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Original Article

The Effect of Recombinant Human Follicle-Stimulating Hormone on Sperm Quality, Chromatin Status and Clinical Outcomes of Infertile Oligozoospermic Men Candidate for Intracytoplasmic Sperm Injection: A Randomized Clinical Trial

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Abstract

Background: Follicle-stimulating hormone (FSH) plays a crucial role in spermatogenesis; in this study, we assessed the effect of recombinant human FSH (rhFSH) on sperm parameters, chromatin status and clinical outcomes of infertile oligozoospermic men candidates for intracytoplasmic sperm injection (ICSI).

Materials and Methods: This interventional randomized clinical trials (IRCT) included 40 infertile oligozoospermic men undergoing ICSI. These individuals were randomized into two groups: 20 men received rhFSH drug for three months and the other 20 men who did not receive rhFSH drug were considered the control group. Before and 3 months after treatment initiation, sperm parameters (using computer-assisted semen analysis) and chromatin status [using chromomycin A3, aniline blue, and sperm chromatin dispersion (SCD) tests] were assessed in these individuals. Furthermore, hormonal profile was assessed using enzyme-linked immunosorbent assay (ELISA). Clinical outcomes of ICSI were also compared between the two groups.

Results: The rhFSH treated group showed a significant increase in the level of FSH, luteinizing hormone (LH), testosterone (T) and prolactin (PRL), as well as significant improvements in sperm parameters compared to the control group. Also, after administration of rhFSH, there was asignificant reduction in the percentage of sperm DNA damage, protamine deficiency and chromatin immaturity, while such a reduction in these parameters was not observed in the control group. Moreover, the percentage of embryos with grade Aquality, was significantly higher in the rhFSH group compared to the control group. The pregnancy rate in the rhFSH group was higher than the control group but the difference was insignificant.

Conclusion: Administration of rhFSH improves sperm quality in infertile oligozoospermic men and results in higher rates of good quality embryos post-ICSI (Registration number: IRCT20170923036334N2).

Keywords: Follicle-Stimulating Hormone, Intracytoplasmic Sperm Injection, Male Infertility, Oligozoospermia

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Introduction

Reduced sperm count termed based on World Health Organization (WHO) criteria as "oligozoospermia", is known as one of the major causes of male infertility and its prevalence shows regional variations (1). Commonly, this abnormality is accompanied by reduced percentage of sperm motility. Previous studies showed that oligozoospermia is a multifactorial condition in which genetic factors, such as chromosomal and single gene alterations, account for 20-30% of the cases (2, 3). In addition, other factors including hormonal imbalance, environmental factors, varicocele, sexually transmitted diseases, obstruction, testicular trauma, secondary testicular failure, infection and inflammation may be considered other etiological factors for the condition (3-5). Among the aforementioned etiologies, hormonal imbalance due to improper function of hypothalamic-pituitary-gonadal (HPG) axis, is considered one of the main underlying reasons for reduced sperm production leading

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to oligozoospermia. In male, this axis controls sperm production and is governed by release of gonadotropin-releasing hormone (GnRH) from the hypothalamus to the anterior pituitary gland leading eventually to release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which results in testicular production of estrogen and testosterone (T) hormone which are required for spermatogenesis (6-8). In this context, it was shown that aging- derived structural changes in median eminence, alter GnRH release and can affect FSH and LH production (9). Another cause of oligozoospermia is reduced FSH level in conditions such as Kallmann syndrome, isolated FSH deficiency or hyperprolactinemia. Moreover, environmental and lifestyle factors can also cause FSH reduction (10).

Considering the role of FSH in the process of spermatogenesis, numerous studies have evaluated the effect of FSH administration on improving spermatogenesis with the hope of increasing sperm count. The results of these studies have shown that gonadotropins in normal-low range, are not able to maintain normal spermatogenesis (14, 15) and in several studies, the effectiveness of FSH administration on improving sperm quality, was observed (16, 17). Also, it has been reported thatin both assisted reproductive technique and natural cycles, FSH administration for 3 months, improves pregnancy rate (16).

In this regard, Simoni et al. (18) showed that FSH administration in men with idiopathic infertility, not only improved sperm parameters, it also increased DNA integrity. Similarly, Colacurci et al. (16) demonstrated that after 3 months of FSH therapy, sperm DNA integrity was significantly improved in infertile men with idiopathic oligozoospermia. In addition, Kamischke et al. (19) showed that unlike sperm motility, volume of testis and sperm DNA condensation improved after treatment with daily 150 international units (IU) of rhFSH. Recently, Ding et al. (13) demonstrated that sperm parameters significantly improved after treatment with rhFSH in infertile men especially in individuals with normal- and low-level of inhibin B. Moreover, Paradisi et al. (20) in a pilot study, stated that a high-dose of rhFSH (daily 300 IU) can significantly increase sperm concentration and count, but could not significantly improve sperm motility or morphology.

FSH receptor defects are known as a potential risk factor for spermatogenetic failure. Selice et al. (21) assessed the effect of FSH treatment on individuals with polymorphism in FSH receptor gene and observed a significant improvement in sperm parameters in oligozoospermic men. In addition, a study by Italian Society of Andrology and Sexual Medicine showed that FSH therapy can improve both quantitative and qualitative sperm parameters and pregnancy rate in idiopathic oligoasthenoteratozoospermic men (22).

According to the above-mentioned statements, the aim of this study was to assess rhFSH therapy effects on sperm parameters, chromatin status, reproductive hormones, and clinical outcomes in oligozoospermic infertile men who were candidates for ICSI.

Materials and Methods

Study design

This randomized interventional clinical trials was reviewed and approved by the Ethics Committee of Islamic Azad University- Qom branch (IR.IAU.Qom.REC1396.56), and registered in Iranian registry for clinical trials (IRCT20170923036334N2). Initially, the aim, design, inclusion, and exclusion criteria of the study, were described to couples.

Briefly, the couples were informed regarding the aim of this study; this study aimed at assessment of the effect of rhFSH treatment on sperm parameters, chromatin status and hormonal levels (as the primary aims) as well as clinical outcome of ICSI (as the secondary) in oligozoospermic individuals' candidate for ICSI. The couples were informed that rhFSH therapy may improve semen quality and subsequently, the ICSI outcome. Men with varicocele or a history of varicocele surgery, systemic diseases, chemotherapy or radiotherapy history, or anatomical problems in genitals or those who were taking medications for systemic diseases or/and depression were not eligible to participate in this study. Therefore, the couples who accepted to participate in this study, were enrolled in to the "rhFSH/treatment group" and couples that refused to be treated with rhFSH but provided semen and blood samples for our study and allowed us to use their clinical data for this study, were considered the "control group".

The inclusion criteria were: low levels of FSH (less than 3 mIU/ml), age of 25-45 years, a history of infertility for at least 2 years, sperm concentration $<15\times10^6$ (i.e. oligozoospermia) according to WHO criteria (23). All the individuals conformed with the study and therefore, no couples were excluded from this study. The sample size was calculated based on the following equation:

$$n = \frac{\left(z_{1-a/2}\right)^2 * (p * (1-p))}{(d)^2}$$

Based on a 10% improvement for each parameter of the primary aims and a confidence interval of 1.96, and 0.1 value, a minimum of 20 individuals were calculated to be included in each group. Therefore, the first 20 individuals who accepted to receive 75 IU rhFSH (Gonal-F), three times a week for 3 months according to a previous study (24), were included in the rhFSH/treatment group, and the first 20 individuals who refused to be treated with rhFSH but provided semen and blood samples for our study and allowed us to use their clinical data for this study, were considered the "control group" (Fig.1).

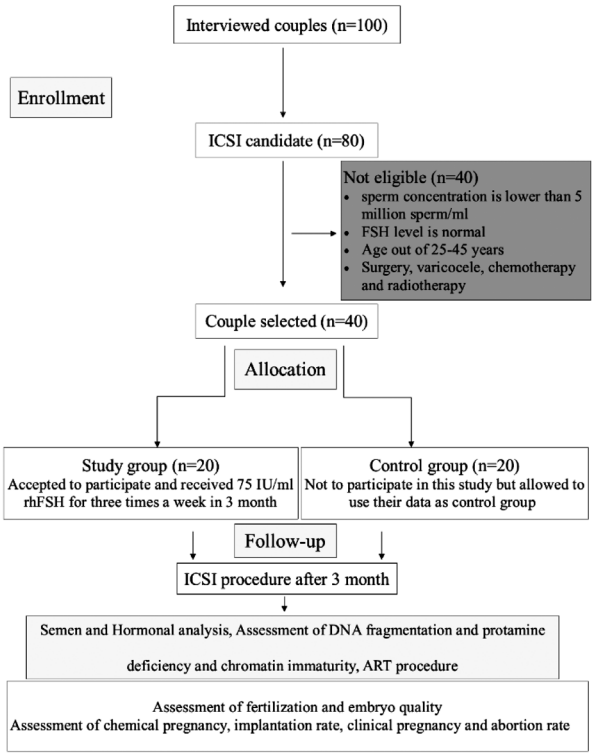

Fig.1: Flow diagram of study design. ICSI; Intracytoplasmic sperm injection and ART; Assisted reproductive technology.

Semen analysis

Semen analysis was performed after liquefaction of samples obtained by masturbation after 3-5 days of sexual abstinence. Each parameters and the number of leucocytes were assessed respectively by CASA software and peroxidase staining according to WHO criteria (23).

Hormonal analysis

After collection, blood samples were immediately centrifuged for 10 minutes at 3000 rpm (Hettich, EBA20, UK) and sera were separated and stored at -70°C. FSH, LH, PRL and total T levels were determined by ELISA method (Demeditec Diagnostics GmbH, Germany). LH and FSH are expressed as mIU/ml while total T and PRL are expressed as ng/ml.

Assessment of DNA fragmentation

DNA fragmentation was assessed using an improved sperm chromatin dispersion (SCD) test (the Halosperm kit, INDAS laboratories, Spain). For each sample, a minimum number of 500 sperm was evaluated under the ×100 objective of an optical microscope. The following five patterns of halo around sperm head were detectable: i. Sperm with large halos (halo width of sperm equal to or higher than the minor diameter of the core), ii. Sperm with medium size halos (halo width of sperm between large and small halo), iii. Sperm with very small size halo (halo width of sperm smaller than one-third of the minor diameter of the core), iv. Sperm without a halo, and v. Degraded sperm. Sperm with small halos, without halos and degraded sperm were considered sperms with fragmented DNA, and data calculated for each sample was reported as percentage (25).

Assessment of protamine deficiency and chromatin immaturity

For evaluation of protamine deficiency, initially, the semen sample was washed with PBS and then, fixed using methanol and glacial acetic acid (3:1 ratio) and stained with chromomycin A3 (CMA3, Sigma, USA) according to Nasr-Esfahani et al. (26). In addition, we assessed chromatin immaturity by aniline blue staining according to Terquem and Dadoune (27). The results are expressed as percentage of protamine deficiency and chromatin immaturity.

Assisted reproductive technology procedure

For ovulation induction, the agonist protocol was used. On day 2 of the cycle, a vaginal ultrasound was carried out to confirm absence of any active follicle and presence of thin endometrium. Following confirmation of basal characteristics, gonadotrophins were administered at 150-225 IU/ml daily. A second ultrasound was carried out on day 6 to monitor follicular growth and adjust drug dose. Suppression using a GnRH antagonist started on day 7 when leading follicles were around 12-14 mm. Triggering was done by 10000 IU/ml human chorionic gonadotropin (hCG) when 3 mature leading follicles were greater than 17 mm.

Thirty-six hour post triggering, oocytes were recovered by transvaginal ultrasound and then, the standard ICSI protocol was performed.

At 16-18 hours after ICSI, fertilization was confirmed by the presence of two pronuclei (2PN) and rate of fertilization was calculated as follows: the number of 2PNs divided by the number of MII oocytes in seminated by ICSI procedure. Three-day post ICSI, embryo quality was assessed based on a three-point scoring system (28, 29): i. Absence or fragmentation of <25% on embryonic surface, ii. Equality of blastomere's size and shape, and iii. Blastomere cell number greater or less than 7. Embryos presenting all above-mentioned criteria were scored as "A", embryos having only two criteria were scored as "B" and embryos presenting only one criteria were scored as "C". All three group (A+B+C) were chosen and the percentage of top-quality embryos was calculated.. The embryos were selected for transfer based on availability, with the following priority: A, B and finally, C. All patients received progesterone supplementation as luteal phase support. Two weeks after embryo-transfer, chemical pregnancy was confirmed by assessment of serum β-hCG. Clinical pregnancy was diagnosed by ultrasonographic visualization of one or more gestational sacs (30).

Statistical analysis

Statistical analysis was performed using SPSS version

21.0 (SPSS Inc. Chicago, IL). For comparison of study parameters between the control and rhFSH groups before and after 3 months, paired sample t test was used. Chi-square was used for comparison of pregnancy outcomes and implantation rate between the two groups. P≤0.05 were considered significantly different.

Results

In this study, the average age of oligozoospermic men was 33.85 ± 4.5 years old in the rhFSH group and 36.25 ± 5.22 years old in the control group with no significant differences between the two groups. The mean of body mass index (BMI) showed no significant difference in rhFSH (28.8 ± 1.8 vs. 29.09 ± 1.8, respectively, P=0.498) or control groups (29.36 ± 1.79 vs. 29.33 ± 1.86, respectively, P=0.683) when comparing before treatment and after 3-month treatment values. The comparison of before treatment and after 3-month treatment values in terms of sperm function and hormonal parameters in the rhFSH and control groups, is shown in Table 1 and Figure 1. Unlike the control group, all the sperm parameters (sperm count, concentration, motility, and morphology), chromatin status (sperm DNA fragmentation, protamine deficiency, and chromatin immaturity) and hormonal profile (FSH, LH, T and PRL) significantly improved after treatment of oligozoospermic men with rhFSH (P<0.05).

In addition, we followed up the clinical outcomes of oligozoospermic men in both groups and the results are presented in Table 2. Males and females age and the number of oocytes retrieved were not significantly different between the two groups (P=0.13), while the mean number of MII oocytes (P=0.02) and embryo transfer (P=0.02) were significantly higher in the rhFSH group compared to the control group. We found that the mean percentage of fertilization rate, embryos with grade B quality and chemical and clinical pregnancy rates were higher in the rhFSH group than the control couple, but differences were not significant. Only, the mean percentage of embryos with grade A quality was significantly higher in the rhFSH group compared to the control group (P=0.026).

Table 1: Comparison of sperm parameters and hormonal profile before and after 3 months in the rhFSH and control group

Parameters	rhFSH n=20		Control group n=20	
	Before	After	Before	After
Sperm concentration (×10⁶/ml)	7.6 ± 2.5	18 ± 3.5[a]	7.9 ± 3.9	8.03 ± 3.8
Total sperm count (×10⁶/ejaculate)	26.9 ± 17.6	65.2 ± 28.9[a]	13.4 ± 7.1	14.2 ± 7.1
Total sperm motility (%)	19.75 ± 10.7	33.5 ± 14.1[a]	14.25 ± 6.9	14 ± 6.2
Progressive sperm motility (%)	4.5 ± 5.3	17.5 ± 10.4[a]	6.5 ± 2.3	6.8 ± 2.05
Immotile sperm (%)	80.25 ± 10.7	66.5 ± 14.15[a]	85.75 ± 6.9	86.25 ± 6.4
Sperm abnormal morphology (%)	98.7 ± 0.5	97 ± 0.8[a]	99 ± 0.00	98.85 ± 0.4
FSH (mIU/mL)	1.83 ± 0.5	3.88 ± 0.9[a]	1.76 ± 0.3	1.79 ± 0.3
LH (mIU/mL)	4.99 ± 1.9	6.048 ± 2.1[b]	5.03 ± 1.7	5.06 ± 1.6
Testosterone (ng/mL)	3.034 ± 1.03	4.56 ± 1.1[a]	3.5 ± 1.05	3.55 ± 1.0
Prolactin (ng/mL)	10.56 ± 2.5	12.69 ± 2.7[a]	9.69 ± 3.6	9.58 ± 3.5

Data are presented as means ± SD. [a]; P<0.001, [b]; P<0.05 indicate significant differences when comparing before and after treatment values. Paired sample t test was used for comparison of parameters before treatment and after three-month treatment. rhFSH; Recombinant human follicle-stimulating hormone, and LH; Luteinizing hormone.

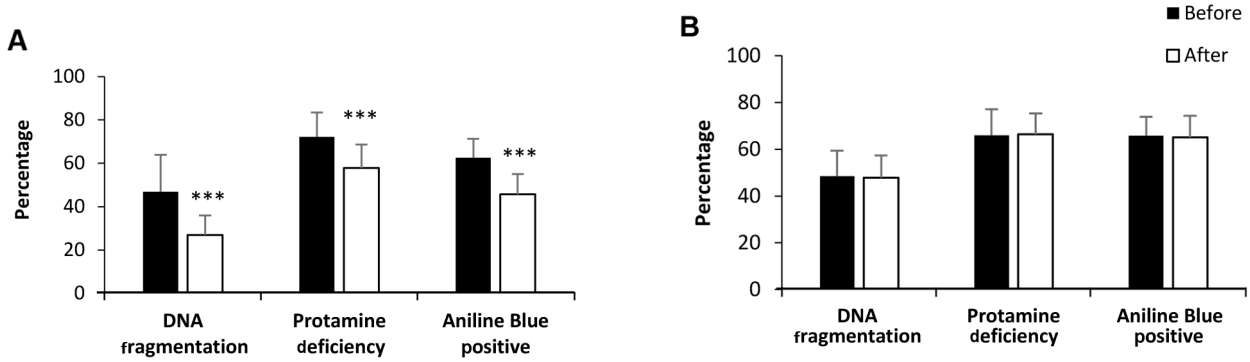

Fig.2: Sperm genomic content comparison. Comparison of mean percentage of sperm DNA fragmentation, protamine deficiency, and chromatin immaturity before treatment and after three-month treatment in the **A.** rhFSH and **B.** Control groups. Paired sample t test was used for comparison of parameters between the two groups. All data are presented as mean ± SD. ***; Show significant differences at P<0.001 and rhFSH; Recombinant human follicle-stimulating hormone.

Table 2: The comparison of, males and females age, the number of oocytes, and ICSI clinical outcomes between the rhFSH and control groups

Parameters	rhFSH group	Control group	P value
Male age (Y)	33.85 ± 4.5	36.25 ± 5.22	0.13
Female age (Y)	28.8 ± 4.9	31.65 ± 4.72	0.07
Number of oocytes	10.55 ± 5.763	7.05 ± 4.123	0.33
Number of MII oocytes	7.5 ± 4.007	4.9 ± 2.8	0.02
Number of embryo transfers	2.06 ± 0.8	1.137 ± 1.8	0.02
Fertilization rate (%)	65.8	59.7	0.56
Embryo quality grade A (%)	16.6	9.7	0.026
Embryo quality grade B (%)	73.4	64.5	0.69
Embryo quality grade C (%)	10	25.8	0.85
Biochemical pregnancy rate (%)	50	25	0.13
Clinical pregnancy rate (%)	35	20	0.48

Data are presented as mean ± SD or percentage. ICSI; Intracytoplasmic sperm injection and rhFSH; Recombinant human follicle-stimulating hormone. Independent samples t test was used for comparison of these parameters between the two groups, except for pregnancy rate compared by Chi-square test.

Discussion

The results of the current study clearly showed that mean percentage of sperm parameters, protamine deficiency, DNA fragmentation, and chromatin immaturity were significantly improved in oligozoospermic men treated with rhFSH for three months compared to untreated oligozoospermic men. In addition, hormonal profile of these individuals was significantly improved compared to the control group. These results showed that administration of rhFSH was effective in improving spermatogenesis function in oligozoospermic men. In this regard, several clinical trials, and a study in a monkey model revealed an increase in testicular volume after FSH treatment, indicating that FSH hormone could increase germ cell proliferation in seminiferous tubules (19, 31, 32). In the light of these considerations, we assessed the effect of rhFSH on sperm functional parameters and clinical outcomes in infertile oligozoospermic men candidate for ICSI.

With regard to sperm DNA fragmentation, Ruvolo et al. (17) showed that FSH administration improves sperm DNA damage in men with hypogonadotropic hypogonadism and idiopathic oligozoospermia with high DNA fragmentation. Unlike the results of current study, others (19) did not observe any improvement in sperm parameters following administration of rhFSH in infertile men. The only parameters that was improved in foregoing study were testicular volume and sperm DNA fragmentation compared to the placebo group. The difference in the results of Kamischke et al. (19) study and our study could be related to the type of selected patients as we only focused on oligozoospermic individuals while they included cases with previous failed fertilization, azoospermic individuals undergoing testicular biopsies, *in vitro* fertilization (IVF) and ICSI.

In addition to sperm functional parameters, in the current study, clinical outcomes of ICSI in both groups were assessed and it was interesting that mean percentage of good quality embryos was significantly higher in the rhFSH compared to the control group. Although, the mean values for percentages of clinical and chemical pregnancy improved in the rhFSH compared to the control group, but the difference was not significant. Considering the impact of DNA fragmentation on development, several studies have shown associations between sperm DNA fragmentation and early embryonic development. But the association between fertilization rate and sperm DNA fragmentation remains controversial (31-34). Indeed, some authors have shown that even treatment of sperm with H_2O_2 does not preclude pronuclei formation (35). Most researchers believed that, due to DNA repairing mechanism in oocyte and early embryos, the effect of DNA damage on development should be observed after maternal-zygotes genomic transition which takes place at around 6-8-cell stage in human (36). Therefore, some studies have observed a significant effect for sperm DNA fragmentation on the quality of embryos on day 3 (32) while others believed that the effect of sperm DNA fragmentation shall be observable at around blastocyst stage when zygotic genome activation is more complete (37-39). In this study, since the common day for embryo transfer is day 3, we could not assess the effect of sperm DNA fragmentation on blastocyst formation rate, but we observed a significant improvement of sperm DNA fragmentation after rhFSH therapy, and a significant rate of good quality embryos on day 3 which is consistent with previous studied in this filed (31, 32).

If sperm DNA fragmentation is not repaired by oocyte or embryo, the embryos may have a reduced chance of implantation and inducing a pregnancy. In this study, we also showed that rhFSH therapy insignificantly improved the pregnancy rate. Although an improvement in pregnancy rate was expected, but not asignificant improvement was observed in this respect that is probably due to small sample size of this study. In addition, the mean number of MII oocytes was significantly higher in the rhFSH group compared to the control group. Obviously, this wasnot related to rhFSH in male and is regarded as a "bias" in the current results which is likely related to sample size and could doubtlessly impact the clinical outcomes of these couples. Therefore, further studies are needed to assess the effect of rhFSH therapy on clinical outcomes in oligozoospermic men in large populations. On the other hand, we highlighted that therapy with rhFSH in infertile men with oligozoospermia was effective in terms of spermatogenesis level, sperm function parameters, and hormonal profile. According to the literature, administration of rhFSH in idiopathic male infertility, could improve spermatogenesis thought he eventual benefits of supra-physiological FSH on spermatogenesis remain unclear. In this regard, Santi et al. (40) stated that the supporting effect of rhFSH on spermatogenesis should be assessed throughevaluation of

sperm parameters as the primary endpoint. Therefore, our results confirm this theory.

Conclusion

Taken together, based on the results of this study, treatment of idiopathic oligozoospermic individuals with rhFSH not only improves sperm parameters sperm chromatin integrity and hormonal profile, but also significantly improves embryo quality post ICSI and insignificantly improves the pregnancy rate.

Acknowledgements

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Authors' Contributions

A.V., M.H.N.-E., M.F., M.T.; Performed the investigations. A.V.; Semen analysis, prepared samples, carried out experimental, and collected data. M.F.; Took part in designing the research and analyzing data. M.T., M.H.-N.E.; Analyzing data and writing the manuscript. M.H.N.-E.; Supervised the study. All authors read and approved the final version of the manuscript.

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Original Article

The Effect of Daily Intake of Selenium, Vitamin E and Folic Acid on Sperm Parameters in Males with Idiopathic Infertility: A Single-Blind Randomized Controlled Clinical Trial

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Abstract

Background: Male infertility may originate from a wide spectrum of conditions while in 30-40 percent of cases, no significant reason can be identified. Thereby, it is recognized as male idiopathic infertility. This study was undertaken to investigate the effect of daily intake of selenium, vitamin E and folic acid on sperm parameters in males with idiopathic infertility.

Materials and Methods: Seventy infertile men were selected to participate in this single-blind, randomized controlled clinical trial using convenience sampling. They were equally divided into two groups via permuted block randomization method. The intervention group received selenium tablet (200 µg per day, oral), vitamin E capsule (400 IU per day, oral) and folic acid tablet (5 mg per day, oral).The placebo group received matching placebo for three months. Semen volume, total sperm motility, sperm concentration, progressive sperm motility, normal sperm morphology, sperm motility index (SMI) and functional sperm concentration (FSC) were assessed by sperm quality analyzer-v (SQAV) before and after the intervention. Paired t test, and independent t test were used to compare the results within and between the groups, respectively. The IBM SPSS V.16.005 was used for data analysis. A $P<0.05$ was considered statistically significant.

Results: After three months, according to within-group analysis, a significant difference was found in mean SMI (P=0.007) and FSC (P=0.001) in the intervention group. According to between-group analysis, no significant difference was found in mean semen volume (P=0.610), sperm concentration (P=0.126), total sperm motility (P=0.765), progressive sperm motility (P=0.767), normal sperm morph (P=0.403), SMI (P=0.556) or FSC (P=0.706) between the groups.

Conclusion: Consumption of selenium, vitamin E and folic acid in infertile men with asthenozoospermia was not effective (Registration number: IRCT2017012432153N1).

Keywords: Folic Acid, Parameters, Selenium, Spermatozoa, Vitamin E

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Introduction

Infertility is characterized as "not being able to get pregnant, after at least one year of regular unprotected sex" (1). In fact, 15% of couples worldwide suffer from infertility (2). Male factor contributes to 20 to 70% of infertility cases and the percentage of infertility solely due to male factor is estimated to be 2.5 to 12% (3). Male infertility may be originated from a wide spectrum of conditions such as anatomy and genetic disorders, neurological or systemic diseases, trauma, infection, iatrogenic injury, gonadotoxins and formation of sperm antibodies (4). In nearly 30 to 40% of infertility cases, there is an abnormality in semen parameters in terms of motility, morphology or concentration, in at least two semen analyses while no significant reason can be detected. Therefore, this type of infertility is identified as male idiopathic infertility (4, 5). Accumulating research has clarified the fundamental role of low levels of reactive oxygen species (ROS) in intracellular signaling which is responsible for spermatozoa maturation, capacitation, hyperactivation, acrosomal reaction and oocyte fusion (6). Several studies have reported that elevated seminal ROS levels exist in 30-80% of infertile men (7). In fact, spermatozoa membrane is rich in poly unsaturated fatty acid; hence, it is vulnerable to the detrimental effects of excessive amounts of ROS which lead to lipid peroxidation, loss of membrane integrity, increased membrane permeability, reduction of sperm motility, structural DNA damage and apoptosis (8). Experimental investigation on sub-fertile

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men have indicated lower levels of antioxidants in the semen as compared with fertile men (9). According to recent research, to confront excessive generation of ROS, various intrinsic antioxidants and extrinsic antioxidants have been applied which controlled the detrimental production of ROS and prevented their side effects (10). Proper spermatogenesis requires two selenoproteins: phospholipid peroxide glutathione peroxidase (PHGPX) and selenoprotein P. In the testis, selenium works in the form of PHGPX known as a selenium-dependent antioxidant enzyme. The most considerable role of this agent is protecting plasmatic membrane of mature spermatozoa against the attack of free radicals. This protein also organizes 50% of the material of mitochondrial mid-piece of spermatozoa; thus, in cases of selenium deficiency, reduced motility of spermatozoa due to abnormality in the morphology of spermatozoon mid-piece are detected (11). Vitamin E is a fat-soluble vitamin that restrains free radicals which induce damage to cell membranes, prevents lipid peroxidation and improves the activity of other antioxidants, thereby decreasing seminal ROS in infertile males. Also, there are some epidemiological data that support a direct relation between improvement of seminal parameters and increased dietary intake of vitamin E (12). Folic acid, as a synthetic form of folate, efficiently scavenges free radicals and has been introduced as an effective factor for reduction of ROS in seminal fluid (13). Therefore, the present study aimed to investigate the effects of daily intake of selenium, vitamin E and folic acid as probably the most effective antioxidant components on sperm parameters in males with idiopathic infertility.

Materials and Methods

This single-blind randomized controlled clinical trial was carried out on 70 men who met the inclusion criteria, and were diagnosed as idiopathic infertile patients attending the clinics of urology (affiliated to Shiraz University of Medical Sciences, Shiraz, Iran) from June 2016 to September 2018. The approval ID for this interventional study was obtained from the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1395.160) and the study was registered with clinical trial registry number IRCT2017012432153N1. Written informed consent was taken from all the study participants.

According to the WHO guideline (2010), patients were respectively diagnosed as oligozoospermia, asthenozoospermia, teratozoospermia, if:

- Sperm concentration (sperm numbers per one milliliter of semen) lower than 15 M/ml and higher than 5 M/ml.

- Total sperm motility lower than 40% or progressive sperm motility lower than 32%.

- Percentage of sperm with normal morphology <4%.

Patients with oligo, astheno, terato or oligoasthenoteratospermia [Based on the WHO guideline (2010)] who attended the clinics of urology were recruited if they met the following inclusion criteria: willingness to participate in the study; not being able to get pregnant after at least one year of regular unprotected sex; abnormal seminal analysis results [confirmed after two semen analyses within 3-4 week intervals done after the same sexual abstinence periods (3-5 days)]; absence of underlying causes screened according to pre-testicular, testicular and post-testicular factors (Table 1) (4). We started antioxidant treatment for cases with a history of varicocelectomy at least 3 months later. Also, varicocele recurrence was ruled out again.

Table 1: Pre-testicular, testicular and post-testicular factors

Pre-testicular factors	Testicular factors	Post - testicular factors
Kallmann syndrome	Varicocele	Coital
Hyperprolactinaemia	Cryptorchidism	Pharmacological
Pharmacological	Testicular cancer	Retrograde ejaculation
	Radiation	Congenital bilateral absence of the vas deferens
	Chemotherapy	Ejaculatory duct obstruction
	Pharmacological	Seminal vesicle dysfunction
	Genetic azoospermia or Oligospermia	Vasectomy
	Y-chromosome microdeletions	Iatrogenic injury to the vas deferens
	Klinefelter syndrome	Young's syndrome
	Environmental	Nerve injury
	Anti-sperm antibodies	Spinal cord injury
	Injury or trauma	Retroperitoneal lymph node
	Infection	Systemic disease

The exclusion criteria were: participant's unwillingness to continue, urogenital infection with antioxidant properties, symptom of an allergy to antioxidant therapy, diagnosis of pre-testicular, testicular or post-testicular factors.

Study design

Patients who met the inclusion criteria were grouped as either intervention (n=35) or placebo group (n=35), through permuted block randomization method. Patients in the intervention group received selenium tablet (200 µg per day, oral), vitamin E capsule (400 IU per day, oral) and folic acid tablet (5 mg per day, oral).The placebo group received matching placebo (250 mg per day, oral) for three months. The placebo was made in the laboratory of the School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran and sodium starch glycolate 100% was used to make the placebo. In this study, selenium was the product of Webber Naturals Company, Canada, vitamin E was the product of Zahravi pharmaceutical company, Iran, and folic acid was the product of Jalinous pharmaceutical company, Iran. During this period, the patients were trained to limit consumption of red meat and food rich in phytoestrogen such as soybeans (due to their effect on reducing sperm count), and stop smoking (due to the toxic chemicals which can cause mutations) and alcohol consumption (which reduces testosterone level and testicular atrophy). They were also taught not to be

exposed to radiation, prolonged heat and the environment of a sauna or Jacuzzi (14). Besides, they were trained to refrain from using a cell phone for more than 11-13 hours per day (15). Also, they were trained to limit consumption of caffeine due to its probable detrimental impact on sperm DNA integrity (16).

After three months, the participants were asked to follow sexual abstinence for 3-5 days, and repeat their semen analysis. Sperm concentration, total sperm motility, progressive sperm motility, sperm morphology, sperm motility index (SMI), and total functional sperm concentration (FSC) were assessed. FSC is the concentration of progressively motile spermatozoa with normal morphology in a semen sample. This is a very difficult parameter to measure manually, since it is required to kill the progressive cells to assess the morphology.

Semen analysis

For assessment of sperm parameters, the WHO 2010 guidelines were considered and sperm quality analyzer- v (SQAV) instrument and optical microscope (for evaluating morphology) were used. In this study, all the sperm parameters except morphology, were evaluated by SQAV.

By evaluating the accuracy of automated computerized semen analyzer instrument (SQAV and CASA) and conventional manual method according to accuracy and precision, various results were reported.

The advantages of using automated semen analyzer include standardization, speed, precision, automated data recording, fewer human errors and less need for high-skilled professionals to perform the analysis. The accuracy of the instrument lies in the fact that it uses a larger sample volume (0.5 ml) in contrast with the volume used in manual analysis and CASA instrument (10-50 µl). The main disadvantage is the inability to carry out a concrete differential morphology assessment. However, this problem has been resolved by integrating manual assessment to detect normal morphology (17).

Statistical method

Continuous variables with normal distribution, and variables with non-normal distribution are reported as mean ± SD and median (IQR), respectively. The categorical data are presented as numbers (%). For checking the normality of data, Shapiro-Wilks test was used. The difference within and between the two groups was investigated using Paired t test, and independent t test, respectively. Mann-Whitney and Wilcoxon's tests were used when the assumption of normality was violated. Moreover, we used chi-squared test to compare the distribution of categorical data [The SPSS V. 16.0. with significance level of 0.05 used for data analysis, the product of International Business Machines Corporation (IBM), Chicago, USA]. A $P<0.05$ was considered significant.

Results

A total of 70 patients (aged 18-55 years) with idiopathic infertility, participated in the present study; however, after three months, only 62 patients completed the study: 30 patients in the intervention and 32 in the placebo group (Fig.1).

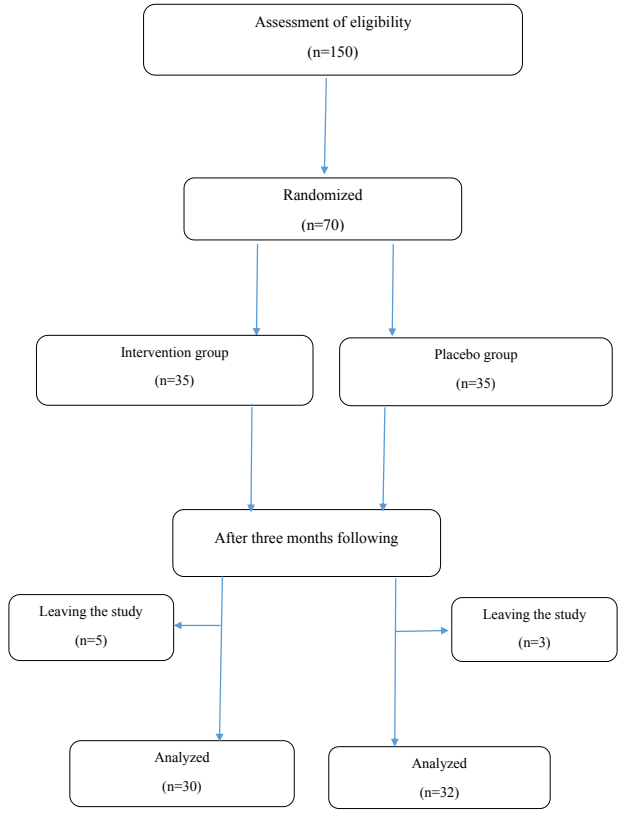

Fig.1: The flowchart of patient selection in this single-blind randomized controlled clinical trial.

Table 2 presents demographic and baseline characteristics of the study participants. No significant difference was found in demographic and baseline characteristics among the participants, and the two groups were well balanced: age (P=0.737), duration of marriage (P=0.392), duration of infertility (P=0.070), smoking (P=0.352), alcohol consumption (P=0.591), and level of education (P=0.186).

Table 3 summarizes the quality of sperm parameters from the baseline to the end of the intervention. No significant difference was found in semen volume (P=0.097), sperm concentration (P=0.270), total sperm motility (P=0.331), progressive sperm motility (P=0.130), normal sperm morphology (P=0.315), SMI (P=0.059) and FSC (P=0.057) between the intervention and control groups at the beginning of the study.

At the end of the intervention (i.e. after three months), within-group analysis indicated significant improvements in SMI (P=0.007) and FSC (P=0.001), but not in other variables in the intervention group. Difference in differences method was used as a statistical technique to investigate the effect of the present intervention by comparing the average change (over time) in the outcome variable in

both intervention and placebo groups (i.e.between-group analyses). No significant difference was found in sperm parameters between the intervention and placebo groups at the end of the study (Fig.2, Table 3).

Table 2: Demographic and baseline characteristics

Variable	Intervention group n=30	Placebo group n=32	P value[a]
Age (Y)	37.23 ± 7.09	36.65 ± 6.41	0.737
Duration of marriage	9.31 ± 6.23	7.87 ± 5.1	0.392
Duration of infertility	6.03 ± 4.35	4.28 ± 3.05	0.070
Smoking			0.352
Yes	8 (26.66)	11 (34.38)	
Never	22 (73.33)	21 (65.62)	
Alcohol consumption			0.591
Yes	5 (16.66)	5 (15.62)	
Never	25 (83.33)	27 (84.37)	
Level of education			0.186
Primary school	1 (3.3)	0 (0)	
Guidance school	3 (13.3)	2 (6.3)	
High school	16 (53.3)	12 (37.5)	
Higher	9 (30)	18 (56.3)	

Data are expressed as mean ± SD or n (%). [a]; Independent t test or chi-squared test.

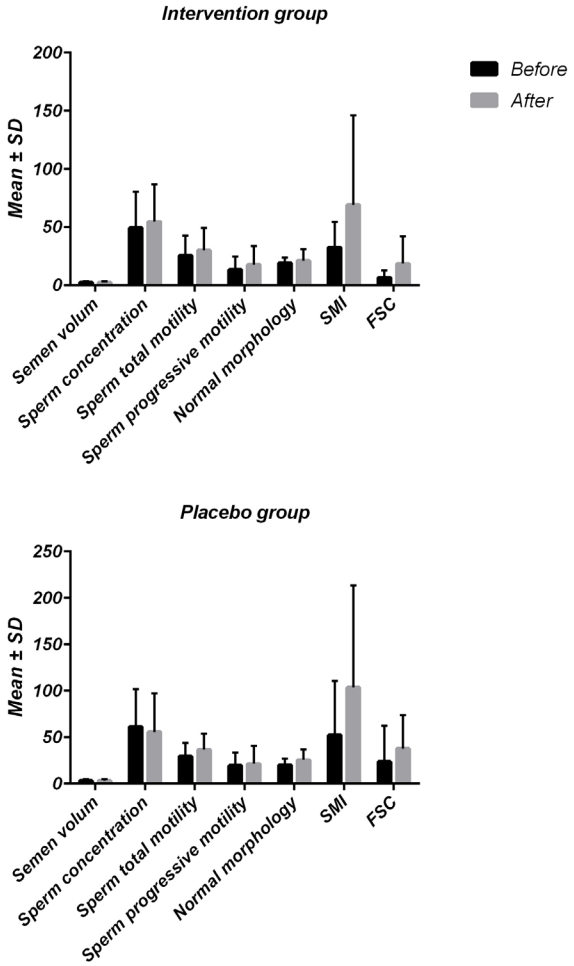

Fig.2: The within and between groups variation of sperm parameters at the end of the study in both groups groups (data expressed as mean ± SD). SMI; Sperm motility index and FSC; Functional Sperm Concentration.

Table 3: The effects of selenium, vitamin E and acid folic on sperm parameters in infertile men

Variable	Intervention group n=30	Placebo group n=32	P value[b]
Volume			
Baseline	2.50 ± 1.13	3.18 ± 1.62	0.097
End	2.52 ± 1.06	3.23 ± 1.43	0.038
Change	0.02 ± 0.84	0.04 ± 0.96	0.610
P value[a]	0.957	0.430	
Concentration (10^6/ml)			
Baseline	49.52 ± 30.98	61.31 ± 40.67	0.270
End	54.67 ± 32.07	55.79 ± 41.39	0.780
Change	5.15 ± 28.99	-5.52 ± 23.01	0.126
P value[a]	0.28	0.170	
Total motility			
Baseline	25.62 ± 17.11	29.52 ± 14.25	0.331
End	30.28 ± 19.27	36.68 ± 17.19	0.171
Change	4.66 ± 17.9	7.15 ± 16.9	0.765
P value[a]	0.166	0.020	
Progressive motility			
Baseline	13.5 ± 11.19	19.51 ± 13.92	0.130
End	17.99 ± 16	21.27 ± 19.19	0.460
Change	4.1 ± 14.21	1.75 ± 17.61	0.767
P value[a]	0.120	0.530	
Normal morph			
Baseline	19.48 ± 4.3	20.02 ± 6.9	0.315
End	21.18 ± 9.9	25.62 ± 11.33	0.486
Change	2.69 ± 8.96	5.6 ± 11.7	0.403
P value[a]	0.069	0.339	
SMI			
Baseline	32.8 ± 21.56	52.33 ± 58.41	0.059
End	69.2 ± 76.94	103.58 ± 109.79	0.375
Change	36.4 ± 66.52	51.25 ± 100.38	0.556
P value[a]	0.007	0.039	
FSC			
Baseline	6.74 ± 5.98	23.70 ± 38.71	0.057
End	18.73 ± 23.36	37.74 ± 36.1	0.087
Change	11.98 ± 20.71	14.04 ± 36.05	0.706
P value[a]	0.001	0.007	

Data are expressed as mean ± SD. [a]; Paired t test or Wilcoxon test, [b]; Independent t test or Mann-Whitney U test, SMI; Sperm motility index, and FSC; Functional sperm concentration.

Discussion

This study aimed to investigate the effect of daily intake of selenium, vitamin E and folic acid on sperm parameters in idiopathic infertile men. At the end of the study, significant changes in total motility were observed in the placebo group as compared to the intervention group. In fact, small sample size and high heterogeneity may have had a significant effect on this finding. The difference in differences method was used and the mean changes in total motility in the placebo and the intervention group

were compared. Finally, based on our results, no significant difference was found between the intervention and placebo groups. It seems that our interventions did not have significant effect on this parameter as compared with the placebo group.

Consistent with our results, Keskes-Ammar et al. (18) showed that the serum concentration of vitamin E in selenium (225 µg/day for three months) and vitamin E (400 mg/day for three months) treated group, significantly increased and malondialdehyde (MDA) significantly decreased. But no significant effects on serum MDA level and serum vitamin E concentration was detected in vitamin B treated group. However, no significant difference in sperm concentration was reported between the two groups. According to Keskes-Ammar et al. (18), dietary content of selenium (64.3 ± 17.7 µg/day) was lower than the amount reported in other studies (80-110 µg/day). Thus, sample size and short duration of follow-up may have a significant role as compared with other similar studies.

In another study, Scott et al. (19) indicated that sperm concentration in the selenium-treated group increased by 22%, in contrast with little or no increase in the group which received selenium + vitamin E + vitamin C + vitamin A and in the placebo group. They also reported that variations in response, small sample size and low concentration of administered selenium, prevented significant difference in sperm concentration among mentioned groups as compared with similar studies.

In another consistent study, Greco et al. (20) investigated the efficacy of two-month daily intake of 1g vitamin E and 1 g vitamin C on sperm parameters in infertile men. At the end of the study, no significant improvement in sperm motility was found. Small sample size, short duration of intervention and high-dose of antioxidants (which has a reverse effect) may have affected their results.

On the other hand, in consistent with our results, in a case-control study, Eroglu et al. (21) indicated lower serum and seminal concentration of selenium and semen total antioxidant capacity (TAC) in patients with oligozoospermia as compared with men with normozoospermia. In fact, selenium has a significant role in the production of mature spermatozoa from immature spermatids. Also, it contributes to the formation of glutathione peroxidase as an important enzyme in the mid-piece of human spermatozoa and protecting spermatozoa against oxidative stress, finally improving sperm quality.

Another study showed that vitamin E combined with clomiphene citrate, as antioxidant and anti-estrogen therapy, were more efficient in improving sperm concentration in idiopathic oligoasthenozoospermia compared with each one individually (22). It was concluded that vitamin E as an antioxidant acts more efficiently in combination of an antiestrogenic hormone such as clomiphene citrate, in improvement of sperm concentration in comparison with its individual administration.

Our study showed no significant improvement in total sperm motility and progressive sperm motility between the two groups.

Consistent with our study, Hassani-Bafrani et al. (23) investigated the effect of vitamins E and B and their combination on sperm motility in a rat varicocele model. They find no significant improvement in sperm motility in varicocelized rats treated with vitamin E. Meanwhile, a significant improvement in vitamin B and vit B+E varicocelized treated group was detected. It is likely that these improvements were due to B complex specially B12 rather than vitamin E alone. In fact, vit B12 plays an important role in the regeneration of one carbon cycle and methionine synthesis and thermoregulation of scrotal and finally improvement of sperm parameters.

In consistence with our results, Moslemi and Tavanbakhsh (24) indicated a significant improvement in sperm motility of asthenoteratozoospermia patients who received daily supplements of selenium and vitamin E for at least 100 days (compared with the baseline). In fact, selenium and glutathione contribute to production of phospholipid hyperoxide GSH-Px, a structural protein that contains more than 50% of mitochondrial capsule of spermatozoa mid-piece, the deficiency of which leads to instability of spermatozoa mid-piece and finally, asthenozoospermia. It seems that the difference in results may partly be due to the larger sample size and longer period of this study in comparison with our study.

Another study showed that total sperm motility increased by 40 and 34% in selenium-treated and B complex-treated groups, respectively. Also, it was reduced by 15% in the placebo group, however, differences among these three groups were not significant. When selenium-treated and B complex-treated groups were combined and compared with the placebo group, a significant improvement in the selenium-treated group was found in comparison with the control group (19). Significant improvements in sperm motility in a larger sample size of men taking selenium supplementation, were indicated.

In agreement with our study, Hawkes et al. (25) evaluated the effect of 48-weekdaily intake of 300 µg of selenium on sperm parameters in healthy volunteer men. Although selenium concentration increased to 61% in blood plasma and 49% in seminal plasma, selenium supplementation had no significant improving effect on serum androgen concentration or sperm motility. It was concluded that testes are as well-protected from selenium excess as well as selenium deficiency. Hence, consumption of a high level of selenium does not change selenium content of sperm and sperm parameters.

Consistent with our results, a systematic review and meta-analysis reported no significant difference in the sperm motility in the folate supplemented group compared with the control group. Also, there was no significant difference in sperm motility in folate plus Zn group in comparison with the control group. Zinc deficiency reduces

the absorption and metabolism of dietary folate because it works as a cofactor for the folate-metabolizing enzymes dihydrofolate reductase and y-glutamyl hydrolase. Therefore, a combination of folic acid and zinc work more efficiently than when they are taken alone (26).

In our study, no significant difference was found in sperm normal morph between the intervention and placebo groups.

Compatible with our results, Raigani et al. (27) showed that although seminal concentration of folic acid in folic acid and folic acid plus zinc sulfate groups was significantly improved as compared with B complex treated group, there was no significant difference in sperm normal morph among groups. One could say that the results of the above study are probably due to lack of control over the nutritional status. In other words, inappropriate diets, smoking, alcohol consumption and exposure to environmental contaminants have certainly had significant roles in the above study.

In consistence with our study, Mohammadi et al. (28) investigated the effect of Condensyl (B vitamins, N-acetyle cysteine, zinc, small amount of vitamin E and quercetine) as a complex of antioxidants to improve sperm parameters, two months after surgical varicocele induction in rats. A significant improvement in testis characteristics and considerable improvements in sperm morphology, were indicated. Folic acid in combination with B2, B3, B6 and B12, supports the one carbon cycle homocysteine re-methylation and increase the efficiency of one carbon metabolic cycle, and finally, reduction of spermatozoa damage in infertile men.

However, another study showed a significant improvement in sperm normal morph after selenium and vitamin E consumption in patients with asthenoteratozoospermia (24). In fact, the large sample size in the mentioned study and longer period of intervention in comparison to our study may have a considerable role in this significant statistical result.

The beneficial effect of antioxidant therapy to treat oxidative-stress-induced male infertility has been indicated in some studies. On the other hand, it can be claimed that reductive stress can be dangerous to cells as oxidative stress. It would be accrued due to ignoring the assessment of the redox status in infertile patients and over use of antioxidants. In these cases, subsequent to antioxidant therapy, endogenous oxidants which are necessary for sperm maturation reduced considerably (10).

In an evidence-based review by Ahmadi et al. (29), it was shown that administration of supplementations such as vitamin E and C, selenium and L-carnitine may ameliorate sperm concentration, motility, morphology and sometimes DNA integrity, but further clinical researches are recommended in order to determine appropriate antioxidant component and efficient antioxidant dose.

Similar to our study, Ardestani Zadeh et al. (13) investigated the effect of daily intake vitamin E) 400 mg, oral, daily) and folic acid (5 mg, oral, daily) and selenium (200 µg, oral, daily) on sperm parameters in 64 infertile men who underwent varicocelectomy and finally, contrary to our results, a significant increase in sperm concentration and motility was reported in the intervention group. This discrepancy with our results may be due to the type of patients studied, duration of intervention and the difference in the semen analysis device. They used anoptical microscope for semen analysis and infertile men were studied and treated with antioxidants for six months.

In another similar study, Moslemi and Tavanbakhsh (24) investigated the effect of daily intake of selenium (200 µg, daily) and vitamin E (400 mg, daily) given for at least 100 days, on sperm parameters in people with idiopathic infertility. Unlike our findings, they found a significant increase in sperm motility and normal morphology compared to before the intervention. Contrary to our study, their study had a higher sample size (690 people), and a longer period of antioxidant therapy but did not have a control group and treatment with placebo. Moreover, they used an optical microscope.

Using an effective component of antioxidants based on their special mechanism, blinded participants and using SQAV as an analytical medical device which performs a complete quantitative evaluation of semen quality and semen parameters in less than 2 minutes. SQAV is also a high-performance analyzer that incorporates technology in electro-optics, computer algorithms and video microscopy and provides a quick, precise and accurate automated semen analysis.

Short duration of intervention, small sample size, lack of access to combined antioxidant supplementation of selenium, vitamin E and folic acid at the mentioned dose and lack of access to similar shape and structure placebo.

Conclusion

Our findings indicated that consumption of selenium, vitamin E and folic acid in infertile men with asthenozoospermia was not effective. However, further prospective randomized controlled trials with a larger sample size, that evaluate semen oxidative status before starting antioxidant therapy, are recommended to confirm the effectiveness of antioxidant therapy on sperm parameters in males with idiopathic infertility.

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Authors' Contributions

R.B.; Designed the study, significantly contributed to data gathering, analysis, and preparing the manuscript. M.S.; Had a role in study design and data analysis. S.A.; Designed the study and supervised data analysis. A.A.; Supervised the study design and data gathering. Sh.H.; Had a role in data gathering and preparing the manuscript. All authors read and approved the final manuscript.

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Original Article

Evaluation of Total Antioxidant Activity and Oxidative Stress in Seminal Plasma from Dogs Supplemented with Fish Oil and Vitamin E

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Abstract

Background: We evaluated the effect of fish oil (FO) and FO in addition to vitamin E (VE) supplementation on total antioxidant activity of dog seminal plasma, and further assessed oxidative stress. Additionally, we measured the effect of this supplementation on hematological parameters and serum biochemistry.

Materials and Methods: In this experimental study, six male dogs were assigned to one of the following three groups for a period of 60 days using a replicated 3×3 Latin square design: control (CG), FO (FOG) and FO in addition to VE (FOEG). On days 0 and 60 of the trial, semen and blood samples were obtained. 2,2V-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) and ferric reducing antioxidant power (FRAP) assays were used to determine total antioxidant activity. Oxidative stress was determined by measuring total sulfhydryl group (T-SH).

Results: Dogs supplemented with FO alone had a lower total antioxidant activity in seminal plasma (ABTS: -59.86% vs. CG and -57.3% vs. FOEG; and FRAP: -37.3% vs. CG and -40.5% vs. FOEG), and higher oxidative stress (T-SH: +53.0% vs. CG and+60.2% vs. FOEG) compared with the other two groups ($P<0.05$). Serum triglyceride (TG) concentration decreased in FOG and FOEG compared with CG, on day 60 ($P<0.01$).

Conclusion: We concluded that total antioxidant activitydecreased and oxidative stress increased in seminal plasma of dogs after FO supplementation for 60 days.

Keywords: Antioxidants, Dog, Fish Oil, Oxidative Stress, Vitamin E

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Introduction

Omega-3 polyunsaturated fatty acids (ω-3 PUFA) are lipids with important biological functions that are found in fish oils (FO). They primarily include eicosapentaenoic acid (EPA, C20:5 ω-3) and docosahexaenoic acid (DHA, C22:6 ω-3). In dogs, FO supplementation improved sperm parameters and increased the percentage of EPA and totalω-3 PUFA in semen (1). However, PUFA such as EPA, DHA and arachidonic acid (AA, C20:4 ω-6) have a high risk of oxidation related to the number of double bonds in their molecules (2). PUFA oxidation can increase oxidative stress in cells. It been studied that the relationship among dietary fatty acid supplementation, sperm PUFA composition and total antioxidant activity, affects semen quality in animals' species (3). In order to balance high dietary ω-3 PUFA intake, adequate antioxidant concentrations are needed.

Antioxidants can be endogenous or exogenous; the latter can be obtained from the diet or dietary supplements. In this context, vitamin E (VE) is among non-enzymatic antioxidants in the body and plays an important role in protecting many different cells and organs against oxidative stress. Reactive oxygen species (ROS) are by-products of cellular metabolism which regulate physiological processes at low-to-moderate levels. However, antioxidant defenses may be inadequate to inactivate ROS, thereby, oxidative stress occurs and damages nucleic acids, lipids and proteins (4). Animals develop enzymatic and non-enzymatic antioxidant systems to reduce or prevent potential oxidative damage. In seminal plasma of dogs, antioxidant enzymes are produced by the testis, epididymis and accessory reproductive organs (5). Although a small amount of ROS is needed for capacitation, hyper activation, motility and acrosome reaction of the sperm as well as fertilization (6), the impactof ROS overproduction on antioxidant defense mechanisms may result in oxidative stress (7).

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We previously observed that sperm lipid peroxidation in dogs decreased with FO alone or FO plus VE supplementation, indicating a potentially protective effect of ω-3 PUFAand VE (8). However, antioxidant activity and oxidative stress in seminal plasma of dogs supplemented with FO alone or FO plus VE, have not been reported thus far. Consequently, here, we evaluated such effects for the first time in dog seminal plasma, and additionally determined the effect of the same supplementations on hematological and biochemical parameters. We hypothesized that FO supplementation would produce lower total antioxidant activity and higher oxidative stressthan FO in addition to VE, in dog seminal plasma.

Materials and Methods

Animals and treatments

In this experimental study, the Institutional Animal Care and Use Committee of the School of Veterinary Sciences, National University of La Plata, Buenos Aires, Argentina approved the study protocol (No. T34-1-13). Healthy male fertile dogs (age range 2-5 yr. and body weight [BW] range 20-30 kg), were used. Body condition score was 3 on a 5-point scoring scale (9). Clinical examination data (clinical record and physical exam), routine blood and biochemical test results and semen parameters were used to assess health status. Dogs were adapted to a standard commercial food (control diet) 15 days before the study. The nutrient composition of the commercial food described on a dry matter basis (%) was as follows: 3.7 metabolizable energy (kcal/g), 30.04 crude protein, 15.02 fat, 1.72 neutral detergent fiber, 7.83 ash, 1.50 calcium, 1.07 phosphorus, 0.048 mineral and vitamin mixture with: 12.39 VE, 0.20 vitamin K, 0.82 vitamin B1, 0.82 vitamin B2, 0.82 vitamin B6, 0.0004 vitamin B12, 0.12 folic acid, 0.10 nicotinic acid, 2.06 calcium pantothenate, 0.02 biotin, 82.1 choline, 0.01 copper, 0.01 iron, 0.02 zinc, 0.003 iodine, 0.01 manganese and 0.0002 selenium). Main ingredients of the extruded pelletwere: chicken meal, wheat, beef meal, rice, chicken oil, micronized soybean meal, gluten meal, corn, beet-root pulp, hydrolyzed chicken protein, fish oil, beer yeast, zeolite, salt, vitamin C, inulin, Yucca schidigera extract, antioxidants, potassium sorbate, yeast walls, yeast nucleotides, sodium hexametaphosphate, methionine and lysine.

Daily dietary intake was controlled and calculated according to the maintenance energetic requirements [MER=132 kcal×kg metabolic BW (BW0.75)] (10). Dogs had free access to water.

We conducted a randomized controlled trial using the random list generated by the computer soft ware and a replicated 3×3 Latin square design. Six crossbreed dogs were assigned to one of the following three groups: i. Control (CG; daily intake of the control diet), ii. FO (FOG; daily intake of the control diet plus a capsule containing the FO supplement dose of 54 mg FO/kg BW0.75), and iii. FO+VE (FOEG; daily intake of FOG plus 400 mg VE (8)). Individual FO dose was calculated as reported by Risso et al. (1). The FO capsule was administered daily with the first meal. Each experimental stage lasted 60 days. Finally, a 60-day washout among periods of treatments was included to avoid the carry-over effect of supplementation; during this period dogs were only given the control diet. This washout time was checked and evaluated in a previous study (8).

Dog owners gave written informed consent and committed to complying with the study protocol, i.e. feeding their dogs with the CG, FO or FOG diets. Furthermore, dogs were taken to the School of Veterinary Sciences everyweek during the experimental period to register their food intake and body weight. All evaluations were carried out by masked independent investigators.

Seminal plasma sample collection

Dogs were trained for semen collection by manual stimulation before starting the study. After sample collectionon days 0 and 60,1-ml aliquotsfrom each sample were centrifuged at 800g for 10 minutes to separate sperm from seminal plasma. After centrifugation, seminal plasma was snap-frozen and stored at -83°C until use (11).

Total antioxidant activity

Two assays were used to determine total antioxidant activityin dog seminal plasma: 2,2V-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS) and ferric reducing ability of plasma (FRAP), as described by Katalinic et al. (12), with some modifications. An ascorbic acid curve was established and the results were expressed as µg of ascorbic acid equivalents per ml. All measurements were performed in duplicate.

Oxidative stress

Oxidative stress in seminal plasma was determined by measuring total sulfhydryl groups (T-SH) using Ellman´s method with some modifications (13). A reduced glutathione (GSH) calibration curve was constructed; results were expressed as micrograms of GSH equivalents per milligrams of protein. All measurements were made in duplicate. Total seminal plasma protein concentration was determined using Bradford´s assay (14).

Blood samples

Venous blood samples for hematological and biochemical analyses were collected on days 0 and 60. Dogs were not fed or given waterfor at least 12 hours before the blood collection. Whole blood (5-ml) was drawn through venipuncture of the cephalic vein using a 21G needle and transferred to 1-mlEDTA tubes and 4-ml tubes without additives. Serum was separated by centrifugation at 1400 g for 5 minutes immediately after collection and then, transferred to another tube for biochemical analysis. Samples collected in tubes with or without EDTA were stored at 4°C for subsequent hematological and biochemical analyses within 6 hours after blood withdrawal.

Hematological and biochemical analyses

Hematological analysis was performed using an automated cell counter (Sysmex-KX-21) for the following variables:

erythrocytes, leukocytes, platelets, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration.

Serum biochemical analysis of total solids, total protein, albumin, globulins, glucose, alanine aminotransferase, urea, creatinine and triglyceride (TG) concentrations was performed on an Intelligent Clinical Chemistry Analyzer (INCAA, DICONEX, Argentina).

Standard commercial food analysis

Once pooled, standard commercial food was analyzed to determine first: dry matter (at 80ºC for 48 hours), and then, neutral detergent fiberfraction (NDF, Ankom 200 Fiber Analyzer, ANKOM Technology, Fairport, NY), crude protein fraction (CP, KjeldahlN x 6.25), lipids fraction (in the ether extract, XT101 ANKOM Technology Method 2) and finally ash (15).

Fatty acid composition of the standard commercial food and fish oil

For lipid extraction, samples of the standard commercial food and FO were studied as described by Folch et al. (16). Fatty acid composition was measured using gas chromatography working with a 30-mm capillary column [Omega Wax 250; Supelco, Bellefonte, PA, USA (Table 1)].

Table 1: Fatty acid content (%) of the standard commercial food and the fish-oil supplemented one

Fatty acid	Standard food	Fish oil
Tetradecanoic (14:0)	0.8	1.8
Palmitic (16:0)	20.9	24.0
Palmitoleic (16:1)	6.0	10.1
Stearic (18:0)	6.3	3.4
Oleic (18:1 ω-9)	27.9	22.3
Vaccenic (18:1 ω-7)	-	1.3
Linoleic (18:2 ω-6)	27.8	2.0
Gamma-linolenic (18:3 ω-6)	1.4	-
Alpha-linolenic (18:3 ω-3)	3.4	2.1
Eicosenoic (20:1 ω-9)	0.1	2.5
Dihomo-gamma-linolenic (20:3 ω-6)	0.2	-
Eicosatrienoic (20:3 ω-3)	0.4	-
Arachidonic (20:4 ω-6)	1.4	2.0
Eicosatetraenoic (20:4 ω-3)	-	0.8
Eicosapentaenoic (20:5 ω-3)	0.1	9.6
Docosapentaenoic (22:5 ω-6)	0.2	-
Docosapentaenoic (22:5 ω-3)	0.2	1.2
Docosahexaenoic (22:6 ω-3)	0.2	16.9
Σ Saturated fatty acids	28.0	29.2
Σ Monounsaturated fatty acids	34.0	36.2
Σ Polyunsaturated fatty acids	35.3	34.6
Σ ω-6	31.3	4.0
Σ ω-3	4.3	30.6

ω-9; Omega 9, ω-7; Omega 7, ω-6; Omega 6, and ω-3; Omega 3.

Statistical analysis

Areplicated 3×3 Latin square was considered. All the squares had two dogs that received three treatments. Data were registered in an Excel® database (Microsoft, USA) and processed using GraphPad Prism 4 for Microsoft Windows® (GraphPad Software, USA). Considering that a previously checked wash out period between treatments was included (8), data of antioxidant activity and oxidative stress were analyzed using one-way ANOVA followed by Tukey-Kramer test for multiple comparisons. Results of total antioxidant activity and oxidative stress assays areexpressed as means ± standard deviation (SD).

Data of hematological and biochemical parameters were analyzed by SAS PROC MIXED (version 9.4; SAS Institute Inc., Cary, NC, USA), with repeated measurements. The model contained within the random effect of dogs, the period of replicated Latin square design, the fixed effect of time (0 and 60 days), treatment (CG vs. FOG vs. FOEG), replication and time by treatment interaction. The time points with significant differences in time by treatment interaction were detected with the slice option of SAS. Significance was set at $P<0.05$. Data of hematological and biochemical analyses areexpressed as least square means (LSM) with standard error of means (SEM).

Results

The average age of dogs was 3.1 ± 1.4 years and they weighed 26.3 ± 2.1 kg.

Total antioxidant activity

Total antioxidant activity of dog seminal plasma did not differamongthe groups on day 0 ($P>0.10$). Conversely, it was lower in FOG on day 60, as shown by lower ABTS radical discoloration (CG, 1.52 ± 0.80 Eq µg ascorbic acid/ml; FOG, 0.61 ± 0.44 Eq µg ascorbic acid/ml; and FOEG, 1.43, ± 0.69 Eq µg ascorbic acid/ml) and lower FRAP (CG, 1.66 ± 0.63 Eq µg ascorbic acid/ml; FOG, 1.04 ± 0.41 Eq µg ascorbic acid/ml; and FOEG, 1.75 ± 0.79 Eq µg ascorbic acid/ml) compared toCG and FOEG (treatment effect, $P<0.05$, Figs.1, 2).

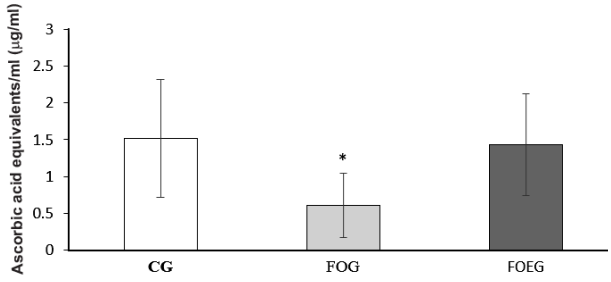

Fig.1: Total antioxidant activity in dog seminal plasma determined by 2,2V-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS) assay in the control (CG, n=6), fish oil (FOG, n=6) and fish oil in addition to vitamin E group (FOEG, n=6) on day 60. *; Treatment effect, $P<0.05$.

Table 2: Hematological and biochemical parameters in samples from dogs assigned to the control (CG, n=6), fish oil (FOG, n=6) and fish oil in addition to vitamin E group (FOEG, n=6), on days 0 and 60

Blood parameters	Day 0				Day 60			
	CG	FOG	FOEG		CG	FOG	FOEG	
	LSM	LSM	LSM	SEM	LSM	LSM	LSM	SEM
Erythrocytes ($\times10^6$/µl)	7.07	7.35	6.27	0.46	7.27	7.15	7.02	0.40
Leukocytes ($\times10^3$/µl)	10.29	11.11	7.98	1.48	12.43	11.83	10.43	1.26
Platelets ($\times10^5$/µl)	2.86	2.61	2.65	0.76	3.82	1.97	2.78	0.74
Hemoglobin (%)	15.72	15.76	15.21	0.70	16.49	17.18	17.67	0.65
Hematocrit (%)	46.53	48.37	47.48	2.00	45.73	47.55	48.27	1.75
MCV (fl)	67.88	67.92	70.28	0.82	68.80	67.91	68.13	0.70
MCH (pg)	24.11	24.30	24.75	0.45	24.22	23.95	23.65	0.38
MCHC (%)	35.66	35.74	35.82	0.61	35.10	35.28	34.19	0.56
Total solids (g/dl)	6.52	6.34	6.86	0.39	6.12	6.22	6.58	0.35
Total protein (g/dl)	5.97	6.39	6.30	0.35	6.05	6.31	6.27	0.28
Albumin (g/dl)	3.08	2.86	2.35	0.25	3.35	2.85	2.60	0.19
Globulin (g/dl)	3.00	3.21	3.72	0.37	2.72	3.22	3.48	0.31
Glucose (g/l)	0.81	0.69	0.83	0.10	0.76	0.87	0.82	0.12
ALT (U/l)	32.80	40.24	47.37	8.69	30.29	44.92	34.29	9.29
Urea (g/l)	0.59	0.56	0.70	0.10	0.58	0.61	0.66	0.11
Creatinine (mg/dl)	1.01	0.97	1.18	0.35	0.95	0.90	1.38	0.38
Triglycerides (g/l)	0.88	1.04	1.07	0.16	1.15	0.75*	0.56*	0.15

LSM; Least square mean, SEM; Standard error of means, MCV; Mean corpuscular volume, MCH; Mean corpuscular hemoglobin, MCHC; Mean corpuscular hemoglobin concentration, ALT, Alanine aminotransferase, and *; Treatment × time interaction, P<0.01. The remaining parameters were not significantly different (P>0.05).

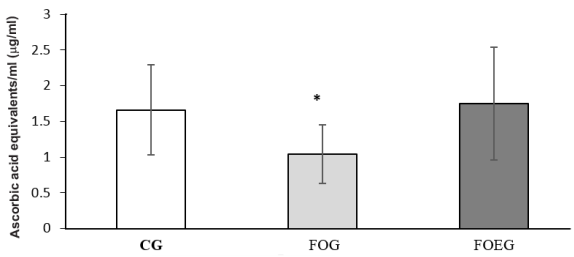

Fig.2: Total antioxidant activity in dog seminal plasma determined by ferric reducing ability of plasma (FRAP) assay in the control (CG, n=6), fish oil (FOG, n=6) and fish oil in addition to vitamin E group (FOEG, n=6) on day 60. Values are means and standard deviation. *; Treatment effect, P<0.05.

Oxidative stress

In seminal plasma, T-SH content assessment showed that oxidative stress was higher in FOG (1.92 ± 0.72 Eq µg glutathione/mg protein) compared to CG (4.09 ± 1.59 Eq µg glutathione/mg) and FOEG (4.83 ± 1.86 Eq µg GSH /mg) after 60-day supplementation (treatment effect, P<0.05, Fig.3).

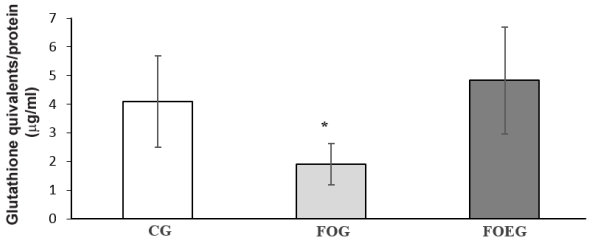

Fig.3: Oxidative stress determined in terms of total sulfhydryl groups (T-SH) in seminal plasma of dogs in the control (CG, n=6), fish oil (FOG, n=6) and fish oil in addition to vitamin E group (FOEG, n=6), on day 60. Values are means and standard deviation. *; Treatment effect, P<0.05.

Hematological and biochemical parameters

Serum TG concentration decreased in FOG and FOEG compared to CG on day 60 (treatment×time interaction, P<0.01). No effect for FO alone or FO plus VE supplementation was observed on the other hematological and biochemical parameters (P>0.05, Table 2).

Discussion

In this study, the effect of FO alone or FO with VE supplementation on total antioxidant activity and oxidative stress was assessed in seminal plasma of dogs. Additionally, the effect of both supplements on hematological and biochemical parameters was evaluated. In accordance with our hypothesis, FO supplementation decreased total antioxidant activity after 60 days, while increasing oxidative stress.

Seminal plasma is composed of proteins, amino acids, enzymes, carbohydrates, lipids, major minerals and trace elements (17). The lipid fraction only contains 2.5% fatty acids, from which, 85% are saturated fatty acids and 15% are unsaturated fatty acids (13.3% monounsaturated fatty acids and1.7% PUFA) (18).

In previous studies on dietary FO supplementgiven to dogs, we observed an increase in AA, EPA and total ω-3 PUFA concentrations in semen. Although in the present study we did not evaluate PUFA composition in seminal plasma, its concentration could have been affected by the ω-3PUFA content in the FO-supplemented diet (1, 8). Such increases in seminal plasma ω-3 PUFA concentrations

could be the result of an imbalance between the antioxidant and oxidant systems, with concomitant decreases in total antioxidant activity and increases in oxidative stress.

VE has been shown to directly neutralize superoxide anion, hydrogen peroxide and hydroxyl radical (19). In this sense, Domosławska et al. (17) reported that supplementation with selenium and VE for 60 days enhanced the antioxidant status of sperm in dogs. In the present study, the higher VE content in FOEG could have maintained total antioxidant activity/oxidative stress balance in seminal plasma.

Another report showed the effects of PUFA consumption on oxidative status in dogs. Dogs received PUFA-rich diets with the proposed of modifying oxidative stress markers in blood; however, the concentration used was not enough to cause an imbalance between the generation and elimination of reactive species by the antioxidant defense systems of dogs. In the cited report, treatments contained 20% acid hydrolyzed fatand 60% PUFA inDHA-enriched soybean oil, and 18% PUFA in bovine tallow (20). In agreement with those results, the PUFA content in the diets of the present study met the maintenance requirements for dogs recommended by the National Research Council 2006 (10).

The National Research Council 2006 (10) cites that diets with more than 50% fatty acid dry matter, alter the antioxidant system. Although in the present study, oxidative stress was not determined directly in blood samples, the evaluated hematological and biochemical parameters (i.e. albumin and total protein) did not show differences among the study groups. On the other hand, the results obtained in seminal plasma may reflect a different stage from that observed in blood results. Consequently, simultaneous evaluation of total antioxidant activity and oxidative stress in blood and seminal plasma, would be useful for a better interpretation of results.

Regarding the lower serum TG concentrations found in FOG and FOEG,our results support the already reported role of ω-3 PUFA in lipid metabolism (21). Thus, the FO dose currently used could be consideredsafe for treatment of hyperglycemia in dogs.

Conclusion

We observed that the decreased antioxidant activity and increased oxidative stress found in seminal plasma of dogs supplemented with FO, would be related to an increased risk of oxidative stress due to higher amounts of PUFA in the diet. Supplementation with FO in addition to VE would exert a protective role against ROS. Further studies are needed toclarify the mechanisms responsible for such total antioxidant activity decrease and possible short- and long-term consequences.

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Authors' Contributions

A.R., F.J.P.; Performed the experiments, analyzed and discussed the results, and wrote the manuscript. Y.C.; Provided the commercial food and collaborated in the experiments. G.S.; Collaborated in the experiments and analyzed and discussed the results. All authors edited and approved the final version of this paper for submission.

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Original Article

Association of *MTHFR* C677T and *MTRR* A66G Gene Polymorphisms with Iranian Male Infertility and Its Effect on Seminal Folate and Vitamin B12

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Abstract

Background: The relation between key enzymes in regulation of folate metabolism and male infertility is the subject of numerous studies. We aimed to determine whether 5, 10-methylenetetrahydrofolate reductase (*MTHFR*) C677T and methionine synthase reductase (*MTRR*) A66G genotypes are associated with male infertility in Iranian men and to evaluate its effect on seminal levels of folate and vitamin B12.

Materials and Methods: In this retrospective study, semen and peripheral blood samples were collected from 254 men with oligoasthenoteratozoospermia (OAT) and 77 normozoospermic men who attended Avicenna infertility clinic. Single nucleotide polymorphism (SNP) analysis was carried out in genomic DNA by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method for *MTHFR* C677T and *MTRR* A66G gene polymorphisms.

Results: In *MTHFR* C677T, our founding showed that T carrier was conversely lower in OAT than normozoospermic men (χ^2-test=7.245, P=0.02) whereas in *MTRR* A66G, A and G carrier showed no significant difference between the two groups (χ^2-test=1.079, P=0.53). The concentration of seminal folate was not different between normozoospermic (18.83 ± 17.1 ng/ml) and OAT (16.96 ± 14.2 ng/ml) men (P=0.47). The concentration of vitamin B12 was slightly higher in normozospermic men (522.6 ± 388.1 pg/ml) compared to OAT men (412.9 ± 303.6 pg/ml, P=0.058).

Conclusion: The *MTHFR* C677T and *MTRR* A66G have no effect on the concentrations of seminal folate and vitamin B_{12}. The present study showed that two SNPs of *MTRR* A66G and *MTHFR* C677T cannot be seen as a risk factor for male factor subfertility.

Keywords: Folate, Male Infertility, *MTHFR*, *MTRR*, Vitamin B12

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Introduction

Infertile men are adult individuals who fail to achieve pregnancy after one year of having intercourse without any birth control. Almost 50% of fertility problems are related to male factors and most of the affected men exhibit low sperm quality (1). Folates participate in DNA, RNA and histone methylation reactions -via involvement in homocysteine metabolism-which can be involved in spermatogenesis (2). 5, 10-methylenetetrahydrofolate reductase (MTHFR) is the basic regulatory enzyme of folate metabolism. *MTHFR* gene is located on chromosome 1 (1p36.22) (3). Methionine synthase reductase (*MTRR*) gene, on chromosome 5, also plays a vital role in DNA synthesis. It is well known that folate and methionine metabolism play essential roles in both DNA methylation and synthesis (4).

MTHFR and MTRR play key interrelated roles in folate metabolism. MTHFR catalyzes the regulation of cellular methylation through the conversion of 5, 10-methylene tetrahydrofolate (THF) to 5-methyl-THF, the primary circulating form of folate metabolism. MTRR is required for the reductive methylation of vitamin B12, also known as cobalamin, an activated cofactor for methionine synthase (MTR), which catalyzes the methylation of homocysteine to methionine (5).

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There are two important polymorphisms in the *MTHFR* gene; C677T (6) and A1298C (7) mutations which can affect its biological activity. Numerous researchers have focused on a C>T mutation at nucleotide position 677 in exon 3 of the *MTHFR* gene which changes alanine to valine (8). The *MTHFR* C677T mutation decreases the corresponding protein activity, so that it impairs the ability to process folate. This mutated gene leads to increase amino acid homocysteine level (9). *MTHFR* polymorphisms are prevalent in terms of the local area C677T and its distribution differs among different population. The prevalence of heterozygous and homozygous state is about 40% and 10% in Caucasians, respectively (10).

The homozygous C667T in the *MTHFR* gene is reported to be associated with the risk of a number of diseases in humans, including some cardiovascular abnormalities (atherosclerosis, cardiovascular) (11), cancers and neural tube disorders (4). Previous studies have shown that activity of MTHFR is much higher in testis instead of the other major organs in adult male mouse. So it was suggested that might play an important role in spermatogenesis (12).

One of the most prevalent polymorphisms in the *MTRR* gene is A66G, which results in an amino acid substitution from methionine to isoleucine at codon 22 (M22I) (4). *MTRR* 66A>G also affects conversion of homocysteine to methionine, which adversely influences enzyme activity and thus is considered as a genetic risk factor for hyperhomocysteinemia. *MTRR* A66G polymorphism may also induce DNA hypomethylation by regulating homocysteine (Hcy) levels (5).

Functional studies indicated that individuals possessing both mutations showed the lowest enzyme activities (4). As a result, both DNA methylation and DNA synthesis may be altered by interacting with homocysteine, vitamin B12 and folate (13). Polymorphisms in the *MTHFR* gene, 677C>T, 1298A>C and the *MTRR* gene, 66A>G, are associated with male infertility.

In the previous study, we demonstrated that administration of folic acid and co-administration of folic acid and zinc sulphate during randomized, double-blind dietary program did not improve the quality of sperm in infertile men with severely compromised sperm parameters, oligoasthenoteratozoospermic (OAT) (1). The present study aimed to assess and compare the mutations in *MTHFR* gene 677C>T, as well as *MTRR* gene 66A>G in men with OAT and normozospermia. Furthermore, we investigated the correlation of these genetic variants with seminal folate and vitamin B12 levels.

Materials and Methods

Patients

In this retrospective study, semen analysis was done for 254 men with OAT and 77 men with normozospermia who attended Avicenna Infertility Clinic (AIC; Tehran, Iran). In all samples, semen parameters, sperm concentration, sperm motility, viability and morphology were evaluated in accordance with the WHO guidelines (14) and they were classified as normozoospermic and OAT samples. Infertile men with sperm concentrations of $<20\times10^6$ ml^{-1}, sperm motility <50% (grades a, b, c) and normal sperm morphology <30% were included as OAT and infertile men with normal sperm parameters were classified as normozospermia.

Patients with leukocytospermia (leukocyte concentration greater than 1×10^6/ml), varicocele, chronic systemic diseases, autoimmune disorders or history of smoking, in addition to excessive alcohol and drug consumption were excluded from the study.

Ethical approval

Each participant provided a written informed consent before the collection of their biological samples. Additionally, the study was approved by Ethics Committee of the Avicenna Research Institute. The ethics code (85.3496) was allocated for our project. All procedures were done in accordance with the ethical Helsinki standards.

Preparation of semen samples

The semen samples were collected by masturbation only 48-72 hours after sexual abstinence and ejaculated into a clean plastic specimen cup. Samples were delivered to the laboratory within 1 hour of collection at 20-40°C. Following complete liquefaction, standard semen analysis was conducted for all participant according to WHO guidelines (14). The remnant semen was centrifuged at 2000 rpm for 5 minutes and supernatant was divided in several aliquots and stored at -20°C for future analysis.

Blood sampling and DNA extraction

Genomic DNA was extracted from white blood cells according to sodium salting out extraction method. The DNA purity and concentrations were determined by measurement of absorbance at 260/280 nm.

Genotyping *MTHFR* C677T

The C677T SNP (rs#1801133) of *MTHFR* gene was studied by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method.

We employed the
F: 5'-CATCCCTATTGGCAGGTTACCC-3' and
R: 5'-TGCGAGGACGGTGCGGTGAGA-3'

PCR primers which were designed using Gene Runner software. PCR cycles were as follow: 32 cycles of 94°C for 30 seconds, 58.4°C for 30 seconds and 72°C for 30 seconds. A 271 bp PCR product was digested overnight by HinfI restriction enzyme endonuclease (New England Biolabs, USA) with the recognition sequence 5'-GˆAGTC-3' at 37°C. The PCR products were visualized on 2% agarose gel. A 271 bp fragment was seen in the absence of mutation [wild-type (CC)], 271, 177 and 94 bp fragments were observed in the presence of one allele mutation [heterozy-

gotes (CT)], and 177 and 94 bp fragments were seen in the presence of two allele mutations [homozygotes (TT)].

Genotyping *MTRR* A66G

The PCR-RFLP assay was employed for identifying *MTRR* A66G polymorphisms.

The primers for *MTRR* A66G:

F: 5'-AGGCAAAGGCCATCGCAGAAGA**C**AT-3' and
R: 5'-GGCTCTAACCTTATCGGATTCACTA-3'

were designed using Gene Runner software. The forward primer was comprised of a mismatch (underlined base C in the primer sequence), generating an NdeI restriction site, 5'-CAˆTATG-3', when the polymorphic allele was present. PCR cycles were as follow: 35 cycles of 94°C for 30 seconds, 58.4°C for 30 seconds and 72°C for 30 seconds. The anticipated PCR product of 98 bp was digested into fragments of 74 and 24 bp by NdeI restriction enzyme endonuclease (NEB, USA) in the presence of the G allele but remains uncut in the presence of the A allele (15). It means, a 98 bp fragment was seen in the absence of mutation [wild-type (AA)], 98, 74 and 24 bp fragments were observed in the presence of only one mutated allele [heterozygotes (AG)], and 74 and 24 bp fragments were seen in the presence of two mutated alleles [homozygotes (GG)].

Measurement of seminal vitamin B12 and folate concentration

Semen folate and vitamin B_{12} levels were measured using radioimmunoassay (RIA) method according to the manufacturer's instructions (MP BiomedicalsSimul-TRAC-SNB Radioassay Kit VITAMIN B12[57Co]/ folate[^{125}I]; MP Biomedicals, USA).

Statistical analysis

Results of the two groups were compared using SPSS 16.0 for Windows software (SPSS Inc, Chicago, IL, USA). Two-Sample Kolmogorov-Smirnov test (K-S test) revealed that our data follow a normal distribution and thus parametrical test was employed. Monte Carlo test was conducted to compare *MTHFR* C>T and *MTRR* A>G mutations in normozoospermic and OAT groups. Folate and vitamin B12 concentrations in two studied groups were compared using parametric tests such as Independent-Samples t test and unconditional logistic regression model to calculatethe odds ratios (OR) and 95% confidence intervals (95% CI). Fisher's exact test was employed in analyzing association of *MTHFR* and *MTRR* mutations with semen concentration of folate and vitamin B12. Differences were considered significant if P value was<0.05.

Results

Demographic characteristic information, including age and infertility period in OAT and normozoospermia men, are given in Table 1, showing that there is no significant difference between them. Therefore, they could not be considered as confounding effect.

Table 1: Demographic characteristic information

Groups	Number	Age (Y)	Length of infertility (Y)
Oligoasthenoteratozoospermic group	254	37.07 ± 7.26	5.89 ± 6.4
Normozoospermic group	77	32.33 ± 4.03	5.24 ± 5.5
t test, P value		-1.59, 0.12	-0.4, 0.69

Data are presented as mean ± SD. The parameters were compared using Independent-Samples t test.

Figure 1 shows the results of PCR-RFLP for MTHFR C677T and MTRR A66G gene polymorphisms with regard to digestion using HinfI and NdeI, respectively.

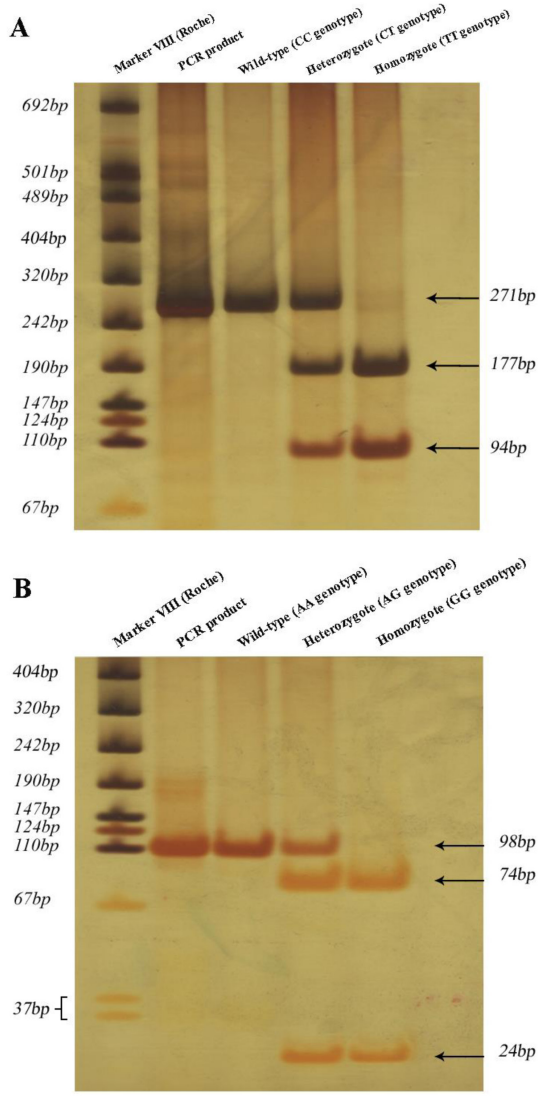

Fig.1: Polymorphisms of *MTHFR* and *MTRR* genes. **A.** Analysis of *MTHFR* C677T gene polymorphism with respect to digestion using HinfI restriction enzyme endonuclease to recognize 5'-GˆAGTC-3' sequence. Wild-type allelesof *MTHFR* gene were not digested with HinfI, but mutated alleles were digested using HinfI, resulting in two fragments. **B.** The result of PCR-RFLP for *MTRR* A66G gene polymorphism with respect to digestion using NdeI restriction enzyme endonuclease to recognize 5'-CAˆTATG-3' sequence. Wild-type alleles of *MTRR* gene were not digested with NdeI, but mutated alleles were digested using NdeI, resulted in two fragments. To detect fragments, we performed a 10% polyacrylamide gel with silver nitrate staining. PCR-RFLP; Polymerase chain reaction-restriction fragment length polymorphism.

Analysis of *MTHFR* gene polymorphism indicated that frequency of CC genotype in OAT men was higher than in men with normozoospermia (59.8 vs. 42.9%), while the frequency of CT genotype was lower in OAT men (46.8 vs. 34.3%). In addition, frequency of TT genotype was lower in OAT men (10.3 vs. 5.9%, Table 2).

As given in Table 2, analysis of *MTRR* gene polymorphism in normozoospermic and OAT men also revealed that frequency of AA genotype in normozoospermic group was higher than in OAT group (26 vs. 22%). Frequency of AG genotype in the two studied groups was almost identical (61 vs. 60.6%) and frequency of GG genotype in OAT group was higher than normozoospermic group (17.4 vs. 13%).

Table 2: Frequency of *MTHFR* and *MTRR* genotyping

Groups	MTHFR genotyping		
	CC	CT	TT
Normozoospermic group	33 (42.9)	36 (46.8)	8 (10.3)
Oligoasthenoteratozoospermic group	152 (59.8)	87 (34.3)	15 (5.9)
	***MTRR* genotyping**		
	AA	AG	GG
Normozoospermic group	20 (26)	47 (61)	10 (13)
Oligoasthenoteratozoospermic group	56 (22)	154 (60.6)	44 (17.4)

Data are presented as n (%). Comparing between cases and controls was performed using Monte Carlo test.

According to Table 3, comparison of C (CC) and T carrier (CT+TT) in *MTHFR* C677T between normozoospermic and OAT groups showed that T carrier was conversely lower in OAT group than normozoospermic group (χ^2-test=7.245, P=0.02). Additionally, our analysis did not show significant difference (χ^2-test=1.079, P=0.53) in A (AA) and G carrier (AG+GG) from *MTRR* A66G between normozoospermic men and OAT men (Table 3).

Table 3: Association between normozoospermic group and OAT group in *MTHFR* and *MTRR* SNPs

Groups	*MTHFR* SNP number		χ^2-test, P value
	C carrier	T carrier	
Normozoospermic group	33	44	7.245, 0.02
Oligoasthenoteratozoospermic group	152	102	
	***MTRR* SNP number**		
	A carrier	G carrier	
Normozoospermic group	20	57	1.079, 0.53
Oligoasthenoteratozoospermic group	56	198	

Comparing the cases with controls was performed using Monte Carlo test. OAT; oligoasthenoteratozoospermia and SNP; Single nucleotide polymorphism.

According to Table 4, the mean value of folate was 18.83 ± 17.1 ng/ml in normozoospermic group. So that 44.2% of the subjects had lower concentration than normal folate levels. In OAT group, the mean value of folate was 16.96 ± 14.2 ng/ml. Thus 36.1% of the subjects had lower concentration than normal folate levels. However, there was no significant difference in semen folic acid content between the two groups (P=0.47, OR=0.99, 95% CI=0.97-1.02). But, the concentration of vitamin B12 was slightly higher in normozospermic men compared to OAT men (522.6 ± 388.1 vs. 412.9 ± 303.6 pg/ml, P=0.058, OR=0.999, 95% CI=0.998-1.00). Low vitamin B_{12} concentrations were identified in 19.5 and 20.5% of the normozoospermic and OAT men, respectively.

Furthermore, seminal folate and vitamin B12 concentrations were compared between C and T carrier in MTHFR C677T. They were also compared between A and G carrier in MTRR A66G, while none of them was statistically different (Table 5).

Table 4: Comparison of seminal folate and vitamin B_{12} concentration in oligoasthenoteratozoospermia and normozoospermic men

Groups	Folate concentration (ng/ml)	P value	Vitamin B_{12} concentration (pg/ml)	P value
Normozoospermic group	18.83 ± 17.1	0.47*	522.6 ± 388.1	0.058**
Oligoasthenoteratozoospermic group	16.96 ± 14.2		412.9 ± 303.6	

Data are presented as mean ± SD. The parameters were compared using Independent-Samples t test. *; OR: 0.99, 95% CI: 0.97-1.02 and **; OR: 0.999, 95% CI: 0.998-1.00.

Table 5: Comparison of seminal folate and vitamin B12 levels between C>T carrier in *MTHFR* C677T and A>G carrier in *MTRR* A66G

Concentration	*MTHFR* SNP number		P value
	C carrier	T carrier	
Folate (ng/ml)	16.88 ± 14.24	19.56 ± 17.59	0.31
Vitamin B_{12} (pg/ml)	427.51 ± 398.81	280.39 ± 191.14	0.17
	***MTHFR* SNP number**		
	A carrier	G carrier	
Folate (ng/ml)	16.71 ± 13.41	18.59 ± 16.65	0.54
Vitamin B_{12} (pg/ml)	506.89 ± 276.32	350.80 ± 360.95	0.25

Data are presented as mean ± SD. The parameters were compared using Fisher's exact test.

Discussion

Folate and other vitamins are vital for DNA synthesis and establishment of epigenetic modifications like DNA/histone methylation (6). Spermatogenesis produces male haploid germ cells that involves distinct cellular and chromatin changes. Folate and normal activity of the corresponding enzymes play important role in nucleotide synthesis, methylation, maintenance of genomic integrity and prevention from DNA damage (12). Consequently, polymorphisms in folate metabolic genes have significant effect on spermatogenesis by inducing DNA hypomethylation and inducing mistakes in DNA repair, strand breakage and chromosomal abnormalities effect on the quality of sperm (16). There is substantial experimental evidence that folate metabolism pathway enzymes are essential for male spermatogenesis. Several SNPs which affect folate metabolism have been recognized, which in turn are associated with the cause of some defects (8). There was another study revealed that male mice lacking *MTHFR* suffered from severe reproductive defects. In these mice, spermatogenesis was failed during early postnatal development and resulted in total infertility (17).

But our analysis regarding the association of *MTHFR* C677T SNP and male infertility showed that T carrier is significantly lower in OAT men compared tonormozoospermic men. Liu et al. (3) found that the C677T mutation might affect the stability of RNA by performing a secondary structure of *MTHFR* mRNA sequence. Some studies showed that *MTHFR* C677T, A1298C and *MTRR* A66G polymorphisms are the risk factors with susceptibility to male infertility (4, 7, 18). However, the other study could not find any evidence for an association between reduced sperm counts and polymorphisms in enzymes involved in folate metabolism in the French population (19).

In the present study, no significant difference was observed in *MTRR* A66G variant between normozoospermic and OAT men. Similar studies were also developed by Kurzawski et al. (2) and Ni et al. (8), showing no significant difference in genotype frequencies of the gene polymorphisms in folate pathway between infertile and fertile men. They demonstrated that these genes in folate pathway were not risk factors for non-obstructive male infertility in the Polish and Chinese population (2, 8). In contrary, Liu et al. (3) proved using meta-analysis with trial sequential analysis that the genetic mutations in the folate-related enzyme genes played an important role in male infertility. However, the results of previous studies regarding this subject remain conflicting rather than definitive.

We found no statistical difference in seminal vitamin B12 and folate concentrations between normozoospermic and OAT men. But another study by Crha et al. (20) reported that folate and cobalamin were higher in seminal plasma from obstructive azoospermia than non-obstructive azoospermia patients.

The current study also demonstrated no association between *MTHFR* C677T and *MTRR* A66G polymorphisms with seminal vitamin B_{12} and folate concentrations innormozoospermic and OAT men. Similar study was also developed by Murphy et al. (21) while no significant correlation was observed between vitamin B_{12}, folate, total homocysteine (tHcy) concentrations and any semen parameters in fertile and infertile men. They also found that infertile men had lower serum folate concentrations than fertile men, but there was no significant difference in red blood cell folate (RCF), B_{12} or tHcy. However, another study showed that adequate intake of vitamins B_9 and B_{12} affects sperm parameters in men with different *MTHFR* polymorphisms, especially T allele genotypes (22).

As anticipated, OAT men had overall poorer semen parameters than normozoospermic controls. The results showed that infertile subjects had lower semen concentration, greater percentage of abnormal sperm morphology, higher percentages of non-motile sperm and more incidences of DNA fragmentation than fertile controls.

Some limitations of this study were low number of normozoospermic men in comparison with OAT men and absence of some demographic data regarding individuals that participated in the current study.

Conclusion

The present study donot reveal the *MTHFR* C677T and *MTRR* A66G gene polymorphisms as risk factor for male factor subfertility in Iranian population. In addition, there is no statistical difference in seminal vitamin B_{12} and folate concentrations between normozoospermic and OAT men. Moreover, this study indicated no association between *MTHFR* C677T and *MTRR* A66G polymorphisms with seminal vitamin B_{12} and folate concentrations in normozoospermic and OAT men. However, larger sample size and well-designed studies are needed to compare the effect of other folate-related enzyme genes in the Iranian population. Larger sample size could generate better statistical results and sufficient data to confirm the improvement of secondary outcomes.

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Authors' Contributions

M.R; Wrote the manuscript and performed experiments. M.R.S.; Designed the research. M.R., N.L.; Obtained ethics approval and involved in the collection and analysis of the data. N.L., M.R.S.; Read the manuscript and commented about different sections of manuscript. M.S., B.J.; Contributed to utilize new reagents/analytical tools. All authors read and approved the final manuscript.

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Original Article

Genetic Determinants of Premature Menopause in A Mashhad Population Cohort

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Abstract

Background: Premature menopause is characterized by amenorrhea before age of 40 years, markedly raised serum luteinizing hormone (LH) level, follicle-stimulating hormone (FSH) level and reduced serum level of estradiol. Genome-wide analysis suggested several loci associated with premature menopause. Here, we aimed to analyze association of variants at the *MCM8*, *FNDC4*, *PRRC2A*, *TLK1*, *ZNF346* and *TMEM150B* gene loci with premature menopause.

Materials and Methods: In this cross-sectional study, a total of 117 women with premature menopause were compared to 183 healthy women. Anthropometric indices were measured in all participants: height, weight, body mass index (BMI), waist circumference (WC) and wrist circumference. Eight single-nucleotide polymorphisms (SNPs) of the indicated genes (rs16991615, rs244715, rs451417, rs1046089, rs7246479, rs4806660, rs10183486 and rs2303369) were identified from the literature. Genotyping was performed using tetra-ARMS polymerase chain reaction (PCR) and ASO-PCR methods.

Results: T allele of the rs16991615, rs1046089, rs7246479 and rs10183486, C allele of rs244715, rs451417 and rs4806660 as well as TT genotype of rs2303369 were associated with an increased risk of premature menopause, likely causing susceptibility to primary ovarian insufficiency (POI) in comparison with C allele. We also found an association between the rs16991615 SNP with premature menopause. Frequency of the minor allele in cases was increased for all SNPs in comparison with controls. All minor alleles, except for rs2303369, showed a statistically significant increased odds ratio (OR). However, after Bonferroni correction for multiple testing, none of the P values were remained significant.

Conclusion: The selected polymorphisms in *MCM8*, *FNDC4*, *PRRC2A*, *TLK1*, *ZNF346* and *TMEM150B* genes may potentially affect susceptibility to premature menopause, although replication of the results in larger cohort could clarify this.

Keywords: Association Studies, Genetic Polymorphisms, Haplotype, Premature Menopause

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Introduction

Premature menopause or primary ovarian insufficiency (POI) is the occurrence of menopause before age of 40 years (1). This condition is characterized by amenorrhea, increased luteinizing hormone (LH) level, follicle-stimulating hormone (FSH) level and reduced level of estradiol. POI may be idiopathic or associated with autoimmune and genetic abnormalities (2). Other than that, factors such as infections, chemo and radiotherapy, surgery and adverse effects of drugs could also be contributory

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(3, 4). POI may lead to increase of all-cause mortality, cardiovascular diseases, depression, type 2 diabetes and increased risk of bone fracture (5-8).

Genetic factors appear to be responsible for approximately 50% of the variations in the onset of menarche and menopause (9). Several studies have found different genetic loci associated with age at natural menopause (ANM) (10-14). DNA repair genes such as *EXO1*, *HELQ*, *UIMC1*, *FAM175A*, *FANCI*, *TLK1*, *POLG* and *PRIM1* along with genes like *IL11*, *NLRP11* and *PRRC2A* which are involved in immune function, are located at these loci (14).

Mutations in some of these genes have been indicated to be involved in POI. For example, Tenenbaum-Rakover et al. (15) found two new homozygous mutations in the *MCM8* gene, a frameshift (c.1469-1470insTA) and a splice site mutation (c.1954-1G>A). These mutations were shown to increase the risk of chromosomal breakage after mitomycin C exposure. The authors also found that *MCM8* plays a pivotal role in gonadal development. Desai et al. (16) also found that mutations in *MCM8* and *MCM9* could be a significant risk factor for POI. Besides, several other studies have replicated findings obtained from rs16991615 polymorphism, located in *MCM8*, as a significant single-nucleotide polymorphism (SNP) associated with ANM in different populations (17, 18). Llácer et al. (19) showed that *IL11*- rs11668344 and *PRRC2A*-rs1046089 are associated with poor ovarian response indicating that women carrying these polymorphisms have decreased oocyte production in response to controlled ovarian hyper stimulation. On this basis, we selected eight different SNPs in the genes more commonly associated with POI. They have not previously been studied in the Iranian population. We aimed to determine whether these SNPs are involved in the development of premature menopause and to find their association with anthropometric characteristics in a population sample from Mashhad city in Iran.

Materials and Methods

Studying participants and anthropometric indices

In this cross-sectional study, a total of 117 women who were originally enrolled in the Mashhad stroke and heart atherosclerotic disorder (MASHAD) study were recruited and compared to 183 healthy women. "MASHAD study" is a 10-years cohort study of a total of 9704 individuals aged 35-65 years, from an urban population in eastern part of Iran. They were selected using a stratified cluster random sampling design. The healthy group was chosen from women without history of menopause before the age of 40 years to match with the cases group in terms of age. The cases were also selected from this study cohort based on their history of premature menopause. A complete history was taken for all subjects, including any surgical procedures, acute or chronic diseases, chemotherapy or radiation therapy, smoking habit, medications as well as the results of periodic examinations. Women who were not willing to enroll in the present study, were excluded. We included all eligible and available volunteer women who met the study criteria. To clarify this, 9704 people participated in "MASHAD study" project. 5838 of the cases were women. 2747 women reached menopause by the time of starting study. Furthermore, 895 women were removed from the project (due to hysterectomy, oophorectomy and other secondary causes). Out of the 1852 remaining women, 117 (all the cases existed in the population) cases were below the age of 40 years at their menopause. They were selected according to the POI definition, including: i. Women going through menopause before the age of 40 years, ii. 12 months of consecutive menstrual cessation or iii. Elevated serum FSH levels >40 IU/L (repeated at four-week intervals) (1). Other inclusion criteria were provided as follows: women with POI, women younger than 40 years old, women without history of diseases affecting menstruation, women without any genetically confirmed diseases or syndromes that early menopause was part of their manifestations, women without any previous surgeries affecting menstruation (oophorectomy, hysterectomy) and women who were not using any drugs affecting menstruation. Hence, 117 women were considered eligible to be enrolled in the present study. Anthropometric indices such as height, weight, body mass index (BMI), waist circumference (WC) and wrist circumference were measured in all participants. Informed consent was obtained from all participants. The approval number from the constituted review board, the Ethics Committee of Mashhad University of Medical Sciences is IR.MUMS.MEDICAL.REC.1398.658.

Genotyping

DNA extraction and quality control

Total genomic DNA was extracted from 200 µl blood or buffy coat using a DNA extraction kit (Parstous, Iran). After DNA extraction, the samples were loaded and run on agarose gel (Parstous, Iran). Quantification of the DNA samples was evaluated by Nanodrop 2000 (Thermo Fisher Scientific, USA) at wave length of 280 and 260 nm.

Tetra-ARMS polymerase chain reaction

Tetra-ARMS polymerase chain reaction (PCR) was carried out in 15 µl reaction volume containing 1 µl of each primer (0.6 pM of each primer), 7.5 µl master mix (Parstous, Iran), 2 µl genomic DNA and 1.5 µl water. Primers were designed using Primer 1 software. Tetra-ARMS PCR was conducted with an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 40 seconds. Final extension step was performed at 72°C for 5 minutes. The sequences of all primers are summarized in Table S1 (See Supplementary Online Information at www.ijfs.ir). Sanger sequencing was performed to confirm the results.

Allele-specific oligonucleotide polymerase chain reaction

Allele-specific oligonucleotide PCR (ASO-PCR) was performed in a 15 µl reaction volume containing 1 µl of each primer (4 µM), 7.5 µl master mix (Parstous, Iran), 2 µl genomic DNA and 1.5 µl water. Primers were designed using Primer 3 software. PCR was carried out with the following condition: one cycle of initial denaturation at 95°C for 7 minutes, followed by 35 cycles including 95°C for 30 seconds, annealing for 30 seconds, 72°C for 30 seconds, followed by one cycle of 7 minutes for the final extension. The sequences of all primers are summarized in Table S1 (See Supplementary Online Information at www.ijfs.ir). The sample data are presented in Figure 1.

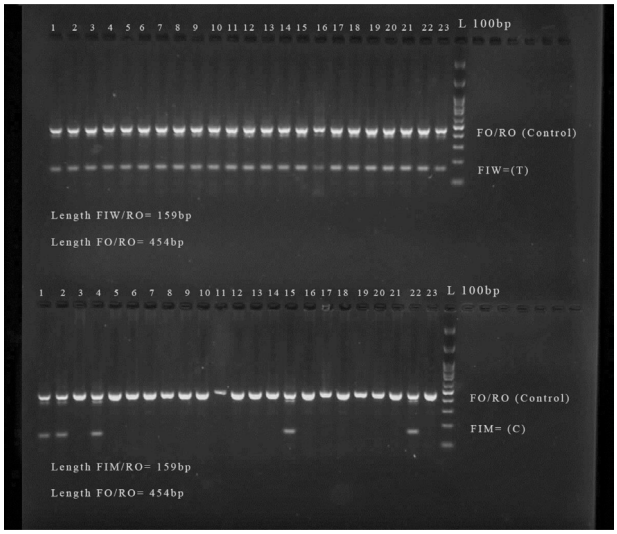

Fig.1: The RS4806660 Gel electrophoresis. Tetra- ARMS polymerase chain reaction (PCR) products were loaded and run on 2% agarose gel.

Statistical analysis

All experiments were performed in triplicate and reported values were displayed as mean ± standard deviation (SD). All data were analyzed using Microsoft Excel and SPSS version 24 (SPSS Inc., USA). t test statistical analysis was applied for comparing mean differences between two independent groups, and One-way ANOVA test was used to compare mean differences between more than two groups. Chi-square test was performed to analyze the Hardy-Weinberg equilibrium. Binary logistic regression test was used to calculate the odds ratio (OR) and confidence interval (CI) based on genotype data. Univariate and multivariate analysis were applied for the polymorphisms. Significance level is shown with *P<0.05, ** P<0.01 and ***P<0.001. Where applicable, Bonferroni correction was used to correct for multiple testing.

Results

Study of population characteristics

The mean age was 55.21 ± 5.56 years for the cases and 54.62 ± 2.89 years for the controls (P=0.411). A significant difference was observed between the average heights, waist circumference, weight, waist/height ratio and waist/hip ratio (WHR) of both groups. The controls were significantly heavier (70.96 ± 10.80 Kg) than the cases (67.11 ± 12.03 Kg, P<0.001). In contrast, the waist/height ratio was higher in the patients (63.55 ± 8.41) compared to the healthy subjects (60.30 ± 6.84, P=0.001). WHR was also greater in patients (0.93 ± 0.08) than controls (0.89 ± 0.07, P=0.001). The data are summarized in Table 1.

Table 1: Demographic features and characteristics of the study population

Indices	Case	Control	P value
Age (Y)	55.21 ± 5.56	54.62 ± 2.89	0.411
Height (m)	1.53 ± 0.06	1.56 ± 0.06	0.001
Waist circumference (cm)	96.96 ± 12.19	93.70 ± 10.44	0.020
Weight (kg)	67.11 ± 12.03	70.96 ± 10.80	<0.001
Hip circumference (cm)	104.71 ± 9.85	105.57 ± 8.78	0.430
Waist/height ratio	63.55 ± 8.41	60.30 ± 6.84	0.001
Waist/hip ratio	0.93 ± 0.08	0.89 ± 0.07	0.001
Mid upper circumference (cm)	30.69 ± 4.47	31.02 ± 4.61	0.550
Body mass index (kg/m^2)	28.78 ± 5.06	29.34 ± 4.22	0.300

Data are presented as mean ± SD.

Association of the single nucleotide polymorphisms and premature menopause

A total of eight SNPs were investigated in this study: rs16991615, rs244715, rs451417, rs1046089, rs7246479, rs4806660, rs10183486 and rs2303369. The genotype and allele frequencies of these SNPs are shown in Table 2. All SNPs conformed to the Hardy-Weinberg equilibrium. We found that the T allele of rs16991615, rs1046089, rs7246479 and rs10183486 as well as the C allele of rs244715, rs451417 and rs4806660 were associated with increased risk of premature menopause, in addition to the TT genotype of rs2303369 which may increase the susceptibility to POI in comparison with C allele careers. Besides, using a recessive model, genotypes of all SNPs were associated with an elevated risk of the disease (Table 3). Our results also suggested that CT genotype of rs7246479 was associated with increased susceptibility to premature menopause in comparison with CC genotype (OR=1.92, 95% CI=1.16-3.18, P=0.01). Furthermore, dominant model showed that genotype frequency of CC+TC was associated with increased risk of premature menopause for rs244715 (OR=1.70, 95% CI=1.06-2.72, P=0.027) in comparison with TT, while the T allele careers of rs1046089 (OR=1.71, 95% CI=1.05-2.79, P=0.03) and rs7246479 (OR=2.13, 95% CI=1.30-3.47, P=0.002) showed a greater association with risk for POI in comparison with CC. The additive model also indicated that TT genotype of rs16991615, rs1046089, rs10183486 and rs2303369, as well as CC genotype of rs451417 and rs4806660, were associated with increased risk of premature menopause, more than 100%. We acknowledge that although in the initial analysis some of these SNPs seemed to be associated with premature menopause, after correction for multiple testing none of them remained significant, as explained in Table 4.

Table 2: Genotype distribution and allele frequencies of the single nucleotide polymorphism (SNPs)

SNP	Genotype frequency										HWE
	Case (117)			Alleles		Control (183)			Alleles		P value
	AA	Aa	aa	A	a	AA	Aa	aa	A	a	
rs16991615 (G>A)	44	39	29	0.57	0.43	96	79	18	0.66	0.33	0.95
rs244715 (A>G)	59	46	12	0.70	0.3	116	61	6	0.80	0.20	0.83
rs451417 (A>C)	48	36	33	0.56	0.43	87	76	20	0.68	0.32	0.85
rs1046089 (G>A)	37	59	21	0.56	0.44	81	89	13	0.69	0.31	0.22
rs7246479 (G/T)	36	67	14	0.60	0.4	89	86	8	0.72	0.28	0.07
rs4806660 (T>C)	50	53	14	0.65	0.34	94	82	7	0.74	0.26	0.10
rs10183486 (C>T)	47	55	15	0.64	0.36	93	81	9	0.73	0.27	0.25
rs2303369 (C>T)	42	54	21	0.59	0.41	77	90	16	0.67	0.33	0.35

Table 3: OR based on different models

SNP	Additive model (aa vs. Aa) OR (95% CI)	P value	Dominant model (aa+Aa vs. AA) OR (95% CI)	P value
rs16991615 (G>A)	3.26 (1.61-6.58)	0.001	1.52 (0.95- 2.45)	0.078
rs244715 (A>G)	2.65 (0.92-7.59)	0.069	1.70 (1.06-2.72)	0.027
rs451417 (A>C)	3.48 (1.76-6.89)	<0.001	1.30 (0.81-2.08)	0.269
rs1046089 (G>A)	2.43 (1.13-5.24)	0.023	1.71 (1.05-2.79)	0.03
rs7246479 (G/T)	2.24 (0.89-5.66)	0.087	2.13 (1.30-3.47)	0.002
rs4806660 (T>C)	3.09 (1.17-8.16)	0.023	1.41 (0.88-2.25)	0.145
rs10183486 (C>T)	2.45 (1.00-6.00)	0.050	1.53 (0.96-2.46)	0.070
rs2303369 (C>T)	2.18 (1.05-4.55)	0.040	1.29 (0.80-2.09)	0.290
	Allelic model (a vs. A)		Homozygote contrast (aa vs. AA)	
rs16991615 (G>A)	1.79 (1.27-2.53)	0.001	3.51 (1.76-6.99)	0.001
rs244715 (A>G)	1.71 (1.17- 2.50)	0.005	3.93 (1.40-11.00)	0.009
rs451417 (A>C)	1.66 (1.18-2.33)	0.003	2.99 (1.54-5.77)	0.001
rs1046089 (G>A)	1.65 (1.17-2.32)	0.004	3.53 (1.59-7.81)	0.002
rs7246479 (G/T)	1.76 (1.25-2.50)	0.001	4.32 (1.67-11.19)	0.003
rs4806660 (T>C)	1.48 (1.042-2.12)	0.028	3.76(1.42-9.91)	0.007
rs10183486 (C>T)	1.53 (1.08-2.18)	0.020	3.29 (1.34-8.09)	0.010
rs2303369 (C>T)	1.39 (0.99-1.95)	0.060	2.40 (1.13-5.10)	0.020
	Heterozygote contrast (Aa vs. AA)		Recessive model (aa vs. Aa+AA)	
rs16991615 (G>A)	1.07 (0.63-1.81)	0.781	3.39 (1.78-6.46)	0.001
rs244715 (A>G)	1.48 (0.90-2.43)	0.119	3.37 (1.22-9.24)	0.018
rs451417 (A>C)	0.85 (0.50-1.45)	0.573	3.20 (1.73-5.92)	0.001
rs1046089 (G>A)	1.45 (0.87-2.41)	0.152	2.86 (1.37-5.96)	0.005
rs7246479 (G/T)	1.92 (1.16-3.18)	0.010	2.97 (1.20-7.32)	0.018
rs4806660 (T>C)	1.21 (0.74-1.97)	0.433	3.41 (1.33- 8.74)	0.010
rs10183486 (C>T)	1.34 (0.82-2.19)	0.240	2.84 (1.20- 6.73)	0.020
rs2303369 (C>T)	1.1(0.66-1.82)	0.710	2.28 (1.13-4.58)	0.020

SNP; Single nucleotide polymorphism, OR; Odds ratio, and CI; Confidence interval.

Table 4: Association between the SNPs and demographic characteristics

Polymorphism		Height (m)	Waist circumference (cm)	Weight (kg)	Hip circumference (cm)	Waist/ height ratio	Waist/ hip ratio	Mid upper circumference (cm)	BMI (kg/m^2)
rs16991615									
AA (n=134)	Mean	1.54604	95.5396	69.8507	105.3455	61.8738	0.9069	30.7632	29.2723
	SD	0.059774	11.59249	11.74	9.636	7.79393	0.07493	4.06007	4.89012
Aa+aa (n=166)	Mean	1.54366	94.4811	69.1659	105.1543	61.2964	0.8992	30.9902	29.0056
	SD	0.058927	10.96122	11.19606	8.85304	7.52282	0.07828	4.93033	4.29641
	P value*	0.730	0.420	0.608	0.859	0.517	0.386	0.670	0.617
rs244715									
AA (n=172)	Mean	1.54413	94.932	68.443	104.625	61.5679	0.9077	30.4241	28.722
	SD	0.058418	11.08496	10.86428	8.78544	7.605	0.07768	2.99326	4.37843
Aa+aa (n=128)	Mean	1.54556	94.9913	70.881	106.0802	61.5399	0.8958	31.5143	29.6764
	SD	0.060525	11.49888	12.06058	9.70565	7.71405	0.07526	6.01161	4.77505
	P value	0.838	0.964	0.069	0.178	0.975	0.186	0.042	0.075
rs451417									
AA (n=135)	Mean	1.5443	95.0711	68.9215	105.0119	61.6286	0.9056	30.6828	28.8994
	SD	0.061006	11.20656	10.88902	8.65259	7.47528	0.07947	3.80482	4.27702
Aa+aa (n=165)	Mean	1.54509	94.8626	69.9313	105.4294	61.496	0.9002	31.058	29.3128
	SD	0.057888	11.30614	11.87232	9.64887	7.7933	0.0746	5.09652	4.79884
	P value	0.908	0.874	0.449	0.697	0.882	0.549	0.481	0.438
rs1046089									
AA (n=108)	Mean	1.53991	94.9944	69.0157	105.1648	61.7733	0.9031	30.5252	29.1215
	SD	0.055895	10.98759	11.2755	8.80033	7.57492	0.0696	3.06539	4.63562
Aa+aa (n=192)	Mean	1.54747	94.9358	69.7342	105.2832	61.4326	0.9024	31.0937	29.1279
	SD	0.061005	11.41384	11.53757	9.43923	7.69138	0.08073	5.20816	4.53979
	P value	0.290	0.966	0.603	0.915	0.712	0.938	0.303	0.991
rs7246479									
AA (n=125)	Mean	1.53992	95.5864	70.3928	105.2984	62.132	0.9078	30.722	29.6871
	SD	0.060728	10.95757	11.09858	9.25444	7.25764	0.06692	3.85928	4.40168
Aa+aa (n=175)	Mean	1.54821	94.5023	68.8098	105.1983	61.1399	0.899	31.0064	28.7198
	SD	0.058037	11.45425	11.64944	9.18376	7.89665	0.08315	4.99693	4.65319
	P value	0.234	0.412	0.239	0.926	0.269	0.329	0.597	0.071
rs4806660									
AA (n=144)	Mean	1.54222	96.3625	67.6604	104.7625	62.5597	0.92	30.5895	28.4628
	SD	0.056426	11.34757	11.4973	8.87954	7.68153	0.07821	3.12603	4.67753
Aa+aa (n=156)	Mean	1.54708	93.6429	71.1695	105.687	60.6176	0.8864	31.1673	29.7452
	SD	0.061813	11.0187	11.13753	9.4933	7.50157	0.07191	5.5638	4.38612
	P value	0.480	0.037	0.008	0.387	0.028	**<0.001**	0.276	0.015
rs10183486									
AA (n=140)	Mean	1.55436	96.3093	71.2586	106.7629	62.054	0.9025	31.3266	29.491
	SD	0.061752	10.84882	11.72958	9.3348	7.45174	0.07029	4.26855	4.52896
Aa+aa (n=160)	Mean	1.5362	93.7589	67.8924	103.8911	61.1148	0.9028	30.5	28.8017
	SD	0.055702	11.48176	10.95238	8.8882	7.79695	0.0823	4.77225	4.59034
	P value	0.008	0.050	0.011	0.007	0.290	0.975	0.119	0.194

Table 4: Contineud

rs16991615		Height (m)	Waist circumference (cm)	Weight (kg)	Hip circumference (cm)	Waist/ height ratio	Waist/ hip ratio	Mid upper circumference (cm)	BMI (kg/m^2)
rs2303369									
AA (n=119)	Mean	1.54345	96.8336	67.5714	104.9563	62.8264	0.9232	30.7568	28.3835
	SD	0.054779	11.18619	11.66716	8.84538	7.69302	0.08058	2.98854	4.79756
Aa+aa (n=181)	Mean	1.54559	93.7095	70.7385	105.4291	60.7116	0.889	30.9753	29.6188
	SD	0.062135	11.1368	11.12204	9.44499	7.50472	0.07112	5.35162	4.35084
	P value	0.760	0.019	0.019	0.665	0.019	<0.001	0.687	0.022

*; Due to multiplecomparison, Bonferroni correction has been applied and the level of significance is set to <0.006 and SNP; Single-nucleotide polymorphism.

Table 5: Univariate multivariate analysis of the polymorphisms between case and control groups

SNP	Univariate analysis	Multivariate analysis
rs16991615	0.312	0.162
rs244715	0.008	0.019
rs451417	0.270	0.589
rs1046089	0.208	0.095
rs7246479	0.286	0.600
rs4806660	0.005	0.024
rs10183486	0.072	0.096
rs2303369	0.008	0.028
Age (Y)	0.163	
Height (m)	0.310	
Waist circumference (cm)	0.439	
Weight (kg)	0.830	
Hip circumference (cm)	0.938	
Waist: height ratio	0.550	
Waist:hip ratio	0.348	
Mid upper circumference (cm)	0.332	
Body mass index (kg/m^2)	0.463	

Univariate and multivariate linear regression were used to indicate the polymorphisms correlations. *; Due to multiple comparison, Bonferroni correction was applied and the level of significance was set to <0.003.

Association of the single nucleotide polymorphisms with demographic characteristics

We found no significant association between rs16991615, rs451417 and rs1046089 polymorphisms and demographic characteristics of the patients. Additionally, only the rs244715 showed an association with Mid-Upper Arm Circumference (MUAC) in which the dominant homozygous genotype had lower MUAC (P=0.042). WC and weight did not seem to be associated with rs4806660, rs10183486 and rs2303369 (P=0.05). In all of these three SNPs, the dominant homozygous genotype had a greater WC. On the other hand, people with the dominant homozygous genotype of rs10183486 were heavier, while the same genotype for rs2303369 and rs4806660 had lower weight. Hip circumference only showed association with rs10183486, as dominant homozygous genotype. Furthermore, waist/height and waist/hip ratios were significantly higher for the dominant homozygous genotype of rs4806660 and rs2303369. Finally, women having TT and CC genotypes for the rs4806660 and rs2303369, respectively, had significantly higher BMI. The association of these SNPs and demographic characteristics are summarized in Table 4. However, after correcting for multiple testing using Bonferroni correction, they were not remained significant. Moreover, univariate regression analysis (model 1) was used to analyze the association between genetic variants and risk of POI. While, multivariate regression analysis (model 2) was adjusted for sex, age, smoking status, BMI, WC, waist/hip, waist/height.

Discussion

We evaluated association of eight different SNPs including rs16991615, rs244715, rs451417, rs1046089, rs7246479, rs4806660, rs10183486 and rs2303369 with premature menopause. All of these SNPs except for rs244715, rs7246479 and rs4806660 were previously identified in GWAS. These SNPs are mapped to different loci in the genome. rs16991615 and rs451417 are mapped to *MCM8* while rs2303369, rs1046089, rs10183486 and rs244715 are mapped to *FNDC4*, *PRRC2A*, *TLK1* and *ZNF346*, respectively. Besides, rs7246479 and rs4806660 are both mapped to *TMEM150B*.

Several studies have established the role of genetic variations in premature menopause. One of the loci is located on chromosome 19 containing MCM8, which encodes a mini-chromosome maintenance protein called DNA replication licensing factor MCM8. MCM8 plays role in DNA repair (16). Moreover, this protein has an important role in gametogenesis as *MCM8* knocked-out mice showed sterility and arrested primary follicles in males and females, respectively (20). Additionally, *MCM8* expression was increased during the follicular phase of menstrual cycle in humans (21). Mutations in this gene have been linked to ovarian failure as well as chromosomal instability (15, 22). Recently, novel mutations of this gene were also identified to associate with POI (23, 24). Large scale studies were previously conducted and they found that rs16991615 was associated with ANM in American populations with Hispanic, African and Indian descent, in addition to European

women (17). This SNP was discovered to ramp up the risk of premature menopause (25). Bae et al. (26) and Day et al. (27) studies revealed association of rs16991615 with age at menopause at a genome-wide significance. The rs16991615 association with age at menopause was also replicated in the Iranian population (28). We identified that a minor allele of rs16991615 and TT genotype is not associated with increased risk of POI in our population. Similar to our results, Setti et al. (29) found no significant association between rs16991615 and poor ovarian response in Brazilian women. Moreover, other studies including Desai et al. (16) could not find any association between rs16991615 and POI. Recently, the minor allele of rs16991615 has been demonstrated to increase age-adjusted inverse anti-mullerian hormone (AMH), suggesting that factors affecting age at premature menopause could have a role in AMH regulation in ovarian reserves (30). For other SNPs including rs451417, we found that CC genotype was associated with an increased risk of POI by more than two-fold in comparison with careers of T allele.

We also investigated association of premature menopause with the rs244715, located in the *ZNF346* gene. *ZNF346* encodes a double-stranded RNA binding protein and takes part in apoptosis regulation (31). We confirmed that the minor allele of rs244715 could have an association with increased risk of the disease and MUAC; however, the study on Brazilian women failed to find any association between rs244715 and poor ovarian response (29).

Furthermore, Perry et al. (32) and Stolk et al. (14) found an association between rs1046089, located in the *PRRC2A* gene that encodes the HLA-B associated transcript, with age at menopause. Variations in this gene have been linked to different phenotypes, including BMI and height. Consistent with this, we noticed that the TT genotype of rs1046089 compared to C allele careers could be linked with increased risk of POI by more than 150%. However, Llácer et al. (19) found an increased risk of poor ovarian response in patients with CT and CC genotype, in comparison with TT genotype.

The rs7246479 and rs4806660 are located on chromosome 19 in the *TMEM150B* gene, which gives rise to transmembrane protein 150B, a member of TMEM150/Damage-Regulated Autophagy Modulator (DRAM) family. DRAM proteins play a role in apoptosis and autophagy (33). We found that women carrying GG genotype of rs7246479 were younger and had a lower rate of POI. Moreover, this SNP is also associated with age at menopause in the Chinese population (13). Furthermore, Setti et al. (29) found that rs4806660 has a protective effect against poor ovarian response. They further identified that the minor allele of rs4806660 was associated with enhanced controlled ovarian stimulation. However, the association was not statistically significant. Nonetheless, we demonstrated that C allele in the recessive model could associate with an increased risk of POI.

Another SNP associated with early menopause is rs2303369. The gene containing this variant encodes a protein called *FNDC4*. FNDC4 belong to the fibronectin type III domain family of proteins. FNDC4 binds to macrophages and monocytes. Administration of this could have therapeutic applications in inflammatory diseases (34). FNDC4 can also activate Wnt/β-catenin signaling pathway (35). Although Laisk-Podar et al. (36) established the association of rs2303369 with early follicular FSH, they could not find any association between rs2303369 and mean ovarian volume. On the other hand, we found that TT genotype of rs2303369 was associated with a significantly increased risk of POI in comparison with the major allele careers.

rs10183486 in the Tousled-like kinase 1 (*TLK1*) gene, encoding a serine/threonine kinase, is suggested to be involved in chromatin assembly, DNA repair and ovarian aging (37). Overexpression of *TLK1* in mouse embryonic stem cells has resulted in developmental arrest and apoptosis, as well as a decrease in pluripotency factors (38). We found that the minor allele of rs10183486 in comparison with the C allele and TT genotype in comparison with C allele careers (CT+CC) were associated with increased susceptibility to POI which is in concordance with the findings of Stolk et al. (14) study, reported an association between rs10183486 SNP and age at menopause.

Limitations of the current study include use of a population derived from a single region of Iran, hence it may not be possible to extrapolate our findings more widely. Secondly, we were unable to completely verify premature menopause by biochemical testing. Thirdly, the sample size of this study was quite small, which may affect the power of this study. Hence, there is a need to conduct further studies in different ethnicities with large sample sizes and to consider more comprehensive criteria for assessing the correlation between variants and premature menopause.

Conclusion

The rs16991615, rs244715, rs451417, rs1046089, rs7246479, rs4806660, rs10183486 and rs2303369 polymorphisms may potentially influence premature menopause. Although ethnic differences may result in diverse outcomes in association studies, more commonly recognized loci related to age at menopause could be subjected to replication in different populations. This could warrant further investigations with larger sample sizes to get a more comprehensive understanding of variations in different geographic regions.

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Authors' Contributions

M.R.M., N.Kh., G.A.F., A.P., M.Gh.-M., T.H.;

Participated in study design, data collection and evaluation, drafting and statistical analysis. M.R.M., R.Kh., S.R.H.; Performed blood sample and demographic data collection to this component of the study. H.Gh., M.R.; Conducted molecular experiments and RT-qPCR analysis. All authors performed editing and approved the final version of this manuscript for submission.

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Original Article

Serum Progesterone Levels with The Use of Four Different Types of Vaginal Progesterone in Frozen-Thawed Embryo Transfer Cycles and Related Pregnancy Outcomes

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Abstract

Background: Luteal phase support (LPS) is essential for hormone replacement therapy (HRT) for frozen-thawed embryo transfer (FET). However, the optimal dose and serum progesterone (P4) levels required for pregnancy are controversial. We attempted to determine the association between pregnancy outcomes and serum P4 levels administered via vaginal suppository for HRT-FET cycles on embryo transfer day.

Materials and Methods: This was a secondary analysis of the dataset from the EXCULL trial, which prospectively investigated pregnancy outcomes of four different P4 vaginal suppositories (Lutinus, Utrogestan, Luteum, and Crinone) for HRT-FET. It was conducted at a private fertility clinic between December 2016 to December 2017. During this trial, 235 cycles were divided into four groups based on serum P4 values (quartile [Q] 1 group: <7.8 ng/mL; Q2 group: 7.8-10.8 ng/mL; Q3 group: 10.8-13.7 ng/mL; Q4 group: >13.7 ng/mL). We investigated clinical pregnancy rate (CPR), positive fetal heart rate (FHR), live birth rate (LBR), and miscarriage rate (MR) for each group. A logistic regression analysis was performed using age, body mass index (BMI), and transferred embryos as covariates.

Results: Serum P4 values (ng/mL) of each drug were as follows: Lutinus, 13.3 ± 4.9; Utrogestan, 9.3 ± 3.3; Luteum, 13.6 ± 4.2; and Crinone, 8.7 ± 3.2 (mean ± standard deviation, P<0.001).The percentages of Utrogestan and Crinone were higher in the Q1 group, while the percentages of Lutinus and Luteum were higher in the Q4 group. Nonetheless, there were no statistical differences between the Q1 and Q4 groups in CPR, FHR, LBR, and MR.

Conclusion: When vaginal P4 was used for FET, although serum P4 levels on transfer day differed based on the drug that was administered, no relationship was observed between serum progesteronelevels and pregnancy outcomes (Registration number: UMIN000032997).

Keywords: Embryo Transfer, Luteal Phase, Pregnancy Outcome, Serum Progesterone, Vitrification

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Introduction

Currently, there is a growing preference for the use of frozen-thawed embryo transfer (FET), rather than fresh embryo transfer (fresh ET) (1). This is because FET may have a higher pregnancy rate as the hormonal status of the endometrium provides an environment that is suitable for implantation (2, 3), and also because ovarian hyperstimulation syndrome can be avoided (4). FET involves two methods: the use of the natural ovulation cycle (natural embryo transfer [N-ET]), as well as the use of the hormone replacement cycle (hormone replacement therapy [HRT] FET [HRT-FET]). HRT-FET provides a better management schedule; although, the replacement of external estrogen and progesterone (P4) is required until at least week 10 of pregnancy, at which point hormone production by the placenta should be sufficient (5-7). The required P4 replacement can be administered intramuscularly, orally, or transvaginally. However, vaginal suppositories are increasingly favored worldwide due to their ease of use and effective drug delivery to the endometrium of the uterus (8).

Currently, four different P4 vaginal suppositories are available for use: Lutinus (Ferring Pharmaceuticals, Saint-Prex, Switzerland), Utrogestan (FUJIFILM Phar-

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maceuticals, Japan), Luteum (ASKA Pharmaceutical, Japan), and Crinone (Merck Serono, Germany). The dosage and administration route of each drug have been determined through clinical trials of fresh ET (9-12). All four of these suppositories have been reported to be effective for fresh ET (13). Because FET has been recently developed, there are insufficient data regarding the optimal P4 dosage for each of these four vaginal suppositories to successfully induce clinical pregnancy. Moreover, the clinical significance of measuring serum P4 levels for FET remains to be established (5). Therefore, some infertility facilities increase the dose of the drug when the serum P4 level is low.

Recently, several studies have reported an association between serum P4 levels and pregnancy outcomes with HRT-FET and have shown a correlation between poor pregnancy outcomes and low serum P4 levels (14-17). From December 2016 to December 2017, we conducted a prospective, randomized, controlled study (the exploratory test) to investigate the clinical pregnancy rates (CPR) after using four types of vaginal P4 medications, Lutinus vaginal tablet, Utrogestan vaginal capsule, Luteum vaginal suppository, and Crinone vaginal gel (the EXCULL study). Outcomes of this trial indicated that the CPR, ongoing pregnancy rate (OPR), and miscarriage rate (MR) among the four suppositories were not significantly different (18). Therefore, we performed a secondary analysis of this data and examined the association between serum P4 levels on embryo transfer day and CPR, positive fetal heart rates (FHR), live birth rates (LBR), and MR for HRT-FET.

Materials and Methods

Study design

This was a secondary analysis of data from our previously published EXCULL trial (18). The EXCULL study was a randomized, controlled trial that prospectively investigated the pregnancy outcomes of four different P4 vaginal suppositories (Lutinus, Utrogestan, Luteum, and Crinone) for HRT-FET. Since this study was an exploratory study, no particular sample size was established. Patients with contraindications listed on the medication package were excluded. This was the only exclusion criterion. We surveyed the number of patients that can be assigned to our clinic in one year.

Patients underwent egg retrieval at our hospital. Embryos were cryopreserved using vitrification, followed by transfer using a single cleavage-stage embryo or a blastocyst derived from the patient's own egg. The HRT protocol was started with administration of transdermal estrogen (E2) tape (Estrana tape; Hisamitsu Pharmaceutical Co., Inc., Tosu, Saga Prefecture, Japan) or E2 gel (L'estrogel 0.06%; FUJIFILM Pharmaceuticals, MA), and its dosage was gradually increased. Luteal phase support (LPS) was then achieved using one of the four vaginal P4 suppositories. The four types of P4 suppositories were administered as follows: Lutinus, 100 mg three times daily; Utrogestan, 200 mg three times daily; Luteum, 400 mg two times daily; and Crinone, 90 mg once daily.

During this analysis, all 235 cycles included in the EXCULL study were analyzed. Endpoints were observation of a gestational sac at 5 weeks of gestation using transvaginal ultrasound (which was defined as CPR), positive FHR at 7 weeks of gestation, LBR, and MR.

Blood sampling

Day (D) 0 represents the date on which the P4 vaginal suppository was initiated for LPS. Blood samples to quantify serum P4 levels were collected on D2, D3, or D5, which corresponded to the embryo transfer dates, from patients undergoing cleavage-stage embryo or blastocyst transfer, respectively. All blood samples were obtained an average of 4 hours after administration of the last vaginal suppository. The P4 concentration was measured at the hospital using an electro-chemiluminescence immunoassay (Cobas e 411 analyzer; Roche Diagnostics GmbH, Germany) according the manufacturer's instructions. The detection limit of P4 was 0.0337 ng/mL.

Statistical analysis

Pairwise comparisons among the four groups were performed using the analysis of variance (ANOVA) or Kruskal-Wallis test for continuous variables and the Chi-squared test for categorical variables. Associations between serum P4 levels and CPR, FHR, LBR, and MR were first analyzed by simple logistic regression analysis and then by multiple logistic regression analysis. The variables included were serum P4 levels, age, body mass index (BMI), pregnancy history, endometrial thickness, previous transfers, participation in the study, type of transferred embryos, and quality of blastocyst. Variables showing statistical significance were included as adjusting potential confounders in the multiple logistic regression analysis. The Hosmer-Lemeshow test was used to assess the goodness-of-fit of the logistic regression model. All statistical analyses were performed using statistical software (SAS 9.4; SAS Institute Inc., Cary, NC, USA). The level of significance was set at $P<0.05$.

Ethical considerations

The EXCULL study was approved by the Ethics Committee of Kinutani Women's Clinic (ethical review number: 2016-1110-1) and was registered with the Clinical Trials Registry (registration number: UMIN000032997). All women who agreed to participate in the study provided informed written consents.

Results

The 235 cycles involved in the EXCULL study were divided into four groups with the lowest serum P4 levels on the embryo transfer day as follows: quartile (Q) 1 group, <7.8 ng/mL; Q2 group, 7.8-10.8 ng/mL; Q3, 10.8-13.7 ng/mL; and Q4 >13.7 ng/mL. Table 1 shows the baseline characteristics of all four groups (Table 1). The four groups

did not differ with regard to age, BMI, pregnancy history, endometrial thickness, number of previous transfers, number of study participants, transferred embryos, or blastocyst quality. However, the proportion of vaginal agent used was significantly different. For Q1 group, the following rates were found: Lutinus, 10.2%; Utrogestan, 42.4%; Luteum, 5.1%; and Crinone, 42.4%. For Q2 group, the rates were as follows: Lutinus, 23.7%; Utrogestan, 27.1%; Luteum, 15.3%; and Crinone, 33.9%. For Q3 group, the rates were as follows: Lutinus, 25.4%; Utrogestan, 25.4%; Luteum, 35.6%; and Crinone, 13.6%. For Q4 group, the following rates were found: Lutinus, 48.3%; Utrogestan, 6.9%; Luteum, 39.7%; and Crinone, 5.2% (P<0.001).

The proportions of Utrogestan and Crinone were high in Q1 group, and those of Lutinus and Luteum were high in Q4 group. Because the ratios of drugs differed in Q1 and Q4, serum P4 values for each drug were confirmed (Table 2, Fig.1). P4 levels (mean ± standard deviation [SD]) were lower for Utrogestan (9.3 ± 3.3 ng/mL) and Crinone (8.7 ± 3.2 ng/mL) than for Lutinus (13.3 ± 4.9 ng/mL) and Luteum (13.6 ± 4.2 ng/mL) (P<0.001). A logistic regression analysis was used to detect an association among the four P4 groups and CPR, FHR, LBR and MR. In performing the logistic regression analysis, Hosmer-Lemeshow was used to evaluate the fitness of the regression model for each pregnancy outcome. We selected age, BMI, and transferred embryo as covariates for the multiple logistic regression analysis, because these factors had affected pregnancy outcomes (CPR, FHR, LBR) consistently and significantly in simple logistic regression analysis. Regarding MR, none of the variables showed statistical significance due to the small number of data. However, it was assumed that the same confounder as CPR, FHR, and LBR existed, so, it was adjusted with the same covariates. After adjusting for confounding factors (age, BMI, and type of transferred embryo), no association among the four groupsand CPR, FHR, LBR, and MR was identified (Table 3).

Table 1: Demographic characteristics and laboratory parameters of the patients

Serum P4 group	Q1 (<7.8 ng/mL) n=59	Q2 (7.8-10.8 ng/mL) n=59	Q3 (10.8-13.7 ng/mL) n=59	Q4 (>13.7 ng/mL) n=58	P value
Age (Y)	35.3 ± 4.5	36.1 ± 4.0	37.3 ± 4.0	36.6 ± 4.6	0.09[1†]
BMI (kg/m^2)	20.5 ± 2.9	20.9 ± 2.4	20.8 ± 3.1	20.1 ± 1.8	0.361[†]
Drug					<0.001[§]
Lutinus	6 (10.2)	14 (23.7)	15 (25.4)	28 (48.3)	
Utrogestan	25 (42.4)	16 (27.1)	15 (25.4)	4 (6.9)	
Luteum	3 (5.1)	9 (15.3)	21 (35.6)	23 (39.7)	
Crinone	25 (42.4)	20 (33.9)	8 (13.6)	3 (5.2)	
Pregnancy history					0.768[§]
Primary	26 (44.1)	26 (44.1)	31 (52.5)	27 (46.6)	
Secondary	33 (55.9)	33 (55.9)	28 (47.5)	31 (53.4)	
Endometrial thickness (mm)	11.1 ± 1.7	11.1 ± 2.2	11.2 ± 1.9	11.1 ± 1.7	0.992[†]
Previous transfers					0.468[§]
0	25 (42.4)	19 (32.2)	16 (27.1)	15 (25.9)	
1	12 (20.3)	16 (27.1)	17 (28.8)	20 (34.5)	
≥ 2	22 (37.3)	24 (40.7)	26 (44.1)	23 (39.7)	
Participation of study					0.563[§]
1	45 (76.3)	42 (71.2)	36 (61.0)	38 (65.5)	
2	9 (15.3)	14 (23.7)	18 (30.5)	15 (25.9)	
≥ 3	5 (8.5)	3 (5.1)	5 (8.5)	5 (8.6)	
Transferred embryo					0.132[§]
Cleavage-stage	28 (47.5)	32 (54.2)	31 (52.5)	20 (34.5)	
Blastocyst	31 (52.5)	27 (45.8)	28 (47.5)	38 (65.5)	
Quality of blastocyst					0.781[§]
High	21 (67.7)	21 (77.8)	21 (75.0)	26 (68.4)	
Poor	10 (32.3)	6 (22.2)	7 (25.0)	12 (31.6)	
Clinical pregnancy rate	17 (28.8)	22 (37.3)	19 (32.2)	25 (43.1)	0.394[§]
Fetal heart beat rate	14 (23.7)	19 (32.2)	16 (27.1)	18 (31.0)	0.731[§]
Live birth rate	12 (20.3)	19 (32.2)	15 (25.4)	16 (27.6)	0.530[§]
Miscarriage rate	5 (29.4)	3 (13.6)	4 (21.1)	9 (36.0)	0.340[§]

BMI; Body mass index, P4; Progesterone, [†]; ANOVA, and [§]; Pearson's chi-square test, Q1-Q4 groups were divided according to the serum P4 level. Data are presented as mean ± SD or as number and count (%).

Table 2: Serum progesterone (P4) levels for each drug on the embryo transfer day

Drug	Lutinus n=63	Utrogestan n=60	Luteum n=56	Crinone n=56	P value
P4 levels (ng/mL)	13.3 ± 4.9	9.3 ± 3.3	13.6 ± 4.2	8.7 ± 3.2	<0.001

Data are presented as mean ± SD.

Table 3: Logistic regression analysis of reproductive outcomes for the four progesterone groups

Type of analysis	Unadjusted analysis		Adjusted analysis	
	OR (95% CI)	P value	OR (95% CI)	P value
Clinical pregnancy				
Q1	1		1	
Q2	1.47 (0.68-3.18)	0.329	1.85 (0.81-4.22)	0.144
Q3	1.17 (0.54-2.57)	0.689	1.63 (0.70-3.78)	0.259
Q4	1.87 (0.87-4.03)	0.109	2.02 (0.89-4.57)	0.092
Fetal heart beat				
Q1	1		1	
Q2	1.53 (0.68-3.44)	0.307	1.92 (0.82-4.52)	0.135
Q3	1.20 (0.52-2.74)	0.673	1.61 (0.67-3.90)	0.288
Q4	1.45 (0.64-3.28)	0.377	1.56 (0.66-3.638)	0.311
Live birth				
Q1	1		1	
Q2	1.86 (0.81-4.29)	0.146	2.36 (0.98-5.71)	0.056
Q3	1.34 (0.56-3.17)	0.512	1.79 (0.72-4.46)	0.214
Q4	1.49 (0.63-3.51)	0.36	1.59 (0.65-3.89)	0.308
Miscarriage				
Q1	1		1	
Q2	0.38 (0.08-1.88)	0.236	0.33 (0.06-1.75)	0.193
Q3	0.64 (0.14-2.92)	0.565	0.59 (0.11-3.10)	0.537
Q4	1.35 (0.36-5.08)	0.657	1.17 (0.30-4.60)	0.822

Data shows OR of groups Q2, Q3, and Q4 when Q1 is set to 1. Adjusted variables: age, body mass index, and transferred embryo.

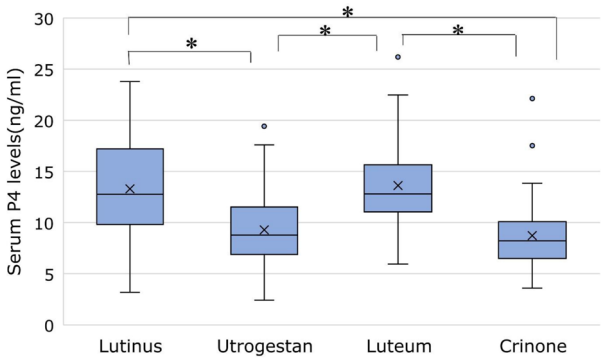

Fig.1: Box whisker plot depicting the serum progesterone levels (ng/mL) for each drug on embryo transfer day. *; P<0.001.

Discussion

We determined whether P4 levels measured on embryo transfer day were related to pregnancy outcomes involving the use of four vaginal P4 suppositories for LPS. We did not identify an association among serum P4 levels and CPR, FHR, LBR and MR.

As previously noted, recent studies have reported that low P4 levels are associated with poor pregnancy outcomes (14-17). However, this study showed the opposite results. We identified three studies that specifically assessed the use of Utrogestan. The first of these was a prospective study of patients who underwent donor egg transfer and Utrogestan (400 mg) administered twice daily. In that study, CPR and OPR decreased when serum P4 levels on the embryo transfer day were <9.2 ng/mL (14). Because we did not use the same protocol for Utrogestan administration, a direct comparison of these findings with those of our study was not feasible. The area under the curve (AUC) of the receiver-operating characteristic (ROC) curve in that previous study was 0.59, indicating that P4 levels had a minimal ability to predict OPR. The second study of Utrogestan was a retrospective evaluation of patients who underwent euploid embryo transfer and preimplantation genetic testing for aneuploidies and were administered Utrogestan at a dose of 200 mg, three times daily (15). When P4 levels were <8.06 ng/mL on the day of embryo transfer, although there was no difference in CPR, the LBR decreased and the MR increased. The finding that the P4 level did not affect CPR is consistent with our findings in the current study. However, they did not proceed with embryo transfer if the P4 level was <5 ng/mL, which was different from our study. Therefore, comparisons between the results of that study and those of ours are not appropriate. The third study was a

retrospective evaluation of patients receiving 200 mg of Utrogestan three times daily (16). In that study, serum P4 levels were <10 ng/mL before the transfer date led to lower CPR, OPR, and LBR. Moreover, these studies showed that when the P4 level was <10 ng/mL, increasing the dose to 400 mg three times daily failed to modify the pregnancy rate. However, the AUC in that study was only 0.62, indicating that the P4 level has low predictive ability for pregnancy outcomes. One retrospective study assessed the outcomes of blastocyst transfer with the administration of 90 mg of Crinone three times daily (17). CPR and OPR decreased when serum P4 levels on the pregnancy determination date were <35 nmol/L (approximately 11.1 ng/mL) while the 50th percentile of P4 levels was 34 nmol/L (10.8 ng/mL). Of note, despite the fact that the dose of Crinone was three folds higher than that used in our study, there was minimal difference from our mean P4 level of 8.7 ng/mL.

Our findings revealed differences in serum P4 levels across the four different types of P4 vaginal suppositories, however, all four drugs had a median P4 level of approximately 10 ng/mL and an overlap in their respective interquartile ranges. All four medications contain natural progesterone, but differ regarding the daily recommended dose: Crinone, 90 mg/day; Lutinus, 300 mg/day, Utrogestan, 600 mg/day; and Luteum, 800 mg/day. Despite these large differences in daily dosages, the difference in measured serum P4 levels was with in 5 ng/mL. Possible reasons include differences in the solubility and absorption rates of the drugs. Specifically, Lutinus and Crinone may have better absorption despite their lower P4 content. The measured P4 levels for these four types of suppositories in our study did not vary greatly from previously reported levels (14-17). With the dosage and usage currently suggested by drug manufacturers, all four drugs are anticipated to produce a serum P4 level of approximately 10 ng/mL.

A systematic review of previous studies regarding the use of vaginal P4 for in vitro fertilization cycles indicated the efficacy and safety of using Crinone, Lutinus, Utrogestan, and Luteum for fresh ET (13). However, the effective dosage and usage of vaginal suppositories for HRT-FET remain unclear due to insufficient data (5). Shapiro et al. (5) stated that monitoring serum P4 levels is ineffective due to the uterine first-pass effect, wherein the transvaginal administration of these drugs yields sufficiently higher endometrial concentrations than intramuscular injection despite the low serum P4 levels (19-21).

In this study, the lowest serum P4 level that could result in a live birth was 4.06 ng/mL, and there were only three cycles with P4 levels <4 ng/mL. We also showed that the AUC of the ROC curve predicting CPR was 0.58, suggesting that serum P4 levels were poorly predictive of CPR.

We have shown in previous studies that there is no difference in pregnancy outcome when the four aforementioned drugs are used (18). In this study, four groups were created based on serum P4 levels, and pregnancy outcomes were examined. The P4 values of the four medications differed, but there was no difference in pregnancy outcomes among the groups. Therefore, there is no need to increase or decrease the medicine based on the serum P4 level, when each of the four drugs were used for HRT-FET as currently indicated by each pharmaceutical company. Although, the dosage and administration level were determined after a trial of fresh-ET. An advantage of our study is that it is a prospective trial with no patient selection bias. We also did not interrupt the study nor did we increase the amount of each drug with a low blood P4 level. High blood P4 levels may lead to drug leaks into the blood vessels instead of the endometrium, so the exclusion of patients with low P4 might be wrong.

This study had some limitations. The data used in our analysis were obtained from a single clinic in Japan, therefore, a patient bias effect cannot be denied. Furthermore, only Japanese patients were included. Compared to women in western cultures, Japanese women tend to have a lower BMI. Therefore, they require a lower dosage of P4 supplementation to attain target levels. In addition, because the serum P4 values of all the drugs are concentrated at approximately 10 ng/mL, it is not known whether there is an optimal serum P4 value that allows pregnancy. Furthermore, this is an exploratory study and we did not set the sample size, so increasing the sample size might make a significant difference in the pregnancy rates among the four groups. Furthermore, large-scale, multicenter, prospective cohort studies are needed to elaborate our findings.

Conclusion

When vaginal P4 was used for FET, despite the fact that serum P4 levels on transfer day differed amongst the experimental groups based on the administered drug, no relationship was observed between serum progesterone levels and pregnancy outcomes. However, the relationship between serum P4 levels and pregnancy outcomes is still controversial and requires further studies.

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Authors' Contributions

R.S., M.K., S.O., M.I., R.K., Y.K.; Contributed to conception and design. R.S., E.F., Y.H.; Contributed to acquisition of data. R.S., R.K., Y.K.; Contributed to analysis and interpretation of data. R.S., M.K., S.O.; Contributed to drafting of the manuscript. R.S., R.K.; Contributed to critical revision of the manuscript for important intellectual content. M.K., S.O., E.F., Y.H.; Contributed to administrative, technical, or material support. M.K., S.O.; Contributed to supervision. All authors read and approved the final manuscript.

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Original Article

Correlation of Anti-Mullerian Hormone Level and Antral Follicle Count with Oocyte Number in A Fixed-Dose Controlled Ovarian Hyperstimulation of Patients of *In Vitro* Fertilization Program

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Abstract

Background: This study was conducted to determine the correlation of anti-Mullerian hormone (AMH) level and antral follicle count (AFC) with oocyte count in women who had received controlled ovarian hyperstimulation in an *in vitro* fertilization (IVF) program.

Materials and Methods: We retrospectively gathered the data of 42 patients who underwent IVF during 2005-2017 at Aster Clinic in Dr. Hasan Sadikin Hospital and Bandung Fertility Center Limijati Hospital, Indonesia. Details of the subjects such as identity, characteristics, history of illness, history of previous therapy, levels of ovarian reserve markers examined (AFC and AMH), follicle-stimulating hormone (FSH) dose given, and number of oocytes produced were recorded.

Results: A significant positive correlation between AMH (P≤0.001, r=0.530), AFC (P≤0.001, r=0.687), and AMH-AFC combination (P≤0.001, r=0.652), and the number of oocytes was found at the FSH dose of 225 IU.

Conclusion: AFC and AMH are able to reliably predict ovarian response to FSH.

Keywords: Anti-Mullerian Hormone, *In Vitro* Fertilization, Ovarian Response

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Introduction

Primary infertility affects 8-12% ofreproductive-age couples globally, and the proportion is estimated to vary from 4.5 to 30% across countries, with the highest percentages found in developing countries (1). In Indonesia, it was estimated that 12.3% of reproductive-age couples suffered from infertility, whereas another survey estimated a prevalence rate of 10-15% (2). As such, the demand for assisted reproductive technology, such as *in vitro* fertilization (IVF) has risen in recent years.

Adequate follicle growth, achievable through follicle stimulating hormone (FSH) administration in controlled ovarian hyperstimulation protocols, is crucial for the success of an IVF cycle (3, 4). However, the varieties inindividual characteristics and response to FSH stimulation among infertile patients havemade it difficult to generate a dose cut-off applicable for both low-responders and high-responders while avoiding the risk of ovarian hyperstimulation syndrome. FSH in an IVF cycle is therefore generally administered based on a fixed dose, and the standard dose used in our clinic was 225 IU. The two best known ovarian reserve markers to predict ovarian response to FSH are mean antral follicle count (AFC) and anti-Mullerian hormone (AMH), although there is a lack of data to conclude which of the two markers served better to predict ovarian reserve (5, 6). AFC is the number of follicles measuring 2-10 mm in size from both ovaries. AMH is detected in the primordial follicle and achieves peak level in the small antral follicle. The AMH level indicates the number of growing follicles, and this level can be used to determine the prognosis of fertility. The number of oocytes obtained will be probably low if the predicted AMH level is low, whereas extreme ovarian response complications can be expected when the predicted AMH level is excessive. Currently available ovarian reserve markers,

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including the AMH and AFC, invariably still show varied results, especially in clinical practice where AMH and AFC level could be at odds with each other (5-7).

It has been previously suggested that ethnicity may influence ovarian reserve markers (8-10). Furthermore, ethnicity may influence the manner with which ovarian reserve markers interact with factors such as age and weight. Age is negatively correlated with AMH and AFC in Caucasian, African-American, Hispanic, and Asian women, however BMI was only negatively correlated with serum AMH level in Caucasian women (8). A cross-sectional study comparing Indian and Spanish women showed that AFC is declined in younger Indian women compared to Spanish women (11). To date, there are few studies that have examined ovarian reserve markers in Indonesian women, much less inthose who received controlled ovarian hyperstimulation in an IVF program. Therefore, it is necessary to study the association of AMH and AFC to obtain an optimal ovarian response in this population. This study was conducted to determine the correlation of AMH and AFC with the number of oocytes in women who hadreceived controlled ovarian hyperstimulation in an IVF program with an FSH dose of 225 IU, which is the most frequently used dose in Indonesian health facilities.

Materials and Methods

In this retrospectively study, the data were obtained from the medical records of patients who underwent the IVF program with the FSH dose of 225 IU at Aster Clinic, Hasan Sadikin Hospital, and Bandung Fertility Center Limijati Hospital, Indonesia. The sample size in this study was calculated by a sampling formula for unpaired analytic categorical study, set at α=0.5 and 1-β=90%. Proportion of the population (P_1 and P_2) were assumed to be 50 and 10%, respectively. The formula yielded a minimum of 26 samples. The inclusion criteria were patients who underwent the IVF program, aged ≤40 years, were given a constant exogenous FSH dose throughout the cycle, and whose medical record included complete patient characteristics, physical examination, AMH and AFC levels throughout the cycle. AMH and AFC measurements were done on the second or third day of the menstrual cycle and this was done consistently. The exclusion criteria were the presence of a history of ovarian surgery, polycystic ovary syndrome, endometriosis, ovarian cyst, orendocrine disease. We also recorded the patients' identity, characteristics, previous medical history, previous medical therapy, levels of ovarian reserve markers (AFC and AMH), and the number of oocytes produced (the oocyte numbers in this study represent numbers for all oocytes aspirated). In this study, we selected patients as a whole, which means that all patients underwent the same treatment regimen using a short protocol with recombinant FSH and human chorionic gonadotropin, and all sperm used had normal parameters.

Ethics approval

This study protocol was approved by Faculty of Medicine, Universitas Padjadjaran, Ethics Committee Review Board (LB.04.01/ACS/TC/066/III/2018) and all study participants gave informed consent, patients consent to participate was written. All authors hereby declare that all patients have been examined in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Statistical analysis

Numerical data arepresented as mean, SD, median and range. Data normality was assessed using the Shapiro-Wilk or Kolmogorov-Smirnov test. The subjects' characteristics were compared using an unpaired t test or a Mann-Whitney test, as appropriate. Correlation between the variables was assessed using Pearson or Spearman correlation test. A P≤0.05 was considered statistically significant. SPSS v24.0 (IBM Corporation, USA) was used to perform statistical analysis.

Results

Of 356 patients enrolled during 2005-2017, 42 patients met the study criteria. Mean age of the patients investigated in this study was 34.8 ± 2.8 years (range: 29-39 years, median: 35 years) and the body mass index (BMI) was 24.4 ± 4.1 kg/m^2 (range: 14.3-33.3 kg/m^2, median: 23.6 kg/m^2). Baseline demographics of the study subjects are shown in Table 1. Normality test results showed that AFC and AMH-AFC levels were normally distributed, which were then analysed using Pearson correlation test, whereas AMH levels and oocyte amount were not normally distributed and analysed by Spearman correlation test.

Table 1: Baseline demographics and study subject characteristics

Variable	Statistical measure		
	Mean ± SD	Median	Range
Age (Y)	34.8 ± 2.8	35	29-39
BMI (kg/m^2)	24.4 ± 4.1	23.6	14.3-33.3
AMH (ng/ml)	3.34 ± 2.28	2.66	0.50-9.90
AFC	9.81 ± 3.99	10	0.50-9.90
Oocyte retrieved	7.4 ± 3.4	7	2-22
AMH-AFC	13.1 ± 5.6	12.3	2.5-27.9

BMI; Body mass index, AMH; Anti-mullerian hormone, and AFC; Antral follicle count.

The largest number of oocytes (4-15) was produced at a range of AMH of 1.2-4 ng/ml and AFC 4-15 (Table 2). There were 37 patients who produced 4-15 oocytes and they were classified as normo-responders. One patient was a hyper-responder due to the production of >15 oocytes; this excessive response was predicted as she had an AFC of >15. Furthermore, four patients produced <4 oocytes and they were classified as hypo-responders.

AMH levels were analysed using Spearman correlation test, AFC and AMH-AFC were analysed by Pearson correlation test. A significant positive correlation was found between AMH (r=0.530, P≤0.001), AFC (r=0.687, P≤0.001), and AMH-AFC combination (r=0.652, P≤0.001) and the number of oocytes. To reduce bias from

possible confounding by age, Spearman's correlation was used to determine the correlation between age and number of oocytes. There was an insignificant negative correlation between age and number of oocytes produced (P=0.129 and r=-0.179). Pearson's correlation test was used to determine the correlation between BMI and AMH, and between BMI and the number of oocytes retrieved; an insignificant positive correlation was found between the two variables (P=0.216, r=0.123, and P=0.452, and r=0.19, respectively).

Table 2: Serum AMH and AFC according to ovarian stimulation response groups

Variable	Oocyte number		
	0-3	4-15	>15
	n=4	n=37	n=1
AMH			
<1.2 ng/mL	1	4	0
1.2-4 ng/mL	3	20	1
>4 ng/mL	0	13	0
AFC			
<4	1	1	0
4-15	3	33	0
>15	0	3	1

AMH; Anti-mullerian hormone and AFC; Antral follicle count.

Discussion

Our study showed that AMH, AFC, and AMH-AFC are significantly correlated with the number of oocytes produced. This is in agreement with previous studies that investigated the relationship between AMH levels and the number of oocytes. Asada et a.l (12) observed a positive correlation between AMH level and oocyte count among Japanese women. AMH can effectively predict ovarian responses and allow clinicians to avoid iatrogenic complications and choose optimal stimulation strategies (13). However, AMH levels showed variations when examined by different examination kits and among different populations. Although we found a relatively strong and significant correlation between AMH levels and the number of oocytes with the FSH dose of 225 IU in this study, these potentially confounding factors should be considered.

Fertility begins to decrease at the age of 30 years and further decreases significantly after the age of 35 years (14, 15), which made our subjects' age (34.8 ± 2.8) a significant potential confounder inour study. In the correlational analysis, however, we did not observe a significant interaction between age and the number of oocytes produced.

How BMI influences ovarian reserve markers and the number of oocytes retrieved, is still unclear. In ameta-analysis, Moslehi et al. (16) concluded that AMH is significantly lower in obese women. On the other hand, a study of 402 women in Turkey, categorized based on ovarian reserve patterns (poor, <7 baseline AFC; adequate, ≥7 baseline AFC, and high ovarian reserve) and BMI group, revealed that serum AMH and FSH levels were similar across all categories (17). Another study of women receiving controlled ovarian stimulation for assisted reproductive technology reported that BMI did not negatively affect the number of oocytes retrieved (18). In our study, we did not find a significant correlation between BMI and AMH or BMI and oocyte count.

We observed a relatively strong positive correlation between AMH-AFC combination and the number of oocytes. In a sequential order, it can be observed that AFC correlates best with the number of oocytes, followed by AMH–AFC combination and AMH. The results of this study are in contrast to the result of a study conducted by Nelson et al. (19), which compared the predictive value of live births that indirectly represents the association between the number of oocytes and AMH, AFC, and AMH-AFC combination only with age, in the UK. The authors reported that AMH showed the best predictive value, followed by the combination of AMH-AFC and AFC only. In the present study, the strongest relationship was observed between the number of oocytes and AFC, and the results were not much different from those of the combination of AMH-AFC. This suggests that, in women without discordant ovarian marker, AFC may be a better choice compared to AMH in predicting ovarian reserve. This agrees with the results of Jayaprakasan et al. (20), who found that AFC predicts ovarian response better than AMH or a combination of AFC and AMH. This is further reinforced by the results of Liao et al. (21) whose study on 8269 women undergoing IVF/ intracytoplasmic sperm injection (ICSI) treatment showed a strong association between AFC, number of oocytes retrieved, and clinical pregnancy rate. These data imply that in the absence of AMH examination, AFC may suffice, as it is well-correlated with the number of oocytes in clinical practice. AFC is easier, and relatively inexpensive, and offers almost immediate results.

In the present study, we found a relatively strong positive correlation between AFC and the number of oocytes at the FSH dose of 225 IU. The AFC measurement performed using ultrasound was effective, easy to use, safe, and non-invasive. Therefore, estimating the number of antral follicles can be used as a predictive test of

ovarian function, ovarian reserve, and ovarian response.

Conclusion

Significant positive correlations of AMH levels, AFC, and AMH-AFC with the number of oocytes were found in this study. These correlations werestrong enough at the FSH dose of 225 IU. AFC is a better ovarian reserve marker compared to AMH and the combination of AMH-AFC in predicting the number of oocytes. Existing data on variations in infertility causes and longevity suggest that an analysis free of infertility including confounding variables and duration, would be preferable. Our study limitations could be overcome by multivariable analysis, but a larger sample size is needed.

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Authors' Contributions

W.P., M.W.F., D.T., T.D.; Examined, treated, observed and followed up the subject of this study. W.A.I.; Was responsible for data analysis, drafting and critically reviewing the manuscript. All authors read and approved the final manuscript.

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Original Article

Effect of Vitamin E on Serum Levels of Vascular Endothelial Growth Factor and Angiopoietin-1 in Women with Polycystic Ovary Syndrome: A Pilot Randomized, Placebo-Controlled Trial

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Abstract

Background: Angiogenesis disturbances are common in women with polycystic ovary syndrome (PCOS). Vitamin E has antiangiogenic properties. Data on the effects of vitamin E on angiogenesis in PCOS is limited, so the current study was conducted to evaluate its effects on angiogenic indices in PCOS patients.

Materials and Methods: This randomized, double-blind, placebo-controlled trial was performed on 43 women aged 20-40 years, diagnosed with PCOS (Rotterdam criteria). It was performed at the referral clinic affiliated to Tabriz University of Medical Sciences, Tabriz, Iran, from April 2017 to September 2017. Patients were randomly assigned into two groups to receive either 400 IU/day vitamin E -as alpha tocopheryl acetate- (n=22) or placebo (n=21), for 8 weeks. Anthropometric, and angiogenic parameters including body weight, fat mass and fat free mass, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiopoietin-1 (Ang-1), and angiopoietin-2 (Ang-2) were measured by standard methods at the beginning and at the end of study. Statistical Package for Social Science version 25 was used for statistical analysis and $P<0.05$ were considered significant.

Results: After adjusting for potential confounders, we observed that vitamin E supplementation significantly reduced body weight, fat mass, Ang-1, Ang-1/Ang-2 ratio and VEGF ($P<0.01$). We did not observe any considerable effect for vitamin E on Ang-2 level or bFGF.

Conclusion: Vitamin E supplementation for 8 weeks in the PCOS women had beneficial effects on body weight, Ang-1, Ang-1/Ang-2 ratio, and VEGF level (Registration number: IRCT201610193140N18).

Keywords: Angiopoietins, Basic Fibroblast Growth Factor, Polycystic Ovary Syndrome, Vascular Endothelial Growth Factor, Vitamin E

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most complex endocrine disorders that causes infertility due to ovulation failure in women (1). Approximately 6-25% of women of reproductive age, are influenced by PCOS (2). The prevalence of PCOS in Iranian women was reported as 19.5% based on the Rotterdam criteria (3). The clinical symptoms of PCOS include menstrual dysfunction, hyperandrogenism, polycystic ovaries, and subfertility (2). Additionally, PCOS can cause obesity and metabolic disorders such as insulin resistance, dyslipidemia, raised levels of inflammatory factors, and endothelial dysfunction. Long-term consequences

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of PCOS are endometrial cancer, diabetes mellitus, hypertension, and cardiovascular disorders (4, 5). The etiology of PCOS remains largely unknown, however, there is accumulating evidence suggesting that angiogenesis dysregulation might play the main role in the pathogenesis of PCOS (4). Angiogenesis is a complex physiological process where new vessels develop from preexisting vasculature (5). Angiogenesis in the ovary is an important part of the process of the menstrual cycle (6). An essential role of the formation of the new vessels in the ovary, is the provision of nutrients and hormones for development of the corpus luteum and follicular growth (4).

Vascular endothelial growth factors (VEGFs) and angiopoietins are among the most important angiogenic markers. Other indices include basic fibroblast growth factor (bFGF) -also known as fibroblast growth factor-2 (FGF2)- and platelet-derived growth factor (PDGF). Angiopoietin-1and -2 (Ang-1 and Ang-2, respectively) as well as VEGF play major roles in the regulation of angiogenesis in the ovary (4).

It was suggested that PCOS women have imbalances in angiogenic/antiangiogenic indices with partial dominance of pro-angiogenic markers. In this regard, increased ovarian expression of VEGF and bFGF has been reported in PCOS women. In addition, elevated levels of Ang-1 were shown in PCOS women compared to the healthy controls (4, 7). The abnormal alterations can cause cysts in the ovary, and disrupt and reduce ovulation rates. The recovery of proper blood vessel development in the ovaries, could improve follicular growth as well as development and ovulation among patients with PCOS (8).

Several studies have suggested that tocopherols reduce the processes of inflammation and angiogenesis (9). In addition, vitamin E levels in the blood of women with PCOS were lower than those of healthy subjects (10). Rahmani et al. (11) reported that vitamin E co-supplementation with omega-3 fatty acids, significantly regulated lipid profile and reduced oxidative stress products in PCOS women. Vitamin E and D co-supplementation was been shown to improve pregnancy outcome in PCOS women (12). In another study, vitamin E supplementation inhibited VEGF-A-mediated angiogenesis (13). In addition to the role of vitamin E in angiogenesis, some evidence indicated that vitamin E has an association with obesity (14). It seems that the mentioned effects are not due to the antioxidant mechanism of vitamin E (12).

Considering data scarcity in this subject, the present study was conducted to evaluate the effect of vitamin E on serum VEGF, bFGF, Ang-1, and Ang -2 as well as Ang-1/Ang-2 ratio in PCOS women. We hypothesized that vitamin E supplementation might have an effect on the angiogenic markers and imbalances in patients with PCOS.

Materials and Methods

This double-blinded, placebo-controlled clinical trial was part of a lager study approved by the Ethics Committee of Tabriz University of Medical Sciences (Ethics approval No. IR.TBZMED.REC.1395.777) and registered at Iranian Registry of IRCT (IRCT201610193140N18). It was performed at the referral clinic affiliated to the Tabriz University of Medical Sciences, Tabriz, Iran from April 2017 to September 2017. The study was advertised in different clinical and therapeutic centers. For the present study, the sample size was calculated based on the results of blood VEGF concentration reported by Mondul et al. (13) by using G*Power (version 3.1.2, Germany). The number of participants was calculated as at least 16 subjects in each group. Considering dropout and to ensure a sample size sufficiently large to enable reliable estimates, we enrolled 22 and 21 subjects in vitamin E and placebo groups, respectively.

The volunteers were given more details on the study by the first author, and then, a written consent form was signed by all of the participants. The participants were able to withdraw from the study at any time.

The inclusion criteria of the study were women within the age range 20-40 years who were diagnosed with PCOS in accordance with the Rotterdam criteria (15). On the other hand, menopause, pregnancy or lactation, diabetes, having hepatic, renal, thyroid, coagulation or cardiovascular disorders, elevated levels of prolactin, smoking, alcohol consumption, fat malabsorption, receiving oral anticoagulants, ovulation induction agents or drugs affecting hormonal profile such as oral conceptive pills (OCP), or having taken antioxidant supplements or adopted a diet or a particular plan for physical activity within the last 3 months, were considered exclusion criteria (16).

Trial design

Trial design was parallel. Initially, forty-three PCOS women with $25 \leq$ body mass index (BMI) < 35 kg/m^2, enrolled in to the study. The participants were randomly assigned into one of the two groups (in a 1:1 ratio), using the Random Allocation Software. The subjects in vitamin E and placebo groups received 400 IU/day vitamin E -as alpha tocopheryl acetate- (n=22), or cellulose capsules (n=21), for 8 weeks.

Vitamin E capsules were produced by Nature Made Pharmaceutical Company (USA, Batch number: 1143156) and provided by Pourateb Pharmaceutical Company (Iran). The placebo capsules were made by Barij Essence Pharmaceutical Corporation (Iran). The capsules of vitamin E and placebo were similar in size and shape. The patients and researcher were blind to allocations until the end of the study. Based on the guidelines (17), all patients received metformin at the dose of 1500 mg (500 mg 3 times daily). At the baseline of the study, the patients were asked to keep their physi-

cal activity and diet unchanged within the 8weeks of intervention.

Adherence to the study

To assess the compliance, the participants were requested to bring the medication containers. All patients were monitored by a weekly phone call and encouraged to consume the supplement. Short Message Service was sent to the patients' cell phones every day. To check the adherence to treatments, the participants were asked to bring the unused capsules. The subjects who had incomplete consumption of the drugs (less than 90% consumption) were excluded from the study.

Evaluation of anthropometrics

Body weight (following overnight fasting) was measured by a digital scale (Seca, Hamburg, Germany) with an accuracy of ± 0.1kg. Height was measured by a non-elastic strip (Seca, Germany) with a precision of 0.1 cm. Further, BMI was calculated as weight in kilograms divided by squared height in meters. The body composition indices including body fat mass percentage (FM%), fat mass (FM), and fat free mass (FFM), were evaluated by a bioelectrical impedance analyzer (8-electrode, TanitaBC-418 MA; Tanita Co., Japan).

Assessment of dietary intake and physical activity

Dietary intake was assessed by 24-hour recall, which was completed on three different days of the week (two weekdays and one weekend). To assess the nutrient intake of the patients, Nutritionist IV software (First Databank, CA) edited for Iranian foods, was used. To control the confounding effects of physical activity, international physical activity questionnaire-short form- (IPAQ-S) was employed for evaluation of physical activity (18). The validity and reliability of the Persian translation of IPAQ in previous studies on the Iranian populations, were tested and approved (19). We assessed physical activity and dietary intakes at the baseline and at the 8th week of intervention.

Laboratory analysis

At the beginning and the end of the study, the patients were instructed to refer to the laboratory on days 3 to 5 of normal menstrual cycle or menstrual induced by progesterone. Fasting blood samples were obtained from the participants. To separate the serum, centrifugation at 1200 rpm for 12 minutes, was done. The samples were kept at -80°C for subsequent experiments (20). Serum levels of Ang-1 and Ang-2, VEGF, and bFGF were measured using the commercial Enzyme-Linked Immunosorbent Assays (ELISAs, Bioassay Technology Laboratory, Shanghai Korain Biotech, China) according to the manufacturer's instructions. Coefficients of variation of the intra-assay and inter-assay assays were less than 10%.

Statistical methods

Data analysis was performed by an intention-to-treat procedure where missing values were treated based on Last-Observation-Carried-Forward method. To identify within-group differences (pre- and post-intervention), we utilized paired samples t tests. To determine between group differences, independent sample t test and Mann-Whitney U test were used for comparison of normally and abnormally distributed variables, respectively. To identify the impacts of vitamin E supplementation on anthropometric and biochemical variables, analysis of covariance adjusted for age, physical activity, BMI, and baseline values, was employed. We used Statistical Package for Social Science version 25 (SPSS Inc., Chicago, Illinois, USA) for all statistical analyses, with $P<0.05$ considered significant.

Results

Four subjects dropped out the study because of pregnancy (n=1) or in vitro fertilization (n=3). Finally, 39 participants remained in the study. However, intention-to-treat analysis was used, so, data for 43 PCOS women were included in the final analysis (Fig.1). No major side effect was observed following taking vitamin E supplement. Hyperandrogenism (clinically) and PCOS (by ultrasound) were seen is nearly all of the subjects. Ninety percent of the participants had oligo-anovulation. The compliance rate of the studied groups was more than 90%. The baseline characteristics of the participants are shown in Table 1. There were no significant differences between the two groups in terms of age, height, and physical activity. Baseline measures for follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were not different between the vitamin E and placebo groups. More details on the hormonal status of the subjects are given in another published article (16).

Table 1: Baseline characteristics of the studied subjects in the vitamin E and placebo groups

Variables	Vitamin E n=22	Placebo n=21	P value*
Age (Y)	27.18 ± 5.77	26.0 ± 4.53	0.68
Height (cm)	162.27 ± 6.86	159.81 ± 6.06	0.22
Physical activity (MET-minute/week)			
Low	8 (36.4)	10 (47.6)	
Moderate	11 (50)	7(52.4)	0.54**
Vigorous	3(13.6)	4 (19)	

Data are presented as mean ± SD or n (%).*; Assessed by independent t test and **; Chi-square test, and METs; Metabolic equivalents.

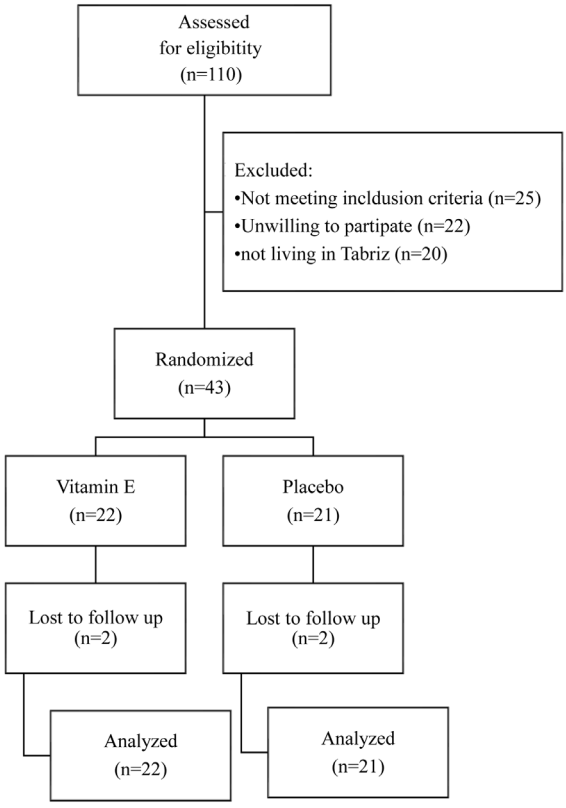

Fig.1: Flowchart of the study.

Dietary intake

Table 2 presents dietary intakes of the studied subjects. There were no significant differences in the dietary intakes of energy and nutrients between the two studied groups.

Table 2: Dietary intakes of the study participants throughout the study in the vitamin E and placebo groups

Variables	Vitamin E n=22	Placebo n=21	P value*
Energy (Kcal/day)	1698.46 ± 215.88	1745.87 ± 308.00	0.56
Carbohydrate (g/day)	214.89 ± 28.03	227.63 ± 52.57	0.32
Protein (g/day)	66.65 ± 13.66	56.9 ± 12.77	0.36
Fat (g/day)	68.05 ± 15.75	63.57 ± 18.47	0.39
SFAs (g/day)	16.09 ± 7.71	15.28 ± 7.48	0.73
PUFAs (g/day)	13.37 ± 5.89	12.69 ± 6.66	0.72
MUFAs (g/day)	17.2 ± 6.3	16.94 ± 6.59	0.89
Cholesterol (mg/day)	194.98 ± 58.55	206.21 ±65.66	0.56
Fiber (g/day)	20.73 ± 4.79	20.31 ± 4.71	0.77
Vitamin E (mg/day)	6.09 ± 3.1**	6.85 ± 3.23	0.47
Vitamin A (RE/d)	440.12 ± 81.93	422.65 ± 132.51	0.60
Vitamin C (mg/d)	66.45 ± 12.05	73.80 ± 20.09	0.15
Selenium (µg/d)	43.39 ± 4.26	48.29 ± 20.80	0.30
Zinc (mg/day)	5.23 ± 1.34	5.21 ± 0.99	0.94

Data are presented as mean ± SD. SFA; Saturated fatty acid, PUFA; Polyunsaturated fatty acid, MUFA; Monounsaturated fatty acid, *; Assessed by independent t test, and **; Vitamin E level is estimated based only on dietary consumption, in the absence of the study supplement.

Anthropometric measurements

No significant difference was found at the baseline of the study in the assessed anthropometric indices except for FM which was significantly higher in the vitamin E group. In within-group analysis, all assessed anthropometric indices had significant changes in the vitamin E supplemented group (P<0.01). In between-groups comparisons, except for FFM, the assessed anthropometric indices were reduced in the vitamin E-supplemented group compared to the placebo group (Table 3).

Table 3: Baseline and 8 weeks after intervention values of the anthropometric indices in the vitamin E and placebo groups

Variables	Vitamin E n=22	Placebo n=21	P value
Weight (kg)			
Before	76.95 ± 10.61	73.23 ± 7.58	0.19[b]
After	75.96 ± 10.3	73.29 ± 7.3	0.01[c]
P value[a]	0.003	0.82	
BMI (kg/m^2)			
Before	29.45 ± 5.35	28.80 ± 3.71	0.64[b]
After	29.07 ± 5.16	28.83 ± 3.70	0.01[c]
P value[a]	0.003	0.75	
FM (kg)			
Before	29.57 ± 4.41	27.08 ± 3.55	0.05[b]
After	28.25 ± 4.45	26.87 ± 3.84	0.001[c]
P value[a]	0.001	0.34	
FFM (kg)			
Before	46.86 ± 4.26	44.92 ± 2.73	0.08[b]
After	47.57 ± 4.14	44.86 ± 2.93	0.22[c]
P value[a]	0.004	0.83	
FM (%)			
Before	36.51 ± 5.54	34.24 ± 2.85	0.09[b]
After	34.85 ± 5.38	33.89 ± 2.85	0.001[c]
P value[a]	0.001	0.90	

Data are presented as mean ± SD. BMI; Body mass index, FM; Fat mass, FFM; Fat free mass, [a]; P value for paired t test, [b]; P value for Independent sample t test, and [c]; P value for ANCOVA: adjusted for total calorie intake, dietary vitamin E intake, age, physical activity and baseline values.

Angiogenic markers

The effects of vitamin E on angiogenic indices are shown in Table 4. The basal values of the angiogenic markers were not different between the two groups. In within-group analysis, VEGF, bFGF, Ang-1, and Ang-1/Ang-2 all had significant reductions in the vitamin E-supplemented group. In between-group comparisons, after adjustment for age, BMI, physical activity, total calorie intake, dietary vitamin E intake, and baseline values, supplementation with vitamin E had significant effects on VEGF, Ang-1,andAng-1/Ang-2 ratio (P=0.01, P=0.001 and P=0.03, respectively).

Table 4: Baseline and 8 weeks after intervention values of the serum angiogenic markers in the vitamin E and placebo groups

Variables	Vitamin E n=22	Placebo n=21	P value
VEGF (pg/mL)			
Before	733.15 (678.03, 1332.15)	423.40 (240.45, 1879.55)	0.96[b]
After	329.85 (290.00, 1381.06)	420.00 (274.15, 1628.72)	0.01[c]
P value[a]	0.005	0.48	
bFGF (pg/mL)			
Before	345.20 (305.99, 631.65)	370.10 (301.35, 590.45)	0.76[b]
After	314.18 (231.95, 318.80)	386.00 (303.7, 642.75)	0.24[c]
P value[a]	0.003	0.66	
Ang-1 (pg/mL)			
Before	1627.16 (1381.54, 2814.50)	1461.80 (1175.90, 1811.05)	0.28[b]
After	864.80 (645.90, 1627.16)	1305.01 (1305.01, 1774.45)	0.001[c]
P value[a]	0.001	0.87	
Ang-2 (pg/mL)			
Before	427.35 (247.78, 590.10)	432.49 (238.00, 493.45)	0.68[b]
After	436.65 (250.70, 554.70)	410.91 (332.75, 410.91)	0.83[c]
P value[a]	0.81	0.49	
Ang-1:Ang-2			
Before	3.44 (2.97, 5.27)	3.41 (2.72, 5.12)	0.49[b]
After	2.63 (1.46, 3.74)	3.52 (3.17, 4.78)	0.03[c]
P value[a]	0.03	0.61	

Ang-1; Angiopoietin-1, Ang-2; Angiopoietin-2, VEGF; Vascular endothelial growth factor, bFGF; Basic fibroblast growth factor, [a]; P value for Wilcoxon test, [b]; P value for Mann-Whitney U-test, and [c]; P value for ANCOVA: adjusted for total calorie intake, dietary vitamin E intake, age, physical activity and baseline values. Data are shown as median (25^{th}, 75^{th}).

Discussion

The present study was conducted to investigate the effect of vitamin E supplementation on the angiogenic markers in patients with PCOS. As far as we know, the present clinical trial is the first to examine the effects of vitamin E supplementation on serum angiogenic markers and anthropometric parameters in patients with PCOS. The results of this study revealed a significant reduction in weight and fat mass after eight weeks of supplementation with vitamin E among patients with PCOS. Both groups had lower energy intakes than daily estimated energy requirements (EER) for moderately active women. Low energy intake is considered a way of weight reduction, so, it is possible that the study subjects had reduced their calorie intakes for weight reduction. Only in the vitamin E group, weight reduction was significant. Few studies had assessed the effects of vitamin E supplementation on body composition components. There is some evidence about an inverse association between serum vitamin E concentration and adiposity (21). It was found that vitamin E is involved in the expression of some genes, like as leptin and peroxisome proliferator-activated receptor-γ (PPARγ), which are related to the glucose and lipid metabolism (14, 22). Leptin regulates food intake and energy balance thus plays a key role in the regulation of body fat mass (14). PPARγ is an adipogenic factor and acts as a regulator of adipogenesis (23). Increased PPARγ activity may have a positive effect on body weight gain and FM (22). Vitamin E down-regulates the expression of PPARγ (24).

Our study results indicated that vitamin E significantly lowered serum Ang-1 levels, while no change was observed in Ang-2 concentration in PCOS women. There is some evidence on angiopoietin disturbances in PCOS women. Scotti et al. (7) investigated angiopoietins of follicular fluids and reported an increase in Ang-1 but no changes in Ang-2.

In our literature review, there were no studies on vitamin E effects on the Ang-1 levels. The probable mechanism of reducing Ang-1 level by vitamin E may be linked with the reactive oxygen species (ROS). The increasing effects of ROS on the level, signaling and biological effects of Ang-1 were shown. Vitamin E has antioxidative properties, so, by scavenging of ROS, it decreases ROS and therefore, Ang-1 levels (25, 26).

In our study, the Ang-1/Ang-2 ratio was decreased. Restoration of the increased level of Ang-1/Ang-2 enhances vascular progression, which in turn, promotes proper follicular evolution and increased ovulation (27). The exact mechanism(s) by which vitamin E exerts these regulatory effects are still unknown, though some possible mechanisms have been proposed. It was stated that oxidants stimulate angiogenesis while antioxidants counteract angiogenesis (28). In addition, tocopherols exert their anti-angiogenic and anti-proliferative effects through preventing signaling and activation of PI3K/PDK/Akt signaling pathway, and inhibiting tube formation of endothelial cells (29).

Our study suggested a lowering effect for vitamin E intake on VEGF in PCOS women. There is some evidence indicating VEGF roles in the pathophysiology of PCOS (4, 30). VEGF, through neovascularization in the ovaries of PCOS patients, supports the increase in ovarian mass. Elevated levels of VEGF have been reported in women with PCOS (31). In addition, endocrine gland-VEGF, as an endothelial cell mitogen, has been shown to be over expressed in the PCOS patients' ovaries (32). Many studies assessed the effects of vitamin E on VEGF. These studies showed different effects for tocopherol on VEGF expression and angiogenesis (33, 34). It seems that the effects are dependent on the phosphorylation status of α-tocopherol (35). In an in vitro study, phosphorylated α-tocopherol (αTP) stimulated VEGF generation, while non-phosphorylated (αT) form did not (36). This is the outcome of PI3K/Akt signaling pathway stimulation or inhibition. Tocopherol phosphorylation and dephosphorylation may indirectly influence pro-angiogenic or anti-angiogenic activities. Creation of αTP in vivo probably describes pro-angiogenic effects of vitamin E. Placenta creation, inhibition of ischemia/reperfusion injury in the brain or cardiovascular system and promotion of wound healing are pro-angiogenic activities. Further, the pro-angiogenic ability of αTP is important in terms of expansion of solid tumors (37).

Our study did not demonstrate the effect of vitamin E on the bFGF in PCOS patients. In contrast to VEGF, little is known about agents influencing bFGF. bFGF has important functions in the ovarian angiogenesis. bFGF is a follicle-stimulating hormone, expressed in theca and granulosa cells leading to promotion of follicular growth and managing its activity (38). bFGF enhances angiogenesis by different mechanisms including stimulation of endothelial cell reproduction, chemotaxis and formation of matrix repairing enzymes such as plasminogen activator and collagenase (39). bFGF has been associated with obesity which is a common characteristic of PCOS. In addition, Artini et al. (40) reported higher levels of bFGF in serum and follicular fluids of patients with PCOS. Thus, correction of bFGF alterations in the biological fluids of PCOS women should be further examined.

Our study had some limitations. The most important limitation of the study was lack of measurement of vitamin E concentration at the baseline and at the end of study. In our study, the duration of the disease was not assessed. Another limitation was the small sample size. We could not provide a sonographic evaluation of ovarian masses at follow-up. Additionally, self-reported dietary intakes -which have the probability of under/over-reporting- and short duration of the intervention were other limitations for our study.

Conclusion

In patients with PCOS, vitamin E supplementation has useful effects on some anthropometric measurements and Ang-1, VEGF and Ang-1/Ang-2 ratio in blood. These findings suggest possible beneficial effects for vitamin E on PCOS. Concerning our study limitations, further studies are recommended to explore the potential effects of vitamin E in the management of angiopoietins disturbances among PCOS patients.

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Authors' Contributions

S.S., Sh.T., A.I., M.P.; Participated in data collection, analysis of data, and drafting the manuscript. B.P.G.; Was involved in the theory and objective of the study, and preparation final revision of the manuscript. All authors read and approved the final version of the manuscript.

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Original Article

Synbiotic Supplementation Improves Metabolic Factors and Obesity Values in Women with Polycystic Ovary Syndrome Independent of Affecting Apelin Levels: A Randomized Double-Blind Placebo - Controlled Clinical Trial

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Abstract

Background: This research investigated the symbiotic supplement influences on serum glycemic indices and lipids as well as apelin rates and obesity values in polycystic ovary syndrome (PCOS) patients.

Materials and Methods: A total of 68 obese or overweight patients (20-44 years old) with PCOS were enrolled to conduct a randomized double-blinded placebo-controlled clinical trial. A total of 34 people in the synbiotic group received a synbiotic supplement and 34 people in the placebo group received placebo, daily for 8 weeks. Fasting blood specimens, anthropometric measurements and dietary intake data were gathered three times during the study. The information was analyzed by independent t test, paired t test, analysis of covariance and chi-square test.

Results: Synbiotic supplementation significantly decreased serum fasting glucose (P=0.02), insulin (P=0.001), homeostatic model assessment for insulin resistance (IR, P=0.001), weight (P=0.02), body mass index (BMI, P=0.02), waist circumference (WC, P=0.01), hip circumference (HC, P=0.02), and waist-to-height ratio (WHtR, P=0.02) but significantly increased high-density lipoprotein (HDL) cholesterol (P=0.02) compared to the placebo. At the end of the trial, no significant differences were seen in serum total cholesterol, triglyceride (TG), low-density lipoprotein (LDL) cholesterol, or apelin levels as well as waist-to-hip ratio (WHR) between the two groups.

Conclusion: Synbiotic supplementation improved glycemic indices, lipid profile and obesity values in women with PCOS. These beneficial effects were not related with alterations in serum apelin levels (Registration number: IRCT20100408003664N19).

Keywords: Apelin, Metabolic Factors, Obesity, Polycystic Ovary Syndrome, Synbiotic

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Introduction

Polycystic ovary syndrome (PCOS) is a momentous endocrine disarray in reproductive age women that leads to infertility and an enhancement in the occurrence of abortion, gestational diabetes and pre-eclampsia (1, 2). The prevalence of PCOS is estimated to be 4 to 21% worldwide (3). These patients indicate an irregular menstruation period, an ovulatory cycle, and androgen excess (4).

PCOS is contemplated as a multifactorial disease that is often accompanied by metabolic disorders including obesity, insulin resistance (IR), dyslipidemia and increased levels of androgens. PCOS is a risk factor for type 2 diabetes, cardiovascular difficulties and endometrial cancers (2, 5).

More than 50% of patients with PCOS are obese (6). Adipose tissue generates several proteins that are called adipokines that have a hormonal function (7). Studies have shown that adipokines derived from fatty tissue, can contribute to the pathogenesis of PCOS (8). Apelin (APLN-13or -17) is an adipokine located on the Xq25-q26 chromosome and it contains 77 amino acids. Adipose tissue is not the only determinant of serum apelin levels. Other organs such as the ovary, breast, gastrointestinal system, and central nervous system can also contribute to apelin secretion (9). It has been proposed that apelin has a function in regulating glucose metabolism, lipolysis and food intake (10). Some studies stated

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that apelin has a significant function in controlling IR in diabetes mellitus type 2 mice and humans (11, 12).

The implication of apelin in the pathogenesis of PCOS seems to be more intricate, involving a disrupted synthesis of androgens (13, 14). Lifestyle modification such as weight loss and using oral contraceptive pills and metformin are the most common treatments for PCOS (5, 15). Recently, modulation of intestinal microbiota equilibrium using probiotics and prebiotics has been suggested as an effective approach for some diseases such as PCOS. The intestinal microbiota imbalance can lead to increased ovarian androgen production and prevents the spread of the natural follicles of the ovary through chronic inflammatory response and IR (15). Living microorganisms called probiotics concede a well profit up on the hostess until used with adequate quantities (16). On the other hand, prebiotic is as "a non-digestible food component" that lucratively affects the host via selectively promoting the development and/or activity of one or a confined number of bacteria in the colon (17).

A composition of probiotic and prebiotic that augment the viability of probiotics in the intestinal tract by stimulating growth or betterment metabolic activity, is called symbiotic (18). Samimi et al. (19) presented that synbiotic supplementation for 12 weeks, declined IR and serum levels of insulin, very-low-density lipoprotein (VLDL) cholesterol and triglyceride (TG) in PCOS patients. Karimi et al. (20) reported that synbiotic supplementation at a dose of 1000 mg for 12 weeks, reduced serum apelin-36 levels in PCOS patients, although changes in IR, blood glucose and insulin levels were not significant. In another study, synbiotic supplementation for 12 weeks led to a notable reduction in serum insulin levels and IR in patients with PCOS (21). There is no further study about the effect of synbiotics in patients with PCOS.

Since variations in gut microbiota combination have been reported in subjects with PCOS (22), synbiotics can be considered as remedial agents (15). However, controversies have been seen in the outcomes of studies (19, 21). Moreover, the effects of synbiotics supplementation on obesity values have not been assayed in these patients. Accordingly, we designed a study to assess the effects of synbiotics on glycemic indices, serum lipids, and apelin levels and anthropometric values in PCOS patients.

Materials and Methods

In this randomized double-blinded placebo-controlled clinical trial study, out of 68 people suffering from PCOS within the age range 20-44 years and with body mass index (BMI) ranged 25-40 kg/m^2 cooperated in this randomized double-blinded placebo-controlled clinical trial in Alzahra Hospital in General Gynaecology Clinic Department, in Tabriz, between June and December 2018.

The sample size was calculated based on information obtained from the research conducted by Ahmadi et al. (23) on IR. The sample size was calculatedas 30 in each group, for the confidence intervals of 95% and a power of 80%. The projected dropout rate was set at 34 with the increase in sample size per group. 2003 Rotterdam criteria determined cognitive performance in PCOS due to three dimensions such as: idiopathic amenorrhea oroligomenorrhea, presenting the hyperandrogenism (convenient clinical and/or biochemical assessments) and PCOS via sonography (8).

Criteria for exclusion included: thyroid gland disorders, diabetes, high levels of serum prolactin (hyperprolactinemia), gestation and lactation, liver or kidney disease, Cushing's syndrome diseases, cardiovascular diseases, high blood pressure, drug consumption including hydrochlorothiazide, insulin therapy, using beta blockers, low-density lipoprotein (LDL) cholesterol medicine, addiction, fertility treatments available, using cortisone-like medicine, following a special diet as well as being athlete or sport in orderly array (longer than the standard 2-week), antibiotic use during the last month and at the time of the study, use of any dietary supplements in the last 2 months or throughout the study, receiving probiotics, prebiotics and synbiotics in the last three months and simultaneous of the study, regular consumption of probiotic products and sensitivity to symbiotic orprobiotic capsules.

The research protocol was approved by the Research Ethics Committee of Tabriz University of Medical Sciences (code: IR.TBZMED.REC.1396.1080) and registered at the IRCT website (Registration number: IRCT20100408003664N19). Written informed consent was gained from all participating women before the study.

Based on the age and BMI, the participants were randomly divided into two groups by a size 2 block randomization technique. In this technique, patients had to retain a normal diet and physical activity throughout the trial.

Public information was obtained for each participant. Body weight and height were measured using a scale (Seca, Germany), and a mounted tape, respectively. BMI was estimated by dividing the weight in kilogram by height in (m)^2. Soft measuring tape in standing up position was used to obtain hip circumference (HC) as well as waist circumference (WC) (24). WC was measured in the narrowest section across the costaarch and the crest of the iliumand HC as the distance around the largest part of hips. Waist-to-hip ratio (WHR) and waist-to-height ratio (WHtR) were respectively calculated as follows: WC in centimeter divided by HC in centimeter and WC in centimeter divided by height in centimeter.

Validated International Assessment of Physical Activity Questionnaire (IPAQ) was used to estimate the level of physical activity (25).

Also, 24-hour method was used to acquire data on daily intake of energy and macronutrients (2 week days and 1 weekend day). Questionnaires were completed before starting the study, at the end of the fourth week and the end of the study. The average energy and macronutrients intakes of all patients were analyzed using Nutritionist 4 software (FDBInc., California).

For eight weeks, patients in the treatment group (n=34) received one capsule of synbiotic and placebo group (n=34) received placebo capsule that was essential in daily use after lunch.

Each synbiotic capsule (500 mg, Zist-Takhmir Co., Iran), included seven strains of helpful bacteria (*Lactobacillus casei* 3×10^9 colony-forming units (CFU)/g, genus Lactobacillus *Lactobacillus rhamnosus* 7×10^9 CFU/g, *Lactobacillus bulgaricus* 5×10^8 CFU/g, genus Lactobacillus acidophilus 3×1010 CFU/g, *Bifidobacterium longum* subsp1×10^9 CFU/g, (strainACS-071-V-Sch8b) 2×10^{10} CFU/g and *Streptococcus thermophilus* subsp3×10^8 CFU/g and inulin-type prebiotics (Fructooligosaccharides (FOS)). The placebo capsule contained starch with identical color and form.

The compliance of the participants with the study protocol was evaluated via phone talks once per week and by assessment of returned capsules every 2 weeks.

Blood sampling and biochemical assays

Blood samples (5 mL) were collected after 12-hour overnight fasting, in the morning. The serum was separated via centrifugation and stored at -70°C up to subsequent research. The standardized enzymatic method using a commercially available Kit (Pars Azmoon, Iran) was employed to evaluate blood glucose. ELISA method using Monobind kit (Monobind Inc., CA, USA) was used to measure the serum insulin level and IR was defined via Homeostasis Model Assessment (HOMA) equations using the following relation: HOMA-IR was estimated by multiplying the fasting insulin (μIU/mL) and fasting glucose (mg/dL) divided by 405 (26).

Standardized enzymatic approach using a commercially available Kit (Pars Azmoon, Iran) was employed to evaluate the total blood cholesterol (TC), TG, and high-density lipoprotein (HDL) cholesterol. Serum concentration of LDL cholesterol was quantified via Friedewald formula (FF): LDL cholesterol=TC-(HDL cholesterol+TG/5) (27). Enzyme-linked Immunosorbent Assay (shortened as ELISA) using Mediagnost kit (Cat No. E2037Hu; Shanghai Crystal Day-Biotech Co., Ltd) was performed to specify blood apelin ratio. The inter-and intra-assay coefficients of variation toward apelin were considered lower than 8 and 10%, respectively.

Finally, all the body measurements, and biochemistry assessments were reassessed at the end of the study.

Statistical analysis

Statistics SAGE IBM® SPSS® Statistics 23 software was used to analyze the data (supplied by SPSS Inc., USA) and findings are presented as mean ± SD. The distribution of variables was normal as assessed by Kurtosis-Skewness statistics. Independent t test was used for comparing primary evaluations of all variables in the two groups at the baseline and (χ^2) criterion was also used for categorical and numeric variables. Changes in body measurements and blood parameters of patients were measured between pre-test and post-test by paired-samples t test. Analysis of covariance (ANCOVA) was applied to recognize any discrepancies between the two groups after the supplementation, adjusting for baseline measurements and confounders. Repeated measures ANOVA were exerted to assess the within-group changes in dietary intake. The following equation determined the variable alterations after intervention by percentage: [(subtraction of after and before values) divided by before values] multiplied by 100. Statistical significance was considered at $P<0.05$.

Results

All participants [the synbiotic group (n=34) as well as the placebo group (n=34)] were ended the study (Fig.1). The adoption of the study was performed well and 95% of the prescribed supplements were consumed during the study. No complication or symptoms were reported following supplementation.

Public characteristics of the patients are shown in Table 1. There were no significant discrepancies between the two groups in the means of age, weight, BMI and physical activity levels at baseline. No groups had significant changes ($P<0.05$) in the rate of women's physical activity during the study.

Table 1: General features of women with PCOS participated in this trial

Variable	Measurement period	Placebo group n=34	Synbiotic group n=34	MD (95% CI), P value
Age (Y)	Baseline	28.6 ± 4.82	30.4 ± 5.82	
Weight (kg)	Baseline	73.67 ± 10.89	76.15 ± 14.97	2.47 (-3.8 to 8.82), 0.438
BMI (kg/m^2)	Baseline	28.47 ± 3.55	29.43 ± 5.69	0.95 (-1.34 to 3.26), 0.409
Physical activity	Baseline			0.564*
	Low	19 (55.9)	16 (47.1)	
	Moderate	8 (23.5)	14 (41.2)	
	High	7 (20.6)	4 (11.8)	
	After intervention			0.328*
	Low	18 (52.9)	14 (41.2)	
	Moderate	9 (26.6)	15 (44.1)	
	High	7 (20.5)	5 (14.7)	

Data are presented as mean ± SD or n (%). PCOS; Polycystic ovary syndrome, CI; Confidence interval, MD; Means difference, BMI; Body mass index, and *; P value is reported based on the chi-square test. MD (95% CI); P value is reported based on the analysis of independent sample t test.

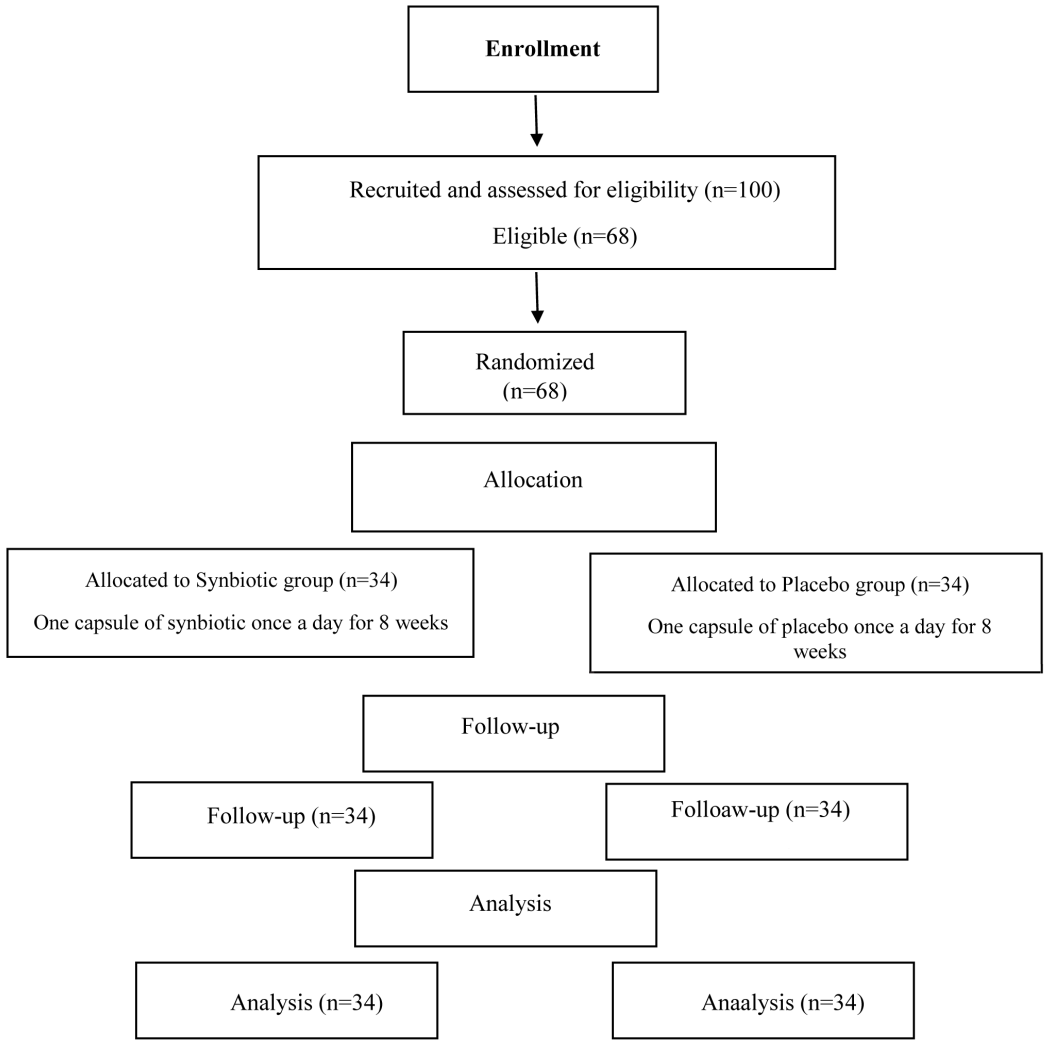

Fig.1: Participants flow diagram.

Table 2: Dietary intakes of women with PCOS participated in this trial at baseline and after 8 weeks of intervention

Variable	Measurement period	Placebo group n=34	Synbiotic group n=34	P value
Energy (kcal/day)	Before	1528.19 ± 300.87	1759.08 ± 511.30	0.027*
	Fourth week	1682.55 ± 360.35	1847.06 ± 387.35	0.517**
	After	1636.36 ± 286.46	1796.08 ± 423.39	0.566**
	P value†	0.021	0.391	
Carbohydrate (g/day)	Before	217.46 ± 50.16	257.47 ± 94.69	0.033*
	Fourth week	230.94 ± 60.64	259.65 ± 59.78	0.347
	After	235.58 ± 54.12	256 ± 67.51	0.804
	P value	0.143	0.954	
Protein (g/day)	Before	51.40 ± 12.74	58.54 ± 20.83	0.093
	Fourth week	56.68 ± 15.68	61.78 ± 14.89	0.610
	After	53.06 ± 13.70	60.17 ± 15.46	0.207
	P value	0.109	0.519	
Total fat (g/day)	Before	52.80 ± 14.69	57.31 ± 15.48	0.223
	Fourth week	61.68 ± 16.83	64.18 ± 22.86	0.905
	After	56.04 ± 12.84	62.03 ± 20.70	0.316
	P value	0.015	0.097	

Data are presented as mean ± SD. PCOS; Polycystic ovary syndrome, *; Significant difference between the two groups at baseline (P<0.05, independent sample t test), †; P value is reported based on the analysis of the repeated measures, and **; P value is reported based on analysis of covariance (adjusted for baseline values).

Table 3: Biochemical parameters of women with PCOS participated in this trial, at baseline and after 8 weeks intervention

Variable	Measurement period	Placebo group n=34	Synbiotic group n=34	MD (95% CI), P value
Glucose (mg/dL)	Baseline	89.02 ± 9.05	91.32 ± 8.07	2.29 (-1.86 to 6.45), 0.274
	After intervention	94.44 ± 9.49	90.08 ± 7.90	-4.52 (-8.50 to -0.54), 0.026†
	MD (95% CI), P value	5.41 (2.38 to 8.43), 0.001**	-1.2 (-4.58 to 2.11), 0.459	
Insulin (μIU/mL)	Baseline	9.46 ± 4.64	13.36 ± 4.89	3.89 (1.58 to 6.20), 0.001*
	After intervention	13.17 ± 5.29	11.50 ± 4.75	-3.90 (-6.18 to -1.62), 0.001†
	MD (95% CI), P value	3.70 (2.06 to 5.34), 0.000**	-1.85 (-3.41 to -0.29), 0.021**	
HOMA-IR	Baseline	2.10 ± 1.12	3.06 ± 1.35	0.95 (0.35 to 1.55), 0.002*
	After intervention	3.08 ± 1.31	2.58 ± 1.15	-0.93 (-1.50 to -0.36),0.001†
	MD (95% CI), P value	0.97 (0.56 to 1.39), 0.000**	-0.47 (-0.90 to -0.04), 0.032**	
TC (mg/dL)	Baseline	197.91 ± 39.80	209.41 ± 33.16	11.50 (-6.25 to 29.25), 0.200
	After intervention	210.11 ± 39.17	208.55 ± 38.92	-6.93 (-22.58 to 8.71), 0.379
	MD (95% CI), P value	12.20 (3.09 to 21.31), 0.010**	-0.85 (-13.92 to 12.21), 0.895	
TG (mg/dL)	Baseline	140.76 ± 71.22	139.29 ± 69.92	-1.47 (-35.64 to 32.70), 0.932
	After intervention	149.14 ± 83.83	137.97 ± 68.61	-11.88 (-31.79 to 8.03), 0.238
	MD (95% CI), P value	8.38 (-5.23 to 22), 0.219	-1.32 (-15.20 to 12.55), 0.847	
LDL-cholesterol (mg/dL)	Baseline	121.61 ± 31.64	135.75 ± 28.40	14.14 (-0.41 to 28.71), 0.057
	After intervention	136.05 ± 32.60	133.84 ± 36.07	-8.34 (-24.11 to 7.42), 0.294
	MD (95% CI), P value	14.44 (5.77 to 23.10), 0.002**	-1.91 (-14.74 to 10.92), 0.764	
HDL-cholesterol (mg/dL)	Baseline	48.14 ± 10.22	45.79 ± 12.05	-2.35 (-7.76 to 3.06), 0.389
	After intervention	44.23 ± 10.73	47.11 ± 12.73	5.39 (0.74 to 10.05), 0.024†
	MD (95% CI), P value	-3.91 (-7.91 to 0.09), 0.055	1.32 (-1.25 to 3.90), 0.304	
Apelin (nmol/mL)	Baseline	28.12 ± 23.56	20.06 ± 13.78	-8.06 (-19.53 to 3.41), 0.164
	After intervention	32.93 ± 25.88	21.86 ± 14.87	-0.81 (-10.88 to 9.25), 0.871
	MD (95% CI), P value	4.80 (-0.96 to10.57), 0.098	1.80 (-5.47 to 9.07), 0.613	

Data are presented as mean ± SD. PCOS; Polycystic ovary syndrome, CI; Confidence interval, MD; Means difference, HOMA-IR; Homeostatic model assessment for insulin resistance, TC; Total cholesterol, TG; Triglyceride, LDL; Low-density lipoprotein, HDL; High-density lipoprotein, BMI; Body mass index, *; P value is reported based on the analysis of independent sample t test, **; P value is reported based on the analysis of paired sample t test, and †; P value is reported based on the analysis of covariance, adjusted for energy intake, BMI and baseline values.

Daily dietary intakes of patients throughout the study are shown in Table 2. There were significant differences (P<0.05) between the two groups in average of daily energy and carbohydrate intakes at baseline. Diversities at other macronutrients intake were not notable between the two groups at baseline. There was a significant increase in energy and whole lipid intake in the placebo group during the research (P=0.02 and P=0.01, respectively). Changes in dietary intakes were not considerable in the synbiotic group. No significant differences were detected in energy and macronutrients intakes between the two groups at the end of the trial (P>0.05).

Metabolic parameters of patients at the beginning and after 8-weeks supplementation are shown in Table 3. There were significant distinctions between the two groups in means of serum insulin (P=0.001) and HOMA-IR (P=0.002) at baseline. No significant differences were seen between the two groups in levels of other biomarkers at baseline.

Results of analysis of covariance indicated statistically considerable variations between the two studied groups in serum levels of glucose (P=0.02), insulin (P=0.001), HOMA-IR (P=0.001) and HDL cholesterol (P=0.02) finally, set toward energy intake, BMI as well as initial amounts. There were no significant alterations in blood TC, TG, LDL cholesterol and apelin levels.

Supplementation with symbiotic reduced by respectively 1.35, 13.92 and 15.68% at blood ratios of glucose, insulin and HOMA-IR and 2.88% increase in HDL cholesterol, in comparison to the placebo group.

Table 3 shows a substantial reduction in insulin and HOMA-IR (by 13.92%, P=0.02 and 15.68%, P=0.03, respectively) in the synbiotic group at the end of the trial in comparison to the baseline values. Also, serum ratios for glucose increased within the placebo group (by 6.08 %, P=0.001). Serum apelin concentrations stayed unchanged in the two groups at the end of the study. The baseline and post-intervention values for apelin levels which had a wide SD were 28.12 ± 23.56 nmol/mL and 32.93 ± 25.88 nmol/mL in the placebo group and 20.06 ± 13.78 nmol/mL and 21.86 ± 14.87 nmol/mL in the synbiotic group, respectively.

Anthropometric measurements of women with PCOS

Table 4: Anthropometric characteristics of subjects with PCOS participated in this trial, at baseline and after 8 weeks of intervention

Variable	Measurement period	Placebo group n=34	Synbiotic group n=34	MD (95% CI), P value
Weight (kg)	Baseline	73.67 ± 10.89	76.15 ± 14.97	2.47 (-3.8 to 8.82), 0.438
	After intervention	74.22 ± 11.14	75.08 ± 15.35	-1.58 (-2.91 to -0.24),0.021†
	MD (95% CI), P value	0.55 (0.19 to 0.90), 0.003**	-1.07 (-2.36 to 0.21), 0.099	
BMI (kg/m^2)	Baseline	28.47 ± 3.55	29.43 ± 5.69	0.95 (-1.34 to 3.26), 0.409
	After intervention	28.72 ± 3.63	29.00 ± 5.76	-0.63 (-1.18 to -0.09), 0.021†
	MD (95% CI), P value	0.24 (0.08 to 0.39), 0.003**	-0.43 (-0.94 to 0.08), 0.101	
WC (cm)	Baseline	93.08 ± 11.49	93.44 ± 11.77	0.35 (-5.28 to 5.98) , 0.901
	After intervention	93.75 ± 11.71	91.08 ± 11.41	-2.94 (-5.25 to -0.64), 0.013†
	MD (95% CI), P value	0.66 (-0.93 to 2.25), 0.406	-2.35 (-4.09 to -0.61), 0.009**	
HC (cm)	Baseline	108.79 ± 7.74	110.19 ± 10.69	1.39 (-3.13 to 5.92), 0.540
	After intervention	109.32 ± 8.27	108.86 ± 11.01	-1.81 (-3.38 to -0.24), 0.024†
	MD (95% CI), P value	0.52 (-0.24 to 1.30), 0.176	-1.32 (-2.68 to 0.03), 0.056	
WHR	Baseline	0.85 ± 0.06	0.84 ± 0.05	-0.007 (-0.03 to 0.02), 0.643
	After intervention	0.85 ± 0.07	0.83 ± 0.05	-0.01 (-0.03 to 0.00), 0.110
	MD (95% CI), P value	0.00 (-0.01 to 0.01), 0.678	-0.01 (-0.02 to 0.00), 0.090	
WHtR	Baseline	0.57 ± 0.06	0.57 ± 0.07	0.00 (-0.03 to 0.03), 0.974
	After intervention	0.57 ± 0.07	0.56 ± 0.07	-0.01 (-0.03 to -0.00), 0.027†
	MD (95% CI), P value	0.00 (-0.00 to 0.01), 0.460	-0.01 (-0.02 to -0.00), 0.028**	

Data are presented as mean ± SD. PCOS; Polycystic ovary syndrome, CI; Confidence interval, MD; Means difference, BMI; Body mass index, WC; Waist circumference, HC; Hip circumference, WHR; Waist to hip ratio, WHtR; Waist- to- height ratio, **; P value is reported based on the analysis of, paired sample t test, and †; P value is reported based on the analysis of covariance, adjusted for energy intake and baseline values.

at baseline and after 8-weeks supplementation are shown in Table 4. There were no considerable differences between the two groups in weight, BMI, WC, HC, WHR and WHtR at baseline.

Results of analysis of covariance illustrated a statistically significant discrepancy between the two studied groups in weight (P=0.02), BMI (P=0.02), WC and HC (P=0.01, P=0.02, respectively) and WHtR (P=0.02) at the end of the study, adjusted for energy intake and baseline values. Changes in WHR was not significant between the two groups at the end of the study (P>0.05).

Significant decreases (by 2.52% and 1.75%, respectively) were observed in WC and WHtR of subjects in the synbiotic group after the intervention compared to the baseline values (P=0.009 and P=0.02, respectively). Changes in other anthropometric variables were not significant within the synbiotic group. Weight and BMI increased within the placebo group (respectively by 0.74%, P=0.003 and 0.87%, P=0.003).

Discussion

The application of probiotics and prebiotics can ameliorate the contrast between intestinal microbiota and host metabolism in obesity and associated metabolic diseases (28). Few studies assessed potential influences of synbiotics in subjects with PCOS. To the best of our knowledge, impacts of synbiotics on lipids profile and obesity values have not been investigated in subjects with PCOS by a supplement similar to that used in our study with respect to form, dose, strains, and duration of use. In a previous study about synbiotic supplementation in PCOS patients that assessed glycemic indices and apelin levels,1000 mg dosage of the capsule (20) was used, which was different from our study (i.e. 500 mg).

According to findings of present trial, synbiotic supplementation reduced fasting blood glucose, HOMA-IR and insulin in patients during eight weeks of supplementation. Our results are in accordance with the findings reported by Samimi et al. (19) which showed that the use of one synbioticcapsules (genera *Lactobacillus Lactobacillus acidophilus*, *L. casei,* and *B. bifidum,* 2×10^9 CFU/g together with 800 mg inulin) per day for 12 weeks, reduced serum insulin and HOMA-IR in PCOS women. Esmaeilinezhad et al. (29) reported that consumption of synbiotic pomegranate juice and synbiotic beverage (2 L/week) for 8 weeks, lowered HOMA-IR in women with PCOS.

It was suggested that synbiotics may play a momentous role in the metabolism of carbohydrates by producing short-chain fatty acids (SCFAs). SCFAs bind to G protein-coupled receptors and increase the secretion of glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), from enteroendocrine L-cells which can trigger insulin production by pancreatic β cells, inhibit glucagon secretion, decrease hepatic gluconeogenesis, and raise insulin sensitivity (17). Synbiotics also improve bowel function, elevate the production of mucin and diminish the amount of gram-negative (inappropriate) bacteria in the colon. These changes decrease the transmission of

lipopolysaccharides (LPS) along the mucous wall and metabolic endo-toxaemia, which can ultimately lead to improvements in insulin receptor function, lower insulin levels, and increased normal ovarian function (15).

Our results confirmed improving effects of synbiotic administration on glycemic indices by lowering HOMA-IR and subsequent lowered blood glucose and insulin in the studied women. However, in a study by Karimi et al. (20) use of synbiotic supplement did not affect these parameters in women with PCOS. Non-effectiveness of synbiotics on glycemic indices has been also reported in a study on subjects with nonalcoholic steatohepatitis (30). Differences in clinical and metabolic characteristics of participants, as well as varying strains and doses of probiotics and type of prebiotics, treatment period and host gut microbiota, might contribute to controversial findings.

HDL cholesterol is considered the helpful cholesterol due to its function of transporting cholesterol in the shape of cholesteryl esters to the liver for rather a hydrolysis (31). According to our results, the mean serum HDL cholesterol levels of studied subjects were lower than 50 mg/dl (normal cut-off based on National Cholesterol Education Program Adult Treatment Panel III) (32) in both groups at baseline and its level elevated in the synbiotic group at the end of the study in comparison with the placebo group. Samimi et al. (19) in their research concluded that the intervention with symbiotic for 12 weeks in PCOS patients, had significant alleviations of serum TG and VLDL cholesterol levels. No other study is available about possible effects of synbiotics on lipid profiles in PCOS.

It was proposed that probiotics may interfere in the removal of cholesterol by reducing cholesterol absorption from the intestine (33). Liong and Shah (34) showed that cholesterol conjugation to the cell wall of probiotics and their special abilities in enzymatic biliary acid decontamination lead to a decrement of serum cholesterol. Moreover, it was demonstrated that the cholesterol-lowering effects of probiotics increase with the use of prebiotics, concurrently (33).

In the present study, along with improvement in serum HDL cholesterol in the synbiotic group, no significant change was detected in other serum lipids. So, further studies are needed to investigate longer synbiotics administration effects and their precise effects on lipid metabolism.

Obesity displays a considerable role in the progress of metabolic disease in women with PCOS. Studies have indicated that the gut microbiota as an environmental factor was altered in obesity and leads to its spread (35, 36).

Our results indicated that synbiotic supplementation reduced weight and BMI and central obesity indices including WC, HC, and WHtR in the intervened group compared to the placebo group. As previously mentioned, no other study investigated possible effects of synbiotics in the form of our supplement on anthropometric characteristics in women with PCOS.

Studies showed that intestinal microbiota modifies the biological system, which results in the regulation of nutrient availability, energy storage, spread of fat mass and inflammation in the host, both of which are associated with obesity (17). The intestinal microbiota is also effective in regulating nutrient intake via the SCFA signaling function (36).

Women participated in our research were asked to follow their previous diet and physical activity, and our analysis showed that there were no significant changes in these variables during the intervention. As a result, it seems that improved obesity values in the group intervened with synbiotics, were not induced by changes in food intake or physical activity. It was possible that the reduction in obesity values in the synbiotic group might be related to improvement in HOMA-IR, at least in part. Evidence suggests that higher insulin sensitivity reduces hyperglycemia, hepatic lipid synthesis and fat accumulation in adipose tissues (37). Additionally, changes in anthropometric measurements were not mediated through apelin levels. Since, synbiotic supplementation did not affect serum apelin levels in our study.

It was suggested that synbiotics by changing the balance of intestinal microbiota (DOGMA), may affect endocannabinoid and apelinergic system. Intestinal microbiota imbalance enhances the permeability of the intestinal epithelium and consequently, the influx of LPS into the circulation, which finally leads to metabolic endotoxemia, activation of the immune system and induction of inflammation. These conditions promote the activity of the apelinergic system in the adipose tissue and the level of apelin in the serum. Thus, changes in intestinal microbiota with symbiotic play an important role in reducing apelin levels in PCOS patients (38). Karimi et al. (20) reported a significant decrease in serum apelin levels in women with PCOS following synbiotic supplementation from 27 ± 21 nmol/l at baseline to 14.4 ± 4.5 nmol/l at the end of study. Changes in the placebo group were not significant (26 ±15 nmol/l and 18.4 ± 2.9 nmol/l, at the beginning and the end of study, respectively). In present study, as described in results section, wide SD distribution of apelin might have contributed to non-considerable changes in concentration of this adipokine. It was also probable that dose or duration of supplementation was not adequate to affect the aplin levels in our trial. As mentioned previously, Karimi et al. (20) applied a two-time higher supplement dosage (1000 mg) for a longer period (12 weeks) and obtained significant changes in apelin levels. No other study is available about possible effects of synbiotics on apelin levels in PCOS or other diseases.

It should be noted that the exact normal range for serum apelin has not been documented yet. To date, a few studies have measured apelin in subjects with PCOS, and their results are incompatible. In the study by Olszanecka-Glinianowic et al. (24) no significant difference was seen

in serum levels of this adipokine in PCOS and non-PCOS women. Plasma apelin-36 levels was significantly higher in normal-weight women than the obese PCOS women (3.1 ± 2.2 vs. 1.2 ± 0.7 μg/l, respectively). In another study, serum apelin levels were correlated positively with BMI, IR, serum insulin and TG in women with PCOS. Apelin levels were lower in women with PCOS than controls (194.1 ± 50.7 pg/ml vs. 292.1 ± 85.6 pg/ml, respectively) (39). Inconsistent results among the findings of the available studies may be related to the design and the demographic and genetic specifications of populations as well as the nature of apelin.

In our study, no association was found between serum apelin concentrations and values of obesity or biochemical parameters before or after interventions in either group (data are not shown). As a result, it seems that favorable changes detected in glycemic indices and lipids profile in our study were not mediated via apelin. Subsequent studies are needed to evaluate the intestinal microbiota impacts on circulating apelin as well as the role of this adipokine in the pathogenesis of PCOS.

The strength of our study was the double-blind placebo-controlled design with no drop-outs. However, the present study had some limitations such as its short study duration of 8 weeks. Also, bacterial flora changes and SCFAs were not assessed through analysis of the stool. This research included overweight or obese patients. Therefore, our findings cannot be generalized to low-weight and/or normal-weight PCOS women, various intervention periods and other kinds of synbiotics. Additional studies are warranted to identify the impacts of synbiotics on other serum adipokines and androgen status in women with PCOS.

Conclusion

It can be said that synbiotic supplementation improved glycemic indices, serum HDL cholesterol levels and obesity values in subjects with PCOS and may be useful in the control of metabolic factors and reducing adiposity in these patients. Synbiotic administration in this study did not affect serum apelin levels. It is offered that the physiopathological function of apelin and metabolic effects of synbiotics in PCOS patients be evaluated more in future studies.

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Authors' Contributions

M.R.; Contributed to the study design and conceived the clinical trial progressing as a principal supervisor, prepared the manuscript, and reviewed the whole project drastically. S.D.; Contributed to the data collection and interpretation as a principal investigator, prepared the manuscript, and reported the final results. M.A.-J.; Performed the statistical analysis and data interpretation. L.F.; Participated in study design and patients recruitment. All authors read and approved the final version of the manuscript.

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Original Article

Sexual Self-Concept in Fertile and Infertile Women: A Comparative Study

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Abstract

Background: Sexual self-concept has a considerable impact on mental and sexual health. However, the relationship between sexual self-concept and infertility is unknown. This study aimed to compare sexual self-concept between fertile and infertile women.

Materials and Methods: This cross-sectional study was conducted on a sample of 250 fertile and 250 infertile women who had referred to 9 health centers affiliated to Medical universities in Tehran and Royan infertility treatment clinics in Tehran, Iran in 2017. Sexual self-concept was measured using the Multidimensional Sexual Self-Concept Questionnaire (MSSCQ) consisting of 20 subscales. Analysis of covariance (ANCOVA) was performed to compare sexual self-concept between the two groups.

Results: The mean age of fertile and infertile women was 34 ± 5.62 and 29.74 ± 5.29 years, respectively. The highest score in both groups was for the sexual self-schemata subscale (mean score for fertile=3.21 ± 0.68 and for infertile=3.42 ± 0.62). The lowest score was for sexual-depression subscale (mean score for fertile=0.59 ± 0.81 and for infertile=0.61 ± 0.76). After adjustment for the age of each subject, the husband's age, duration of marriage, and women's education, we analyzed the sexual-satisfaction, the power-other sexual control, and the fear-of-sex subscales, which were found to be significantly lower in infertile women ($P<0.05$). No other significant differences between the fertile and infertile groups were observed.

Conclusion: We observed significant differences between fertile and infertile women in terms of sexual-satisfaction, the power-other sexual control, and the fear-of-sex, but not in other sexual self-concept subscales. These findings suggest that there is need to improve sexual self-concept among both fertile and infertile women. Indeed implementation of educational and counseling programs by reproductive health specialists might play an important role in enhancing sexual self-concept among these populations.

Keywords: Fertility, Infertility, Self-Concept, Sexual Health

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Introduction

Sexual health is a broad concept that contains many dimensions including sexual self-concept (1). Sexual self-concept refers to the totality of an individual as a sexual being, which includes positive and negative believes and emotions (2). In fact sexual self-concept refers to the feelings, concepts, and behaviors about sexual relationships (3). Thus, sexual self-concept plays an important role in sexual behavior and reproductive health (4), and is a key indicator of successful sexual activity (5). Indeed sexual self-concept might influence sexual life in many ways both in men and women.

Although studies on sexual self-concept are limited, existing evidence suggests that negative sexual self-concept could be associated with lower sexual activity, low sexual satisfaction, fear of sex, and other sexual-related psycho-

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logical problems. For instance, a recent review on factors affecting sexual self-concept indicated that age, gender, sexual transmitted infections, body image, sexual abuse in childhood, and mental health might be associated with sexual self-concept (6). A study among a sample of post-menopausal women found that there were a significant association between sexual self-concept and stress, anxiety and depression (5).

A pile of evidence exists on sexual health in infertile couples, but little is known about sexual self-concept and infertility. It is argued that since infertility by itself imposes several emotional and psychological problems, the addition of impaired sexual self-concept among infertile couples could deepen the problem even further (7, 8). In fact, sexual self-concept as one of the prominent indicators of sexual function is expected to be highly affected in infertile women (9) and thus merits investigation. Additionally, since in some societies such as Iran women are more vulnerable to infertility problems than other countries (10), it seems that studying sexual self-concept in infertile women is of major importance. Infertility brings negative feelings for women such as guilt and shame. They usually feel that their integrity as a woman is compromised and cannot play their feminine role as expected. They are worried that their marital life will be at risk. Generally, men will cope more easily with infertility and are less emotionally affected by it compared to women (11). However, to our best knowledge, no study exists to address sexual self-concept and infertility. Thus, the present study aimed to assess sexual self-concept among a sample of fertile and infertile women. The findings of this study could contribute to the existing literature regarding sexual health and may help improve related policies and practices.

Materials and Methods

Design and participants

This cross-sectional study was conducted on a sample of fertile and infertile women in 2017. The infertile women were recruited from the Royan Institute by a convenience method while the fertile women were recruited from health centers affiliated to medical universities in Tehran, Iran (Iran University of Medical Sciences, Shahid Beheshti University of Medical Sciences, and Tehran University of Medical Sciences). A list of appropriate health centers was provided and based on the required sample size, preferably from different geographical areas, three centers affiliated to each university were selected (a total of 9 centers). The centers affiliated to each university were coded and a colleague who was not connected to the study randomly drew three centers. Then, proportional to the population a convenience sample of fertile women from each center was entered into the study. The required sample size for the study was estimated based on the smallest mean score difference between the two groups derived from a pilot study. We estimated that 234 women are needed for each group in order to be able to detect at least 0.15 mean score difference for sexual self-concept between the two groups considering a standard deviation of 0.82 with 80% statistical power at 5% significance level. However, allowing for a 10% drop out a sample of 250 was chosen for each group using the following formula:

$$n = \frac{\left(z_{\alpha/2} + z_{\beta}\right)^2 \sigma^2}{\left(\mu_1 - \mu_2\right)^2}$$

$n = (1.96+0.84)^2 \times 0.82^2/(1.60-1.45)^2$

$n = 7.84 \times 0.67/0.0225 = 234$

Inclusion criteria for fertile women were: having at least one healthy child, using a contraceptive method, not having a nursing infant and no history of infertility. The infertile women were included if they had a primary infertility problem. However, the inclusion criteria for all participants were: being of Iranian origin, aged between 18-45 years, and with no medical or psychological disorders. In addition, they would not have any records of alcohol or drug abuse and would not have to be taking libido effective drugs.

Measures

The data were collected using a short questionnaire containing items on demographic information and the multidimensional Snell's Sexual Self-Concept Questionnaire (MSSCQ). The questionnaire was designed and validated by Snell (12). The MSSCQ is a self-report instrument, measuring the various dimensions of sexual self-concept with 100 items and 20 subscales. The questionnaire measures both positive and negative concepts. The positive concepts refer to those such as sexual motivation, sexual satisfaction and sexual schemata. For instance, sexual schemata is defined as a cognitive framework that organizes and guides the processing of information about the sexual-related aspects of oneself. The negative concepts refer to concepts such as sexual anxiety, sexual depression, and power-other sexual control. The later subscale (power-other sexual control) is defined as the belief that the sexual aspects of one's life are controlled by others who are more powerful and influential than oneself. The detailed descriptions of the subscales are explained by Snell (12). Each subscale contains 5 items and is rated on a five-point Likert scale with scores ranging from 0 to 4. The mean score for each subscale is considered as the cut-off point and a score above the mean indicates a higher tendency for that subscale. The psychometric properties of the questionnaire are described previously. The Cronbach's alpha coefficient for the questionnaire was reported to be between 0.72 and 0.94. Reliability and validity of the Persian version of the questionnaire are well documented in previous studies. The Cronbach's alpha coefficient for the questionnaire varies from 0.41 to 0.87 (13, 14).

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp.,

Armonk, NY, USA). The continuous variables were expressed as the mean and standard deviation (SD), and the categorical variables were expressed as numbers and percentages. When appropriate, Chi-square test and independent t test were used to compare baseline characteristics between fertile and infertile women. Analysis of Covariance (ANCOVA) was used to compare sexual self-concept between groups while adjusting for the subject's age, her husband's age, duration of their marriage, and her level of education. All statistical tests were two-tailed and the level of significance was set at $P \leq 0.05$.

Ethical considerations

The Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran, approved this study (IR.SBMU.PHNM.1394.263). The participants were informed of the purpose of the study and were assured of confidentiality. After signing a consent form and agreeing to participate, all women completed the questionnaires.

Results

The mean age of fertile and infertile women was 34 ± 5.62 and 29.74 ± 5.29 years, respectively. The educational level of most fertile and infertile women and their husbands was at secondary and university levels. In both groups, the majority of women were housewives and the economic status of households was moderate. The mean duration of infertility was 5.54 ± 3.868 years. The demographic characteristics of the participants are presented in Table 1.

Table 1: Distribution of some demographic variables in fertile and infertile women

Variables	Fertile women n=250	Infertile women n=250	P value
Age (Y)	34 ± 5.62	29.74 ± 5.29	<0.001*
Husband's age (Y)	38.88 ± 6.41	33.99 ± 5.29	<0.001*
Duration of marriage (Y)	12.46 ± 5.78	7.12 ± 3.91	<0.001*
Education			0.001**
Elementary	28 (11.2)	37 (14.8)	
Secondary	132 (52.8)	91 (36.4)	
University	90 (36)	122 (48.8)	
Job			0.740**
Housewife	200 (80)	197 (78.8)	
Employed	50 (20)	53 (21.2)	
Economic status			0.213**
Weak	30 (12)	39 (15.6)	
Moderate	176 (70.4)	179 (71.6)	
Good	44 (17.6)	32 (12.8)	

Values are given as mean ± SD or n (%). *; Derived from independent t test and **; Derived from Chi-square test.

The highest and the lowest mean scores for sexual self-concept in both groups were for the sexual self-schemata (mean score=3.21 ± 0.68 and 3.42 ± 0.62) and the sexual-depression subscales (mean score=0.59 ± 0.81 and 0.61 ± 0.76), respectively, indicating that both groups were well prepared for processing of information about the sexual-related aspects of oneself and similarly had a low level of sexual related depression. Comparing sexual self-concept between fertile and infertile women, which was performed after carrying out adjustments for wife's age, husband's age, duration of marriage, and education of the women, showed that the mean scores of sexual-satisfaction, power-other sexual control, and fear-of-sex subscales were significantly lower in infertile women ($P<0.05$). There were no significant differences between the fertile and infertile women for other subscales ($P>0.05$). The comparison of the sexual self-concept subscales between fertile and infertile women is summarized in Table 2.

Table 2: Comparison of the subscales of sexual self-concept in fertile and infertile women

Subscales of sexual self-concept	Fertile women n=250	Infertile women n=250	P value*
Sexual-anxiety	0.69 ± 0.75	0.80 ± 0.72	0.200
Sexual self-efficacy	2.54 ± 0.68	2.51 ± 0.64	0.741
Sexual-consciousness	2.59 ± 0.66	2.60 ± 0.60	0.896
Motivation to avoid risky sex	3.12 ± 0.65	3.11 ± 0.72	0.245
Chance/luck sexual control	0.71 ± 0.60	0.83 ± 0.61	0.152
Sexual-preoccupation	0.77 ± 0.67	0.84 ± 0.67	0.758
Sexual-assertiveness	1.82 ± 0.55	1.80 ± 0.51	0.523
Sexual-optimism	1.78 ± 0.50	1.82 ± 0.47	0.826
Sexual problem self-blame	1.58 ± 0.78	1.47 ± 0.71	0.065
Sexual-monitoring	1.08 ± 0.62	1.12 ± 0.64	0.684
Sexual-motivation	2.19 ± 0.79	2.31 ± 0.70	0.673
Sexual problem management	2.33 ± 0.69	2.42 ± 0.60	0.386
Sexual-esteem	2.53 ± 0.74	2.54 ± 0.74	0.902
Sexual-satisfaction	2.66 ± 0.84	2.59 ± 0.83	0.033
Power-other sexual control	0.89 ± 0.65	0.77 ± 0.63	0.008
Sexual self-schemata	3.21 ± 0.68	3.42 ± 0.62	0.090
Fear-of-sex	1.67 ± 0.53	1.52 ± 0.60	0.012
Sexual problem prevention	2.90 ± 0.66	2.88 ± 0.71	0.736
Sexual-depression	0.59 ± 0.81	0.61 ± 0.76	0.440
Internal-sexual-control	2.46 ± 0.63	2.46 ± 0.62	0.741

Data are presented as mean ± SD. *; Derived from ANCOVA adjusted for age, husband's age, duration of marriage, and education as covariates.

Discussion

The results of this study indicated that some aspects of sexual self-concept were lower among the infertile women as compared to the fertile women. Infertility and the associated sexual problems can cause sexual dysfunction and sexual dissatisfaction (15). Since sexual pleasure is more a product of the mind than of the body, it is very likely that sexual pleasure could be affected by the consequences of infertility such as depression, which in turn interferes with sexual satisfaction (16). Another

explanation for such observation is that infertile women appear to be mainly concerned about occurrence of pregnancy during the sexual relationship, and not sexual satisfaction or enjoyment, which can interfere with sexual pleasure among infertile women. Similar findings were reported by other investigators where they reported that sexual dysfunction was lower among infertile women when compared to a sample of fertile couples (17-19).

Since all infertile women in the present study were under treatment and their sexual relationship was mostly based on the schedules of their treatment plan, their sexual activity was mostly planned accordingly and not based on their natural desire. Therefore, the role of the husband in their relationship has been diminished with regard to coercion or control of sexual activities in this group. The results of other studies were also similar. Fear-of-sex was lower in infertile women, which is probably due to the women's high desire to become pregnant. While, in fertile women, fear of unwanted pregnancy might unconsciously lead to fear of sexual relationship. The mean score of this subscale in the other studies was consistent with the present study (3, 20).

The mean scores of sexual-anxiety and sexual-depression in fertile and infertile women were not significantly different. It appears that infertility alone cannot increase sexual-anxiety and sexual-depression in infertile women. Potki et al. (6) found that various aspects of the sexual self-concept change over a 4-year period. In this evolutionary period, the sexual anxiety of individuals decreases and they feel more sexually friendly so that in the first two years, women's sexual anxiety could be reduced by 70%. Furthermore, as the age and length of the marriage increase, sexual intercourse experiences increase as people feel more comfortable and have more sexual compatibility, resulting in less sexual-anxiety (21). In the present study, the mean duration of marriage was over four years in the fertile and infertile women, which might explain the low level of sexual-anxiety in both groups. Sexual-depression had the lowest score in both fertile and infertile groups, which meant that women had a good acceptance of themselves as a sexual entity and an attractive wife.

There was no significant difference between the fertile and infertile women in terms of sexual self-efficacy and sexual-consciousness. The mean score of these subscales in studies by Hasanzadeh (20) and Zaheri et al. (21) were consistent with those in the present study. Another study, however, showed that sexual self-efficacy in fertile women was higher than that of infertile women (15). Differences in these results can be due to differences in cultural characteristics and the lifestyles of the participants.

There was no significant difference for motivation to avoid high-risk sexual behavior between fertile and infertile women (15). It appears that the high motivation to avoid high risk sex in both groups is probably due to the formation of sexual behaviors within the family framework based on cultural and social values in Iranian context, which in turn lead to the motivation to avoid risky sexual behaviors (22). The low score of self-blame in sexual problems and the high score of sexual problem management and sexual problem prevention in both groups suggest that women do not solely consider themselves responsible for the incidence of sexual problems and dysfunction, but they consider these issues as mutual responsibilities of spouses, and at the same time, have fare management when facing sexual problems.

According to our findings both fertile and infertile groups had low scores in sexual-preoccupation, sexual-assertiveness and sexual-optimism subscales. The low score of sexual-preoccupation suggests that extreme thinking about sex is not a major concern for women. Extreme thoughts about sex will distance people from moderate conditions and may create serious behavioral issues for them. This was not observed in the present study, which might be due to being married and the definition of sexual activities within a family environment. These findings of the present study were in line with the findings of other studies (3, 20).

The low score of sexual-assertiveness reflects the low level of self-confidence with regards to sexual activities in both groups. According to Bui et al. (23) most women express that they are in a lower rank than men, and they had low sexual-assertiveness and sexual self-efficacy. Sexual assertiveness is not only a way of expressing feelings and communicating about sexual experiences, but it also potentially affects sexual health and satisfaction (24). Sexual optimism is positively correlated with sexual health. Addressing the positive aspects of sexual relationships affects how one perceives his or her sexual future, and this promotes other dimensions of health (25). Given the low scores of sexual-optimism and sexual-assertiveness in both groups and considering the important role of these subscales in sexual health, it is strongly recommended to increase the attention of health professionals to these subscales of sexual self-concept and to provide counseling to women in this regard.

There was no significant difference between fertile and infertile women in terms of sexual-esteem and sexual-motivation. According to other studies, women with an integrated sexual identity are more likely to achieve sexual self-esteem (6). Also high sexual-esteem can be a protective factor against high-risk sexual behaviors (26).

The low score of sexual-monitoring in both groups suggests that the individuals do not pay enough attention to the attitudes and reactions of others about their sexual attitudes and consider sex as a private issue between themselves and their partners. Interestingly, we observed that the importance of privacy in marital sexuality increased the mean score of internal-sexual-control in both groups.

Overall one might argue that there were no significant differences between fertile and infertile women in most measures of sexual self-concept. Two reasons may explain such observations. Firstly, it is possible that sexual self-concept is an abstract concept and is less associated with reproductive conditions. Secondly, since infertile women usually seek help for their sexual health thus the difference between fertile and infertile women are limited to those

aspects that rarely could be dealt with among infertile women. As such, future studies should focus on assessing sexual-satisfaction, power-other sexual control, and fear-of-sex among both fertile and infertile women.

Of the strengths of this study was the selection of fertile women from various health centers in Tehran, which included people with various socio-economic backgrounds. Nonetheless, the main limitation of this study relates to its cross sectional design.

Conclusion

The present study showed that the highest and lowest sexual self-concept scores in both fertile and infertile groups were sexual self-schemata and sexual-depression, respectively. Infertile women scored lower on sexual-satisfaction, power-other sexual control and fear-of-sex compared to fertile women. There was no difference between the two groups in other subscales. Since the low level of sexual satisfaction in infertile women can affect their sexual life and ultimately the quality of their marriage, the enhancement of this subscale of sexual self-concept in infertile women is considered as an important factor in promoting sexual health. To moderated fear-of-sex and power-other sexual control in fertile women, educational-counseling programs can improve the sexual health of women in these subscales. It appears that it is essential to encourage clinicians and providers in infertility and gynecology centers to address the issues of sexual health and sexual problems of both fertile and infertile women and to provide advice on these issues.

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Authors' Contributions

H.L.; Participated in the study design and data collection. H.R.; Designed the study and supervised it. R.O.-S.; Contributed extensively in interpretation of the data and the conclusion. S.M.; Participated in data analysis and interpretation. A.M.; Contributed to analysis and provided the final draft. All authors read and approved the final manuscript.

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Original Article

Differences in and Correlates of Sexual Function in Infertile Women with and without Polycystic Ovary Syndrome

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Abstract

Background: The aim of this study was to examine sexual function and its correlates among infertile women with polycystic ovary syndrome (PCOS) in comparison with their non-PCOS counterparts.

Materials and Methods: In this case-control study, 209 infertile women (116 PCOS and 93 non-PCOS) from Tehran, Iran, were evaluated in February and March 2018. Female sexual function index (FSFI), hormonal status, and documented reports of hyperandrogenic manifestations of the patients were investigated.

Results: The mean age of the patients was 32.00 ± 5.00 years old. Eighty-four (40.2%) patients including 42.2% of the PCOS patients and 37.6% of the non-PCOS cases (P>0.05), were suspected of female sexual dysfunction (FSD). The most impaired functions in both groups were desire and arousal. Sexual function was not significantly different between the groups. However, PCOS women had more orgasm problems and acne worsened their sexual function. Total FSFI was positively associated with prolactin level but negatively associated with central obesity in the non-PCOS group; it was negatively correlated with marital duration in the PCOS group. Luteinizing hormone (LH) and pain, prolactin level and lubrication, and central obesity and arousal were correlated in the non-PCOS women. Prolactin level and orgasm, marital duration and arousal, and marital duration and the total FSFI were correlated in the PCOS women.

Conclusion: Sexual function was similarly low in infertile PCOS and non-PCOS women. However, orgasm problems and the negative effect of acne varied between the two groups. Further investigations may target how hormonal profile may affect sexual function. Practitioners should scrutinize the specific impaired sexual domains and their correlated conditions in PCOS women, notably orgasm, acne, and prolactin level. Interventions should be well tailored based on particular needs of infertile PCOS women.

Keywords: Infertility, Polycystic Ovary Syndrome, Sexual Dysfunction, Women

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Introduction

Infertility is a disease of the reproductive system that is defined as "the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (1). In the Iranian population, recent estimations indicated that lifetime primary infertility rates, based on clinical, epidemiological, and demographic definitions set by the World Health Organization, are respectively 20.2, 12.8, and 9.2%, while that of secondary infertility is 4.9% (2). One of the common disorders linked to infertility and associated with ovulation problems is polycystic ovary syndrome (PCOS). The definition of PCOS by the United States National Institutes of Health entails anovulation and hyperandrogenemia. However, the European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM) defines PCOS as polycystic ovaries diagnosed based on ultrasound (3). Studies have reported that up to 83% of infertile Iranian women have PCOS, and an infertility rate of 8-73% in PCOS patients was shown (4).

Sexual life of infertile women is a pivotal research area because infertility is associated with an increased risk of female sexual dysfunction (FSD) (5). Sexual function involves various domains, including sexual desire, arousal,

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vaginal lubrication, experiencing orgasm, coital satisfaction, and feeling of pain during intercourse (6). Infertile couples are more prone to develop sexual dysfunction. A recent estimation in infertile Iranian women, for instance, found that 64.3% of the patients developed sexual dysfunction, with sexual desire and vaginismus having the highest and lowest rates, respectively (7). Another study suggested that the sexual behavior of infertile Iranian women is severely narrowed to produce pregnancy (8) and that infertility causes Iranian women to suffer from serious problems in various domains, including sexuality (9). More to the point, sexual dysfunction may considerably affect mental health as well as the sexual quality of life (10). Furthermore, women's sexual problems may even escalate if they concurrently suffer from PCOS (11). That is, the sexuality of women with PCOS may become even more compromised, especially as a result of obesity, hirsutism, baldness, and acne (12). Moreover, sexual problems are suggested as the possible causes of discrepancies in perceptions of infertile women with and without PCOS towards their sexual life and function (13).

Although various studies have addressed the sexual problems experienced by women with PCOS (14, 15), few studies have focused on sexual issues and their correlates when such patients simultaneously suffer from infertility. Recent meta-analyses have determined similar sexual function between PCOS women and healthy controls (16, 17). However, it can be argued that further studies investigating possible effects of comorbid PCOS and infertility are warranted, especially in the Iranian population. Moreover, investigating possible differences in sexual problems and their correlates between infertile PCOS patients and their non-PCOS counterparts may provide more knowledge about the specific role that comorbid PCOS and infertility play in Iranian women life.

The present study, therefore, investigated the differences between infertile Iranian women with and without PCOS in terms of sexual function. It also aimed to evaluate the degree to which hormonal, anthropomorphic, and hyperandrogenic manifestations may be correlated with the sexual function of these groups of women.

Materials and Methods

Study design and sampling

This case-control study involved infertile PCOS women as the case group and their infertile non-PCOS counterparts as the control group using a convenience sampling method. Patients visiting two infertility centers in Tehran, Iran were recruited. The study was introduced and explained to the patients to obtain their informed consent. The questionnaires were administered using the interviewer-administered method. Overall, 216 infertile women were recruited in February and March of 2018.

The sample size was determined for two independent samples using G*Power software V.3 (18). The estimation setting was set to a medium effect size (0.50), a significance level (α) of 0.05 (two-tailed), a power of 0.80, and an allocation ratio of 1. The calculation suggested that a sample size of 128 participants (64 for each group) could achieve the actual power of 0.803. The inclusion criteria were as follows: age above 18 years and diagnosis of infertility for both groups, and diagnosis of PCOS for the PCOS group. PCOS was diagnosed based on the international evidence-based guideline for the assessment and management of PCOS, 2018 (19). This guideline identifies the condition in adult women if two of the three conditions of androgen excess, ovulatory dysfunction, and polycystic ovarian morphology are present. Ultrasound is required if either androgen excess or ovulatory dysfunction is not present. Certain disorders, including thyroid disease (based on thyroid-stimulating hormone (TSH) level), hyperprolactinemia (based on prolactin level), and non-classic congenital adrenal hyperplasia (based on 17-hydroxyprogesterone (17-OHP) level), were ruled out by clinical judgment.

The following exclusion criteria were considered: psychiatric disorders; severe emotional problems in the past six months; consumption of oral contraceptive pills, gonadotropin-releasing hormone (GnRH) agonists, or insulin sensitizers in the past six months; chronic cardiovascular diseases; primary or secondary vaginismus and dyspareunia; pelvic mass; active genital infection; external vaginal anomalies; pelvic endometriosis; and partner's sexual dysfunction.

Measures

A checklist was devised to survey the participants' demographic information, including the patient's age, occupation, and education, the spouse's age and education, and the duration of their marriage. Clinical information including duration of infertility, duration of treatment, central obesity (waist-to-hip ratio), body mass index (BMI), and hyperandrogenic manifestations, including the presence of acne, hirsutism (Ferriman-Gallwey score), and baldness (i.e. male-pattern hair loss), was also collected.

On the third day of the menstrual cycle (induced by 100-200 mg progesterone in oil injection in amenorrheic patients), a baseline vaginal ultrasound examination was performed, and serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), TSH, and prolactin levels were measured using immunoradiometric assays (Izotop, Budapest, Hungary). Dehydroepiandrosterone sulfate (DHEAS), 17-OHP, and total testosterone (TT) were measured using enzyme immunoassays (Diagnostics Biochem Canada Inc., London, Canada).

The Female Sexual Function Index (FSFI) (20) was employed to assess the patients' sexual function. This 19-item questionnaire assesses six domains of sexual desire (two items, domain factor 0.6), arousal (four items, domain factor 0.3), lubrication (four items, domain factor 0.3), orgasm (three items, domain factor 0.4), satisfaction (four items, domain factor 0.4), and pain (three items, domain factor 0.4) for a comprehensive evaluation of the female sexual response cycle. Each domain's raw score is multiplied by

its domain factor, yielding a possible score in the range of 0 to 6, except for desire, which has a range of 1.2 to 6. According to the score which ranges 1.2-36, higher total scores indicate better sexual function. The questionnaire can identify women who had no sexual encounters during the preceding four weeks. The Persian version of the FSFI was approved as a valid and reliable screening and assessment instrument, indicating a Cronbach's alpha of 0.93 and test-retest reliability of 0.83 (21). The instrument showed a Cronbach's alpha of 0.89 in the current dataset. The second and fifth authors administered the questionnaires using the interviewer-administered method by asking patients to choose the response that best described their status.

FSD was identified based on FSFI scores. In an original study by Rosen, scores lower than 26.55 indicated a diagnosis of FSD with a specificity of 0.73 and a sensitivity of 0.89 (20). In addition, a raw score below 3.9 indicated sexual dysfunction in each domain (22).

Ethical considerations

This study was performed according to the Declaration of Helsinki, and the Ethics Committee of Iran University of Medical Sciences approved the study protocol (ID: IR.IUMS.FMD.REC.1396.9311290023). Informed consent was obtained from all participants.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 24.0 (IBM Corp., Armonk, NY, USA) (23). Missing data were handled using the pairwise deletion method. Since the scores showed a non-normal distribution, non-parametric statistics was adopted. The Mann-Whitney U test and the Chi-square test for comparing the groups, as well as logistic regression for predicting FSD via hyperandrogenic manifestations, were conducted. Spearman's Rho was also calculated to determine the correlation of patient's age, duration of infertility, duration of treatment, BMI, central obesity, levels of FSH, TSH, LH, prolactin, TT, DHEAS, and 17-OHP (all in mcg/L), and LH/FSH ratio with the total FSFI score and the domains. Two-tailed P value was set at <0.05.

Results

Sample characteristics

Among the 216 patients recruited in this study, seven (0.03%) patients were sexually inactive based on the FSFI and were excluded. Therefore, 209 infertile women, including 116 PCOS patients and 93 non-PCOS patients, remained in the study.

Table 1 presents the demographic and clinical characteristics of the patients. The mean age was 32.00 ± 5.00 years, with the PCOS group being older (P<0.001). Central obesity was more frequent in the PCOS group (P<0.01), and there was a higher degree of anovulation as the cause of infertility (P<0.001) in this group. Conversely, infertility in the non-PCOS group was more often found to be unexplained or due to tubal causes (P<0.01).

Evaluation of female sexual function

Table 2 presents the sexuality domains for both groups. Arousal had the lowest score in the PCOS group (3.69 ± 1.23) and non-PCOS group (3.67 ± 1.32). On the other hand, the highest mean score was related to satisfaction in the PCOS group (5.06 ± 1.00) and non-PCOS group (5.11 ± 0.95). The total FSFI for in our sample population was 27.15 ± 4.30, with 26.97 ± 4.73 in the PCOS group and 27.38 ± 3.72 in the non-PCOS group. FSD was diagnosed in 40.2% of the participants, including 42.2% of the PCOS patients and 37.6% of the non-PCOS patients.

Sexual function comparison

Table 3 presents comparisons between the PCOS and non-PCOS groups in terms of sexual function. Based on the raw scores for sexual function, there were no significant differences between the two groups (P=0.325 to 0.975). Also, the two groups had no significant difference in terms of FSD (P=0.500). Based on the categorization of sexual dysfunction in the domains (score<3.9), the two groups only showed a significant difference in orgasm problems (P=0.035).

Hyperandrogenic manifestations and sexual function

According to Table 3, acne (51.7%), baldness (41.4%), and hirsutism (57.8%) were more commonly found in the infertile PCOS patients (P<0.001). Acne was the sole impactful hyperandrogenic manifestation, increasing the odds of FSD by 1.87 [1.02, 3.43] (P=0.042) in the total sample and 2.18 [1.01, 4.68] (P=0.046) in the PCOS group.

Hormone comparison

Table 4 presents the results of the hormone tests in both groups. Group differences were seen in FSH, which was higher in the non-PCOS group, and LH, which was higher in the PCOS group (P<0.001). The LH/FSH ratio was higher in the PCOS group to a statistically significant degree (P<0.001).

Sexual function correlates

In the non-PCOS patients, there were significant relationships between LH and pain (n=87, rho=0.26, P=0.015), prolactin level and lubrication (n=85, rho=0.22, P=0.048), prolactin level and total FSFI (n=85, rho=0.22, P=0.045), and central obesity and arousal (n=93, rho=-0.26, P=0.013). In the PCOS group, marital duration and arousal (n=103, rho=-0.31, P=0.001), marital duration and total FSFI (n=103, rho=-0.25, P=0.013), and prolactin level and orgasm (n=114, rho=-0.23, P=0.012) were correlated.

Confounding effect of age

Because the mean ages of the two groups were statistically significantly different (P<0.001, Table 1), the analyses were repeated using linear regression analysis, including the PCOS group as the dummy variable and age as the covariate. There were no significant changes in the

pattern of the comparisons. Additionally, controlling for age, partial correlations were estimated between each pair of hormones and sexuality domains. Consequently, the results indicated only negligible changes to the zero-order correlations. Therefore, the previously reported differences and correlations were shown to be valid.

Table 1: Demographic and clinical information of the study sample

Variables	Total n=209	PCOS n=116	Non-PCOS n=93	Test statistic	P value
Patient's age (Y)	32.00 ± 5.00	31.00 ± 5.00	34.00 ± 6.00	z=-4.06	**<0.001**[a]
Spouse's age (Y)	36.00 ± 6.00	35.00 ± 6.00	37.00 ± 6.00	z=-3.57	**<0.001**[a]
Duration of marriage (Y)	6.70 ± 4.40	6.40 ± 4.20	7.10 ± 4.70	z=0.96	0.339[a]
Duration of infertility (Y)	4.20 ± 3.40	4.00 ± 3.50	4.40 ± 3.40	z=-1.50	0.133[a]
Duration of treatment (Y)	0.80 ± 0.61	0.79 ± 0.64	0.82 ± 0.58	z=0.98	0.329[a]
BMI	26.63 ± 4.10	26.66 ± 3.85	26.58 ± 4.43	z=0.76	0.448[a]
Central obesity	0.90 ± 0.06	0.91 ± 0.06	0.89 ± 0.06	z=2.61	**0.009**[a]
Patients' education					
Illiterate	4 (1.9)	2 (1.7)	2 (2.2)	$\chi^2[5] = 8.33$	0.139[b]
Primary	13 (6.2)	6 (5.2)	7 (7.5)		
Secondary	29 (13.9)	20 (17.2)	9 (9.7)		
Diploma	74 (35.4)	40 (34.5)	34 (36.6)		
University	53 (25.4)	34 (29.3)	19 (20.4)		
Missing data	36 (17.2)	14 (12.1)	22 (23.7)		
Patients' job					
Housewife	149 (71.3)	85 (73.3)	64 (68.8)	$\chi^2[3] = 5.00$	0.172[b]
Home-based	2 (1.0)	2 (1.7)	0 (0.0)		
Employed	26 (12.4)	16 (13.8)	10 (10.8)		
Missing data	32 (15.3)	13 (11.2)	19 (20.4)		
Spouses' education					
Illiterate	4 (1.9)	3 (2.6)	1 (1.1)	$\chi^2[5] = 9.12$	0.104[b]
Primary	14 (6.7)	7 (6.0)	7 (7.5)		
Secondary	45 (21.5)	27 (23.3)	18 (19.4)		
Diploma	65 (31.1)	36 (31.0)	29 (31.2)		
University	41 (19.6)	28 (24.1)	13 (14.0)		
Missing data	40 (19.1)	15 (12.9)	25 (26.9)		
Infertility type					
Primary	125 (59.8)	75 (64.7)	50 (53.9)	$\chi^2[2] = 2.95$	0.229[b]
Secondary	63 (30.1)	31 (26.7)	31 (33.3)		
Missing data	21 (10.0)	9 (7.8)	12 (12.9)		
Previous treatment					
None	51 (24.4)	33 (28.4)	18 (19.4)	$\chi^2[1] = 3.57$	0.168[b]
Induce	65 (40.4)	44 (46.8)	21 (31.3)	$\chi^2[1] = 3.89$	0.049[b]
IUI	65 (40.4)	32 (34.0)	33 (49.3)	$\chi^2[1] = 3.76$	0.052[b]
IVF/ICSI	29 (18.0)	15 (16.0)	14 (20.9)	$\chi^2[1] = 0.65$	0.442[b]
Missing data	48 (23.0)	22 (19.0)	26 (28.0)		
Infertility cause[c]					
Anovulation	128 (61.2)	110 (94.8)	18 (19.4)	$\chi^2[1] = 123.87$	**<0.001**[b]
Tubal	26 (12.4)	6 (6.0)	19 (20.4)	$\chi^2[1] = 9.82$	**0.002**[b]
Unexplained	17 (8.1)	3 (2.6)	14 (15.1)	$\chi^2[1] = 10.73$	**0.001**[b]
Missing data	42 (20.1)	0 (0.0)	42 (45.2)		

Data are presented as mean ± SD or n (%). IUI; Intrauterine insemination, IVF; *In vitro* fertilization, ICSI; Intrauterine insemination, PCOS: Polycystic ovary syndrome, BMI; Body mass index, [a]; Mann-Whitney U (Asymp. P), [b]; Pearson's Chi-square test (2-sided), and [c]; Anovulation and tubal categories are not mutually exclusive. The bolded P indicates a significant difference.

Table 2: Comparisons of sexual function

Variables	Total n=209			PCOS n=116			Non-PCOS n=93			z statistic	P[a]	χ^2[1]	P[b]
	M ± SD	Min, Max	FSD n (%)	M ± SD	Min, Max	FSD n (%)	M ± SD	Min, Max	FSD n (%)				
Desire	3.79 ± 1.05	1.20, 6.00	117 (56.0)	3.78 ± 1.01	1.20, 6.00	67 (57.8)	3.81 ± 1.09	1.20, 6.00	50 (53.8)	-0.24	0.792	0.33	0.563
Arousal	3.68 ± 1.27	1.20, 6.00	91 (43.5)	3.69 ± 1.23	1.20, 6.00	56 (48.3)	3.67 ± 1.32	1.20, 6.00	35 (37.6)	-0.29	0.775	2.38	0.123
Lubrication	4.99 ± 1.12	1.20, 6.00	34 (16.3)	4.92 ± 1.15	1.20, 6.00	21 (18.1)	5.07 ± 1.07	1.20, 6.00	13 (14.0)	-0.99	0.325	0.65	0.442
Orgasm	4.60 ± 1.08	1.20, 6.00	48 (23.0)	4.52 ± 1.17	1.20, 6.00	33 (28.4)	4.68 ± 0.95	1.20, 6.00	15 (16.1)	-0.61	0.539	4.43	**0.035**
Satisfaction	5.08 ± 0.98	2.40, 6.00	26 (12.4)	5.06 ± 1.00	2.40, 6.00	15 (12.9)	5.11 ± 0.95	2.40, 6.00	11 (11.8)	-0.34	0.738	0.06	0.810
Pain	5.01 ± 1.05	1.60, 6.00	34 (16.3)	5.00 ± 1.09	1.60, 6.00	22 (19.0)	5.04 ± 1.00	1.60, 6.00	12 (12.9)	-0.03	0.978	1.39	0.238
FSFI	27.15± 4.30	15.90, 36.00	84 (40.2)	26.97 ± 4.73	15.90, 36.00	49 (42.2)	27.38 ± 3.72	16.60, 34.50	35 (37.6)	-0.29	0.774	0.46	0.500

PCOS; Polycystic ovary syndrome, M; Mean, SD; Standard deviation, Min; Minimum, Max; Maximum, FSFI; Female sexual function index, FSD; Female sexual dysfunction (FSFI below 26.55 for global FSD and below 3.9 for domain FSD), [a]; Mann-Whitney U (Asymp. P) on the raw scores, and [b]; Pearson's Chi-square test (2-sided) on the FSD categorizations. The bolded P indicates a significant difference.

Table 3: Predicting FSD based on hyperandrogenic manifestations

Variables	Total n=209	PCOS n=116	Non-PCOS n=93	Total		PCOS	
				Unadjusted[a]	Adjusted[b]	Unadjusted[a]	Adjusted[b]
	n (%)			Odds [95%CI]			
Acne							
None	135 (64.6)	54 (46.6)	81 (87.1)	**1.87 [1.02, 3.43]**	**2.15 [1.12, 4.11]**	**2.18 [1.01, 4.68]**	**2.22 [1.00, 4.89]**
Mild	48 (23.0)	44 (37.9)	4 (4.3)				
Moderate	13 (6.2)	13 (11.2)	0 (0.0)				
Severe	3 (1.4)	3 (2.6)	0 (0.0)				
Baldness							
None	151 (72.2)	66 (56.9)	85 (91.4)	0.89 [0.45, 1.73]	0.77 [0.38, 1.56]	0.77 [0.36, 1.64]	0.62 [0.28, 1.27]
Mild	28 (13.4)	28 (24.1)	0 (0.0)				
Moderate	14 (6.7)	14 (12.1)	0 (0.0)				
Severe	6 (2.9)	6 (5.2)	0 (0.0)				
Hirsutism							
None	129 (61.7)	47 (40.5)	82 (88.2)	1.34 [0.74, 2.42]	1.44 [0.77, 2.71]	1.23 [0.57, 2.63]	1.22 [0.56, 2.67]
Mild	44 (21.1)	41 (35.3)	3 (3.2)				
Moderate	18 (8.6)	18 (15.5)	0 (0.0)				
Severe	8 (3.8)	8 (6.9)	0 (0.0)				

FSFI; Female sexual function index, FSD; Female sexual dysfunction (FSFI below 26.55), PCOS; Polycystic ovary syndrome, CI; Confidence interval, [a]; The unadjusted models included each hyperandrogenic manifestation as an independent variable and FSD as a dependent variable, and [b]; The models were adjusted for age. Bolded results indicate significant (P<0.05).

Table 4: Comparisons of laboratory results

Hormones[a]	Total n=209			PCOS n=116			Non-PCOS n=93			z statistic	P[b]
	n	M ± SD	Min, Max	n	M ± SD	Min, Max	n	M ± SD	Min, Max		
FSH	201	4.93 ± 3.27	0.50, 24.00	110	4.14 ± 2.34	1.30, 18.10	91	5.97 ± 3.96	0.50, 24.00	**-4.73**	**<0.001**
TSH	202	1.98 ± 1.07	0.09, 7.00	111	1.95 ± 0.96	0.20, 6.20	91	2.01 ± 1.19	0.09, 7.00	0.19	0.848
LH	201	5.75 ± 4.39	0.05, 42.30	111	6.77 ± 4.96	0.70, 42.30	91	4.40 ± 3.05	0.05, 16.10	**5.13**	**<0.001**
Prolactin	199	14.57 ± 7.79	1.00, 61.10	109	14.30 ± 5.42	2.30, 35.80	91	14.93 ± 10.17	1.00, 61.10	0.80	0.427
Testosterone	141	1.69 ± 0.73	0.40, 3.80	93	1.79 ± 0.83	0.40, 3.80	46	1.60 ± 0.60	0.40, 3.10	1.44	0.150
DHEAS	117	1.72 ± 0.85	0.30, 5.80	80	1.87 ± 1.07	0.40, 5.80	37	1.55 ± 0.48	0.30, 2.70	0.85	0.395
17-OHP	120	2.0 ± 0.68	0.40, 4.00	80	1.93 ± 0.63	0.40, 3.50	40	2.07 ± 0.73	0.50, 4.00	-0.91	0.364
LH/FSH	201	1.41 ± 0.97	0.02, 5.19	87	1.78 ± 0.95	0.22, 5.19	114	0.91 ± 0.74	0.02, 3.73	**7.11**	**<0.001**

PCOS; Polycystic ovary syndrome, M; Mean, SD; Standard deviation, Min; Minimum, Max; Maximum, FSH; Follicle-stimulating hormone, TSH; Thyroid-stimulating hormone, LH; Luteinizing hormone, DHEAS; Dehydroepiandrosterone sulfate and 17-OHP: 17-hydroxyprogesterone, [a]; All values are in microgram per liter, and [b]; Mann-Whitney U (Asymp. P). Bolded results indicate a significant difference.

Discussion

The aim of this study was to evaluate sexual function and its correlates among infertile patients with and without PCOS. The findings suggest that infertile women with and without PCOS considerably suffered from diminished sexual function. A recent meta-analysis reported domains of lubrication, orgasm, and satisfaction to be the sources of difference between infertile and fertile women (24), while these three domains were proportionately better in the two groups evaluated in the current study.

It should be noted that domain-specific FSD was determined by a relatively higher cut-off point (< 3.9), which was originally applied to infertile women (25); however, some other studies employing slightly lower cut-off points, reported a relatively higher degree of domain-specific FSD in healthy Iranian women (26). Nevertheless, similar to the aforementioned study (26), desire and arousal problems not only showed a marked prevalence but were also the most problematic sexual domains in the current study.

In addition, the literature suggests that women with and without PCOS in the general population (16, 17) and infertile Iranian women with and without PCOS (27) do not differ in terms of sexual function. Although PCOS women compared with their healthy counterparts, may mainly have dissatisfaction with their sex life, rather than with sexual activity (28), the current study indicated a diminished level of sexual function in both groups, with the orgasm domain being a specific source of difference. This indicates a crucial need for further attention paid to the sexuality of infertile PCOS women.

Furthermore, while some studies could not determine the effects of hyperandrogenic manifestations on sexual function (29), others indicated that acne-related concerns could reduce sexual satisfaction in both PCOS women and their spouses (30). Also, in our study, although infertile PCOS patients featured higher levels of LH and FSH, as well as an elevated LH/FSH ratio, it was the non-PCOS group that showed a significant association between LH and pain. In addition, an earlier study found no association between LH and quality of life of PCOS patients (31). However, LH was previously shown to be connected with orgasm problems in healthy, postmenopausal women (32) and with sexual function in PCOS patients in the general population (33). LH contributes to the circulation of reproductive hormones, including androgens and estrogens (34), and it is suggested to make women apt to love and intimacy (35). In addition, studies in which significant hormonal correlations were seen with LH in PCOS patients, emphasized the multifactorial nature of human sexual function which can be influenced by a variety of psychosocial and cultural factors (33). Thus, more studies are needed to provide supports for the results observed in the current study.

The current study revealed a significant contrast between the two groups in the relationship between prolactin level and sexual function. Other studies have also reported that among various relevant hormones, there was only a negative association between prolactin level and function of orgasm in PCOS women (36). Elevated prolactin levels were found to be associated with sexual problems in the general population (37). Also, women with PCOS can have mildly elevated levels of prolactin (38). Thus, the current results in line with the previous findings (36), indicated the diminished function of orgasm to be associated with higher prolactin levels in infertile PCOS women.

Contrary to expectations, higher central obesity which is marked in PCOS women (11, 12), indicated lower sexual arousal in infertile non-PCOS women only. Additionally, although some studies reported that the age of infertile women had a negative association with sexual function (39), the current findings instead, suggest the negative impact of marital duration on sexual arousal and total FSFI in infertile PCOS women. Thus, the current results indicate that obesity and marital characteristics could also be sources of difference in sexual function between infertile PCOS women and those without PCOS.

Ultimately, the distinctions raised in the current study suggest that infertile PCOS and non-PCOS women may need more well-tailored research on their specific biological, hormonal, and psychological dimensions. It is suggested that future studies include spouses assessments to further examine the relational nature of sexual desire and arousal in patients. More importantly, some studies have suggested implementing educational interventions to enhance the sexual function of infertile women (40). The current study, which indicates the exclusively negative effect of prolactin level, acne, and marital duration on the sexual function of infertile PCOS women, implies that interventions may need to be modified accordingly to educate patients on how to manage their specific problems. Last but not least, policymakers concerned with the family structure and sexual health of the Iranian population, should focus on and facilitate particular needs and problems of the patients in order to maintain their marriage as socially stable and psychologically fruitful as the wider population does.

This study lacked a control group of fertile women. Therefore, it failed to find any possible differences between fertile and infertile women, especially those who may seek professional help for their sexual problems. Moreover, since this study had a cross-sectional design, caution needs to be taken in making any generalizations or considering causal implications.

Conclusion

This study demonstrated diminished sexual function in infertile Iranian women, especially in terms of the desire and arousal domains. The PCOS and non-PCOS groups were not significantly different in terms of sexual function, while orgasm dysfunction was higher in the PCOS women. In addition, acne increased sexual dysfunction in the PCOS women. The infertile non-PCOS women

with higher levels of prolactin had lower dyspareunia and those with higher LH had lower total FSFI and lubrication problems, while the higher the central obesity the higher their arousal problems. However, infertile PCOS women mainly showed orgasm dysfunction as a result of lower levels of prolactin, and lower total FSFI and arousal as a result of marital duration.

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Authors' Contributions

A.A.S., F.K., H.Z.; Contributed to the conceptual design. B.T., F.G.; Contributed extensively to data collection. M.A.T., H.Z.; Contributed to statistical analysis and provided the initial draft. All authors contributed to editing and approving the final version of this manuscript for submission.

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Ethics, Legal, Social, Counseling Article

Qur'anic Views on Human Cloning (I): Doctrinal and Theological Evidences

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Abstract

Background: Human cloning is a recent occurrence that is not confined to bio-issues; rather, it has provoked numerous questions worldwide and presented scientific and religious challenges. These series of articles aim to examine the proposed approaches and analyze the aspects of human cloning in terms of tenets, morals, jurisprudence, and laws. In this paper, we analyze the ideological and theological evidences, regardless of scientific, ethical and legal problems that exist in the reproduction method.

Materials and Methods: We used a descriptive-analytical method to consider the challenges of human cloning according to the "system of Divine creativity" and "the will of God", as well as the "pairing system" and "diversity in nature" with emphasis on the Holy Qur'an and Qur'anic commentaries.

Results: According to the Qur'an, although any type of physical changes and retouching of the human body are forbidden, the alteration of God's creation may not prove the prohibition of cloning. Cloning is not contradictory to the principle, precedent, and rule of coupling, and it may be one of the hidden precedents of creation. Therefore, not only does a clone not contradict the precedent of the variety of men, but this variety is a sign for men and not a precedent predominated over the order of nature.

Conclusion: It is proven that cloning does not give life; instead, it utilizes the life bestowed by God. This technique does not contradict the precedents of existence. It is a way to discover some precedents of God and is under the order of cause and effect of the world. Cloning is not considered as a challenge to human beliefs, nor is it a change in Divine creation. Moreover, cloning does not contradict the theological teachings and concepts of the Holy Qur'an and Shiite Muslims.

Keywords: Clone, Human Cloning, Theological

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Introduction

Once the news of the first mammal (a sheep named Dolly) cloned by a group of Scottish scientists supervised by Ian Wilmut was broadcast (1), the probability of human cloning came into existence (2) and created anxiety among humans. Accordingly, some countries have adopted new laws that pertain to cloning whereas others made changes to their current laws (3). The reaction of monotheists regarding cloning, however, brought serious concerns in many scientific and religious circles. Scholars, on the one hand, have concerns about human cloning and, and on the hand, do not consider it to be a contradiction between science and religion (4-6). Some Shiite scholars, while denying the contradiction of human cloning to Qur'anic verses, state that Almighty God has created man some how, He can reach the secrets of the universe and take possession of them; of course all are inside the casualty rule and the will of God (7). Therefore, if cloning exists in humans, it does not oppose the Unity of God (Tawhid) nor the system of creation (8). However, its legal permission depends on how one uses cloning and the medical syndromes that are to be cured through this technique (9). It must be taken into consideration that the most important principle and source of knowledge about Divine injunctions, as the basis of Islam, is the Holy Qur'an, to the extent that legal and social laws and regulations are rooted in Qur'anic verses and conducts of the Holy Prophet of Islam (pbuh) (8). Therefore, without consideration and research in this area, Muslim scholars cannot legitimize a new area

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for future study. Numerous verses of the Holy Qur'an call for reflection and ponder, after which Muslims must deeply consider new matters that have not previously existed in order to reach high peaks of new areas. Human cloning consists of different aspects of theology, ethics, and law, each of which may be studied independently. Undoubtedly, this issue is engaged in the fundamental issues of human nature and value, which must be examined through recognizing the angles and various aspects of it. What is considered here is not legal or ethical matters, regardless of their effects. The present research examines and criticizes the most important issues of human cloning in the field of theology, based on the Holy Qur'an and Ahl al-Bayt (pbut).

Materials and Methods

In this descriptive-analytical study, to reach a reasonable conclusion, our main sources are related to Islamic texts and statements of Muslim scholars. The first and basic source is undoubtedly the Holy Qur'an, the holy book of Muslims revealed from God (10), whose orders and words are accepted by all Muslims. Secondly, the prophetic words (Hadith) and practices (Sunnah), as well as, his family's (Ahl al-Bayt) statements are considered to be a confirmed source of practicing and issuing fatwas (Islamic legal statements) in Islam. Therefore, by giving general details on human cloning, we divided the Qur'anic views (verses) into four sections. In each section, we discuss and review the statements of the Prophet and Imams (pbut), as well as great Muslim scholars (especially Shia) in order to have a sound analysis and respond to the questions of the writing. It is not our intent to prove or reject cloning; the Qur'anic references are studied to reach a common theory between Muslim scholars and provide a possible solution for the present challenges of cloning. We used a descriptive-analytical method with a critical approach in each section. The Results and Conclusion are based on a critical study of the materials.

Among all Islamic documents, only doctrinal and theological evidences related to the issue of cloning were considered instead of the jurisprudential and ethical evidences. Reproductive cloning contains various aspects of studies and each must be reviewed in its appropriate place or position. In this manuscript, apart from all negative aspects of human cloning, if it were to be examined on mankind, we consider it doctrinally and theologically and believe that no scientific discovery is contrary to the eternal power of God, cause of firm beliefs of believers worldwide, or contrary to Muslims' tenets. Therefore, our aim is an open discussion and forum on this topic by taking into consideration Qur'anic verses and Islamic ideology. Here we discuss the following questions. i. Is cloning an alteration of Divine creation? ii. Is cloning contradictory to the Creatorship of God? iii. Is cloning contradictory to the principle, precedent, and rule of coupling? iv. Is cloning contradictory to the precedent of the variety of men?

Results

Literal interpretation of cloning

The definition of cloning is "to make an exact copy of an animal or plant". In English, it is termed "cloning", whereas in French, it is "clonage", and "Istinsakh" in Arabic or copying. Clone is derived from the Greek root "klon" that means a bud and slip of a plant stem used by horticulturists to produce new plants. In other words, clone means the asexual reproduction of a single cell (11).

The similarity between cloning and propagation by a slip of a plant stem is that in both the act of reproduction is carried out in the absence of fecundation. In the first, a slip or root is detached from a tree and planted elsewhere and in the latter, reproduction is also performed in the absence of impregnation.

Technical aspects of cloning

The asexual reproductive process of a group of living beings that are all genetically the same is called cloning. Briefly, cloning is a series of processes to produce a clone or a copy. Somatic cell nuclear transfer (SCNT) is a technique by which the nuclear substances of a somatic cell are transferred into an enucleated oocyte (the genetic material is removed) to be reprogrammed and initiate development. Finally, an embryo is produced that can be transferred to a surrogate uterus (reproductive cloning) or can be used to produce embryonic stem cells (reproductive cloning) (12, 13). In reproductive cloning, this new living being is a copy of the individual from which the somatic cell was removed (14).

The goals of human cloning

Today human cloning is divided into two categories: reproductive and therapeutic.

The aim of reproductive cloning is to produce a complete copy of an individual whereas therapeutic cloning aims to produce cells or tissues via stem cell technology for transplantation into an individual with a matched genetic identity. Along with these two applications, cloning has many other implications. Initially, mammalian cloning was performed to answer two fundamental biological questions. Does the nucleus of a well-differentiated somatic cell have all the genetic information for development of an individual? Can a fully differentiated cell be reprogrammed to a totipotent cell? The birth of Dolly provided the answers to these two questions in mammals (15). Subsequently, with the advent of stem cell technology (16), cloning was thought to be the only possible path to produce a patient's specific stem cells (17) to prevent immune rejection. However, with the advent of induced pluripotent stem cells (IPS) from a well-differentiated cell (18), this aim of cloning subsided and researchers focused on the other use for cloning, which was the production of transgenic animals through SCNT for production of recombinant proteins (19) which were approved by the FDA and other international regulatory agencies. In addition, SCNT has been used for conservation of endangered species in a process called in-

terspecies SCNT (ISCNT) (20).

History of cloning

Approximately 100 years prior to the birth of Dolly, a scientist named Jacques Loeb (1859-1924) focused his research on the process of parthenogenesis. Subsequently, Hans Spilman (1983) a German embryologist whose work on the salamander enjoyed universal fame, undertook the first animal cloning experimentations. He succeeded in dividing the embryo of a salamander until the 16th stage. In fact, he demonstrated that the cells reproduced from the first stages of an embryo could once more begin dividing to produce a complete salamander. In 1962, the English biologist John Gurdon (15), cloned a frog by taking the intestinal cells of a tadpole and transferring it to an oocyte whose genetic contents had been removed. Although the extent of success of these experimentations was limited, they positively persuaded researchers to attempt the same procedures on mammals. In 1978, the birth of Louise Brown (21) in England through *in vitro* fertilization opened a new horizon in infertility and experimental biology and paved the way for mammalian cloning (22). On July 5, 1996 at 5 pm, Dolly was born. This ewe was the first mammal created through SCNT. However, this event was not announced to the public until February 23, 1997 when Dolly was seven months old. The reputation of Dolly, however, resulted from the impending probability of human cloning. Due to the seriousness of the idea of mammalian cloning, there were various reactions worldwide (23).

Study of doctrinal and theological evidences of human cloning

Is cloning an alteration of Divine creation?

The Holy Qur'an (4:118-119), in discussing with polytheists or nonbelievers, mentions Satan or the evil (Shaytan) who claimed that he would mislead people and order them to change creation (10):

"Allah has cursed Satan who said, 'I will surely take of Your servants a settled share, and I will lead them astray and give them [false] hopes, and prompt them to slit the ears of cattle, and I will prompt them to alter Allah's creation.' Whoever takes Satan as a guardian instead of Allah has certainly incurred a manifest loss."

In the Age of Ignorance, the desert settlers of Arabia used to cut their animals' ears in order to forbid people from riding them and these animals became useless. This manner was used to clarify what was unlawful and what was lawful. The above verse was revealed to show this great sin and their heresy (24).

Some consider cloning as one of the examples of this practical change of creatures by Satan. They state that cloning changes human nature and thus breaks the boundaries, by interfering and retouching Divine laws that regulate man's creation, as well as it is submission to the apparent and hidden devils. They emphasize the phrase: "and I will prompt them to alter Allah's creation". Besides this verse, in the verse 30:30, it is forbidden to change creation:

"The nature made by Allah in which He has made men; there it is no altering of Allah's creation, that is the right religion, but most people do not know" (10).

They concluded from these two verses that "altering Allah's creation is forbidden (unlawful or haram)."

These verses are the most fundamental examples for the prohibition of human cloning, which are variously interpreted by most critics. These critics believe that it is unlawful because an alternation of God's creation occurs with human cloning (25, 26). One jurist who exemplified this verse stated that altering God's creation meant every change or action disproportionate to man's nature [i.e., God prescribed certain tasks for bodily organs and altering them would result in infidelity (kufr)]. Therefore, this is unlawful.

In contrast, some jurists (27, 28) do not rely on these verses; rather, they present many proofs to reject this reasoning and state that it is incorrect to prove prohibition of cloning through these verses.

A contemporary scholar says that cloning in the general sense is not altering Divine creation but it is applying a type of Divine creation by way of discovery which is correspondent with the Divine creation. Thus suggesting that the procedure of cloning or SCNT is not a Divine creation. However, the process is similar to insemination and *in vitro* or *in vivo* fertilization, which results in the development of healthy individuals (27). Similarly, another scholar who rejected this idea has stated that there is no reason to forbid alternation of the creature in general because if it were forbidden, then any type of alternation in nature should be forbidden as well (28).

In a criticism of the above mentioned, it must be stated that, first, a literary review of "khalk-al Allah" (God's Creation) in the mentioned verse is given followed by remarks from Sunni and Shiite scholars who regard the verses in question.

The author of the commentary Mukatil bin Sulayman interprets khalk-ul Allah (God's creation) as din-ul Allah (religion of God) (29). Moreover, in Lisan-ul Arab for the word khalk by virtue of the verse changing Allah's creation (falayughayyirunnakhalk-al Allah) it is interpreted as din-ul Allah (changing the religion of God) (30).

Furat al-Kufi (31), in his Tafasir under this verse, narrated from Imam Bakir (pbus): "The connotation of khalk-ul Allah is religion and commands of Allah…". Sheikh Tusi, after quoting different views on this issue, states: "The most compelling explanation of the verse, considering the verse falayughayyirunnakhalk-al Allah (changing Allah's creation) (verse 30:30), is din-ul Allah (changing the religion of Allah)" (32). Tabrisi comments that alternation of the creature as alternation of the lawful and unlawful of God (33). Bin Kathir quoted from bin 'Abbas, 'Akramah

and Mujdahid that, by the virtue of the verse 30:30, khalk-ul Allah means din-ul Allah (34).

Suyuti in his al-Durr al-Manthur reports from bin ‘Abbas that it means religion of God (35). AllamahTabatabai has stated: "The example of this is the Qur’anic verse 30:30 where Allah states: latabdila li khalk-il Allah. There is no altering Allah's creation, and then the altering Allah's creation means to come out from the injunction of nature and to leave the right religion"(24). In Tafsir-iNimuni this phrase is interpreted as: "to change the (genius) Divine nature (to exchange the monotheistic nature by polytheism)" (36). In Ruh all-Maani it is commentated as altering Divine nature, considering the Qur’an: 30:30 (37).

In conclusion, from these points of views of many scholars, this verse and its key words, (falayughayyirunnakhalk-al Allah [to alter Allah’s creation]), which is considered to be cloning by some scholars, is referred to as the alteration of Divine religion and commands and not physical changes. Moreover, it is not mentioned in general to cover all changes with the result of turning many permitted acts (mubah or lawful) into unlawful ones. Breeding plants and animals are common practices throughout history, which could be interpreted as altering God's creation according to this theory. Therefore, *in vitro* fertilization and cloning, which are implications of natural biological mechanisms, are generally considered to be permissible acts by the majority of jurists. In conclusion, according to this verse, any type of physical changes and retouching of the human body are the subjects of this injunction. According to the Qur’anic verses 4:117-119 (10), possibly the direct conclusion drawn from this verse is not correct and an interpretation of one verse should be based on the other verses. Therefore, altering God's creation may not prove the prohibition of cloning.

Is cloning contradictory to the Creatorship of God?

In many verses the Holy Qur’an attributes creation and existence to God. In verse 7:54, it is explicitly written (10):

"Look! All creation and command belong to Him. Blessed is Allah, the Lord of all the worlds."

In verse 2:258:

"Have you not regarded him who argued with Abraham about his Lord, because Allah had given him kingdom? When Abraham said, My Lord is He Who gives life and brings death, he replied, I [too] give life and bring death. Abraham said, Indeed Allah brings the sun from the east; now you bring it from the west. There at the faithless one was dumb founded. And Allah does not guide the wrongdoing lot."

Some consider cloning as bringing a new creature into existence. They claim that this procedure leads to the doubt that humans can make alternations in a creature that has been created; thus, he can be a creator. People conceive this to be the process of creating living beings. This doubt has persuaded a group of scholars to use this idea as a reason to prohibit cloning. They regard cloning incompatible to the nature of Islamic beliefs. An ignorant person would imagine that men can create in the way that God creates. This false impression would lead to the weakness of simple-hearted men (38). Rabitat-ul Alam al-Islami, in a letter, refers to this question and declares that cloning is not compatible with human nature and it results in a hesitation (25).

On the other hand, some jurists (39, 24) contradict this theory: "On the basis of this sense, people always retouch Divine work; God, the Almighty, says: 'Allah-u Yatawaffaal-anfusahinamawtiha', If God brings death, so no one can bring death for anyone! These are contradictory to each other. In fact, the work done by God's servant is different from God's work. The work of a farmer also is interference in God's work. The Holy Qur’an says: 'afaraaytummatahruthun? aantumtazraunahu am nahn-ulzariun?' indeed, it is the Creator, the Almighty God, and not the farmer, that gives it birth or replenishes life. Human knowledge hopes to discover the laws of creation and implement them for his aims. Even after myriads of years, man would not be able to create a being out of the rule of creation."One Muslim scholar declares that cloning is creativeness (takhlik) that can be performed by man but creatorship is only for Allah. Cloning is not "creation of life", as some assume or imagine, but is profiting from a life given by Allah (39).

Another scholar, through an explanation of the meanings of khalk (creation) in the Holy Qur’an and determination of the difference between creating from nothing (as refereed in the Qur’an by “fatir”) and the creation or formation of beings according to the sole role of nature, tried to respond to this misconception as follows: "khalk is applied in Qur’anic verses in three meanings: i. kadkhalaktuka min kabluwa lam takushayan; Certainly I created you before when you were nothing (verse 19:9). Here khalk means creation from nothing. ii. innakhalaknahum min tininlazib; 'Indeed We created them from a viscous clay (verse 37:11). In this case it means to create a living being from a lifeless thing. iii. thummakana alakatanfakhalakafasawwa; 'Then he became a clinging mass; then He created [him] and proportioned [him]'(76:38). In this case it means alternation from another form and not coming into existence from nothing" (24).

In a criticism of the above mentioned, it must be stated that as human knowledge extends to discover creation, man is unable to create a being outside the rules of creation (or rule of Allah). In fact, cloning is not creating a new being, but innovation by scientists with existing biological material and a role set by Allah Himself. This is also true for creation of cells by aid of computer (40).

Furthermore, scientists do not have knowledge regarding characteristics of the human spirit, of which God says: "They question you concerning the Spirit. Say, The Spirit is of the command of my Lord" (verse 17:85). Scientists are unable to make a living cell, out of nothing, much less create a human being.

The manner of the Holy Qur'an is all affairs in the universe are undertaken solely by God's will and is fulfilled by its chain of causes. If knowledge reaches these causes, it puts in the length and process of creation. Cloning is not a misconception in religion, for every scientific advantage could affect and threaten people's (common) beliefs. Many scholars do not count it as a doubt on religion; instead, they consider it to be an explicit example of the Resurrection, the creation of Eve, and the birth of Jesus (41). In conclusion, since this technology is not out of the cause and effect order of the universe nor out of the will of God (8), cloning is not against Monotheism nor the order of creation.

Is cloning contradictory to the principle, precedent, and rule of coupling?

The Holy Qur'an, verse 49:13 states:

"Indeed we created you from a male and a female, and made you nations and tribes that you may identify yourselves with one another."

The existence is based on the creation of male and female. "In all things we have created pairs so that you may take admonition" (verse 51:49). The world of beings is based on the pairing pre-resumption and it predominates not only throughout the human world but also in all of the animals and plants. The Holy Qur'an frequently points to this phenomenon. "And [We] create you in pairs" (verse 78:8). "And that is He who created the mates, the male and the female, from a drop of [seminal] fluid when emitted" (verse 53:45-46). The Divine precedent of creating mankind is via marriage between a man and woman. According to some people, human cloning alters and annihilates this precedent. Such individuals state that human cloning is on the basis of satisfaction with one sex without the need for the other sex. Rabitat-ul Alam al-Islami on the strength of verse 49:13 mentions human cloning as a violation of the rule of coupling (25).

A group of scholars believe that throughout the Holy Qur'an, whenever it is written about the creation of man and woman, it is referred to in the usual sexual way where the majority of people come into existence through the joining of sperm and ovum. This does not oppose the fact that a human may be born through other methods, as Jesus who did not have a father (42). It is also said that perhaps these verses speak about the common and usual way of reproduction, but they do not refute other methods. One Muslim thinker has stated that if the precedent of coupling is common and covers the entirety of this material world; thus a human would not be able to break it even if he desires because only the legislative laws can be violated and not the ontological ones. Therefore, cloning is not a violation of the rule of the world of being. If it were so, then humans would have power equal to God's and could break the laws by which God created nature. Moreover, if the creation of Adam and the birth of Jesus are miracles, which is a precedence that has dominated over existence, then human cloning may be one example of a hidden precedence that confirms cloning (43).

In a criticism of the above, it must be referred that Ahkamal-Kuran, under verse 49:13 writes: "Allah created people of males and females. If He willed, He would be able to create man without them, as creating Adam and Eve or Jesus, without male. In fact, Allah is able to do all these" (44).

In some commentaries such asal-Balagh (45) and Nimune (36), under the verse we read that creation of people from a male and a female refers to the generation of men that originate from Adam and Eve. The source of all is one and there is no superiority of someone to others. Indeed, piety is the true criterion. Al-Tafsir al- Mubin declares that this verse is not among the verses of injunctions. It indicates that all men and women benefit from equal rights. It does not indicate making laws and violating them (46). Thus, although it is true that many Qur'anic verses emphasize coupling, no verse indicates that it is infinite to this precedent. The coupling precedent is the common method of nature, but not the absolute law. For example, coupling is contradictory to parthenogenesis (another type of reproduction which is well-observed in nature) and opposite to the creation of Adam, who came from soil, Eve, and Jesus who only had a mother. Despite their unusual creations, they came into this world under the precedents that controlled existence. In one word, cloning may be one of these hidden precedents of creation.

Precedent (sunnah in Arabic) literally means way, method, habit, character, tradition, and nature, and technically the Divine precedents or Sunnahs of God are firm rules that comprise the root and base of the world order dictated by the Wise and Exalted God. These rules must exist for the World Order to not be disarranged. The divine precedent in philosophy is called "the World Order" or "the Causes Rule".

Many verses of the Holy Qur'an emphasize that these precedents are firm and changeless. For instance, in verses 30:62 and 48:23 it says:

"And you may not see any change in the divine precedent."

This content is repeated twice in verse 35:43:

"But no change wilt thou find in Allah's way (of dealing): no turning off wilt thou find in Allah's way (of dealing)."

Is cloning contradictory to the precedent of the variety of men?

God the Almighty, inverse 30:22, regards the variety of beings in the world as one of His signs.

"Among His signs is the creation of the heavens and the earth, and the difference of your languages and colors. There are indeed signs in that for those who know."

Various verses mention this individuality and count it as a sign of God (16:130; 35:27; and 39: 21).

Many scholars state that cloning requires creating iden-

tical copies of man and they consider cloning to be opposed to the law of variety. On this issue, Rabitat-ul Alam al-Islami states that variety and distinction are precedents of God in the process of creating man, Who enriches human life through evolution, while cloning deprives the cloned human from distinctive attributes that make him unique among his fellow creatures (25).

Criticizing what was mentioned above, it must be referred that Allamah Tabatabai under verse 22 of Surah Rum (10) says: "The difference of languages here apparently means difference of words which one is Arabic and the other is Persian, and the difference of colors means difference of races according to colors."

Scholars who study about macrocosm exemplify exact verses. They believe that the world of creation and making with its order is only from the All-Wise God. Zuhayli, also in al-Munir, states that the variety of men regarding their sole origin (one father and one mother) is remarkable (47).

This opposition is not a fair assessment of the fact, for the cloned person is not entirely like the original one. The word "clone" (copy) here is applied indulgently because scientifically the cloned person is different from his origin in three aspects:

i. In SCNT, only the somatic genome is from the original (who gives the nuclear substances) and the other genome that exists in the cytoplasm, including the mitochondria, are from donor cytoplasm or the oocyte.

ii. The wombs in where an original and a clone grow are different.

iii. After birth, we could not limit the personal identity of a person to his genetic identity (8). This fact is stated in Article 3 of the International Declaration of Human Genetic Data:

"Each individual has a characteristic genetic make-up. Nevertheless, a person's identity should not be reduced to genetic characteristics, since it involves complex educational, environmental and personal factors and emotional, social, spiritual and cultural bonds with others and implies a dimension of freedom" (48).

Also, as explained for the abovementioned verses, this variety is a sign for men and not a precedent that predominates over the order of nature. In parthenogenesis, some living beings reproduce by transferring the same genetic structure. A fairly complete similarity is observed in twins from one egg. Harris (49) names God as the Greatest Artisan Portraitist. Consequently, as we mark the difference among beings as a sign of God, why would clones not be counted as a sign from Him? When an entire code of a human being is in a cell by which he can be reproduced and created, therefore it is a sign of God, which indicates that every cell of a man is also a man.

Discussion

Through general details on human cloning, the Qur'anic views (verses), statements of the Prophet and Imams (pbut), and the great Shiite Muslim scholars were reviewed and discussed in order to reach reasonable conclusions and have appropriate analyses at hand. Then, by aiming not to prove or reject cloning, we studied Qur'anic references to reach a common theory between Muslim scholars and provide a possible solution for the present challenges of cloning. Apart from all negative aspects of human cloning, we considered cloning from doctrinal and theological aspects and believe that no scientific discovery is contrary to the eternal power of God, cause of firm beliefs of believers worldwide, or contrary to Muslims' tenets. The following questions were discussed. i. Is cloning an alteration of Divine creation? ii. Is cloning contradictory to the Creatorship of God? iii. Is cloning contradictory to the principle, precedent, and rule of coupling? iv. Is cloning contradictory to the precedent of the variety of men? In each section, after a detailed discussion, some of the main sources and documents were studied and criticized, especially different aspects by the Shite scholars. In the first section, it was proved that *in vitro* fertilization and cloning is generally considered a permissible act by the majority of jurists and according to the Qur'an. Any type of physical changes and retouching of the human body are the subjects of this injunction and altering God's creation may not prove the prohibition of cloning. In the second section, it was proved that, since this technology is not out of the cause and effect order of the universe nor out of the will of God, it is not against Monotheism nor the order of creation. In the third section, we proved that, not only is cloning not contradictory to the principle, precedent, and rule of coupling, but also it may be one of the hidden precedents of creation. Finally, in the fourth section, it was proved that a clone is not contradictory to the precedent of the variety of men; rather, this variety is a sign for men and not a precedent predominated over the order of nature. The differences among beings is a sign of God; therefore, why are clones not counted as signs from Him? When an entire code of a human being is in a cell by which he can be reproduced and created; therefore, it is a sign of God, which indicates that every cell of a man is also a man.

Conclusion

The above mentioned reasons are most of the proofs considered in relation to human cloning and its relative doctrinal problems. Our study of the views and comparing them to the Qur'anic contemporary and non-contemporary exegeses of Shiite and Sunnite Muslims enabled us to prove that:

i. Cloning is not giving life but it is utilizing the life bestowed by God, the Almighty.

ii. This technique does not contradict the precedents of existence. It is a way to discover some precedents of God and is under the order of cause and effect of the world.

iii. Since Islam calls mankind for reflecting and thinking, this technique is not considered to be a challenge to human beliefs and it is not a change in Divine creation.

iv. Finally, human cloning is not contradictory to any theological teachings and concepts of the Holy Qur'an and Shiite Muslims.

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Authors' Contributions

Kh.A.-Kh., M.H.N.-E., A.H.; Participated in the research design, data collection and evaluation, drafting, and critical analysis.

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