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Original Article

Effects of Dextran-Coated Superparamagnetic Iron Oxide Nanoparticles on Mouse Embryo Development, Antioxidant Enzymes and Apoptosis Genes Expression, and Ultrastructure of Sperm, Oocytes and Granulosa Cells

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Abstract

Background: Although application of superparamagnetic iron oxide nanoparticles (SPIONs) in industry and medicine has increased, their potential toxicity in reproductive cells remains a controversial issue. This study was undertaken to address the response of sperm, oocyte, and resultant blastocyst to dextran-coated SPIONs (D-SPIONs) treatment during murine *in vitro* fertilization (IVF).

Materials and Methods: In this experimental study, murine mature oocytes were randomly divided into three groups: control, and low- and high-dose groups in which fertilization medium was mixed with 0, 50 and 250 µg/ml of D-SPIONs, respectively. Sperm and/or cumulus oocyte complexes (COCs) were cultured for 4 h in this medium for electron microscopic analysis of sperm and COCs, and assessment of developmental competence and genes expression of *Gpx1*, *Sod1*, *catalase*, *Bcl2l1* and *Bax* in the resultant blastocysts.

Results: Ultrastructural study of sperm, oocyte, and granulosa showed destructed mitochondria and membranes in spermatozoa, vacuolated mitochondria and distorted cristae in oocytes, and disrupted nuclei and disorganized cell membranes in granulosa in a dose-dependent manner. Data showed that cleavage and blastocyst rates in the 250 µg/ml of D-SPIONs were significantly lower than in the control group ($P<0.05$). Gene expression of *GPx1*, *Sod1*, *catalase*, *Bcl2l1* and *Bax* in resultant blastocysts of the high-dose group and catalase and Bax in resultant blastocysts of the low-dose group, was higher than the controls.

Conclusion: There is considerable concern regarding D-SPIONs toxic effects on IVF, and mitochondrial and cell membrane damage in mouse spermatozoa and oocytes, which may be related to oxidative stress and apoptotic events.

Keywords: Apoptosis, Nanoparticles, Oocytes, Oxidative Stress, Spermatozoa

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Introduction

Nowadays, there is great interest towards using nanotechnology due to its increasing application in all aspects of life, including agriculture, industry, medicine and public health (1, 2). All nanoparticles have a common characteristic: nanoparticle synthesis leads to remarkable changes in their chemical, physical and biological properties when compared to their original counterparts (3). Despite the beneficial properties of nanomaterials, potential risks of these materials are a matter of concern. Since some nanomaterials are used in medicine, there is concern about possible toxicity of these nanomaterials for human health (4, 5). Important toxicological concerns regarding the engineered nanomaterials are related to their redox potential, and transport of some particles across the biological cell membranes, particularly into the mitochondria (6). Toxicity of nanoparticles to the female reproductive system and fertility has been confirmed in some studies (7, 8). Likewise, titanium

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dioxide nanoparticle induced testis and sperm lesions, and diminished sperm numbers and sperm motility in male mice (9). Therefore, further studies are required to examine the biocompatibility and safety of these new materials in greater detail.

Superparamagnetic iron oxide nanoparticles (SPIONs) have magnetic, electronic and optical properties, which make them suitable for medical and scientific applications such as in vitro diagnostic tests, SPION-based contrast enhancement in magnetic resonance imaging, magnetic hyperthermia treatment and magnetic drug targeting for diagnosis and therapy of cancer and other diseases (10).

A considerable body of evidence indicates that SPIONs have toxic activities. Toxicity and reactive oxygen species (ROS) production in response to uptake of metal oxide nanoparticle, are caused by generation of hydroxyl radicals by strong catalytic impacts of nanoparticle surfaces such as content of iron oxide, and release of iron ions into an aqueous phase, and result in superoxide-driven Fenton reaction (11). Also, promotion of intracellular free iron levels leads to a ROS-antioxidant imbalance due to stimulation of ROS generation over Fenton and Haber-Weiss reactions. Consequently, SPION induces oxidative damage by a ROS -mediated mechanism (12) and leads to apoptosis by affecting the mitochondria, death receptors and endoplasmic reticulum. The mitochondrial pathway of apoptosis is mediated by the B-cell-lymphoma protein 2 (Bcl-2) family which includes two main groups: anti-apoptotic (Bcl-2, Bcl2l1, Bcl-W, Bcl-B, A1 and Mcl-1) and pro-apoptotic (Bax, Bak and Bok) proteins. Maintaining a balance between these groups is critical for cell protection against apoptosis (13).

Nanoparticle coating with biocompatible polymers such as chitosan or dextran, may act as a barrier against SPIONs' toxic potential and hugely protect cellular molecules, such as lipids, proteins, and DNA, from oxidative stress (14). Such coating also increases the colloidal stability, aggregate size, cellular interaction and biocompatibility, and iron oxide cores (15). Thus, these polymers have dire effects on the fate and level of SPIONs uptake in different cells. Stroh et al. (16) showed that citrate-coated SPIONs could dramatically promote protein oxidation and oxidative stress, but do not affect cell viability.

Although our knowledge about SPIONs toxicity has improved in recent years, the effects of this nanoparticle on fertilization are still a major concern, because iron oxide nanoparticles have the capacity to penetrate the placenta and aggregate in the fetus (17). Moreover, small nanoparticles could cross the blood-testis barrier and appear in the testes (18). Thus, in this paper, the potential risks of D-SPIONs for murine *in vitro* fertilization (IVF) were investigated by transmission electron microscopy (TEM) in sperm, granulosa cells and oocytes. Then, the developmental competence and changes in antioxidant enzymes (glutathione peroxidase 1 (*GPx1*), superoxide dismutase 1 (*Sod1*) and catalase (*Cat*), *Bcl2l1* (apoptotic inhibitor) and Bax (apoptotic activator) gene expression, were evaluated in the resultant blastocysts.

Materials and Methods

In this experimental study, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, USA) and Gibco (Grand Island, USA), unless stated otherwise.

Preparation of dextran-coated nanoparticle suspension

The starting materials, $FeCl_3 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$, and NH4OH solution were purchased from Merck. Magnetite nanoparticles were synthesized according to the literature with some modifications (19) through the alkaline coprecipitation method using iron (II) and (III) chlorides. Briefly, 1.6 g $FeCl_3 \cdot 6H_2O$ and 0.6 g $FeCl_2 \cdot 4H_2O$ were grinded and then added to a beaker containing 50 ml deionized water. The beaker was kept in an ultrasonic bath for 30 minutes. The prepared solution was transferred into a three-neck flask and agitated vigorously under nitrogen gas atmosphere. After 5 minutes' agitation, 30 ml NH4OH was added dropwise during 45 minutes. Finally, the suspension was kept at 75-80°C for 80 minutes. The nanoparticles were separated magnetically and washed several times to adjust the pH. The collected iron oxide nanoparticles were dispersed in a 5% dextran solution and stirred for 5 hours at 75°C. The solution containing dextran-coated iron nanoparticles, was centrifuged at 11000 rpm for 15 minutes to eliminate the larger particles.

Evaluation of D-SPIONs characterization

Phase analysis was performed by a Philips X-ray diffractometer (model PW3710) using Cu-Kα radiation at a wavelength of 1.54 A in the 2θ range of 5-80°. Fourier-transform infrared spectroscopy (FTIR) of the sample was done by a PerkinElmer spectrometer in the range of 400-4000 cm-1. TEM experiments were conducted on a Philips CM30 TEM with an operating voltage of 200 kV. The TEM sample preparation was done according to the literature (20). The nanoparticles containing aqueous solution, were sonicated and then, a drop of the solution was placed on the carbon-supported Cu grid. The nanoparticles on the grid, were used for the experiment after solvent evaporation.

Animals

Fifty mature female BALB/c mice (6-8 weeks old) were superovulated by an intraperitoneal (IP) injection of 10 IU pregnant mare serum gonadotropin (PMSG, GONASER®, HIPRA, Amer, Spain) followed 48 hours later by injecting 10 IU human chorionic gonadotropin (hCG, Organon, Oss, The Netherlands). Sperm samples for IVF and TEM, were obtained from the caudae epididymides of fifteen mature (12-week-old) male BALB/c mice. All females and males were kept under controlled temperature and humidity conditions with a 12-hour light/dark schedule. Animals had *ad libitum* access to food and water. All animal care and procedures were approved by the Ethics Committee

of Shiraz University of Medical Sciences (Approval No. IR.SUMS.REC.1396.S356).

Experimental design

To evaluate possible toxic effects of D-SPIONs on sperm, granulosa cells, oocytes and resultant blastocysts, three groups were considered according to the level of D-SPIONs added to IVF medium [G-IVF PLUS (Vitrolife, Gothenburg, Sweden)]; Group I (control), conventional IVF medium without any treatment (G-IVF PLUS); Group II and Group III conventional IVF medium supplemented with 50 µg/ml and 250 µg/ml of D-SPIONs, respectively. In all groups, sperm or COCs were incubated for 4 hours in IVF medium and fixed with glutaraldehyde for electron microscopic analysis. To evaluate the effects of this nanoparticle on developmental competence, IVF was done in G-IVF PLUS supplemented with 0, 50 and 250 µg/ml of nanoparticles. Then, the presumptive zygotes were cultured until expanded, reaching the blastocysts stage in G1/G2 PLUS without nanoparticle. The resultant expanded blastocysts were used for gene expression analysis.

Sperm capacitation

Spermatozoa were collected from the cauda epididymides of mature male mice and capacitated by preincubation at 37°C with 5% CO_2 for 1 hour in 200 µl of G-IVF PLUS drops under mineral oil (Reproline Medical GmbH, Rheinbach, Germany). These spermatozoa were used for IVF and TEM assay.

Sperm preparation for TEM

Spermatozoa were randomly divided into three groups and incubated for 4 hours in G-IVF medium with different concentrations (0, 50 and 250 µg/ml) of nanoparticles under mineral oil. After incubation, 0.5 ml of semen was transferred into a micro tube, washed with phosphate buffered saline (PBS) twice and centrifuged at 400 g for 10 minutes. Then, the supernatant was removed and each sample was fixed with 2.5% glutaraldehyde (pH 7.4) overnight. The samples were centrifuged for 10 min at 400 g at room temperature and washed in sodium cacodylate for 3 times (5 minutes each) and centrifuged for 10 minutes at 400 g. Each sample was post-fixed in 1% buffered osmium tetroxide for 60 minutes. Post-fixed samples were centrifuged for 10 minutes at 400 g, and the supernatant was discarded; then, samples were washed in sodium cacodylate for 3 times (5 minutes each) and embedded in 1% agar. After that, embedded samples were dehydrated in ascending concentrations of 30-100% ethanol. Finally, the samples were embedded in resin (agar 100) and polymerized at 60°C overnight. Thick sections (0.5-1 µm) were stained with toluidine blue and examined by light microscope (Axioskop, Carl Zeiss Microscopy GmbH, Göttingen, Germany). Thin sections (60-90 nm) were contrasted with uranyl acetate and lead citrate and examined by TEM (21).

COCs collection

The COCs were immediately harvested from the oviductal ampulla 13-14 hours post-hCG injection. These COCs were used for IVF and TEM assay.

COCs preparation for TEM

COCs were exposed to different concentrations (0, 50 and 250 µg/ml) of nanoparticle in the G-IVF medium under mineral oil for 4 hours at 37°C with 5% CO_2. Then, they were washed twice in PBS to remove culture medium and nanoparticles. COCs were immersed in 2.5% glutaraldehyde overnight. Then, COCs were washed in sodium cacodylate for 3 times (5 minutes each). Following fixation in 1% buffered osmium tetroxide for 30 minutes, COCs were washed in sodium cacodylate for 3 times (5 minutes each) and dehydrated in ascending concentrations of 30-100% ethanol. Each COC was embedded in resin (agar 100) and polymerized at 60°C overnight. Thick sections (0.5-1 µm) were stained with toluidine blue and examined by light microscope. Thin sections (60-90 nm) were contrasted with uranyl acetate and lead citrate and examined by TEM (21).

In vitro fertilization and embryo culture

COCs were inseminated *in vitro* with 1×10^6 spermatozoa/ml in 100 µl of G-IVF PLUS containing 0, 50 or 250 µg/ml of D-SPIONs, for 4 hours. The presumptive zygotes were cultured in G1 PLUS for 1.5 days, and then, the embryos were transferred to G2 PLUS under mineral oil at 37°C in a humidified incubator with 5.0 % CO_2, and the rates of cleavage and blastocyst were recorded in at least 4 replicates.

RNA extraction, cDNA synthesis and quantitative real-time RT-PCR

Total RNA was extracted from 3 pools of 15 expanded blastocysts per group, using the RNeasy Micro Kit (Qiagen, Hilden, Germany) following the manufacturer instructions. First-strand cDNA synthesis was carried out using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Real-time reverse transcription - polymerase chain reaction (RT-PCR) was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, USA). The PCR amplification was conducted in a final volume of 25 µl consisting of 1 µl of the cDNA template, 12.5 µl of RealQ Plus 2x Master Mix Green Low ROX (Ampliqon A/S, Odense, Denmark), and 1 µl of each primer (10 pmol/µl). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as a reference (22). The gene expression of *GPx1*, *Sod1* and *Cat* as main antioxidant enzymes, and *Bcl2l1* and *Bax* in expanded blastocysts, was analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers used for RT-PCR are listed in Table 1.

Table 1: Details of primers used for quantitative real-time reverse transcription - polymerase chain reaction (RT-PCR)

Gene	Nucleotide sequences (5′–3′)	Fragment size (bp)	Accession number
GPx1	F: CAGGAGAATGGCAAGAATGAAGAG R: GGAAGGTAAAGAGCGGGTGA	136	NM_008160.6
Sod1	F: GGGTTCCACGTCCATCAGTAT R: GGTCTCCAACATGCCTCTCTT	121	NM_011434.1
Cat	F: CTCAGGTGCGGACATTCTACA R: AATTGCGTTCTTAGGCTTCTCAG	206	NM_009804.2
Bcl2l1	F: GCAGGTATTGGTGAGTCGGA R: CTCGGCTGCTGCATTGTTC	130	NM_001289716.1
Bax	F: TGGAGATGAACTGGACAGCAAT R: TAGCAAAGTAGAAGAGGGCAACC	155	NM_007527.3
Gapdh	F: TGTTTCCTCGTCCCGTAGA R: ATCTCCACTTTGCCACTGC	106	NM_001289726.1

Statistical analysis

Before any statistical analysis, the normality of data and homogeneity of variances were evaluated by the Shapiro-Wilk test and means of Bartlett's test, respectively. Developmental competence and real-time RT-PCR data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test using SPSS 20 (IBM Corp., Armonk, N.Y., USA). Data is expressed as mean ± standard deviation (SD). Differences were considered significant at $P<0.05$.

Results

D-SPIONs characterization

D-SPIONs were characterized by X-ray Diffraction (XRD) pattern, TEM and FTIR spectrum (Fig. 1). The XRD pattern of the synthesized nanoparticles showed that all the peaks corresponded to Fe3O4 and no other peak from other phases, could be detected. In XRD analysis, the major XRD peak was calculated at 2θ = 35.6 and other peaks were observed from 0 0 1, 1 1 2, 1 0 3, 0 0 4, 2 0 4, 3 2 1, 2 2 4 and 4 1 3. The full width at half maximum (FWHM) of the 1 0 3 peak was used to estimate the average crystallite size of D-SPIONs using the Scherrer method. The average size of D-SPIONs was 17.44 nm.

The FTIR spectrum of the synthesized D-SPIONs, is shown in Figure 1B. The peak at 578 cm^{-1} corresponded to the Fe-O bond absorption. The peak at 1622 cm^{-1} is an indication of C=O stretching vibrations. Peaks at 1016 cm-1 and 1149 cm^{-1} corresponded to the C-OH alcoholic hydroxyl stretching vibrations and the peak at 3380 cm^{-1} showed the presence of the hydroxyls in the dextran-coated nanoparticles (23). The bands seen around 2900 cm^{-1} and 1240-1460 cm^{-1}, showed the νC–H and the δC–H vibrational modes of the dextran (24).

The TEM images of D-SPIONs are presented in Figure 1C. The sample consisted of monodisperse coated nanoparticles with particle size in the range of 20-30 nm. The coating of the particles was almost uniform with rounded shapes which might result in better biocompatibility.

D-SPIONs destroyed the mitochondria and membranes of spermatozoa in a dose-dependent manner

The degree of D-SPIONs effect was clearly dependent on their concentration. After co-incubation with D-SPIONs, the nano-treated spermatozoa and control sperm cells were subjected to TEM. As shown in Figure 2, some of the sperm mitochondria in the low-dose (50 µg/ml) D-SPIONs group, were swollen but cell membrane was normal. Most of the spermatozoa mitochondria in the high -dose (250 µg/ml) D-SPIONs group, were swollen, with completely distorted mitochondrial cristae, and cell membrane in the midpiece was disorganized and/or distorted, whereas in the control group, spermatozoa mitochondria were regular in shape. Axoneme and longitudinal fiber microtubules in the tail regions were normal.

D-SPIONs had negative effects on oocyte mitochondria and nuclei and membranes of granulosa cells in a dose-dependent manner

As the nanoparticles' dose increased, the effect of nanoparticles on the granulosa cells and mitochondria in the ooplasm, became more obvious. As shown in Figure 3, some granulosa cells in the low-dose (50 µg/ml) D-SPIONs group, had dense nuclei and cell membranes were disorganized and/or distorted. Most of the granulosa cells in high-dose (250 µg/ml) D-SPIONs group, had disrupted nuclei, disorganized cell membranes and aggregation of nanoparticles, clearly discerned between the granulose cells. In the ooplasm, mitochondria were vacuolated and cristae were distorted, whereas in the control group, mitochondria had regular shape and cortical granules were seen.

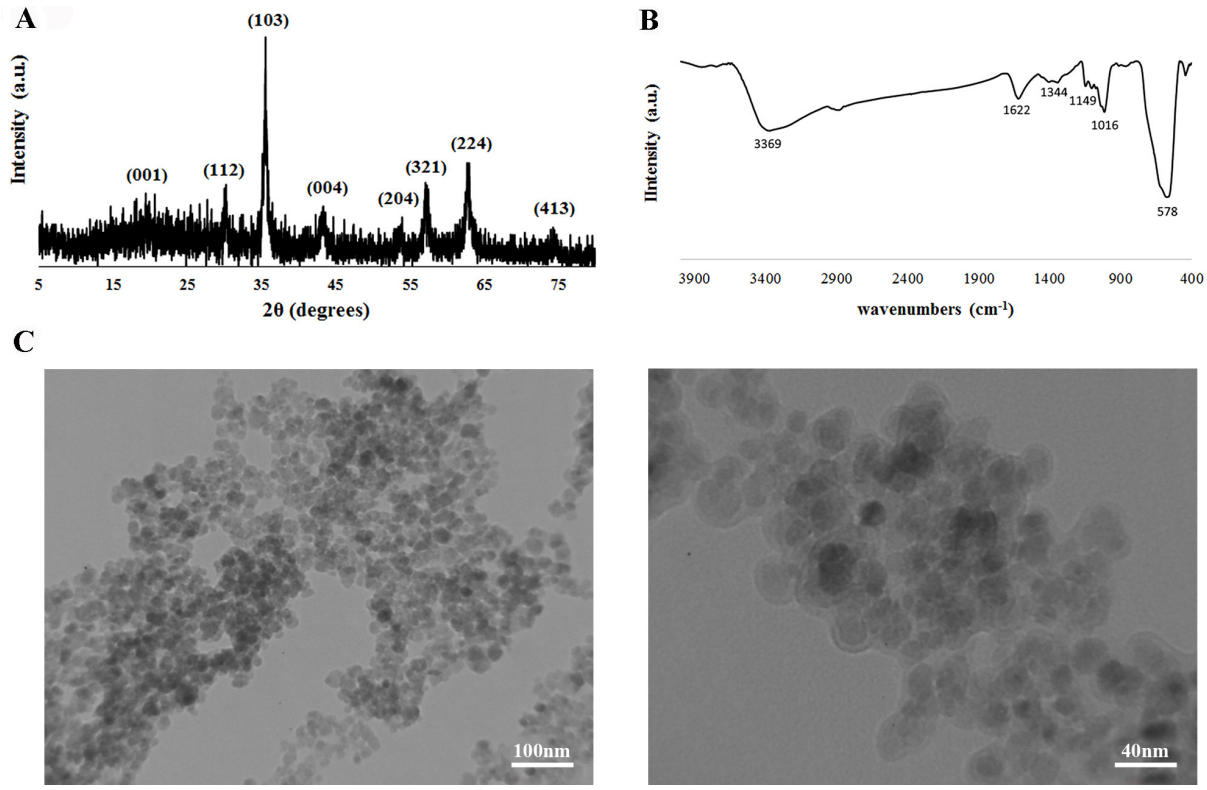

Fig 1: Evaluation of dextran-coated superparamagnetic iron oxide nanoparticles (D-SPIONs) characterization. **A.** XRD pattern of the synthesized D-SPIONs shows that all peaks correspond to the magnetite. **B.** FTIR spectrum for synthesized D-SPIONs. **C.** TEM images of D-SPIONs show that the sample consists of core/shell monodispersed nanoparticles with particle size in the range of 20-30 nm.

D-SPIONs reduced oocyte developmental potential in the high-dose group

The developmental competence of MII oocytes after D-SPIONs treatment, was evaluated by IVF and culture in G1/G2 media until the blastocyst stage. As shown in Table 2, the rate of embryo cleavage at 250 µg/ml of D-SPIONs was significantly lower than that of the control group, with 89.79 ± 2.68%, 76.62 ± 6.10%, and 69.79 ± 6.15% ($P<0.05$) in the control, and 50 and 250 µg/ml of D-SPIONs, respectively. The proportion of oocytes that developed to the blastocyst stage, was significantly lower ($P<0.05$) at 250 µg/ml of D-SPIONs (34.99 ± 11.42%) compared to the control group (67.08 ± 4.90%).

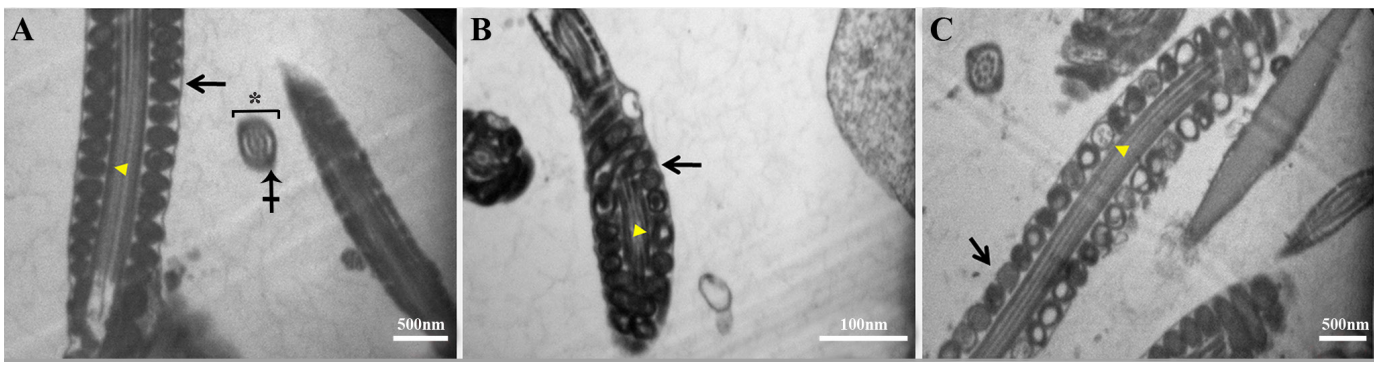

Fig 2: Transmission electron microscopy (TEM) analysis in sperm. **A.** Control group with intact cell membranes (arrow) and mitochondria with normal shape (arrowhead). Axoneme (asterisk) and longitudinal fiber microtubules (crossed arrow) in tail regions are normal. **B.** In 50 µg/ml D-SPIONs-treated group, some of the sperm mitochondria are swollen (arrowhead) but cell membrane was normal (arrow). **C.** Due to internalization or binding of D-SPIONs in the high dose group (250 µg/ml), most mitochondria are swollen (arrowhead), have entirely distorted mitochondrial cristae, and the cell membrane in the midpiece is disorganized and/or distorted (arrow).

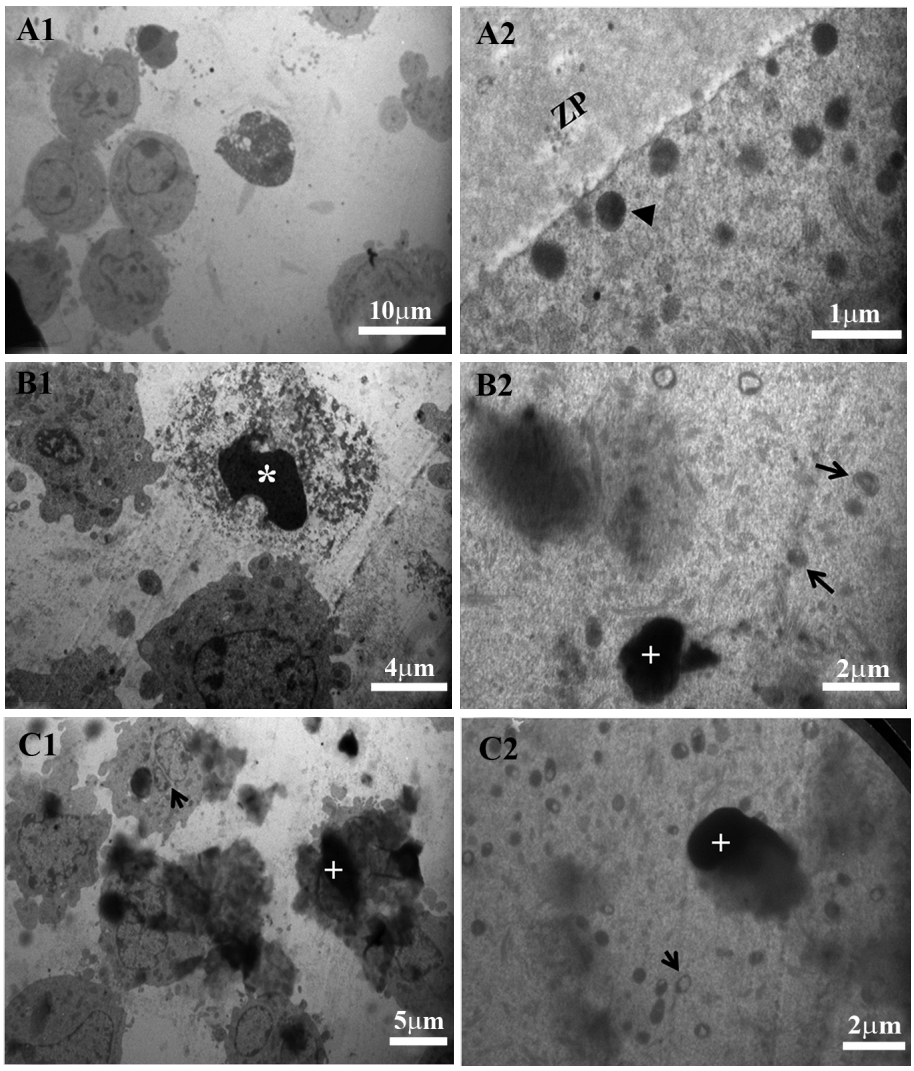

Fig 3: TEM analysis in granulosa and oocyte. **A1.** Normal granulosa cells in the control group. **B1.** Some granulosa cells in 50 μg/ml dextran-coated superparamagnetic iron oxide nanoparticles (D-SPIONs), with dense nuclei and disorganized and/or distorted cell membranes (star). **C1.** Most of the granulosa cells in 250 μg/ml D-SPIONs with disrupted nuclei show disorganized cell membranes (arrow) and aggregation of nanoparticles between the granulose cells (plus). **A2.** Mitochondria with normal shape and cortical granules (arrowhead) in the oocytes of the control group. **B2.** Ooplasm in 50 μg/ml D-SPIONs has few vacuolated and cristae-distorted mitochondria and several normal mitochondria (arrow). **C2.** Ooplasm in 250 μg/ml D-SPIONs have vacuolated and cristae-distorted mitochondria (arrow). The plus sign denotes D-SPIONs. ZP; Zona pellucida.

Table 2: Effect of different concentrations of D-SPIONs in IVF medium, on developmental potential

D-SPIONs (μg/ml)	No. of oocytes	Cleavage rate n (% ± SD)[1]	Blastocyst formation n (% ± SD)[1]
0	157	139 (89.79 ± 6.00)	101 (67.08 ± 10.95)
50	269	214 (76.62 ± 12.21)	146 (51.92 ± 13.63)
250	240	157 (69.79 ± 12.30)*	81 (34.99 ± 22.83)*

[1]; The ratio of cleavage and blastocyst embryos per MII oocytes from at least 4 replicates, *; Mean percentage marked by an asterisk in each column, is significantly different from the control group (P < 0.05), SD; Standard division, and IVF; *In vitro* fertilization.

High-dose D-SPIONs increased the expression of antioxidant enzyme genes

In order to investigate whether the addition of D-SPIONs to the IVF medium changes the expression of three main antioxidant enzymes, we quantified the transcripts of glutathione peroxidase 1 (GPx1), superoxide dismutase 1 (Sod1) and catalase (Cat) genes in each group on the expanded blastocysts. As shown in Figure 4, transcript levels of the GPx1 gene were significantly increased by 250 μg/ml of D-SPIONs (1.44 ± 0.10) when compared to the control group (1.00 ± 0.07, P<0.05). The result of real-time RT-PCR indicated that the relative Sod1 mRNA expression was upregulated by 250 μg/ml of D-SPIONs (1.58 ± 0.06) in the expanded blastocysts, compared with 50 μg/ml of D-SPIONs (1.20 ± 0.09, P<0.05) and the control group (1.00 ± 0.06, P<0.01). Transcript abundance of Cat was significantly increased in 50 and 250 μg/ml of D-SPIONs groups (1.65 ± 0.14, P<0.05 and 1.88 ± 0.08, P<0.01, respectively) in comparison to the control group (1.02 ± 0.12).

High levels of Bcl2l1 transcript were observed in 250 μg/ml of D-SPIONs group (1.65 ± 0.07) when compared to 50 μg/ml of D-SPIONs (1.24 ± 0.10, P<0.05) and control (1.00 ± 0.04, P<0.01) groups. Gene expression of Bax as an apoptotic activator was promoted in both of the D-SPIONs groups (1.35 ± 0.04, P< 0.01 and 1.46 ± 0.05, P< 0.001 for 50 and 250 μg/

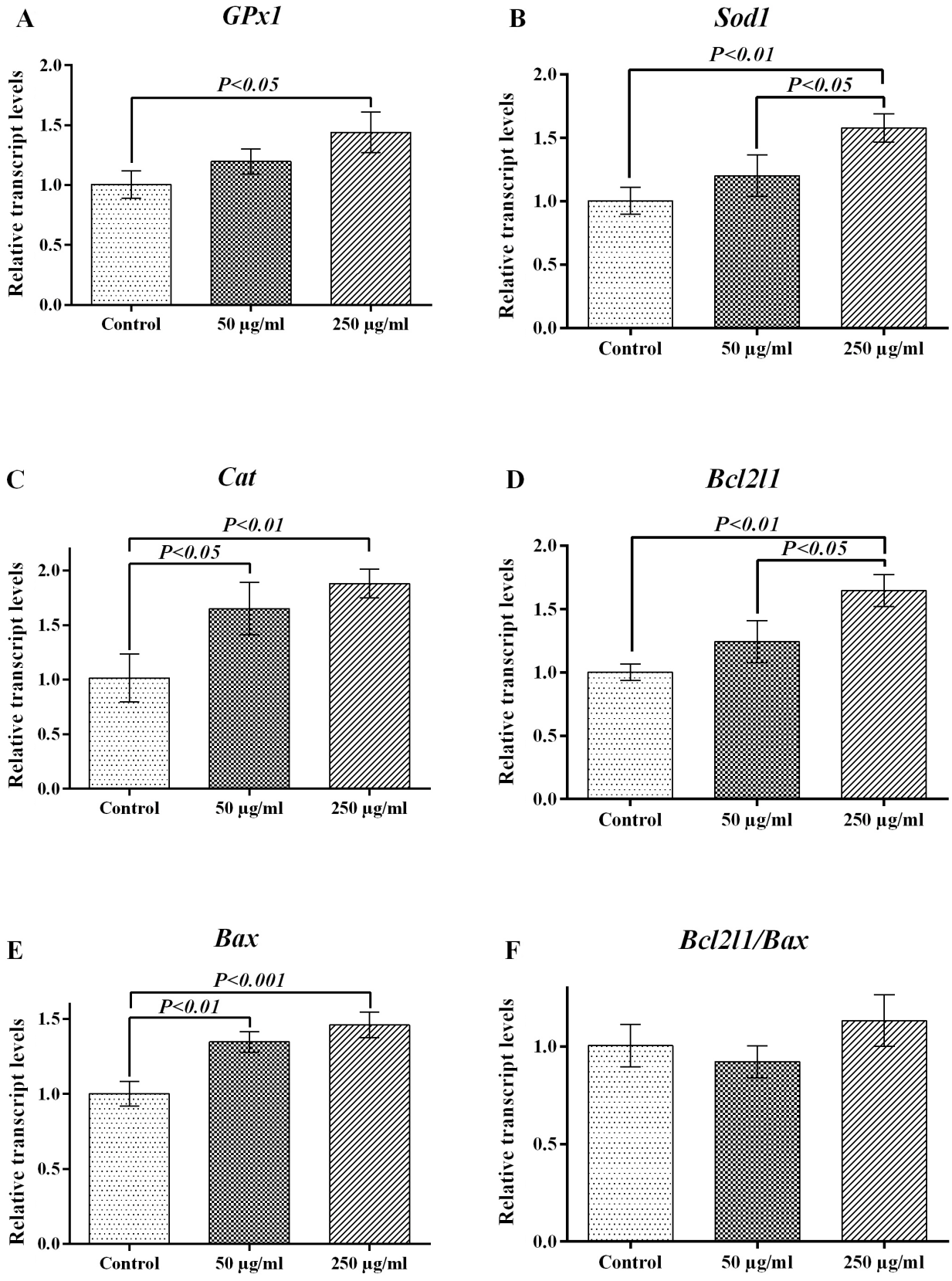

Fig 4: Addition of dextran-coated superparamagnetic iron oxide nanoparticles (D-SPIONs) to the *in vitro* fertilization medium alters gene expression of antioxidant enzymes and apoptotic genes in resultant expanded blastocysts. **A.** Glutathione peroxidase 1 (*GPx1*), **B.** Superoxide dismutase 1 (*Sod1*), and **C.** Catalase (*Cat*) are the main antioxidant enzymes. **D.** *Bcl2l1* and **E.** *Bax* are apoptosis inhibitor and activator, respectively, and **F.** Is ratio of *Bcl2l1* to *Bax*.

ml of D-SPIONs group, respectively) compared to the control group (1.00 ± 0.05), the ratio of Bcl2l1 to Bax was not significantly different among the groups (P>0.05).

Discussion

Despite the potential benefits of nanoparticles, some literature has demonstrated that nanoparticles might have negative impacts on biological systems based on their size and properties (25). Although several surface modifications have been applied to make these nanoparticles more biocompatible, their potential toxic effects remain a matter of concern. The purpose of this investigation was to evaluate the interaction of D-SPIONs and sperm, oocyte and granulosa cells, as well as the probability of changes in oxidative stress enzymes and apoptotic genes in resultant blastocysts in a dose-dependent manner (50 and 250 µg/ml) in an *in vitro* mouse model.

TEM in the midpiece sperm, which is responsible for the vigor of sperm, revealed that D-SPIONs destroyed most of the spermatozoa mitochondria and membranes at high dose (250 µg/ml), while a few of the sperm mitochondria and no membranes were affected by low dose (50 µg/ml) D-SPIONs. Jeng and Swanson (26) also revealed that high concentration of SPIONs had a negative e-ect on mitochondrial function. Oral administration of high dose (200 mg/kg/day) polyvinyl pyrrolidone-coated silver nanoparticles could induce adverse effects on sperm morphology (18). It has been indicated that swelling of the midpiece and mitochondrial enlargement led to a disruption in redox metabolism, enhancement of ROS generation and induction of apoptosis (27). Similar to our findings for sperm, our results indicated that disruption in oocyte and granulosa cell was directly correlated with dose-dependent increases in D-SPIONs. Liu et al. (28) reported that calcium phosphate nanoparticles could penetrate human granulosa cells, and enter lysosome and mitochondria. Likewise, Courbiere et al. (29) in their study on the mouse oocyte, showed that cerium dioxide nanoparticles were capable of penetrating the oocyte zona pellucida, and the accumulation of nanoparticles led to in vitro toxicity. Another study found toxic effects of cerium dioxide nanoparticles on mouse spermatozoa and oocytes (30). These studies and our results contrast a previous investigation which reported that human granulosa cells (HLG-5) treated with different coatings of SPIONs showed no toxic effects and results indicated ameliorated biocompatibility properties (31). This discrepancy may be related to the kind and concentration of nanoparticles, species and experimental condition.

The results of the present study showed that exposure of sperm and oocyte to D-SPIONs for 4 hours in fertilization medium, caused a significant decrease in cleavage and blastocyst rates in a concentration-dependent manner. This finding was in agreement with a previous study showing a significant reduction in cleavage rate by treatment of fertilization culture with cerium dioxide nanoparticles (30). Hsieh et al. (32) reported adverse impacts of CdSe-core quantum dots (QDs) on mouse oocyte maturation, and fertilization and on embryo early development, but that was not the case for ZnS-coated CdSe QDs. They concluded that surface modification of CdSe-core QDs with ZnS, significantly inhibits their toxicity. In contrast, our results showed that surface modification of D-SPIONs with dextran could not effectively prevent the negative impacts of this nanoparticle. It seems that the oxidative stress response to SPIONs could be produced by at least four sources: 1. generation of ROS from the surface of this nanoparticle, 2. production of ROS via leaching iron ions from the surface degradation, 3. disrupting mitochondrial and other organelle functions, and 4. induction of cell signaling pathways which triggered the production of ROS (33). Thus, as explained above, these mechanisms, by generation of ROS, could influence fertilization. Oxidative stress not only promoted lipid peroxidation by damaging the cell membrane (34), but also induced DNA fragmentation in sperm which triggered a reduction in fertilization rate. Sperm DNA damage led to a disruption in zona pellucida binding which subsequently resulted in a low rate of fertilization (35). Furthermore, oxidative stress induced by nanoparticles is associated with DNA damage and had a negative effect on oocyte quality in mouse oocyte (29).

In our study, the levels of *GPx1*, *Sod1* and catalase transcripts as antioxidant enzymes, in the high dose group were significantly higher than that of the control group in resultant blastocysts. It has been demonstrated that *GPx1* is related to lipid peroxidation. *GPx1* and *Sod1* have an important role in the spermatozoa membrane integrity (36). Interaction between iron and some free radicals such as superoxide through the Haber - Weiss reaction leads to production of highly toxic hydroxyl radicals (12). Thus, these results may suggest that after exposing the oocyte and sperm to SPIONs, these antioxidant enzyme genes, as a ROS scavenger, significantly increased over time to protect the resultant embryos from oxidative stress. Another possible reason may be related to higher mitochondria dysfunction in the high-dose D-SPIONs group. It has been demonstrated that upregulated *GPx1* also leads to mitochondrial dysfunction, and a reduction in cellular proliferation, mitochondrial potential and ATP production. Thus, GPx1, by regulating mitochondrial function, may moderate redox-dependent cellular responses (37).

This study, surprisingly, showed a significant increase in the anti-apoptotic *Bcl2l1* gene in the high-dose D-SPIONs group when compared to the low-dose and control groups, while pro-apoptotic *Bax* gene expression in both nanoparticle groups was significantly higher than that of the control group. The *Bcl2l1/Bax* ratio in this study was not significantly different among groups. BCL-2 family proteins play an important role in regulating the mitochondrial-related apoptosis pathways; it seems that surviving blastocysts with promotion of mitochondrial antioxidant enzymes, upregulation of *Bcl2l1* and

maintenance of *Bcl2l1/Bax* ratio, prevented DNA damage and cell death. Ilani et al. (4) by IP administration of titanium dioxide nanoparticles in female mice, found that rates of fertilization and blastocysts were not affected; however, levels of *Bcl2l1* and Bax expression respectively decreased and increased by titanium dioxide nanoparticles, which may be related to the apoptotic effect of this nanoparticle in resultant blastocysts. It has been confirmed that *Bcl2l1* prevents apoptosis by binding to the BH3 domains of BAX and BAK1 to prevent their activation (38), therefore, probably in response to *Bax* overexpression, the amount of *Bcl2l1* increased to inhibit apoptotic effects of Bax.

Conclusion

This study, for the first time, found that despite massive use of D-SPIONs in various fields of science such as medicine, considerable concern exists regarding their toxicity towards IVF, and mitochondrial and cell membrane damage in mouse spermatozoa and oocytes, as well as overexpression in oxidative enzymes and apoptotic genes in the resultant blastocysts. Therefore, it is beneficial to examine possible toxicity of this nanoparticle before its application in various fields of nanotechnology. Future studies are needed to understand more details about the mechanisms and molecular pathways of interaction between D-SPIONs and reproductive cell damage. It is also essential to evaluate its biocompatibility and possible toxic effects on other cells, tissues and organs.

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Authors' Contributions

S.A.; Designed the experimental study. A.B., S.N.; Carried out the oocyte and sperm collection and IVF. A.B., E.K.A.; Collected the experimental data. A.B.; Carried out the gene expression and statistical analysis. E.K.A.; Performed TEM analysis. A.B., E.K.A., F.M.; Interpreted the data. A.B., S.A.; Wrote the first draft of the manuscript. E.M., M.J.M.; Synthetized and completed the characterization of D-SPIONs nanoparticle. All authors read and approved the final manuscript.

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Original Article

The Effect of N-Acetyl-Cysteine on *NRF2* Antioxidant Gene Expression in Asthenoteratozoospermia Men: A Clinical Trial Study

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Abstract

Background: One of the important factor associated with male infertility is high production of reactive oxygen species (ROS). The main function of Nuclear factor erythroid 2-related factor 2 (*NRF*2) is to activate the cellular antioxidant response by inducing the transcription of a wide array of genes that can combat the harmful effects of factors such as oxidative stress. The purpose of this study was to evaluate the effect of N-acetyl-L-cysteine (NAC), as an antioxidant drug, on NRF2 Gene Expression in Asthenoteratozoospermia Men.

Materials and Methods: In this randomized, blinded clinical trial study, included 50 infertile men with asthenoteratozoospermia, who received NAC (600 mg, three times daily). Sperm parameters analyzed according to the world health organization (WHO; 2010). Sperm DNA fragmentation, relative *NRF*2 expression, and seminal plasma level of antioxidant enzymes were measured by TUNEL assay, reverse transcription polymerase chain reaction (RT-PCR) and ELISA test, respectively.

Results: After NAC treatment, findings showed a significant increase in sperm concentration and motility compared to pre-treatment status, whereas the percentage of abnormal morphology and DNA fragmentation was significantly decreased ($P<0.05$). A significant improvement in expression of *NRF*2 gene and antioxidant enzyme levels were observed compared to pre-treatment by NAC ($P<0.05$). Significant correlations were observed between NRF2 mRNA expression level, specific sperm parameters and level of antioxidant enzymes ($P<0.05$).

Conclusion: The results demonstrated that NAC oral supplementation protected against oxidative stress by enhancing *NRF*2 expression. This could improve semen parameters quality parameters in asthenoteratozoospermia men (Registration number: IRCT20170830035998N4).

Keywords: Factor Erythroid 2-Related Factor 2, Nuclear Asthenoteratozoospermia, N-Acetyl-Cysteine, Oxidative Stress

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Introduction

One of the main causes of infertility in men is oxidative stress or high production of reactive oxygen species (ROS). It can also be provoked from reduced antioxidant capacity of semen and spermatozoa creating the conditions termed oxidative stress (1). Oxidative stress contributes to damage to various sperm parameters such as sperm morphology, sperm count and sperm DNA fragmentation associated with reducing fertility (2). Although, low amounts of ROS is essential for physiological and functional processes (such as acrosome reaction, capacitation and perm-oocyte penetration), excessive production of ROS can negatively impact the sperm quality and subsequently hampers fertility (3). Naturally, excessive production of ROS is counterbalanced by enzymatic and non-enzymatic antioxidants present in male reproductive tract (4). Production of antioxidant enzymes are regulated by a common regulatory factor-like nuclear factor erythroid 2-related factor 2 (*NRF2*) (5). *NRF2* regulates gene transcriptions containing antioxidant response elements (AREs) (6) like catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX).

In normal conditions, *NRF*2 is repressed by the negative regulator protein Keap1, largely localized in the cytoplasm. In this condition, *NRF2* is targeted by ubiquitination and proteasome degradation. Under oxidative stress condition *NRF2* is phosphorylated. This phenomenon disrupts formation of the Keap1- *NRF2* complex. Subsequently, *NRF2* is translocated in the nucleus and the level of enzymes containing this regulatory element is up-regulated (7).

NAC is derived from amino acid L-cysteine containing sulfhydryl groups that has free radical scavenging activ-

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ity (8-10). Therefore, it is supplemented to alleviate glutathione (GSH) depletion during oxidative stress. Despite the well-known antioxidant capacity of NAC in different oxidative stress conditions (including male infertility) the correlation between NAC-induced oxidative protections and signaling transduction pathway remains to be elucidated (11-12).

Therefore, we investigated expression of *NRF2* in the sperm of asthenoteratozoospermia individuals treated with NAC. In addition, we studied relationship of *NRF2* expression with protein level of antioxidant enzymes, including CAT, SOD and GPX.

Materials and Methods

A randomized, blinded clinical trial was designed for this study. A total of 50 infertile men with idiopathic asthenoteratozoospermia, at the age of 25 to 40 years old, were enrolled. Patients were referred to ACECR Infertility Research Center (Qom, Iran) from July 2018 to November 2018. None of the infertile couples had previously achieved pregnancy.

Inclusion criteria were infertile men with no history of varicocele, obstruction, cancer and chemotherapy as well as abnormal testes, leukospermia, cigarette smoking and alcohol consumption. Infertile patients were considered as male individuals with "asthenoteratozoospermia", according to the world health organization (WHO) guidelines (13). A normal female partner was defined as a woman with regular menses, normal hormonal profile and hysterosalpigogram. The male individuals were defined as asthenozoospermic, if their total sperm motility was below 40% and/or their progressive motility was below 32%. Most of our participants had absolute asthenozoospermia and both parameters were below the WHO criteria. During this study, the patients received NAC (600 mg daily, for three months). Variables sperm parameters, DNA fragmentation index, *NRF*2 gene expression and level of the antioxidant enzymes in seminal plasma were measured before and after intervention.

Semen analysis and preparation

Sperm analysis was performed according to the WHO guidelines criteria, 2010 (14). All Semen samples were collected by masturbation after 3-4 days of abstinence and allowed to liquefy for 15-30 minutes at room temperate. Total and progressive motility were analyzed using the computer-aided sperm analysis (CASA) system (LABOMED, SDC313B, and Germany). Sperm morphology was stained with Papanicolaou and 200 sperms were evaluated per slide (15). Sperm number was counted by a sperm counting chamber and expressed as million/ml. Samples with more than 1 million leukocytes in 1 ml of semen were excluded from the study. Semen samples were washed by Ham's F-10 solution. The resulting sperm pellet was divided into several aliquot parts and they were kept frozen at -80ºC for subsequent analyses of RNA and biochemical factor levels.

Assessment of DNA fragmentation (TUNEL assay)

Sperm DNA fragmentation analysis was determined using the in-situ cell death detection kit (Roche, Germany) based on the labeling of DNA strand breaks (TUNEL technology) (16). At least 200 stained sperms per field were assessed under an epifluorescent microscope (BX51, Olympus, Japan) at ×100 magnification. Percentage of the sperms with DNA-damaged was considered as number of TUNEL-positive (green fluorescence) and percentage of the sperms with intact DNA was considered as number of TUNEL-negative (red fluorescence).

Assessment of *NRF*2 by reverse transcription–polymerase chain reaction

After complete liquefaction, the cells in 1 ml of every sample were pelleted by centrifugation (6000 rpm). Total cellular RNA extraction was performed by using RNeasy Plus Micro Kit (Qiagen, Germany) according to the manufacturer's instruction.

To remove DNA contamination, the extracted RNA samples were treated with DNase I. cDNA was reverse transcribed from 2µg of total RNA using M-MLV reverse transcriptase (Fermentase Corporation, Lithuania) and the corresponding oligonucleotide primers. Polymerase chain reaction (PCR) was carried out using 2µg cDNA specific primers for the both *GAPDH* and *NRF*2 genes (Table 1).

Table 1: Primers used for RT-PCR analysis

Transcript	Sequence (5'-3')	Length of DNA product (bp)
GAPDH	F:TGGCTACAGCAACAGGGTG R: CTCTTGTGCTCTTGCTGGG	104
NRF2	F:AGCACATCCAGTCAGAAACC R:TAGCCGAAGAAACCTCATTG	203

Real-time PCR program consisted of enzyme activation at 95°C for 30 seconds, followed by 40 cycles of a two-step program, including template denaturation at 95°C (5 seconds) and annealing/extension at 58°C (30 seconds). The PCR product sizes were 203bp for *NRF*2 and 104bp for GAPDH. The $2^{-\Delta\Delta Ct}$ method was calculated to represent the relative quantification of mRNA expression of *NRF2* after normalization to that of GAPDH, where ΔCT= (CT, *NRF2* antioxidant genes-CT, *GAPDH*).

Assessment of semen biochemical factors

For the biochemical factors analysis, we separated seminal plasma and stored it at -80°C until use. Total antioxidant capacity (TAC) and Malondialdehyde (MAD) of the plasma for all samples were measured using the commercial kits (Zell Bio GmbH, Wurttemberg and Germany). The level of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) was assessed by ELIZA kit (Abnova Corporation, Taiwan).

Statistical analysis

The statistical software SPSS (Version 20, USA) was used for data analysis. Data are presented as mean ±

standard error of the mean (SEM). The paired sample t-test was used for comparison of the samples before and after NAC treatment. Correlation between different variables was studied using the Pearson correlation coefficient. A P<0.05 was considered statistically significant.

Ethical considerations

This clinical trial study was registered in the Iranian Registry of clinical trials (Registration number: IRCT20170830035998N4) and it was approved by the Ethics Committee for Research Involving Human Subjects at Science and Research Branch of Azad Medical University (Tehran, Iran). An informed consent was obtained from each participant and this study was in continuation of previous study (17).

Results

Effect of N-acetyl-L-cysteine treatment on sperm parameters

Sperm concentration, sperm motility (total and progressive motilities), sperm morphology were significantly different at end of the study (Fig. 1). After NAC supplementation, mean sperm concentration and percentage of motile sperm were significantly increased compared to the samples before NAC treatment (P<0.05). The results showed significant improvement in the samples with abnormal morphology (P<0.05). Additionally, significant improvement was observed in sperm DNA fragmentation after treatment by NAC (P<0.01).

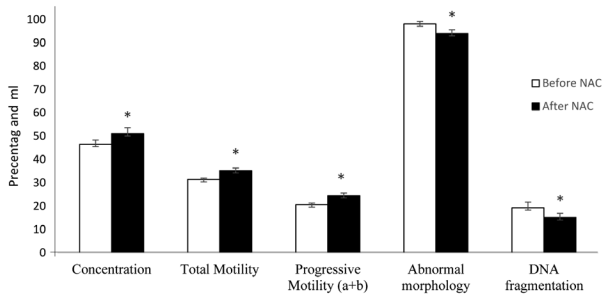

Fig 1: Comparison of sperm parameters before and after NAC treatment. *; significant difference before and after treatment, and NAC; N-acetyl-cysteine.

Effect of N-acetyl-L-cysteine treatment on *NRF2* gene expression

To explore role of NAC in regulating the expressions of *NRF2*, we analyzed relative expression of *NRF2* gene in sperm cells using RT-PCR method. As shown in Figure 2, expression of *NRF2* gene after treatment was significantly higher than before treatment. The results indicated that after intervention, NAC significantly increased *NRF2* expression level (1.00 ± 0.14 vs. 1.79 ± 0.18 respectively, P=0.01).

Effect of N-acetyl-L-cysteine tr eatment on biochemical factors

A higher level of TAC on seminal plasma was observed after NAC supplementation. Moreover, the level of MDA on seminal plasma was significantly lower in infertile men after treatment with NAC compared to with before treatment with NAC (P<0.05). In addition, the results demonstrated that CAT, GPX and SOD levels were significantly increased in NAC treated group (P<0.05, Table 2).

Correlation analysis showed that *NRF2* mRNA expression was correlated with sperm parameters (sperm abnormality, total motility and DNA fragmentation). Additionally, *NRF2* gene expression was negatively correlated with MDA, while it was positively correlated with seminal plasma TAC and other antioxidant enzymes levels (including CAT, SOD and GPX) were detected both before and after NAC treatment (P<0.05 for all tests, Table 3).

Table 2: Comparison of biochemical factor before and after NAC

Biochemical factors	Before NAC (n=50)	After NAC (n=50)	P value
TAC(μM)	1.82 ± 0.11	2.51 ± 0.13	0.01*
MDA(μM)	2.36 ± 0.10	1.97 ± 0.09	0.01*
CAT(U/ml)	13.44 ± 2.63	18.04 ± 1.79	0.005*
SOD(U/ml)	0.14 ± .014	0.18 ± .006	0.01*
GPX(U/ml)	344 ± 12.68	378 ± 13.25	0.04*

Data are shown as mean ± SD, *; Significant differences between before and after NAC treatment, TAC; Total antioxidant capacity, CAT; Catalase, SOD; Superoxide Dismutase, GPX; Glutathione Peroxidase, MDA; Malondialdehyde, and NAC; N-acetylcysteine.

Table 3: Correlations between NRF2 mRNA level, sperm parameters and level of antioxidant enzymes before and after NAC

Correlations	*NRF2*	
	r	P value
Sperm abnormal morphology (%)		
Before NAC	-0.436	**0.02**
After NAC	-0.473	**0.01**
Total Motility (%)		
Before NAC	0.399	**0.04**
After NAC	0.499	**0.01**
DFI (%)		
Before NAC	-0.389	**0.05**
After NAC	-0.430	**0.03**
MDA(μM)		
Before NAC	-0.441	**0.001**
After NAC	-0.438	**0.001**
TAC (μM)		
Before NAC	0.488	**0.05**
After NAC	0.408	**0.02**
CAT(U/ml)		
Before NAC	0.226	**0.05**
After NAC	0.326	**0.03**
SOD(U/ml)		
Before NAC	0.664	**0.01**
After NAC	0.815	**0.000**
GPX(U/ml)		
Before NAC	0.194	0.094
After NAC	0.255	**0.05**

CAT: Catalase; DFI: DNA Fragmentation Index; GPX: Glutathione peroxidase; MDA: Malondialdehyde; NAC: N-acetylcysteine; NRF2: Nuclear factor erythroid 2-related factor 2; SOD: Superoxide dismutase; TAC: Total antioxidant capacity, and significant differences in bold.

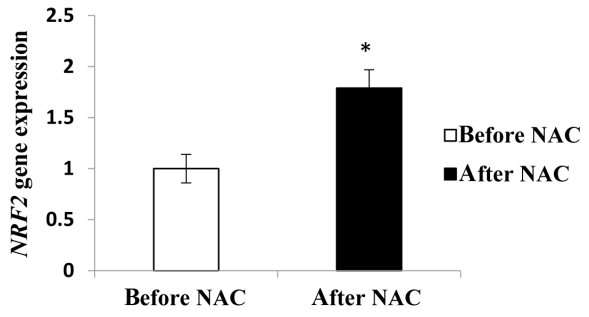

Fig 2: Comparison of relative expression of NRF2 before and after NAC treatment. NAC; N-acetyl-cysteine, NRF2; Nuclear factor erythroid 2-related factor 2, and *; Significant difference before and after NAC treatment.

Discussion

The presence large number of mRNAs in human spermatozoa may effect on the events of spermatogenesis and sperm quality (18). Correlation between sperm quality and mRNA expression has previously been investigated in animals (19). Therefore Analysis of testicular genes may be an essential marker to study the role of antioxidant genes in spermatogenesis and diagnosis of male infertility.

The main results of our study revealed the role of *NRF2* gene on sperm quality through NAC supplementation *in vivo*. Enhancement of *NRF* 2 gene expression by NAC may account for the improved antioxidant capacity induced by NAC. NAC is a thiol compound which can provide sulfhydryl substance. It should be taken into account that NAC has antioxidant properties. It acts via increasing the intra-cellular concentration of cysteine/GSH and scavenging free radical (20, 21). GSH plays important role in physiological functions and protection against oxidative stress (22, 23). NAC, a known antioxidant drug, can protect cells from oxidative stress through regulating *NRF* 2 signaling pathway by regulating GSH synthesis and maintaining the level of GSH in cells (24, 25).

Our results showed a significant improvement in the sperm parameters after 12 weeks treatment with NAC, compared to the pre-treatment baseline. The results of this study revealed that there was a relationship between *NRF* 2 mRNA levels and specific sperm functional parameters including, (motility, abnormal morphology and DNA fragmentation) after NAC treatment. Excessive oxidative stress directly contributed to the damage of sperm DNA by initiating apoptosis via inducing caspase-mediated enzymatic degradation of sperm DNA (26). Antioxidant administration, such as NAC, may help decrease ROS and improve sperm DNA fragmentation (27,28). A significant correlation was observed with *NRF2* mRNA expressions and sperm quality showed that the effect of NAC on sperm parameters might be mediated through *NRF*2. Several studies determined low sperm quality in humans associated with abnormal mRNA content of the certain gene (29). Yu et al. (30) showed that functional discrepancy in the *NRF* 2 gene promoter was correlated with abnormal spermatogenesis in humans. Previous studies showed that long term cigarette smoking can cause male infertility through inhibiting *NRF* 2 gene expression and sperm DNA fragmentation (31). Therefore, disruption of *NRF* 2 mRNA level might be one of the molecular signaling pathways of disruptive sperm function.

Defect in expression of *NRF2* transcription factor is known to be critical in regulating the major determinants of the defense system against oxidative stress leading to harmful effects (32, 33). Results from the recent study demonstrated that mouse testes germ cell and Leydig cell were protected from oxidative stress in the process of heat treated-induced oxidative stress by activation of *NRF2* (34). In presence of oxidative stress, *NRF2* releases Keap1-mediated repression and is translocated to the nucleus. In addition, it binds to ARE located in the promoter of many antioxidant enzymes and activates the expression of ARE-dependent genes (35, 36). NAC acts to reduce glutathione (GSH) precursor and increasing of glutathione reductase (GR) levels by up-regulation of NRF2 expression, attenuating the ability to scavenge free radicals and oxidative stress damage (37). In this study, NAC administration increased TAC and decreased MDA levels in seminal plasma. These effects of NAC are consistent with the results obtained from previous study, indicating that NAC could improve lipid metabolism through *NRF2* signaling pathway in patients with renal ischemia/reperfusion injury (38).

The obtained negative correlation between *NRF* 2 gene expression and MDA, in addition to the positive correlation of this gene expression with TAC suggests a possible associating effect. Previous studies reported that *NRF* 2-knockout mouse had low total antioxidants levels as well as high testicular and epididymal lipid peroxidation (MDA) levels which resulted in lower sperm motility than normal males 6). According to our results, NAC significantly increased level of the antioxidant enzymes such as CAT, SOD and GPX. It was declared that there is direct correlation between *NRF*2 gene expression and antioxidant enzyme levels (CAT, SOD and GPX) in seminal plasma. In fact, role of *NRF* 2 is to maintain homeostasis between oxidative stress and antioxidant system (37).

In contrast to these results, several studies confirmed that *NRF* 2 knockout decreased antioxidant genes expression and increased oxidative injury in mouse, indicating that the *NRF* 2/ARE pathway is a key regulator of the body's redox state. It was reported that activity of many antioxidant enzymes (e.g. SOD and CAT) decreased in *NRF2*-/- mouse (39). Therefore, men with low sperm quality are likely to decrease *NRF* 2 mRNA and level of antioxidant enzymes. These correlations were further improved after NAC.

Conclusion

In the present study, we observed beneficial effect of NAC, which improves sperm parameters, decreases MDA production and increases antioxidant enzyme levels, in addition to increasing *NRF*2 levels. Accordingly, normal human spermatogenesis requires an integrated antioxidant capability as reduced antioxidant enzyme levels may be attributed with defective sperm function. Thus, antioxidant therapy, such as NAC, may induce sperm

function by up-regulating *NRF*2 expression level.

Acknowledgements

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Authors' Contributions

R.J., M.N., K.P., N.H.; Contributed to prepare concept, design and draft the manuscript. Registration in IRCT, ethical committee approval, data collection and statistical analysis was carried out by R.J. All authors approved the final version of the manuscript.

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Original Article

Effect of Human Testicular Cells Conditioned Medium on *In Vitro* Maturation and Morphology of Mouse Oocytes

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Abstract

Background: Testicular cell conditioned medium (TCCM) has been shown to induce female germ cell development *in vitro* from embryonic stem cells (ESCs). Testicular cells (TCs) secrete a variety of growth factors such as growth differentiation factor-9 (GDF-9), bone morphogenetic protein 4 (BMP-4), stem cell factor (SCF), leukemia inhibitory factor (LIF), and other, that could improve oocyte maturation. Here we have investigated the effect of human TCCM (hTCCM) on *in vitro* maturation (IVM) and morphology of mouse oocytes.

Materials and Methods: In this experimental study, 360 germinal vesicle (GV) oocytes were obtained from NMRI mice, aged 4-6 weeks that had received 5 IU pregnant mare's serum gonadotropin (PMSG) 48 hours before. GV oocytes were subjected to IVM. 120 GV oocytes were cultured in each medium; hTCCM as the test group, DMEM + 20%FBS as the control group and Ham's F10 + HFF medium as the sham group. The rates of the IVM and perivitelline space (PVS) changes were recorded at 8, 16 and 24 hours after culture. The metaphase II (MII) oocytes were subjected for *in vitro* fertilization (IVF) and the fertilization rate was evaluated after 1, 2, and 3 days.

Results: There was a significant difference between the maturation rates in hTCCM (31.67% MII) and the control [0% MII, $P<0.05$, (7.5% MI, 52.5% deg. and 40%GV)] groups but there was not a significant difference between the maturation rates in hTCCM and the sham group (53.33% MII, $P>0.05$). IVF success rate for MII oocytes obtained from IVM in the hTCCM group was 28.94% (n=11). Our data showed that hTCCM is an effective medium for GV oocyte growth and maturation compared to the control medium.

Conclusion: Our findings show that TCCM supports oocyte IVM in mice and affect oocyte morphology.

Keywords: Conditioned Medium, *In Vitro* Fertilization, *In Vitro* Maturation, Perivitelline Space, Testicular Sperm Extraction

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Introduction

The last 40 years has witnessed major improvements in curing infertility using assisted conception procedures such as hormonal induction of ovulation; *in vitro* fertilization (IVF), embryo transfer (ET), intracytoplasmic sperm injection (ICSI), and gamete and embryo vitrification. For many patients, the assisted reproductive technologies (ART) can be helpful to treat infertility. Nevertheless, a failure in germ cell development, which is mainly caused by age, disease or a toxic therapy (e.g. chemotherapy), providing an actual treatment is often difficult for the clinicians (1). *In vitro* maturation (IVM) of germinal vesicle (GV) oocytes is an effective method to supply mature oocytes. This method, as a helpful treatment for infertility, is

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of higher up importance for ART. IVM is a cost-effective and simple treatment for certain infertile couples with few side effects for gonadotropin stimulation (2).

Successful pregnancy and live birth rates fter intracytoplasmic sperm injection of *in vitro* matured GV oocytes was originally reported in 1996 (3). The main challenge in IVM is the preparation of an adequate medium, which provides the most similar microenvironment to the in vivo condition (4, 5). Recent studies have shown that the conditioned medium (CM) from mesenchymal and embryonic stem cells (MSCs and ESCs, respectively) used for IVM, can significantly improve the oocyte maturation and embryo development rates (6, 7, 8).

The preliminary data has shown the effects of testicular cell conditioned medium (TCCM) from rat testis on *in vivo* PGC differentiation of MSCs (9). Furthermore, it was reported that co-culture of ESCs derived primordial germ cell-like cells (PGCLCs) with testicular somatic cells and sequential exposure to morphogens and sex hormones mimics key marks of meiosis (10). Moreover, microarray analysis showed that sertoli cell conditioned medium (SCCM), which contains effective factors for *in vitro* germ cell differentiation could facilitate germ cell progression in human ESCs (hESCs) (11,12). All of these findings reveal the fact that TCCM contains growth factors secreted from various sources of testicular cells (13, 14) that affect PGC formation and meiotic accomplishment in spermatogenesis.

Interestingly there are other reports on the effects of TCCM on *in vitro* oogenesis using ESCs in mouse, buffalo and human (14-16). In 2006, Lacham-Kaplan and co-workers claimed the formation of artificial ovaries containing oocyte-like structures from mouse embryonic stem cells (mESCs) following culture with TCCM (14). Later, in 2016 the supportive effect of the conditioned medium from testicular cells to provide a better female germ cell developmental niche was shown using buffalo ESCs and the gene expression profile assessment of the differentiated cells (15). In summary as IVM is the final part of the *in vitro* oogenesis, these reports indicate the supportive effect of TCCM on *in vitro* oogenesis using gene expression profile assessments, which means there are supportive elements or growth factors within TCCM which can be used in IVM, too.

These findings indicate that factors secreted by testicular cells such as BMP, SCF, epidermal growth factor (EGF), insulin growth factor (IGF), growth differentiation factor-9 (GDF-9) and many others growth factors and cytokines support female germ cell development in mammals (14-15). According to previous studies, it has been proven these factors are also involved in oocyte growth and maturation (16-20), therefore we hypothesized that CM obtained from TESE-derived cell cultures may improve IVM. The present study is the first report to investigate whether human testicular cell conditioned medium (hTCCM) can improve the IVM in mice based on the reports of *in vitro* oogenesis using TCCM.

Materials and Methods

Preparation of human testicular cell conditioned medium

In this experimental study, human TCCM was collected from TESE cell cultures as explained elsewhere (21). TESE samples that contained sperm from individuals with non-obstructive azoospermia disorder were used after fully-informed patient consent. This study has 2 Ethical numbers: 1. IR.SSU.REC.1394.102 is for the testicular cell conditioned medium, and 2. IR.SSU.REC.1397.087 is for the IVM part of the project using testicular conditioned medium). After washing the TESE samples in Dulbecco's Modified Eagle Medium + 20% fetal bovine serum (DMEM + 20% FBS, Invitrogen, UK)) medium, tissue fragments were minced into small pieces mechanically with a 19-gauge needle, following enzymatic digestion and were pelleted by centrifugation for 3 minutes at 200 g. The supernatant was removed and the pellet was seeded in tissue culture flasks containing DMEM + 20% FBS medium. Collection of the CM was performed 4 days after each passage with 80-90% confluency, then filtered through 0.22-mm syringe filter and stored at -20°C (13).

Animals

The Naval Medical Research Institute (NMRI) mice from Yazd Reproductive Sciences Institute animal house were used for gamete collection for IVM and IVF. Mice were maintained on a 12 hours light/12 hours dark cycle, a temperature range of 22-25°C, 40-60% humidity and free access to food and water. All animals were treated according to the ethical guidelines provided by the Yazd Reproductive Sciences Institute Ethical Committee for animal studies.

Collection of germinal vesicle oocytes and their *in vitro* maturation

Fifteen 4-6 weeks old female mice received an injection of 5 IU pregnant mare serum gonadotropin (PMSG). At 48 hours post-injection, immature GV oocytes from the ovaries of these mice were extracted. The GV oocyte retrieval was performed by scratching the ovaries with a sterile 28-gauge needles while visualized under a stereomicroscope (Olympus, Japan; Fig. 1I, II).

GV oocytes (Fig. 1III) were individually cultured in microdrops (Fig. 1IV) of hTCCM, DMEM + 20% FBS (control group), or Ham's F10 + HFF (sham group). In total, 360 GV oocytes were used in this study (120 per group). GV oocytes were incubated at 37°C in a humidified chamber with 5% CO2 for 24 hours. Oocyte maturation, shape and PVS changes were evaluated at 8, 16, 24 hours by a stereomicroscope. Only those oocytes that displayed distinct first polar bodies were classified as metaphase II (MII) oocytes. The MII oocytes were selected for IVF and embryo development.

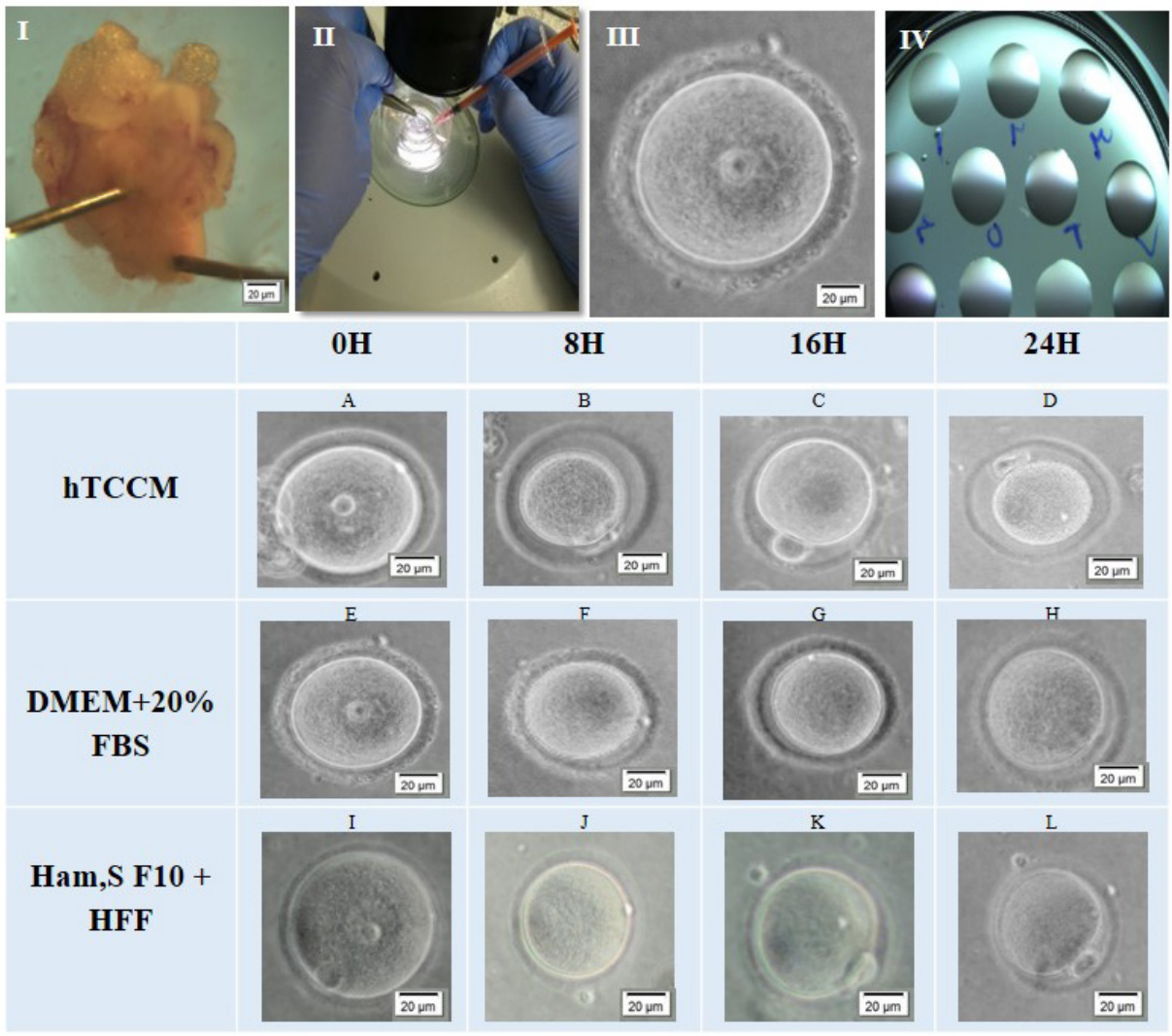

Fig 1: Egg retrieval process in mouse for IVM. **I.** First ovaries were obtained from stimulated mice. **II.** Using the stereo microscope ovary was scratched. **III.** Each individual GV oocyte was recovered and **IV.** Then cultured separately in a small microdrop for IVM. The GV oocyte within each drop was coded for the follow up and data analysis. IVM progress steps at different times in hTCCM and control and sham groups. After 8, 16 and 24 hours in hTCCM and sham condition, some of the GV oocytes **(A, I)** developed further and become mature MII oocytes **(B-D, K-L)**. Nevertheless, in DMEM + 20% FBS condition GV oocytes **(E)** could develop to MI oocytes **(F-H)** but none of them could complete the IVM process to form MII oocytes.
IVM; *In vitro* maturation, GV; Germinal vesicle, hTCCM; Human testicular cell conditioned medium, MII; Metaphase II, DMEM; Dulbecco's Modified Eagle Medium, and FBS; Fetal bovine serum.

In vitro fertilization and embryo development

The developmental potential of the oocytes that reached to the MII stage via IVM in hTCCM was evaluated by IVF. Sperms were taken from the caudal epididymis of mature NMRI males and capacitated for 1 hour at 37°C. MII oocytes were incubated with spermatozoa for 4 hours in G-IVF medium (Vitrolife, Sweden). Then, the oocytes were washed to remove extra spermatozoa and then cultured in a microdrop of G1-plus medium (Vitrolife, Sweden) at 37°C in a humidified chamber containing 5% CO_2 for three days. Their developmental stages were determined by morphological evaluations conducted every 24 hours under a stereomicroscope. Fertilization rate was scored as the number and percentage of 2-cell and 4-cell cleavage embryos observed at 24 and 48 hours after insemination.

Statistical analysis

Maturation rate, shape and PVS changes and developmental competence in mouse oocytes were evaluated for each developmental stage category and compared between the test and control groups. The data was analyzed according to the two-sample test and statistical analysis was performed using the chi-square test with R V.3.1.0 software. $P \leq 0.05$ was considered statistically significant.

Results

Effects of hTCCM on the maturation of mouse germinal vesicle oocytes

Murine GV oocytes were cultured in hTCCM (test group, Fig. 1A) and DMEM + 20% FBS (control group, Fig. 1E) and Ham's F10 + HFF medium (sham group, Fig.1I) for further IVM. The IVM of the mouse oocytes was assessed for 24 hours; specifically, at 8 (Fig. 1B, F, J), 16 (Fig. 1C, G, K), and 24 (Fig. 1D, H, L) hours. Resumption of meiosis from GV to the MII stage was considered to be oocyte IVM. Significant differences were seen in IVM

rates between hTCCM at different hours; 8.33% (8 hours), 26.67% (16 hours), and 31.67 % (24 hours) compared to the control medium group after 8, 16, and 24 hours ($P<0.05$). However, there was no significant difference in IVM rates between hTCCM at different hours compared to the sham group after 8, 16, and 24 hours: 6.66%, 25%, 53.33%, respectively ($P>0.05$). Table 1 shows the number and percentages of degenerated, MI, and MII oocytes in the test group (hTCCM), control group (DMEM + 20% FBS) and sham group at different hours.

In the three groups some of the GV oocytes were developed further to MI after 8 hours. Interestingly, the number of the degenerated oocytes (Fig. 2A) in hTCCM group (n=21) was less than the control group (n=29), but it was more than the sham group (n=9). The number of MI (Fig. 2B) oocytes in hTCCM group (n=65) was significantly higher than the control group (n=11), but it was less than the sham group (n=68). The number of the MII oocytes (Fig. 2C) in hTCCM (n=10) was also significantly higher than the control group (n=0). It was also higher than the sham group but not significantly (n=8, Table 1).

After 16 hours, IVM was checked in the three groups and as a result the degenerated oocytes in hTCCM group (n=30) were less than those in the control group (n=40), but it was more than the sham group (n=11, Fig. 2A). Interestingly, the number of MI and MII oocytes in the hTCCM group (n=48 and n=32, respectively) was higher than the control group (n=9 and n=0, respectively), but the number of MI and MII after 16 hours was in the sham group n=74 and n=30, respectively (Fig. 2B, C, Table 1). The degenerated oocytes after 24 hours in hTCCM (n=37) were less than degenerated oocytes in the control group (n=63), but it was higher than sham group (n=31) (Fig. 2A). After 24 hours almost 1/3 of oocytes progressed to MI (n=37) and MII (n=38) in the hTCCM group, whereas in the control group only 9 oocytes and in the sham group 20 oocytes developed further to MI stage. None of the GV oocytes progressed to MII stage in the control group and 64 oocytes reached to MII in the sham group (Fig. 2B, C, Table 1).

To summarize the data, the degeneration rate in the three groups increased after 24 hours. However, the ratio was lower in the hTCCM and sham groups (Fig. 2A). Moreover, all three conditions have favored further development of the GV oocytes to MI stage after 24 hours. This progress was significantly higher in hTCCM group (Fig. 2B). MI formation rates in hTCCM and sham groups have decreasing trends, which might be because of the maturation of the oocytes to MII after 24 hours (Fig. 2C). Also, in the control group MI formation decreased after 24 hours, which was either due to the degeneration or arrest between 16 and 24 hours (Fig. 2B, Table 1). It is noteworthy that the oocyte maturation to MII increased in the test group after 24 hours (Fig. 2C), which indicates the positive effect of time on oocyte IVM in hTCCM (Table 1). Despite development of oocytes to MI stage in the control group, no complete IVM to MII stage happened after 24 hours (Fig. 2C).

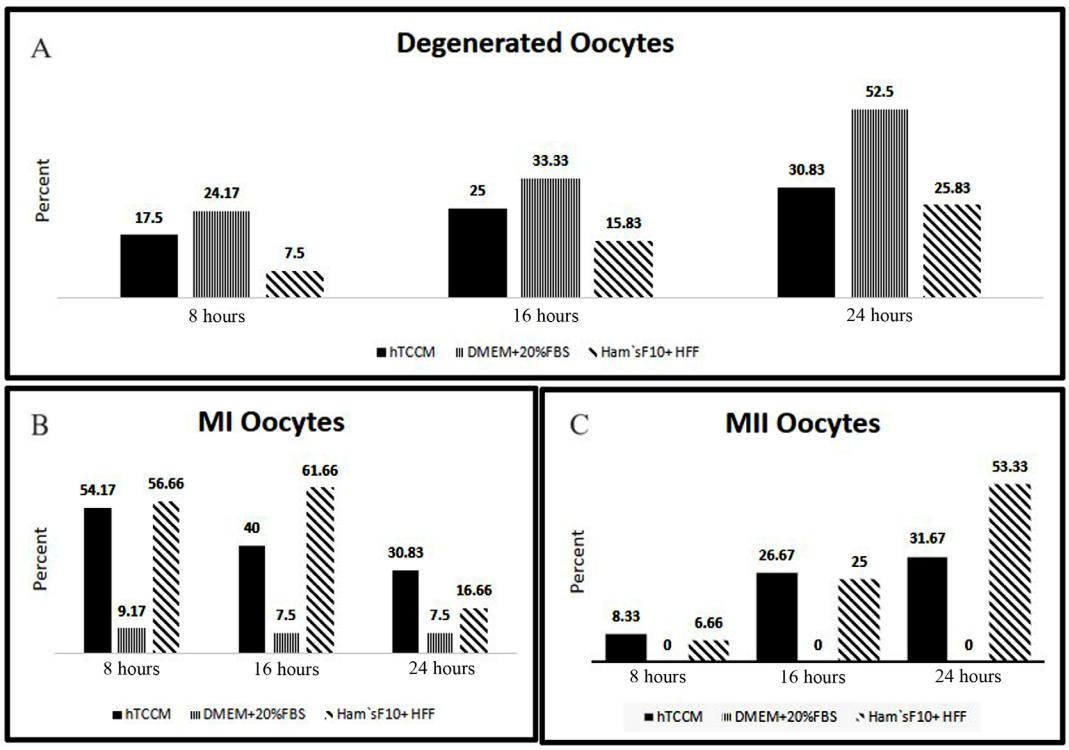

Fig 2: Rate of the degeneration, MI and MII oocytes formation in hTCCM, DMEM + 20% FBS and Ham's F10 + HFF media after 8, 16 and 24 hours. **A.** Rate of the degeneration increased in three media during 24 hours. The highest rate of the degeneration was in the control medium. **B.** In hTCCM group and Ham's F10 + HFF medium the number of MI oocytes decreased after 16 and 24 hours due to further maturation to MII oocytes. In the control group MI oocytes decreased after 16 hours due to degeneration. In the control group none of the GV oocytes developed to MII stage. **C.** hTCCM and Ham's F10 + HFF medium seem to have a supportive progressive effect on the IVM rate to MII stage after 8, 16 and 24 hours. IVM; In vitro maturation, GV; Germinal vesicle, hTCCM; Human testicular cell conditioned medium, MI; Metaphase I, MII; Metaphase II, DMEM; Dulbecco's Modified Eagle Medium, FBS; Fetal bovine serum, and HFF; Human follicle fluid.

Table 1: IVM rates at 8, 16, and 24 hours in the three groups

Group	MI n (%) In different time			MII n (%) In different time			Deg. n (%) In different time		
	8 H	16 H	24 H	8 H	16 H	24 H	8 H	16 H	24 H
hTCCM	65 (54.17)	48 (40)	37 (30.83)	10 (8.33)	32 (26.67)	38 (31.67)	21 (17.5)	30 (25)	37 (30.83)
DMEM + 20% FBS	11 (9.17)	9 (7.5)	9 (7.5)	0 (0)	0 (0)	0 (0)	29 (24.17)	40 (33.33)	63 (52.5)
Ham's F10 + HFF	68 (56.66)	74 (61.66)	20 (16.66)	8 (6.66)	30 (25)	64 (53.33)	9 (7.5)	11 (15.83)	31 (25.83)
* P value		< 0.05			<0.05			0.7	
** P value		< 0.05			0.11			0.1	
*** P value		< 0.05			<0.05			0.27	

*; P value between hTCCM and DMEM + 20% FBS, **; P value between hTCCM and Ham's F10 + HFF medium, ***; P value between Ham's F10 + HFF medium and DMEM + 20% FBS, H; Hours, n; Number, hTCCM; Human testicular cell conditioned medium, MI; Metaphase I, MII; Metaphase II, Deg.; Degenerated, DMEM; Dulbecco's Modified Eagle Medium, FBS; Fetal bovine serum, HFF; Human follicle fluid, and IVM; *In vitro* maturation.

Oocyte morphology assessment

Oocyte morphology was evaluated during IVM process under an inverted microscope and was characterized on intra and extra cytoplasmic properties. In this study, we evaluated only some extra cytoplasmic abnormalities: wide PVS and irregular shape. Our results show that there are significant differences in the rates of wide PVS and irregular shapes between the hTCCM and control groups (P<0.05, Fig. 3). Table 2 show number and percentages of oocytes that have wide/normal PVS and irregular/normal shapes following IVM in hTCCM and control groups at different time points.

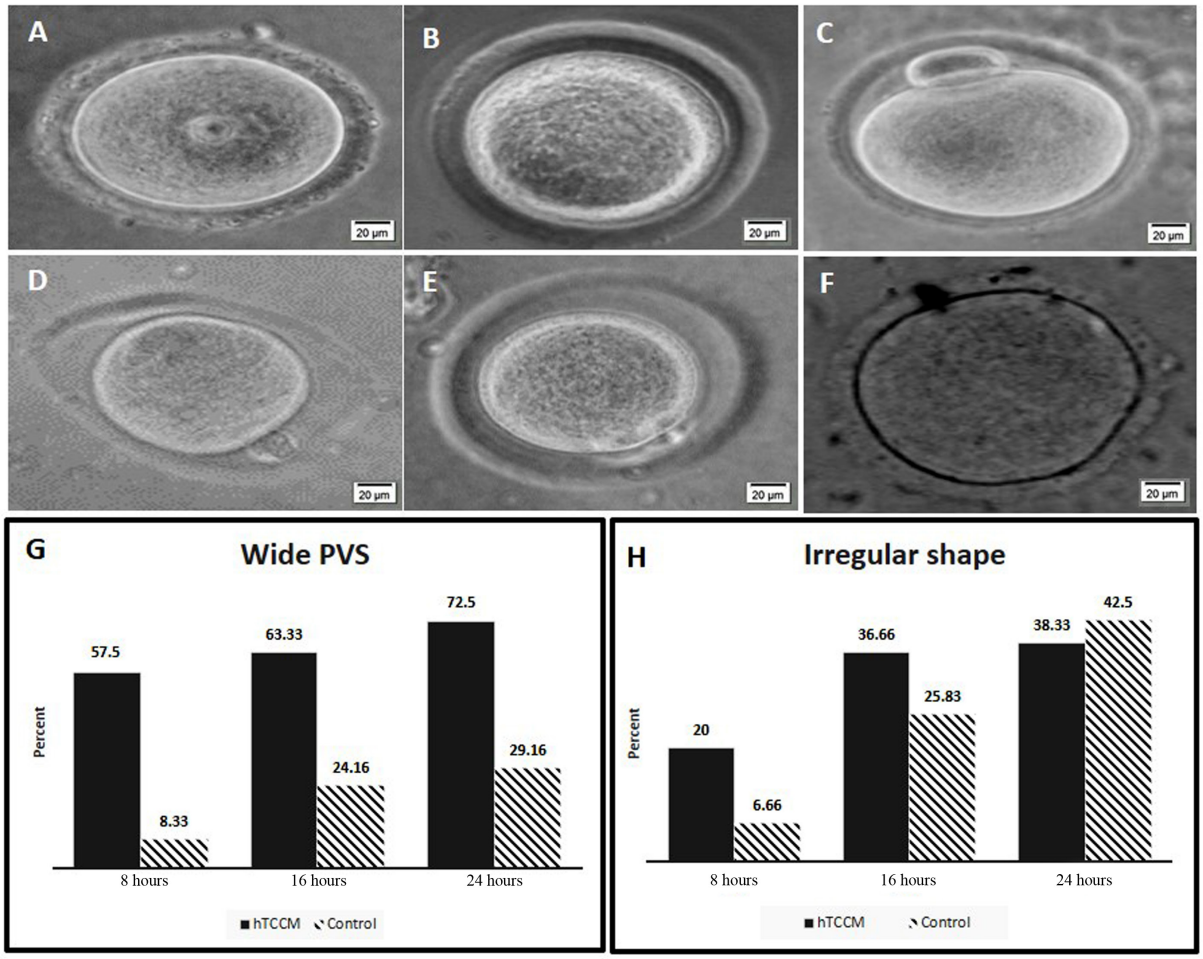

Fig 3: The oocytes shape and PVS change rates in hTCCM and control media during IVM process. **A.** Normal GV, **B.** Normal MI, **C.** Normal MII, **D, E.** Wide PVS, **F.** Irregular shape, and **G.** Rate of wide PVS oocytes formation during IVM. In hTCCM and control groups the percent of wide PVS oocytes increased after 8, 16 and 24 hours. But this increasing is higher in hTCCM medium and different is significantly. **H.** Rate of irregular oocytes formation during IVM. In hTCCM and control groups the percent of irregular oocytes formation increased after 8, 16, 24 hours and there was significant difference between two groups. PVS; Perivitelline space, IVM; *In vitro* maturation, hTCCM; Human testicular cell conditioned medium, MI; Metaphase I, and MII; Metaphase II.

Table 2: Rate of oocytes wide/normal PVS and irregular/normal shape following IVM in hTCCM and control group at different time points

Group	hTCCM			DMEM+20%FBS			P Value
	8 H	16 H	24 H	8 H	16 H	24 H	
Wide PVS n (%)	69 (57.5)	76 (63.33)	87 (72.5)	10 (8.33)	29 (24.16)	35 (29.16)	0.02*
Normal PVS n (%)	51 (42.5)	48 (36.66)	33 (27.5)	110 (91.66)	91 (78.83)	85 (70.83)	0.53
Irregular Shape n (%)	24 (20)	44 36.66)	46 (38.33)	8 (6.66)	31 (25.83)	51 (42.5)	0.02*
Normal Shape n (%)	96 (80)	76 (63.33)	74 (61.66)	112 (93.33)	89 (74.16)	69 (57.5)	0.52

*; Significant level <0.05, H; Hours, n; Number, hTCCM; Human testicular cell conditioned medium, PVS; Perivitelline space, DMEM; Dulbecco's Modified Eagle Medium, FBS; Fetal bovine serum, and IVM; *In vitro* maturation.

Embryo development following *In vitro* fertilization of *In vitro* maturation oocytes

The developmental competence of oocytes following IVM in the hTCCM group was assessed by IVF and subsequent embryo culture to the 2-cell and 4-cell stages at 24, 48 and 72 hours (Fig. 4A-C). The percentages of 2-cell embryos after 1, 2, and 3 days were 28.94, 34.21, and 28.94, respectively. Moreover, the percentages of 4-cell stage embryos after 1, 2, and 3 days were 10.52, 21.05 and 28.94, respectively.

The developmental rates of embryos to 2-cell stage embryos increased until the second day after IVF, then decreased on the third day. Instead, the number of the 4-cell stage embryos increased up to the third day after IVF (Fig. 4C).

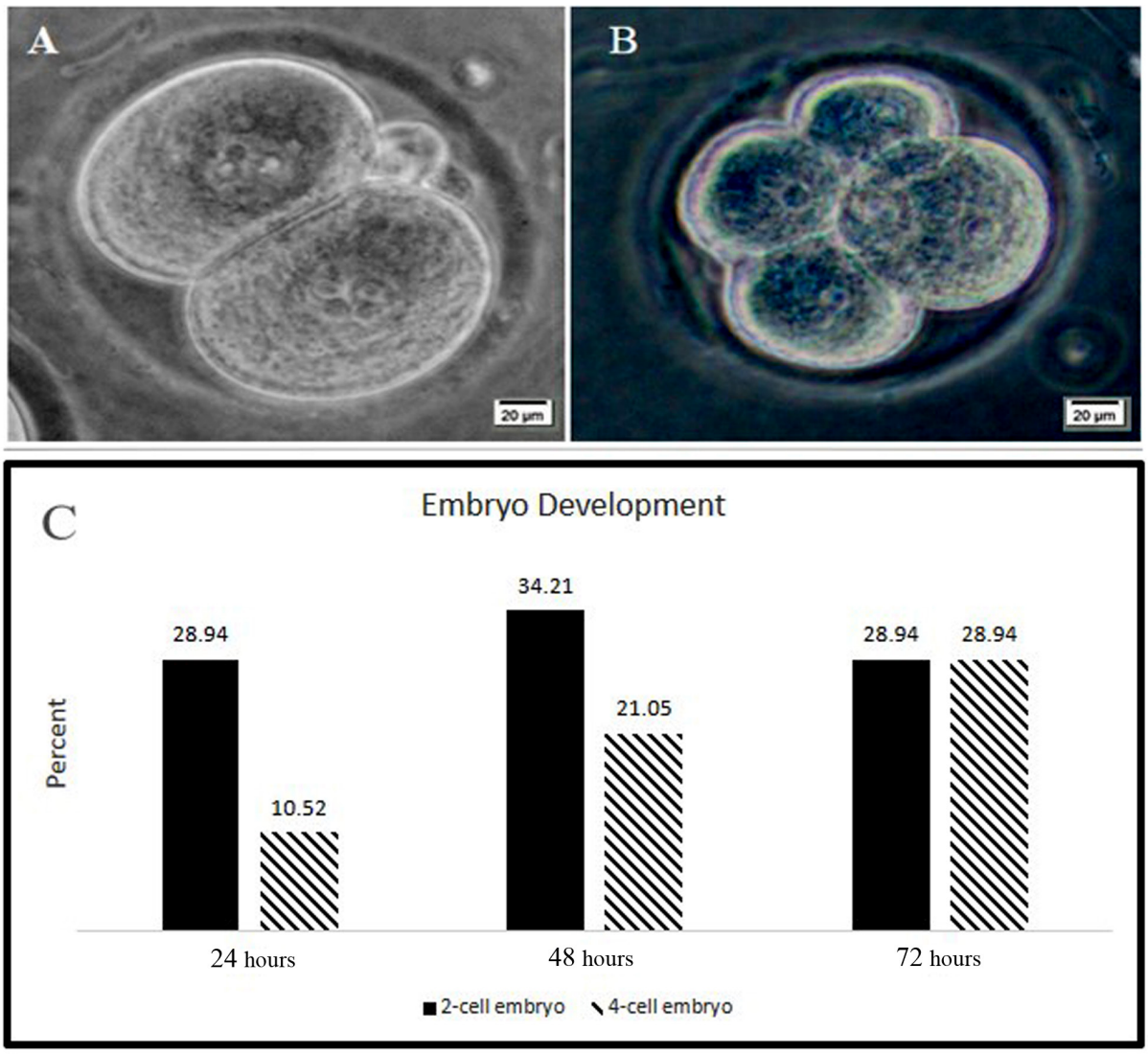

Fig 4: Embryo development following IVF of MII oocytes from the hTCCM group. **A** and **C.** Following IVF from 38 oocytes, after 24 hours, 11 of fertilized oocytes developed to 2 cell embryos and **B and C.** 4 of the oocytes become 4 cell embryos. **A** and **C.** After 48 hours, 13 embryos developed to 2-cell stage embryos and **B and C.** 8 of the MII oocytes formed 4-cells embryos. At 72 hours following IVF, 11 embryos were arrested at 2-cell stage and in total 11 embryos were observed at 4-cell stage. The embryos did not follow further but after three days none of them formed blastocyst. IVF; *In vitro* fertilization, hTCCM; Human testicular cell conditioned medium, and MII; Metaphase II.

Discussion

This study evaluated the impact of the conditioned media collected from human testicular cell cultures on IVM rates of the GV. The results of this study showed that hTCCM has positive effects on IVM (31.67% MII, n=38) of mouse immature oocytes compared to the DMEM + 20% FBS as the control group [0% MII; (7.5% MI; 52.5% deg. and 40% GV)]. Furthermore, the matured oocytes obtained by IVM in hTCCM were IVF in G-IVF medium, from which 28.94% (n=11) grew and reached to the 4-cell stage embryo in G1 medium.

Human TCs are cultured in DMEM + 20% FBS, thereby, for preparation of hTCCM, the conventional IVM medium (Sage; Cooper Surgical) and recently routine culture medium for IVM (TCM-199; Sigma, USA) were not used in our study. Consequently, DMEM + 20% FBS was used as the control medium and Ham's F10 + HFF medium was used as the sham medium. Similarly, Ling and colleagues have used DMEM, α-MEM, and HTF as the controls to investigate the effects of mesenchymal stem cell (MSC) conditioned medium on the IVM. Interestingly the rate of IVM in DMEM in their study is higher than HTF (6). Likewise, embryonic stem cell growth medium (ESGM) was used as the control to investigate the effect of the embryonic stem cells conditioned medium (ESCM) on IVM (7). We cannot precisely explain why the results of IVM in our control group (DMEM + 20% FBS) was 0%. Nevertheless, the reason for this difference might be related to the various sources of the serum that were used. In our study, DMEM was supplemented with 20% FBS, whereas Ling et al. used 10% FCS. Moreover, Ling et al. cultured immature oocytes together with granulosa cells, but we did IVM followed by denudation. The other issue might be the effect of group culture for IVM in some studies (7), though single oocyte culture for IVM was done in our study. Further studies are underway using routine standard IVM medium.

About 15% of the oocytes obtained in ovarian stimulation cycles are immature (22). The success rate of pregnancy resulting from embryos of IVM oocytes is very low compared to the embryos that are obtained from immature oocytes resulting from ovulation stimulation (23). Few studies have reported successful fertilization and embryo development from these oocytes that lead to live birth (24). Therefore, many studies have been conducted based on the selection of more suitable factors to modify the culture condition to improve the oocyte IVM efficacy in different species such as porcine (25), bovine (16), and human (26). For instance, it has been shown that by adding some growth factors such as EGF or IGF to IVM medium, the maturity rate and also embryo development rate improve significantly (26, 18, 19). The beneficial effects of EGF on IVM have been demonstrated in different species, including mice (27), humans (28) and deer (29). Similarly, it has been shown that growth and differentiation factor-9 (GDF9), support the folliculogenesis in animal research and also in human organ culture studies (20, 30, 31).

Furthermore, conditioned medium is an important culture supplement device in the IVM process. Several studies have used various types of conditioned medium to improve IVM (31, 32). Similar to our report, cross species studies have indicated the beneficial effect of conditioned medium of one species for IVM of another species (33, 34). It was verified that canine oocytes were able to effectively progress to MII while cultured in bovine cumulus oocyte complex (COC) conditioned medium (34). Similarly, conditioned medium of EC-SOD transgenic mouse embryonic fibroblasts (Tg-CMEF) supports canine oocyte IVM (33). Human bone marrow mesenchymal stem cell (hBM-MSC) conditioned medium was shown to have supportive effect for IVM of mouse oocytes (8).

It has also been shown that IVM using granulosa cell conditioned medium (GCCM) improves the MII oocyte formation rate with a higher expression of genes involved in oocyte maturity (32). Testicular cells are believed to secrete various growth factors that activate signaling pathways finally leading to gametogenesis (13). Moreover using the gene expression profile assessments of the specific markers it was reported that TCCM can support *in vitro* development of ESCs from mouse and buffalo into ovarian structures formation containing oocyte-like cells (14, 15). Thereby, TCCM contain the factors that play a role in oocyte maturation, which can be used to develop a new condition to improve IVM outcomes.

The other issues, which might have an effect on the outcome of IVM are the oocyte retrieval methods and the basal medium used for IVM (35). Here, we have used one method for oocyte retrieval. DMEM+20% FBS was used as basal medium for both control and test groups to keep the condition as consistent as possible during the study.

One of the main determinants of oocyte quality is the morphology of the oocytes, such as: PVS and shape properties (36). Some studies have verified that oocyte morphology has an important role in embryo development (37, 38). Also, it has been informed that great quality embryos are acquired following IVM if normal oocytes are used (38). Perivitelline space anomalies are among the most important abnormalities of the extra cytoplasmic component. It has been suggested that a large PVS may be related to increased oocyte degeneration (38) and lower fertilization rates (39). On the other hand, it was shown that embryo development rate was significantly higher in oocytes that had a PVS abnormality compared to the normal oocytes (40).

This report demonstrates that IVM oocytes cultured in hTCCM may achieve a better meiotic competence and a higher developmental capability than those cultured in DMEM + 20% FBS medium.

Conclusion

For the first time, our data indicated that hTCCM, which contains putative growth factors, could efficiently

improve IVM of mouse GV oocytes. The IVF/IVC of the MII oocytes was assessed for three days until formation of 4-cell stage embryos. Our findings suggest the supportive role of hTCCM in improving IVM conditions as a new insight in infertility treatments.

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Authors' Contributions

M.A., B.A.; Designed the proposal of the study. M.A.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. F.A.; Contributed to TCCM production and collection. A.K.; Contributed to animal lab experiments. B.A., M.D.A., S.M.S.; were responsible for overall supervision. M.A.; Drafted the manuscript, which was revised by B.A. All authors read and approved the final manuscript.

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Original Article

Associations of Major Dietary Patterns and Dietary Diversity Score with Semen Parameters: A Cross-Sectional Study in Iranian Infertile Men

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Abstract

Background: This cross-sectional study pointed to assess the relationship between major dietary patterns and dietary diversity score with semen parameters, in infertile Iranian males.

Materials and Methods: In this cross-sectional study, 260 infertile men (18-55 years old) who met the inclusion criteria, entered the study. Four Semen parameters, namely sperm concentration (SC), total sperm movement (TSM), normal sperm morphology (NSM) and sperm volume were considered according to spermogram. A 168-item food frequency questionnaire (FFQ) was used to collect dietary intakes and calculate dietary diversity score. Factor analysis was used to extract dietary patterns.

Results: The following four factors were extracted: "traditional pattern", "prudent pattern", "vegetable-based pattern" and "mixed pattern". After adjusting potential confounders, those in the highest quartile of the traditional pattern had 83% less odds for abnormal concentration, compared with the first quartile (OR=0.17, 95% CI: 0.04-00.73); however, subjects in the highest quartile of this pattern had 2.69 fold higher odds for abnormal sperm volume as compared with those of the first quartile (95%Cl: 1.06-6.82). Men in the second quartile of prudent pattern had 4.36 higher odds of an abnormal sperm volume in comparison to the reference category (95%CI: 1.75-10.86), after considering potential confounders. With regard to mixed pattern, men in the second, third and fourth quartile of this pattern had respectively 85 (5%Cl: 0.03-0.76,), 86 (95%Cl: 0.02-0.75) and 83 % (95%Cl: 0.034-0.9) less odds of abnormal concentration, compared with the first quartile. Additionally, no significant association was found between dietary diversity score and sperm quality parameters.

Conclusion: Higher intake of the traditional diet was linked to lower abnormal semen concentration and poorer sperm volume. Also, the mixed diet was associated with reduced prevalence of abnormal semen concentration.

Keywords: Dietary Diversity Score, Dietary Pattern, Infertility, Spermogram

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Introduction

Infertility is described as inability of a couple to conceive within 12 months or more of regular unprotected intercourse (1). It has been a global issue and a clinical problem in recent decades affecting 15% of all couples in reproductive ages (2). A meta-analysis reported a 10.9% infertility rate in Iranian population. In approximately 40% of infertile couples, male factors are the only or a contributing reason in the inability to have a successful pregnancy(3).

Some disorders such as varicocele, anatomical or hormonal problems, genetic anomalies and infections were shown to contribute to male infertility. Moreover, circumferential factors such as air pollution, industrial chemicals, depression, alcohol use and smoking have been considered potential risk factors reducing sperm quality parameters in developed countries (4). Studies have suggested associations between semen quality and lifestyle factors, including physical activity and dietary intakes(5). According to results of human and animal studies, direct correlations exist between reactive oxygen species (ROS)

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production in spermatozoa and antioxidant status (6).

However, several studies focused on associations between food components, such as antioxidants and semen quality , and analysis of dietary pattern revealed a new and comprehensive outlook for assessment of the association between diet and the chance of chronic diseases (7). This approach has examined the influences of whole diet, instead of considering individual nutrients or foods, and could be more useful for prediction of risk factors (8). In Thai men, a western pattern was correlated with poorer sperm density and normal sperm morphology while a healthy pattern was not related to any sperm quality parameters (9). Also, a prudent pattern was correlated with a reduced risk of asthenozoospermia in Iranian males while the western pattern was related with increased odds of asthenozoospermia(10).

Dietary diversity score (DDS) is another index to evaluate the quality of diet. Recent researches suggested that, more various diets could be protective against some chronic diseases such as cancers or cardiovascular diseases. A varied diet is associated with higher intake of micronutrients and macronutrients such as fiber, vitamins and calcium, all of which being negatively associated with obesity(11). As we know, the linkage between dietary diversity score and infertility has not been studied. Furthermore, the findings of previous investigations were controversial; so, more researches especially in Middle Eastern populations are needed. It is worth noting that unlike other subfertility risk factors, diet is an adjustable factor and can be considered in counseling of infertile men. Therefore, this study was designed to assess the association of premier dietary patterns and dietary diversity score with semen parameters, in Iranian infertile male.

Materials and Methods

Participants

This cross-sectional study was performed in 2018 in Isfahan, a central province in Iran. In total, 270 participants who referred to a major infertility clinic, with primary or secondary history of infertility and aged between 18-55 years old, were selected. Before entering the study, all patients signed the consent letter. Those with history of disorders (infection, azoospermia, genital surgery or other genital diseases, anatomical disorders, or endocrinopathy), or metabolic diseases, or those receiving hormone therapy, supplements, cytotoxic drugs or immunosuppressant were not included in the study. Also, patients with history of psychiatric or physiological disease that could affect sperm quality, were not included (12). Furthermore, 10 participants with incomplete information or caloric intakes more than 4200 kcal/day were excluded from the study. Finally, 260 subjects who met the inclusion criteria entered the study. The study was ethically approved by Ethics committee of Isfahan University of Medical Sciences (IUMS), Isfahan, Iran (No.397387).

Assessment of semen parameters

After three days of abstinence, semen samples were obtained. The samples were collected into sterile containers and liquefied at 37°C for 30 minutes before analysis. Samples were analyzed according to the 5th edition of World Health Organization (WHO) laboratory manual for the evaluation of human semen. Four semen parameters including sperm concentration (SC), total sperm movement (TSM), normal sperm morphology (NSM) and sperm volume, were assessed. Evaluation of the motility of sperm was done based on WHO criteria and the motility was scored from A to D. A+B is defined as total progressive motility, C is defined as non-progressive motility, A+B+C is defined as total motility and D is defined as immotile sperm (13).

Assessment of dietary intakes

A validated 168-item food frequency questionnaire (FFQ) was used to evaluate dietary intakes of the individuals. This questionnaire has previously been shown to be valid for evaluating Iranian food intakes (14). All participants noted their average consumption of each food item in terms of the specified serving size over the past year. The specific categories were: 6 times or more a day, 3-5 times a day, 2-3 times a day, every day, 5-6 times /week, 2-4 times /week, once a week, 1-3 times a month and less than once a month. Nutritionist IV software, which is modified for Iranian meals, was used to extract dietary intakes of participants based on their FFQ.

Dietary diversity score

In this study, DDS was calculated according to the study by Kant et al.(15). In this method, food items are categorized into 5 categories: 1. bread and cereals, 2. vegetables, 3. fruits, 4. meat and substitutes, and 5. dairy products. Then, each group was divided into several subgroups. Bread and cereals were divided into seven subgroups: white bread and refined grains, whole bread branny biscuits, macaroni, breakfast cereals, rice and flour. Vegetables were classified into seven groups: fines herbs, potato, starchy vegetables, tomato, yellow vegetables, green vegetables and legumes. Fruits consisted of berries and citrus fruits, and other fruits. Meat and substitutes were divided into four subgroups including: red meat, poultry, sea food and egg. Dairy products included three groups: milk, yogurt, cheese and dried whey. Each group was given a score from 0 to 2, so the maximum score was 10. To calculate the score for each group, the whole subgroups consumed by a person were divided by total subgroups and then multiplied by 2. For example, if a participant consumed four subgroups of cereals, the score was calculated as$(4\div7)\times2=1.14$.

Assessment of other variables

A structured questionnaire was used to collect other demographic data, medical history, alcohol or cigarette use and supplements intake. Subjects were interviewed face-to-face. Weight (with accuracy of 0.1 kg) and height (with accuracy of 0.5 cm) were measured. Body mass index

(BMI) was then calculated in kilograms per meter square.

Statistical analysis

Continuous variables are reported as mean (± SD or SE). The normality of the data was assessed by using the Shapiro–Wilk test. In this study, we categorized the 168 food items into 32 food groups to facilitate the analysis. The classification was based on the nutrient profiles resemblance, or recipe of foods (10, 16). Major dietary factors were identified by using factor analysis with varimax rotation method. Considering the Eigen value >1.9 and scree plot, four factors were selected.

We applied the quartiles of major dietary patterns or DDS scores to assess the relationships between dietary intakes and sperm parameters. One-way analysis of variance (ANOVA) (or Kruskal Wallis test as a non-parametric test) or chi-square tests were used to assess general characteristics and dietary intakes of study participants across different quartiles of dietary intakes. Differences in the sperm parameters versus food intake amounts were compared using chi-square test. Abnormal semen parameters were defined as Oligospermia : SC<20 M/ml, TSM<60%, sperm volume<3 ml and NSM<65% (12). As we were not able to analyze data with NSM<65% in our study, NSM<4% as the world health organization (WHO) cut point was considered for normal morphology (13). The frequencies of abnormal semen quality parameters in each quartile, were compared by the chi-square test or fisher exact test if required. Multiple logistic regression [odds ratios (ORs) with 95% confidence interval (CI)] was used to assess the relationship between major dietary patterns and DDS and sperm quality parameters. In adjusted model, potential confounding variables including age, BMI, education, total energy intake, alcohol use, smoking and vitamin-mineral use were justified by analysis of covariance (ANCOVA). In all models, the first quartile was considered the reference level. For all analyses, SPSS software (Version 25) was used and significance level was considered P<0.05.

Results

Based on Eigen values and scree plot, we selected four factors. Factor-loading matrixes of foods and food group classification are provided in Table 1. A positive loading in a factor showed a linear association with the factor, while a negative loading shows that the food group was reversely correlated with the factor. The "traditional pattern" was defined by high intakes of organ meat, dairy products, saturated fats, fruits, fruit juice, legumes, sugary beverages, deserts and sweets. The "prudent pattern" was defined by high intakes of nuts, olive oil, red meat, dried fruits, fruit juice, fish and low intake of refined grains. The "vegetable-based" pattern was defined by high intakes of fruits, leafy vegetables, yellow vegetables, legumes, tomato and other non-starchy vegetables. The "mixed pattern" was defined by high intakes of green/black tea, vegetable oils, and potato, and low intake of whole grains and soy bean. Each participant was given scores for the traditional, prudent, vegetable-based and mixed major dietary patterns according to his/her consumption of items.

Table 1: Loadings of foods and food groups across major dietary patterns

	Component			
	1	2	3	4
Sugary beverage	0.659 *			
Organ meat	0.640			
Processed food	0.627			
Pickles	0.614			
Sauce	0.613			
Saturated fat	0.596			
Dairy products	0.521			
Fruits	0.411		0.523	
Sweets and deserts	0.512	0.446		
Snacks	0432	0.398		
Red meat		0.696		
Dried fruits		0.793		
Olive oil		0.679		
Refined grains		-0. 525		0.309
Nuts		0.511		
Fruit juice	0.422	0.475		
Fish		0.397		
Poultry			0.450	
Legumes	0.305		0.449	
Leafy vegetables		0.319	0.564	
Yellow vegetables			0.438	
Tomato			0.678	
Non starchy vegetables			0.615	
Cheese			0.428	
Skim fat dairy			0.349	
Egg			0.335	
Potato	0.345			0.403
Salt				0.625
Vegetable oils				0.505
Tea(green/black)			0.398	0.480
Soy bean				-0.385
Whole grain				-0.325

Factor loadings less than 0.3 were omitted for simplicity. Principal Component Analysis as the extraction method and Varimax with Kaiser Normalization as the rotation method, were applied. 1, 2, 3, 4; These numbers show four categories of food patterns.

Mean age, BMI and TEE of study participants and semen quality parameters are shown in Table 2. Demographic data showed that 36.5% of participants smoked cigarette and 20.3% used alcoholic drinks. Additionally, the education of 22.6% of participants was less than diploma. Dietary intakes of selected nutrients and energy intake of study participants among different quartiles of dietary patterns, are reported in Table 3. Intakes of energy (P=0.01), protein

(P=0.002), total fiber (P<0.001), eicosapentaenoic acid (EPA) (P=0.008), docosahexaenoic acid (DHA) (P=0.002), folate (P<0.001), vitamin E (P=0.001) and selenium (P<0.001) were significantly different across quartiles of traditional pattern. General characteristics and energy intake of individuals among different quartiles of DDS are shown in Table 4. The distribution of energy intake (P<0.001) and BMI (P=0.018) was different among quartiles of DDS.

To clarify major dietary patterns-semen quality relation or DDS-semen quality relation, prevalence of abnormal sperm parameters in quartiles of different dietary patterns or DDS was evaluated (Table 5). The prevalence of abnormal SC was lower in the top quartile of traditional dietary intake, compared with the bottom quartile (P=0.015). Also, the abnormal SC prevalence was reduced in the second quartile of mixed diet in comparison to the first category (P=0.041). Moreover, a significant difference in terms of abnormal sperm volume prevalence was found across categories of prudent dietary intake (P=0.035). However, there was no significant relationship between different quartiles of DDS and prevalence of abnormal semen parameters.

Multivariable- adjusted odds ratio for abnormal semen quality across quartiles of premier dietary patterns and DDS, are shown in Table 6. After adjusting potential confounders, those in the highest quartile of the traditional dietary pattern, odds were 83% less for abnormal SC, compared with the first quartile (OR=0.17, 95% confidence interval (95% CI); 0.04-00.73); however, subjects with the highest quartile of this pattern had a 2.69 fold higher odds for abnormal sperm volume as compared with the first quartile (95% Cl: 1.06-6.82). Men in the second quartile of prudent dietary pattern had 4.36 higher odds of an abnormal sperm volume in comparison to the reference category (95% CI: 1.75-10.86), after considering potential confounding variables. With regard to mixed dietary pattern, men in the second, third and fourth quartile of this pattern had respectively 85 (95% Cl: 0.03-0.76), 86 (95% Cl: 0.02-0.75) and 83 % (95% Cl: 0.034-0.9) less odds for abnormal SC, compared with the first quartile.

Furthermore, there was no significant relationship between DDS and semen parameters.

Table 2: Characteristics of participants

Characteristics	Mean ± SD
Age(Y)	31.24 ± 4.33
BMI (Kg/m^2)	26.94 ± 4.09
Energy (Kcal)	2516.51 ± 686.94
MET (MET-h/week)	29.2 ± 2.12
Sperm parameters	
Density (Mol/ml)	13.11 ± 16.01
Volume(ml)	4.13 ± 2.06
Total motility (%)	29.76 ± 18.08
Normal. Morphology (%)	4.23 ± 10.68
DDS	5.09 ± 1.29

SD; Standard deviations, BMI; Body mass index, and DDS; Dietary diversity score as assessed by Analysis of variance (n=260).

Table 3: Dietary intakes of energy and selected nutrients of study participants among different quartiles of dietary patterns

	Traditional			Prudent			Vegetable based			Mixed		
	Q1	Q4	P	Q1	Q4	P	Q1	Q4	P	Q1	Q4	P
Energy (Kcal/d)	2306 ± 678	2622 ± 605	0.01	2630 ± 732	2482 ± 658	0.39	2501 ± 612	2427.79 ± 654	0.40	2450 ± 672	2481.06 ± 631	0.42
Proteins (% of energy)	15.90 ± 2.40	14.30 ± 2.10	0.002	14.67 ± 2.44	15.28 ± 2.76	0.44	15.79 ± 2.42	14.76 ± 2.66	0.01	15.53 ± 2.49	15.02 ± 2.56	0.42
Fats (% of energy)	29.10 ± 6.40	29.70 ± 5.30	0.06	30.13 ± 5.77	31.07 ± 6.82	0.23	29.54 ± 5.84	30.72 ± 6.18	0.37	30.67 ± 6.49	29.11 ± 5.74	0.09
Carbohydrates (% of energy)	58.55 ± 8.09	59 ± 5.60	0.06	58.03 ± 6.50	57.35 ± 7.02	0.38	57.97 ± 6.95	58.01 ± 7.34	0.86	57.22 ± 7.83	58.86 ± 7.48	0.39
Total fiber (g/d)	34.30 ± 11.30	48.80 ± 2.10	<0.001	44.66 ± 17.45	39.61 ± 14.64	0.19	41.62 ± 16.98	38.46 ± 15.40	0.57	37.14 ± 14.61	43.30 ± 17.39	0.17
DHA (mg/d)	0.23 ± 0.21	0.13 ± 0.12	0.002	0.18 ± 0.17	0.22 ± 0.26	0.60	0.27 ± 0.27	0.18 ± 0.17	0.05	0.23 ± 00.24	0.20 ± 0.21	0.56
EPA (mg/d)	0.07 ± 0.07	0.03 ± 0.04	0.008	0.05 ± 0.05	0.06 ± 0.08	0.55	0.08 ± 0.09	0.05 ± 0.05	0.07	0.07 ± 0.08	0.05 ± 0.06	0.53
SFA (g/d)	26.07 ± 11.50	27.12 ± 8.80	0.38	28.05 ± 9.85	37.35 ± 12.59	0.28	26.39 ± 9.11	26.99 ± 10.46	0.30	28.19 ± 11.13	25.94 ± 10.31	0.42
Folate (mg/d)	518 ± 117	613 ± 163	<0.001	593.23 ± 177.16	541.35 ± 118.73	0.15	574.47 ± 159.23	532.66 ± 131.85	0.37	530.88 ± 111.85	567.90 ± 147.01	0.40
Zinc (g/d)	13.50 ± 4.60	14 ± 4.10	0.34	14.58 ± 4.86	14.14 ± 4.20	0.79	14.35 ± 4.45	13.53 ± 4.15	0.58	14.43 ± 4.85	13.45 ± 3.81	0.37
Selenium (g/d)	97.40 ± 28.60	131 ± 54	<0.001	131.43 ± 52.68	114.08 ± 38.67	0.01	118.48 ± 51.67	111.99 ± 38.90	0.80	102.10 ± 26.89	118.11 ± 52.18	0.02
Vitamin C (mg/d)	228 ± 134	242 ± 139	0.73	237.51 ± 138.1	244.23 ± 125.60	0.97	248.45 ± 112.62	237.71 ± 141.08	0.92	245.35 ± 147.06	241.65 ± 136.93	0.96
Vitamin E (IU)	10.70 ± 5.00	14.70 ± 5.70	0.001	14.19 ± 5.70	13.67 ± 6.32	0.41	12.62 ± 4.54	13.34 ± 5.72	0.12	12.46 ± 5.45	12.54 ± 4.41	0.01

All data presented as means ± SE. SE; Standard error, P; P value as assessed by analysis of variance (ANOVA) test, DHA; Docosahexaenoic acid, EPA; Eicosapentaenoic acid, SFA; Saturated fatty acids (n=260), Q1; first quartile of intake, and Q4; fourth quartile of intake.

Table 4: Characteristics of participants across quartile of dietary diversity score (DDS)

	Q1 (<4.2) (n=66)	Q2 (4.2-5.15) (n=64)	Q3 (5.16-5.9) (n=68)	Q4 (>5.9) (n=62)	P
Age (Y)	30.6 ± 3.70	31.16 ± 3.55	30.7 ± 4.39	32.53 ± 5.33	0.142[a]
PA (MET/h)	29.63 ± 2.03	29.23 ± 2.28	28.92 ± 2.24	29.24 ± 1.84	0.421[b]
WC (cm)	92.95 ± 8.56	93.63 ± 10.61	95.50 ± 9.74	96.01 ± 12.25	0.221[a]
BMI (kg/m^2)	25.87 ± 3.38	26.54 ± 3.82	27.39 ± 4.32	27.98 ± 4.53	0.018[b, c]
Energy intake (kcal)	2175 ± 578.10	2370 ± 610.30	2620 ± 599.80	2916 ± 737.6	<0.001[a, d]

All data presented as means ± SD. SD; Standard deviations, P; P value: [a]; Assessed by Kruskal-Wallis test, [b]; Assessed by ANOVA test, [c]; Body mass index (BMI) was significantly different between the first and forth quartile of DDS (P=0.018), [d]; The distribution of energy intake was different between the first and third, first and fourth, second and fourth quartile of DDS (P<0.001), PA; Physical activity, and WC; Waist circumference.

Table 5: The associations of abnormal semen quality with dietary patterns and DDS

Quartiles		Concentration (<20 M/ml versus ≥20)	Total motility (<60% versus≥60)	Normal morphology (<4% versus≥4)	Volume (<3 ml versus≥3)
Traditional diet	Q1	93.8	92.3	83.1	30.8
	Q2	92.3	95.4	80	21.5
	Q3	84.6	93.8	75.4	29.2
	Q4	76.9	95.4	80	33.8
	P	0.015	0.85	0.75	0.45
Prudent diet	Q1	81.5	96.9	84.6	21.5
	Q2	89.2	92.3	84.6	41.5
	Q3	86.2	96.9	76.9	30.8
	Q4	90.8	90.8	72.3	21.5
	P	0.41	0.30	0.21	0.035
Vegetable-based	Q1	90.8	93.8	83.1	21.3
	Q2	89.2	95.5	75.4	32.3
	Q3	83.1	89.2	83.1	32.3
	Q4	84.6	95.4	76.9	27.7
	P	0.51	0.15	0.57	0.60
Mixed Diet	Q1	96.5	93.8	81.5	27.7
	Q2	81.5	95.4	81.5	32.3
	Q3	83.1	90.8	81.5	32.3
	Q4	86.2	96.9	73.8	23.1
	P	0.041	0.48	0.62	0.60
DDS	Q1	89.4	97	80.3	28.8
	Q2	87.5	92.2	78.1	34.4
	Q3	86.8	95.6	88.2	26.5
	Q4	83.8	91.9	71	25.8
	P	0.83	0.52	0.10	0.70

All values are presented by percentage, P; P value as assessed by chi square test, DDS; Dietary diversity score (n=260), Q1; first quartile of intake, Q2; second quartile of intake, Q3; third quartile of intake, and Q4; fourth quartile of intake.

Table 6: Multivariable- adjusted odds ratio for abnormal semen quality across quartiles of major dietary patterns and DDS

		Concentration <20 M/ml	P	Total motility<60%	P	Normal morphology<4%	P	Volume<3 ml	P
Traditional dietary pattern									
Crude	Q1	Reference		Reference		Reference		Reference	
	Q2	0.71 (0.17-2.97)	0.64	2.17(0.43-10.78)	0.34	0.83(032-2.14)	0.70	0.78 (0.33-1.84)	0.58
	Q3	0.31 (0.08-1.17)	0.08	1.26(0.27-5.88)	0.76	0.62(0.24-1.58)	0.32	1.42 (0.62-3.26)	0.40
	Q4	0.16 (0.04-0.66)	0.01	1.57(0.28-8.84)	0.60	0.80(0.29-2.24)	0.68	2.33 (0.95-5.72)	0.06
Adjusted model	Q1	Reference		Reference		Reference		Reference	
	Q2	0.76 (0.17-3.36)	0.72	1.96 (0.35-10.96)	0.44	1.26(0.53-2.95)	0.59	0.79 (0.3-1.89)	0.6
	Q3	0.33 (0.08-1.32)	0.11	3.44 (0.50-23.25)	0.20	0.7(0.3-1.60)	0.04	1.65 (0.69-3.92)	0.25
	Q4	0.17 (0.04-0.73)	0.01	3.35 (0.44-25.43)	0.24	1.08(0.44-2.67)	0.85	2.69 (1.06-6.82)	0.03
Prudent dietary pattern									
Crude	Q1	Reference		Reference		Reference		Reference	
	Q2	0.77 (0.23-2.54)	0.67	0.38(0.06-2.53)	0.32	1.01(0.35-2.86)	0.98	4.45 (1.80-10.96)	0.001
	Q3	0.70 (0.24-2.05)	0.51	0.97(0.12-7.70)	0.98	0.59(0.23-1.56)	0.29	2.50 (1.04-6.01)	0.03
	Q4	1.97 (0.65-5.96)	0.22	0.25(0.04-1.34)	0.10	0.51(0.21-1.26)	0.14	1.20 (0.50-2.85)	0.68
Adjusted model	Q1	Reference		Reference		Reference		Reference	
	Q2	0.69 (0.2-2.38)	0.56	0.27 (0.03-2.36)	0.24	1.18(0.5-2.82)	0.69	4.36 (1.75-10.86)	0.002
	Q3	0.79 (0.24-2.57)	0.69	0.80 (0.07-8.81)	0.85	1.13(0.49-2.60)	0.76	2.39 (0.97-5.92)	0.05
	Q4	1.89 (0.59-6.08)	0.28	0.17 (0.02-1.18)	0.07	1.18(0.54-2.59)	0.66	1.13 (0.46-2.75)	0.77
Vegetable-based dietary pattern									
Crude	Q1	Reference		Reference		Reference		Reference	
	Q2	0.83 (0.24-2.87)	0.77	4.95 (0.50-48.28)	0.16	0.60 (0.24-1.48)	0.27	1.68 (0.74-3.82)	0.21
	Q3	0.50 (0.15-1.59)	0.24	0.61 (0.14-2.56)	0.50	0.90 (0.34-2.37)	0.84	1.69 (0.73-3.89)	0.21
	Q4	0.51 (0.15-1.65)	0.26	1.36 (0.27-6.63)	0.70	0.64 (0.26-1.56)	0.33	1.29 (0.56-2.95)	0.53
Adjusted model	Q1	Reference		Reference		Reference		Reference	
	Q2	0.83 (0.23-2.95)	0.77	3.73 (0.34-40.03)	0.27	0.75 (0.33-1.71)	0.50	1.69 (0.73-3.87)	0.21
	Q3	0.54 (0.16-1.83)	0.32	0.61 (0.11-3.25)	0.56	0.59 (0.26-1.34)	0.21	1.78 (0.75-4.19)	0.18
	Q4	0.49 (0.14-1.66	0.25	2.15 (0.34-13.31)	0. 40	0.73 (0.32-1.65)	0.45	1.14 (0.49-2.67)	0.74
Mixed dietary pattern									
Crude	Q1	Reference		Reference		Reference		Reference	
	Q2	0.15 (0.03-0.77)	0.02	1.28 (0.25-6.49)	0.76	1.16 (0.45-2.94)	0.75	1.26 (0.56-2.84)	0.56
	Q3	0.15 (0.03-0.74)	0.02	0.69 (0.15-3.10)	0.63	1.02 (0.40-2.59)	0.96	1.16 (0.52-2.62)	0.70
	Q4	0.17 (0.03-0.89)	0.03	2.22 (0.37-13.34)	0.38	0.68 (0.28-1.61)	0.38	0.69 (0.30-1.59)	0.39
Adjusted model	Q1	Reference		Reference		Reference		Reference	
	Q2	0.15 (0.03-0.76)	0.02	2.27 (0.33-15.51)	0.4	0.87 (0.39-1.89)	0.72	1.29 (0.56-2.98)	0.53
	Q3	0.14 (0.02-0.75)	0.02	1.10 (0.17-6.92)	0.91	1.58 (0.70-3.56)	0.26	1.22 (0.53-2.79)	0.62
	Q4	0.17 (0.034-0.9)	0.03	3.43 (0.44-26.64)	0.23	1.74 (0.78-3.91)	0.17	0.73 (0.31-1.69)	0.46
DDS									
Crude	Q1	Reference		Reference		Reference		Reference	
	Q2	0.83 (0.28-2.44)	0.73	0.36 (0.06-1.97)	0.24	0.72 (0.34-1.50)	0.38	1.29 (0.61-2.72)	0.49
	Q3	0.77 (0.27-2.22)	0.63	0.67 (0.10-4.18)	0.67	1.31 (0.60-284)	0.48	0.89 (0.41-1.90)	0.76
	Q4	0.61 (0.21-1.73)	0.36	0.35 (0.06-1.90)	0.22	0.78 (0.37-1.66)	0.53	0.86 (0.39-1.87)	0.70
Adjusted model	Q1	Reference		Reference		Reference		Reference	
	Q2	0.75 (0.24-2.31)	0.62	0.4 (0.07-2.37)	0.31	0.83 (0.34-1.98)	0.67	1.43 (0.66-3.08)	0.35
	Q3	0.82 (0.26-2.52)	0.73	1.1 (0.15-7.76)	0.91	1.56 (0.57-4.27)	0.38	1.01 (0.44-2.27)	0.98
	Q4	0.57 (0.17-1.90)	0.36	0.99 (0.13-7.33)	0.99	0.52 (0.21-1.32)	0.17	1.04 (0.43-2.49)	0.90

All data presented as means ± SD. SD; Standard deviations, P; P value: [a]; Assessed by Kruskal-Wallis test, [b]; Assessed by ANOVA test, [c]; Body mass index (BMI) was significantly different between the first and forth quartile of dietary diversity score (DDS, P=0.018), [d]; The distribution of energy intake was different between the first and third, first and fourth, second and fourth quartile of DDS (P<0.001), PA; Physical activity, and WC; Waist circumference.

Discussion

We found that a higher traditional diet intake was correlated with reduced abnormal sperm concentration and poorer sperm volume in Iranian infertile men. Furthermore, the mixed diet showed a significant relationship with lower levels of abnormal sperm concentration. The novelty of our study was the evaluation of the relationship between DDS and sperm quality parameters in infertile men, even though there was no significant association between DDS and sperm quality parameters in our study.

The findings of some recent studies on the association between diet and sperm parameters agreed with the present study while some others did not. One study was conducted among sub-fertile men referring to an in vitro fertilization clinic in the Netherlands; in this study, the "health-conscious" dietary pattern and the "traditional Dutch" pattern were extracted. There was a reverse association between the health-conscious diet and DNA fragmentation index, while the traditional diet, as seen in our study, was positively related with sperm concentration and folate level in red blood cells. Nevertheless, the authors did not find any association between dietary patterns and sperm movement (17). Another study done at the University of Rochester on healthy men, indicated that prudent pattern was only related with percentage of sperm with progressive motility, while the Western pattern was not correlated with any sperm quality parameters (18). A systematic review and meta-analysis of six observational studies on 8207 participants, declared that individuals with the highest adherence to healthy dietary pattern versus those with the lowest adherence, had significantly higher level of sperm concentration. However, in this analysis, there was no significant association between eating dietary patterns and other sperm parameters (19) . Another research done in Poland, suggested that a pro-healthy pattern was not related with any sperm quality parameters. Similarly, in our study, the prudent dietary pattern was only related to sperm volume (20).

In the present study, the traditional dietary pattern was defined by high intakes of dairy products, saturated fats, fruits, sugary beverages and sweets. Animal products such as meat and dairy products are major sources of protein and micronutrients. Trans fatty acids (TFAs), saturated fatty acids (SFAs) and preservative agents or hormonal residues like xenobiotics or anabolic steroids, may affect sperm quality (21). Previous investigations showed that total dairy food intake was reversely associated with NSM and among physically active young men, whole-fat dairy intake was related to a significantly lower PRM (progressive motility), whereas intake of low-fat milk was specifically related to a higher progressive motility and sperm concentration. Consumption of low-fat and skimmed milk was also related with higher levels of insulin and insulin-like growth factor 1 (IGF-1) (22). With regard to dietary fat food intakes, fat-rich foods, such as hydrogenated fat and saturated fat, might also reduce the sperm quality in humans (21). Based on the results of a systematic review of 17 randomized trials, antioxidant supplementation improved sperm movement in most trials. Additionally, there are some important minerals with antioxidant role such as zinc, selenium and vitamin E ,that can be received via diet instead of supplements(23, 24). In our study, intakes of folate, selenium and vitamin E were significantly higher in top level of traditional diet. Furthermore, fruits and vegetables, which are the main source of fiber intake, can directly bind to unconjugated estrogens and reduce the estrogen level of plasma.

Additionally, the mixed diet was significantly related to lower abnormal SC; this might be possibly due to the presence of catechins and the aflavins in green tea (GT) and black tea (BT) (components of mixed diet), respectively. These bioactive phytochemicals could be related to the antioxidant activity (25). Low intake of SFAs or TFAs in vegetable oils as well as low intake of soy bean in this dietary pattern, could be responsible for the observed associations. High concentration of phytoestrogens in soy foods can be responsible for their negative effects on male fertility. Phytoestrogens are known to have destructive effects on the male endocrine system with unfavorable effects on fertility. The results of a study on Caucasian subjects showed that lower sperm concentration was related to a higher intake of soy foods (26).

Surprisingly, a vegetable-based pattern mostly including fruits and vegetables, was not related to any sperm quality parameters, which is in contrast with the findings of some previous studies. However, fruits and vegetables contain large amounts of some minerals such as selenium, vitamin C and vitamin E, which may indirectly improve semen quality through their anti-inflammatory and protective role against free radicals. The presence of environmental contaminants including chlorinated pollutants and pesticides might be a possible explanation for this observation. Therefore, the antioxidative role of fruits and vegetables could be diminished by possible toxic effects of pollutants and pesticides (27).

DDS is an index to evaluate the quality of diet. Moreover, it can represent intake of micronutrients or energy. A previous study among Tehranian women showed that increasing diversity score in cereals was related to higher intake of carbohydrates, proteins and calcium; however, increasing fruits and vegetables scores were related to higher intake of vitamin A and C and lower intake of energy(28). Therefore, it is important to note that intake of which food groups increases the DDS.

Strengths of this study included the use of dietary pattern analysis, instead of nutrient or whole food analysis, which more closely reflects overall diet and interaction between all components and the ability to adjust multiple potential confounders (29). Some limitations should be noted while interpreting the results of the study. The design of the study was the main one as determining the direction of association in cross-sectional studies is impossible. Another limitation of the study was the use of FFQ to evaluate habitual dietary

intake. Although a validated FFQ with adequate validity and reproducibility was used, it could be prone to measurement error, which usually leads to debilitation of the associations of interest.

Conclusion

Higher intake of the traditional diet was linked to a lower abnormal semen concentration and poorer sperm volume. Also, the mixed diet was associated to reduced prevalence of abnormal semen concentration. Because of changes in food availability and variation in eating patterns among different socioeconomic status, ethnic groups and cultures, more prospective investigations are needed to explain the correlation between dietary habits and infertility.

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Authors' Contributions

M.SH.; Participated in study design, data collection and evaluation. M.N.; Participated in data collection and evaluation. MR.M.; Participated in statistical analysis of data. H.A.; Contributed extensively in interpretation of the data and the conclusion. GH.A.; Contributed to all experimental work, and interpretation of data. P.S.; Participated in interpretation of the data and wrote the final manuscript. All authors read and approved final the manuscript.

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Original Article

Diet and The Risk of Endometriosis in Iranian Women: A Case-Control Study

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Abstract

Background: Endometriosis is one of the most common pelvic diseases associated with dyspareunia, pelvic pain, and infertility. The primary aim of this study is to evaluate the role of diet on the risk of endometriosis among Iranian women.

Materials and Methods: This case-control study was conducted in two health research centres between 2015 and 2016. There were 207 women with endometriosis (case) and 206 women without endometriosis (control) who were evaluated by laparoscopy. The women were asked about their frequency of consumption per week of portions of selected dietary items in the Iranian diet in the year before the interview.

Results: The results indicated that intake of green vegetables (odds ratio [OR]=0.39, 95% confidence interval [CI]=0.21–0.74, Ptrend=0.004), red meat (OR=0.61, 95% CI=0.41–0.91, Ptrend=0.015) and dairy products (milk [OR=0.65, 95% CI=0.47–0.92, Ptrend=0.014], cheese [OR=0.53, 95% CI=0.37–0.76, Ptrend<0.001]), fresh fruit (OR=0.68, 95% CI=0.50–0.93, Ptrend=0.015) and grain legumes (OR=0.59, 95% CI=0.47–0.77; Ptrend<0.001) had a significant association with lower risk of endometriosis. Consumption of carrots, green tea, fish, eggs and oil was not significantly related to the risk of endometriosis.

Conclusion: This study suggests that certain types of dietary components may be related to the risk of endometriosis.

Keywords: Diet, Endometriosis , Risk

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Introduction

Endometriosis is one of the most common hormone-dependent gynaecological diseases. It is a chronic inflammatory gynaecological condition that is characterized by dyspareunia, pelvic pain, and infertility (1). Despite the high association with morbidity, the prevalence, incidence, aetiology and risk factors of endometriosis remain elusive (2). Endometriosis may be related to genetic, hormonal, anatomic, immune, inflammatory, environmental and lifestyle factors (3). The role of diet in several chronic diseases has been determined (4). Oestrogen activity is a common denominator for many known endometriosis risk factors and an association has been demonstrated between diet and oestrogen dependency; therefore, endometriosis may also be affected by diet (1, 5). The role of diet in the incidence and progression of endometriosis has become a growing field of interest in recent years (6, 7) and summarized in a recent review (8). Diet may play a role in its etio-pathogenesis (9), and can be influenced through multiple pathways that include effects on oxidative stress, prostaglandin (PG) metabolism, smooth muscle contractility, inflammation, immune function and estrogenic effects (10). Huang et al. (11) reported that dietary factors altered serum sex-hormone concentrations and activity. A few human studies explored the relationship between diet and endometriosis risk, but had conflicting results (1, 2, 12). Parazzini et al. (12) found no association between the consumption of milk, cheese, carrots, fish and whole grain foods and the risk of endometriosis; however, there was a significant association between the intake of green vegetables, fresh fruit, and red

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meat with endometriosis risk. Alternatively, Yamamoto et al. (7), in a large cohort study, reported a significant association between the consumption of red meat and the risk of endometriosis, while consumption of fish, poultry, and eggs were unrelated to endometriosis risk. Another large US cohort study revealed that total fat consumption was not associated with the risk of endometriosis (2). Of note, most published studies supported the association used animal models of endometriosis and extrapolated the results to humans (13). According to the limited number of studies on this topic and inconsistencies between these prior study findings, the present analytic research was conducted to further investigate if the selected diet factors affect the occurrence of endometriosis in Iranian women.

Materials and Methods

Study Population

This case-control study was carried out at two referral centres, Royan Institute and Vali-Asr Reproductive Health Research Centre (both in Tehran, Iran), between April 2015 and March 2016. The Institutional Review Board and the Medical Ethics Committee of Tehran University of Medical Sciences approved this study. All procedures performed in this study were in accordance with the Declaration of Helsinki and an informed consent was obtained from all individual participants included in the study.

The control and case groups were from both centres. A total of 510 women who underwent diagnostic laparoscopy were recruited for this study. The main indications for laparoscopy were symptoms of endometriosis (dysmenorrhea, dyspareunia and pelvic pain), uterine abnormality, tuboperitoneal disorder and unexplained factor in infertility. Women (n=97) who had adhesions, leiomyomas, fibromas, and/or uterine abnormalities at laparoscopy were excluded from the study. Finally, we included 413 women who were divided into two groups according to the laparoscopy findings of endometriosis (case group) or normal pelvis (control group). Following surgery, the stage of the disease was defined according to the classification system of the revised American Society for Reproductive Medicine (rASRM) as stage I (minimal), stage II (mild), stage III (moderate) and stage IV (severe) (14). Histologic confirmation was obtained in 79.6% of the women with endometriosis.

Dietary Assessment

The required data were collected using a structured questionnaire for information on demographic variables and reproductive characteristics. For each selected dietary item in the Iranian diet, the participants were asked about their frequency of consumption per week (i.e., 14 meals) in the year before the interview. The questionnaire was a structured questionnaire similar to that reported by Parazzini et al. in their study (12) on diet habit; specifically, the selected dietary items were green vegetables (0-6, 7-12, ≥13 portions/week), fresh fruits (≤6, 7-13, ≥14 portions/week), carrots (0, 1, ≥2 portions/week), grain legumes (0, 1, ≥2 portions/week), red meat (0-3, 4-6, ≥7 portions/week), fish (0, 1, ≥2 portions/week), milk (0, 0.5-6, ≥7 portions/week), cheese (≤2, 3-5, ≥6 portions/week), eggs (0, 1, ≥2 per week) and green tea (yes or no). The items of green vegetables and fruits included all types, specifically all of the main sources in the Iranian diet such as spinach/other greens, kale, green salads, broccoli, cauliflower, citrus fruit, apples, peaches, melons, strawberries/cherries, bananas and pears.

In terms of content validity, we requested that 10 experts in the fields of nutrition, midwifery, reproductive health and gynaecology review the questionnaire and assess each item based on four criteria: relevancy, clarity, simplicity, and necessity. The content validity ratio (CVR) was calculated based on the responses to the necessity of questions (nE) according to the following formula.

$$CVR= (nE-N/2)/ (N/2)$$

Lawshe's table was used to determine the CVR cut-off point (15). According to Lawshe, for 10 professionals, the minimum required CVR for each item is 0.94. The content validity index (CVI) for this questionnaire was based on the Waltz and Bausell CVI (16). The CVI for each item was obtained by dividing the number of professionals who ranked the items as compatible or full compatible for each criterion (relevancy, clarity, simplicity, and necessity) to the total number of professionals. The average value of the three criteria was used as the total CVI for each item. The minimal required amount of CVI for each item was 0.90 (17). A test-retest analysis with an interval of 15 days was approved in a pilot study of 30 women with endometriosis. We assessed the test-retest reliability of the questionnaire by using two correlational measures, Spearman's correlation and Cohen's kappa, to show the similarity in the responses to an item on test and retest (18). According to Field (18) and Cade et al. (19) large correlation coefficients of 0.5 or greater indicate high reliability. The value of Kappa identifies the strength of the agreement according to the categories reported by Masson et al. (20) of poor (<0.20), fair (0.21-0.40), moderate (0.41-0.60), good (0.61-0.80), and very good (0.81-1.00). Table 1 shows that all correlation coefficients are above 0.6, which indicates good reliability.

Table 1: Spearman's r and Cohen's kappa correlational measures between test and retest

Food item consumption	Spearman's r [b]	Cohen's kapp[a]
Green vegetables	0.768	0.627
Fresh fruits	0.960	0.880
Carrots	0.873	0.827
Grain legumes	0.847	0.759
Green tea	0.768	0.627
Type of oi1[a]	-	0.655
Fish	0.967	0.948
Eggs	0.606	0.663
Meat	0.789	0.701
Milk	0.703	0.660
Cheese	0.818	0.748

[a]; Mode in test and retest is reported, [b]; Because this is a nominal variable, and Cramer's V correlation coefficient was used.

Statistical analysis

The sample size was calculated according to the studied variables and based on the "rule of thumb" method. We considered at least 10 samples per variable; therefore, the sample size was estimated to be 200 women (21). The chi square or t-tests, when appropriate, were employed using SPSS software (version 22, USA) to compare categorical and continuous variables, respectively. We used logistic regression to determine the risk factors associated with endometriosis. In addition, logistic regression was used to adjust for age, education levels and body mass index (BMI). The odds ratio (OR) and 95% confidence interval (CI) were outputted for each of the calculated factors. In order to test the linear trend for ordinal variables such as green vegetables, fresh fruits, carrots, grains legumes, fish, eggs, meat, milk and cheese, we reported the OR and P value for the trend using category medians. P values less than 0.05 were considered to be statistically significant.

Results

The distribution of cases and controls according to age and selected characteristics is presented in Table 2. The severity of disease was staged according to the rASRM classification of endometriosis. Endometriosis was staged as minimal (rASRM stage I) in 56 (27.1%), mild (rASRM stage II) in 24 (11.6%), moderate (rASRM stage III) in 44 (21.3%), and severe (rASRM stage IV) in 83 (40.1%). When the demographic characteristics of the women with endometriosis were compared with the control group, there were no significant differences detected in the age at menarche, parity, occupation, cigarette smoking and history of infertility. In contrast, there was a significant difference concerning age, education and BMI between the two groups. The mean age was 31.50 ± 5.52 years in the endometriosis group and 30.30 ± 5.96 years in the control group (P=0.02).

Table 2: Comparison of demographic characteristics of endometriosis cases and controls.

Parameters	Control N (%)	Case N (%)	P Value*
Age (Y)			
≤24	34 (16.5)	17 (8.2)	0.02
25-34	127 (61.7)	131 (63.3)	
≥35	45 (21.8)	59 (28.5)	
Age at menarche (Y)			
<12	17 (8.3)	11 (5.3)	0.47
12	53 (25.7)	58 (28.0)	
>12	136 (66.0)	138 (66.7)	
Parity			
0	160 (77.7)	154 (74.4)	0.54
1	40 (19.4)	43 (20.8)	
≥2	6 (2.9)	10 (4.8)	
Education levels			
Under diploma	76 (36.9)	45 (21.7)	0.004
Completed high school	74 (35.9)	91 (44)	
University	56 (27.2)	71 (34.3)	
Occupation			
Housewife	170 (82.5)	157 (75.8)	0.09
Employed	36 (17.5)	50 (24.2)	
BMI (kg/m^2)			
<20	7 (3.4)	20 (9.7)	0.001
20-24.9	68 (33.0)	92 (44.4)	
25-29.9	93 (45.1)	74 (35.7)	
≥30	38 (18.4)	21 (10.1)	
Smoking			
No	202 (98.1)	204 (98.6)	0.69
Yes	4 (1.9)	3 (1.4)	
History of infertility			
Yes	182 (88.3)	182 (87.9)	0.89
No	24 (11.7)	25 (12.1)	

*** Chi square test, BMI: Body mass index, and Y; Year.**

Table 3 shows the relationship between selected food intake and the risk of endometriosis. Our results indicated that intake of green vegetables (Ptrend=0.004) and red meat (Ptrend=0.015) were significantly associated with a lower risk for endometriosis. Fresh fruits (Ptrend=0.015), dairy (milk [Ptrend=0.014] and cheese [Ptrend<0.001]), and grain legumes (Ptrend<0.001) were associated with a decreased risk for endometriosis. Table 3 shows an OR greater than one for those with zero or one portion of grain legumes per week compared to those with two portions of grain legumes per week. Decreased grain legume intake appears to be a risk factor whereas higher grain legume intake appears to be protective. Consumption of carrots, green tea, fish, eggs, and oil were not significantly related to the risk for endometriosis.

Table 3: Risk of endometriosis and selected food intake

Food item consumption No. of servings	Control N (%)	Case N (%)	OR unadjusted (95% CI)[a]	OR adjusted (95% CI)[a]	P Value*
Green vegetables					
≤6 portions/week	176 (85.4)	193 (93.2)	1†	1†	0.03
7-12 portions/week	25 (12.1)	13 (6.3)	0.47 (0.23-0.95)	0.47 (0.22-1.02)	
≥13 portions/week	5 (2.4)	1 (0.5)	0.18 (0.02-1.57)	0.13 (0.01-1.21)	
P value for trend**	-	-	0.45 (0.25-0.82)	0.39 (0.21-0.74)	0.004
Fresh fruits					
≤6 portions/week	127 (61.7)	143 (69.1)	1†	1†	0.04
7-12 portions/week	43 (20.9)	47 (22.7)	0.97 (0.60-1.56)	0.92 (0.54-1.58)	
≥13 portions/week	36 (17.5)	17 (8.2)	0.41 (0.22-0.78)	0.43 (0.22-0.85)	
P value for trend**	-	-	0.71 (0.54-0.94)	0.68 (0.50-0.93)	0.015
Carrots					
0 portions/week	93 (45.1)	77 (37.2)	1†	1†	0.40
1 portions/week	55 (26.7)	57 (27.5)	1.25 (0.77-2.01)	1.25 (0.75-2.07)	
≥2 portions/week	58 (28.2)	73 (35.3)	1.52 (0.96-2.40)	1.38 (0.84-2.27)	
P value for trend**	-	-	1.23 (0.98-1.55)	1.16 (0.91-1.47)	0.232
Grain legumes					
0 portions/week	76 (36.9)	89 (43.0)	1†	1†	<0.001
1 portions/week	40 (19.4)	75 (36.2)	1.60 (0.98-2.61)	1.54 (0.91-2.63)	
≥2 portions/week	90 (43.7)	43 (20.8)	0.40 (0.25-0.65)	0.32 (0.19-0.54)	
P value for trend**	-	-	0.66 (0.52–0.84)	0.59 (0.47–0.77)	<0.001
Green tea					0.51
Yes	47 (22.8)	50 (24.2)	1†	1†	
No	159 (77.2)	157 (75.8)	1.07 (0.68-1.69)	1.17 (0.71-1.93)	
P value for trend**	-	-	1.07 (0.68-1.69)	1.01 (0.63-1.65)	0.951
Type of oi1					
Animal	6 (2.9)	3 (1.4)	1†	1†	0.32
Vegetable	185 (89.8)	182 (87.9)	1.96 (0.48-7.98)	2.15 (0.47-9.80)	
Animal and vegetable	15 (7.3)	22 (10.6)	2.93 (0.63-13.59)	3.42 (0.65-17.82)	
Fish					
0 portions/week	118 (57.3)	122 (58.9)	1†	1†	0.49
1 portions/week	59 (28.6)	63 (30.4)	1.03 (0.66-1.59)	0.97 (0.61-1.56)	
≥2 portions/week	29 (14.1)	22 (10.6)	0.73 (0.39-1.34)	0.67 (0.35-1.30)	
P value for trend**	-	-	0.90 (0.68-1.18)	0.85 (0.63-1.14)	0.284
Eggs					
0 portions/week	45 (21.8)	37 (17.9)	1†	1†	0.07
1 portions/week	36 (17.5)	47 (22.7)	1.58 (0.85-2.93)	1.80 (0.94-3.47)	
≥2 portions/week	125 (60.7)	123 (59.4)	1.19 (0.72-1.97)	1.21 (0.71-2.06)	
P value for trend**	-	-	1.04 (0.82-1.33)	1.04 (0.81-1.35)	0.734

Table 3: Continued

Food item consumption No. of servings	Control N (%)	Case N (%)	OR unadjusted (95% CI)[a]	OR adjusted (95% CI)[a]	P Value*
Meat					
0-3 portions/week	144 (69.9)	166 (80.2)	1†	1†	0.03
4-6 portions/week	50 (24.3)	35 (16.9)	0.60 (0.37-0.98)	0.53 (0.31-0.90)	
≥7 portions/week	12 (5.8)	6 (2.9)	0.43 (0.15-1.18)	0.47 (0.15-1.38)	
P value for trend**	-	-	0.63 (0.44-0.91)	0.61 (0.41-0.91)	0.015
Milk					
<1 portions/week	117 (56.8)	141 (68.1)	1†	1†	0.04
1-6 portions/week	33.0	57 (27.5)	0.69 (0.45-1.06)	0.67 (0.42-1.06)	
≥7 portions/week	21 (10.2)	9 (4.3)	0.35 (0.15-0.80)	0.39 (0.16-0.93)	
P value for trend**	-	-	0.64 (0.47-0.88)	0.65 (0.47-0.92)	0.014
Cheese					<0.001
≤2 portions/week	74 (35.9)	118 (57)	1†	1†	
3-5 portions/week	120 (58.3)	79 (38.2)	0.41 (0.27-0.62)	0.39 (0.25-0.61)	<0.001
≥6 portions/week	12 (5.8)	10 (4.8)	0.52 (0.21-1.27)	0.52 (0.21-1.33)	<0.001
P value for trend**	-	-	0.52 (0.37-0.73)	0.53 (0.37-0.76)	

†; Reference category, *; P values are adjusted for age, education levels and body mass index (BMI), **; Determined using category medians, [a] OR; Odds ratio, and CI; Confidence interval.

Discussion

Selected Dietary Items

Vegetables/Fruits

The findings of the present study indicate that higher intake of green vegetables (OR=0.39, 95% CI=0.21–0.74; Ptrend=0.004) and fresh fruits (OR=0.68, 95% CI=0.50–0.93; Ptrend=0.015) can lower the risk of endometriosis. In three investigations, the relationship between servings per week or day of fresh fruit and green vegetable intake and endometriosis risk were evaluated (15, 16, 27). Similar to our study, Parazzini et al. (12) reported that intake of fresh fruits and green vegetables decreased the endometriosis risk. In contrast, Trabert et al. (1) found that vegetable consumption was not related to risk of endometriosis; however, higher disease risk was associated with increased fruit intake. The study's authors posited that the results could be associated with a higher percentage of pesticide consumption in the cultivation of fruit, which might produce reactive oxygen species and decrease the antioxidant capacity of fruits and vegetables. The use of organochlorine pesticides in fruits should not prohibit their use. Rather, the use of organic fruits or removing the peels from contaminated fruits should be considered (22). Harris et al. (23) prospectively assessed data collected from 70 835 premenopausal women and found that fruit intake was associated with a decreased endometriosis risk; among these, citrus fruits had strongest association and it was suggested that the presence of beta-cryptoxanthin in these foods was probably responsible for this phenomenon. Unlike our finding in green vegetables, they reported that consumption of some vegetables such as cruciferous vegetables, corn and peas or lima beans were related to a higher risk of endometriosis. This positive association was observed among women with no history of infertility, which might explain the inconsistency between our results and those reported by Harris et al. (23), given that the highly selected population (almost 90%) in our study consisted of infertile women.

Populations on vegetarian diets usually have elevated sex-hormone binding globulin (SHBG) levels (24). A low-fat diet also decreases the levels of oestrogen in both pre-menopausal and post-menopausal women (25). Oestrogen conjugates enter the hepatic circulation through the bile, and are interrupted by dietary fibre; this encourages faecal oestrogen elimination (26). Increased SHBG or reduced serum levels of oestrogen can decrease oestrogenic stimulation of the endometrium, and restrict the proliferation of tissues that produce PGs (12). Hormonal agents are a potential connection between endometriosis and diet, as unopposed oestrogens can increase endometriosis risk. More difficult to explain in biological terms is the protective effect of a fruit and green vegetable-rich diet. High levels of carotenoids, folic acid, vitamin C and lycopene in a diet rich in fruits and green vegetables may cause inhibition of cell proliferation (27). In addition, it seems that dietary fruits and green vegetables may be surrogates for fibre. Fibre, as mentioned previously (28), decreases enterohepatic circulation and may thereby decrease the risk or severity of endometriosis.

Red Meat/ Fish

The findings in this study were inconsistent with the results of many similar studies in other countries (1, 7, 12, 13, 29). Our results showed a decreased endometriosis risk for those with 4-6 portions of meat per weak compared to those with 0-3 portions of meat per week (OR=0.61, 95% CI=0.41–0.91; Ptrend=0.015). Four studies analysed the risk of endometriosis with frequent red meat consumption

(1, 12, 13, 29, 30), which is a rich source of saturated fat. In an Italian case–control study (12) and a prospective cohort study (7), high intake of red meat increased the risk for endometriosis. In a Belgian matched case-control study with prospective recruitment, meat consumption was not linked with the risk of peritoneal endometriosis (29). In another case-control study (1), no association was found between the risk of endometriosis and increased servings of red meat. The effect of red meat consumption reported by Parazzini et al. (12) could be connected to the fat content and type of fat in meat. Meat diet contains large amounts of fat, which can further increase oestrogen levels (7), and is comparably higher in omega-6 fatty acids (FAs), which stimulates the production of pro-inflammatory PGs (31). Fung et al. (32) have reported that a high intake of meat is associated with elevated serum concentrations of oestrogen sulphate and oestradiol; consequently, its consumption might directly contribute to increased levels of circulating steroid hormone (33) and to the maintenance of the disease. This is an arguable topic in spite of comparable serum levels of oestradiol in women with and without endometriosis (34), although the likelihood of elevations in local oestrogen synthesis with increased red meat consumption cannot be excluded (13). In a most recent study, Yamamoto, et al. (7) demonstrated that the effect of high intake of meat among endometriosis women with no report of infertility was partly related to the relationship between heme iron intake and endometriosis, with the mechanism of inflammation triggered by oxidative stress which involved in pathophysiology of the endometriosis.

There are several possible explanations for the inconsistency between our results and those obtained by other studies. One of the reasons for this discrepancy could be the kind of meat consumed in Iran and other countries. Beef and lamb are the most widely consumed meats in Iran, whereas in most other countries, pork is one of the highly consumed meats. Dioxin contamination in food products and animal food in Italy that occurred during the entire study is another factor that could impact the study results. The method for cooking meat is one of the most effective factors that varies in different nations and cultures (35). Another possible explanation for this might be the different slaughter procedures for sheep and calves in Iran (ritual cutting) and other countries (captive bolt stunning). Schulze et al. (36) explained that in ritual cutting, animals suffer much less pain and less stress hormones. Also, as the heart of animals killed by this way works much later, more blood and other materials, including hormones, will be removed from the animals (36); this could possibly be corroborated by other studies in which indicated an association between heme iron intake and endometriosis risk (7) and also reported lower concentrations of hemopexin, which is the major vehicle for the transportation of heme iron (37).

In the present study, we found no association between the consumption of fish and endometriosis risk. This finding was consistent with the results of similar studies conducted by Parazzini et al. (Italy), Trabert et al. (Belgium) and Heilier et al. (USA) (1, 12, 29). Harel et al. (38) reported that fish consumption had the potential to reduce PGE2 and PGF2α concentrations and could be a possible risk-reducing factor for endometriosis.

Dairy products

Our findings suggest that dairy product consumption was associated with a reduced risk of endometriosis. As higher intake of milk (OR=0.65, 95% CI=0.47–0.92; Ptrend=0.014) and intake of 3-5 portions of cheese (OR=0.53, 95% CI=0.37-0.76; Ptrend<0.001)] was associated with a decreased risk of endometriosis. Few studies have examined the association between the intake of dairy foods and nutrients with risk of endometriosis. In the first human study that evaluated intake of dairy, Parazzini et al. (12) reported no association between milk or cheese consumption and risk of endometriosis. Alternatively, Trabert et al. (1) found a non-significant inverse correlation between dairy intake and risk of endometriosis. The results of a prospective cohort study revealed that high consumption of dairy products, specifically yogurt and ice cream during adolescence, was associated with a lower risk of endometriosis (6).

It has been shown that serum and peritoneal fluid pro-inflammatory cytokine concentrations are elevated in women with endometriosis (8). Dairy products may be related to the endometriosis-associated inflammatory responses (8, 10). Zemel et al. (39) stated that a milk diet decreased inflammatory markers and oxidative stress, including interleukin-6 (IL-6) and tumour necrosis factor-alpha receptor 2 (TNF-α R2). Another hypothesis for this association is the ability of calcium and vitamin D to down-regulate insulin-like growth factor-I (IGF-1), which plays a role in the growth-promoting process and up-regulation of transforming growth factor beta (TGF-β) acts as a negative autocrine growth factor (1).

Grains

Our result shows an OR lower than one for those with ≥ 2 portions (OR=0.59, 95% CI=0.47-0.77; Ptrend<0.001) of grain legumes per week compared to the reference group. Higher grain legumes intake appears to be protective. An analysis of the number of servings per week of grain did not show any association with the risk of endometriosis according to Trabert et al. (1). Similarly, no association was reported between the intake of grain and endometriosis risk (12, 30). Refined cereals can influence glycaemic load (GL) and glycaemic index (GI). These variables are used to estimate the rate of carbohydrate absorption and subsequent insulin demand. When insulin binds to its receptor in the endometrium, it is able to induce the growth of endometrial stromal cells. Moreover, hyper-insulinaemia increases the level of oestrogens through reducing the serum level of SHBG and increases the level of IGF-1 by lowering the serum level of insulin-like growth factor-binding protein 1 (IGFBP-1). Both IGF-1 and oestrogens stimulate endometrial cell proliferation

(31). Accordingly, cereal consumption could be correlated with endometriosis risk.

Our data do not support an association between endometriosis risk and intake of any of the other nutrients or food groups evaluated in this study (e.g., carrots, green tea, oil, and eggs).

In the present results, oil intake was not associated with endometriosis risk (p-trend=0.32); this finding was similar to our previous study (30) performed on 156 infertile patients (p-trend=0.21). In the Italian case–control study, no association was found between oil consumption and risk of endometriosis (12).

This study has some limitations. An inevitable limitation is that case-control studies in nutritional epidemiology may be at a potential risk for recall bias. Information depends entirely on memory and there may be possible variations in recall bias for cases versus controls. Cases may associate their disease to their bad dietary habits and may over-report consumption of foods considered unhealthy. However, we believe that since the majority of women interviewed were probably unaware of the possible relationship between diet and endometriosis, the effect of recall bias was relatively low. As approximately 90% of our study population in both groups were infertile, this might limit the generalisability of results to all endometriosis women. Thus, our findings have implications for women with endometriosis from infertility clinic-based studies. Another weak point of our study was that we did not use the food frequency questionnaire (FFQ) due to the disadvantages of longer food lists and an additional respondent burden (40). The strong point of our study was the detailed availability of all records for the 413 participants. All of the participants completed the questionnaire.

Conclusion

Despite the limitations, this research demonstrates that there is some association between intake of green vegetables, red meat, dairy products, cheese, fresh fruit and grain legumes with lower risk of endometriosis. These results highlight the necessity for appropriate extensive prospective evaluations to study these factors in fertile women with endometriosis to increase generalization of the findings.

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Authors' Contributions

M.A., Sh.JS., F.A.; Study concept and design. Sh.JS., N.J.; Drafting of the manuscript and interpretation of data. F.A.; Acquisition of data, administrative, technical, and material support. N.J., M.A., Sh.JS.; Critical revision of the article for important intellectual content. M.A.; Statistical analysis. All authors read and approved the final manuscript.

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Original Article

Alterations of CD4+T Cell Subsets in Blood and Peritoneal Fluid in Different Stages of Endometriosis

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Abstract

Background: Endometriosis is a chronic inflammatory disorder with known immune disturbances. The aim of this study was to compare the frequency of different CD4+ T cells [T helper (Th)1, Th2, Th17 and regulatory T cells (Tregs)] in peripheral blood (PB) and peritoneal fluid (PF) of patients that have early and advanced stages of endometriosis with a control group.

Materials and Methods: In this case control study, PB and PF samples were collected from women aged 24-40 years who underwent laparoscopy procedures. The frequency of CD4+ T subsets were analysed by flow cytometry and compared between three study groups; early endometriosis (stage I, II), advanced endometriosis (stage III, IV) and control (no endometriosis). T cell numbers were compared between the PB and PF in each of the aforementioned groups.

Results: No statistically significant difference was found between the study groups regarding the numbers of Th1, Th2 and Th17 cells in PB. The PF of patients with advanced endometriosis had increased numbers of Th17 cells compared to the control group (P=0.003), with P values of 0.059 and 0.045 in both menstrual phases. Increased numbers of Th2 cells in PF from early compared to advanced stages of endometriosis were detected exclusively in the luteal phase (P=0.035).
The control group had increased numbers of Treg and Th2 cells in the PF compared to PB (both, P value=0.046). However, in the early stages of endometriosis there were more Th2, Th17 and Treg cells in the PF compared to PB (P values: 0.005, 0.047 and 0.013, respectively), while the number of Th17 cells was higher in the PF compared with PB in the advanced stages of endometriosis (P= 0.013).

Conclusion: There were increased numbers of Th17 cells in the PF of patients with advanced stages of endometriosis, which could be related to the severity of this disease.

Keywords: Endometriosis, Regulatory T Cell, T helper 1 Cell, T helper 2 Cell, T helper 17 Cell

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Introduction

Endometriosis, characterized by presence of endometrial tissue outside of uterine cavity, is a chronic inflammatory disorder that involves 6-10% of reproductive age women (1, 2).

Despite numerous investigations regarding the pathogenesis of endometriosis, the definite aetiology remains undetermined. Many factors such as genetic predisposition, hormonal imbalance, environmental factors and, especially, immune system disturbances are potential aetiologic factors (3-5).

Systemic and local changes in immune responses that include impaired CD4+ T cells have been frequently reported as contributing factors in endometriosis pathogenesis (6-8).

CD4+ T cells exert their potential role in endometriosis

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through cytokines that are involved in implantation and proliferation of ectopic endometrial cells, inflammation and angiogenesis (7, 9-12). Aberrations in CD4+ T cell populations have been assessed in several endometriosis studies (11-16); however, only one or two of these CD4+ T cells were investigated in each of these studies. To date, the trend in changes in all four CD4+ T subsets in early and advanced stages of endometriosis and between peripheral blood (PB) and peritoneal fluid (PF) in each stage of this disease have not been assessed in a single study. Hence, the current study was designed and implemented to answer the following questions:

1. Are there deviations in the numbers of T helper (Th)1, Th2, Th17 and regulatory T (Treg) cells in peripheral blood between stages I, II and III, IV of endometriosis and a control group?
2. Are there deviations in the numbers of Th1, Th2, Th17 and Treg cells in PF between stages I, II and III, IV of endometriosis and a control group?
3. Are there any changes in the number of Th1, Th2, Th17 and Treg cells between blood and PF in endometriosis stages I, II and III, IV and control group?

Materials and Methods

Participants and specimens

This case-control study enrolled 20 women with endometriosis confirmed by observation of endometriotic lesions during laparoscopy and pathological confirmation of disease in biopsies that were taken from endometriotic foci as the case group and a control group comprised of 10 women with no evidence of endometriosis during laparoscopy. All participants were 24 to 40 years of age. The presence of endometriosis was confirmed by a gynaecologist during laparoscopy. According to the revised American Society for Reproductive Medicine classification of endometriosis (17), we divided the endometriosis group into two subgroups - 10 women with early stage endometriosis (stage I, II) and 10 women with advanced stage of this disease (stage III, IV). All control women underwent laparoscopy for other diseases (dermoid or follicular cysts) and endometriosis was not detected in any of these women. Women who had a history of autoimmunity, inflammatory disorders (including allergies) or other gynaecological diseases (e.g., polycystic ovary syndrome) and those who received hormonal treatment during three months before taking samples were excluded from the study.

The study was approved by the Ethics Committee at Tehran University of Medical Sciences (TUMS), Tehran, Iran (Ethics code: IR.TUMS.MEDICINE.REC.1395.1073). The samples were taken from women who referred to Yas and Arash hospitals, both of which are TUMS affiliated women's hospitals. All women signed an informed consent form for study participation before entering the study. A total of 5 mL of peripheral blood (PB) was collected from the antecubital vein of each patient before they underwent general anaesthesia. PF was aspirated by the surgeon after insertion of the second trocar at the beginning of the laparoscopic procedure. The volume of PF varied from 2 to 8 mL in different cases. In each group, the samples were classified as follicular or luteal phase based on the date of the patient's last menopausal period, which was reported by each patient at the time of sampling and confirmed by pathologic reports in cases where samples of endometriotic lesions were obtained for pathological investigations.

Separation of mononuclear cells

PB and PF were collected in heparinized tubes and transferred in sterile, cold conditions to the laboratory where they were diluted with phosphate-buffered saline (PBS) at a 1:1 ratio. The diluted PB or PF were layered on Ficoll-Hypaque (Inno-train, Germany) and centrifuged (1000 g, 20 minutes). The cells in the interphase layer were collected and transferred into new tubes and washed completely with PBS. After discarding the supernatant, the precipitated mononuclear cells were suspended in culture medium, and the number and viability of these cells were determined by vital staining.

Culture and staining process

PB or PF mononuclear cells were divided into two portions. One part was used for detection of Treg cells and the other for stimulation and recognition of Th1, Th2 and Th17 cells.

Treg cells were considered to be CD4+CD25+CD127-FOXP3+ cells. For determination and evaluation of the Treg cells, we stained the mononuclear cells with FITC-labelled anti-CD4, PE/Cy7-labelled anti-CD25, and APC-labelled anti-CD127 antibodies (Biolegend, CA, USA); after cell fixation and permeabilisation, the cells were stained with PE-conjugated anti-FOXP3 antibody.

For detection of Th1, Th2 and Th17 cells in PB or PF, the mononuclear cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 (Biosera, France) supplemented with 10% FBS (Gibco, UK), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, UK) in 24-well cell culture plates and stimulated with phorbol myristate acetate (PMA, Sigma, St. Louise, MO, USA) at 50 ng/mL and ionomycin (Sigma-Aldrich, St. Louise, MO, USA) at 1 µg/mL concentrations and incubated at 37°C and 5% CO_2. After an hour, 10 µg/mL Brefeldin A (eBioscience, San Diego, CA, USA) was added. The cells were harvested after a 5-hour incubation period by using PMA, Ionomycin and Brefeldin A, followed by staining with FITC-conjugated anti-CD4 antibody. After fixation and permeabilisation, intracellular cytokines were stained with PE-Cy7-conjugated anti-interferon gamma (IFNγ), APC-conjugated anti-interleukin 4 (IL-4) and PE-conjugated anti-IL-17 antibodies. CD4+IFNγ+, CD4+IL-4+ and CD4+IL-17+ cells were considered to be Th1, Th2 and Th17 cells, respectively. Isotype-matched fluoro-

phore-conjugated antibodies were used as the controls.

The stained cells (10^5 cells) were investigated by BD FACSCalibur instrument (Becton Dickinson, CA).

Gating method

The lymphocytes were gated according to forward and side scatters, which were representative of the cell size and granularity. In the group of stimulated cells, we considered Th1 cells to be CD4+IFNγ+, Th2 were CD4+IL-4+ and Th17 were CD4+IL-17+. For Treg cell discrimination, first the CD4+CD127- cells were gated from the lymphocytes. Then, from these gated cells, CD25+FOXP3+ were specified. The percentage of the CD4+CD25+FOXP3+CD127- cells from the lymphocyte population was defined as the frequency of the Treg cells. These percentages were calculated using FlowJo software (Version 7.6.1). The gating procedure was similar for both PB and PF (Fig. 1); however, the percentage of the lymphocytes was different in PB and PF.

Statistical analysis

Because of the non-normal distribution of the samples, we used the Kruskal-Wallis test to compare the frequency of the Th1, Th2, Th17 and Treg cells in PB and PF samples between the three groups. The Wilcoxon test was used to compare the percentage of each T cell population in each group between PB and PF. P values <0.05 were considered to be statistically significant. SPSS version 19 and GraphPad prism Version.6 software was used for data analysis and for drawing the plots.

Results

Participants′ characteristics

After considering the inclusion and exclusion criteria, 10 samples were selected in each group. We had six follicular and four luteal phase samples in each of the control and advanced endometriosis (stage III, IV) groups; however, in the early stages of endometriosis (stage I, II), we collected seven follicular and three luteal phase samples. The ages (median, minimum-maximum) of women in the three groups were similar: control (33.5, 27-40 years), endometriosis stage I, II (32, 24-38 years) and endometriosis stage III, IV (32, 25-38 years) (P value: 0.875). The age medians were similar between groups when compared according to menstrual phase of sampling.

Comparison of the frequency of CD4+ T cells in blood between the three groups

Our results indicated similar numbers of Th1, Th2 and Th17 in blood samples of the three groups (P values: 0.78, 0.298, and 0.228, Fig. 2). The Kruskal-Wallis test results showed different numbers of Treg cells between the three groups (P=0.042) with mean ranks of 13.10 (control), 12.20 (stage I, II), and 21.20 (stage III, IV); however, a pairwise comparison between each pair of groups indicated that none of the P values were statistically different and the adjusted P values were 0.067 (stage I, II vs. stage III, IV of endometriosis), 0.119 (control vs. stage III, IV of endometriosis) and 1 (control vs. stage I, II of endometriosis). Our results showed no significant differences in the four blood CD4+ T cell subsets when they were compared based on menstrual phases.

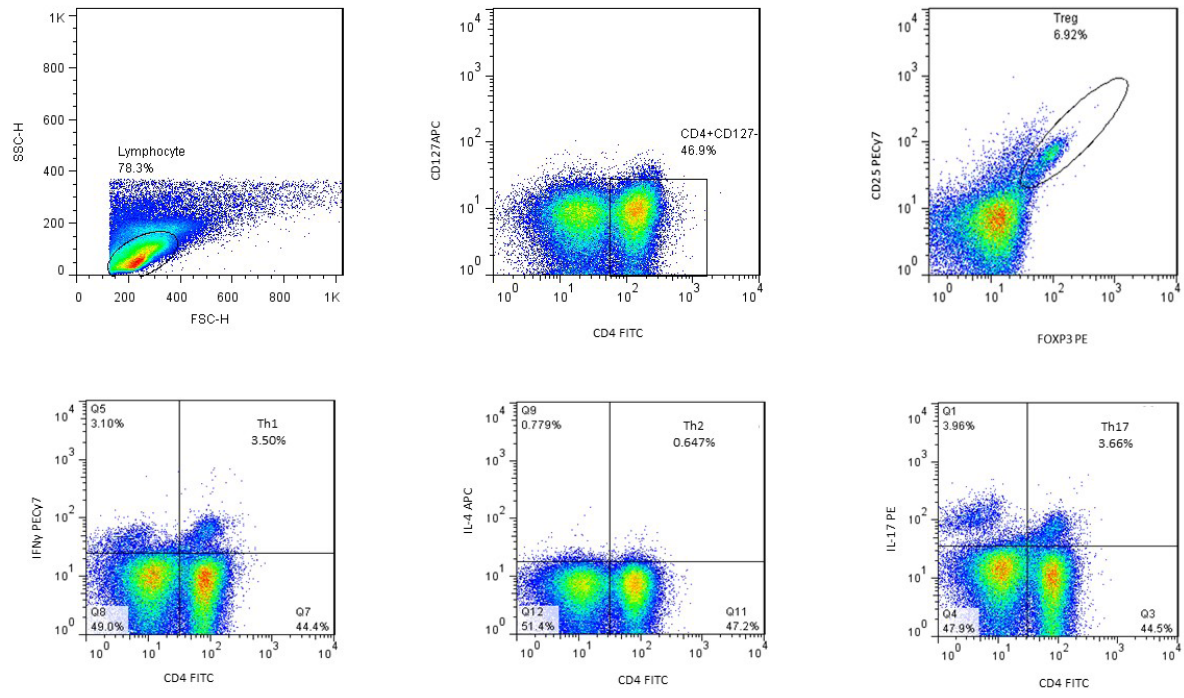

Fig. 1: Gating strategy for the detection of regulatory T cells (Treg) (upper row) and T helper (Th)1, Th2 and Th17 cells (lower row) in peripheral blood (PB). The sample is from a patient in stage III, IV endometriosis (follicular phase). CD4+CD25+FOXP3+CD127- were defined as Treg cells. CD4+IFNγ+ were considered to be Th1 cells, CD4+IL-4+ were Th2 cells and CD4+IL-17+ were considered to be the Th17 cells.

Comparison of the frequency of CD4+ T cells in peritoneal fluid between the three groups

The numbers of Th1, Th2 and Treg cells were not different in the PF of the three groups; however, there was only an increased number of Th2 cells in the PF of endometriosis stage I, II (mean rank=10) compared to stage III, IV (mean rank=3.5) in the luteal phase (P=0.035). There were increased numbers of Th17 cells in the PF of advanced endometriosis (stage III, IV) cases compared to controls (adjusted P=0.003, mean rank of 22.4 in advanced endometriosis vs. 9.30 in the control). This trend was seen in both the follicular (P=0.059) and luteal [P=0.045, mean ranks were 8.25 (advanced endometriosis) and 2.75 (control)] phases. The frequency of Th17 cells did not differ between the other groups (Figs. 2, 3).

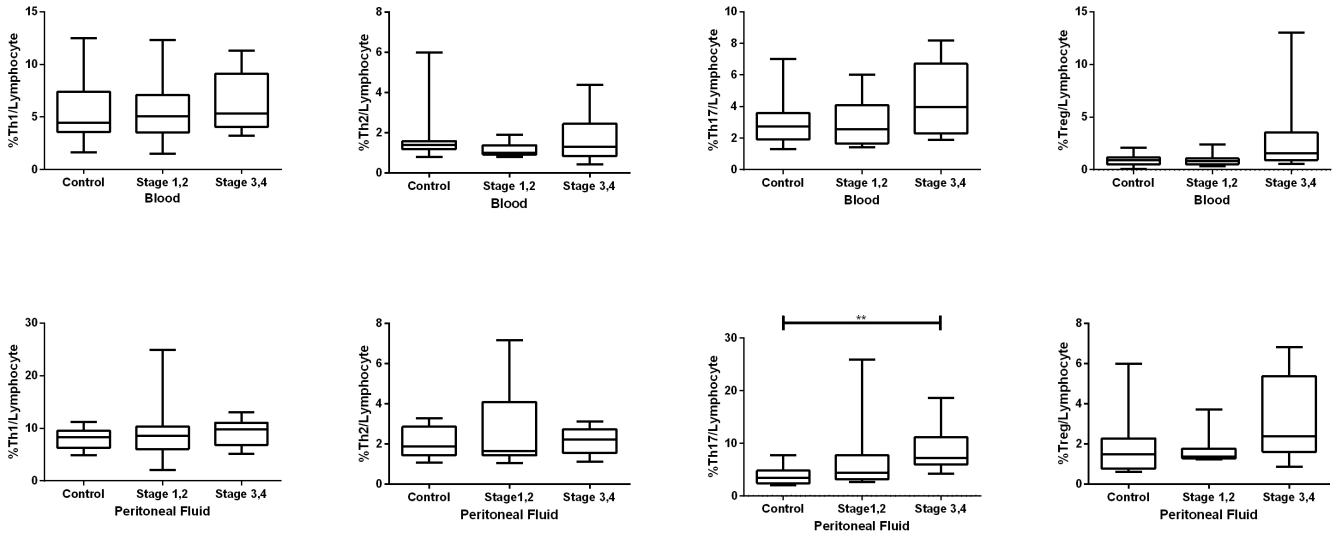

Fig 2: Frequency of T helper (Th)1, Th2, Th17 and regulatory T (Treg) cells compared in peripheral blood (PB) (upper row) and peritoneal fluid (PF) (lower row) amongst the control, early stages of endometriosis (stage I, II) and advanced endometriosis (stage III, IV) groups. Each box plot represents 25-75% quartiles with median.

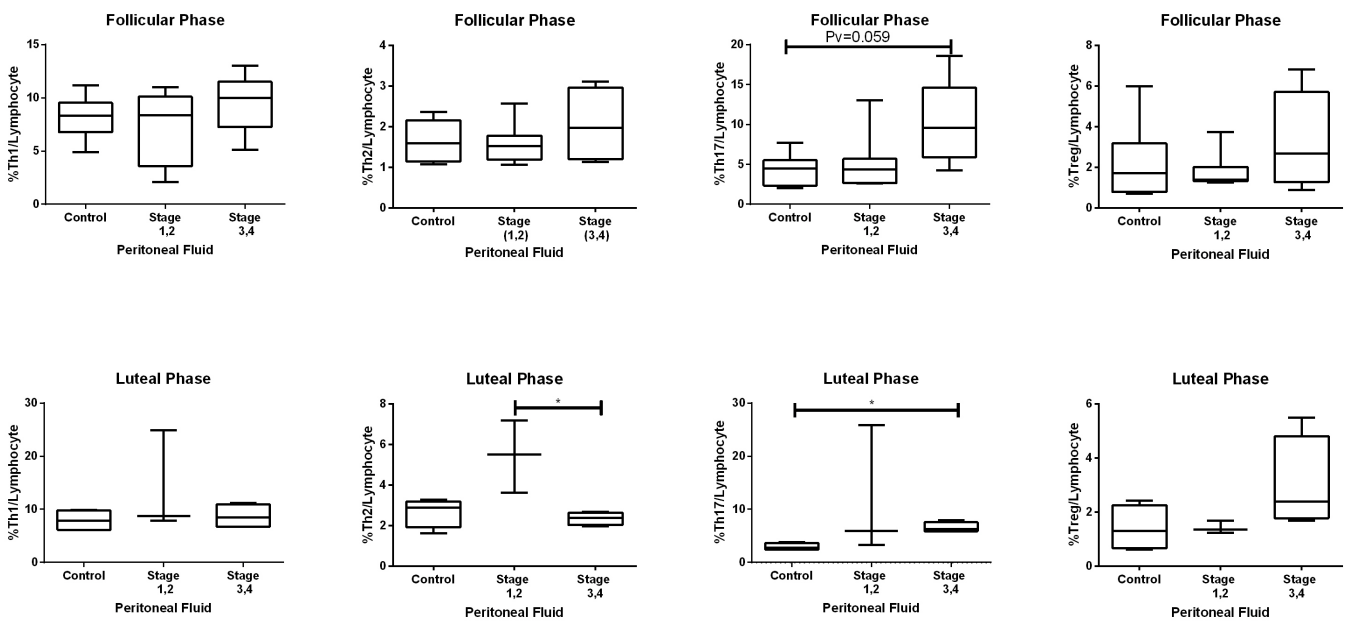

Fig. 3: Frequency of T helper (Th)1, Th2, Th17 and regulatory T (Treg) cells in peritoneal fluid (PF) in the control, early endometriosis (stage I, II) and advanced endometriosis (stage III, IV) groups in the follicular (upper row) and luteal (lower row) phases. Each box plot represents 25-75% quartiles with the median. *; P value<0.05.

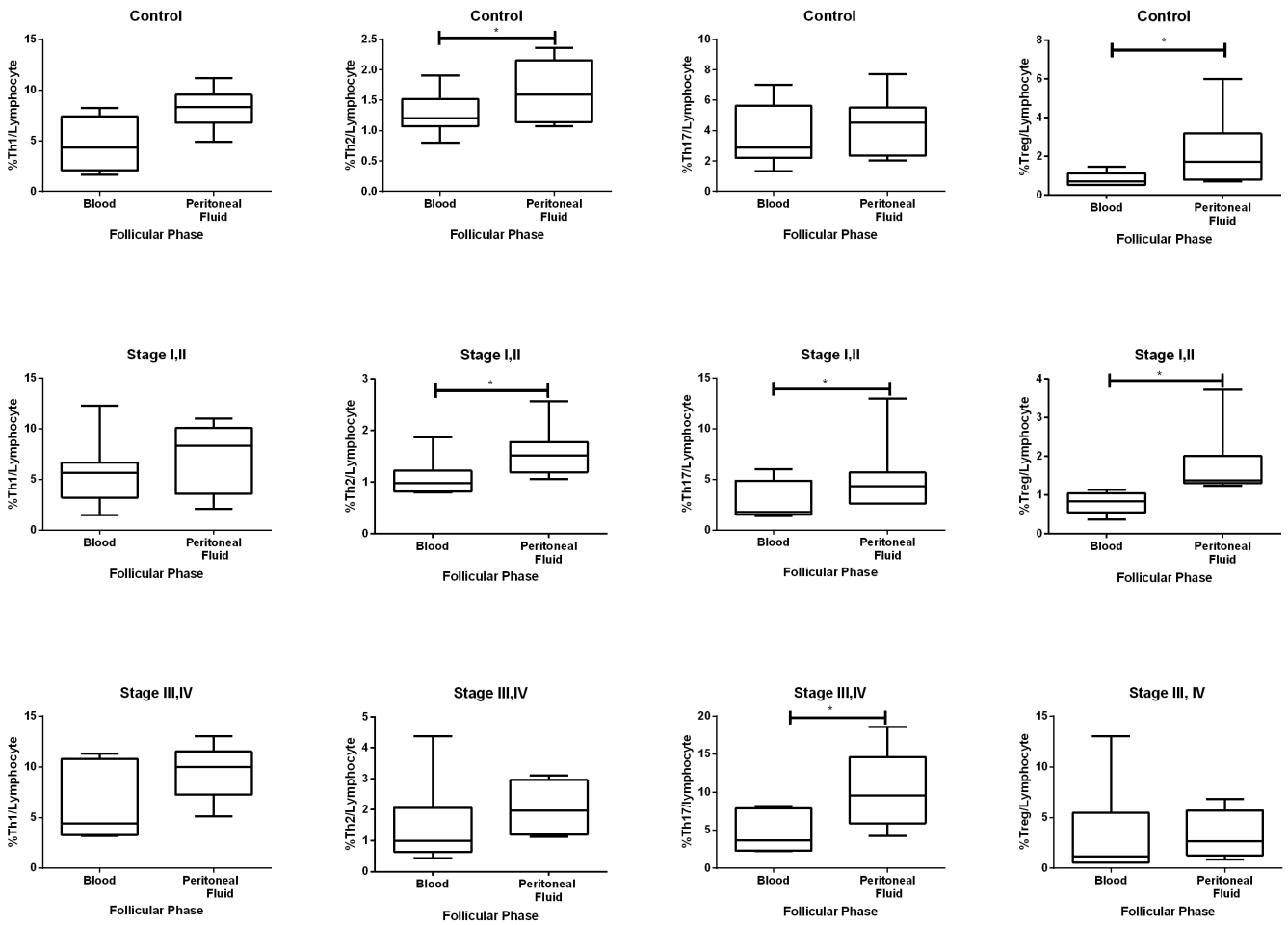

Fig. 4: Frequency of T helper (Th)1, Th2, Th17 and regulatory T (Treg) cells were compared in peritoneal fluid (PF) with peripheral blood (PB) in the control (upper row), early endometriosis (stage I, II) (middle row) and advanced endometriosis (stage III, IV) (lower row) groups in the follicular phase. Each box plot represents 25-75% quartiles with median. *; P value<0.05.

Comparison of the number of CD4+ T cell subsets between peritoneal fluid and peripheral blood in the three groups

The control group had increased numbers of Treg and Th2 cells in the PF compared to PB (both, P=0.046). This up-regulation was only detected in the follicular phase when compared with the luteal phase (Fig. 4).

In the early stages (I, II) of endometriosis, we observed increased numbers of Th2, Th17 and Treg cells in the PF compared to PB (P values were 0.005, 0.047 and 0.013, respectively). This observation was only noted in the follicular phase (P-values were 0.018, 0.028 and 0.018, respectively). Th17 cells were the only CD4+ T cell subsets that increased in the PF of stage III, IV endometriosis (P=0.013, Fig. 4). This change was also observed in the follicular phase (P=0.046). We did not observe any change in quantity of these T cell subsets between the PB and PF in the luteal phase in any of the groups.

Discussion

Endometriosis is a complex disorder with variable immune disturbances (18, 19); however, the role of CD4+ T lymphocytes deviation in different stages of disease is not completely elucidated.

In this study, we compared the frequencies of Th1, Th2, Th17 and Treg cells between early and advanced stages of endometriosis, and in women without endometriosis, as a control group. Because the population of immune cells varies according to hormonal changes in the menstrual cycle (20), we also compared these cells in the follicular and luteal phases. We found no systemic deviation in frequency of CD4+ T subsets in different stages of endometriosis; however, Th17 cells were increased in the PF in patients with advanced endometriosis compared to controls.

First, we compared the numbers of four CD4+ T subsets in peripheral blood between the three groups. Our results did not show any significant differences in frequency of blood Th1, Th2, Th17 and Treg cells between the different groups. The systemic deviations of CD4+ T cell numbers between three groups were not reflected in our study. We found one study by Takamura et al. (18) that compared all four CD4+ T subsets concurrently in blood from patients

with stage III, IV endometriosis (10 samples; 6 follicular and 4 luteal phases) with 10 normal individuals (4 follicular and 6 luteal phases). Similar to our results, they indicated no systemic changes in Th2, Th17 and Treg cells between the endometriosis and control subjects (18). However, these researchers confirmed an increased number of Th1 cells in the blood of patients with endometriosis (18). This finding differed from our results.

Regarding Th1 and Th2 cells, Szyllo et al. reported diminished IFN-γ levels and augmented IL-4 production by stimulated blood T cells of endometriosis patients; however, the authors did not divide the T cells into CD8 or CD4 cells (21). Also, the results of another study confirmed increased systemic CD4+IL-4+ and CD4+IL-10+ cells in patients with endometriosis compared to a control group (10). Our findings about circulatory Th2 frequencies were not consistent with these studies (10, 21).

In terms of blood Th17 cells, three independent studies demonstrated no significant differences in PB Th17 cell numbers between endometriosis and control groups (14, 18, 22), which supported our findings.

There are contradictory results concerning the frequency of Treg cells in blood samples between endometriosis and healthy women (11, 12, 18, 22). Although Olkowska-Truchanowicz et al. have reported decreased numbers of blood Treg cells in endometriosis (12), other investigators (11, 18, 22) reported that blood Treg cells remained unchanged between endometriosis and controls, which supported our results. Olkowska-Truchanowicz et al. compared the number of Treg cells in 17 blood samples obtained during the follicular phase from women with advanced stages (III, IV) of endometriosis to 15 samples from the follicular phase in control women without endometriotic foci. They introduced CD4+CD25highFoxP3+ cells as the Treg cell population (12). On the other hand, Gogacz et al. investigated the numbers of Tregs in two groups with endometriosis (15 samples in early stages and 7 samples with advanced endometriosis) and 20 women with unexplained infertility without any evidence of endometriosis. These researchers introduced CD4+CD25+FoxP3+ cells as Tregs and reported similar numbers of Treg cells between mild and advanced endometriosis (11). Khan et al. evaluated the frequency of Tregs amongst three groups – early stage endometriosis (n=15), advanced stage endometriosis (n=24) and control women (n=21), who were in different menstrual phases. Like other researchers, this group introduced CD4+CD25+FoxP3+ as Tregs and reported similar numbers of blood Tregs in endometriosis and control samples (22).

Next, we compared the number of different CD4+ T subsets locally in the PF. Th17 cells were the only CD4+ T cells increased in the PF of stage III, IV endometriosis relative to the control group. This trend was observed in both the follicular and luteal phases. We could not find any article that compared the number of all four CD4+ T subsets in the PF between endometriosis and control groups; however, Takamura et al. compared all four subsets in endometriotic and normal endometrial tissues, and reported increased Th17 cells in ectopic tissue compared to eutopic endometrium (18). Gogacz et al. compared the numbers of CD3+CD4+IL-17+ T cells between 22 infertile women with endometriosis (mild endometriosis n=15; severe endometriosis n=7) and 20 infertile women without any evidence of endometriosis. All samples were taken during the follicular phase. These researchers reported that the percentage of Th17 cells in the PF was higher in the higher stages of endometriosis (III, IV) compared to the lower stages (I, II) in the follicular phase; however, they did not observe any significant difference in the number of Th17 cells in the PF between the endometriosis and healthy groups. They concluded that a correlation existed between the frequency of Th17 in PF and severity of endometriosis (14). Chang et al. reported increased numbers of Th17 cells in the PF in endometriosis (stage I, II and stage III, IV) compared to control women. In comparison with other studies, the sample size of their study was more similar to our study and the results that pertained to the number of Th17 cells between stage III, IV and controls were concordant with our results (23). Our results suggested predominant Th17 responses in stage III, IV of endometriosis relative to the control group.

Th17 cells play prominent roles in induction of inflammation and development of endometriosis. IL17 stimulates secretion of IL-8 by endometrial stromal cells (ESCs), expression of cyclooxygenase 2 and proliferation of ectopic endometrial cells. IL-8 could induce the adhesion of stromal cells to fibronectin. In this way, IL-17 could increase endometriotic lesions (24-26).

Although some studies demonstrated increased levels of CD4+CD25+FoxP3+ cells in the PF (12, 22, 27) and ectopic tissue (28) of endometriosis subjects, we did not detect this increase in our study. On the other hand, some researchers reported unvarying numbers of Tregs in PF (11) or ectopic lesions (18, 29) in endometriosis, which supported our results.

We did not detect any significant difference in frequency of Th2 cells in the PF from different stages of endometriosis and controls; however, our results indicated higher numbers of Th2 cells in the PF exclusively in the luteal phase in the early stages of endometriosis compared to advanced stages. There are studies that illustrated deviation to Th2 immune responses, directly through investigating the expression of Th2 related cytokines or transcription factors (10, 30) or, indirectly (18) through deviation of Th1/Th2 proportions in endometriosis.

In the third step, we compared the frequency of different CD4+ T cells between the PF and PB in the different groups. We performed this comparison based on the menstrual cycle. We observed a fluctuation in CD4+ numbers between PB and PF only in the follicular phase. This finding could be related to the dominancy of oestrogen in the follicular phase and of progesterone

in the luteal phase (31). Endometriosis is characterized by a dependency on oestrogen (32) and resistance to progesterone (33), which may guide us to observed alterations in follicular phase.

Our findings indicated that Th2 and Treg cells increased in the PF of the control group relative to blood. High doses of oestrogen could deviate immune responses from Th1 to Th2 (34). On the other hand, oestrogen levels increase from the pre-ovulatory to the end of the menstrual phase in the PF compared to blood (35, 36). Thus, in the last days of the follicular phase, which was compatible with the time of sampling, high peritoneal doses of oestrogen might cause shift of immune responses to Th2 (34). This could confirm the up-regulation of Th2 cells in the PF in control samples from the follicular phase. On the other hand, oestrogen could bind to its receptor on the surface of CD4+CD25-T cells and convert them to CD4+CD25+T cells that express FOXP3 and IL-10 (37). Increased oestrogen levels in PF in the pre-ovulatory phase could lead to upraised levels of Treg cells in PF compared to blood in the control samples. Compatible with our results, Khan et al. reported a modest increase in Treg cells in PF of normal subjects compared to PB (22).

The number of Th17 cells, as well as Th2 and Treg cells, in the early stages of endometriosis (stage I, II) were elevated in the PF compared to blood. The increased levels of Th17 cells in the PF compared to blood in stage (I, II) endometriosis could be explained by two assumptions. The first is related to production of C-C chemokine ligand 20 (CCL20) by ectopic endometrial cells. CCL20 is stimulated by inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and IL-1β in the local microenvironment and it can attach to C-C chemokine receptor type 6 on Th17 cells, which leads to recruitment of these cells to the PF (19, 38). The second assumption is related to increased differentiation of naïve T cells to Th17 at the local site of endometriotic tissue, which is rich in transforming growth factor beta (TGF-β), IL-23 and IL-6 (9, 39, 40).

With progression of endometriosis to stage III or IV, we found that only Th17 cells were increased in PF compared to PB. Similar to our findings, Gogacz et al. reported that there were elevated levels of Th17 cells in the PF of stage III, IV endometriosis, which was proportional to blood (14); however, other researchers reported no changes in the numbers of Th17 cells between the PF and PB in endometriosis subjects (22). It seems that Th17 cells could be involved in endometriosis development by production of inflammatory cytokines and angiogenic factors. Chang et al. confirmed that IL-27 from macrophages or ESCs could stimulate production of IL-10 by Th17 cells, which induces progression of endometriosis (23). In the higher stages of endometriosis, Th17 cells cause cyclooxygenase 2 induction, inflammation and cell adhesion. In this way, these cells could promote the development of endometriosis.

We did not locate any article that compared all four subsets of CD4+ T cells in both PB and PF between early and advanced stages of endometriosis and controls. However, our study had some limitations such as the small sample size. In addition, we did not use anti-CD3 antibody for more specific determination of Th and Treg cells.

This study provided a comprehensive view of systemic and local changes in Th1, Th2, Th17 and Treg cells in early and advanced stages of endometriosis. Identification and application of local factors that affect Th17 cells can provide novel approaches for future treatments of advanced endometriosis.

Conclusion

In this study, apparent changes in systemic CD4+ T cells were not found between different stages of endometriosis and the controls; however, our results indicated a predominance of Th17 cells in the local microenvironment of the PF. Th17 superiority could be related to disease progression because we observed a higher number of Th17 cells in the more advanced stages of endometriosis. However, future studies should be conducted to evaluate this relationship at the cellular and molecular levels.

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Authors' Contributions

F.P.; Participated in data collection, doing technical procedures, analysis and interpretation of data. H.A.; Contributed in doing technical procedures. F.N.A.; Had a role as an administrative technical support. F.D.T., R.H., Z.A.; Provided the samples. R.M., S.R.; As supervisors, participated in conception and design, analysis and interpretation of data. S.R.; Had an extra role in drafting the manuscript and statistical analysis. All Authors read and approved the final manuscript.

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Original Article

Predictive Value of Endometrial Length Measurement by Transvaginal Ultrasound and IVF/ICSI Outcomes

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Abstract

Background: The purpose of this study to determine the relationship between endometrial length and positive pregnancy test in patients who underwent assisted reproductive technology (ART).

Materials and Methods: This cross-sectional study included patients who were referred for in vitro fertilisation/intracytoplasmic sperm injection (IVF/ICSI) therapy from 2013 to 2016. All nulliparous women who met the inclusion criteria were between 20-38 years of age and presented for ultrasound measurements prior to fresh embryo transfer (ET). Endometrial length was measured by transvaginal ultrasound (TVS) with a Medison Accuvix device on the day of human chorionic gonadotropin (hCG) administration. The relationship between endometrial length and treatment success was assessed. The independent sample t test, receiver operating characteristic (ROC) curve and the area under the curve (AUC) index and chi-square test were used for data analysis. P values <0.05 were statistically significant.

Results: There was a significant relationship between endometrial length (41.5%) and treatment success (P<0.05). The endometrial length of 41.5(mm) with a sensitivity of 66.7%, specificity of 50.6%, positive predictive value of 46.8%, negative predictive value of 69.4%, and efficiency of 56.62% can be used as a proper cut-off point with an AUC of 0.63.

Conclusion: The value of 41.5(mm) for endometrial length can be used as a proper cut-off point for prediction of a higher ART success rate. We recommend TVS as the first step for assessment of uterine and endometrium receptivity in the ART cycle.

Keywords: ART, Endometrial Length, Ultrasonography

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Introduction

Infertility is considered as a global public health issue that affects almost 186 million people worldwide (1). Infertility is a consideration in one out of eight reproductive age women and one out of ten men of reproductive age (2). Despite improvements in assisted reproductive technology (ART), only less than 50% of patients achieve success in terms of live-birth deliveries (3).

A successful pregnancy outcome for patients who undergo ART depends on embryo quality, favourable intrauterine environment, and a skilful *in vitro* fertilisation (IVF) laboratory (4). Another important factor is endometrial receptivity (5, 6).

There are several studies that discuss predictive value of endometrial characteristics in terms of ART success, such as Echo pattern and endometrial thickness (7). However, in women without uterine abnormalities, little is known about uterine and endometrial length (8). In recent studies, a catheter or hysterometer has been used for uterine length measurements. In a similar study, the researchers used transvaginal ultrasound (TVS) and compared implantation and clinical pregnancy rates between groups that had with uterine lengths >7.0 cm, 7-7.9 cm and >9.0 cm. The results were varied and there was much controversy in the findings (9-11).

The question arises as to whether an association exists between the endometrium length (from the internal ostium of the cervix to the uterine fundus) and the incidence of clinical pregnancy in women with normal uterine anatomy who undergo IVF or intracytoplasmic sperm injection (ICSI).

This study aimed to determine the relationship between

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endometrial length and positive pregnancy in ART patients. We hypothesized that an association exists between uterine length and positive pregnancy test in patients who underwent IVF/ICSI.

Materials and Methods

Study population

This cross-sectional study included 166 patients who referred to Royan Institute, Tehran, Iran for IVF/ICSI therapy and ET from 2013 to 2016. The study and protocols were approved by the Royan Institute Ethics Committee (Ethics number: IR.ACECR.ROYAN.REC.1396.146) and informed written consent was obtained from the patients.

All nulliparous women aged 20-38 years of age who presented for ultrasound measurements prior to fresh ET were considered for inclusion. The inclusion criteria consisted of: first IVF/ICSI treatment cycle, absence of any anomaly in the endometrium or myometrium, and no histories of abortion or curettage, hysteroscopy, polypectomy or myomectomy. Patients who had endometrial lengths between 7-14 mm were enrolled. Finally, 166 patients who met the eligibility criteria were selected.

Uterine length measurements

The OCP-LD cycle was begun for all patients from the second to fifth days of menstruation. From day 17 of menstruation, the patients received daily subcutaneous injections of GnRH to 500 µg/d. At 12-14 days after the GnRH-a, the dose was reduced to 200 µg/d. Then, FSH stimulation was begun according to ovarian reserve and the patient's age. Subsequently, the dosage was increased or decreased according to the patient's needs.

Assessment of follicle growth and endometrial condition were performed by vaginal ultrasound. If the patient had at least three follicles that were ≥18 mm in two ovaries, we measured the endometrial length (from the internal ostium of the cervix to the fundus) of the uterus by TVS on the day of the human chorionic gonadotropin (hCG) administration (Fig. 1).

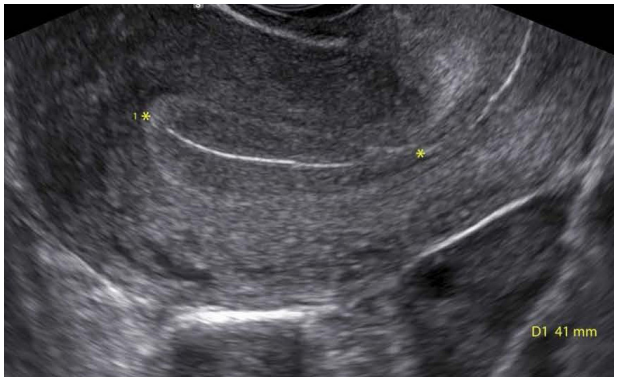

Fig. 1: Evaluation of endometrial length of uterus by vaginal ultrasound.

Outcome measures

Chemical pregnancy was defined as cycles that resulted in the identification of serum beta-hCG but without the subsequent development of a gestational sac. Clinical pregnancy denoted cycles that resulted in ultrasound confirmation of an intrauterine gestational sac. We considered clinical pregnancy as the positive outcome of IVF/ICSI.

Statistical analysis

Cases were divided into two groups - positive and negative for pregnancy. Variables of age, ET number and endometrial thickness, weight, height and body mass index (BMI) were similar in the positive and negative pregnancy groups.

Data was entered into SPSS Version 21 software for statistical analysis. The relationship between endometrial length and treatment success was assessed. We used the independent sample t test, multiple logistic regression, receiver operating characteristic (ROC) curve and the area under the curve (AUC) index for data analysis. P values<0.05 were considered to be statistically significant.

Results

Overall, 166 cases (IVF or ICSI) that met the inclusion criteria entered the study. Patients were between 20 and 38 years of age with a mean age of 29.08 ± 4.24 years. The overall pregnancy rate was 39.8%.

The adjusted P value was obtained by adjusting for age, height, weight, BMI, ET and thickness for the relationship between endometrial length and pregnancy (positive and negative). The results showed that weight, height and BMI had a significant effect on this relationship (Table 1).

Table 1: Multiple logistic regression results

Variables	B(SE)	P value	OR	95% CI for OR	
				Lower	Upper
Age (Y)	0.002 (0.046)	0.965	1.002	0.916	1.096
Height	-0.763 (0.284)	0.007	0.466	0.267	0.813
Weight(kg)	0.866 (0.338)	0.010	2.379	1.227	4.612
BMI (kg/m^2)	-2.242 (0.887)	0.012	0.106	0.019	0.605
ET number	-0.361 (0.282)	0.201	0.697	0.401	1.212
Endometrial thickness	0.040 (0.103)	0.698	1.041	0.851	1.273
Endometrial length	-0.094 (0.041)	0.022	0.910	0.839	0.987
Constant	127.632 (46.008)	0.006	26.910	-	-

BMI; Body mass index, ET; Embryo transfer, CI; Confidence interval, OR; Odds Ratio, B; Beta, and SE; Standard error.

The variables of age, ET number, endometrial thickness, weight, height and BMI were similar in the positive and negative pregnancy groups (Table 2).

Table 2: Independent t test results for comparing the demographic and laboratory characteristics of the positive and negative pregnancy groups

Variables	Pregnancy				P value*
	Positive (N=66)		Negative (N=100)		
	Mean	SD	Mean	SD	
Age (Y)	29.23	3.76	28.98	4.55	0.714
Height(cm)	161.56	5.90	160.25	6.21	0.177
Weight (kg)	66.62	8.49	66.32	11.52	0.847
BMI (kg/m^2)	25.58	3.42	25.74	3.61	0.772
ET number	2.34	0.55	2.19	0.72	0.167
Endometrial thickness (mm)	11.4	2.1	10.82	2.3	0.08

*; Independent sample t test (statistical significance level: 0.05), BMI; Body mass index, ET; Embryo transfer, and SD; Standard deviation.

The endometrial length range from 28-58 mm and thickness range was 7-14 mm. We initially used the ROC curve and AUC index to determine the cut-off length of the endometrium in terms of treatment success and positive pregnancy (Fig. 2).

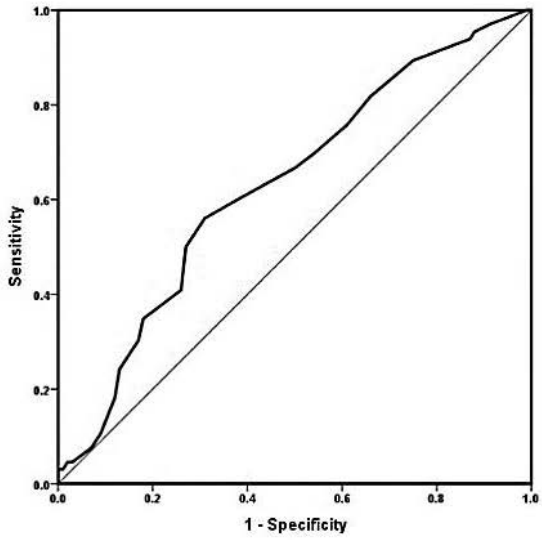

Fig. 2: Receiver operating characteristic (ROC) curve.

We determined that the AUC was 0.63, which was acceptable and significant because it was greater than 0.6 (P<0.05, Fig. 2, Table 3). Thus, the cut-off point was determined to be 41.50, which could be used as a proper cut-off point.

Table 3: AUC index results

AUC	SE	P value	95% CI	
			Lower bound	Upper bound
0.630	0.044	0.0044	0.544	0.716

AUC; Area under the curve, CI; Confidence interval, and SE; Standard error.

There was a significant relationship between endometrial length and treatment success (P<0.05, Table 4).

We determined that the 41.5 value had a sensitivity of 66.7%, specificity of 50.6%, positive predictive value of 46.8%, negative predictive value of 69.4%, and efficiency of 56.62% and could be used as a proper cut-off point.

Table 4: Chi-square test results for new cut of point

Endometrium length		Pregnancy		Total	P value
		Positive (n=66)	Negative (n=100)		
≤ 41.5	N	22	50	72	0.034
	Endometrium length (%)	30.6%	69.4%	100%	
	Pregnancy rate (%)	33.3%	50%	43.4%	
> 41.5	N	44	50	94	
	Endometrium length (%)	46.8%	53.2%	100%	
	Pregnancy rate (%)	66.7%	50%	56.6%	

We determined that the 41.5 value had a sensitivity of 66.7%, specificity of 50.6%, positive predictive value of 46.8%, negative predictive value of 69.4%, and efficiency of 56.62% and could be used as a proper cut-off point.

Discussion

Infertility treatments are expensive. Because the implantation rates are low, it is necessary to find a way to predict the success of an ART cycle (12).

There are limited studies about the diagnostic value of endometrium length using TVS in infertile women who undergo IVF/ICSI and the determination of its cut-off. We found that the IVF/ICSI success was higher in cases that had greater endometrial length. In this study, we noted that the value of 41.5 with a sensitivity of 66.7% and specificity of 50.6% could be used as a proper cut-off point with an AUC of 0.63. This was the first time that an endometrial length cut-off point for IVF/ICSI has been assessed.

A similar study that assessed the predictive value of endometrial length and success of the IVF/ICSI confirmed our result. Abdel et al. stated that the depth of ET is one of the most important factors in IVF/ICSI adaption (13). An appropriate endometrial length is necessary. In the current study, we concluded that IVF/ICSI success was higher in cases that had higher endometrial lengths.

As a physiological view, we noted the effects of oestrogen on the endometrium and success of the ART cycle, which correlated with uterine size (9). Increased endometrial length observed by TVS might be an index for a sufficient hormonal level and appropriate environment of uterine, and would result in a better ART outcome.

Hawkins et al. measured the uterine length (from the fundus to the external ostium of the cervix) in ART cycles before ET. They noted that the implantation rate and clinical pregnancies were higher in cases with uterine lengths between 7 and 9 cm, which were consistent with our finding, although it was not statistically significant (8). In contrast, Firouzabady et al. did not report any association

between uterine length and IVF/ICSI adaption (10, 11).

There were some differences between our study and previous studies. We measured the endometrial length with TVS; however, we omitted the cervix length from the measurement. Therefore, our assessment and its relationship with ART outcome would be more logical. The endometrial length was measured by an experienced sonographer in order to eliminate any inter-observer bias.

Some studies confirmed the positive effect of endometrial thickness in success of IV. For this, we math cases in term of endometrial thickness. Momeni conducted a meta-analysis and reported that women, who underwent IVF which resulted in positive pregnancy outcomes, had higher mean endometrial thicknesses compared with a non-pregnant group (14).

We suggest that additional studies be conducted with larger sample sizes. The combined uterine index cut-off points and profile for ART success that includes thickness, echo pattern, position and length might improve the ART outcome. More studies should evaluate these findings.

Conclusion

We determined that the value of 41.5 for endometrial length had appropriate sensitivity, specificity, positive predictive value, negative predictive value and efficiency, and could be used as a proper cut-off point for prediction of a higher ART success rate. We recommend TVS should be performed as the first step for uterine and endometrium receptivity assessment in the ART cycle.

Acknowledgements

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Authors' Contributions

F.A., F.P.; Participated in study design, data collection and evaluation. F.A.; Performed Ultrasound examinations. F.P; Participate in follow up the outcomes of the patients after ET. A.H.M.; Contributed extensively in interpretation of the data and the conclusion. All authors read and approved the final version of the manuscript.

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Original Article

Influence of Catheter Type and Tenaculum Use on Intrauterine Insemination Outcome

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Abstract

Background: We investigated the impact of the choice of catheter type and tenaculum use on pregnancy related outcomes in intrauterine insemination (IUI) treatments.

Materials and Methods: A total of 338 consecutive IUI cycles were assessed in this retrospective study. Participants were divided according to the insemination technique - soft catheter (group 1; n=175), firm catheter (group 2; n=100), or tenaculum (group 3; n=63). Clinical, laboratory, semen parameters and pregnancy related outcomes were compared.

Results: Demographic characteristics and laboratory parameters were similar between the groups (P>0.05). The clinical pregnancy rate (CPR) was significantly higher in the firm catheter (19%, 19/100) and tenaculum (31.7%, 20/63) groups compared to the soft catheter group (5.1%, 9/175, P<0.001). There were no significant differences between the groups in live birth and miscarriage rates per clinical pregnancy (P>0.05).

Conclusion: Our findings indicate that the use of a firm catheter or tenaculum for IUI might result in a higher CPR, but might not have a considerable effect on the live birth rate (LBR). Further prospective randomized studies are required to determine the long-term effects of the catheter type or tenaculum use on IUI success.

Keywords: Catheter, Clinical Pregnancy Rate, Intrauterine Insemination, Live Birth Rate, Tenaculum

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Introduction

Intrauterine insemination (IUI) is an effective and widely used treatment that is mainly recommended for male factor, minimal and mild endometriosis, cervical factor or unexplained infertility cases. The term unexplained infertility includes infertile pairs whom ovulatory function, tubal passage and semen analysis are normal. The procedure involves the direct delivery of washed spermatozoa in order to bypass the cervix and increase the sperm volume at the site of fertilisation (1-4).

In the literature, the pregnancy rate reported in IUI cycles varies widely from 4-40% (5, 6). This great variation might be related to female age, type and duration of infertility, sperm parameters and technical aspects (7, 8). Under the heading of technical aspects, in particular, the catheter type can possibly influence pregnancy outcomes for IUI (9). In many recent *in vitro* fertilisation (IVF) studies, the consistency of the embryo transfer (ET) catheter has been determined to be a considerable factor in the success of ET, whereas the influence of catheter type in IUI is still controversial(10).

In a meta-analysis of 1871 IUI cycles, it was reported that endometrial scratch injury was associated with higher clinical pregnancy and ongoing pregnancy rates (11). The authors suggested that the local endometrial trauma and subsequent acute inflammatory process might have prompted decidualization and improved the implantation rate. On the other hand, Balci et al. reported that the immediate uterine contractions induced by tenaculum application to the cervix during IUI might enhance sperm transport to the ampulla and result in a higher pregnancy rate (12). In this study, we aimed to investigate whether firm catheter introduction or tenaculum use for IUI might affect pregnancy related outcomes through local endometrial injury, induced myometrial contractions, or in via other means as suggested above.

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Materials and Methods

This retrospective study was conducted on a total of 338 IUI cycles carried out at the Department of Obstetrics and Gynaecology, Zekai Tahir Burak Women's Health Education and Research Hospital, Ankara, Turkey between 2015 and 2017. Written informed consent was obtained from the participants for future use. The patients were assigned to three groups - IUI performed with a soft catheter (group1, n=175); firm catheter (group 2, n=100); or with the assistance of a tenaculum to ease the introduction (group 3, n=63). The Ethics Committee of Zekai Tahir Burak Women's Health Education and Research Hospital, Ankara, Turkey approved this study (reference number: 2017/20), which was conducted in accordance with the Declaration of Helsinki 2013 Brazil version (20796219-724.087).

Inclusion criteria for IUI consisted of unexplained infertility with a minimum duration of one year, age under 35 years, normal uterine cavity, at least one patent tube, basal follicle stimulating hormone (FSH) <10 mIU/mL, no history of gynaecologic surgery and at least 5 million motile spermatozoa for the male partner. The first and subsequent cycles were admitted to the study. Exclusion criteria were diminished ovarian reserve and male infertility.

Ovarian stimulation was achieved by recombinant FSH (recFSH; follitropin alfa, Gonal-F, Serono, Turkey, Istanbul; follitropin beta Puregon, Organon, Turkey) and human menopausal gonadotropin (hMG; Ferring, Turkey) based on the patient's historical and clinical factors. recFSH andhMG were administered in a low-dose step up stimulation protocol that began on the second day of the menstrual cycle. Ovarian response was recorded through ultrasound examination of antral follicles and by determination of serum oestradiol (E2) levels. Ovulation was triggered by human chorionic gonadotropin (hCG) (u-hCG, Pregnyl, Organon,Turkey; rec-hCG, Ovitrelle, Serono, Turkey) when one or two follicles reached a diameter of ≥18 mm.Finally, IUI was carried out after 36 hours of hCG administration.

Semen was collected by masturbation after 3-5 days of sexual abstinence and a few hours prior to the scheduled insemination time. The spermatozoa were washed free from the seminal liquid and prepared for insemination by the swim-up technique. The difficulty of the insemination was determined with respect to the comments of two physicians with the same techniques. For the initial attempt to cannulate the cervix, a soft catheter (Allwin Medical Devices, CA, USA) was preferred; thereafter, due to the difficulty degree of introduction, a firm catheter (Technocath Medical Scientifics, Ankara, Turkey) or tenaculum were used for the insemination. Finally, the sperm sample (0.5-1 mL) was slowly injected through the catheter into the uterine cavity.

Approximately two weeks after insemination, all participants underwent pregnancy tests. The endpoints of the study were the clinical pregnancy rate (CPR), which was defined as evidence of a gestational sac after more than six weeks gestation confirmed by ultrasound and the live birth rate (LBR), which was defined as the delivery of a live foetus after 20 weeks of gestational age.

Statistical analysis

Statistical analysis was performed using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to examine continuous variables with normal and abnormal distributions. One-way analysis of variance was used for normally distributed continuous variables and the Kruskal-Wallis test for abnormally distributed continuous variables. Nominal variables were analysed by Pearson's chi-square or Fisher's exact test, when applicable. Continuous variables are presented as mean-standard deviation (SD) or median (min–max), and categorical variables are presented as the number of cases and percentage. A P value of <0.05 was considered to be significant. Power analysis and sample size calculations were carried out using the G*Power 3.0.10 program (Franz Faul, Universität Kiel, Kiel, Germany).

Results

From the 361 initial participants, 22 (6.09%) dropped out of the study. Therefore, 338 participants were included in the study: 175 in group 1 that used a soft catheter, 100 in group 2 that used a firm catheter and 63 in group 3 that used a tenaculum to ease the introduction (Fig. 1).

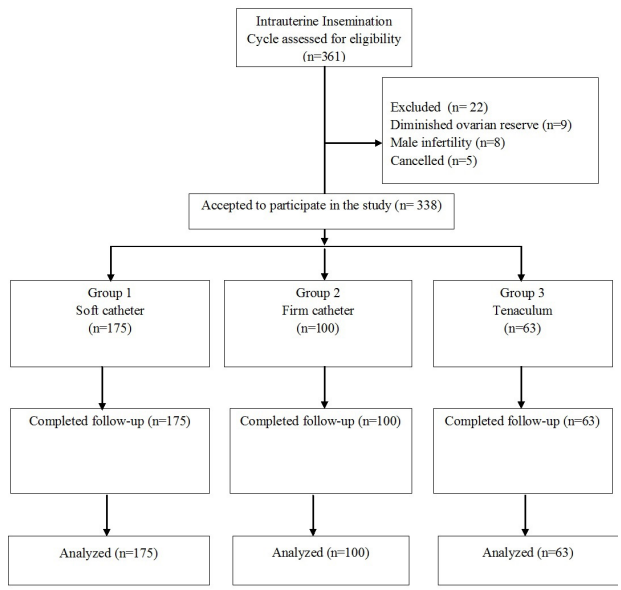

Fig. 1: Enrollement and follow-up of the study subjects.

Table 1 lists the participants' demographic characteristics and laboratory parameters. There were no significant differences between groups regarding age, body mass index (BMI), baseline hormone profiles, type and duration of infertility. Dose and type of gonadotropin (recFSH versus hMG), u-hCG versus rec-hCG utilization for trigger, luteal phase support, antral follicle count, number of follicles >17 mm and endometrial thickness on hCG day were comparable in all groups. Total progressive motile sperm count (TPMSC) and sperm morphology were also similar between the groups (P>0.05).

Table 1: Demographic characteristics and laboratory parameters of the patients

	Group 1 Soft (n=175)	Group 2 Firm (n=100)	Group 3 Tenaculum (n=63)	p
Age (Y)	26.53 ± 4.51	27.05 ± 4.98	27.97 ± 4.33	0.103[a]
BMI (kg/m^2)	25.05 ± 3.46	24.66 ± 3.44	24.05 ± 3.19	0.135[a]
Primary infertility (%)	126 (72.0)	80 (80.0)	48 (76.2)	0.322
Secondary infertility (%)	49 (28.0)	20 (20.0)	15 (23.8)	
Duration of infertility (Y)	3 (1-12)	3 (1-14)	3 (1-16)	0.589[b]
Baseline FSH (IU/L)	6.78 ± 1.72	7.01 ± 1.88	6.44 ± 1.35	0.120[a]
Baseline LH (IU/L)	4.51 ± 1.56	4.77 ± 1.75	4.66 ± 1.85	0.454[a]
Baseline E2 (pg/mL)	41.21 ± 17.50	40.52 ± 14.04	41.19 ± 16.16	0.941[a]
Antral follicle count	10 (6-16)	10 (7-16)	10 (4-16)	0.115[b]
hMG(%)	85 (48.6)	45 (45.0)	23 (36.5)	0.165
rFSH (%)	90 (51.4)	55 (55.0)	40 (63.5)	
Duration of stimulation (D)	5 (5-16)	5 (5-13)	5 (5-16)	0.478[b]
rFSH dose (IU)	75 (37.5-225)	75 (37.5-187.5)	75 (37.5-112.5)	0.522[b]
hMG dose (IU)	112.5 (75-150)	150 (75-225)	75 (75-225)	0.251[b]
Number of cycle	2 (1-5)	2 (1-5)	2 (1-5)	0.723[b]
Number of >17 mm follicles	1 (1-3)	1 (1-4)	1 (1-3)	0.763[b]
Trigger Pregnyl (%)	151 (86.3)	81 (81.0)	54 (85.7)	0.498
Ovitrelle (%)	24 (13.7)	19 (19.0)	9 (14.3)	
E2 on hCG administration day (pg/mL)	398.54 ± 154.85	338.13 ± 15.01	537.92 ± 375.66	0.196[a]
TPMSC (x10^6)	51.37 ± 22.17	53.35 ± 25.78	52.62 ± 25.57	0.801[a]
Morphology	6.94 ± 1.77	7.04 ± 1.60	6.98 ± 1.70	0.903[a]
Endometrial thickness on hCG day (mm)	8.94 ± 1.73	8.96 ± 1.72	9.02 ± 1.82	0.954[a]
Trilaminer sign (%)	159 (90.9)	94 (94.0)	56 (88.9)	0.476
Luteal phase support (%)	38 (21.7)	26 (26.0)	21 (33.3)	0.194

Data are presented as mean ± SD or n(%). SD; Standard deviation, [a]; One-way ANOVA test, [b]; Kruskal Wallis test, BMI; Body mass index, FSH; Follicle stimulan hormone, LH; Luteinizan hormone, E2: Estradiol, hMG; Human menopausal gonadotropine, hCG; Human corionic gonadotropine, and TPMSC; Total progressive motile sperm count. P<0.05 is statistical significant.

Table 2 summarizes the pregnancy related outcomes. There were 48 clinical pregnancies with a CPR of 14.2% (48/338) and the LBR per cycle was 11.53% (39/338), which was comparable to recent data (12). The CPR was significantly higher in the firm catheter (19%, 19/100) and tenaculum groups (31.7%, 20/63) compared to the group that used the soft catheter (5.1%,9/175) (P<0.001). Both the live birth/clinical pregnancy [84.2% (16/19), 80.0% (16/20), 77.8% (7/9); P=0.736] and miscarriage/clinical pregnancy [15.8% (3/19), 20.0% (4/20), 22.2% (2/9); P=0.736] were comparable in all groups (Fig. 2).

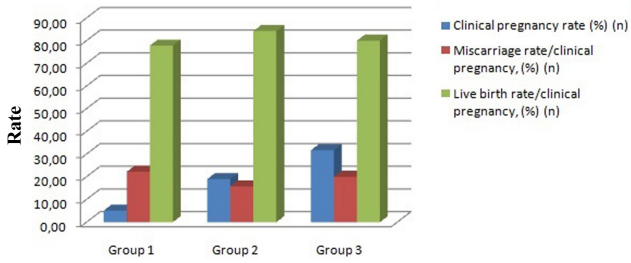

Fig. 2: Perinatal outcomes of the groups. Group 1; Soft, Group 2; Firm, and Group 3; Tenaculum.

Table 2: Pregnancy related outcomes of soft, firm catheter and tenaculum applied patients undergoing IUI treatment

	Group 1 Soft (n=175)	Group 2 Firm (n=100)	Group 3 Tenaculum (n=63)	P value
Clinical pregnancy rate)	5.1 (9/175)[a, b]	19 (19/100)[a]	31.7 (20/63)[b]	<0.001*
Miscarriage rate/clinical pregnancy	22.2 (2/9)	15.8 (3/19)	20.0 (4/20)	0.736
Live birth rate/clinical pregnancy	77.8 (7/9)	84.2 (16/19)	80.0 (16/20)	

Data are presented as n (%). *; Statistically significant, IUI; Intrauterine insemination, [a]; Group 1 versus Group 2, and [b]; Group 1 versus 3.

Discussion

IUI is a commonly used cost-effective line of treatment for infertility (1, 13). In the literature, the pregnancy rate of IUI widely varies (e.g., 4-40%) (5, 6). This variation in pregnancy rates might be related to many factors, including the type of catheter used. The consistency of the ET catheter has been considered a determining factor in the success of ET procedures, whereas the impact of catheter type on IUI has been not been thoroughly investigated and limited data are available (10, 14).

In a study conducted by Smith et al., the pregnancy rates were not statistically different between the soft and firm catheter groups when a gentle technique was used and the technician did not touch the top of the fundus with the catether. (15). Lavie et al. observed by sonography that the firm catheters disrupted the three layer pattern of the endometrium in some patients who underwent IUI; however, they reported the same overall pregnancy rate with soft catheters (16). Similar outcomes were obtained in other related IUI studies (13, 17, 18). The results of a Cochrane data analysis indicated that there was no evidence of any significant difference between soft and firm catheters for IUI in terms of pregnancy related outcomes or adverse events (19).

Park et al. reported no significant differences in the CPR between non-using and using a tenaculum during intrauterine insemination (20). In contrast, Balci et al. suggested that uterine manipulation by applying a tenaculum to the cervix increased immediate uterine contractility and resulted in a higher pregnancy rate when they used ultrasound guidance to record the frequency of uterine contractions after insemination (12). Similarly, in our study, there was significantly greater CPR in the firm catheter and tenaculum groups compared to the soft catheter group. This difference in the success of the IUI and IVF treatments depended on the catheter type, and might be due to the difference between the location and timing of events during both procedures. In IUI, fertilisation takes place at the ampulla, away from the endometrium that is presumed to be damaged by a firm catheter. If any negative effect occurs in the uterine cavity during IUI, it may be achieved both by the volume of inseminated sperm and by the period of time until implantation, which is enough for natural recovery. Furthermore, in the course of artificial insemination, the uterine contractions induced by tenaculum application or by introduction of firm catheter might cause an immediate increase in passage of the sperm to the fallopian tubes, shorten the arrival time to the ampulla, and might disappear just before the fertilisation (14, 19).

On the other hand, endometrial scratch injury is a technique suggested by several studies to improve implantation rates in women who undergo in vitro fertilisation and have histories of recurrent implantation failure (RIF). Its application in IUI is less common. This procedure consists of applying a local endometrial travma to induce an acute inflammatory process and release of growth factors or proinflammatory cytokines, which arepresumed to improve decidualization and a subsequent successful implantation (21, 22). In a meta-analysis of 1871 IUI cycles, it was reported that endometrial scratch injury was associated with a higher CPR (OR 2.27) and ongoing pregnancy rate (OR 2.04) in comparison with the controls (11). Therefore, we suggest that inserting a firm catheter into the uterine cavity might have induced a local endometrial trauma and a subsequent inflammatory cascade, which resulted in a higher pregnancy rate compared to the gentle touch with the use of a soft catheter.

The limitations of this study are its retrospective design and small sample size. The primary aim of this study was to determine the difference in CPR between groups. According to the post hoc power calculation, ourgroup sample sizes of 175, 100 and 63 achievedan 80% power to detect a difference of 0.039 between the null hypothesis, which both group proportions were 0.124 and the alternative hypothesis that the proportion in the other group was 0.254 with a significance level of 0.05.

Conclusion

This study showed that the application of a tenaculum or insertion of a firm catheter during the IUI might result in a higher CPR but does not alter LBR results. Further randomized prospective studies would be necessary to assess the long-term effects of catheter type and tenaculum use on IUI outcome.

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Authors' Contributions

P.G.C., A.S.O., M.K.P., N.Y.; Participated in study design, data collection and evaluation, drafting and statistical analysis. P.G.C., H.A.I., N.H.; Performed ovarian stimulation and prepared the participants for IUI pertaining to this component of the study. P.G.C., A.S.O., H.A.I.; Contributed extensively in interpretation of the data and the conclusion. All authors read and approved the final manuscript.

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Original Article

Evaluation of *Muc1* Gene Expression at The Time of Implantation in Diabetic Rat Models Treated with Insulin, Metformin and Pioglitazone in The Normal Cycle and Ovulation Induction Cycle

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Abstract

Background: Mucin-1(Muc1) is one of the first molecules in the endometrium that confronts implanting embryos. There is insufficient knowledge about the impacts of diabetes and drugs developed for diabetes treatment on expression of this molecule at the time of implantation. Therefore, this study aimed to investigate the impacts of diabetes and insulin, metformin and pioglitazone on Muc1 expression at the time of implantation.

Materials and Methods: This experimental study was conducted on a total of 63 female Wistar rats divided into 9 groups. To induce type 1diabetes, streptozotocin (STZ) and for induction of type 2 diabetes, nicotinamide (NA) and STZ were injected intraperitoneally. For superovulation, human menopausal gonadotropin (HMG) and human chorionic gonadotropin (HCG) were used. Insulin, metformin and pioglitazone were administered for two weeks. Finally, the endometrial expression of Muc1 was evaluated by quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR).

Results: *Muc1* expression was non-significantly increased in type 1 and type 2 diabetic groups compared to the control group (P=0.61 and 0.13, respectively); also, it increased in insulin-treated type 1 diabetic group compared to the control group (P=0.0001). Its expression was increased in insulin-treated type 1 diabetic group compared to untreated diabetic group (P=0.001). The expression level of *Muc1* was significantly reduced in superovulated and insulin-treated type 1 diabetic group compared to the insulin-treated type 1 diabetic group (P=0.001).

Conclusion: One of the causes of fertility problems in diabetes, is changes in *Muc1* expression during implantation. On the other hand, the use of insulin in these patients can even lead to overexpression of this gene and worsen the condition. However, these changes can be partially mitigated by assisted reproductive technology (ART) such as superovulation. Also, treatment with metformin and pioglitazone can restore *Muc1* expression to near normal levels and has beneficial effects on implantation.

Keywords: Diabetes Mellitus, Embryo Implantation, *Muc1*, Ovulation Induction

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Introduction

Diabetes mellitus is a metabolic disorder which is basically characterized by a chronic hyperglycemic condition. Type 1 diabetes predominately affects individuals of younger ages and type 2 diabetes as the most common type of diabetes, was previously thought to affect the ages of 40-60 years (1). The onset of type 2 diabetes occurs at younger ages (fertility age) today and it is predicted to occur at even younger ages in the future (2). Diabetes affects women in many ways and the association between diabetes and infertility was shown (3). Numerous studies observed that the incidence of infertility is higher in women with diabetes than in healthy women (4, 5).

Increased maternal blood glucose caused by diabetes can have detrimental effects on the expression of genes involved in the implantation process (6). However, the exact mechanism contributing to early pregnancy failure and recurrent spontaneous abortion in diabetes, remains

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largely unknown (7). Based on the emerging investigations, implantation failure is the main reason of about 75% of pregnancy losses (8). Embryo and uterus molecular crosstalk is the key factor for a successful pregnancy in the implantation process (9).

Muc1 as an anti-adhesion and antibacterial protein, is mainly expressed in the luminal and glandular endometrial epithelium at different stages of the menstrual cycle (10). Increment of *Muc1* expression before implantation leads to prevention of the embryo adhesion. Then, at the initiation of receptivity period of endometrium, *Muc1* is reduced and endometrium comes into contact with blastocyst. Therefore, timely inhibition of *Muc1* expression plays an important role in the uterine receptivity (11, 12). Actually *Muc1* hides the expression of cell adhesion molecules that are important for blastocyst attachment and plays an important role in regulating endometrial acceptance for blastocyst implantation (12). *Muc1* expression during implantation window in the endometrium of recurrent implantation failure women, is significantly lower than normal women (13). *Muc1* is an important factor in determining uterine receptivity and its endometrial expression is required for selection and implantation of the high-quality and active blastocysts. Significant decreases in Muc1 can impair endometrial embryo selection and lead to subfertility (11). On the other hand, increases in Muc1 in cell surface can inhibit cell-cell adhesion (14).Therefore, dysregulation of the mechanisms involved in the expression of *Muc1* at the time of implantation, may prevent implantation and establishment of early pregnancy.

Ovulation induction or superovulation in a controlled manner, is the most common method of assisted reproductive technology (ART). Various studies observed that infertile patients undergoing ART such as ovulation induction, experience molecular changes in their endothelium which can impair the expression of genes engaged in the embryonic implantation (15). Medications used to control diabetes include insulin for type 1 diabetes and oral medications such as metformin and pioglitazone and ultimately insulin for type 2 diabetes (16).

Studies demonstrated that *Muc1* expression in the endometrium is very important at the time of implantation, but there is insufficient knowledge about how this gene is expressed under diabetic conditions and the impacts of diabetes treatment and superovulation on the expression of this gene, need further assessments. Therefore, this study aimed to investigate the impacts insulin, metformin and pioglitazone as well as superovulation on the expression profile of *Muc1* during the implantation process, by using experimental rat diabetes model (type 1 and type 2 diabetes).

Materials and Methods

This experimental study was done in female Wistar rats (6-8 weeks old; 200-250 g; obtained from Pasteur Institute, Iran). Animals were exposed to standard conditions, 12 hours light/dark cycle and 20-2°C, and they had free access to standard water and food. They were housed in the central animal laboratory of Isfahan University of Medical Sciences, Isfahan, Iran. All experimental processes were approved by the Institutional Animal Ethics Committee of Isfahan University of Medical Sciences (IR.MUI.REC.1396.3.366).

Diabetes induction

To induce type 1 diabetes, streptozotocin (STZ, Sigma-Aldrich, Germany) was administered intraperitoneally at a dose of 60 mg/kg. For induction of type 2 diabetes, nicotinamide (NA, Sigma-Aldrich, Germany) was injected intraperitoneally at a dose of 200 mg/kg and after 15 minutes, STZ 60 mg/kg was given (17). To confirm diabetes induction, fasting blood sugar (FBS) was determined 3 days after the injection(s) by a glucometer (HemoCue Glucose 201+, Sweden) in samples collected from the dorsal vein of rats. In this study, in case of an FBS> 250 mg/dl, diabetes induction was confirmed (18).

Ovulation induction

Human menopausal gonadotropin (HMG; N. V. Organon, The Netherlands) and human chorionic gonadotropin (HCG, N. V. Organon, The Netherlands) was used for ovulation induction. Three days before mating, first, HMG was injected intraperitoneally at 7.5 I.U. and 48 hours later, HCG was injected at 7.5 I.U. in the same manner (19).

Study design and sample collection

Diabetic and normal rats were randomly divided into 9 groups: control (healthy animals that received no treatments), type 1 diabetic rats induced by STZ that received no treatments, insulin-treated type 1 diabetic rats, superovulated rats induced by HMG/HCG, superovulated type 1 diabetic rats, superovulated and insulin-treated type 1 diabetic rats, type 2 diabetic rats induced by NA-STZ that received no treatments, 20 mg/kg/day pioglitazone (Sobhan, Iran)-treated diabetic rats (20), and 100 mg/kg/day metformin (Sobhan, Iran) -treated diabetic rats (21). There were 7 rats in each group and animals were kept in diabetic conditions for 4 weeks (for more than one sex cycle), and administered with drugs for 4 weeks. During all diabetic conditions and treatments, FBS levels were monitored by a glucometer (HemoCue Glucose 201+, Sweden) and glucose reagent strips (ACCU-CHEK Active, Germany), every 4 days.

Four days earlier than the end of the treatment period, two female rats of each group were mated with a male rat and vaginal plug was checked in the following morning. The day when the vaginal plugs were observed or vaginal smears showed spermatozoa, was considered the first day of pregnancy. Rats were fasted overnight during the 3rd night and anesthetized through intraperitoneal injection of ketamine hydrochloride (50 mg/kg; ROTEXMEDICA, Germany) and xylazine hydrochloride (7 mg/kg; Daroupakhsh, Iran) on the following day; then, they were sacrificed under sterile conditions on the 4th day of gravidity (the day

of implantation). Uterine horns were surgically separated and snap-frozen in liquid nitrogen and stored at -80°C for further investigations.

Total ribonucleic acid isolation and complementary DNA synthesis

Total ribonucleic acid (RNA) was extracted from endometrial tissue by RNX-plus (Sinaclon, AryoGen Biopharma Complex, Iran) according to the manufacturer's protocol. Purity was defined by 1% agarose gel electrophoresis. The total RNA concentration was measured using a Nanodrop device (Nanolytic, Germany) at a density of 260 nm. DNase I treatment was accomplished in order to remove genomic DNA in the RNA samples by DNase I set (Fermentas, Lithuania). Complementary DNA (cDNA) synthesis was conducted using 1 µg of total RNA, by means of PrimeScriptTM RT reagent Kit (TaKaRa, Kusatsu, Japan) as reported in the protocol (22).

Quantitative real-time reverse transcription polymerase chain reaction

The relative expression level of *Muc1* gene was measured by real-time reverse transcription polymerase chain reaction (RT-PCR) in comparison with *β-actin* as a reference gene. The primers were planned using GeneRunner software (Version 4.0; Hastings Software Inc., Hastings, US) and the specificity of each primer was tested by BLAST (http://blast. ncbi.nlm.nih.gov/Blast.cgi). The list of primers is presented in Table 1 (23).

RT-PCR was performed by Applied BiosystemsStepOnePlus™ instrument using RealQ Plus ×2 Master Mix, green (high ROX) (AMPLIQON, Denmark) (24). Standard cycling protocol was utilized to perform RT-PCR, as follows: denaturation at 95°C for 10 minutes, denaturation at 95°C for 15 seconds, annealing at the specific temperature for each gene (Table 1) for 60 seconds, and finally, an extension was done for 15 seconds at 72°C for 40 cycles. Gene expression determination was carry out using the $2^{-\Delta\Delta CT}$ method (25).

Statistical analysis

All statistical analyses were done by using SPSS software, version 20.0 (SPSS Inc., US). To analyze the normality of the data, Kolmogorov-Smirnov test was applied. RT- PCR was repeated three times and the final results are shown as means ± standard error of the mean. One-way Analysis of Variance (ANOVA) with post hoc LSD multiple comparisons were accomplished to recognize statistical significance. Statistical significance was set at P<0.05.

Results

Muc1 gene expression in type 1 diabetic and superovulated groups compared to the control group

Relative expression of *Muc1* was increased in type 1 diabetic and insulin-treated type 1 diabetic groups compared with the control group; however, statistically significant differences were only found for insulin-treated type 1 diabetic group (P=0.0001, 0.61; respectively). The other groups (superovulated, superovulated type 1 diabetic and superovulated and insulin-treated type 1 diabetic groups) did not show a significant difference when compared with the control group (P=0.51, 0.78, 0.95, respectively).

Muc1 expression in insulin-treated type 1 diabetic group increased compared to the untreated diabetic group (P=0.001). In superovulated and insulin-treated type 1 diabetic groups, relative expression of *Muc1* gene was significantly reduced compared to the insulin-treated type 1 diabetic group (P=0.001, Fig.1).

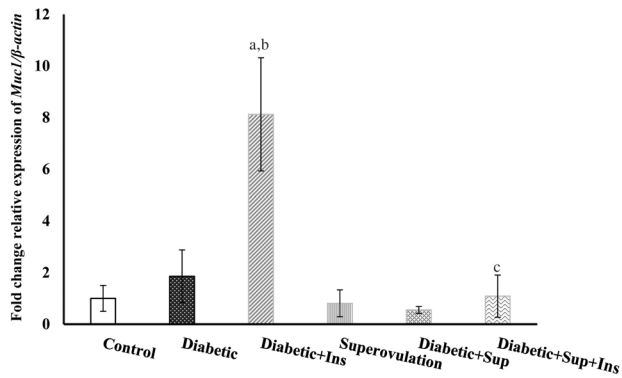

Fig 1: Relative expression of *Muc1* in the endometrium of type 1 diabetic rats at the time of implantation. The relative expression of *Muc1* was normalized against *β-actin* using $2^{-\Delta\Delta CT}$ method. All values are presented as mean ± SEM. A P<0.05 was considered statistically significant. SPSS software was used to analyze the data. Lowercase letters indicate a statistical significance as follows: a: Compared to control, b: Untreated diabetic, c; Insulin-treated diabetic groups. Sup; Superovulation, and Ins; Insulin.

Muc1 gene expression in type 2 diabetic groups compared to the control group

Type 2 diabetic group showed increment (though not significantly) of the expression of *Muc1* compared to the control group (P=0.13). Relative expression level of *Muc1* was not significantly different between metformin-treated and pioglitazone-treated type 2 diabetic groups, and the control group (P=0.94, 0.75; respectively).

Table 1: PCR primer sequences

Primers	Sequence	Tm (ºC)	Annealing temperature (ºC)	Amplicon size (bp)
βactin	F: 5′-GCCTTCCTTCCTGGGTATG-3′ R: 5′-AGGAGCCAGGGCAGTAATC-3′	63.4 63	60	178
Muc1	F: 5′- ATCAAGTTCAGGTCAGGCTC -3′ R: 5′- AGAGGAAGGGAACTGCATC-3′	60.1 59.9	57	171

Muc1 expression was non-significantly reduced in type 2 diabetic groups treated with metformin and pioglitazone compared to untreated type 2 diabetic group (P=0.11, 0.07; respectively, Fig.2).

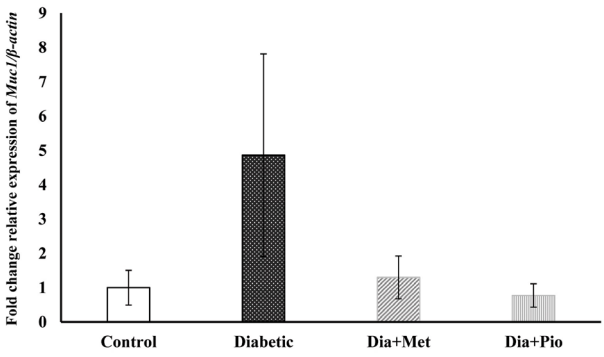

Fig 2: Relative expression of *Muc1* in the endometrium of type 2 diabetic rats at the time of implantation. The relative expression of *Muc1* was normalized against *β-actin* using 2-*ΔΔCT* method. All values are presented as mean ± SEM. A P< 0.05 was considered statistically significant. SPSS software was used to analyze the data. Dia; Diabetic, Met; Metformin, and Pio; Pioglitazone.

Discussion

According to the results of the present study, induction of type 1 and type 2 diabetes increased the expression of *Muc1* in rats' endometrium at the time of implantation. Both metformin and pioglitazone had positive effects on restoration of *Muc1* expression to normal levels but insulin caused overexpression of *Muc1*. However, ovulation induction partially moderated the effect of insulin and *Muc1* expression level became closer to normal.

The results of the current study showed that induction of type 1 and type 2 diabetes increased *Muc1* gene expression in rats' endometrium. *In vitro* studies indicated that Muc1 is reduced in humans and mice specifically in the area where the blastocyst implants in the uterus. It is hypothesized that low level of Muc1 in the blastocyst implantation area during implantation window, is an important factor for successful embryo-endometrial interaction. High expression of Muc1 may damage cell-cell and cell-matrix adhesion, probably leading to implantation failure (26). Aktug et al. (14) study showed that induction of diabetes affects cleaved junctions, cell adhesion molecules and related proteins. They fertilized the oocytes isolated from the healthy and diabetic rats and found that Muc1 expression was increased in a group of blastocysts in which, oocytes were separated from diabetic rats. Albaghdadi et al. (27). also observed the overexpression of *Muc1* in the uterus of diabetic mice at the time of implantation. In fact, the present study also confirmed these observations and showed that diabetes can increase *Muc1* expression during implantation which can lead to implantation failure.

The present study showed that treatment with insulin in type 1 diabetic rats, increased *Muc1* expression to a higher level compared to untreated diabetic rats, which may result in prevention of blastocyst contact with uterine epithelium and prevention of implantation. In Seregni et al. (28) study, insulin was found to increase the level of Muc1 expression in the blood of patients with breast cancer. The present study, consistent with these results, indicated that Muc1 overexpression caused by treatment with insulin during implantation can lead to implantation failure.

In the present study, treatment with either metformin or pioglitazone was effective in reducing *Muc1* expression levels in diabetic rats treated with metformin or pioglitazone compared with untreated diabetic rats. No studies were found on the effect of metformin or pioglitazone on *Muc1* expression at the time of implantation, under diabetic conditions. However, there is some evidence that metformin reduces MUC1 protein in women with breast cancer (29).

Furthermore, the results of the present study showed that ovulation induction in all induced groups including healthy, diabetic and insulin-treated diabetic rats, reduced Muc1 expression, although it was not significantly different from the control group. However, comparing insulin-treated diabetic rats with superovulated insulin-treated diabetic rats may be important since ovulation induction may possibly modulate insulin-induced increment of *Muc1* expression. Inyawilert et al. found that ovulation induction attenuated *Muc1* mRNA expression in the rat uterus on day 3.5 of the estrous cycle (30). Contrary to the present study, Wang et al. found that *Muc1* expression was artificially increased in ovine following ovarian stimulation, that may be due to difference in method of superovulation and the type of drug used to induce ovulation (31). Nonetheless, further studies are required to determine the effects of ovulation induction on *Muc1* expression and implantation.

According to the results of the current study, it can be concluded that type 1 and type 2 diabetes alter the expression of *Muc1* gene in the rat uterus at the time of implantation. Because of the importance of proper expression of *Muc1*, its aberrant expression may affect uterine receptivity and lead to implantation failure and subsequent infertility.

Both anti-diabetic drugs metformin and pioglitazone, had positive effects on restoration of *Muc1* expression to its normal levels. Inevitable treatment with insulin in type 1 diabetes caused overexpression of *Muc1*; however, ovulation induction partially moderated such effects and restored *Muc1* levels closer to normal values. However, ovulation induction alone may have adverse effects on the expression of this molecule.

There were limitations in this study, and the examined effects should be assessed in a larger number of rats in future works; also, follow-up of animal pregnancy to investigate the effects of medications on pregnancy outcomes was not possible in the current study.

Conclusion

The use of insulin by diabetic patients, can even lead to overexpression of *Muc1* and worsen the condition. However, these changes can be partially mitigated by ART such as superovulation. Also, treatment with metformin and pioglitazone can restore *Muc1* expression closer to normal levels and have beneficial effects on implantation. Therefore, it can be said that diabetes can alter *Muc1* gene expression which can disrupt the implantation process and consequently induce infertility. However, treatment with metformin and pioglitazone as well as ovulation induction, can be helpful.

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Authors' Contributions

R.Z., P.N., B.R., N.E, R.A. ; Participated in study design, data collection and evaluation, drafting and statistical analysis. R.Z., P.N.; Conducted molecular experiments and RT-qPCR analysis. All authors read and approved the final manuscript.

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Original Article

Zinc Protects against MDMA-Induced Apoptosis of Sertoli Cells in Mouse via Attenuation of Caspase-3

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Abstract

Background: 3,4-Methylenedioxymethamphetamine (MDMA) disrupts function of the endocrine system and different organs such as heart, blood vessels, kidney, liver and nervous systems. This study was conducted to evaluate impact of MDMA on apoptosis and Zinc in the MDMA-induced apoptosis of cultured Sertoli cells by measuring *Caspase-3* gene expression.

Materials and Methods: In this experimental study, Sertoli cells were incubated with MDMA (0, 0.5, 1, 3, 5 mM), Zinc (0, 8, 16, 32, 64 μM) and Zinc (8 μM) prior to adding MDMA (5 mM) for 24 and 48 hours. MTT assay was used for evaluating impacts of these conditions on the viability of Sertoli cells. *Caspase-3* gene expression level was detected using quantitative reverse transcription PCR (qRT-PCR) in all of the tested groups.

Results: Finding showed that cellular viability was decreased and level of *Caspase-3* mRNA was increased in MDMA treated cells. Additionally, pre-treatment with Zinc (8 μM) attenuated MDMA-induced apoptosis and down-regulated caspase-3. The mean of caspase-3 mRNA level (fold change ± SE) was 3.98 ± 1.18, 0.31 ± 0.28, and 1.72 ± 0.28 in respectively MDMA (5 mM), Zinc (8 μM), and Zinc+MDMA groups vs. control group. The mean of *Caspase-3* mRNA (fold change) was not statistically different in the tested groups ($P>0.05$), unless MDMA (5 mM) group ($P = 0.008$).

Conclusion: We suggest that MDMA toxicity could be involved in apoptosis of Sertoli cells. In addition, Zinc could reduce MDMA-induced apoptosis by down-regulation of *Caspase-3* mRNA levels.

Keywords: Apoptosis, *Caspase-3*, MDMA, Sertoli Cells, Zinc

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Introduction

3,4-Methylenedioxymethamphetamine (MDMA) as an illicit drug is used by young adults in the world (1). Since MDMA is consumed mainly by the young population, its effects on the reproductive system would be important to be taken into consideration (2,3). This hallucinogenic drug induces apoptotic cell death in different organs (4,5). MDMA affect the endocrine system and gonads (6). Prolonged usage of MDMA has several adverse effects on the pituitary-gonadal axis. This condition may affect the process of spermatogenesis (7). MDMA results in testicular edema and sperm DNA damage, low sperm count, immature/abnormal spermatozoa and asthenozoospermia. Poor semen quality can leads to male infertility (6). It has been demonstrated that numerous mechanisms are responsible for MDMA toxicity, including oxidative stress, metabolic compromise and inflammation (8). The deleterious impact of free radicals is termed oxidative stress causing serious biological damage (9). There is an excess of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in biological systems regarding deficiency of antioxidants. The role of oxidative stress in MDMA toxicity has been observed by reducing the activity of endogenous antioxidants glutathione peroxidase, catalase (CAT) and superoxide dismutase (10). A Sertoli cell regulates spermatogenesis via controlling germ cell proliferation and apoptosis (11). Caspase-3 as a general activated death protease can catalyze the cleavage of cellular macromolecules such as DNA and proteins (12). Zinc as a fundamental trace element is essential to the structure and function of over 300 enzymes in endocrine system, immune system as well as anti-cancer defense mechanisms (13,14). Deficiency of Zinc leads to intrauterine growth retardation (delayed growth), appetite loss, pyrexia, cytokine storm and impaired reproductive capacity (15).

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Zinc get involved in NADPH oxidase generating ROS (16). Zinc induces production of metallothionein (MT) (17). Zinc deficiency has been associated with oxidative stress. In this regard, intracellular Zinc contents such as metallothionein regulate the oxidative stress. Zinc intake is associated with decreased level of ROS production in activated human neutrophils and decrease malone dialdehyde (MDA) (18). Here, we evaluated the effect of MDMA on apoptosis and Zinc on MDMA-induced apoptosis of cultured Sertoli cells by measuring *Caspase-3* gene expression.

Materials and Methods

This experimental study was approved by research ethics committee of Urmia Medical Sciences University (Urmia, Iran; IR.UMSU.REC.1397.448). TM4 mouse cell line (Sertoli cells) was purchased from the Pasteur Institute of Iran and used at this study. TM4 cells were grown in DMEM/Nutrient Mixture F-12 Ham (Biosera, France), with 10% FBS (Gibco, USA) and 1% (v/v) Penicillin/Streptomycin mixture (Gibco, USA). They were incubated at 37°C and 5% CO_2 condition. The culture medium was replaced every 24 to 48 hours. The cultured cells were trypsinized after reaching 80% confluence by 0.25% trypsin-EDTA solution (Gibco, USA). Then, the cells were transmitted and cultured again on a new medium twice per week. The cells were cultured at a density of 5×10^3 cells per well in 96-well culture plate (SPL, Korea). This was followed by incubation at 37°C and 5% CO_2. After 24 -hours incubation, the cells were treated with MDMA (0, 0.5, 1, 3 and 5 mM; Sigma-Aldrich, USA) for 24 and 48 hours. Additionally, the cells were treated with 0, 8, 16, 32, 64 µM Zinc sulfate (Sigma, USA for 24 and 48 hours. After that, the supernatant was replaced with 100 µl PBS including 1 mg/ml MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Sigma, USA). The samples were incubated at room temperature for 4 hours. Then, 100 µl dimethyl sulfoxide (Merck, Germany) was added and they were kept at room temperature for 15 minutes. Absorbance of each sample was measured at 590 nm with a microplate reader (Bio-Tek, USA). Data were reported as relative viability (% control).

Subsequently, the effective concentrations of MDMA and Zinc were determined. Then, TM4 cells were cultured in four groups and examined. They groups include MDMA, Zinc, Zinc pretreatment, and control group. These groups were subdivided in two subgroups regarding the 24 and 48 hours exposure time. The cells were cultured in 96-well plate at a density of 5×10^3 cells / well, duplicately. The cells were twice cultured at the same time with identical density. After 48 hours exposure, the old medium was replaced by medium containing Zinc. After that time, the medium was replaced with MDMA-medium for the wells containing pre-treatment and MDMA groups, Zinc- medium for Zinc group and a free medium for control group. For the wells with 24 hours exposure, the same protocol 48 hours exposure period was applied, unless the starting pretreatment one day later. In addition, MDMA and Zinc exposure times were 24 hours. Zinc pre-treatment time was similar for both 24 and 48 hours subgroups, and only the treatment exposure time was different. MTT assay was then performed to evaluate cell viability of the different tested groups. To determine effective concentrations of MDMA and Zinc, these concentrations at 48 hours exposure time were used for the following experiments. In each set of triplicate cultures, 10^5 TM4 cells/wells were seeded in 6 well plates for 24 hours, followed by treatment consistent with the experimental main groups: MDMA (5 mM), Zinc (8 µM), pre-treatment and control (one well for each group). Following stabilization period of 24 hours, the well with pre-treatment group was exposed to Zinc and the other wells were exposed to free medium for the following 48 hours. The medium was replaced with a fresh medium in the midpoint of culture. The day after, the medium was aspirated from each well and replaced with MDMA containing medium for the wells regarding tested conditions. Forty eight hours after the last treatment, the cells were trypsinized and the cells pellet was used to extract RNA after centrifugation at 3000 rpm for 10 minutes. RNA extraction was carried out by the RNX Plus Solution Kit (SinaClon, Iran). In this study, forward and reverse primers for the target gene (*Caspase 3*) and reference gene (*β-actin*) were as follow:

Caspase-3 (136 bp PCR product):

F: 5'-GCA GCT TTG TGT GTG TGATTC-3'
R: 5'-AGT TTC GGC TTT CCA GTC AG-3'

β-actin (250 bp PCR product):

F: 5'-TAG GCG GAC TGT TAC TGA GC-3'
R: 5'- GCT CCA ACC AAC TGC TGT C-3' (19).

Concentration of the RNA was confirmed for all samples and synthesis of cDNA with the same RNA concentration was performed by the following compounds: total RNA was used to produce cDNA using two-steps reverse transcription polymerase chain reaction (RT-PCR) kit (Thermo Scientific, Sweden) as following: 10 µg RNA, 1 µl random Hexamers, 2 µl dNTPs mix (10 mM), 1 µl RiboLock RNase Inhibitor (20 U/µl), 4 µl 5X Reaction Buffer, 1 µl reverse transcriptase (RT) enzyme (200 U/µl). Final volume was reached to 20 µl, using nuclease-free water. The cDNA synthesis was carried out in a thermocyclor at 25°C for 5 minutes and 42°C for 60 minutes. Then, the synthesized cDNA was used to perform quantitative reverse transcription polymerase chain reaction (qRT-PCR). PCR program includes 95°C for 10 minutes (initial denaturation), 40 cycles of 95°C for 20 seconds (denaturation) and 60°C for 45 seconds (annealing). All samples were replicated two times. The relative expression level of Caspase-3 was normalized to β-actin. The results are reported based on Mean fold differences ± standard error (SE). All of the treated groups were compared to the control group. To analyze data, SPSS version 20 software was used. The statistically

significant data was determined by one-way analysis of ANOVA and Tukey's test. To determine significance of the results, Threshold of P value was considered as 0.05. The qRT-PCR results were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Results

Cell viability and proliferation were determined using MTT assay. These were significantly reduced in the TM4 cells incubated with different concentrations of MDMA (0, 0.5, 1, 3, 5 mM) for 24 and 48 hours. In these two of exposure times, MDMA dose dependently decreased cell viability with IC50 values of 5 mM for 24 hours and 3 mM for 48 hours exposure (Fig. 1).

The cells were treated with various concentrations of Zinc (0, 8, 16, 32, 64 µM) for 24 and 48 hours. Cell viability was increased in the lower concentrations of Zinc in 24 and 48 hours exposure times, compared to the control Cell viability and proliferation were decreased as concentration of Zinc was increased (Fig. 2).

After analysis of the represented primary experiment data, 5 mM MDMA and 8 µM Zinc were considered as effective concentrations. The TM4 cells were exposed to 8 µM Zinc for 48 hours prior to adding MDMA. Cellular viability was then assessed and compared to the groups exposed to each of MDMA and Zinc. As shown in Figures 3 and 4, MDMA (5 mM) decreased cell viability, whereas cellular viability was increased in the Zinc (8 µM) group and those receiving Zinc before adding MDMA, in comparison with the control.

Amplification efficiency was set at 90-105%. The results showed that the mean (± SE) of fold change was 3.98(± 1.18), 0.31(± 0.28), and 1.72(± 0.28) in MDMA (5 mM), Zinc (8 µM), and Zinc+MDMA groups, respectively. Mean of Caspase-3 mRNA expression level (fold change) was not statistically different in the tested groups (P value>0.05), unless in MDMA (5 mM) group (P value=0.008). In this regard, cellular viability and mean of Caspase-3 mRNA expression level (fold change) were similar in the control and Zinc+MDMA groups. These results implied that Zinc had a protective effect against MDMA induced-Sertoli cell apoptosis in mouse. Figure 5 shows a gel image of RT-PCR products in this study.

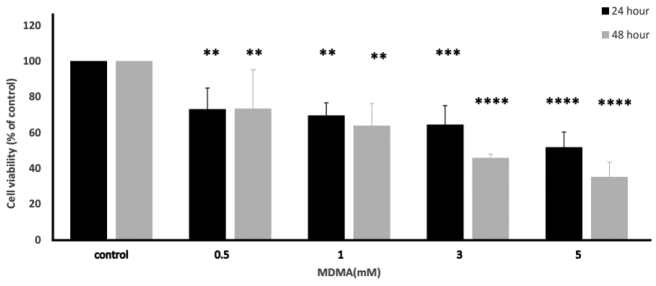

Fig 1: Impact of MDMA on TM4 cells. Evaluation of MDMA effect on TM4 cells in the tested groups showed significant differences in tested groups versus controls after 24 and 48 hours. **; P<0.01, ***; P<0.001, ****; P<0.0001, and MDMA; 3,4-Methylenedioxymethamphetamine.

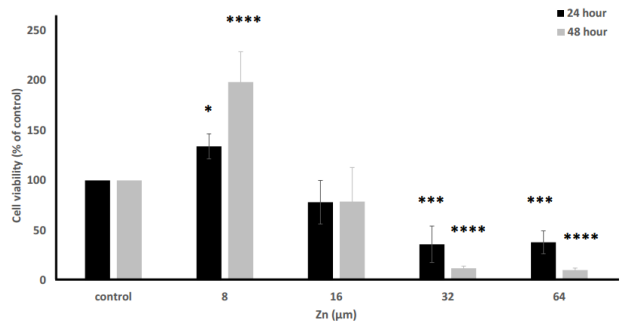

Fig 2: The impact of Zinc on TM4 cells. Analysis of the Zinc effect on the tested groups showed significant differences in the tested groups versus. controls after 24 and 48 hours. *; P<0.05, ***; P<0.01, and ****; P<0.001.

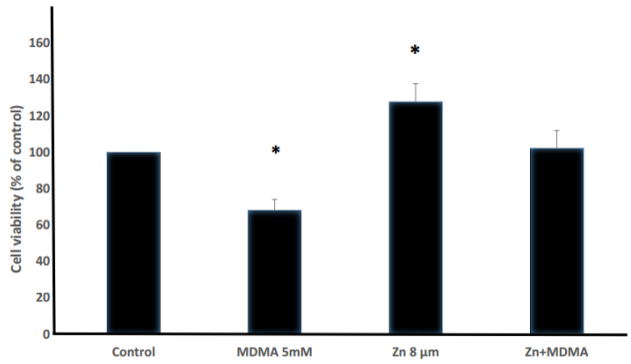

Fig 3: Comparison of cell viability in the tested groups versus control group. The experimental groups were treated with MDMA (5 mM) and/or Zinc(8 µM). In the Zinc+MDMA group, the cells were pre-treated with Zinc (8 µM) for 48 hours, before MDMA (5 mM) exposure. *; P<0.05, and MDMA; 3,4-Methylenedioxymethamphetamine.

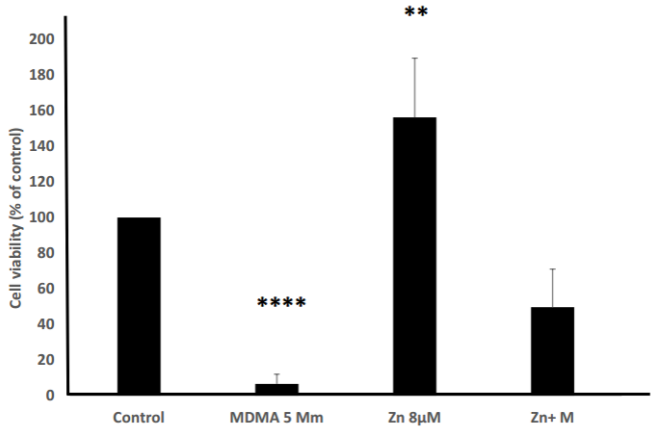

Fig 4: Comparison of the tested groups versus control group regarding cellular viability. The experimental groups were treated with MDMA (5 mM) and Zinc(8 µM). In the Zinc+MDMA group, the cells were pretreated with Zinc (8 µM) for 48 hours, before MDMA (5 mM) exposure. **; P<0.01, ****; P<0.001, and MDMA; 3,4-Methylenedioxymethamphetamine.

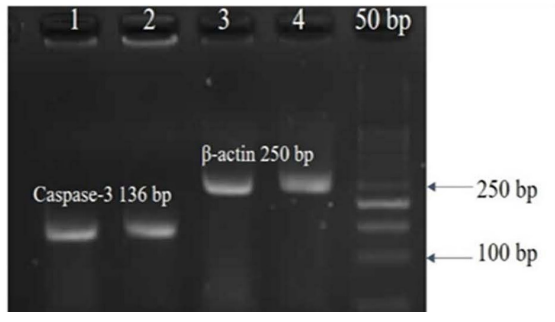

Fig 5: Reverse transcription polymerase chain reaction (RT-PCR) Analysis of tested genes by electrophoresis in a 2.5% agarose gel.

Discussion

We investigated cell viability as well as *Caspase-3* mRNA expression level in the TM4 cells, incubated with MDMA, Zinc and Zinc+MDMA. We demonstrated that exposing cultured TM4 Sertoli cells to MDMA decreased cellular viability, while low concentration (8 μM) of Zinc reversed this condition. At high concentrations (16, 32 and 64 μM), Zinc had toxic effects and caused a high cellular death rate. Pre-treatment with Zinc, prior to MDMA exposure, attenuated MDMA-induced cellular toxicity. In addition, *Caspase-3* gene expression level was increased in MDMA-treated cells, while it was decreased in low concentration Zinc-treated cells. Pre-treatment with Zinc decreased MDMA-induced elevation of *Caspase-3* mRNA expression levels. These findings showed that MDMA had cytotoxic and apoptotic effects on TM4 Sertoli cells, whereas Zinc (8 μM) had anti-apoptotic effects and attenuated MDMA-induced cellular toxicity. It is well known that MDMA administration can induce oxidative stress (6, 8). It can harm the function of endocrine system and gonads (20). MDMA could influence male reproductive organs, cause sperm DNA damage. It also alters sperm count, sperm maturation and sperm mobility (6). MDMA induces cellular oxidative stress and apoptosis (21). While Sertoli cells have critical role in spermatogenesis (22), apoptosis of these cells leads to loss of germ cells. In the present study, our findings implied that MDMA had cytotoxic effect on Sertoli cells. It is well known that expression levels of caspase genes family is crucial in determining susceptibility of cells to apoptotic stimuli. *Caspase-3*, in particular, is a key player in this process and has many cellular targets, activation of which lead to apoptosis (23). Our study revealed MDMA-induced up-regulation and increase in *Caspase-3* mRNA expression level in the Sertoli cells. Decreased cellular viability and high level of *Caspase-3* gene expression in the MDMA treated-Sertoli cells may be defined as testicular injury that resulting in enhanced Sertoli cell apoptosis. It may be partly involved in infertility associated with MDMA consumption. Excessive ROS production and disrupting antioxidant defense of cells are associated with apoptotic effect of MDMA (24). In biological systems, the antioxidant properties of Zinc have been clearly demonstrated (25-27). Zinc supplementation induces metallothionein in diverse organs such as liver, kidney and intestine (28). Metallothionein has antioxidant effects (29). Zinc is known as a cofactor for Zn/Cu superoxide dismutase (SOD) enzyme, which acts as an ROS scavenger. This enzyme catalyzes alteration of O_2 radical into the less harmful components of O_2 and H_2O_2 (30). The protecting effect of Zinc against MDMA induced-Sertoli cell apoptosis seems to be related to its antioxidant properties. MDMA exerted cytotoxic property and induced-apoptosis through over-expression of *Caspase-3* in TM4 Sertoli cells. However, all of these parameters reversed or they recovered when the MDMA exposure was pretreated with Zinc. Only at 8 μM concentration of Zinc, we observed elevated cell viability after 24 and 48 hours exposure times. Maintaining right concentration of Zinc in the right physiological conditions can prevent oxidative stress in each cell of the body (30). In this study, high concentrations of Zinc had severe toxic effects on Sertoli cells and this finding is compatible to the previous studies.

Conclusion

It can be concluded that Sertoli cells treatment with MDMA lead to decreased level of cell viability and induction of apoptosis by over-expression of *Caspase-3* mRNA level. Pre-treatment of Zinc, in advance to MDMA exposure attenuated MDMA-induced Sertoli cell apoptosis via inhibition of *Caspase-3* gene expression.

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Authors' Contributions

M.B.; Designed experiments, analyzed data and co-wrote the paper. M.B., N.H., M.L., M.N-Z.; Performed experiments, analyzed data and co-wrote the paper. I.A.; Analyzed data and co-wrote the paper. All authors read and approved the final manuscript.

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Original Article

In Vitro and *In Vivo* Determinations of The Anti-GDNF Family Receptor Alpha 1 Antibody in Mice by Immunochemistry and RT-PCR

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Abstract

Background: The glial cell-derived neurotrophic factor (GDNF) family plays essential roles in the maintenance, growth, regulatory and signalling pathways of spermatogonial stem cells (SSCs). In this study, we analysed the expression of anti-GDNF family receptor alpha 1 antibody (GFRa1) by immunohistochemistry (IHC), immunocytochemistry (ICC), Fluidigm real-time polymerase chain reaction (RT-PCR) and flow cytometry analyses.

Materials and Methods: In this experiment study, ICC, IHC, Fluidigm RT-PCR and flow cytometry were used to analyse the expression of the germ cell marker GFRa1 in testis tissue and SSC culture.

Results: IHC analysis showed that there were two groups of GFRa1 positive cells in the seminiferous tubules based on their location and expression shape - a small round punctuated shape on the basal compartment donut shape and a C-shaped expression located between the basal and the luminal compartments of the seminiferous tubules. OCT4 and PLZF positive cells may have similar patterns of expression as the first group. Assessment of the seminiferous tubule sections demonstrated that about 27% of the SSCs were positive for GFRa1. Fluidigm RT-PCR confirmed the significant expression ($P<0.001$) of *GFRa1* in the SSCs compared to testicular stromal cells (TSCs). Flow cytometry analysis demonstrated that about 75% of the isolated SSCs colonies were positive for GFRa1.

Conclusion: The results indicated that GFRa1 had a specific expression pattern both *in vivo* and *in vitro*. This finding could be helpful for understanding the proliferation, maintenance and signalling pathways of SSCs, and differentiation of meiotic and haploid germ cells.

Keywords: Analysis, Embryonic Stem Cells, GFRa1, Pluripotent Stem Cells

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Introduction

In the mammalian testis, spermatogonial stem cells (SSCs) are located on the basal membrane of seminiferous tubules and are essential for normal spermatogenesis. SSCs can be established in adherent and non-adherent culture systems. The self-renewal and maintenance of SSCs during an in vitro culture depends on the presence of soluble growth factors and adhesion molecules. SSCs express different surface markers, including anti-GDNF family receptor alpha 1 antibody (GFRa1) (1, 2) α6 (CD49) and β1 (CD29) integrins (3, 4), CD9 (5), E-cadherin (6), and THY-1 (CD90) (7, 8). The GFRa1 receptor is expressed in undifferentiated spermatogonia cells in rodents and has been used as a marker for the isolation of undifferentiated SSCs (9). GFRa1 is a co-receptor that recognizes the glycosylphosphatidylinositol-linked glial cell-derived neurotrophic factor (GDNF) family of ligands. GDNF is a main growth factor for *in vitro* cultivation of SSCs and supports the survival of neuronal cells throughout the regulation of cyclic adenosine 3′, 5′-monophosphate (cAMP)-dependent signalling pathways (10, 11). In mammalian testes, GDNF affects the target cells by binding to a receptor complex that consists of receptor tyrosine kinase Ret (C-RET) and GFRa1 (12). During in vitro cultivation of testicular germ stem cells, the GDNF molecule regulates both self-renewal and proliferation of SSCs, prevents SSC differentiation and activates the in vivo maintenance of the stem cell pool (13-16). When two soluble growth factors, GFRa1 and fibroblast growth factor 2 (FGF2), are combined with GDNF, they enhance both proliferation and long-term expansion of cultivated germline stem cells (GSCs) (13). Similarly, GFRa1 combined with the growth fac-

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tors FGF, LIF and GDNF supports the short-term cultivation of rat SSCs (17).

The aim of the present investigation was to understand the localization and pattern of *GFRa1* gene expression in the testis section and in generated SSCs and testicular stromal cells (TSCs). The results showed that GFRa1 expression was distributed above the base membrane of the testicular lumen, which suggested that GFRa1 plays a crucial role in the proliferation and self-renewal of germ stem cells in testes. The GFRa1 expression pattern would be valuable for innovative future researches in the fields of reproductive biology and biotechnology.

Materials and Methods

Digestion, characterization and culture of testicular cells

In this experimental study, the ethical committee of Amol University of Special Modern Technologies (IR.AUMST.REC.1398.03.07) approved the animal experiments. Testis cells from C57BL/6 mice (7-week-old) were placed in an enzymatic digestion solution that contained DNAse (0.5 mg/ml, Sigma Aldrich, USA), collagenase (0.5 mg/ml, Sigma Aldrich, USA) and dispase (0.5 mg/ml, Sigma Aldrich, USA) in an HBSS buffer (PAA, USA). After characterizing the SSCs, digested testicular cells were filtered through a cell strainer and were cultured in GSCs culture media at 37°C and 5% CO_2 in air. This media contained StemPro-34 medium, 1% L-glutamine (PAA, USA), 1% N2-supplement (Invitrogen, USA), 6 mg/ml D + glucose (Sigma Aldrich, USA), 1% penicillin/streptomycin (PAA, USA), 5 µg/ml bovine serum albumin (Sigma Aldrich, USA), 0.1% ß-mercaptoethanol (Invitrogen, USA), 30 ng/ml oestradiol (Sigma Aldrich, USA), 60 ng/ml progesterone (Sigma Aldrich, USA), 1% non-essential amino acids (PAA, USA), 10 ng/ml FGF (Sigma Aldrich, USA), 100 U/ml human LIF (Millipore), 1% MEM vitamins (PAA, USA), 8 ng/ml GDNF (Sigma Aldrich, USA), 20 ng/ml epidermal growth factor (EGF, Sigma Aldrich, USA), 30 µg/ml pyruvic acid (Sigma Aldrich, USA), 1% ES cell qualified FBS, 100 µg/ml ascorbic acid (Sigma Aldrich, USA) and 1 µl/ml DL-lactic acid (Sigma Aldrich, USA).

RNA extraction and real-time polymerase chain reaction analysis

Total RNA was extracted from the SSCs and TSCs with a NucleoSpin® RNA II Kit (Macherey-Nagel, Düren, Germany) for real-time polymerase chain reaction (RT-PCR) analysis. In the next step, RNA samples were decontaminated with DNase I (EN0521, Fermentas, USA) to remove genomic DNA contamination. cDNA was synthesized with oligo (dT)18, total RNA (2 µg) and a RevertAidTM H Minus First Strand cDNA Synthesis Kit (K1622, Fermentas). The PCR reactions were carried out using a Mastercycler gradient machine (Eppendorf, Germany). The cDNA samples were exposed to PCR amplification by GFRa1 primers under the following reaction conditions: initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing temperature at 59-70°C for 45 seconds, extension time for 45 seconds at 72°C, and a final polymerization at 72°C for 10 minutes. The PCR products were observed using 1.6% agarose gel electrophoresis, stained with ethidium bromide solution (10 µg/ml), and then visualized and photographed with a UV transilluminator (UVIDOC, UK). The forward and reverse primer used for *GFRa-1* was as follow:

F: 5′-ACTCCTGGATTTGCTGATGTCGG-3′
R: 5′-CGCTGCGGCACTCATCCTT-3′ (product size: 193 bp) (18, 19).

Gene expression analyses on the Fluidigm Biomark system

The expression level of the *GFRa1* Mm01253716_m1 gene in SSCs and TSCs was examined by the Fluidigm Biomark system. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Mm99999915-g1 was the reference gene for normalization. SSCs and TSCs were picked up with a micromanipulator technique, lysed with a solution of lysis buffer that contained 9 µl RT-PreAmp Master Mix (5.0 µl Cells Direct 2× Reaction Mix, Invitrogen, USA), 2.5 µl 0.2× assay pool and 1.3 µl TE buffer, 0.2 µl RT/Taq Superscript III (Invitrogen, USA). Then, the amount of the amplified product of RNA-targeted copies was examined with TaqMan real-time PCR on a BioMark Real-Time Quantitative PCR (qPCR) system. Samples were analysed in two technical repeats. The Ct values were calculated using Excel and GenEx software (20-22).

Immunocytochemical staining

Isolated SSCs from the testes were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X-100/PBS, blocked with 1% BSA/PBS and incubated with primary antibody GFRa1 (Sigma Aldrich, USA). The process was followed by an overnight incubation (~16 hours) of fluorochrome species-specific secondary antibody at 4°C. The labelled cells were identified by simple nuclear counterstain with 0.2 µg/ml of 4′, 6-diamidino-2-phenylindole (DAPI) dye. The positive cells labelled with antibodies were visualised with a confocal laser scanning microscope Zeiss LSM 700 and images of the cells were obtained using a Zeiss LSM-TPMT camera (20, 23, 24).

Tissue processing for immunohistochemical staining

Testicular tissue was picked up after decapsulation of tunica albuginea, washed with PBS and fixed in 4% paraformaldehyde. The tissue was dehydrated during tissue processing and surrounded in Paraplast Plus. In the next step, the tissue was cut with a microtome, usually with a thickness of around 8-10 µm. Sections from the testes tissues were mounted on Hydrophilic Plus slides and stored at room temperature until use. During the immunohistochemical staining process, the slides were washed by xylene and slowly dehydrated

through a series of decreasing concentrations of ethanol. Before staining, antigen retrieval was done by the heat-induced epitope retrieval (HIER) method at 95°C for 20 minutes and the non-specific binding sites in the tissue sections were blocked with 10% serum and 0.3% Triton in PBS. Then, the tissue sections were incubated with primary antibody *GFRa1* (Sigma, USA) and species-specific secondary antibody. The labelled cells were characterised under a confocal laser scanning microscope Zeiss LSM (20).

Flow cytometry analysis

After determining the cell viability by trypan blue staining, the cells were resuspended in PBS/FBS staining buffer and incubated with cell surface primary antibody to GFRa1 conjugated with fluorochrome (APC, R&D Systems) for one hour. The samples were washed and a flow cytometry analysis was performed with a BD FACSCalibur flow cytometer. The acquired results were analysed with BD CellQuest Pro software.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA). The comparison of GFRa1 expression in the SSCs and TSCs groups was analysed by the independent samples t test. $P<0.05$ was considered to be statistically significant.

Results

The location of the germ cell marker GFRa1 in the seminiferous tubule of the mouse testis was analysed as the first study of this experiment. We observed two distinct populations of GFRa1 positive cells based on their location and pattern of expression in the seminiferous tubules of the mice. The first group of GFRa1 positive cells had a small round punctuated expression and were located at the epithelium of the seminiferous tubules. The number in the first group was much lower than the Second group. The second group of GFRa1 positive cells was situated between the basal and the luminal compartment of seminiferous tubules and had a donut and C-shaped expression. The OCT4 and PLZF positive cells and the first population of GFRa1 positive cells that were located in the basal part of seminiferous tubules might be similar or were possibly the same cells. Down-regulation of GFRa1 positive cells was obvious in the completely differentiated part of the seminiferous tubule, presenting haploid cells.

We counted the GFRa1 positive cells in the testis sections and determined that about 27% of the testicular tubule germ cells expressed GFRa1. High magnification confocal microscopy analysis showed that GFRa1 was negative in the interstitial tissue cells in the seminiferous tubule of the testis (Fig. 1). SSCs and TSCs were cultivated in distinct media to study *GFRa1* gene expression in these cells. SSCs were isolated from the adult testis after enzymatic digestion and the isolated cells were cultivated in the presence of the above mentioned growth factors. The generated SSCs were characterized according to our previous study (20). The immunocytochemistry (ICC) examination demonstrated that the isolated SSCs were positive for the GFRa1 protein whereas the TSCs were negative (Fig. 2). Quantitative mRNA expression by Fluidigm real-time RT-PCR for the *GFRa1* gene indicated significant expression ($P<0.001$) of SSCs in comparison to TSCs (Fig. 3A). Similarly, RT-PCR analysis showed that *GFRa1* was clearly expressed in the SSCs, but not in the TSCs (Fig.3B). Flow cytometry analysis results confirmed the expression of GFRa1 on SSCs and demonstrated that about 75% of isolated SSCs expressed GFRa1 (Fig. 4).

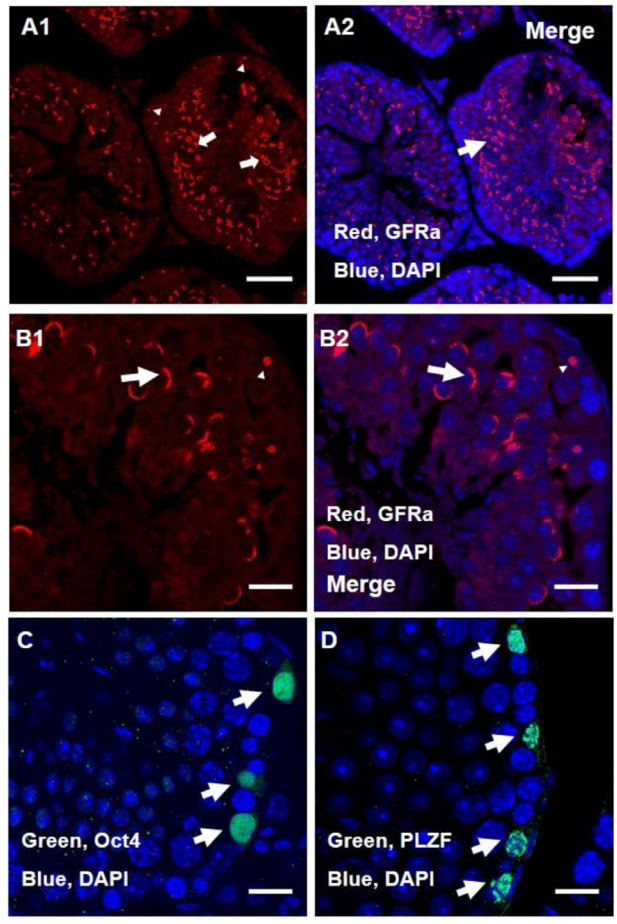

Fig. 1: Immunohistochemistry (IHC) analysis of anti-GDNF family receptor alpha 1 antibody (GFRa1) in a testis section. IHC characterization revealed that there are two distinct populations of GFRa1 positive cells. **A1, B1.** The first population expresses GFRa1 with a small round shape in the basal compartment (arrowhead). The second group is located between the basal epithelium and the luminal compartment. This group shows donut and C-shaped expression of GFRa1 (large arrow). Red GFRa1 merges with blue 4', 6-diamidino-2-phenylindole (DAPI), **A2, B2.** Green OCT4 merges with blue DAPI, **C.** PLZF merges with blue DAPI, **D.** According to sections **C** and **D**, we suggest that PLZF and OCT4 positive cells have similar expression patterns as the first population of GFRa1 positive cells (Scale bar: 50 µm).

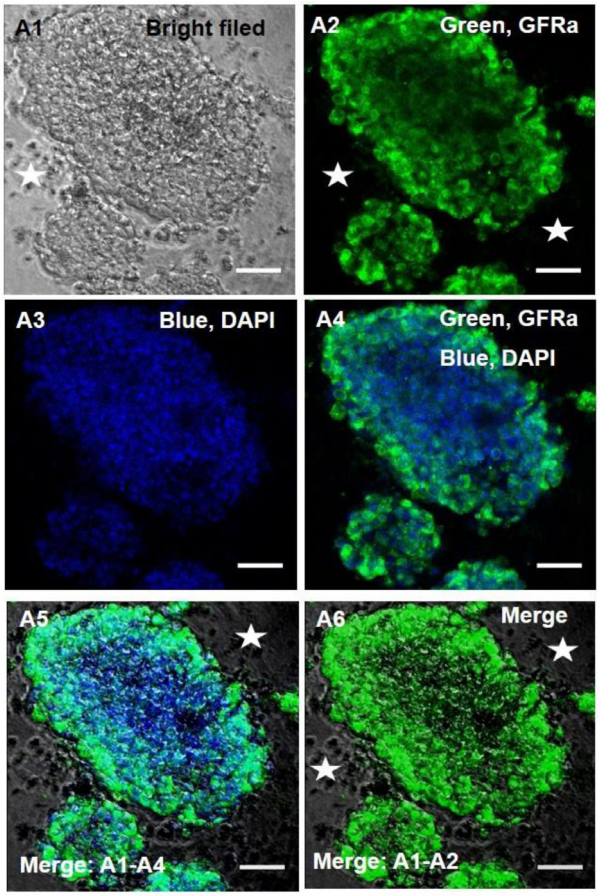

Fig. 2: Immunocytochemical analysis of PLZF in spermatogonial stem cells (SSCs) confirmed the expression of anti-GDNF family receptor alpha 1 antibody (GFRa1) in the SSCs and lack of expression in the testicular stromal cells (TSCs) (star). **A1.** Bright field, **A2.** Green fluorescence for PLZF, **A3;** Blue for 4', 6-diamidino-2-phenylindole (DAPI), and **A4-6.** Merged images (Scale bar: 50 µm).

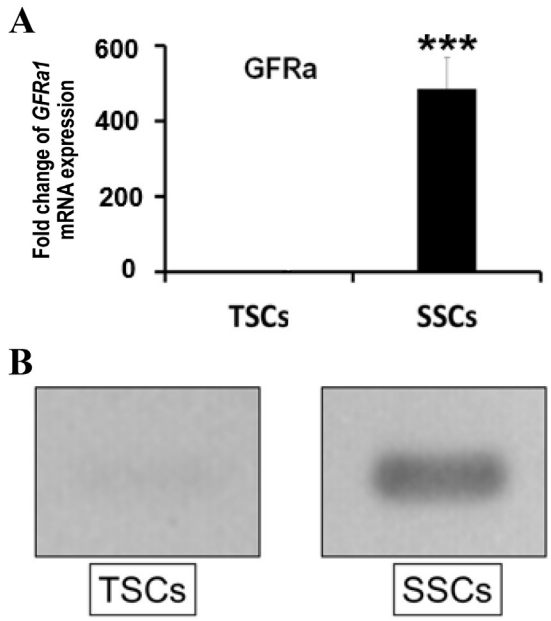

Fig. 3: mRNA expression of the anti-GDNF family receptor alpha 1 antibody (*GFRa1*) gene. **A.** Fluidigm real-time PCR (RT-PCR) analysis for *GFRa1* expression in the spermatogonial stem cells (SSCs) and testicular stromal cells (TSCs, P<0.001). Y-axis shows fold change of *GFRa1* mRNA expression in contrast with mouse embryonic fibroblasts. **B.** Reverse transcription polymerase chain reaction (RT-PCR) analysis of *GFRa1* gene for TSCs and SSCs.

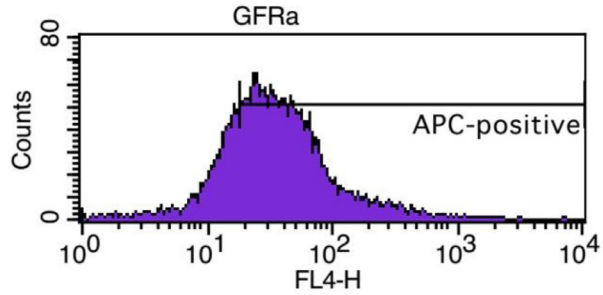

Fig. 4: Flow cytometry analysis for anti-GDNF family receptor alpha 1 antibody (GFRa1) expression in the spermatogonial stem cells (SSCs).

Discussion

Visualization of the testis tissue section by confocal microscopy showed that the germ stem cell marker GFRa1 was localized in these cells above the basement membrane of the testicular lumen. *In vivo* results showed negative expression of GFRa1 in the basement membrane and differentiated part of the testicular lumen. This result showed the heterogeneity of gene expression among undifferentiated spermatogonia during the epithelial cycle (25). A recent study of GDNF regulatory roles on the SSCs fate has shown that GFRa1 and its co-receptor complex, which is located in germ cells, play essential roles during the first wave of spermatogenesis (26). Additionally, as GDNF is involved in SSCs proliferation, it has been suggested that the lack of GDNF or incorrect expression of *GFRa1* would limit colony expansion (27). In a recent study, the results showed elevated GDNF levels when the mitotic activity of undifferentiated spermatogonia was low (28). Kanatsu-Shinohara et al. have suggested that SSCs undergo self-renewal when GDNF is elevated and they undergo differentiation when the GDNF concentration is low (29). Similarly, Sharma and Braun demonstrated that GDNF levels are highest during the stages of SSC proliferation and lowest during the stages of quiescent spermatogonia, which eventually differentiates into A1 spermatogonia (30). While about 27% of testicular tubule cells express GFRa1, negative expression of GFRa1 has been demonstrated in the interstitial tissue cells. Similarly, *in vitro* Assessment revealed that GFRa1 is expressed in SSCs, but not in the TSCs. This finding was confirmed by Fluidigm RT-PCR and ICC. Grisanti et al. reported that 5% of A_{paired} (A_{pr}) spermatogonia expressed GFRa1 asymmetrically while 10% of A_{single} (A_s) did not express GFRa1 (31). As the expression of GFRa1 was obvious between the basement membrane cells and differentiated site of the seminiferous tubules (spermatocytes) of the mouse testes, it seemed that GFRa1 expression was not necessary for the reserved SSCs in the basement membrane and differentiated spermatogonia in the final stage. Therefore, similar to the in vivo model, down-regulation of the GFRa1 germ cell marker might be necessary for the in vitro analysis of SSCs differentiation into sperm. Binding GDNF to the GFRa1 receptor and activating the Ret intracellular signalling pathway regulates the self-renewal and proliferation of SSCs (32). Hasegawa

et al. have reported that the stimulation of GFRa1 in the SSCs triggers activation of ERK1/2, which prevents them from differentiation. Similarly, this group demonstrated that the abolished activation of GDNF signalling by the deletion of GFRa1 decreased SSC proliferation (33). It has been proven that GDNF pushes SSC self-renewal by preventing SSC differentiation and not by stimulating proliferation. Activation of GDNF signalling has been shown to increase the phosphorylation of AKT3 in undifferentiated spermatogonia, which led to SSC self-renewal or progenitor cell expansion (26). By activation of the transcription factors Etv5, Bcl6b and Lhx1 in early spermatogonia, GDNF prevented expression of the c-Kit receptor (34). Production of GDNF and FGF2 by Sertoli cells regulates the self-renewal and proliferation of SSCs, whereas expression of activin A and BMP4 reduces maintenance and promotes differentiation of SSCs (35). During *in vitro* conditions, a different concentration of GDNF (10-100 ng/ml) protein was used for the colony formation of SSCs in culture (35, 36). In the prepubertal testis, interstitial Leydig and peritubular myoid cells express CSF1, which increased the proliferation of undifferentiated SSCs (37).

Conclusion

Analysis of the data confirmed that the GFRa1 germ cell marker is expressed above the basement membrane of the seminiferous tubule of the testis and in the differentiated section. It seems that GFRa1 is expressed during proliferation and differentiation. According to the roles of GDNF in the regulation of SSCs functions and the potential use for SSCs in the clinical setting, it would be of benefit to conduct future studies on GFRa1 against infertility and other male reproductive dysfunctions. Our results would be helpful for future studies to identify in vitro proliferation and differentiation of SSCs by up- or down-regulation of GFRa1 expression in these SSCs.

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Authors' Contributions

H.A.; Carried out and designed the experiment, performed data assembly, data analysis, and wrote the manuscript; A.N.T.; Contributed to the conception and design of the work, wrote the manuscript and performed data analysis, T.S.; Provided critical feedback and data analysis, and edited the manuscript. The authors read and approved the final manuscript.

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Original Article

The Four-Item Patient Health Questionnaire for Anxiety and Depression: A Validation Study in Infertile Patients

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Abstract

Background: The most common mental disorders in infertile patients are depression and anxiety. The four-item Patient Health Questionnaire-4 (PHQ-4) is a widely used tool that consists of the PHQ-2 depression and Generalized Anxiety Disorder-2 (GAD-2) scales. Given that PHQ-4 has not been validated in infertile patients, this study aimed to examine its reliability and validity in this population.

Materials and Methods: Participants in this cross-sectional study consisted of 539 infertile patients from a referral fertility centre in Tehran, Iran. The PHQ-4, Hospital Anxiety and Depression Scale (HADS), World Health Organisation-Five Well-Being Index (WHO-5), Penn State Worry Questionnaire (PSWQ) and demographic/infertility questionnaires were administered to all participants. Factor structure and internal consistency of PHQ-4 were evaluated via confirmatory factor analysis (CFA) and Cronbach's alpha, respectively. The convergent validity of this scale was examined by its relationship with HADS, WHO-5 and PSWQ.

Results: CFA results provided support for a two-factor model of PHQ-4. Internal consistency of the PHQ-4 and its subscales both were elevated with Cronbach's alpha coefficients of 0.767 (PHQ-4), 0.780 (PHQ-2) and 0.814 (GAD-2). Inter-item correlations were between 0.386 and 0.639, and corrected item-total correlations were between 0.576 and 0.687. PHQ-4, PHQ-2 and GAD-2 showed positive correlations with measures of HADS-anxiety, HADS-depression, and PSWQ and negative correlations with WHO-5, which confirmed convergent validity. Among demographic/fertility variables, we observed that gender, infertility duration, and failure in previous treatment were correlated with PHQ-4 and its subscales scores.

Conclusion: The PHQ-4 is a reliable and valid ultra-brief screening instrument for measuring both anxiety and depressive symptoms in infertile patients.

Keywords: Anxiety, Depression, Infertility, Reliability, Validity

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Introduction

Anxiety and depression, which tend to co-occur, are two of the most prevalent mental disorders in both the general population and outpatient settings (1, 2). Anxiety and depression are almost twice as common among people who experience fertility problems compared with the general population (3). This could be explained by the fact that infertility is considered as one of the great stressors in these people's lives, which could lead to serious psychological, social and cultural consequences (4-6). Among these, depression and anxiety are two of the most prevalent psychiatric disorders that adversely affect quality of life, well-being, and marital relationship and satisfaction (7, 8).

The results of a cross-sectional study on 1128 infertile patients showed a prevalence rate of 49.6% for anxiety and 33.0% for depression in Iran (8). A meta-analysis study in Iran also reported that the overall prevalence rate of depression among infertile couples was 0.47 (95% CI: 0.40, 0.55) (9). In another study, the prevalence of generalized anxiety disorder (GAD) was reported to be 28.3% among 1146 infertile patients in a referral fertility centre in Tehran, Iran (10).

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In this regard, screening infertile patients for anxiety and depression could help predict those at risk and provide an opportunity for early intervention in order to improve quality of life in these patients. A brief screening tool that is both reliable and valid seems necessary as the first step of therapy for these disorders, especially in busy settings like referral infertility centres (11, 12).

The nine-item measure Patient Health Questionnaire-9 (the PHQ-9) has demonstrated strong psychometric properties for depressive disorders and has been widely used in numerous investigations (13-16). Similarly, the seven-item measure GAD-7 has shown good psychometric properties in assessing anxiety disorders in both the general population and clinic-based settings (17-19). In order to enhance efficiency and reliability of screening, the four-item ultra-brief PHQ-4 was developed. PHQ-4 consists of a two-item measure (PHQ-2) for depression and a two-item measure for anxiety (GAD-2). PHQ-4 and its subscales have also been shown to be an excellent self-reported screening tool for both depression and anxiety symptoms (12, 20, 21).

Thus far, the PHQ-4 has not been validated in infertile patients. The aim of this study was to assess the validity and reliability of PHQ-4 in infertile patients.

Materials and Methods

Participants and study design

This was a cross-sectional study of 539 infertile patients who were undergoing fertility treatment at the Infertility Treatment Centre of Royan Institute, Tehran, Iran. The data were collected in the evaluation phase of treatment by convenience sampling between May and August 2017. Those who were married were asked to complete the instruments separately from each other and refrain from discussing their answers. We followed the STROBE statement guidelines, whenever applicable, for reporting this study. The sample size was determined using the rule of thumb suggested by Comrey and Lee (22). They suggested that researchers obtain samples of 500 or more subjects whenever possible for factor analysis studies. The eligibility criteria were as follows: 1. suffering from infertility; 2. at least 18 years of age; 3. married and in a heterosexual relationship; and 4. able to read and write in Farsi. Further details of the design and methodology of this study have been described elsewhere (18, 23). This study was conducted after receipt of approval by the Ethics Committee of Royan Institute, Tehran, Iran (Registration Number: IR.ACECR.ROYAN.REC.1395.187), and all participants gave written informed consent to take part in this questionnaire-based study.

Instruments

Demographic/infertility variables of participants that included age (years), sex (male, female), educational level (primary, secondary, university), duration of infertility (years), cause of infertility (self, partner, both/unexplained), failure of previous treatment (no, yes), and history of abortion (no, yes) were collected.

Patient Health Questionnaire-4

The PHQ-4 is an ultra-brief tool for detecting both depression and anxiety disorders, which consists of the first two items of each of the measures PHQ-9 and GAD-7 (20). Hence, the PHQ-4 consists of two, 2-item subscales - one for depression (PHQ-2) and the Generalized Anxiety Disorder-2 (GAD-2) for anxiety. Each item is scored on a 4-point Likert scale that ranges from 0 (not at all) to 3 (nearly every day). The total PHQ-4 score ranges from 0 to 12, and total PHQ-2 and GAD-2 can range from 0 to 6. Higher scores denote greater levels of depression and anxiety. In this study, we used relevant translated items from PHQ-9 and GAD-7, which had been validated in infertile patients (18, 23).

Hospital Anxiety and Depression Scale

The Hospital Anxiety and Depression Scale (HADS) is a commonly used self-administered tool that consists of 14 items. This scale is designed to measure both anxiety (HADS-A) and depression (HADS-D) (24). Each item is scored on a 4-point Likert scale that ranges from 0 to 3. The total HADS-A and HADS-D scores can range from 0 to 21, with higher scores denoting greater levels of anxiety and depression. The Persian version of HADS has demonstrated sound psychometric properties in infertile patients (25). In the present study, the Cronbach's alpha coefficient of the HADS-A and HADS-D were 0.884 and 0.783, respectively.

World Health Organisation-Five Well-Being Index

The World Health Organisation-Five Well-Being Index (WHO-5) is a brief, 5-item self-administered tool that measures well-being during the previous two weeks (26). Each item is scored on a 6-point Likert scale that ranges from 0 (at no the time) to 5 (all of the time). The raw scores are transformed to a score from 0 to 100, with higher scores indicative of better well-being. The Persian version of the WHO-5 has demonstrated sound psychometric properties in infertile patients (27). In the present study, the Cronbach's alpha coefficient of the WHO-5 was 0.858.

Penn State Worry Questionnaire

The Penn State Worry Questionnaire (PSWQ) is a 16-item self-administered tool that measures both frequency and intensity of worry (28). Each item is scored on a 5-point Likert scale that ranges from 1 (not at all typical) to 5 (very typical). The total PSWQ score can range from 16 to 80, with higher scores denoting greater worry. We used the Persian-language version of PSWQ (with some modification in translation), which was validated among students (29). In the present study, the Cronbach's alpha coefficient of the PSWQ was 0.886.

Statistical analysis

The factor structure of the PHQ-4 was examined with confirmatory factor analysis (CFA) using maximum likelihood estimation. Two models were tested. The first model was a one-factor model with all four items loaded on sin-

gle factor, which represented the PHQ-4 total score. The second model was a two-factor model where depression worded items were loaded on the PHQ-2 and the anxiety worded items were loaded on the GAD-2. Overall model fit was assessed using multiple fit criteria as suggested in the literature. Specifically, four goodness-of-fit indices were calculated - chi-square/degree of freedom (χ^2/df), compara¬tive fit index (CFI), root mean square error of approximation (RMSEA), and standardised root mean square residual (SRMR). Values of χ^2/df<2, CFI>0.95, and RMSEA and SRMR<0.08 indicate good fit to the data (30-33). The internal consistency of the PHQ-4 and subscale scores was evaluated by using Cronbach's alpha, inter-item correlation and corrected-item total correlation. Convergent validity was examined by measuring the correlations between the PHQ-4 and measures of HADS-A, HADS-D, WHO-5 and PSWQ. Pearson's correlation coefficient, independent t-test and one-way ANOVA were used to examine the relationship between PHQ-4 scores and demographic/fertility characteristics. Statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA) and LISREL 8.80 (Scientific Software International, Inc., Lincolnwood, IL, USA). A P <0.05 was considered statistically significant.

Results

Participants′ characteristics

A total of 539 infertile patients (249 men and 290 women) participated in this study. The average age and infertility duration of the participants were 32.97 (SD=5.34) and 5.55 (SD=4.07) years, respectively. Table 1 summarizes the other demographic and fertility characteristics.

Descriptive statistics and internal consistency of the Patient Health Questionnaire-4

Table 2 shows the item wording, descriptive statistics and internal consistency reliability of the PHQ-4 and its subscales. The item means ranged from 0.95 to 1.27. The mean (SD) scores were 4.63 (3.29) for the PHQ-4, 2.42 (1.86) for the PHQ-2 and 2.22 (1.82) for the GAD-2. The corrected item-total correlations for the PHQ-4 were in the acceptable range of 0.576 to 0.687. Moderate to strong inter-item correlations were observed among the PHQ-4 items (rs ranged from 0.386 to 0.639). Taking the brevity of the PHQ-4 and its subscales into account, we determined that the internal consistencies of the PHQ-4, PHQ-2 and GAD-2 were satisfactory (Cronbach's Alpha=0.814, 0.767 and 0.780, respectively).

Table 1: Demographic and fertility characteristics of the participants

	Mean ± SD or n (%)
Age (Y)	32.97 ± 5.34
Sex	
Male	249 (46.2)
Female	290 (53.8)
Educational level	
Primary	92 (17.1)
Secondary	175 (32.5)
University	272 (50.4)
Duration of infertility (Y)	5.55 ± 4.07
Cause of infertility	
Self	163 (30.2)
Partner	155 (28.8)
Both/Unexplained	221 (41.0)
Failure of previous treatment	
No	253 (46.9)
Yes	286 (53.1)
History of abortion	
No	382 (70.9)
Yes	157 (29.1)

SD: Standard deviation (n=539).

Table 2: Item wording, descriptive statistics and internal consistency of the PHQ-4

	Mean (SD)	Corrected item total correlation	Alpha if item deleted	Cronbach's Alpha
PHQ-2 items				
1. Little interest or pleasure in doing things	1.19 (1.05)	0.576	0.794	
2. Feeling down, depressed, or hopeless	1.22 (1.01)	0.687	0.741	
GAD-2 items				
3. Feeling nervous, anxious or on edge	1.27 (1.00)	0.674	0.748	
4. Not being able to stop or control worrying	0.95 (1.01)	0.600	0.782	
PHQ-2 total score	2.42 (1.86)			0.767
GAD-2 total score	2.22 (1.82)			0.780
PHQ-4 total score	4.63 (3.29)			0.814

SD; Standard deviation, PHQ-4; Patient Health Questionnaire-4, GAD-2; Generalized Anxiety Disorder-2, and PHQ-2; Patient Health Questionnaire-2.

Convergent validity

As presented in Table 3, there were strong correlations between PHQ-4 and measures of HADS-A (r=0.717), HADS-D (r=0.535), WH0-5 (r=-0.559) and PSWQ (r=0.560). We obtained the same results for both the PHQ-2 and GAD-2. As seen in Table 3, the correlations of PHQ-2 with measures of depression (HADS-D and WHO-5) were higher than the correlations with measures of anxiety (HADS-A and PSWQ). The correlations of GAD-2 with measures of anxiety (HADS-A and PSWQ) were also higher than the correlations with measures of depression (HADS-D and WHO-5)

Table 3: Correlations between PQH-4 and measures of HADS, WHO-5, and PSWQ

	HADS-A	HADS-D	WHO-5	PSWQ
PHQ-2	0.573	0.491	-0.518	0.451
GAD-2	0.700	0.458	-0.475	0.545
PHQ-4	0.717	0.535	-0.559	0.560

PHQ-4; Patient Health Questionnaire-4, PHQ-2; Patient Health Questionnaire-2, GAD-2; Generalized Anxiety Disorder-2, HADS; Hospital Anxiety and Depression Scale, WHO-5; World Health Organisation-Five Well-Being Index, and PSWQ; Penn State Worry Questionnaire. All correlations were significant at the 0.001 level.

Confirmatory factor analysis

The Confirmatory factor analysis (CFA) were used to examine the goodness of fit of the one and two-factor models of PHQ-4. The goodness of fit indices showed that the one-factor model did not fit the data well ($\chi^2(2)=86.25$, $P<0.001$; $\chi^2/df=43.12$; CFI=0.92; RMSEA=0.280 and SRMR=0.060). The result indicated that the two-factor model was a good fit to the data ($\chi^2(1)=0.02$, $P=0.881$; $\chi^2/df=0.02$; CFI=1.00; RMSEA<0.001 and SRMR=0.001). (Fig. 1)

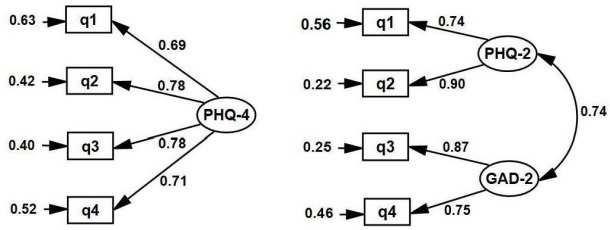

Fig. 1: Confirmatory factor analysis (CFA) for a one- and two-factor model of the Patient Health Questionnaire-4 (PHQ-4).

Relationship of the Patient Health Questionnaire-4 to demographic characteristics

Table 4 shows the relationships of the PHQ-4, PHQ-2 and GAD-2 with demographic/fertility characteristics. As seen in Table 4, women exhibited higher scores of PHQ-2, GAD-2 and PHQ-4 compared to men. Significant, but low, positive correlations were obtained between infertility duration and scores of PHQ-2 (r=0.118), GAD-2 (r=0.128) and PHQ-4 (r=0.139). Patients with previous treatment failures scored higher compared to patients who were undergoing their first treatment. Age, level of education, cause of infertility and history of abortion were not related to scores of the PHQ-4 and its subscales.

Table 4: Item wording, descriptive statistics and internal consistency of the PHQ-4

Variable	PHQ-2		GAD-2		PHQ-4	
	Mean (SD) or r	P value	Mean (SD) or r	P value	Mean (SD) or r	P value
Age (Y)	-0.014	0.741	-0.016	0.707	-0.017	0.691
Duration of infertility (Y)	0.118	0.006	0.128	0.003	0.139	0.001
Sex		<0.001		<0.001		<0.001
Male	1.90 (1.83)		1.78 (1.70)		3.67 (3.11)	
Female	2.86 (1.77)		2.59 (1.86)		5.45 (3.18)	
Educational level		0.731		0.063		0.218
Primary	2.54 (1.91)		2.62 (1.99)		5.16 (3.36)	
Secondary	2.35 (1.83)		2.10 (1.82)		4.46 (3.26)	
University	2.41 (1.86)		2.15 (1.76)		4.56 (3.24)	
Cause of infertility		0.300		0.934		0.554
Self	2.23 (1.90)		2.17 (1.95)		4.40 (3.36)	
Partner	2.49 (1.73)		2.23 (1.83)		4.72 (3.20)	
Both/unknown	2.50 (1.91)		2.24 (1.73)		4.74 (3.25)	
Failure of previous treatment		0.022		0.031		0.012
No	2.22 (1.88)		2.04 (1.79)		4.26 (3.24)	
Yes	2.59 (1.83)		2.37 (1.84)		4.96 (3.26)	
History of abortion		0.157		0.430		0.213
No	2.34 (1.81)		2.18 (1.82)		4.52 (3.21)	
Yes	2.59 (1.95)		2.31 (1.84)		4.90 (3.41)	

PHQ-4; Patient Health Questionnaire-4, PHQ-2; Patient Health Questionnaire-2, GAD-2; Generalized Anxiety Disorder-2, SD; Standard Deviation, and r; Pearson correlation coefficient.

Discussion

To the best of our knowledge, this is the first study that examined the reliability and validity of the PHQ-4 in infertile patients. There is some evidence that infertile patients experience more anxiety and depression than the general population. In this study, the mean PHQ-4 score was 4.63 (SD=3.29), which was considerably higher than reported in the German (M=1.76, SD=2.06) and Colombian (M=1.27, SD=2.01) general population (12, 34), US college students (M=2.98, SD=2.41) (21), patients from primary care clinics in the United States (M=2.5, SD=2.08) (20) and pre-operative surgical patients (M=2.63, SD=2.58) (35).

Taking the brevity of the PHQ-4 and its subscales into account, the internal consistency reliability of the PHQ-4 was relatively high. The obtained Cronbach's alpha values were in line with previously reported values in different populations (12, 20, 21, 35). In addition, the inter-item correlations and corrected item-total correlations were also within acceptable ranges.

Despite the strong correlation between the PHQ-2 and GAD-2 subscales, CFA results demonstrated that these two subscales of the PHQ-4 reflected two separate dimensions (i.e., depression and anxiety). Previous exploratory factor analysis and CFA of the PHQ-4 also yielded two subscales, anxiety and depression (12, 20, 21).

Convergent validity of the PHQ-4 and its subscales was confirmed via its strong correlations with HADS, WHO-5, and PSWQ inventories. In addition, the correlations of PHQ-2 (or GAD-2) with other depression (or anxiety) inventories were higher than the correlations with other anxiety (depression) inventories. These results were compatible with previous studies that reported correlations between PHQ-4 scores and measures of depression, anxiety, quality of life, well-being, hope and self-esteem (12, 20, 21, 34, 35).

We also examined the relationship between demographic/fertility characteristics and the PHQ-4, PHQ-2 and GAD-2. As expected, women exhibited higher scores of PHQ-2, GAD-2 and PHQ-4 compared to men. Empirical evidence supports the view that women express more anxiety and depression than men. Epidemiologic studies in the infertility context also show that anxiety and depression disorders are more prevalent among women than men (25, 36). Contrary to some general population-based studies (12, 34), there were no relationships between age and scores of PHQ-4 and its subscales. However, there were low indirect correlations between infertility duration and anxiety/depression scores. These results were consistent with previous studies (25, 37-39). In addition, there was a similar trend in other studies for measures of well-being, marital satisfaction and quality (25, 39). In our study, patients with unsuccessful treatment outcome obtained higher scores of PHQ-4 compared to patients who underwent their first treatment, which was in line with previous studies on measures of anxiety/depression and related measures such as quality of life, well-being and life satisfaction (39).

Several limitations of the current study should be mentioned. First, this was a single-centre study and the generalization of the findings may be limited. Second, unfortunately, structured diagnostic interviews based on DSM-IV were not performed, which precluded any discussion of the sensitivity and specificity of the scale. Third, because of the cross-sectional setting of the present study, causal inference between PHQ-4 scores and demographic/fertility characteristics could not be determined. Fourth, the test-retest reliability of the PHQ-4 was not assessed in this study. Fifth, we did not have data on infertility-specific instruments such as fertility problem inventory (FPI) (40) and fertility quality of life (FertiQoL) (39) to examine convergent validity of the PHQ-4.

Despite the limitations, the present study provided a number of important implications for both researchers and practitioners. We assessed a sample of patients with infertility; therefore, our assessment of PHQ-4 suggests that this instrument can be used as a quick, reliable and valid primary screening instrument for patients who require in-depth assessment, follow-up for diagnosis and psychological intervention for anxiety and depression symptoms. Health professionals can use this scale to assess large numbers of infertile patients and rapidly screen them for anxiety and/or depression symptoms. Second, this questionnaire also provides a useful assessment tool when data must be collected by telephone or online. Third, clinicians and therapists who work with infertile patients should be aware of the factors associated with anxiety and/or depression symptoms such as female sex, long infertility duration and unsuccessful treatment.

Conclusion

PHQ-4 is a reliable and valid screening instrument that can be used to measure anxiety and depressive symptoms in infertile patients. The scale is an ultra-brief and easy to use tool that can be administered in a few minutes. PHQ-4 provides an economic tool for research and practice. Furthermore, the CFA results provide support for the two-factor structure of the scale (PHQ-2 and GAD-2) and use of these factors as discrete variables.

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Authors' Contributions

A.G., S.M.; Conception and design, data interpretation, and drafting of the manuscript. R.O.S., M.H., M.S.; Conception and design, collection, assembly of the data,

and drafting of the manuscript. S.M.; Analysis of the data. All authors read and approved the final version of the manuscript.

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Original Article

Exploring Infertile Couples' Decisions to Disclose Donor Conception to The Future Child

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Abstract

Background: Despite significant advances in reproductive technology, using donor assisted reproductive technology is a double-edged sword that has numerous challenges. One of the most challenging issues for couples is whether or not to disclose this information to donor offspring. This study, therefore, explored infertile couples' decision to disclose donor conception to their future child.

Materials and Methods: This qualitative study was conducted using content analysis approach in 2012 in the Milad Infertility Centre, Mashhad, Iran. Data were collected through semi-structured interviews with 32 infertile persons including nine couples and 14 women who were selected by purposive sampling. Data were analysed by conventional qualitative content analysis adopted by Graneheim and Lundman using MAXQDA 2010 software.

Results: Two categories were emerged: 'not to disclose information to the child' and 'to disclose information to the child'. The first category consisted of three subcategories: 1. child support from probable harms; 2.to maintain healthy family relationships; and 3. lack of a compelling reason to disclose this information. The second category embraced four subcategories: 1. awareness of the others; 2. emergence of new living conditions; 3. appreciation for the donor; and 4. honesty among family members. The main reason for not disclosing information was to protect the child from probable harm.

Conclusion: Although protecting children from possible harms was a major reason for infertile couples' secrecy, keeping this secret would not be always easy. Therefore, increasing public awareness about the donation process in order to change the beliefs of community and eliminate the infertile couples' concerns would help them to overcome this problem. Additionally, long-term psychological counselling during and after the donation process is highly recommended.

Keywords: Child, Decision Making, Disclosure, Donor Conception, Infertility

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Introduction

Infertility is a global reproductive health problem (1) that affects 8-12% of childbearing couples worldwide (2). Globally, approximately 48.5 million couples suffer from infertility (3). The experience of infertility can cause a wide range of social, psychological, physical and financial problems for couples (4-6). Despite considerable advances in assisted reproductive technology (ART) and new methods for becoming pregnant (7), which include donor conception, its use is compared to a double-edged sword that has many challenges for infertile couples. One of the most challenging issues of donor conception pertains to disclosure of this information to the child (8, 9). In other words, having a healthy baby does not end the challenges faced by the couples who undergo ART (10). Parents are confronted with many difficult questions that include how, what, when, and whether to disclose this information (11). In the past, reproductive endocrinologists advocated confidentiality and, prior to 1980, parents were advised to maintain secrecy regarding assisted reproductive donation procedures. However, the attitudes and approaches regarding these procedures have changed in recent decades and a sincere atmosphere has emerged among specialists due to lower levels of perceived stigma

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following the increased use of ART (10). Concerning the importance of disclosure to the child, countries such as Switzerland, New Zealand and the State of Victoria in Australia enacted laws on access by children to their origin of conception (12). Legislations in some countries have granted these children the right to be informed regarding their biological parents once the child reaches maturity (8, 10). Different countries have varying approaches in terms of matters of confidentiality. Although all forms of ART in Iran are approved by religious leaders, there is only a law for embryo donation and no laws exist for other donation methods. According to implementing regulations of the embryo donation law, the transfer of donated embryos is carried out in strictly confidential conditions. The documents and information on donors and recipients of donated embryos can only be obtained by order of the judicial authorities (13).

Studies around the world have reported different results regarding couples' views on disclosure of this information to the child. Some couples agreed to tell the child, whereas others have a negative opinion of this matter (10). In some studies, most couples who underwent fertilization via donated oocytes either chose to not disclose this information or were undecided (14-16). Conversely, in some other studies, the results were inconsistent. For example, in one study, 78% of donor oocyte recipients had decided to tell the child, 16% initiated the disclosure process, and 6% had planned for non-disclosure or were uncertain (16-18).

In Iran, few studies have reported the views of infertile couples in relation to the issue of disclosure. In a study conducted in Tabriz, most female respondents believed that it would be preferable for children born through surrogacy to not be told about the procedure (19). In a study in Tehran, most women also stated that they had no intention to disclose the gamete donation conception to their children (20).

We undertook this study because in recent years we have seen a sharp increase in the use of donated eggs (21) and, to the best of our knowledge, no qualitative research has been conducted in Iran regarding the decision to disclose this information to the donor children. This is the first Iranian study that has used the qualitative approach to explore decisions by infertile couples who underwent donor conception that pertained to whether they might disclose or not to disclose this information to their future children. It is hoped that the findings of this study will be useful for policy making, planning and providing services to infertile couples in Iran and other countries with similar social and cultural contexts.

Materials and Methods

This qualitative study was conducted using the content analysis approach. Qualitative study, where participants' experiences are extracted, is appropriate for issues where information is limited and no prior research has been conducted (22).

The study was conducted in Mashhad, Iran in 2012. The participants consisted of 32 persons, which included nine infertile couples and 14 infertile women candidates for the following: donor eggs (11 persons), donor embryo (seven persons), donor egg and surrogacy (two persons), and surrogacy (three persons). The participants were purposively selected and interviewed.

The study population included all Iranian, Persian-speaking couples who referred to the Milad Infertility Centre with a diagnosis of infertility due to male, female, both, or unknown reasons. The participants were candidates for donor conception at the time of the study or beforehand and did not have biological children or stepchildren.

The sampling was purposive and we attempted to take into account the maximum variety of participants' choices in terms of age, education, economic status, cause of infertility, and duration of infertility and its treatment.

Data were collected using in-depth semi-structured interviews by the first author. Before starting the interview, each participant was informed about the purpose of the study and the study duration. Participants signed a written informed consent for study participation. The interview started with a general question about the purpose of the study (describe your experience with deciding to disclose the use of donation methods to your future child) and followed with the following questions: 'What factors influenced your decision-making? How did these factors influence your decision making? What were your difficulties and obstacles in making this decision?' Based on the participants' responses, the interviewer asked additional questions or used probing questions to direct the interviews to elicit the participants' experiences. Sampling continued without restriction in the number of participants until data saturation. A second or third interview was arranged to complete the data when the researcher faced gaps in the data during analysis and needed to ask new questions from particular participants. Each interview took between 30-120 minutes. Interviews with infertile couples were conducted separately; if there was a clear disagreement between their responses, a joint interview with the husband and wife was also held.

Data collection and analysis were carried out simultaneously by following the principles of conventional qualitative content analysis adopted by Graneheim and Lundman where coding and categorizing originate directly from the text (23). Conventional content analysis is usually used when the purpose of the study is to describe a phenomenon in which limited studies are available (24). Data were analysed in four stages. In the first stage, all recorded interviews were transcribed verbatim and considered as an analysis unit. In the second stage, by repeated reading of the transcripts, immersion in the data was performed to obtain a general insight. After repeated reading, the text was divided into sentences and paragraphs to identify the meaning units. In the third step, through process of reduction and condensation of meaning units, concepts or key ideas that were hidden within the meaning units, were coded. In the fourth step, similar codes were grouped into categories using

MAXQDA 2010 software (25).

In order to increase the credibility of the study, variations in research participants, repeated reading of the interviews, long-term engagement with participants and the research environment during data collection and feedback from the participants (member check) were used. Dependability of the data was established through feedback from an external observer experienced in qualitative research.

To ensure the confirmability of the findings, the text of few interviews, codes and extracted categories were given to two researchers who were familiar with qualitative data analysis to confirm the accuracy of the process of data analysis. To examine transferability, two women who did not take part in the study, but had the similar profile with the participants, were asked to elaborate their experiences regarding the disclosure of the donor conception to the future child. What they explained was a reflexion of what researchers found in this study.

The study was approved by the Ethics Committees of Shahid Beheshti University of Medical Sciences, Tehran, Iran (Research Project Code: SBUMS. 8717). Prior to the interview, the purpose of the study was explained to the participants who subsequently provided their written informed consent. Participants were assured that their information would be kept confidential. All participants were allowed to withdraw from the study at any time without any change in their care plan. Prior to the interview, the permission of participants for recording their voice was taken; just one participant disagreed, so notes were taken during her interview.

Results

The age range of the women who participated in the study was 23-41 years, the age range of the men who participated was 26-40 years, the duration of marriage ranged from 7-14 years, and the duration of infertility treatment with donation methods ranged from 1-4 years. The education in women ranged from elementary to bachelor's degree and, in men, from diploma to postgraduate.

Couple's decisions about disclosure of the use of donor conception to the child included two categories including not to disclose to the child and to disclose to the child (Table 1).

Table 1: Categories and sub-categories emerged from the data

Sub-Categories	Categories
Child support from possible harms To maintain healthy family relationships No compelling reasons for disclosure	Not to disclose information to the child
Awareness of others Emergence of new living conditions Appreciation for the donor Honesty among family members	To disclose information to the child

Not to disclose information to the child

Data analysis demonstrated that most participants decided not to disclose the donation process to the child. The category of 'Not to disclose information to the child' appeared from three subcategories of 'child support from probable harm', 'to maintain healthy family relationships', and 'no compelling reasons to disclose', which are discussed in detail. The most commonly cited sub-category was 'child support from probable harms'.

Child support from probable harms

Most participants stated that they were mostly concerned about the child poor of acceptance and his possible reaction. The child's limited understanding of the current normal life situation and making him aware of the different aspects of birth would make it very difficult for the child. Therefore, disclosure would be a sensitive issue that could cause emotional and psychological trauma. Secrecy about the origin of the child, infliction of unnecessary emotional burden on the child and the possible harms and confusion could be prevented. One participant stated:

"Major trauma will be imposed on the child when somebody tells him that his current parents are not his genetic parents - even if this situation would happen to me at this age, of course I would be upset and would be generally confused, you know, definitely it is much harder for a young child "(40-year-old male, candidate for embryo donation).

Aside from the social stigma that exists with pregnancy from donation procedures, disclosure of the origin of pregnancy for the reason that the child is seen as different from other children would result in isolation, loneliness, fear, and stress. The child would be haunted by the question, 'Who do I belong to? Are my real parents good or bad?' Awareness of the issue may cause the child to feel shame and his sense of self-worth might be damaged, which would affect the child's self-esteem and feelings of identity, as one participant expressed:

"Once the child knows, he will become isolated and when he is exposed to the society, he will have the impression that he is different from the others and would feel isolated" (31-year-old female, candidate for oocyte donation).

Despite this issue, some couples are concerned that the child will unexpectedly discover the information regarding his identity. One respondent stated:

Even if we have children, our problems are not over and we still have to worry about the child realizing the truth) 34-year-old male, candidate for egg donation).

To maintain healthy family relationships

Couples are always concerned about the destruction of family relationships, in particular the relationships between parents and the child following disclosure of this information. They are concerned that disclosure might

lead to low sense of belonging and family dependency in the child. As a consequence, it causes a change in perception towards the parents and, ultimately, the child's rejection of the parents would happen.

"I'm telling you, once the child knows about the issue, his thoughts will be preoccupied unconstructively and he might say, 'my parents are lying to me and I'm not their child'" (36-year-old female, candidate for egg donation).

No compelling reasons to disclose

Some of the participants stated that there is no need to make the child's life complicated with confusing and stressful information. Telling the truth to the child would not benefit the child because he cannot do anything about it. On the other side, the genetic relationships between the child and the couple would result in some ownership to the couples and, through pregnancy and exchange of blood and nutrients between the mother and the foetus, the biologic relationships and the role of the mother is maintained. Thus, disclosure is not considered to be vital. Breastfeeding can also help maintain this role. One participant stated:

"It is not necessary to disclose this information. The child is mine, the sperm is mine and the child will develop inside my wife's uterus. After birth, my wife will breastfeed the child" (36-year-old male, candidate for egg donation).

There was an initial agreement between the couple's decisions about not disclosing this information to the child. They both decided not to inform the child about the donation procedure.

To disclose information to the child

Regarding the category of to disclose this information to the child', data analysis revealed that only a few couples reported the intent to disclose. This category was derived from four subcategories: 'awareness of the others', 'emergence of new living conditions', 'appreciation for the donor' and 'honesty among family members'. 'Awareness of others' was the subcategory most often mentioned by the participants.

Awareness of others

Awareness of the others plays an important role in the couple's decision. When others are aware of the issue, it becomes important that parents tell the child the truth in order to prevent accidental disclosure by others. Being told the truth by others would result in the child's feelings of distrust toward her/his parents. One respondent commented:

"In my opinion, it is much better that we will tell the truth ourselves because when others know about this issue, the child must also know"(43-year-old female, candidate for surrogacy).

Emergence of new living conditions

Couples who desired secrecy commented that the emergence of a new life situation would cause them to re-evaluate their decisions because disclosure varies depending on the future situation of the community and, with passage of time, peoples' knowledge and awareness of these procedures will increase. This would pave the way for a community easier acceptance and decreased exposure of the child to social stigma. Also, in case the same problems exists for future offspring and disclosure is necessary to protect the child, the couple's decision will change and there is a greater possibility of telling the truth to the child at the appropriate time. One participant explained this idea in the following way:

"In cases where the same problem exists for the child, I would say the fact. I will only disclose the truth when my child becomes wiser, and can identify and be able to understand the situation better"(41-year-old female, candidate for oocyte donation and surrogacy).

Appreciation for the donor

Regarding the use of familiar donors, disclosure to the child at an appropriate age for the purpose of appreciating the donor is predictable, as one participant stated:

"It is much better that the child be told that her auntie is also his rightful mother, and should know that she/he has some duties towards her and is entitled to many rights" (31-year-female, candidate for surrogacy).

Honesty among family members

One of the participants explained that she made use of ethical reasons in making her decision to disclose, and the principle of honesty and the desire for honest and transparent relationships between family members. Although parents are known to be honest; however, most are only willing to partially tell the child about the truth about surrogacy, but not regarding egg donation. This indicates that the issue of absence of genetic relationships is more sensitive than surrogacy. One participant expressed this idea:

"I think that at least I can tell my child about surrogacy and I think it is easier since telling a lie is very difficult for me to do. I hate to lie and I always think of the consequences of lying"(39-year-old female, candidate for oocyte donation and surrogacy).

Most participants, from both urban and rural areas, decided to keep the use of donor conception secret. The results of this study also showed that among the types of donor conception, surrogate candidates were more likely to disclose the donation method to the child.

There was an agreement between the couple's decisions to disclose information to the child, and they both decided to inform the child about the donation procedure under certain circumstances.

Discussion

The results of the present study indicated that most couples decided not to disclose the use of donor conception to their future child. This finding, when compared with studies conducted worldwide, indicated that levels of disclosure to the child in Iran are much lower. Despite general recommendations to parents in relation to disclosure of the donor conception to future offspring (16, 26), results of the studies conducted worldwide about disclosing the origin of pregnancy to the child yielded a wide range of results. A survey of 111 recipient couples that used donor eggs or sperm showed that the majority of participants planned for disclosure and some had begun the disclosure process. Only a few planned for non-disclosure or were not certain. (18). The results of a study in Spain (2014) showed that most participants intended to disclose donor conception to the child, whereas a few participants did not intend to disclose this information or had not yet decided at the time of completing the questionnaire (12). In some studies, most couples who conceived through oocyte donation decided not to disclose this information or were uncertain (14-16).

According our study, the main reason for non-disclosure to the child was to protect the child from probable harms. In some studies, protection of the child from possible harms, including psychological and moral harm, was the most common explanation (27). In a study conducted in Northern California, parents who did not disclose this information believed that nondisclosure to the child protected the child from unnecessary psychological pressure, avoided potential harm and confusion as well as the feeling of isolation (28). Studies that compared the long-term consequences of disclosure and non-disclosure by families have found that there is no difference in child welfare or the parent-child relationships (29-31). In contrast, some studies believe that not telling the truth may increase the child's psychological problems (32).

Another reason expressed by the current study participants for non-disclosure was to support and maintain family relationships. In a study, the most important motivation by couples toward non-disclosure of the donor conception to the child was fear of rejection by the family, social environment and/or the child (33). In some studies, the existence of stigma due to donation methods from the causes of secrecy has been expressed. In some studies, the stigma surrounded the issue of donor conception has been mentioned as a reason for secrecy (11).

Another reason stated by the current study participants for not disclosing was lack of compelling reasons for disclosure. This finding of this study is congruent with another study which found that many mothers decide not to disclose the issue of donor conception to the offspring, as they feel that there is no need to tell the child. For these mothers, genetics were far less important than parenting (10).

According to the findings of this study, awareness of others about this issue was the most common reason given by the couples who intended to disclose donation methods to the future child. There exists a clear relationship between disclosure and non-disclosure in relation to the child and society. Couples who opted for non-disclosure to the child were obviously more secretive towards the society, while couples who intended to disclose this information were more truthful on disclosing it to families and friends. Since the social environment may be aware of the problem of infertility and gamete donation, parents are always concerned about others disclosing this fact to the child. In one study, parents who decided to disclose donor conception expressed concern about accidental disclosure of this subject from someone other than the parents (28). A study in Iran assessed infertile couples' decisions in relation to disclosure of donor conception to others. The results indicated that couples who chose not to disclose this information to others stressed the idea of child protection from accidental disclosure as it could affect the child-parent relationship and create a lack of trust about the parents (13).

Another reason mentioned by this study's participants for disclosure was emergence of new living conditions. Parents who opted not to disclose stated that new living conditions might lead them to reconsider their decision (34).

Although the results of this study indicated that another reason for disclosure was appreciation for the donor, which was the case for familiar donors, differing results have been reported. Another study reported no significant difference between known and unknown oocyte recipients regarding disclosure to the future child (35).

In this study, another reason for disclosure was honesty among family members. This finding was consistent with the results from other studies (10, 11, 28, 33).

The child's right to know has been mentioned as an important factor for disclosure (11) in worldwide research studies; however, in the present study, this was not mentioned by any of the participants.

The fear of disclosure remains a difficult question: 'When the child realizes the truth, does he or she still accept us as his or her parents?

Based on the results of current study, non-disclosure is accompanied by stress. Couples' concern about disclosure remains as a hard question: When the child realizes the truth, does he/ she still accept couple as his or her parents? Worries and apprehension can also harm the sexual and emotional relationships of the couple (36). However, the results of this study also showed that disclosing information to the child may be associated with stress and negative effects on the couple and the child. According to one study, in some people, disclosure of the fertility conditions was like a double-edged sword that put additional pressure on them (37). In such circumstances, providing counselling can help them to cope better with their stressful situation and come to terms with their experiences (38). They should also be encouraged to adopt adaptive coping strategies in order to enhance their self-empowerment and to achieve

a sense of personal wholeness by merging the bio-psycho-social perspectives (39).

The findings of this study showed that among the three types of donation, those who were candidates for surrogacy were more likely to disclose the truth to the child. Some candidates for a traditional surrogacy decided to disclose the use of the surrogate mother to the child, but not disclose the use of the surrogate mother's egg. This might be due to decreased social stigma about surrogacy. This was consistent with the findings reported by Readings et al. (10). Research have shown that the presence or lack of biological communication with the child has a profound effect on the disclosure process in parents (40).

The strengths of this study include the use of a qualitative approach to directly reflect participants' responses. In addition, this study was conducted in a referral centre admitting patients with different socio-cultural backgrounds. The information was obtained from couples rather than only women.

One of the limitations of this study was the lack of cooperation of some participants, which was due to the sensitivity of the issue of donation conception and its stigma.

In this research, the couples' decisions to disclose information to the child was evaluated. However, conducting further studies for long-term assessments would be beneficial because the reported sentiments of the couples in relation to disclosure do not always reflect their future behaviour, and the decision to disclose might be different from the actual disclosure.

Conclusion

The results of the present study indicated that most couples decided not to disclose the use of a donation procedure to their future child in order to protect the child from possible harms. The results of this study were somewhat different from those in other countries. The rate of disclosure to the child in Iran is very low, which might be due to the stigma of using donation methods in Iranian culture.

Therefore, we recommend interventions to change public perceptions to reduce this stigma and present fertility donation methods in a natural way to resolve the infertile couples' concerns. Also, since the use of donor conception and childbirth does not end couples' concerns about disclosure, long-term counselling is recommended for these couples.

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Authors' Contributions

F.H.-T.; Study design, im¬plementation, analysis, and drafting of the manuscript. R.L.R, M.S.; Supervised the study design, im¬plementation, analysis and revised the manuscript. All authors read and approved the final version of manuscript.

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Original Article

Analysing First Birth Interval by A CART Survival Tree

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Abstract

Background: Birth spacing, especially the first birth interval (FBI), is a suitable index to investigate the delayed fertility that results in a low fertility pattern. Non-parametric familiar alternatives to the Cox proportional hazard regression (CPH) model include survival trees that can automatically discover certain types of covariate interactions according to the survival length. The aim of this research is to study FBI influential factors by applying survival trees.

Materials and Methods: In this cross-sectional study, 610 married women (aged 15-49 years), were selected from different regions of Tehran, Iran in the Winter and Spring of 2017. Classification and regression trees (CART) for the FBI survival tree were fitted by taking into consideration the predictors of each woman's age, age at first marriage, educational level, partner's educational level, activity, region, house ownership, kinship, partner's race, marriage time attitude, and expenditure using R packages.

Results: Since the PH assumption of the CPH model was not confirmed for the covariates of age at first marriage (P=0.001), kinship (P=0.000), partner's race (P=0.001), and marriage time attitude (P=0.042), the results of this model were not valid. Thus, a CART survival tree was fitted. The validity of the fitted model in assessing FBI was confirmed by the significant result of the log rank test (P<0.01) for the terminal nodes and the value of the separation measure, which was greater than 1. The fitted tree had 13 terminal nodes and the most vital FBI predictor was women's age. The longest FBI belonged to educated and employed women, ages 30-37 years.

Conclusion: Analysing patterns of birth spacing by selecting the appropriate statistical method provides important information for health policymakers. In order to formulate appropriate demographic policies, it is essential to take into consideration age, educational level and job status of the women, all of which have essential roles on their decision to have children.

Keywords: Cox Proportional Hazards Model, First Birth Intervals, Machine Learning, Survival Analysis

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Introduction

Iran has the lowest fertility rate in the Middle East. In Iran, there has been a reduction in fertility rate from 7 births per woman in 1979 to 1.9 in 2006 and 1.8 in 2011 (1, 2). Delays in childbearing result in a low fertility rate and a decreased fertility pattern in the society. Among a large number of factors that influence the determination of the fertility pattern, it is essential to study the first birth interval (FBI), which is defined as the length of time between two successive live births. FBI is advantageous because the chances for better recall during the post-marriage period or the duration after a woman's marriage; it is easier for women to remember their first pregnancy information. The delay in the menstrual cycle that occurs after childbearing is not observed in this birth interval. Of note, the other birth intervals are heavily affected by irregular changes in FBI. If women deliver their first child during their younger ages and have shorter ideal birth intervals, it could cause them to have their subsequent children sooner. Thus, these women could most have achieve to their ideal number of children and complete the dimension of their family (3).

Survival analysis comprises a branch of statistical methods that analyse event occurrence and time. Survival analysis has been used to study FBI over the past decade in Iran by using Demographic Health Research (DHS) or survey data. According to the DHS data in 2000, the FBI was 2.7 years (3) and increased to 3.5 years in 2010 (4). Survey data from Semnan Province, Iran in 2012 indicated that the FBI was 2.76 years and 90% of the first children were born four years after the marriage date (5); in Tehran, the FBI increased from 2.5 years in 2000 (2) to 3.2 years in 2017 (6).

Most FBI studies applied non-parametric and semi-parametric survival analyses such as Kaplan-Meier (KM) estimations, log-rank tests, and the Cox proportional hazard (CPH) regression model to study factors that impacted

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FBI. Although simple interpretations of the covariate effects and inferences can be readily achieved by non-parametric survival analysis, they suffer from simultaneously studying the effect of covariates on the response variable. Semi-parametric methods that include CPH and its extensions are used to study survival data and they force a particular connection between the covariates and the response variable. When it is not feasible to define the logarithm of the hazard rate as a linear function of the covariates, more adaptable methods such as survival trees are available. Advantages of survival trees include tremendous flexibility and automatic detection of certain types of covariate interactions without any further specification. Therefore, significant predictive groups of covariates can be easily derived from survival trees. Fitting a single tree has been mostly replaced by an ensemble of trees, which often results in more powerful predictive models that are free from selection of single tree challenges. However, a single tree can still be helpful to gain perception and ease of data interpretation (7).

Many authors have proposed tree-based methods for univariate (or uncorrelated) survival data (8, 9). The development of survival trees has recently grown where the goal was mainly to extend existing tree methods to the case of censored survival data. Classification and regression trees (CART) has gained popularity in many application fields due to the handling of a variety of data structures, the requirement for few statistical assumptions, and ease of interpretation of classification and prediction rules. A CART survival tree has been provided by generalizing the CART algorithm for survival data (8). The main aim of the current research is to study FBI influential factors by applying survival trees as a valid substitution the CPH model when the PH assumption is not fulfilled.

Materials and Methods

In this cross-sectional study, we investigated factors that influenced women's FBI so we selected 610 married women, aged 15-49 years from a survey entitled 'The Effect of Socio-economic Dimensions of Rationality on Childbearing behaviour in Tehran' in 2017 (10).

The sample size was chosen by the Cochran formula , which took into consideration a 5% error level, proportion of 0.5 (384 samples), a design effect of 2.5 and nonresponse rate of 1.25. Samples were selected by multi-stage stratified random sampling from different regions of Tehran Province, Iran between February and May, 2017. By applying the hierarchical clustering approach, we clustered the regions of Tehran Province according to the developmental indices into four developmental levels: more developed, developed, middle developed, and developing (11). Therefore, the first developmental level consisted of regions 1, 2, 3, and 6, the second developmental level consisted of regions, 5 and 7, the third developmental level consisted of regions 4, 8, 9, 10, 11, 12, 13, 16, 21, 22, 14, and 20, and the forth developmental level consisted of regions 15, 17, 18, and 19. Then, each of the developmental levels in different regions of Tehran was considered as a class; the regions in each of these classes were proportionally selected according to their size. Finally, 10 regions were selected for the final selection. In each selected region, four large blocks were randomly chosen and the samples were collected by systematic random sampling in each block between February and May, 2017. A structured questionnaire that contained demographic, fertility history and childbearing attitudinal factors was completed (10). The validity of the questionnaire was confirmed by 10 demographers and sociologists. Cronbach's alpha reliability of the questionnaire's factors was at least 0.771. There were no interventions or treatments in this study, and the aim of the study was explained to the respondents prior to the interview process. The participants provided oral consent to participate in this study and the ethical code was supplied by National Population Studies and Comprehensive Management Institute for the questionnaire (code number: 20/18627). The event of interest was the time of the FBI in months and the main aim of this original study was to detect factors that influenced the women's FBI.

According to different studies that investigated influential factors on FBI in Iran, the most important covariates included women's age (6, 12, 13), age at first marriage (1, 14-18), educational level (4-6, 14, 15, 19, 20), partner's educational level (16, 20), activity (14, 16, 20), region (18, 21), house ownership (16, 19), kinship (21, 22), partner's race (14, 20), marriage time attitude (18,21), and expenditure (16). According to the literature, we selected the following covariates of women's age, age at first marriage (<20, 20-29, >30 years), educational level (under diploma, diploma and above), partner's educational level (under diploma, diploma and above), activity (unemployed, employed), region (developing, middle developing, developed and more developed), house ownership of the family (rent, own, other), kinship (family, non-family), partner's race (Fars, Turk, other), marriage time attitude (sooner: those who thought that they married sooner than their desired time; later: those thought that they married later than their desired time; and on-time: those who thought that they married at the same desired time), and expenditure (<2 million tomans, 2–3.5 million tomans, ≥3.5 million tomans) were considered. To evaluate the influence of selected covariates on FBI accurately, a CART survival tree method ("rpart" R package) was used for data analysis. With a survival outcome, the splitting criterion used by rpart is equivalent to the one introduced by LeBlanc and Crowley (9).

Statistical analysis

A CART survival tree can be broadly described as follows:

1. Splitting: Breiman et al. (23) used CART to formalize and generalize the basic idea of recursive binary partitioning of a determined covariate space into smaller regions until a minimum node size could be attained. This

is often achieved by minimizing a measure of node impurity. The concluded regions that contain observations of homogeneous response values are called nodes (parent and children nodes). The final partitions are called terminal nodes. For survival data, Ciampi et al. (24) suggested the use of log-rank statistics to compare the two groups formed by the children nodes. The retained split is the one with the largest significant test statistic value. The use of the log-rank test leads to a split which assures the best separation of the median survival times in the two children nodes.

2. Pruning and selection: Backward and forward methods are two approaches used to select a final tree which is not too large to over-fit the data and fail to generalize well to the population of interest, or too small to miss important characteristics of the relationship between the covariates and the outcome. The backward method builds a large tree and then selects an appropriate subtree by pruning and a forward method uses a built-in stopping rule to decide when to stop splitting a node further. The two most important pruning indices are cost-complexity (23) and split-complexity (25).

At the terminal nodes of the selected tree, appropriate node summaries are usually computed to interpret the tree or obtain predicted values. At these nodes, for a categorical outcome, the node proportions of each value will be reported. For a continuous outcome, the node average will be informed and for a survival outcome, the KM estimate of the survival function or the estimated hazard ratios (HR) calculated by the Nelson-Aalen estimator of cumulative hazard function (CHF) (26) will be reported. There is no commonly accepted approach used to assess the predictive ability of the fitted CART survival tree (27). One common approach is to plot the KM estimates for event-free or overall survival in the g groups made by a predictive classification scheme called risk strata or groups. This figure and also significant P-values of the log-rank test when its null hypothesis tests the equality of the survival functions in the g risk strata are necessary, but do not provide sufficient condition for good predictive ability of the fitted CART survival tree. Another approach is to fit a CPH model using dummy variables for the risk strata, and find the estimated HR of the risk strata with respect to a reference group. Crowley et al. (27) proposed a measure of separation (SEP) according to the proportioned absolute estimated logarithm of HR values of the CPH model for the terminal nodes based on a reference node. For survival data, SEP is the standard deviation estimation of the predicted log HR according to a model that has a dummy variable for each group. The favourable tree-based models have greater than 1 value for SEP.

Results

In this section, the rationale of applying a CART survival tree in analysing FBI is studied. The dataset includes 469 women with at least one child and 141 censored observations (childless women). It is important to note that the KM mean of the FBI was 38 ± 1.06 months and KM survival estimate plot for the women's FBI in Figure 1 indicates that most of the women's the FBI (about 88%) for most women were less than five years.

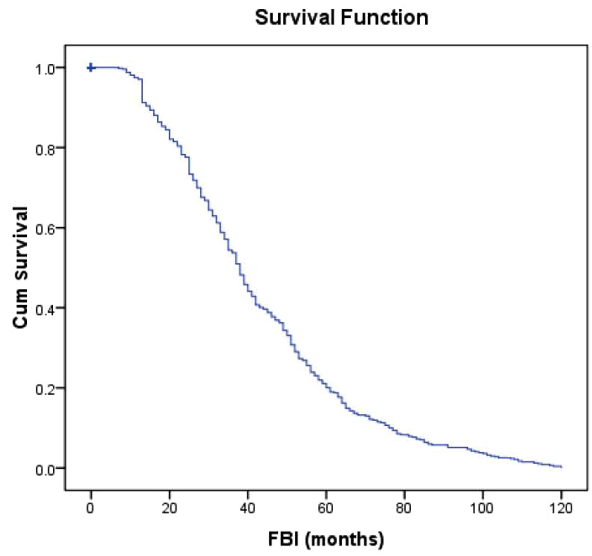

Fig. 1: Survival plot of first birth interval (FBI).

Table 1 indicates the frequency of women's demographic statistics and KM estimates. In order to describe the women's FBI according to the selected covariates in univariate analysis, we used KM estimates and the log-rank test as non-parametric survival tools.

The mean FBI was 42.87 months with a standard deviation of 1.11 months and median of 38 months. The women's mean age was 35.22 ± 7.91 years and the age at first marriage was 22.61±4.6 years. Table 1 lists the KM means (standard errors) and P value of the log-rank tests for the FBI according to the selected covariates. According to these indicators, it would be easy to define the average and significant differences of this variable amongst the various categories of covariates. Table 1 shows that the women's age (P<0.001), educational level (P<0.001), partner's educational level (P=0.001), activity (P=0.014) and region (P=0.020) had significant effects on FBI.

The CPH model was applied to investigate the simultaneous effects of all the covariates on FBI. The PH hypothesis for all covariates was tested by correlating the corresponding set of scaled Schoenfeld residuals with time in order to test for independence between residuals and time. The results are presented in Table 2. A non-significant relationship between residuals and time supports the PH assumption whereas a significant relationship refuses this assumption. Since the PH assumption test of the CPH model was statistically significant for the covariates of age at first marriage (P=0.001), kinship (P<0.001), partner's race (P=0.001), and marriage time attitude (P=0.042), the PH assumption could not be fulfilled. Therefore, it was unrealistic to expect the reported Cox coefficients to be satisfactory indicators of the actual covariate effects on FBI and the results of the fitted CPH model were not valid.

Table 1: Frequency distribution of women's characteristics and their first birth interval (FBI) Kaplan-Meier (KM) estimates

Variables	Frequency	Percent	KM estimates		
			Median survival time (SD)	Log rank test	P value
Age (Y)					
<=29	152	24.9	38 (1.72)	22.290	0.000**
30–39	265	43.4	41 (2.47)		
>=40	193	31.6	34 (1.90)		
Age at first marriage (Y)					
<20 (ref)	209	34.3	34 (1.58)	4.466	0.107
20–29	351	57.5	40 (1.35)		
>30	50	8.2	38 (3.95)		
Educational level					
Under-diploma	81	13.3	32 (1.59)	13.452	0.000**
Diploma and above	529	86.7	39 (1.10)		
Partner's educational level					
Under-diploma	112	18.4	32 (1.65)	10.633	0.001**
Diploma and above	498	81.6	39 (1.12)		
Activity					
Unemployed	415	68.0	37 (1.22)	6.030	0.014*
Employed	195	32.0	42 (4.13)		
Region					
Developing and middle developing	419	68.7	37 (1.07)	5.440	0.020*
Developed and more developed	191	31.3	41 (2.83)		
House ownership					
Renter	307	50.3	38 (1.28)	1.317	0.518
Owner	238	39.0	37 (1.49)		
Other	65	10.7	35 (3.82)		
Kinship					
Family	168	27.5	40 (2.39)	1.328	0.249
Non-family	442	72.5	37 (1.41)		
Partner's race					
Fars	340	55.7	40 (1.40)	5.827	0.054
Turk	160	26.2	37 (2.25)		
Other	110	18.0	34 (2.37)		
Marriage time attitude					
Sooner	63	10.3	33 (2.94)	2.263	0.323
Later	166	27.2	41 (2.85)		
On-time	381	62.5	38 (1.12)		
Expenditure					
<2 million tomans (ref)	362	59.3	37(1.25)	1.765	0.414
2–3.5 million tomans	176	28.9	41 (2.02)		
≥3.5 million tomans	72	11.8	36 (1.76)		
Total	610	100.0	38 (1.06)		

***; Significant at the 0.05 level, and **; Significant at the 0.01 level.**

We sought to accurately evaluate the influence of selected covariates on FBI by applying a CART survival tree method to the data. The final pruned survival tree selected by cross-validation had 13 terminal nodes and is shown in Figure 2. The first line in each terminal node indicates the HR within the group, the second line in each node is the number of events and the whole samples on the selected node, and the third line is the percentages of samples in that node. The terminal nodes in Figure 2, from left to right, are named nodes A to M. According to the confirmed

CART survival tree in Figure 2, the important covariates in analysing FBI were women's age, partner's educational level, region, race partner, kinship, house ownership, educational level, age at first marriage, and activity. The first split is based on women's age. The left node samples are those with age values less than 37 and the right node samples are those with age values greater or equal to 37. Therefore, the terminal nodes that are indicated in Figure 2 by the sorted HR values of FBI are Node C (HR=0.56), Node A (HR=0.6), Node B (HR=0.67), Node D (HR=0.87), Node G (HR=0.92), Node I (HR=0.97), Node J (HR=1.1), Node F (HR=1.2), Node L (HR=1.4), Node H (HR=1.6), Node E (HR=1.9), Node K (HR=2.1), and Node M (HR=2.6). The longest interval between marriage and first birth belongs to the women who were 30-37 years of age and who lived in the developing and middle regions, diploma and above educational level, were owners or had other ownership status, and employed (Node C with an HR=0.56). The shortest interval between marriage and first birth belonged to the 37 years old or older women who had under diploma educational level partner and were renters (node M, with HR=2.6).

Table 2: Cox proportional hazard (CPH) model for first birth interval (FBI).

Variables	CPH model			PH assumption test		
	β	Hazard ratio (HR)	Standard error	P value	Chi Square	P value
Age (Y)	0.018	1.019	0.007	0.011*	2.443	0.118
Age at first marriage (Y)						
<20 (ref)						
20-29	-0.130	0.878	0.114	0.255	10.747	0.001**
>30	0.053	1.054	0.208	0.799	2.166	0.141
Educational level						
Under-diploma (ref)						
Diploma and above	-0.26	0.764	0.170	0.115	1.236	0.266
Partner's educational level						
Under-diploma (ref)						
Diploma and above	-0.162	0.850	0.143	0.257	0.692	0.405
Activity						
Unemployed (ref)						
Employed	-0.168	0.845	0.119	0.158	0.019	0.889
Region						
Developing and middle developing (ref)						
Developed and more developed	-0.148	0.863	0.110	0.180	1.847	0.174
House ownership						
Renter (ref)						
Owner	0.052	1.054	0.109	0.630	0.007	0.932
Other	0.035	1.035	0.174	0.842	0.251	0.615
Kinship						
Family (ref)						
Non-family	0.318	1.374	0.115	0.005**	21.470	0.000**
Partner's race						
Fars (ref)						
Turk	0.241	1.272	0.115	0.037*	0.138	0.710
Other	0.118	1.125	0.132	0.373	10.176	0.001**
Marriage time attitude						
Sooner (ref)						
Later	-0.206	0.814	0.175	0.240	4.150	0.042*
On-time	0.022	1.022	0.162	0.892	0.784	0.376
Expenditure						
<2 million tomans (ref)						
2–3.5 million tomans	-0.005	0.995	0.115	0.964	0.733	0.392
>3.5 million tomans	0.142	1.152	0.176	0.422	0.670	0.413

*; Significant at the 0.05 level and **; Significant at the 0.01 level.

Before further interpretation of the fitted CART survival tree, model validation by sub-group analysis for the terminal nodes was conducted according to two different approaches. Table 3 shows the mean, median and 95% confidence intervals of the FBI along with the log-rank test to compare the FBI in different nodes and significant difference in FBI in the different nodes (P<0.01), which confirmed the validation of the fitted CART survival model according to the first model validation approach.

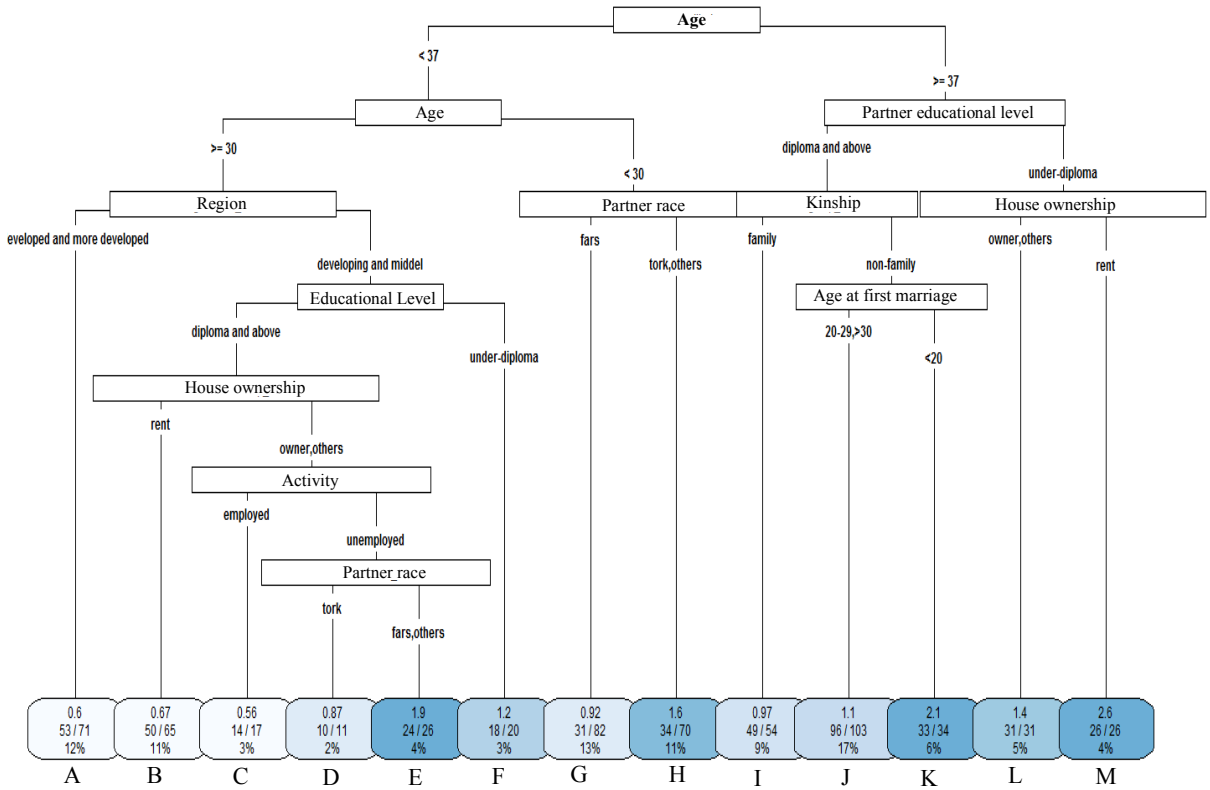

Figure 2: Classification and regression tree (CART) survival tree of first birth interval (FBI). The first line in each terminal node indicates the hazard ratio (HR) within the group. The second line in each node is the number of events and the whole samples on the selected node. The third line are is the percentages of samples in that node. The terminal nodes from left to right are nodes A to M.

Table 3: Mean, median and log rank test for FBI by nodes

Node	Mean survival time		Median survival time		Log rank test	
	Estimate	95% Confidence interval	Estimate	95% Confidence interval	Chi square	P value
A (HR=0.6)	58.98	(51.03, 66.93)	50.00	(40.83, 59.17)		
B (HR=0.67)	55.20	(47.81, 62.59)	53.00	(46.07, 59.93)		
C (HR=0.56)	63.21	(45.14, 81.29)	48.00	(0.00, 110.34)		
D (HR=0.87)	47.80	(31.10, 64.50)	33.00	(12.86, 53.14)		
E (HR=1.9)	30.17	(24.15, 36.18)	26.00	(22.40, 29.60)		
F (HR=1.2)	38.33	(28.82, 47.85)	37.00	(26.61, 47.39)		
G (HR=0.92)	46.29	(39.10, 53.48)	48.00	(32.73, 63.27)	117.82	0.000**
H (HR=1.6)	33.82	(29.10, 38.54)	34.00	(26.86, 41.14)		
I (HR=0.97)	44.65	(39.03, 50.27)	42.00	(33.77, 50.23)		
J (HR=1.1)	41.33	(36.94, 45.73)	38.00	(34.16, 41.84)		
K (HR=2.1)	27.61	(21.81, 33.41)	25.00	(17.79, 32.21)		
L (HR=1.4)	36.68	(31.08, 42.28)	34.00	(30.90, 37.10)		
M (HR=2.6)	25.11	(20.48, 29.75)	23.00	(21.77, 24.23)		
Overall	42.87	(40.69,45.05)	38.00	(35.91, 40.09)		

***; Significant at the 0.05 level, **; Significant at the 0.01 level, and FBI; First birth interval.**

Table 4: Cox proportional hazard (CPH) model first birth interval (FBI) according to the terminal nodes

Variable Node	CPH model				PH assumption test	
	β	Hazard ratio (HR)	Standard error	P value	Chi square	P value
A	-1.793	0.251	0.251	0.000	0.403	0.525
B	-1.622	0.249	0.249	0.000	0.021	0.885
C	-1.895	0.341	0.341	0.000	0.077	0.781
D	-1.353	0.376	0.376	0.000	0.105	0.745
E	-0.390	0.284	0.284	0.170	0.028	0.866
F	-0.910	0.309	0.309	0.003	1.177	0.278
G	-1.230	0.270	0.270	0.000	1.035	0.309
H	-0.591	0.262	0.262	0.024	1.049	0.306
I	-1.166	0.246	0.246	0.000	0.045	0.832
J	-1.065	0.225	0.225	0.000	1.534	0.215
K	-0.317	0.263	0.263	0.228	0.420	0.517
L	-0.758	0.268	0.268	0.005	0.060	0.806

According to Figure 2, after the first split on women's age (<37 and ≥37 years), it was clear that the women whose ages ≥37 (especially in nodes J to M) tended to have lower survival time of childlessness and greater hazard rate (>1.1), which resulted in a shorter FBI. The smallest survival time node (node M) was formed by renter women aged ≥37 years with a less than diploma educational level (HR=2.6). Again, after the second split on women's age, the subjects with a value of between 30 and 37 in nodes A to D tended to have greater survival time of childlessness and lower HR (<0.87), which resulted in longer FBI. The largest survival time node (node C) was formed by women aged 30-37 years who resided in developing and middle regions, had a diploma and above educational level, were owners or had other house ownership status, and were employed (HR=0.56).

In order to study the second model validation approach for the fitted CART survival tree, we computed the SEP measure, which confirms the predictive ability of the fitted model. The CPH model was fitted to the FBI according to the terminal nodes of the resultant tree [Table 4, (27)].

The values of -2log likelihood (4755.845) and P<0.01 of the fitted CPH model indicated the significance of this model. Moreover, the PH assumption test was not statistically significant for all of the terminal nodes, which confirmed the validity of the CPH model (P>0.05). Thus, the resultant coefficient estimations of the CPH model in Table 4 could be valid for computation of the SEP value of the fitted CART survival tree. In order to calculate the SEP value, first, from the second line of terminal nodes in Figure 2, we took into consideration we took into consideration the fractions of the number of risk exposure samples on that node to the whole sample size (this fraction for node A is 71/610 = 0.12). Then, each fraction was multiplied by the coefficients (β) of the CPH model for the terminal nodes in Table 4 and summed. The SEP value could be calculated by computing the exponential of the resultant value. In this study, this value is equal to 2.94 and it is >1, which resulted in the validity of this model (28).

Discussion

By recently decreasing the total fertility rate (TFR) under the replacement level recently in Iran, many researchers investigated the effect of factors on TFR. One of its most influential factors was birth interval (1-3). The median trend of FBI in Tehran from 2009 (23 months) (2) to 38 months (2017) (6) indicated an ascendant, which demonstrated the need for more researches in this field. Most studies on FBI were conducted by applying the CPH model (5, 14). CPH is a semi-parametric, popular technique for analysing survival data. If the PH assumption (which means the logarithm of the hazard rate is a linear function of the covariates) does not fulfil all the covariates in the real data sets, it is unrealistic to expect the reported Cox coefficients to be satisfactory indicators of the actual covariates. Tree-based or recursive partitioning methods, such as survival trees, are popular non-parametric alternatives to the CPH model they need fewer assumptions, have greater flexibility, are easy to understand, can be explained easily, and inevitably they identify different kinds of covariate interactions. Moreover, based on the covariates, they can cluster subjects according to their length of survival patterns (29-31). Survival trees are a very active ongoing area of research (7).

To the best of our knowledge, no studies have considered influential FBI factors by the CART survival tree. The main aim of this paper was to apply the CART survival tree, to analyse the FBI of 610 married Iranian women, as an alternative non-parametric method for situations where the PH assumption of the CPH model was not fulfilled. According to the results, the KM estimator of the FBI was 38±1.06 months; almost 88% of the women delivered their first child more or less over a five-year interval. Although based on the log-rank test, the women's age, educational level, their husband's educational level, region and activity significantly affected their FBI (P<0.05). The results of the fitted CPH model were not reliable due to the unsatisfactory results of the PH assumption test for some

of the covariates. In order to consider the simultaneous effects of all covariates on FBI, a CART survival tree was fitted to the data. The validity of the model was confirmed according to the results of the log-rank test ($P<0.05$) and SEP measure (SEP>1) for terminal nodes of the fitted CART survival tree.

According to the resultant CART survival tree, the root or most influential factor on FBI was the women's age. In some of the studies, increasing age was a contributing factor to women's fertility, which caused an increase in the incidence of problems and diseases during pregnancy and childbearing played a crucial role in the women's fertility (6, 12, 13). Keshavarz et al. (13) assessed 20–49-year-old married women in Isfahan and noted a reverse correlation with the women's age and delay in their childbearing, which was in line with the results of this study. The HR of FBI for women aged ≥37 years of age was almost larger than for the women <37 years of age. Thus, the interval between marriage and childbearing for women decreased by increasing their age.

Another important issue is women's education. Women's views on marriage and fertility can be influenced by education, in particular the longer duration of university studies. Instead of childbearing, it seems that university educated women concentrated on alternative social roles. The probability of tendency, identifying, and entering women into a range of social activities and technical skills could be increased by education (5). On the other hand, educated women have access to information about how to delay their childbearing and are more likely to be engaged in occupations that are not readily compatible with having children (19). The findings of previous studies in other countries (32, 33), and particularly Iran (4), indicated that increasing women's educational level resulted in increased FBI. An assessment of the DHS data from 38 out of 51 countries found that illiterate women were more expected to consider a shorter space between their marriage and childbearing compared with educated women (34). A survey conducted in seven Asian countries indicated a negative relationship between women's educational level and their FBI (33). Iranian studies in Hamedan Province (35), Shiraz Province (15), Ahwaz Province (20), and Tehran Province (6) showed that women's educational levels were one of the important covariates that had a significant effect on FBI. These results were in line with the results of this study. The FBI for women with a 'diploma and above' was almost longer than the under diploma' educational level (5, 14, 15, 19).

The partner's educational level had a significant influence on FBI in this study. Most women with educated partners had longer FBI compared to those whose partners were uneducated. Charmzadeh et al. (20) and Alam reported the same result (16).

The results of some studies indicated that age at first marriage was an important and main determinant of FBI (14). In theory, the marriage age is inversely related to FBI, and women who married at a younger age were more likely to have their first birth later (17). Abbasi-Shavazi et al.(1), in a study in Iran, reported that the delay in marriage for women was not desirable, but delayed motherhood due to contraceptive use after marriage was attributed to achieving their goals (1). Some studies indicated that a higher age at first marriage was associated with a decreased risk of long FBI (14, 18, 22, 27, 33). In the current study, the first marriage age variable in the presence of other covariates, partner's educational level, kinship, and house ownership, in the multivariate analysis influenced FBI but contradicted the above mentioned studies. The covariate of age at first marriage in the fitted CART survival tree was located under the cluster of women aged ≥37 years and was probably due to the interaction effect of partner's educational level, which resulted in a shorter FBI for women who married younger in this cluster. The same results were also obtained by Erfani and McQuillan (18).

The findings of the current study showed that employed women had children later. This finding supported economic theories. Based on the contradiction between childbearing and economic activity, due to barriers of work and childbearing conflicts, and opportunity costs of childbearing, women's employment would be expected to lead to an increased delay in childbearing and decrease in the number of children. Erfani et al. (14), Charmzadeh et al. (20), and Alam (16) reached the same conclusions.

Region was also a significant factor for FBI in this study. The influential effect of this covariate has been studied in fertility researches (18, 21). Erfani and McQuillan (18) concluded that woman who lived in developing regions compared to more developed regions had shorter FBI. Their findings supported the results of our study.

Another influential factor on FBI was partner's race. Other studies like Charmzadeh et al. (20) and Erfani et al. (14) evaluated the effect of race on FBI. However, Erfani et al. (14) reported that this covariate was not significant. Charmzadeh et al. (20) concluded that women whose partners were of the Fars race had longer FBI compared to other women. This result was along the same line as this study for women aged <30. However, for women aged 30 to <37 years of age, the women whose partners were of the Fars race had shorter FBI compared to the other women.

House ownership was a significant covariate for FBI in this study. Charmzadeh et al. (20) indicated that renter women had shorter FBI. Alam (16) and Yohannes et al. (36) also studied women's socio-economic status and its impact on FBI; they concluded that rich women had longer FBI. These studies supported our results in the cluster of women aged ≥37 years. However, in the cluster of women aged <37 years, under the effect of the educational level covariate, we reached a different conclusion. Educated renter women had an almost longer FBI compared to other educated women.

Another significant covariate on FBI was kinship. The influence of this covariate on women's fertility was

studied by Saadati and Bagheri (21) and Bagheri et al. (22). Saadati and Bagheri (21) determined that this covariate did not significantly impact FBI. According to their result, women with family partners had longer FBI compared to women with non-family partners (21).

Conclusion

The delay in childbearing or increased birth intervals, particularly the first childbearing or FBI, are among the main factors that decreased the fertility rat to low levels. Therefore, it is essential to study the factors that affect FBI. Based on the findings of this study, a reduction in the interval between marriage age and childbearing will not be attained unless policy makers and governors provide appropriate socio-economic conditions for the families, especially in terms of the women 's employment and education.

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Authors' Contributions

M.S., A.B.; Contributed substantially and equally to the concept and design of this study, data acquisition, the statistical analysis and interpretation. Both authors read and approved the final manuscript.

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Case Report

Two Rare Cases of Uterine Leiomyosarcomas Originating from Submucosal Leiomyomas Proved by Their Immunohistochemistry Profiles

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Abstract

The most common mesenchymal tumours of the uterine corpus originate from smooth muscle cells. Leiomyomas are commonly found in women of child bearing age; however, leiomyosarcomas occur later in life (50-55 years of age). Most uterine leiomyosarcomas occur de novo, but rare cases of leiomyosarcomas that arise from leiomyomas have been reported. We present two cases of fertile women with submucosal leiomyomas that became malignant and discuss their pathologic features and immunohistochemistry studies for P16, P53 and Ki67.

Keywords: Immunohistochemistry, Leiomyoma, Leiomyosarcoma, Uterus

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Introduction

The most common mesenchymal tumours of the uterus are the smooth muscle tumours (1). The majority of these tumours are leiomyomas. Leiomyosarcomas occur rarely, around 1 in 800 smooth muscle tumour cases (2). Some of these tumours present with diagnostic challenges. The most frequently used parameters to differentiate these tumours are the extent and degree of atypia, coagulative necrosis and mitotic activity of the tumour (1). Tumour cellularity, vascular and myometrial invasion, tumour cell differentiation and presence of giant cells are beneficial. Leiomyomas are very common and present in 20-30% of women aging over 30 years; however, leiomyosarcomas account for only 1.3% of uterine malignancies. Most uterine leiomyosarcomas occur de novo but rare cases of leiomyosarcomas that arise from leiomyomas have been reported (3). In this paper, we report two cases of this rare phenomenon. Both patients gave consent for using their clinical data in research.

Case 1

A 41-year-old woman (gestation 3, labour 3) presented to the gynaecology clinic with menometrorrhagia. Her past medical history and physical examination, including vaginal exam, were normal. On transabdominal ultrasound, the uterine size was 117x68 mm with a homogenous myometrial echo. A solid mass that measured 63x43x52 mm with mixed echogenicity filled the endometrial cavity and was suggestive of a submucosal leiomyoma. The adnexa were normal. No other abnormal abdominopelvic findings were identified. The patient underwent a total abdominal hysterectomy and bilateral salpingo-oophorectomy. During the surgery, an omental adhesion was identified, which the surgeon decided to send for abdominal cytology. On gross examination of the specimen in the uterus, a 7 cm diameter submucosal mass and three (1.5, 0.8 and 0.3 cm diameter) intramural masses were found. The largest mass had a creamy cut surface with areas of haemorrhage and the smaller masses had homogenous creamy cut surfaces (Fig. 1A). A cyst filled with clear watery fluid was identified in the right ovary. Histologic examination of the submucosal mass showed a classic leiomyoma appearance except for multiple foci of nuclear atypical features and a high mitotic index (Fig. 1B, C). The intramural masses were diagnosed as leiomyomas. The cytology was negative. Immunohistochemistry revealed P16 and P53 nuclear staining and a high Ki67 index in leiomyosarcomatous areas, but not in any other areas (Fig. 1D-F).

Case 2

A 35-year-old woman (gestation 2, labour 1, abortion 1) went to the Gynaecology Clinic with abnormal uterine bleeding and spotting. Her drug history revealed consumption of a vaginal herbal suppository and oral contraceptive during the last year. Vaginal examination revealed a mass lesion that projected from the cervical canal. Transvaginal ultrasound showed a uterine that was 102x55x31 mm in size, endometrial thickness of 4 mm and a submucosal mass in the cervical canal that measured 54x46 mm. No abnormality was identified in the adnexa. The patient underwent a transvaginal myomectomy surgery. The surgical specimen was a round, polypoid creamy mass that measured 5.5x4x2.5 cm. The cut surface was homogenous and creamy with a typical whorled pattern (Fig. 2A). Microscopic sections revealed conventional leiomyoma with some foci of increased cellularity, nuclear pleomorphism and atypia with numerous mitotic figures with some atypical ones (Figure 2B, C). Immuno-

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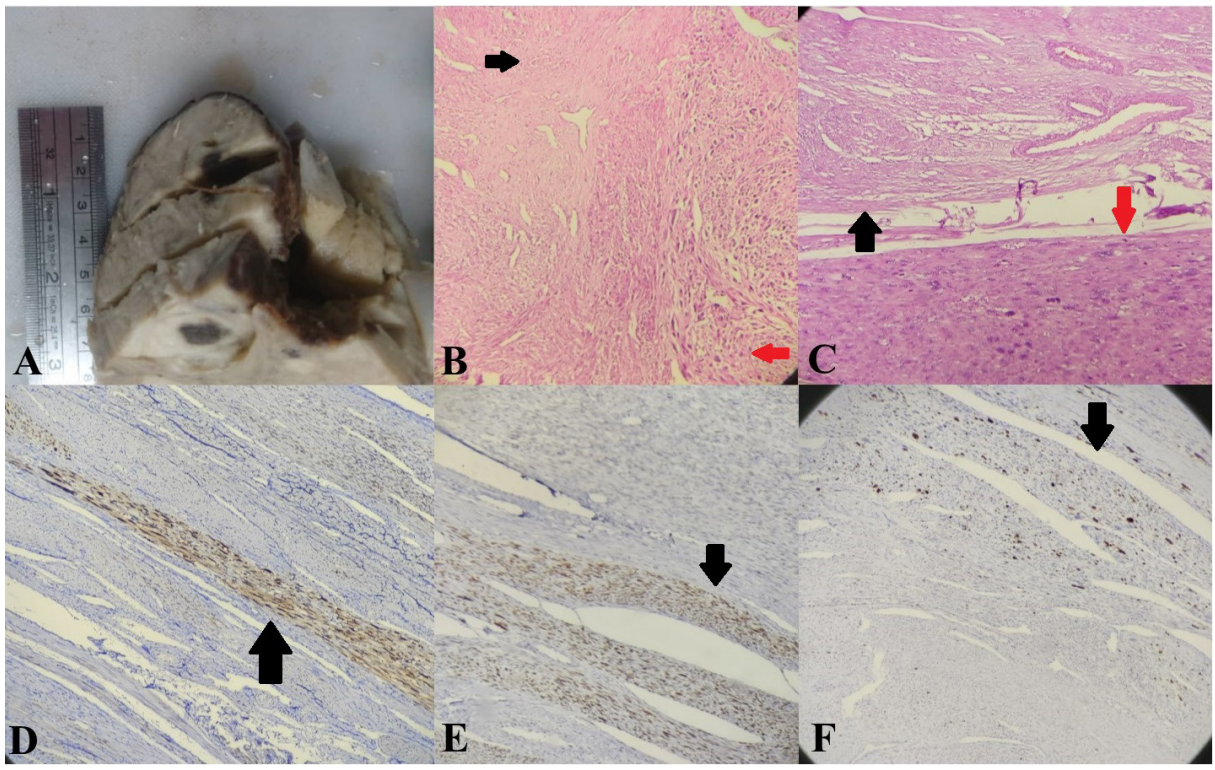

Fig. 1: Case 1. **A.** Gross appearance of the tumour. **B.** Leiomyomatous area on the left (black arrow) opposed to leiomyosarcomatous area on the right (red arrow). Haematoxylin and eosin (H&E) staining, x40. **C.** Leiomyomatous area on top opposed to leiomyosarcomatous area on the bottom (H&E staining, 100x). **D.** P16, **E.** P53, and **F.** Ki67 were all positive in the leiomyosarcomatous areas (arrows).

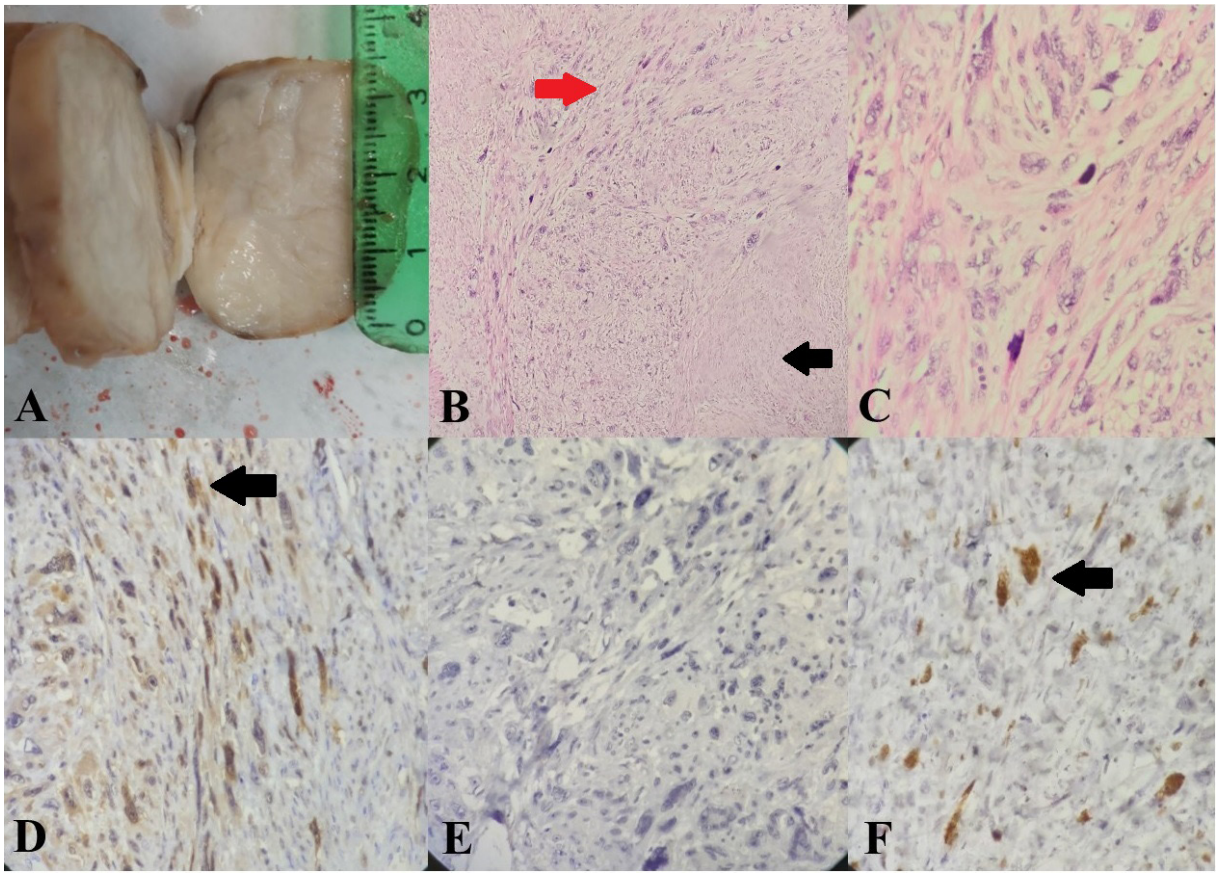

Fig. 2: Case 2. **A.** Gross appearance of the tumour. **B.** Leiomyomatous area on the right (black arrow) opposed to leiomyosarcomatous area on the left (red arrow). Haematoxylin and eosin (H&E) staining, x40. **C.** Leiomyosarcomatous area (H&E staining, x400). **D.** P16, **E.** P53, and **F.** Ki67 (Positive staining are identified by arrows).

histochemistry was positive for P16 nuclear staining and a high Ki67 index in these areas, but P53 was negative (Fig. 2D-F).

Discussion

Two types of acceptable theories for the origin of leyomyosarcomas are presented in the literature: a) uterine leiomyomas have no malignancy potential and leiomysarcomas are a de novo process without pre-existing neoplastic lesions (1) and b) rarely, leiomyomas can undergo malignant changes and become leiomyosarcomas (4).

We report two cases of uterine leiomyosarcomas that came from leiomyomas. The mean age of leiomyosarcoma presentation is 10 years more than leiomyomas (50-55 years) (1) and the most frequent symptoms are abnormal vaginal bleeding, abdominal pain and presence of an intramural mass. Concerning age, both women in our cases were younger than expected, but the initial symptom was abnormal vaginal bleeding in both. Solitary lesions occur more frequently in leiomyosarcomas than in their benign counterparts (3). Like other previously reported cases, one of our cases had multiple leiomyomas (5, 6). Leiomyosarcomas are most commonly intramural masses that measure around 6-9 cm in diameter (1); our cases were localized in the submucosa and were smaller in size compared to most de novo leiomyosarcomas.

The parallel between the current case features and leiomyosarcomas shows the unpredictable appearance that a leiomyoma undergoing malignant changes can take. Fortunately, the limited number of reported cases (with the exception of a few) (6-8), had no recurrences or metastases during long-term follow up (5, 9-11).

The accepted criteria for leiomyosarcoma consists of high mitotic figures, nuclear atypia and coagulative necrosis. In our cases, the necrosis was absent but the presence of the two other features differentiated them from smooth muscle tumours of uncertain malignant potential (STUMP) with regards to the current diagnostic criteria (1). Under these conditions, an appropriate good question is: 'When the tumour does not have all the characteristic features of a leiomyosarcoma, what are the alternative tools to help with diagnosis?' We ordered immunohistochemistry studies for some of the available markers that were used in previous studies.

In 2009, Mittal et al. (12) performed an immunohistochemistry examination of 26 leiomyosarcomas that had benign looking areas. They scored the stainings of P53, oestrogen receptor (ER), progesterone receptor (PR) and Ki67 index in both the leiomyosarcoma and leiomyoma areas. They attempted to detect genetic aberrations by means of high density oligonucleotide array (CGH array). The results showed that ER and PR were lower in leiomyosarcomas areas compared to leiomyomas, but Ki67 index and P53 scores were higher in these tumours. In addition leiomyoma-like areas presented with alterations of numerous oncogenes, transcription factors and tumour suppressor genes. The proposed theory was that not all, but only rare cases, of leiomyomas could be precancerous lesions.

The investigation of p53 mutation in uterine smooth muscle tumours was first conducted by De Vos et al. They sequenced the P53 exons of eight cases of leiomyomas and eight cases of leiomyosarcomas. Point mutations were observed in three cases from the leiomyosarcoma group, whereas none of the leiomyomas showed any alterations (13). Subsequently, many studies used p53 immunohistochemistry as a helpful diagnostic tool; however, it was uncommon to use p16 (6, 10, 11). We performed immunohistochemistry for the P53 and P16 markers, and the Ki67 index for both cases. The results showed positive P16 staining and high Ki67 index coloration in both cases. P53 staining was observed in only one case. The pattern of staining showed that, other than the positive areas, the remaining sections were similar to classic leiomyoma. Therefore, we concluded that the malignancy arose from a benign tumour. In cases with similar features, immunohistochemistry for both P53 and P16 could be a useful tool in proving the malignant nature of these areas. Utilization of both markers was quite unique and there have been few studies. Thus, we recommend further investigations of these cases.

Conclusion

This study used immunohistochemistry for the P53, P16 markers and the Ki67-index to confirm leiomyosarcomas that arose from small submucosal leiomyomas in two young women. These findings raise the possibility of malignant transformation of very benign looking leiomyomas.

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Authors' Contributions

H.G.; Participated in collection of clinical data, evaluation of patient specimens and interpretation of immunohistochemistry results. M.R.; Participated in evaluation of patient specimens and interpretation of immunohistochemistry results. Z.V.; Prepared the manuscript and participated in collection of patient data. All authors read and approved the final manuscript.

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Letter to The Editor

Endometrial Cancer in Women with Adenomyosis: An Underestimated Risk?

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Endometrial carcinoma (EC) has a worldwide incidence of 8.4 per 100,000 persons per year and is a leading cause of cancer mortality in women in developed countries after ovarian and cervical cancer (1). Among the gynecological diseases, polycystic ovary syndrome and uterine leiomyomas have been associated with a potential increased risk to develop EC, whereas less robust data are available for endometriosis and adenomyosis (2). The association between EC and adenomyosis, defined by the presence of ectopic endometrial glands and stroma within the myometrium, is still debated: on the one hand, adenomyosis is found with an incidence of 10 to 18% in EC specimen at final histology after hysterectomy (3). On the other hand, accumulating evidence suggests that these two diseases share several altered molecular pathways, which lead to increased angiogenesis, abnormal tissue growth, and invasion. In details, adenomyosis and EC are both associated with a local microenvironment characterized by high level of vascular endothelial growth factor, platelet-derived growth factor, increased production of reactive species of oxygen and pro-inflammatory cytokines, KRAS mutations and, to a lesser extent, progesterone-resistance, epithelial-mesenchymal transition and fibroblast-to-myofibroblast trans-differentiation (4). All these elements can cause growth and reduced apoptosis rate of endometrial stromal cells. Interestingly, accumulating evidence suggests that EC may arise due to incessant proliferative stress of endometrial stromal cells at the junctional zone endometrium (5), the same anatomical and histological location hypothesized to give origin to adenomyotic foci from oligoclonal stromal cells. In this scenario, recently a large epidemiological dataset compared endometrioid EC co-existing with adenomyosis and endometrioid EC arising from adenomyosis with EC without adenomyosis (6). According to this analysis, EC arising from adenomyosis was associated with significantly younger onset ages and better survival than other cases where adenomyosis was just co-existing. This distinctive behavior between the two conditions may suggest that when EC arises from adenomyotic microenvironment, the degenerated stromal cells could have a less aggressive phenotype and could more susceptible to hormonal influence. Although speculative, the differences between EC arising from, or just co-existing with, adenomyosis may be a key element to understand also the potential different responses to hormonal drugs such as medroxyprogesterone and levonorgestrel-release intra-uterine devices, especially in the scenario of fertility-sparing approach in very selected patients with early-stage disease. A correlation between EC and obesity with metabolic syndrome (i.e. polycystic ovary syndrome) was proven (1), so further studies are required to evaluate a correlation between adenomyosis and these factors. On that basis, we solicit both future large epidemiological studies and molecular investigations to clarify whether with EC arising from adenomyosis or just co-existing with it may need different therapeutic strategies.

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A.S.L.; Drafted the manuscript. M.S.; Revised the manuscript. All authors read and approved the final manuscript.

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Letter to The Editor

COVID-19 Mediated by Basigin Can Affect Male and Female Fertility

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Abstract

Coronavirus disease 2019 (COVID-19) prevalence has caused many problems in society and disrupted many regular aspects of life. COVID-19 contains major structural proteins that among them, S protein can promote fusion of the viral and cellular membranes and facilitate the entry of coronavirus into the host cells. Basigin (BSG) is one of the most important receptors for COVID-19 that mediates its entry to host cells. Also, Basigin has an important role in male and female reproduction. Basigin is expressed in the uterus and plays an important role during embryo implantation and needed for successful implantation. Therefore, disruption or inhibition of Basigin causes to a weakness in embryo implantation. Therefore, if a woman or a man is infected with COVID-19, it is recommended that they do not attempt to conception until their treatment is complete. It is also recommended tests for COVID-19 be performed on infertile couples before using assisted reproductive technology (ART).

Keywords: Basigin, CD147, COVID-19, Fertility

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Basigin (BSG), also known as CD147 is a glycosylated transmembrane protein in human cells. Basigin is a potent inducer for matrix metalloproteinases and vascular endothelial growth factor. Also Basigin is an important regulator of cell metabolism (1).

Role of Basigin in male and female reproduction

BSG is expressed in Sertoli cells, Leydig cells and germ cells and is recognized as a critical factor for spermatogenesis. So, disruption or inhibition of Basigin causes to spermatogenesis failure (2). Expression of BSG has been confirmed in the stroma, cumulus and granulosa cells of ovary. BSG mRNA and protein were detected in granulosa cells in follicles at all stages of development and also in the corpora lutea (1). Some data suggest that BSG may play a role during the follicle development and corpus luteum formation (3).

Basigin and embryo implantation

Basigin is expressed in the uterus and plays an important role during embryo implantation, in the way that embryonic expression of BSG is needed for successful implantation (4, 5). Therefore, disruption or inhibition of Basigin causes to a weakness in embryo implantation of embryos.

Basigin and COVID-19 invasion

BSG is a cellular receptor of COVID-19 which can mediate the entry of virus into the host cells (6). It also has an impact on certain infectious diseases such as malaria, Neisseria meningitides, and HIV-1 (7). COVID-19 contains the major structural proteins Spike (S), envelope (E), membrane (M), and nucleocapsid (N), among them, S protein can promote the fusion of viral and cellular membranes (8). So, it facilitates the entry of coronavirus

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into the host cells. It is reported that COVID-19 creates a novel invasion route with binding of S protein to BSG through which it invades host cells. This novel invasion route for COVID-19 provides a new target for anti-viral drug development (9).

Finally, it is concluded that Basigin is necessary for normal fertility in both males and females. Concerning the invasion route of COVID-19 mediated by Basigin, it is hypothesized that the COVID-19 infection can potentially effect on reproduction. Therefore, if a woman or a man is infected with COVID-19, it is recommended that they do not attempt to conception until their treatment is complete. It is also recommended tests for COVID-19 be performed on infertile couples before using assisted reproductive technology (ART).

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Authors' Contributions

S.M.; Drafted the manuscript. M.Sh., A.M.; Took part in revising the manuscript. All authors read and approved the final manuscript.

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Letter to The Editor

An Overview on Guidelines on COVID-19 Virus and Natural and Assisted Reproductive Techniques Pregnancies

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Abstract

In this article, we reviewed and compared some of COVID-19 and pregnancy guidelines; this can be useful for pregnant women including those with a history of infertility specially those undergone assisted reproductive techniques (ART). The general advice given for prenatal care is to reduce face-to-face visits. All women who refer for prenatal visits should be evaluated for signs of the infection at the time of entry. The triage of suspected women should be done separately from other patients. Outpatient monitoring with a 14-day self-quarantine can be considered for asymptomatic infected women and for those with mild symptoms.
Inpatient management criteria include moderate to severe symptoms and the target level of oxygen saturation is 92 to 95% in different guidelines. In the presence of fever, it is important to conduct a thorough examination of other causes of the fever. It is important to monitor fluid intake and output, maintain fluid and electrolyte balance and prevent fluid overload. Thromboembolic prophylaxis is recommended. Corticosteroid administration is based on obstetrics indications, while in critical ill cases, it should be based on multi-disciplinary teams (MDT) decision. A positive COVID-19 result in the absence of other obstetrics causes, cannot be considered an indication for delivery in mild and asymptomatic cases. In critically ill pregnant women, an individualized decision should be made about delivery time by the MDT. General anesthetic should be avoided unless inevitable for standard procedures such as intubation is an aerosol-generating procedure (AGP). There is agreement on the point that babies born to infected mothers, even if isolated from the mother at birth, should be considered a close contact of the mother and tested for COVID-19 and separated from other neonates. Breastfeeding is encouraged and hand hygiene and face mask during feeding are highly recommended by all guidelines.

Keywords: Corona Virus, COVID-19, Guideline, Pregnancy

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Over the past few months, COVID-19 caused by the coronavirus has become pandemic and has approximately affected all aspects of people's life around the world. The incubation period of COVID-19 infection is approximately estimated to be 5 to 6 days with a maximum of 14 days, and the virus can remain on some surfaces for up to 72 hours (1). In non-pregnant cases, it almost takes 2 week on average from appearance of symptoms to recovery, in mild cases and three to six weeks in severe cases (2) and mortality has been reported between 0.9 and 9% in different populations and countries (3). People around the world have made changes in their daily life and social interactions to reduce the risk of the virus transmission as much as possible. Along with other changes in daily life, all the medical guidelines in all disciplines including obstetrics, have been modified according to the new pandemic conditions.

To date, no effective treatment for the virus has been found, and even little is known about the effects of the virus and how to protect against it or prevent it. Given that there was no previous scientific evidence, researchers around the world began extensive research about the virus and even changed their research field to the virus (4-6) .

Before this pandemic, the guidelines were prepared based on strong scientific documents, but in the case of COVID-19 pandemic, due to an inevitable need for new guidelines and lack of strong evidence, guidelines are being modified based on expert consensus or documents that do not have a strong scientific basis. It should be noted that since pregnant women especially those with a history of infertility are known as high-risk population during COVID-19 pandemic crisis, it is important to have a useful guide for pregnancy. So, in this article, we reviewed and compared some of the reliable guidelines in the field of

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pregnancy and coronavirus including American Society for Maternal-Fetal Medicine (ASMFM), The Royal College of Obstetricians and Gynaecologists (RCOG), Queensland Guideline, The Royal Australian and New Zealand College of Obstetricians and Gynaecologists (RANZCOG), The International Federation of Gynecology and Obstetrics (FIGO), and Government of Western Australia (1, 2, 7-12).

All-purpose principles for prenatal care in all pregnancies in the period of COVID-19 pandemic

Physiological changes, immune suppression, and increased oxygen demands in pregnancy may impact the severity of symptoms and may cause some adverse outcomes (e.g. preterm delivery, restricted fetal growth and fetal and neonatal mortality) in pregnant women with lower respiratory tract infections (2), but based on the available data, it seems that pregnant women are not at higher risk compared to non-pregnant women, and the virus has not been found in the amniotic fluid, vaginal discharge, or breast milk until now (1, 8). The current findings do not indicated an increasing risk of first-trimester miscarriage and vertical transmission (1).

In the COVID-19 pandemic, priority should be given to preventing the spread of the virus, so pregnant women should be told to report any symptoms of a sore throat and fever before a face-to-face visit. If a pregnant woman has suspicious symptoms or has been in contact with an infected person, she should not enter the clinic so that their appointment can be changed. The general advice given for prenatal care in all guidelines is to reduce face-to-face visits and do only essential cares, for example, it is better to perform prenatal care and ultrasound examination on the same day and in one place with no or only one attendant to minimize patient and health care provider risks (1, 2, 7-10, 12).

An ultrasound examination in the first trimester is agreed by all guidelines. It is suggested to perform a single ultrasound between 11 to 14 weeks, to determine the gestational age and nuchal translucency (NT) in parallel with a combined screening test (2, 9, 10, 12).

ASMFM suggested that if somebody prefers to perform cell-free DNA testing, NT scan can be considered optional (10). All guidelines agree upon anomaly scan ultrasound at 18 to 23 weeks and an ultrasound examination at the third trimester to assess fetal growth based on risk factors. In all guidelines, phone or video call, electronic record system or telemedicine is a reasonable option that limits patient exposure. Queensland guideline has suggested that health care providers can also visit patients at homes (2). However, organizations that provide such care must be organized to plan patient visits and appropriate feedback. All guidelines agree upon adjusting the number of prenatal visits, but emphasize that any decision to adjust prenatal care should be made based on regional and individual circumstances and delayed visits should not affect the health and safety of pregnant women. COVID-19 outbreak potentially augments the threat of domestic violence (12). All guidelines emphasize that women should be investigated for mental health at all face-to-face or through telemedicine visits (1, 2, 7-10, 12). So seclusion, grief, economic drawbacks, lack of security and failure to access supportive apparatus, are all commonly acknowledged risk factors for being psychologically unhealthy (12). Women who refer for prenatal visits should be evaluated for signs of the infection at the time of entry, and tested if they have symptoms. They should be asked about fever and respiratory symptom and previous contact with infected people. In the presence of mild and moderate symptoms including new fever and new cough, they should be referred for COVID-19 test and in the presence of more severe symptoms including chest pain and shortness of breath, they should undergo hospitalized evaluation (11). Efforts must be made to provide an equilibrium regarding the risk of unnoticed maternal and fetal complications due to pre-existing co-morbidities against the possible dangers of COVID-19 (12).

Approach for suspected or confirmed COVID-19 pregnancies

Women who are being admitted with any evidence of pneumonia, Acute Respiratory Distress Syndrome (ARDS), or temperature more than 37.8°C and at least one of acute incessant coughing, shortness of breath, hoarseness, wheezing or sneezing nasal discharge or congestion, or sore throat, should be evaluated for COVID-19 (9). The triage of suspected women should be done separately from other patients, and it should be determined whether they need inpatient care or can be monitored on an outpatient basis (8). RCOG recommends sending a full blood count for women with an isolated fever; if lymphopenia is detected, testing for COVID-19 is highly recommended (12). Health care providers should attempt to restrict displacement of the patients from one place to another (11).

Protocols for outpatient care

Outpatient monitoring with a 14-day self-quarantine can be considered for asymptomatic infected women and for those with mild symptoms (10). If outpatient monitoring is needed, the patient should go home by a private vehicle, stay at home, not attend public places such as work, school, or shopping centers, not see visitors and the house windows should be open and she should be separated from other family members. In addition to women with a positive COVID-19 test, self-isolation of 14 days is also recommended while waiting for the test result or being in touch with a confirmed case of COVID-19 (8).

Outpatients women should be monitored closely and given specific instructions about when to contact their health care providers, they should perform daily self-assessments and obstetric care providers should ensure that the medical institution has a reliable feedback mechanism for early detection of a worsening condition (10). Urgent

pre-arranged appointments for high-risk pregnancies will need an individual decision on urgency and potential risks or benefits. In cases where there is an urgent and unplanned need for appointment, it is better to call first and be guided by phone, and if it is deemed that prenatal care cannot be delayed until termination of the advised period of quarantine, it is better to provide facility for local care, ideally at the end of a working day (12). There is no guidance about the timing of occurrence for follow-up outpatient care; nonetheless, it is commonsensical to conduct a follow-up visit at least once within 2 weeks of diagnosis of COVID-19. These visits can either be carried out through telemedicine or specialized COVID-19 clinics where obtainable. Obstetric care providers should engage in outpatient care to screen for potential obstetric complications and maternal and fetal health (10). Routine prenatal cares such as fetal growth scans and Glucose Tolerance Test should be postponed until termination of the quarantine period. Fetal well-being tests such as Non Stress Test and Biophysical Profiles should be performed based on obstetrics indications and, if possible, should be reduced (10). Although the link between intrauterine growth disorder and coronavirus has not yet been established, but considering the data from SARS, RCOG recommends that pregnant women who become infected, have a fetal growth ultrasound two weeks after recovery (12). If a person is infected in the first trimester, although the data in this regard is not complete, she can have a detailed anomaly scan at 18-24 weeks (2, 8). As well, ASMFM recommends that those infected in the first trimester, should undergo a thorough anomaly scan, and those who become infected in the later stages of pregnancy, undergo an ultrasound scan in the third trimester (10).

Protocols for inpatient care

Inpatient management criteria include moderate to severe symptoms, oxygen saturation below 95%, associated underlying diseases and fever higher than 39°C despite taking acetaminophen. ASMFM has also mentioned that increases in work of breathing (respiratory rate greater than 30 bpm, use of accessory muscles, pursing of lips, and need for oxygen supplementation) are also important signs of worsening of the patient's condition; ASMFM has pointed to exertional oxygen saturation as a criterion for inpatient admission (10). Based on The International Federation of Gynecology and Obstetrics (FIGO) statement, hospitalization should be considered for pregnant women with fever and respiratory difficulties (7). The target level of oxygen saturation is considered 92-95% by Queensland guideline, above 94% by RCOG and above 95% by ASMFM. Patients with oxygen saturation during walking oxygen saturation test ≤95% on room air, should be considered for inpatient admission. If hospitalization is required, health care providers including midwifery nurses, obstetricians, anesthesiologists, and neonatologist, should be informed and visits should be limited. All guidelines recommend keeping the patients in negative pressure rooms if possible and isolate them in a room which has a place to change the clothes of the staff and has facilities such as a bathroom; also, personal protection is emphasized in contact of suspected or confirmed patients (1, 2, 7-12). All guidelines agree that suspected or confirmed COVID-19 cannot be the only indication for admission or transfer. However, in case of moderate disease, given the possibility of deterioration of maternal condition and preterm birth, early transfer should be considered and private transport is recommended where possible; during the transfer, the patient should have a face mask. Necessary imaging should be performed regardless of pregnancy and, of course, abdominal shielding should be used on the gravid uterus. Fetal monitoring should also be performed according to obstetrics indication and whenever fetal intervention, including delivery, fetal monitoring should be considered. Treatment and management of hospitalized patients should be done based on clinical symptoms.

When it is highly probable to have be a staggering number of patients infected with COVID-19, health care providers should bear in mind to consider other possible differential diagnoses and care requirements. It is of utmost importance to offer permanence and steadiness in maternity services, and stay alert and concentrated on the common roots of neonatal and maternal morbidity (1). In case of fever, it is important to conduct a thorough examination of other causes of fever and not consider all pyrexia related to COVID-19 (12).

It should be noted that in some cases, even in the presence of hypoxia, tachycardia may not develop; so, it is important to check vital signs, and oxygen saturation along with other vital signs whenever there is an indication (1, 2, 7-11). Frequency of vital sign assessment depends on the severity of illness so that for patients with mild symptoms, it can be performed every 4 to 8 hours and for whom with severe disease, every 2 to 4 hours. For patients with critical illness, continuous pulse oximetry and telemetry should be done and recording should be done every 1 to 2 hours; also, noninvasive and invasive cardiovascular monitoring can be considered if indicated. Patients should be treated based on their clinical symptoms. For the time being, no therapeutic or prophylactic medications for COVID-19 are approved by the U.S. Food and Drug Administration and there is no proven antiviral treatment (10). Several reports illustrated that even after a period of recovery, there might be a possibility of a quick worsening of the situation. Following a woman's improvement, it is reasonable to evaluate heart at least about 24-48 hours later. On discharge, advice should be given to the woman to come back instantaneously whenever she feels any deterioration in her condition (12).

Severe and critically ill patients

ASMFM defined Severe disease by an O_2 saturation ≤93%, a respiratory rate higher than 30 bpm, a ratio of arterial partial oxygen pressure to fraction of inspired

oxygen of <300, or >50% lung involvement on imaging and defined Critical Disease as shock, or respiratory insufficiency which needs mechanical ventilation or high-flow nasal cannula, and multi-organ failure (10). RCOG has emphasized that we should be careful about the symptoms that indicate decompression, such as the number of breaths above 30, need for oxygen above 40% or a decrease in urine output (12) . Deterioration of myalgias, unrelenting or more recurrent fevers, decreased oxygen saturation, deteriorated of dyspnea and increased work of breathing should be considered as early warning signs and in the presence of failure to sustain oxygen saturation ≥95% by supplemental oxygen, rapidly increasing supplemental oxygen need, hypotension (mean arterial pressure MAP <65 mmHg) despite adequate fluid replacement or any document of end-organ dysfunction (e.g. altered mental status, renal failure, hepatic failure, cardiac dysfunction, etc.), management in intermediate acuity setting or intensive care unit admission should be considered (10). Since thrombocytopenia may occur in critically ill COVID-19 patients, aspirin and thromboprophylaxis must be hold in cases who suffer from thrombocytopenia (12).

Intubation timing should be individually considered. Maternal status, preexisting co-morbidities, presence of multi-organ failure, vital oxygen supplementation, and need for transport to a facility with a higher level of care should be taken into account when inserting an ultimate airway. Use of noninvasive positive-pressure ventilation, e.g. Bilevel Positive Airway Pressure (BiPAP) or Continuous Positive Airway Pressure (CPAP) are some alternatives for intubation, but BiPAP and CPAP use are contentious due to concern for aerosolizing infectious particles, although some institutions have employed these modalities in order to evade intubation (10).

Some medications in suspected or confirmed COVID-19 infected pregnancies

Empiric antibiotic therapy

Attention should be paid to bacterial pneumonia co-infection. If community-acquired pneumonia co-infection is suspected, infectious counseling must be done (2, 8); the use of antibiotics is reasonable, culture data should be obtained before initiating antibiotics, and empirical antibiotic treatment may be done while waiting for the results and starting antibiotics should not be delayed for more than 45 minutes in case antibiotics are indicated. Ceftriaxone plus azithromycin or ceftriaxone alone that are commonly used, are not contraindicated in pregnancy (10) . Patients with elevated procalcitonin levels may have a bacterial co-infection. It should be noted that a high procalcitonin level does not exclude COVID-19 infection.

Fluid administration

It is important to monitor fluid intake and output, maintain fluid and electrolyte balance and prevent fluid overload. The guidelines have stressed that invasive fluid therapy should not be performed (1, 2, 7-10) . In hypovolemic cases and NPO status, administration of maintenance intravenous fluids is recommended and in acute resuscitation, in order to avoid worsening of pulmonary edema, a conservative fluid therapy (500-1000 ml bolus of crystalloid fluids), should be considered (10). Since acute respiratory distress syndrome may accompany this infection, in the setting of moderate-to-severe symptoms of COVID-19, hourly monitoring of fluid input and output is reasonable. After 250-500 ml boluses and before proceeding with further volume resuscitation, fluid overload should be assessed and during labor, caution with neutral fluid balance is emphasized (12).

Venous-thromboembolic prophylaxis

Besides pregnancy which is itself a risk factor for thromboembolism, infectious conditions, due to inflammatory mechanisms, stimulate coagulation; also, isolation of infected people leads to sedentary lifestyle and increased chance of coagulation. Thus, all guidelines emphasize on thromboembolic prophylaxis even in the absence of any risk factor. All patients hospitalized due to COVID-19 infection, have to receive anticoagulants except when they are waiting to give birth within the next 12 hours. Following birth, in the absence of postpartum hemorrhage, anticoagulant should be started as soon as possible, and in the setting of regional anesthesia, RCOG states that it should be started four hours after the last injection or after removing the catheter. Where complicated COVID-19 cases are receiving the care of multi-disciplinary teams (MDT), proper low-molecular-weight heparin (LMWH) dosing should be evaluated in an MDT including an obstetrician (12) and ASMFM states that when regional anesthesia is considered, anesthesiologists should be consulted regarding the timing of anticoagulation therapy (10) . According to RCOG, regardless of the delivery type, anticoagulants should be taken for 10 days after delivery. Clearly, risk assessment for venous thromboembolism should be done and if there is another indication to continue, anticoagulants should be continued based on the indication (12).

In critically ill pregnant women and mechanically-ventilated patients, given the increased risk of thromboembolic events, if there is no contraindication, prophylactic anticoagulation is highly recommended, but in terms of therapeutic anticoagulation, there is limited evidence. Moreover, the increased risk of preterm labor in inflammatory illness (spontaneous or iatrogenic) also can increase the risk of peripartum hemorrhage, where anticoagulant therapy may worsen the situation. Importantly, there are no clinical data to support if early, full-dose anticoagulation is advantageous in these patients; so, decision about therapeutic anticoagulation should be individualized (10) . For patients receiving prophylactic dosing, LMWH may be preferred due to the once-daily dosing to reduce exposures to health care providers.

Given the short half-life of unfractionated heparin and its reversibility with protamine sulfate, it is preferred in critically ill cases without confirmed thrombosis. As well, unfractionated heparin is preferred for prophylaxis in pregnancies at risk for preterm labor (10).

Corticosteroid administration for fetal lung maturation

In case of confirmed or suspected infection, corticosteroid administration done based on obstetrics indications should not be changed, but in severe critical ill cases that require intubation and ICU, corticosteroids administration may cause deterioration in their condition and should be done based on MDT decision (2, 7, 8). ASMFM states that corticosteroids should be used with caution due to their potential worsening effect on pulmonary status and viral shedding, and the maximum age for administration should be 34 gestational weeks (10). According to RCOG guideline, corticosteroids for fetal lung maturation must be administered whenever indicated and there is no evidence suggesting it can lead to any damage in the presence of the virus. Obviously, immediate intervention for birth should be implemented for their administration (12).

Magnesium Sulfate

According to some guidelines, no change should be made to its usual indications (2). ASMFM indicate that decision-making should be done based on each individual case, for example, in cases such as fetal neuroprotection, it should be given before 32 weeks. Magnesium sulfate is not contraindicated in women with mild or moderate symptoms (11). However, in patients who are critically ill, magnesium sulfate should be discontinued or the dose should be adjusted, especially if benzodiazepines were taken. Since it is not yet known whether magnesium sulfate can cause pulmonary edema or not, it is essential to conduct an accurate fluid intake and output monitoring (10).

Aspirin

According to all guidelines, aspirin can be continued in cases of clinical indications, such as prevention of preeclampsia.

Tocolytics

There is still no finding in favor of changing tocolytics indications, but it is recommended not to use betamimetics due to the possibility of exacerbation of maternal hypotension, tachycardia and pulmonary edema. The use of nifedipine may be advantageous in infected patients because of some resemblances between high altitude pulmonary edema and lung presentations in COVID-19. NSAIDs (e.g. indomethacin) administration to COVID-19 patients has raised concern, and it can be used be as an alternative of NSAIDs as tocolytic if indicated. Nonetheless, no adequate data is available to advocate that this utilization should be changed during this time.

Mode and timing of delivery

Delivery time

According to all guidelines, in mild and asymptomatic cases, a positive COVID-19 test without other obstetrics causes, is not a reason for delivery and mode of birth would not be affected by the virus, unless the patient's respiratory condition needs immediate intervention for birth. Patients who are currently in quarantine, should undergo an individual evaluation about possibility of delaying a previously-scheduled caesarean birth, or induction of labor, but possibility of an urgent delivery must also be taken into account (12). For cases whose elective caesarean section or induction of labor cannot be postponed, optimal management of the respiratory situation should be established and the woman should be evaluated by the MDT (1). In asymptomatic or mildly symptomatic pregnant patients, positive for COVID-19 at 37 to 38 6/7 gestational weeks without other indications for delivery, expectant management is recommended until 14 days after a positive COVID-19 polymerase chain reaction (PCR) or until 7 days after being symptomatic and 3 days after disappearance of symptoms. This choice can result in reduced exposure of health care providers and the neonate to COVID-19 virus. In an asymptomatic or mildly symptomatic pregnant patient positive for COVID-19 who is at 39 gestational week or more, delivery can be a potential alternative to lessen the risk of worsening maternal status (10). Since the severe peak of symptoms may occur in the second week , it is important to propound delivery before that time in mild and moderate cases not requiring urgent care (11). Data regarding delivery timing and acute respiratory distress syndrome are insufficient and inconclusive.

In critically ill pregnant women, an individualized decision should be made about delivery time by the MDT, according to maternal and fetal condition and potential betterment of the woman following elective birth. Obviously, maternal well-being should always be preferred (12) In critically ill pregnant women, surgical and neonatal equipment for an urgent cesarean section and hemorrhage package including methergine, prostaglandin f2α and misoprostol need to be accessible at the bedside and tranexamic acid, which must be refrigerated, should be available in the ICU. Decisions for delivery time need close correlation between the obstetricians and critical care teams and should be made based on gestational age, maternal status, contemporary pulmonary disease (e.g. cystic fibrosis, asthma, or sarcoidosis), critical illness and possibility of removing the ventilator mechanics. It should be noted that the need for mechanical ventilation alone is not an indication for pregnancy termination. The uterus pressure during third trimester of pregnancy, can effect lung function including expiratory and inspiratory reserve volume, and functional residual capacity and can consequently cause severe hypoxemia specially in critically ill pregnant patients; so, theoretically, improvement of lung function can be achieved by early

delivery and it is logical to think about delivery in the setting of worsening critical illness. After 34 gestational weeks, delivery should be considered for critically ill women, because delivery can lead to optimize respiratory condition (11) . Nevertheless, it is unknown whether delivery results in a substantial improvement of all case. So, if pregnancy termination is considered according to severe hypoxemia, particularly in pregnancies before 30-32 gestational weeks, other options including prone positioning, and even advanced ventilator methods such as extracorporeal membrane oxygenation (ECMO), should also be considered (10).

Delivery mode

Decisions about delivery mode should also be made based on obstetric indications (1, 2, 7-10). However, in the absence of indications, normal vaginal delivery should be encouraged (1). If elective caesarean has been considered, it is important to individually evaluate urgency and the possibility of continued pregnancy by use of frequently fetal surveillance (2, 8). It is necessary to keep in mind that in the virus pandemic, most of pregnant women including those with history of infertility specially who underwent assisted reproductive techniques may be stressful about their childbirth. RCOG recommends that delivery mode should be explained for the women and their preferences should be taken into account (12). Based on the available data, there is no evidence about priority of delivery mode. Therefore, delivery mode should not be affected by the presence of the virus, unless the patient's respiratory situation requires immediate delivery.

Since intubation is AGP, general anesthetic must be kept away unless it is obligatory for standard indications (2, 8).

Intrapartum care

When a COVID-19 infected woman is hospitalized for childbirth, all the team members including neonatologist, obstetrician, midwife-in charge, neonatal nurse in charge, anesthetist and infection control team, should be notified. The time of birth should be noticed to neonatal team to allow them performing personal protective equipment (PPE)(1, 2, 7-10, 12). PPE contains gown, gloves, mask, pinafore, a fluid-resistant surgical gown, and a visor (1) . A negative pressure room for childbirth is preferred. Only one birth support person should be present during birth while wearing surgical mask (8). Low-risk COVID-19 infected women whose home is close to the hospital and have a private vehicle available, may prefer to stay at home in the latent phase of labor (1, 12). The woman should be given surgical mask on presentation; for the first stage of labor, she should be managed under contact and droplet precautions but for the second and third stages, using contact and airborne precautions is recommended if accessible (8) . The number of staff entering the room should be reduced to minimum (12). If it is possible to apply telemetry to provide a safe situation, the 2nd staff is allocated outside the room for double checking medications with the staff in the birth room (2, 8) . Patients should be allowed to have a single, asymptomatic birth partner during their labor. It is necessary that birth partners remain by the woman's bedside, not walking around the ward and wash their hands repeatedly (11, 12). In addition to routine maternal management and standard practice, monitoring respiratory rate and oxygen saturations should be done. RCOG recommends hourly measurement of oxygen saturation, keeping O_2 saturation >94% by O_2 therapy (12) . Since there is no evidence that intrapartum oxygen therapy is beneficial for the fetus, and considering the point that face mask can be impregnated with the patients' respiratory secretions leading to treatment staff contact with the secretions, in the current situation, it is recommended not to use oxygen therapy even though nasal cannula and face mask are not recognized as an APG. Due to the high rate of asymptomatic COVID-19 carriers, this advice should be applied to all women during delivery. Early amniotomy and oxytocin augmentation to prevent dysfunctional labor can be considered. In the second stage of labor, women should be encouraged to push to avoid prolonged labor. Some actions such as massage of perineal body and using warm packs can lead to reduction of the third and four degree lacerations (11). When hypoxia or worn-out occur in a symptomatic woman during labor, individualized decision should be made with regard to using elective instrumental delivery in order to shorten the second stage of labor.

If symptoms worsen, an individual evaluation should be performed to make a decision about carrying out emergent cesarean or continuing the labor especially whenever it is possible to help the effort for resuscitation (12). Due to restricted resources for blood product, it is recommended to manage the third stage of labor by using prophylactic drugs including misoprostol and tranexamic acid to prevent postpartum hemorrhage (11).

In case of fetal compromise or distress, continuous fetal heart monitoring should be done (1, 2, 7-10). For asymptomatic COVID-19 infected women, continuous monitoring of fetal heart is not indicated only because of viral infection, unless in the presence of other medical indications (12).

Australian Guidelines recommend to beware of Fetal Scalp Electrode Monitoring and Fetal Blood Sampling until more data become available. However, if Fetal Scalp Electrode or Fetal Blood Sampling is planned, meditate on the potential hazard of fetal transmission against acknowledged merits of improved assessment of fetal condition (2, 8). However according to RCOG guideline, Fetal Scalp Electrode Monitoring and Fetal Blood Sampling can be performed and they are not contraindicated (12).

In cases where the woman develops a fever, in addition to considering COVID-19, other causes of fever and sepsis should be investigated according to guidelines on sepsis in pregnancy. Inexplicable fever caused by other reasons

during labor or immediate postpartum should be assessed in a typical manner. However, it is recommended that a pregnant women should also be tested and/or monitored for COVID-19 according to institutional policy and guidelines (10). No document indicated that water immersion is contraindicated (2, 8). During the first stage of labor, women can be allowed to be in water (11), but according to some guidelines, since SARS-COV-2 has been detected in stool and accessible PPE is not generally waterproof and there is a potential risk of damage to PPE completeness during urgent procedures, using birthing pools in labor ward should be avoided in COVID-19 infected women (2, 8, 12). According to RCOG, labor and birth in water are not allowed for symptomatic COVID-19 infected women, however, data is inadequate for COVID-19-positive women who are asymptomatic (12).

Pain relief

In terms of regional analgesia, no document showing regional analgesia is contraindicated in COVID-19 infected patients, is available (1, 2, 7-10) ; even epidural analgesia is recommended during labor for COVID-19 infected women given the possibility of urgent cesarean section and to avoid of general anesthesia as an AGP procedure (11, 12) . In terms of nitrous oxide, current information is not sufficient and there is a conflict about filtering, washing and AGP potential in the setting of COVID-19 (2). There is no data that Entonox using is an AGP. According to RCOG, when Entonox is considered, it is important to use a single-patient microbiological filter (12) but according to Queensland and Western Australia guidelines, for healthcare staff protection, administration for suspected or confirmed COVID-19 women should be avoided; however, it should be kept in mind that since asymptomatic women may ask to use analgesia during labor, if nitrous oxide is suggested, face mask instead of mouth piece should be considered(2, 8), and a microbiological filter of <0.05μm pore size should be used (8).

Newborn care

There is no data indicating that delayed cord clamping can lead to increased risk of neonatal infection. When there is no other contraindication, delayed cord clamping is still reasonable because there is no contrary finding until now (1, 2, 8, 12). Cleaning and drying the baby is recommended as usual while the cord is still intact (12). According to RANZCOG, mothers and babies can practice skin-to-skin contact. Whether or not the mother has COVID-19 infection, she and her baby should be together all the time, even without delay after the birth, in order to establish breastfeeding (1). On the other hand, Centers for Disease Control (CDC) suggests that the mother and the baby should be separated from each due to the possibility of neonatal morbidity from maternal transmission (11).

The possibility of vertical transmission has not been completely excluded and even some evidence proposed the possibility of vertical transmission (12); also, intrapartum transmission due to exposure to maternal stool is probable and even mother's respiratory secretions can be potential source of infection for newborns. There is agreement that babies born to infected mothers, because of the close contact with the mother even if isolated at birth, should be tested for COVID-19 and separated from other neonates. Although maternal infection alone is not an indication for neonatal admission, 14 days of quarantine in a shared location with mother is recommended for them. Surely, decision about mother and baby co-location should be made individually (2, 8, 10). However, FIGO has mentioned that according to some recommendations, neonates should be separated from their infected mother and kept in separate rooms or ≥6 feet away from the mother or physical barriers should be used (7).

Breastfeeding

Since COVID-19 virus has not been found in breast milk until now, breastfeeding is not contraindicated (1, 2, 7-10). Really, as a potentially important source of antibody for the baby, breastfeeding is encouraged (7, 10). Shedding infective airborne droplet from the mother is probably the main risk for babies, so hand hygiene and wearing a facemask during feeding, are highly recommended by all guidelines. According to some guidelines, given the lack of enough evidence in this context, mother or baby skin washing is not necessary before close contact or breastfeeding (2, 8) while other guidelines recommend that before expressing breast milk, women should wash not only their hands but also breast (11). It is recommended to provide to current sterilization standards for breastfeeding equipment and formula preparation (2, 8).

Discharge

It is recommended to consider normal discharge criteria. Also, it is suggested to notify the patients about continuing quarantine until completion of 14 days. For discharge home, a negative test is not mandatory. If discharged before completion of 14 days, constant clinical monitoring is advisable until 14 days. It is important to use local capacity such as telemedicine services and home visiting for clinical evaluation after discharge. Necessary advice should be given to the patient about indication for readmission. Most frequent reasons for readmission are respiratory symptoms one to three weeks after discharge (2, 8). Rapid deterioration has been reported even after a period of recovery, so, after improvement of a patient's condition, at least 1-2 day follow-up should be performed; also on discharge, the woman should be enforced to return immediately if she feels bad.

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Authors' Contributions

R.P., M.R., A.A., A.M.; Contributed in reviewing the

guidelines and writing the manuscript. All authors read and approved the final manuscript.

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

Aims and scope

International Journal of Fertility & Sterility is a peer review and quarterly English publication of Royan Institute of Iran. The aim of this journal is to disseminate information through publishing the most recent scientific research studies on Fertility and Sterility and other related topics. Int J Fertil Steril has been certified by the Ministry of Culture and Islamic Guidance since 2007. It has also been accredited as a scientific and research journal by HBI (Health and Biomedical Information) Journal Accreditation Commission since 2008. **This open access journal holds the membership of the Committee on Publication Ethics (COPE) and the International Committee of Medical Journal Editors (ICMJE).**

1. Types of articles

The manuscript in the field of Fertility and Sterility can be considered for publications in Int J Fertil Steril. These manuscripts are as below:

A. Original articles

Original articles are scientific reports of the original research studies. The article consists of English Abstract (structured), Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, Author's Contributions, and References **(Up to 40)**.

B. Review articles

are the articles written by well experienced authors and those who have excellence in the related fields. The corresponding author of the review article must be one of the authors of at least three published articles appearing in the references. The review article consists of English Abstract (unstructured), Introduction, Conclusion, Author's Contributions, and References **(Up to 70)**.

C. Systematic Reviews

Systematic reviews are a type of literature review that collect and critically analyzes multiple research studies or papers. The Systematic reviews consist of English Abstract (unstructured), Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, Author's Contributions, and References **(Up to 70)**.

D. Short communications

Short communications are articles containing new findings. Submissions should be brief reports of ongoing researches. The short communication consists of English Abstract (unstructured), the body of the manuscript (should not hold heading or subheading), Acknowledgements, Author's Contributions, and References **(Up to 30)**.

E. Case reports

Case reports are short discussions of a case or case series with unique features not previously described which make an important teaching point or scientific observation. They may describe novel techniques or use equipment, or new information on diseases of importance. It consists of English Abstracts (Unstructured), Introduction, Case Report, Discussion, Conclusion, Acknowledgements, Author's Contributions, and References **(Up to 30)**.

F. Editorial

Editorial should be written by either the editor in chief or the editorial board.

G. Imaging in reproductive medicine

Imaging in reproductive medicine should focus around a single case with an interesting illustration such as a photograph, histological specimen or investigation. Color images are welcomed. The text should be brief and informative.

H. Letter to editors

Letter to the editors are welcome in response to previously published Int J Fertil Steril articles, and may also include interesting cases that do not meet the requirement of being truly exceptional, as well as other brief technical or clinical notes of general interest.

I. Debate

2. Submission process

It is recommended to see the guidelines for reporting different kinds of manuscripts here. This guide explains how to prepare the manuscript for submission. Before submitting, we suggest authors to familiarize

themselves with Int J Fertil Steril format and content by reading the journal via the website (www.ijfs.ir). The corresponding author ensures that all authors are included in the author list and agree with its order, and they must be aware of the manuscript submission

A. Author contributions statements Sample
It is essential for authors to include a statement of responsibility in the manuscript that specifies the contribution of every one of them. This participation must include conception and design of the manuscript, data acquisition or data analysis and interpretation, drafting of the manuscript and/or revising it for critically important intellectual content, revision and final approval of the manuscript and statistical analysis, obtaining funding, administrative, technical, or material support, or supervision. Authors who do not meet the above criteria should be acknowledged in the **Acknowledgments section.**

B. Cover letter And Copyright Sample
Each manuscript should be accompanied by a cover letter, signed by all authors specifying the following statement: “The manuscript has been seen and approved by all authors and is not under active consideration for publication. It has neither been accepted for publication nor published in another journal fully or partially (except in abstract form). **Also, no manuscript would be accepted in case it has been pre-printed or submitted to other websites.** I hereby assign the copyright of the enclosed manuscript to Int J Fertil Steril”.

The corresponding author must confirm the proof of the manuscript before online publishing. It is needed to suggest three peer reviewers in the field of their manuscript.

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Authors whose first language is not English, encouraged to consult a native English speaker in order to confirm his manuscripts to American or British (not a mixture) English usage and grammar. It is necessary to mention that we will check the plagiarism of your manuscript **by iThenticate Software.** Manuscript should be prepared in accordance with the "International Committee of Medical Journal Editors (ICMJE)". Please send your manuscript in two formats Word and Pdf (including: title, name of all the authors with their degree, abstract, full text, references, tables and figures) and Also send tables and figures separately in the site. The abstract and text pages should have consecutive line numbers in the left margin beginning with title page and continuing through the last page of the written text. Each abbreviation must be defined in the abstract and text when they are mentioned for the first time. Avoid using abbreviation in the title. Please use the international and standard abbreviations and symbols.

It should be added that an essential step toward the integration and linking of scientific information reported in published literature is using standardized nomenclature in all fields of science and medicine. Species names must be italicized (e.g., Homo sapiens) and also the full genus and species written out in full, both in the title of the manuscript and at the first mention of an organism in a paper.

It is necessary to mention that genes, mutations, genotypes, and alleles must be indicated in italics. Please use the recommended name by consulting the appropriate genetic nomenclature database, e.g., HUGO for human genes. In other word; if it is a human gene, you must write all the letters in capital and italic (e.g., OCT4, c-MYC). If not, only write the first letter in capital and italic *(e.g., Oct4, c-Myc).* **In addition, protein designations are the same as the gene symbol but are not italicized.**

Of note, Int J Fertil Steril will only consider publishing genetic association study papers that are novel and statistically robust. Authors are advised to adhere to the recommendations outlined in the STREGA statement (http://www.strega-statement.org). The following criteria must be met for all submissions:

1. Hardy-Weinberg Equilibrium (HWE) calculations must be carried out and reported along with the P-values if applicable [see Namipashaki et al. 2015 (Cell J, Vol 17, N 2, Pages: 187-192) for a discussion].
2. Linkage disequilibrium (LD) structure between SNPs (if multiple SNPs are reported) must be presented.
3. Appropriate multiple testing correction (if multiple independent SNPs are reported) must be included.

Submissions that fail to meet the above criteria will be rejected before being sent out for review.
Each of the following manuscript components should begin in the following sequence:

Authors’ names and order of them must be carefully considered (full name(s), highest awarded academic degree(s), email(s), and institutional affiliation(s) of all the authors in English. Also, you must send the mobile number and full postal address of the corresponding author).

Changes to authorship such as addition, deletion or rearrangement of author names must be made only before the manuscript has been accepted in the case of approving by the journal editor. In this case, the corresponding author must explain the reason of changing and confirm them (which has been signed by all authors of the manuscript). If the manuscript has already been published in an online issue, an erratum is needed.

Title is providing the full title of the research (do not use abbreviations in title).
Running title is providing a maximum of 7 words (no more than 50 characters).

Abstract must include Background, Materials and Methods, Results, and Conclusion (no more than 300 words).

Keywords, three to five, must be supplied by the authors at the foot of the abstract chosen from the Medical Subject Heading (MeSH). Therefore; they must be specific and relevant to the paper.

The following components should be identified after the abstract:

Introduction:
The Introduction should provide a brief background to the subject of the paper, explain the importance of the study, and state a precise study question or purpose.

Materials and Methods:
It should include the exact methods or observations of experiments. If an apparatus is used, its manufacturer's name and address
should be stipulated in parenthesis. If the method is established, give reference but if the method is new, give enough information so that another author can perform it. If a drug is used, its generic name, dose and route of administration must be given. Standard units of measurements and chemical symbols of elements do not need to be defined.

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They must be presented in the form of text, tables and figures. Take care that the text does not repeat data that are presented in tables and/or figures. Only emphasize and summarize the essential features of the main results. Tables and figures must be numbered consecutively as appeared in the text and should be organized in separate pages at the end of the manuscript while their location should be mentioned in the main text.

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Supplementary materials would be published on the online version of the journal. This material is important to the understanding and interpretation of the report and should not repeat material within the print article. The amount of supplementary material should be limited. Supplementary material should be original and not previously published and will undergo editorial and peer review with the main manuscript. Also, they must be cited in the manuscript text in parentheses, in a similar way as when citing a figure or a table.

Provide a legend for each supplementary material submitted.

Discussion:
It should emphasize the present findings and the variations or similarities with other researches done by other researchers. The detailed results should not be repeated in the discussion again. It must emphasize the new and important aspects of the study.

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

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

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Any conflict of interest (financial or otherwise) and sources of financial support must be listed in the Acknowledgements. It includes providers of supplies and services from a commercial organization. Any commercial affiliation must be disclosed, regardless of providing the funding or not.

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