. Therefore, the bleeding and rupture of the vessels were reduced. The joints of the heart along with their coatings were separated from the bone. The right atrium was perforated with a needle while another syringe needle was inserted into the left ventricle and rinse solution was injected until drained out from the right atrium hole and become clear. Then, the fixative solution was injected promptly, and this procedure continued until the hindlimbs and forelimbs of the animals became pale and started to shake. After these signs, the left testicle was removed to perform further procedures as shown in Figure 1 (14).

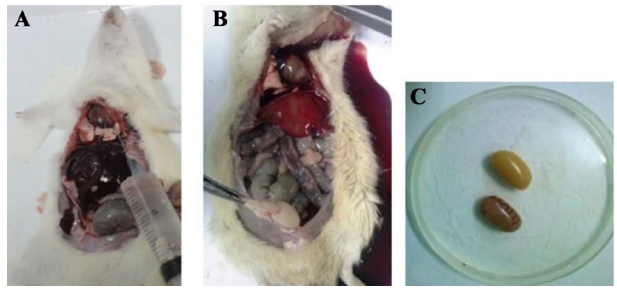

Fig.1: Perfusion phase through the left ventricle. **A.** The Pre-perfusion stage while the gut had a normal appearance, **B.** Post-perfusion characterized by a change in the appearance of internal organs such as intestine, liver, and testicles, showing that perfusion was successful and then the initial fixation happened, and **C.** The testis color changed after the perfusion process.

Sample preparation

To prevent autolysis by lysosomal enzymes and tissue damage by bacteria, as well as to maintain the structural features and the ingredient volume of the tissue, testicles were fixed by 4% paraformaldehyde for 72 hours (considering the size, type of samples, and the fixative quality). After the fixation process, to prepare the tissue for the examination under an optical microscope, the tissue processing was carried out. Finally, the samples were paraffin-embedded. Then, the samples were cut

by a microtome apparatus at 5 µm, and the slides were placed on poly-L-lysine slides. After deparaffinization and hydration by alcohol with descending gradient, samples were examined by PCNA and TUNEL techniques under an optical microscope.

TUNEL assay

The apoptosis rate was evaluated by the TUNEL assay as the staining of apoptotic cells was performed based upon the following protocol (15): samples were deparaffinized with xylol in two steps (5 minutes for each step), placed in ethanol with decreasing gradient as 95, 80, and 70% and washed three times with phosphate-buffered salin (PBS). After washing the slides with PBS three times, the slides were incubated with 50 µL protein kinase K and rinsed three times by PBS. Then, the slides were incubated with TUNEL reaction mixture for 24 hours at 4°C. Samples were then incubated with anti-fluorescein antibody conjugated with horse-radish peroxidase (Converter-POD) and stained with chromogenic diaminobenzidine (DAB).

Control slides: a few numbers of slides were incubated with DNase I solution and rinsed with PBS. The slides were stained according to the previous steps of staining. Since DNase I enzyme causes DNA fragmentation, the stained slides were considered the positive controls. The negative control slides were stained with the omission of terminal transferase.

Cell proliferation examination method

The immunostaining was performed using the PCNA kit and based on the protocol, as previously described (16): this technique is similar to TUNEL. The peroxidase activity within the tissue was blocked by the addition of 3% hydrogen peroxide in methyl alcohol. Then, a few drops of blocking solution were poured (100 µl) on the samples. Next, the samples were incubated with the anti-PCNA biotin-conjugated antibody for 30 to 60 minutes in the humid environment and at room temperature. Finally, the samples were stained with chromogenic DAB. To perform hematoxylin staining, samples were incubated with Alcian blue (or hematoxylin), rinsed with running buffer, and then washed with distilled water. Then, the samples were dehydrated with an increasing gradient of ethanol. Xylene was applied for the transparency of the samples in two steps, and then they were mounted using special glue.

Control slides: some positive control slides (according to the materials of the kit) were stained. The negative control slides were also stained after the omission of antibody.

Stereology method

The numerical density of PCNA-positive and TUNEL-positive cells is calculated in the unit of the surface by unbiased frames grades. For this aim, the testicular sections from different groups were imaged by Olympus optical microscope model BX51. Then, the numerical density of TUNEL- and PCNA-positive cells was calculated in the unit of surface. The average cells in the unit of surface of the rats' testicles in different groups are calculated based on this formula (15):

$$N_a = \frac{\sum Q}{a/f \sum P}$$

In this formula, ΣQ is the number of calculated cells, a/f is the area of each frame, and ΣP is the number of clash points in the frame area.

Statistical analysis

Data were analyzed using the SPSS 16 software. Results were expressed as the means and standard deviations (means ± SD). Statistical analysis was performed with one-way ANOVA followed by Tukey's test to compare the differences between groups. Differences were considered statistically significant if $P<0.05$.

Results

Effect of Vitamin E on cell apoptosis in testis tissue following exposure to diazinon

The effects of DZN and Vit E on cell apoptosis are shown in Table 1. The histological study using the TUNEL assay showed that DZN in the experimental group i significantly increased ($P<0.001$) spermatogonial cells and the rate of apoptosis in primary spermatocytes compared to the control group (Figs.2, 3). The rate of apoptosis was decreased in the experimental group ii, which shows the protective role of Vit E. In the sham and experimental group iii, the number of apoptotic cells was similar to the control group and most of their seminiferous tubules lacked apoptotic cells, and only one or two apoptotic cells was observed in some tubules ($P=1.000$, Fig.3).

Table 1: The effect of DZN and Vit E on PCNA- and TUNEL-positive cells in testes tissue of rats

Group	PCNA positive cells		TUNEL positive cells	
	Spermatogonia	Primary spermatocyte	Spermatogonia	Primary spermatocyte
Control	0.32320 ± 0.027	0.35834 ± 0.037	0.0450 ± 0.011	0.0896 ± 0.014
DZN	0.04989 ± 0.011[a]	0.05888 ± 0.015[a]	0.2174 ± 0.024[a]	0.4241 ± 0.052[a]
DZN+Vit E	0.15868 ± 0.016[a, b]	0.20384 ± 0.026[a, b]	0.1092 ± 0.025[a, b]	0.2008 ± 0.014[a, b]
Vit E	0.33266 ± 0.032	0.36034 ± 0.037	0.0517 ± 0.010	0.0958 ± 0.011
Sham	0.31743 ± 0.031	0.35734 ± 0.037	0.0375 ± 0.010	0.0827 ± 0.012

Values are expressed mean ± SD. [a]; Significantly different from control group ($P<0.001$), [b]; Significantly different from DZN-group ($P<0.001$), DZN; Diazinon, Vit E; Vitamin E, PCNA; Proliferative cell nuclear antigen, and TUNEL; Terminal deoxynucleotidyl transferase end-labeling.

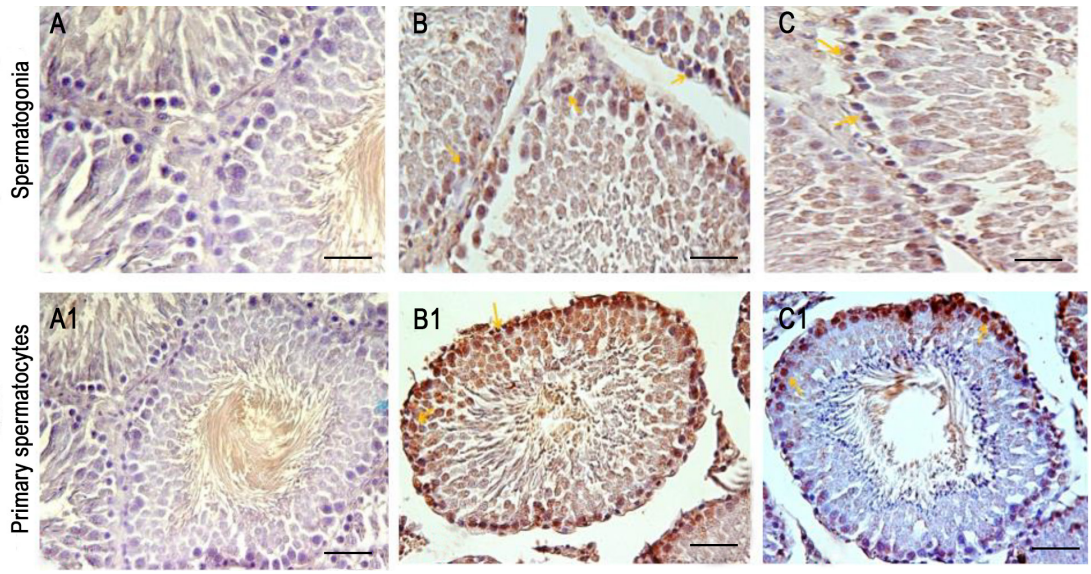

Fig.2: Testicular tissue sections after the TUNEL assay for the detection of apoptosis in primary spermatocytes and spermatogonial cells. **A, A1.** Control group. Most of the seminiferous tubules did not undergo apoptosis, **B, B1.** In the experimental group i. DZN caused the increase in the apoptotic cells compared to the control group, and **C, C1.** In the experimental group ii, vitamin E decreased the number of apoptotic cells compared to the experimental group i. The TUNEL-positive cells are marked by an arrow (scale bar: A, B, C: 5 µm, A1, B1, C1: 10 µm). TUNEL; TUNEL; Terminal deoxynucleotidyl transferase end-labeling and DZN; Diazinon.

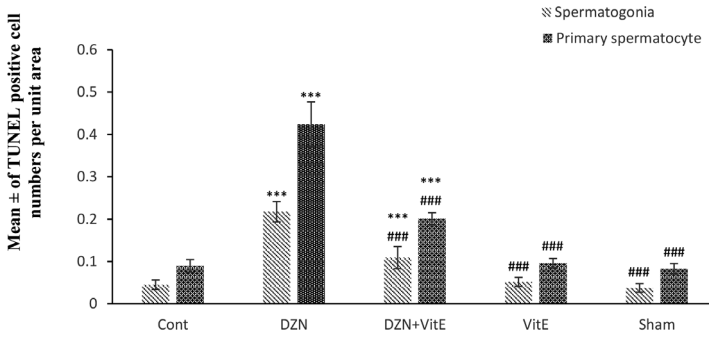

Fig.3: The effect of DZN and Vit E on the apoptosis rate of primary spermatocytes and spermatogonial cell in seminiferous tubules. The results were presented as the mean ± SD. DZN; Diazinon, Vit E; Vitamin E, ***; Compared to the control group (P<0.001), ###; Compared to the DZN-group (P<0.001).

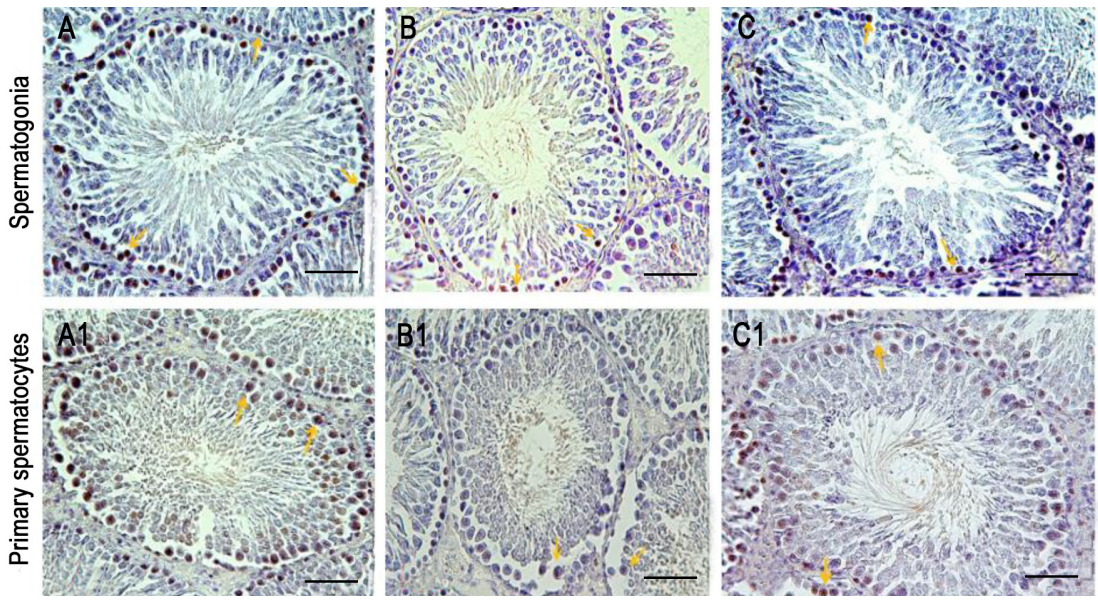

Fig.4: Testicular tissue sections after the PCNA technique. **A, A1.** The control group. Most of the spermatogonial cells and primary spermatocytes are proliferating cells, **B, B1.** In the experimental group i, DZN caused a decrease in the proliferation rate of the cells, and **C, C1.** In the experimental group ii, which was receiving DZN and vitamin E, the number of proliferating spermatogonia and primary spermatocytes were increased compared to the experimental group i. PCNA-positive cells are determined by the arrow (scale bar:10 µm). PCNA; Proliferative cell nuclear antigen and DZN; Diazinon.

Effect of Vitamin E on cell proliferation in testis tissue following exposure to diazinon

The results of DZN on cell proliferation and the protective effect of Vit E are shown in Table 1. Figure 4 indicated the cell proliferation by the PCNA technique. The exposure to DZN in the experimental group i significantly decreased spermatogonial cells and the proliferation of primary spermatocytes compared to control group (P<0.001) and therefore this group had the lowest number of cell proliferation (Fig.5). Vit E, in the experimental group ii, caused the higher rate of proliferation in spermatogonial cells and the primary spermatocytes compared to the experimental group i (P<0.001). As shown in Figure 5, the number of proliferating cells in the control, sham, and experimental group iii were not significantly different (P=0.992).

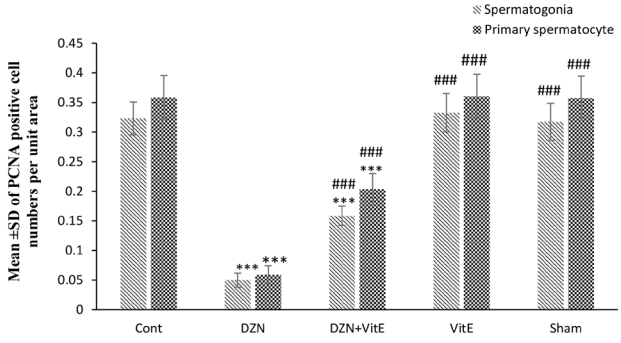

Fig.5: The effect of DZN and Vit E on the proliferation of primary spermatocytes and spermatogonial cells in seminiferous tubules (mean ± SD). DZN; Diazinon, Vit E; Vitamin E, ***; Compared to the control group (P<0.001), and ###; Compared to the DZN-treated group (P<0.001).

Discussion

Several studies conducted on various organophosphates have shown that oxidative stress can cause apoptosis. In this study, the TUNEL assay was employed for the investigation of the effect of DZN on apoptosis of spermatogonial cells and primitive spermatocytes. Histological studies also showed that DZN increased the apoptosis rate of spermatogonial cells and the primary spermatocytes. The administration of Vit E in DZN-treated rats reduced the number of apoptotic cells. Although a majority of absorbed toxins are detoxified and excreted via the detoxification process performed by the liver; however, a portion of toxic compounds may remain and accumulate in different parts of body tissues such as ovary and testis, and therefore affect the reproduction of the animals (17). According to the study performed by Sargazi et al. (15) DZN induced apoptosis in secondary and Graafian follicles while Vit E inhibited DZN-induced apoptosis. In a study carried out by Bustos-Obregon and Gonzalez-Hormazábal (18), they showed that malathion causes apoptosis in type A and B spermatogonia, spermatocyte, and spermatid. Malathion also significantly decreases the activity of serum acetylcholinesterase. According to the study of Razavi et al. (19) sub-chronic exposure to DZN induced caspase-mediated apoptosis, and crocin reduced the toxic effects of DZN by inhibiting apoptosis in aortic tissue. It is also reported that methyl parathion, dichlorvos, and chlorpyrifos increase caspases-3 and -9 in some tissues such as endometrium and retina, and the consumption of vitamin C and E together reduces the pathological effects of DZN and the rate of apoptosis caused by these pesticides (20, 21). Apoptosis is an energy-dependent process that causes the activation of caspases. The transfer of phosphatidyl serine to the outside of the plasma membrane, the changes in the permeability of the mitochondrial membrane, activation and the transfer of caspases to the nucleus, and fragmentation of DNA are the characteristics of apoptosis (22). Also, based on several studies, organophosphates change the gene expression level of gene-related apoptosis including pro-apoptotic Bax and anti-apoptotic Bcl-2 (23, 24). Therefore, organophosphates stimulate apoptosis through the activation of internal and external pathways (7, 8).

As mentioned previously, cellular damages caused by organophosphate compounds have different features, but these detrimental changes mainly affect the structure and the performance of DNA (25). Pina-Guzman et al. (26) confirmed that the toxicity of organophosphate is mediated by phosphorylation of some proteins such as nuclear proteins. Organophosphates have alkylation characteristics which can affect DNA. They also have electrophilic characteristics which can affect nuclear proteins. Therefore, organophosphates including DZN make changes in the chromatin structure and sperm DNA in the spermatogenesis process thereby phosphorylation of nuclear proteins (protamine). According to the research performed by Uzun et al. (27), sperm counts, sperm motility, plasma follicle stimulating hormon (FSH), luteinizing hormon (LH) and testosterone levels were decreased and abnormal sperm numbers were increased in rat testicles. Vitamins E and C had a protective effect on sperm counts, sperm motility and abnormal sperm numbers, but not on plasma FSH, LH and testosterone levels. Correspondingly, organophosphates can affect the normal characteristics of germ cells and their nucleus (28). Therefore, the free radicals produced during the DZN metabolism in the liver can reduce the efficiency of the testicles in the synthesis of hormones and produce low quality germ cells by the elevation of lipid peroxidation and the damage to the cell membrane (12). Vit E can prevent the peroxidation process in biological systems by blocking free radicals. The differences in the type and amount of compounds, as well as the time periods, are different factors that have been considered.

One of the functions of proliferator cell nuclear antigen is the process of DNA polymerase delta and epsilon. PCNA also interacts with other proteins, and by interaction with cyclin-dependent kinases affects the cell cycle. Testicular tissues possess spermatogenetic epithelium in which the germ cells are transferred from margin to the lumen side and developed from spermatogonia to sperm. PCNA expression has a direct association with mitosis. Since spermatogonial cells and spermatocytes are actively dividing in the seminiferous tubules, it could be considered a cellular

proliferation marker for the identification of mitotic cells in seminiferous tubules (29-32). The PCNA method showed a significant reduction in cell proliferation in the experimental group i and the increase in spermatogonial proliferation activity and the primary spermatocyte in the control group, sham and experimental group iii. Also, the administration of Vit E in DZN-treated rats significantly increased the number of spermatogonia and spermatocyte proliferation. Organophosphate compounds along with their metabolites exert their toxicity at micromolar concentrations when used in vitro thereby blocking the synthesis of DNA in glial and neuronal cells (33). DZN-induced decreased cell proliferation showed that organophosphates affect cellular responses to cytokine and the ability of cellular proliferation and gene expression by the elevation in the generation of free radicals. Increasing malondialdehyde level may also cause cellular changes and apoptosis (34). Čolović et al. (35) have shown that DZN at different doses decreased the proliferation of blood lymphocytes and the fibroblasts of human skin in the culture medium and also blocked the acetylcholinesterase activity and increased the malondialdehyde level. Penna-Videau et al. (36) reported that one dose of malathion increased the apoptosis rate of germ cells in seminiferous tubules and decreased the number of sperms and activity of plasma cholinesterase. Therefore, DZN and other organophosphate compounds can damage to testicular tissue by the influence on the different cellular processes such as DNA transcription, breaking DNA or proteins chemical bond, decreasing the cells proliferation, and eventually causing the mutation in spermatozoa by the alteration of the gene contents in spermatocyte (37). Vit E has antioxidant properties and can restrict the activity of free radicals and the rate of lipid peroxidation in the cell membrane; thus reducing the damage to testes and spermatozoa (13). According to the results of the current study and previous reports, the use of other antioxidants and the examination of other specific parts of testicular tissue including extracellular matrix proteins are recommended to identify other mechanisms of toxicity.

Conclusion

DZN caused testicular toxicity by reducing proliferation and increasing apoptosis in testicular germ cells. Vit E could be considered a potential therapeutic agent for testicular toxicity caused by reduced cell proliferation and increased apoptosis. Therefore, Vit E can protect testicular tissues against DZN toxicity. Considering that organophosphates are inevitably used for the quality of agricultural products the use of antioxidant compounds is recommended for those in contact with these toxic agents.

Acknowledgements

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Authors' Contributions

F.R.A.; Conducted the data collection, analysis, the interpretation of the data and writing the manuscript. Z.S.; Participated in the data collection and analysis. M.R.N., M.J., H.R.S.; Contributed to the study design, evaluation and the interpretation of data and the final approval of the manuscript. All authors read and approved the final manuscript.

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

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Conclusion: It emphasizes the new and important aspects of the study. All conclusions are justified by the results of the study.

Acknowledgements: This part includes a statement thanking those who contributed substantially with work relevant to the study but does not have authorship criteria. It includes those who provided technical help, writing assistance and name of departments that provided only general support. You must mention financial support in the study. Otherwise; write this sentence "There is no financial support in this study".

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Example: Phillips SJ, Whisnant JP. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York: Raven Press; 1995; 465-478.

Abstract book:

Example: Nabavi SM. Stem cell therapy for multiple sclerosis. Cell J. 2013; 5 Suppl 1: Os-13.

Thesis:

Name of author. Thesis title. Degree. City name. University. Publication date (year).

Example: Eftekhari Yazdi P. Comparison of fragment removal and co-culture with Vero cell monolayer's on development of human fragmented embryos. Presented for the Ph.D., Tehran. Tarbiyat Modarres University. 2004.

Conferences:

Name(s) of editor(s). Conference title; Holding date; Holding place. Publication place; Publisher name; Publication date (year).

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Internet References

Article:

Surname(s) and first letter of name & middle name(s) of author(s). Manuscript title. Journal title (abbr). Publication date (year); Volume (Issue): Page number. Available from: URL link. (Observation date).

Example: Jahanshahi A, Mirnajafi-Zadeh J, Javan M, Mohammad-Zadeh M, Rohani M. Effect of low-frequency stimulation on adenosineA1 and A2A receptors gene expression in dentate gyrus of perforant path kindled rats. Cell J. 2008; 10 (2): 87-92. Available from: http://www.celljournal.org. (20 Oct 2008).

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Example: Anderson SC, Poulsen KB. Anderson's electronic atlas of hematology.[CD-ROM]. Philadelphia: Lippincott Williams & Wilkins; 2002.

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