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Systematic Review

The Genetics of Non-Syndromic Primary Ovarian Insufficiency: A Systematic Review

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Abstract

Several causes for primary ovarian insufficiency (POI) have been described, including iatrogenic and environmental factor, viral infections, chronic disease as well as genetic alterations. The aim of this review was to collect all the genetic mutations associated with non-syndromic POI. All studies, including gene screening, genome-wide study and assessing genetic mutations associated with POI, were included and analyzed in this systematic review. Syndromic POI and chromosomal abnormalities were not evaluated. Single gene perturbations, including genes on the X chromosome (such as *BMP15*, *PGRMC1* and *FMR1*) and genes on autosomal chromosomes (such as *GDF9*, *FIGLA*, *NOBOX*, *ESR1*, *FSHR* and *NANOS3*) have a positive correlation with non-syndromic POI. Future strategies include linkage analysis of families with multiple affected members, array comparative genomic hybridization (CGH) for analysis of copy number variations, next generation sequencing technology and genome-wide data analysis. This review showed variability of the genetic factors associated with POI. These findings may help future genetic screening studies on large cohort of women.

Keywords: Genetic, Gynecology, Molecular, Precision Medicine

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Introduction

Primary ovarian failure (POF) or primary ovarian insufficiency (POI) is defined as primary or secondary amenorrhea in women younger than 40 years of age with follicle stimulating hormone (FSH) ≥40 IU/L and estradiol levels ≤50 pg/mL (1-3). The anti-mullerian hormone (AMH) is another indicator of POI risk (2). Recently Venturella et al. (4) described a new methodology to quantify ovarian reserve combining clinical, biochemical and 3D-ultrasonographic parameters called ovAGE.

Several causes for POI have been described, including iatrogenic and environmental factor, viral infections and chronic disease as well as genetic alterations (1, 5). Numerical defects of X chromosome, such as 45,X and 47,XXX, are often associated with ovarian dysgenesis and accelerated follicular atresia (1). Recently, single genes causing non-syndromic POI have been evaluated (6). The aim of this review was to collect all genetic mutations associated with non-syndromic POI.

Materials and Methods

Electronic databases were searched from inception of each database, until February 2018 (7). The research was conducted using MEDLINE, EMBASE, Web of Sciences, Scopus, ClinicalTrial.gov, OVID and Cochrane Library as electronic databases. Review of articles also included the abstracts of all references retrieved from the search. We used the following keywords and text words: "Ovarian", "Failure", "POF", "POI", "Genetic", "Genomic", "Syndrome", "Chromosomal", "Premature", "Primary" and "Infertility."

All studies assessing genetic mutations associated with non-syndromic POI, including mutations located in X and autosomal chromosomes, were analyzed. Syndromic POI and chromosomal abnormalities (e.g. numerical defects, X-structural abnormalities, X-autosome translocations and autosomal rearrangements) were not evaluated. Pleiotropic single gene disorders (e.g. Fragile X syndrome), mitochondrial gene diseases (e.g. Perrault syndrome), and multiple malformation syndromes (e.g. cerebellar ataxia) were also excluded.

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Results

Single genes causing non-syndromic primary ovarian insufficiency

Many genes whose product is known playing a role in human folliculogenesis (candidate genes) have been studied (6).

Genes on the X chromosome

Bone morphogenetic protein 15 (BMP15) (Xp 11.2)

BMP15 is a member of the transforming growth factor (TGF) family involved in stimulating folliculogenesis. It promotes follicle maturation by regulating granulosa cell differentiation and proliferation (8). Evidences from animal models primarily suggested the possible involvement of BMP15 in pathogenesis of POI. Bmp15 knockout female mice presented subfertility and defective ovulation processes (9). Concerning human disease, the first evidence was reported in the 2004 (10). They identified a heterozygous p.Y235C missense mutation in two POI patients, caused by decrease of granulosa cell proliferation through a dominant negative effect. Many variants in *BMP15* gene have been described in Caucasian, Indian and Chinese patients with POI (10-17). These variants in *BMP15* lead to impaired dimerization, reducing production of mature BMP15 active protein and subsequent defective granulosa cell signaling, in addition to increased follicle atresia.

Progesterone receptor membrane component 1 (PGRMC1) (Xq22-q24)

PGRMC1 is a putative progesterone-binding membrane receptor, expressed in various tissues (18-22). Authors (21) have identified a mother and daughter with POI carrying an X-autosome translocation [t(X;11)(q24;q13)] and a sporadic missense mutation (p.H165R), in the cytochrome b5 binding domain of *PGRMC1*. These variants are associated with lower levels of PGRMC1, and consequently ovarian cells apoptosis. Wang et al. (23) catalogued a different missense mutation (c.556C.T; p. P186S), however, more research is needed to establish association of this variation with POI.

Androgen receptor (AR) (Xq12)

The *AR* gene is related with reproduction, as well as sex differentiation. AR is present in ovary, precisely in granulosa cells, and it is useful to follicles development. Shiina et al. (24) found that deficiency of AR in female mice may lead to a POI-like phenotype and dysregulation of many important genes involved in folliculogenesis. These results probably indicate that regular folliculogenesis need AR-mediated androgen action. We reported two examples of mutations on *AR* gene linked with POI: CAG repeat length in exon 1 and two missense mutations (p.T650A and p.O658K) (25-30).

Premature ovarian failure, IB (POFIB) (Xq21.2)

POFIB is considered as a region codified by OMIM, located within the critical POI1 region. In a patient with secondary amenorrhea (POI), this region was interrupted by a breakpoint in an X-autosome translocation. Lacombe et al. (31) proved linkage to Xq21 in a family having five patients with POI. A homozygous p.R329Q mutation was identified, leading to decreased ability to bind actin filaments.

Dachshund family transcription factor 2 (DACH2) (Xq21.3)

DACH2 is located on Xq21.3 and involved in POI (32) with two missense mutations, p.R37L and p.F316S (33).

Fragile X mental retardation I (FMRI) (Xq27.3)

FMR1 is an X-linked gene located at Xq27.3 and characterized by CGG repeats in its 5' untranslated region. Full mutation, consisting of >200 CGG repeats, is associated with the fragile X-syndrome in male carriers, but not with POI. CGG repeats ranging from 55 to 199 are known as *FMR1* premutation and recognized as common gene involved in POI. *FMR1* premutations have been identified more frequently in POI patients having a positive family, compared to sporadic forms (34). *FMR1* premutations are more frequently identified in Caucasian patients with POI than general population (35). Compared to Caucasian population, the prevalence of *FMR1* premutations is lower in Asian POI patients (36). Some studies indicate that there is no association between Intermediate range of CGG repeats (41or 45-54 repeats) and POI (37). FMR1 protein is a RNA binding protein highly expressed in fetal ovary germ cells and granulosa cell. *FMR1* premutations associate with decreased size of initial follicular pool (38-40).

Genes on autosomal chromosomes

Growth differentiation factor 9 (GDF9) (5q31.1)

As with *BMP15*, *GDF9* is part of TGF gene family presented in oocytes. These characteristics make it an important candidate gene for POI. New heterozygous variants have been found in European, Caucasian and Asian patients (41-43), but not in Japanese and New Zealand population (44, 45). Norling et al. (46) performed high-resolution array comparative genomic hybridization (CGH) in 26 POI Swedish cases and discovered a partial GDF9 gene duplication with 475 bp length.

Folliculogenesis specific bHLH transcription factor (FIGLA) (2p13.3)

FIGLA, encodes an oocyte- specific, basic helix-loop-helix (bHLH) transcription factor, which is necessary for the first stage of folliculogenesis. It regulates expression of zona pellucida genes. Three variants have been described, by Zhao et al. (47), in 100 Chinese patients with POI, including missense mutation in two women, deletion of p.G6fsX66 in one woman and deletion p.140delN in another woman. The Deletion of p.G6fsX66 may cause POI through a mechanism of haploinsufficiency, while

the deletion of p.140delN may induce impaired heterodimerization. Tosh et al. (48) also identified an intronic variant in 209 Indian patients with POI.

***New ovary homeobox gene (NOBOX)* (7q35)**

NOBOX is an ovary homeobox gene involved in first stages of folliculogenesis. Rajkovic et al. (49) identified NOBOX role, using knockout mouse models. In female mice, they determined fibrous tissues replacing follicles, causing similar phenotype to non-syndromic ovarian failure. Lechowska et al. (50) identified that NOBOX deficiency may lead to POI through a disorder caused due to communication between somatic cells and oocytes during embryonic development. This results in anomalous junctions between joined oocytes within syncytial follicles. Several *NOBOX* mutations have been detected in Caucasian POI patients (51-53). For instance, p.R355H mutation associates with a decrease in *NOBOX* DNA binding activity and a dominant negative effect. No *NOBOX* variant has been found in Chinese women with POI (54).

***Nuclear receptor subfamily 5, group A, member I (NR5AI): Steroidogenic factor1 (SF1)* (9q33)**

NR5A1, *SF1*, is a nuclear receptor involved in early gonadal differentiation. NR5A1 modulates the transcription of genes implicated in steroidogenesis such as AMH, nuclear receptor subfamily 0, group B, member 1 (DAX1), CYP11A, steroidogenic acute regulatory protein (StAR), CYP17A1, CYP19A1 and INHA. *N5RA1* knockout in mouse granulosa cells induced hypoplastic ovaries, reduced number of oocytes and infertility. *NR5A1* mutations have been identified by Lourenco et al. (55) in four families with history of POI and in 2/25 women with sporadic POI. These mutations, not identified in control patients, were associated with altered transactivation activity of factors involved in follicle growth. In a study including 356 Dutch patients with POI, nine different mutations were determined in coding regions of *NR5A1* (56). Jiao et al have identified Py5D mutation, as a non-domain region, in Chinese women with POI.

***Estrogen receptor 1 (ESR1)* (6q25.1)**

ESR1 gene is one of the two estrogen receptor subtypes. It has been considered as a potential candidate gene for POI (57). ESR1 knockout mice models showed an early loss of fertility due to the impaired follicles maturation. Qin et al. (58) analyzed 41 single nucleotide polymorphisms (SNPs) in 400 cases and 800 women controls. They found one SNPs related to POI in *ESR1* (rs2234693) in Korean and Dutch women. They also identified two novel SNPs in *HK3* and *BRSK1* (rs2278493 and rs12611091, respectively), probably involved in POI pathogenesis.

***FSH receptor (FSHR)* (2p21-p16)**

FSH/FSHR signaling has an important role in regular gonadal function. A study, composed of 75 patients with hypergonadotrophic ovarian dysgenesis and primary or secondary amenorrhea cases, discovered homozygous mutations (59-61). These mutations are common (0.96%) in Finnish women, but rare in the other populations (13, 62-70).

***TGF, beta receptor III (TGFBR3)* (1p33-p32)**

Human *TGFBR3* is located at 1p33-p32 and translates the TGF-beta type III receptor. In Chinese women with idiopathic POI, two missense variants, p.E459G and p.P825L were identified. The third one, p.P775S, was discovered in an Indian POI case.

***G protein-coupled receptor 3 (GPR3)* (1p36.1-p35)**

GPR3 gene is an element of the G protein coupled receptor family. In Gpr32/2 mice, higher quantity of the oocytes, in antral follicles, quickly restarted meiosis. Similar variant (c.135G.A; p.V45V) was discovered in one Chinese woman.

***Wingless-type MMTV integration site family, member 4 (WNT4)* (1p36.23-p35.1)**

WNT4 translates a secreted extracellular signaling protein which plays a role in female sex differentiation.

***Inhibins (INH): Inhibin, alpha (INHA) (2q35), Inhibin, beta A (INHBA)* (7p15-p13), Inhibin, beta B (INHBB) (2cen-q13)**

INH is a member of TGF-b family. In New Zealand (7%), Indian (11.2%) and Italian (4.5%) women with POI a common missense variation c.769G.A (p.A257T) was found in the *INHA* gene. Regarding *INHBA*, new missense variations were found only in Indian POI women. Causative variation in *INHBA* and *INHBB* genes has not been found yet.

***POU class 5 homeobox 1 (POU5FI)* (6p21.31)**

In *NOBOX* gene knockout mice, *POU5F1* reproduction factor gene is significantly down-regulated, making it a possible aspirant gene for POI. A study found one non-synonymous mutation (p.P13T) in 175 Chinese women with POI.

***Class 5 homeobox MutS homolog 4 (MSH4)* (1p31) and *MSH5* (6p21.3)**

MSH4 (1p31) and *MSH5* (6p21.3) are members of mammalian DNA mismatch repair genes family. Mouse model carrying an inactivating *MSH4* mutation in the germ line, identified a severe disruption of meiosis in *Msh4* mutant females and males. Furthermore, failure of the chromosome pairing in oocytes caused apoptosis increase, loss of oocyte pool and ovarian structures. A heterozygous mutation p.P29S in the MSH4-binding domain of *MSH5* in Caucasian patients. Guo et al. (36), using whole exome sequencing in a Chinese pedigree with POI, identified a homozygous missense mutation (p.D487Y) in the *MSH5* gene of two sisters with POI. In addition, POI phe-

notype was determined in mice carrying the homologous mutation.

Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (CITED2) (6q23.3)

CITED2 gene encodes a protein inhibiting transactivation of HIF1A-induced genes. *CITED2* mutations may lead to idiopathic POF. Fonseca et al. (30) analyzed 139 patients with POI and 290 controls. They identified a missense mutation p.P202T in *CITED2* of one case. More studies are needed to identify the role of *CITED2* mutations in POI pathogenesis.

***Spermatogenesis and oogenesis specific basic helix-loop-helix transcription factor I (SOHLHI)* (9q31.3) and *SOHLH2* (13q13.3)**

SOHLH1 and *SOHLH2* are only expressed in primordial follicles and they encode testis-specific transcription factors. They are needed for spermatogenesis, oogenesis and early folliculogenesis. Sohlh2 knockout adult female mice are infertile, since differentiation of the oocyte during early oogenesis is compromised and oocytes are quickly lost.

***Phosphatase and tensin homolog (PTEN)* (10q23.3)**

PTEN gene, located on chromosome 10q23.3, encodes protein which negatively regulates intracellular levels of PI3K and consequently AKT/PKB signaling pathway in cells. It has been found essential maintenance of dormancy of primordial follicles. In mice with PTEN deficiency, the entire pool of primordial follicles was prematurely activated and early depleted.

Nanos homolog 1, 2, 3 (Drosophilia): *NANOSI* (10q23.11), *NANOS2* (19q13.32), *NANOS3* (19q13.13)

NANOS gene belongs to a family needed for primordial germ cell (PGC) evolution and conservation. Three homologs of this molecule have thus far been determined: *NANOS1*, *NANOS2* and *NANOS3*. *NANOS2* defect solely cause spermatogonia failure, while defective PGC conservation in both males and females with NANOS3 deficiency was observed. *NANOS3* variants have been studied in 80 Chinese and 88 Caucasian POI patients. No causative variant was detected in coding exons, while a different study found a hypothetically important heterozygous mutation (c.457C.T; p.R153W). In addition, new homozygous variation (c.358G.A; p.E120K) was discovered in two sisters with POI.

***Cyclin-dependent kinas inhibitor IB (CDKNIB)* (12p13.1-p12)**

CDKN1B, also known as P27 or KIP1, translates an inhibitor involved in growth and differentiation of several tissue. It is responsible for follicle atresia. Authors, showed early follicle depletion in *CDKN1B* knockout mice.

***Anti-mullerian hormone receptor, type II (AMHR2)* (12q13)**

AMHR2 translates an AMH receptor. It has a central part in the conservation and growth of reproductive organs in humans (6). Studies found a polymorphism (c.-482 A.G; rs2002555) in *AMHR2* of some populations, but not in the others (58). Recently, two new missense variations in a group of Chinese women with POI was noted by Qin et al. (58).

***Forkhead box protein L2 (FOXL2)* (3q23)**

FOXL2 belongs to forkhead family of transcription factors, expressed in mammalian undifferentiated granulosa cells. It may play a role in fertility conservation. *FOXL2* knockout mouse models presented an early activation and decrease of primordial follicles. Moreover, one study recently identified that *FOXL2* is essential for regulation of AMH. Three *FOXL2* mutations have been found in almost 5% of non-syndromic POI patients, including c.772T>A (p. Tyr258Asp), c.661_690del (p.Ala221_Ala230del) and c.560G>A (p.Gly187Asp).

***Forkhead box 03 (FOX03)* (6q21)**

FOXO3 gene, located at 6q21, belongs to the forkhead family of transcription factors. FOXO3 is involved in oocyte quiescence. It suppresses initiation of follicular maturation and controls the rate of utilization of the reproductive potential. FOXO3 null mice presented an early global activation of primordial follicles, until a premature depletion of the primordial oocyte pool. Several *FOXO3* variants have been identified in different ethnic groups with different frequency.

***Forkhead box 01 (FOX01)* (13q14.1)**

FOXO1, belonging to forkhead family of transcription factors, may be involved in follicular steroidogenesis and plays a role in follicle development by controlling granulosa cell proliferation, apoptosis and differentiation. In 60 patients with POI from New Zealand and Slovenia two variants have been found. These variants included one missense mutation, and one 5' UTR mutation (p.P84L and c.-30C.T, respectively).

***The Wilms tumor 1 (WT1) gene* (11p13)**

WT1 gene is translated to a transcription factor expressed in granulosa cells. Variations of this gene lead to defects in granulosa cell polarity, which could explain the origin of POI and POI phenotype in knockout mice.

***The Splicing Factor 1 (SF1) gene* (11q13.1)**

SF1 has a significant role in ovarian development and it appears to cause POI in Tunisian women by reducing estradiol levels.

***Spalt-like transcription factor 4 (SALL4)* (20q13.2)**

SALL4 encodes a zinc finger transcription factor which

plays role in the developing limbs and motor neurons. *SALL4* might also be involved in conferring totipotency on oocytes. So, 100 Han Chinese women with non-syndromic ovarian failure were screened and two variants were identified in this gene, including p.Val181Met and p.Thr817Ala.

***Meiotic protein covalently bound to DSB (SP011)* (20q13.31)**

SPO11 encodes a protein which is essential for formation of double stranded breaks (DSBs) (or initiation of meiotic DSBs). It is also required for the chromosome segregation. Infertility has been observed in *Spo11* knockout mice due to impaired meiosis and depletion of oocytes.

***DNA meiotic recombinase I (DMCI)* (22q13.1)**

DMC1 encodes a member of the recombinases family (also called DNA strand-exchange proteins). Recombinases are essential in repairing breaks of double-strand DNA.

Genome-wide studies in primary ovarian insufficiency

The candidate gene approach and cytogenetic studies have provided some important results so far. Recently, new strategies have been performed for identifying new genes and unknown pathways associated with POI development. These strategies include linkage analysis in families with multiple affected patients, array-CGH for analysis of copy number variants (CNVs), genome-wide association (GWA) studies (GWAS), genome-wide sequencing of exomes (WES) and the whole genome sequencing (WGS) as well as the next generation sequencing (NGS) (6).

Genome-wide association studies

In genetics, a GWAS consists of the analysis of a genome-wide set of genetic variants to discover their association with a trait. Especially, GWASs focus on SNPs. GWA studies use high-throughput genotyping technologies to investigate the entire genome and common SNPs assay, without any prior hypotheses regarding the mechanism or biological pathways. Then, this analysis consists of studying SNPs in affected and control women.

Thanking GWAS, several potentially POI related loci were identified in Chinese, Korean, and Dutch women, but no interesting finding was confirmed by replicating these studies. The major limitation of GWAS is lack of statistical power, due to population proportions and sample size. POI is actually a rare disease, so it could be difficult to increase the sample size.

Genome-wide association studies based family linkage analysis

Some GWAS studies also showed a dominant pattern of inheritance. A large consanguineous Middle-Eastern family with POI, presenting an autosomal recessive pattern of inheritance, was subject of GWA. They identified two regions including 7p21.1-p15.3 and 7q21.3-q22.2.

Genome-wide association studies based on age of menopause

A new strategy to identify genetic mechanism involved in POI might consist of using evidences from shared genetic susceptibility natural menopause or early menopause women. Studies have identified a significant association between POI and three SNPs including rs2278493 in hexokinase 3 (*HK3*), rs2234693 in estrogen receptor 1 (ESR1) and rs12611091 in BR serine/threonine kinase 1 (*BRSK1*) (6).

Copy number variants

CNVs are a structural variants involving DNA regions >1 kb. They consist of alterations in the copy number of specific regions such as deletions and duplications. They can be either inherited or spontaneously arisen de novo, leading to phenotypic variations and disease. Recently, array-CGH has been used to search CNVs potentially involved in POI. Studies have identified eight statistically significant different CNVs in chromosomal regions (1p21.1, 5p14.3, 5q13.2, 6p25.3, 14q32.33, 16p11.2, 17q12 and Xq28) of five genes playing role in reproduction, includingDynein axonemal heavy chain 5 (*DNAH5*), NLR family apoptosis inhibitory protein (*NAIP*), dual specificity phosphatase 22 (*DUSP22*), nuclear protein 1 transcriptional regulator (*NUPR1*) and AKT serine/threonine kinase 1 (*AKT1*).

Ledig et al. (13) performed array-CGH analysis in 74 German patients with POI and identified 44 rearrangements (deletions and insertions) through several genes involved in meiosis, DNA repair and folliculogenesis. Seventeen novel microduplications and seven novel microdeletions, six of which were located in the coding regions of 8q24.13, 10p15-p14, 10q23.31, 10q26.3, 15q25.2 and 18q21.32 have been identified. Two novel microdeletions were discovered to cause haploinsufficiency for *SYCE1* and *CPEB1* genes playing a role in ovarian failure in knockout mouse models. In 2014, Norling et al. (46) performed a case-control study. They used arrayCGH to identify CNVs in 26 POI patients. Eleven unique CNVs were found in 11 patients, including a tandem duplication in part of the *GDF9* gene promoter region, known as probable causative gene for POI. Further studies in much larger POI samples are needed to identify novel CNVs and to discern the utility of array-CGH in replacing conventional karyotyping.

Whole exome sequencing

WES allows simultaneous analysis of base pairs across the entire exome. This was traditionally defined as the sequence encompassing all exons of protein coding genes in the genome. Six WES was performed in non-syndromic inherited POI. It has been indicated that majority of the candidate genes play role in meiosis and DNA repair, in this study (Table 1).

Table 1: Identified genes using WES associated with non-syndromic inherited POI

Abbreviation	Genes
STAG3	*Stromal antigen 3*
SYCE1	*Synaptonemal complex central element*
eIF4ENIF1	*Eukaryotic translation initiation factor 4E nuclear import factor*
CLPP	*Caseinolytic mitochondrial matrix peptidase proteolytic subunit*
C10ORF2	*Chromosome 10 open reading frame 2*
HFM1	*ATP-dependent DNA helicase homolog*
MCM8 and *MCM9*	*Minichromosome maintenance complex component 8 and 9*
LARS2	*Leucyl-tRNA synthetase 2, mitochondria; C10orf2*
HSD17B4	*Hydroxysteroid (17-beta) dehydrogenase 4*

WES; Whole exome sequencing and POI; Primary Ovarian Insufficiency.

Whole genome sequencing and next generation sequencing

NGS has revolutionized genomic research. Thanks to NGS an entire human genome can be sequenced in a single day and new molecular players in POI can be identified. Fonseca et al. (30) performed a retrospective case-control cohort study including 12 patients with non-syndromic POI and 176 controls. NGS was used to sequence complete coding regions of 70 candidate genes in POI patients and mutations in *ADAMT19*, *BMPR2* and *LHCG* were identified.

Discussion

This review includes almost all genetic abnormalities and genes linked with non-syndromic POI. This exhibits the importance and variability of genetic elements involved in POI genesis and identified by different techniques. Different conclusion can be made based on our review. First, several genes come out as POI candidates, but only a little part of them have been established unequivocally causative factor, by functional tests. Second, remarkable differences in frequency exist among different ethnic groups. New studies with a large sample sizes should more imply disparate ethnic groups. Moreover, interactions between gene-gene and protein-protein are not yet entirely clear. Recent and future advances in sequencing techniques will help find other novel genes involved in POI. Finally, discovering the pathogenesis and molecular bases of POI is useful not only to understand the ovarian physiology, but also to improve genetic and fertility counseling. Once new variants are found, they can help prognosticate the age of menopause. In future, findings from this review may help large genetic screening studies on infertility and may help women plan their fertility.

Conclusion

This review showed variability of the genetic factors associated with POI. These findings may help future genetic screening studies on large cohort of women.

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Authors' Contributions

R.V., V.D.V., A.C., P.D.A., G.S., B.A., F.P.I., D.L., F.Z.; Participated in study design, data collection and evaluation, drafting and statistical analysis. E.R., C.D.M., G.V.; Contributed to conception and design. All authors participated in the protocol development, collection and analysis of the included data, writing of the manuscript and final approval.

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Systematic Review

Rubella Immunity in Pregnant Iranian Women: A Systematic Review and Meta-Analysis

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Abstract

Rubella infection within the first trimester of pregnancy may lead to adverse pregnancy outcomes. The present study was conducted to evaluate the immunity against rubella among the pregnant Iranian women. The steps of meta-analyses were conducted based on the MOOSE protocol and results were reported according to the PRISMA guideline. To review the associated English and Persian literature, a comprehensive search was conducted among the international databases such as Scopus, PubMed/Medline, Science Direct, Embase, Cochrane library, Web of Science and Google Scholar search engine as well as Iranian databases, until April 1, 2018 using the following medical subject headings (MeSH) keywords: 'Pregnant', 'Gestational', 'Prenatal care', 'Complications of pregnancy', 'Pregnancy', 'Rubella infection', 'Prevalence, 'Epidemiology', 'Immunity', 'Immunization', 'Antibody', 'Immunogenicity' and 'Iran'. Cochran's Q test and I^2 index were used to investigate heterogeneity in the studies. Random effects model was used to estimate the rate of rubella immunity. The obtained data were analyzed using Comprehensive Meta-Analysis Ver.2. Fifteen studies constituting 7,601 pregnant Iranian women met the inclusion criteria. The overall pooled rubella immunity rate was 90.1% [95% confidence interval (CI): 86.1-93.1]. Rubella immunity rates were respectively 88.6% (95% CI: 80.6-93.6) and 91.5% (95% CI: 88.1-93.9) before and after national vaccine program. Rubella immunity rates were 91.4% (95% CI: 87.8-94.0) and 87.2% (95% CI: 74.3-94.1) based on the enzyme-linked immunosorbent assay (ELISA) and haemagglutination-inhibition (HAI) methods, respectively. There was no significant association between rubella immunity and vaccination program (P=0.398), diagnostic methods (P=0.355), geographic regions (P=0.286), quality of the studies (P=0.751), occupation (P=0.639), residence (P=0.801), and year of the studies (P=0.164), but it was significantly associated with age (P<0.001).

Despite high rubella immunity among the pregnant Iranian women, anti-rubella antibody screening is recommended for all women of childbearing age.

Keywords: Immunity, Iran, Meta-Analysis, Pregnant Women, Rubella

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Introduction

Rubella virus is an important pathogen worldwide and a member of the genus Rubivirus in the Togaviridae family. This human virus is transmitted through aerosols and usually causes benign infections in children and young adults (1, 2). Rubella virus in adults may also cause severe inflammation and joint pain (3). Moreover, this infection may cause premature birth, low birth weight (4), miscarriage, stillbirth (5) and congenital rubella syndrome (CRS) during the first trimester of pregnancy (4-6). This syndrome is characterized by fetal abnormalities, including mental retardation, blindness, deafness (7), heart defects, cataracts (6), hepatomegaly and jaundice (8). Rubella infection is dangerous during pregnancy, especially during the first trimester. The rate of congenital malformations in newborns is 50, 25 and 17% for the first, second and third months, respectively (9-11).

Currently, there is no antiviral treatment for rubella (2), but an efficient vaccine is available against rubella (2, 3). The World Health Organization (WHO) recommends a comprehensive strategy for rubella and CRS control and eventual elimination in conjunction with rubella elimination, using measles/rubella or measles/mumps/rubella vaccines (12).

According to studies conducted in different regions of the world, the immunity against rubella has been reported to be diverse from 66-100% (13-16). Many studies have been conducted in Iran and these studies have reported the rubella immunity rate of 75-96% in pregnant women (17-20). Given the importance of this subject, the need for a comprehensive study is necessary. The analysis includes study of rubella immunity in pregnant Iranian women before and after introduction of the vaccine and assessing influence factors on sero-status.

A more clear picture of the problem dimensions in the community can be provided through systematic review of all documentation and combining them with meta-analysis (21-23). This study was conducted to assess rubella immunity in pregnant Iranian women.

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Materials and Methods

Study protocol

To identify relevant studies, a systematic review was performed on cross-sectional and case-control studies related to rubella immunity in pregnant women. The review was carried out in accordance with Meta-analysis of observational studies in epidemiology (MOOSE) protocol and results were reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guideline (23). To avoid bias in the study, search, selection of studies, quality assessment and data extraction were independently performed by two researchers. In case of discrepancies in the result of the two researchers, the study was referred to the third researcher.

Search strategy

To evaluate related English and Persian literatures, a comprehensive search was conducted in six national databases including: Iranian Research Institute for Information Science and Technology (IranDoc; https://irandoc.ac.ir), Scientific Information Database (SID; http://www.sid.ir/), Barakat Knowledge Network System (http://health.barakatkns.com), Iranian National Library (http://www.nlai.ir/) and Regional Information Center for Science and Technology (RICST; http://en.ricest.ac.ir/), Magiran (http://www.magiran.com/) and six international databases including: Scopus, PubMed/Medline, Science Direct, Embase, Cochrane Library, Web of Science (ISI) and Google Scholar search engine. The search was done without time limit until April 1, 2018. High-sensitivity search was independently carried out by two researchers who were familiar with searching in databases (Azami M. and Jaafari Z.). Medical Subject Headings (MeSH) keywords were 'Pregnant', 'Gestational', 'Pregnancy', 'Rubella infection', 'Prevalence, 'Epidemiology', 'Immunity', 'Immunization', 'Antibody', 'Prenatal care', 'Immunogenicity' and 'Iran'. The combined search was performed using Boolean operators (AND and OR). Combined search in PubMed database is shown as follows: ("Pregnant"[Title/Abstract] OR "Pregnancy"[Title/Abstract] OR"Prenatal care" [Title/Abstract] OR "Gestational"[Title/Abstract]) AND ("Rubella"[Title/Abstract] OR "Immunity"[Title/Abstract] OR "Immunogenicity"[Title/Abstract] OR "Immunization"[Title/Abstract] OR "Antibody"[Title/Abstract] OR "Prevalence"[Title/Abstract] OR "Epidemiology"[Title/Abstract]) AND ("Iran"[Title/Abstract/Affiliation]).

After the end of search, the title of collected articles was entered into EndNote™ resource management to find similar articles. Manual search was also carried out by reviewing the reference list of relevant articles.

Inclusion and exclusion criteria

The inclusion criteria according to PICO (Evidence Based Medicine) (24) were: i. **P**opulation: pregnant Iranian women, ii. **I**ntervention: serological tests such as enzyme-linked immunosorbent assay (ELISA) or haemagglutination-inhibitory (HAI) methods to confirm immunity against rubella, iii. **C**omparison: it can show the immunity seroprevalence in terms of age, occupation and place of residence, and iv. **O**utcome: estimating the overall seroprevalence of rubella immunity in pregnant women and other risk factors.

Exclusion criteria were: i. Non-random sample for seroprevalence of rubella immunity, ii. Non-pregnant women, iii. Non-Iranian sample, iv. Low-quality studies, v. Duplicate studies, and vi. Review articles, case reports and letters to the editor.

Methodological quality assessment

The researchers evaluated quality of the selected studies using a scoring system, according to the modified Newcastle Ottawa Scale (NOS) for cross-sectional studies (25). The attainable minimum score was five and the articles that received a minimum score underwent quality assessment and Metadata extraction processes.

Data extraction

Data extraction form included the author's name, age (mean ± SD), place of residence, sample size, study design, rubella immunity, before/after national vaccination program, diagnostic method, quality score and the number of event and total in case and control groups or odds ratio (OR) and 95% confidence interval (CI) for risk factors. The extracted data was compared by two researchers and shared with the third researcher in case of discrepancies and finally a consensus was reached to re-examine and compare the results. Specific questions or relevant ambiguities in the articles were asked from the author via email.

Statistical analysis

The binomial distribution was used to estimate the standard error of rubella immunity in each study. OR index was calculated to evaluate the effect of age, occupation and place of residence on rubella immunity. Cochran's Q test and I^2 index were used to investigate heterogeneity in the studies. Interpretation in this regard was as follows: 0-24% indicates low heterogeneity, 25-49% indicates moderate heterogeneity, 50-75% indicates substantial heterogeneity and over 75% indicates high heterogeneity. To estimate the seroprevalence of rubella immunity and to measure the effect of age on rubella immunity rate due to high heterogeneity between studies, random-effects model was used. To measure the effect of occupation and place of residence on rubella immunity rate, due to low heterogeneity, the fixed effects model was used to combine data (26). We also conducted sensitivity analysis by removing one study at the same time to assess the stability of the meta-analysis results. Sub-group analysis and meta-regression of the rubella immunity were used to find the potential sources of heterogeneity. Sub-group analysis were divided based on five regions of Iran, national rubella vaccination program, diagnostic methods and quality of studies. Funnel plot and Egger and Begg's tests were used to examine the publication bias. Finally, data were analyzed using Comprehensive Meta-Analysis software Ver.2 (Biostat, Inc. Company, U.S. and U.K.). The significance level was set at 0.05.

Results

Searching results and characteristics

In this systematic review, 280 articles were found by two researchers, among of which 264 articles were excluded because of the following reasons: duplicates (n=140), irrelevance (n=68), non-observational epidemiological studies (n=12), non-random sample (n=14), the sample size other than pregnant Iranian women (n=18); lack of assessing rubella immunity (n=9), and non-original studies (n=4, Fig.1). Finally, 15 studies comprising 7,601 pregnant woman with a mean age of 26.47 years [95% CI: 23.18-29.76] were entered into the meta-analysis process. The characteristics of each study are shown in Table 1.

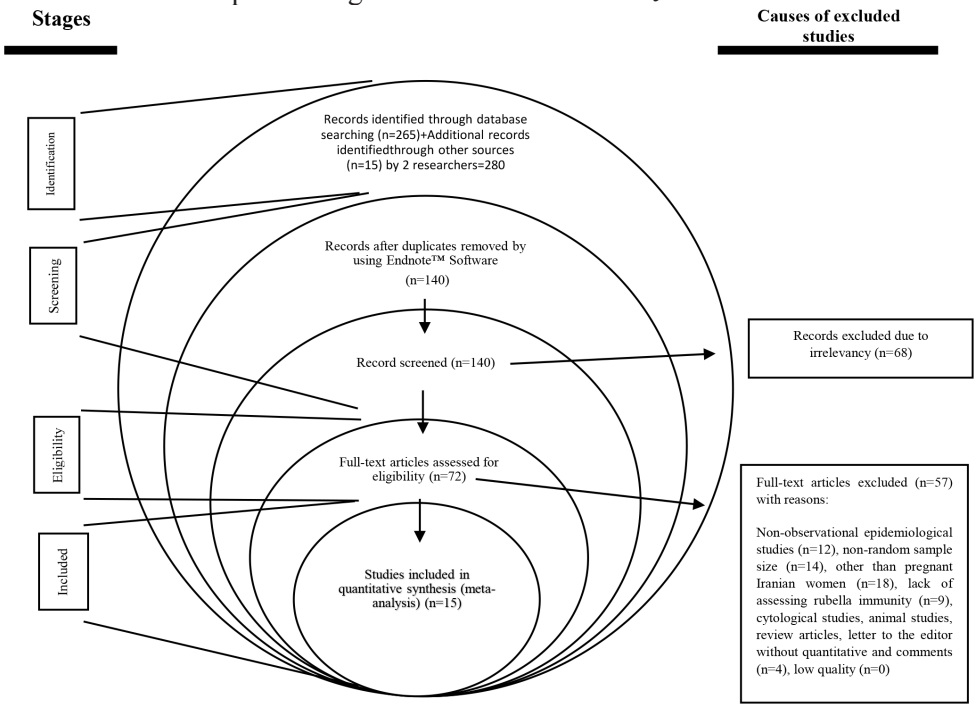

Fig.1: Study flow diagram.

Table 1: Summary of characteristics entered into the meta-analysis

First author, Published year	Year of study	Design	Place	Sample size	Age (mean ± SD)	Method	Immunity (%)	Quality	Ref.
Akbarian et al., 2007	2004	Cross-sectional	Tehran	810	21.9 ± 2.4	ELISA	85.5	High	(17)
Ghafourian Boroujerdnia et al., 2003	2000	Cross-sectional	Ahvaz	250	NR	HAI	92	High	(18)
Doraji et al., 2009	2009	Cross-sectional	Tehran	120	NR	ELISA	91.6	Medium	(19)
Pakzad and Moattari, 1987	1986	Cross-sectional	Ahvaz	100	NR	HAI	90	Medium	(20)
Mokhtari et al., 2010	2007	Cross-sectional	Mashhad	73	26.7 ± 6.5	ELISA	90.4	High	(27)
Amini et al., 1996	2009	Cross-sectional	Tehran	210	NR	ELISA	94.3	Medium	(28)
Ashraf Ganjoei and Mohammadi, 2001	1997	Cross-sectional	Kerman	410	26.58 ± 5.5	ELISA	94.6	High	(29)
Pakzad and Ghafourian, 1995	2004	Cross-sectional	Dezfull	500	NR	HAI	74.8	Medium	(30)
Modarres, 2000	1996	Cross-sectional	Tehran	3008	NR	HAI	94	Medium	(31)
Bagheri Josheghani et al., 2015	1993	Cross-sectional	Kashan	80	30 ± 5.2	ELISA	92.5	High	(32)
Honarvar et al., 2013	2010	Cross-sectional	Shiraz	175	27.3 ± 5.3	ELISA	96	High	(33)
Ghafourian Boroujerdnia, 2001	2011	Cross-sectional	Ahvaz	300	NR	ELISA	78	High	(34)
Majlessi et al., 2008	1990	Cross-sectional	Tehran	965	NR	ELISA	91.1	High	(35)
Eslamian, 2000	2004	Cross-sectional	Tehran	500	NR	HAI	76	Medium	(36)
Ghaderi and Ghaderi, 2016	1995	Cross-sectional	Birjand	100	NR	ELISA	94	Medium	(37)

HAI; Haemagglutination-inhibition, ELISA; Enzyme-linked immunosorbent assay, NR: Not reported. and SD; Standard deviation.

Pooled rubella immunity

The heterogeneity in this study was estimated to be high (P<0.001 and I^2=95.7%). In an analysis of 7,601 pregnant women in Iran, rubella immunity rate was found to be 90.1% (95% CI: 86.1-93.1, Fig.2A). The lowest and highest rates were related to the studies in Dezful [74.8% (95% CI: 70.8-78.4)] (30) and Shiraz [96% (95% CI: 91.8-98.1)] (33), respectively. Forest plot for analysis of sensitivity was performed by removing one study at the same time to test the stability of the pooled. The results are shown in Figure 2B.

Results of the subgroup analysis

Quantity of studies in the South, East and Central regions of Iran were 2, 9 and 4 studies, respectively. Rubella immunity rate for these regions was 93.3% (95% CI: 88.7-96.2), 87.8% (95% CI: 79.0-93.1) and 90.1% (95% CI: 85.4-93.4), respectively. This difference was not significant (P=0.286, Table 2). Sub-group analysis of rubella immunity rate based on quality of the studies was not significant (P=0.751, Table 2).

Rubella immunity rate, based on the ELISA method, was 91.4% (95% CI: 87.8-94.0) and based on the HIA method was 87.2% (95% CI: 74.3-94.1). Sub-group difference was not significant (P=0.355, Table 2). Sub-group analysis of rubella immunity rate based on national vaccination program is shown in Table 2. The difference was not significant (P=0.398).

A

Author (s), Published year	Event rate	Lower limit	Upper limit	Z-Value	p-Value	Total	Relati weig
Akbarian et al., 2007	0.855	0.829	0.878	17.781	0.000	693 / 810	7.4
Ghafourian BoroujerdNia et al., 2003	0.920	0.879	0.948	10.477	0.000	230 / 250	6.8
Doraji et al., 2009	0.916	0.851	0.954	7.260	0.000	110 / 120	6.2
Pakzad and Moattari, 1987	0.900	0.824	0.945	6.592	0.000	90 / 100	6.2
Mokhtari et al., 2010	0.904	0.812	0.954	5.644	0.000	66 / 73	5.8
Amini et al., 1996	0.943	0.902	0.967	9.427	0.000	198 / 210	6.4
Ashraf Ganjoei and Mohammadi, 2001	0.946	0.919	0.964	13.104	0.000	388 / 410	6.9
Pakzad and Ghafourian, 1995	0.748	0.708	0.784	10.562	0.000	374 / 500	7.3
Modarres, 2000	0.940	0.931	0.948	35.839	0.000	2828 / 3008	7.4
Bagheri Josheghani et al., 2015	0.925	0.843	0.966	5.919	0.000	74 / 80	5.6
Honarvar et al., 2013	0.960	0.918	0.981	8.238	0.000	168 / 175	5.8
Ghafourian Boroujerdnia et al., 2001	0.780	0.730	0.823	9.081	0.000	234 / 300	7.2
Majlessi et al., 2008	0.911	0.891	0.927	20.574	0.000	879 / 965	7.3
Eslamian, 2000	0.760	0.721	0.795	11.008	0.000	380 / 500	7.3
Ghaderi and Ghaderi, 2016	0.940	0.873	0.973	6.535	0.000	94 / 100	5.6
	0.901	0.861	0.931	11.049	0.000	6805 / 7601	

Statistics for each study | Event rate and 95% CI | 0.00 | 0.50 | 1.00

Random-effects model

Meta Analysis

B

Author (s), Published year	Point	Lower limit	Upper limit	Z-Value	p-Value
Akbarian et al., 2007	0.905	0.860	0.937	10.008	0.000
Ghafourian BoroujerdNia et al., 2003	0.900	0.856	0.931	10.465	0.000
Doraji et al., 2009	0.900	0.857	0.932	10.566	0.000
Pakzad and Moattari, 1987	0.902	0.859	0.932	10.614	0.000
Mokhtari et al., 2010	0.901	0.859	0.932	10.648	0.000
Amini et al., 1996	0.898	0.854	0.929	10.489	0.000
Ashraf Ganjoei and Mohammadi, 2001	0.897	0.853	0.929	10.480	0.000
Pakzad and Ghafourian, 1995	0.909	0.872	0.936	11.791	0.000
Modarres, 2000	0.896	0.858	0.926	11.699	0.000
Bagheri Josheghani et al., 2015	0.900	0.857	0.931	10.605	0.000
Honarvar et al., 2013	0.896	0.852	0.928	10.497	0.000
Ghafourian Boroujerdnia et al., 2001	0.908	0.868	0.937	10.964	0.000
Majlessi et al., 2008	0.901	0.856	0.933	10.066	0.000
Eslamian, 2000	0.909	0.870	0.936	11.469	0.000
Ghaderi and Ghaderi, 2016	0.899	0.855	0.930	10.561	0.000
	0.901	0.861	0.931	11.049	0.000

Statistics with study removed | Event rate (95% CI) with study removed | 0.00 | 0.50 | 1.00

Meta Analysis

Fig.2: Forest plot for rubella immunity in pregnant Iranian women. **A.** Pooled estimate and **B.** sensitivity analysis.

Table 2: Rubella immunity in pregnant Iranian women subgrouped using regions, quality of studies, diagnostic method and national vaccination program by random effects model

Variable		Study (n)	Sample size (n)	Heterogeneity				95% CI	Pooled estimate (%)
				Q	df	P value	I^2 (%)		
Regions	Center	4	5793	188.24	8	<0.001	95.75	85.4-93.4	90.1
	South	2	483	1.87	1	0.171	46.65	88.7-96.2	93.3
	East	9	1325	57.04	4	<0.001	92.98	79.0-93.1	87.8
	Test for subgroup differences: Q=2.50, df(Q)=2, P=0.286								
Quality of the studies	High	8	3063	71.50	7	<0.001	90.21	86.5-93.6	90.7
	Medium	7	4538	251.62	6	<0.001	97.24	80.3-94.6	89.4
	Test for subgroup differences: Q=0.27, df(Q)=1, P=0.751								
Diagnostic method	ELISA	10	3243	88.14	9	<0.001	88.14	87.8-94.0	91.4
	HIA	5	4358	241.90	4	<0.001	98.34	74.3-94.1	87.2
	Test for subgroup differences: Q=0.85, df(Q)=1, P=0.355								
National vaccination program	Before	8	5278	291.49	7	<0.001	97.6	80.6-93.6	88.6
	After	7	2323	26.67	6	<0.001	79.34	88.1-93.9	91.5
	Test for subgroup differences: Q=0.71, df(Q)=1, P=0.398								

CI; Confidence interval, HAI; Haemagglutination-inhibition, ELISA; Enzyme-linked immunosorbent assay, Q; Q test for heterogeneity, df; degrees of freedom, and I^2; I square.

Association of rubella immunity rate with age, occupation or accommodation place

The rubella immunity among pregnant women was significantly associated with age (≤25 versus >25 years old) [OR=10.31 (95% CI: 5.24-20.27, P<0.001)], but it was not significantly associated with occupation (employed versus housekeeper) [OR=1.06 (95% CI: 0.81-1.38, P=0.639)] and place of residence (urban versus rural) [OR=0.97 (95% CI: 0.80-1.18, P=0.801)] (Fig.3).

Meta-regression

Meta-regression of rubella immunity rate for the year of study was not statistically significant (P=0.164, Fig.4A).

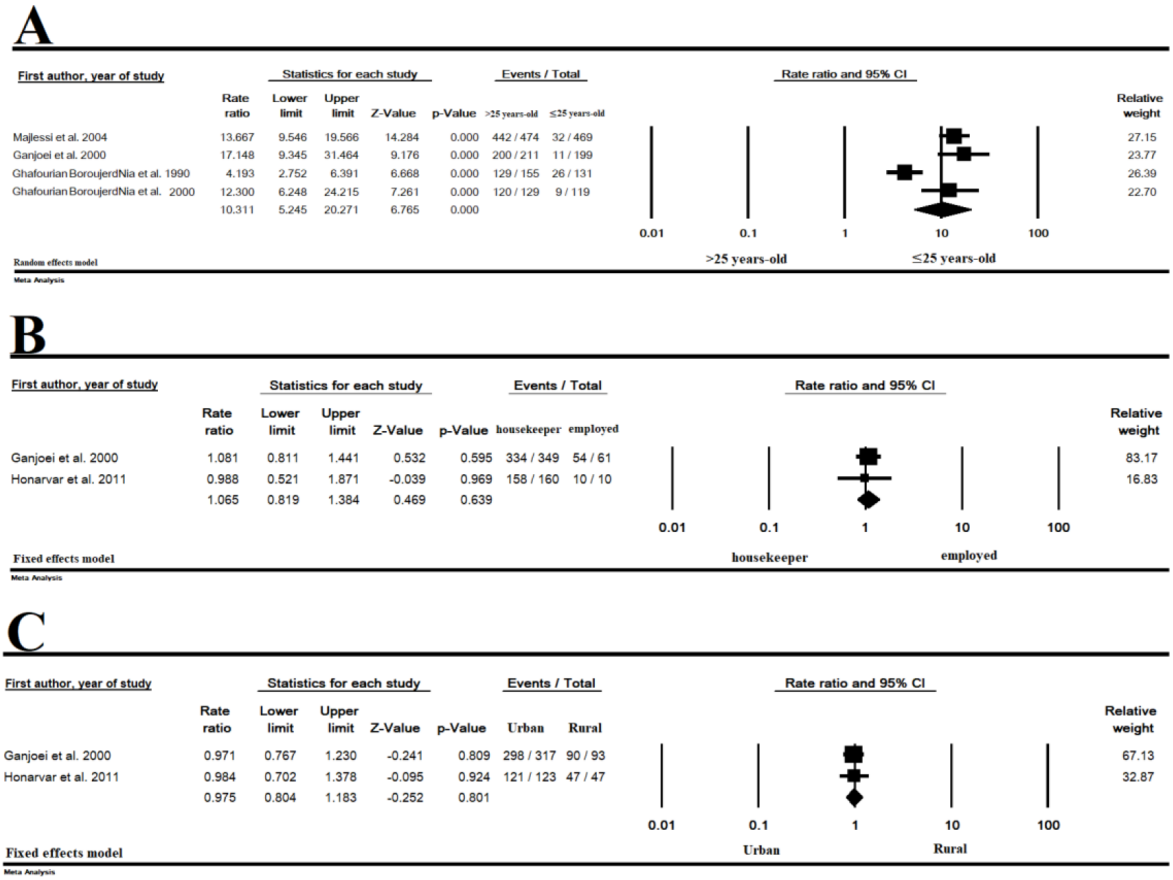

Fig.3: The association between rubella immunity rate and variables. **A.** Age, **B.** Occupation, and **C.** Place of residence.

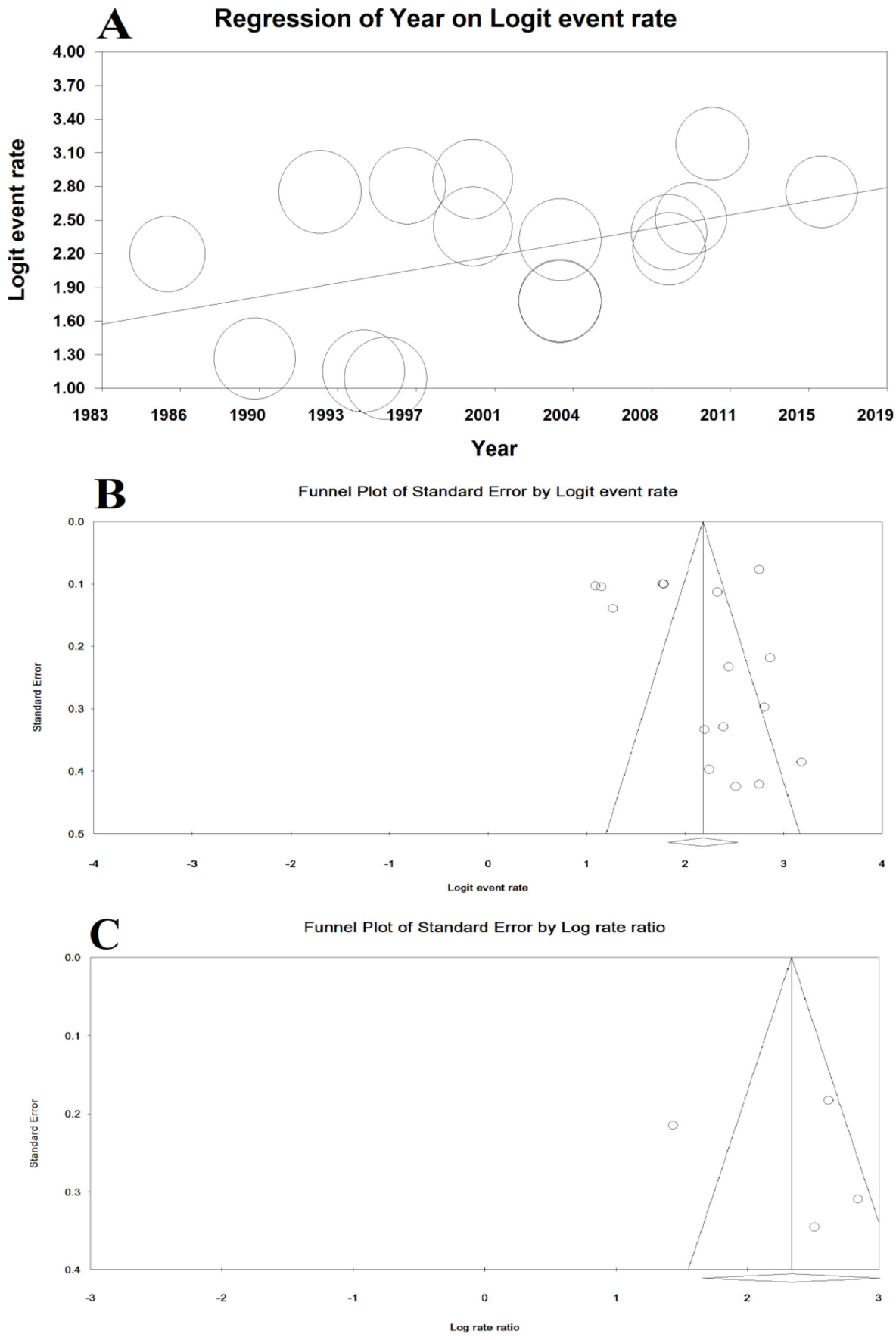

Fig.4: Meta-regression and publication bias. **A.** Meta-regression model of rubella immunity based on the year of the study, **B.** Publication bias for rubella immunity rate, and **C.** Relation between immunity and age.

Publication bias

In the evaluation of publication bias for rubella immunity rate, a funnel plot was drawn and P values based on Egger and Begg's tests were estimated to be 0.45 and 0.75, respectively. In addition, rubella immunity rate were respectively 0.79 and 0.73 for association between rubella immunity rate and age, while they were not statistically significant (Fig.4B, C).

Discussion

The results showed that rubella immunity rate among pregnant Iranian women was 90.1% and rubella immunity was not significantly associated with geographic regions, quality of the studies, diagnostic methods, vaccination program, occupation, place of residence, and year of the studies, occupation, place of residence, and year of the studies, but it was significantly associated with age.

CRS is declining in the world due to increased rubella vaccine coverage (38, 39). However, it remains a threat and a costly disease in some areas, where pregnant women were not immunized and protected against rubella virus. According to WHO, the primary objective of vaccination against rubella is the prevention of CRS. For this reason, immunization with rubella-containing vaccine is recommended for adult girls, women of childbearing age, or both (40).

In Iran, as a member of WHO, prevention and control of measles and rubella (MR) is an important priority (40). Several studies, conducted in various provinces of Iran, revealed that the immunity rate against rubella in women of reproductive age (15-45 years) is 69.9-97% (41, 42), and according to the present study, the immunity rate against rubella in pregnant Iranian women was found to be 86.1-93.1%.

Rubella immunity rate among the pregnant women has been reported in European (74-98%), African (53-95%) and Asian (54-96%) countries (43-53). The probable cause of these different reports may be due to the universal coverage of vaccination against rubella and different diagnostic methods. However, in this study, this difference was not statistically significant.

In the past, MR were endemic in Iran and most of the people were infected until puberty. Therefore, most women acquired immunity against measles, rubella and mumps in their reproductive age. In 2002, the Ministry of Health and Medical Education in Iran established a comprehensive strategy for the elimination of MR. This strategy was launched with the aim of vaccinating 33,579,082 people, aged 5-25 years old, and 98% of the target population were vaccinated. This successful measure led to a decline in the incidence of MR to less than one case per million (41).

In this study, the rubella immunity rate in pregnant women before and after national vaccination program was estimated to be 88.6 and 90.4%, respectively. This difference was not statistically significant. It can be said that the high prevalence of IgG antibody seroprevalence during the years before implementation of vaccination programs is due to the high incidence of rubella and immunity through contact with the virus. In other countries such as Mexico, vaccination coverage was carried out from 1998 and this has been increased in Mexican pregnant women (14, 54).

In the present study, immunity rate against rubella was estimated 91.4% and 87.2%, using respectively ELISA and HAI methods. The specificity and sensitivity of the ELISA method for determining antibody against rubella was reported 61.7% and 95%, respectively (55). Shekarchi et al. (56) also mentioned that ELISA method is as accurate as HAI method and it would reliable, if purified antigens and carefully prepared reagents were used.

In this study, rubella immunity rate was higher in younger pregnant women. In a study performed by Alvarado-Esquivela in Mexico (14), age and socioeconomic level were significant and the other risk factors, such as residence, education level and occupation were not significant. In the study conducted by Hamdan et al. (48) in Sudan, the examined risk factors such as age, education level, gestational age, history of jaundice and body mass index were not significant. In another study in United States, international travel was demonstrated as a risk factor (55). Thus, it can be stated that each region has its own set of risk factors.

Limitations of the present study, including: i. The Iranian databases could not be used for advance search and ii. Many risk factors such as the year of birth and etc., were neglected.

Conclusion

This meta-analysis provides information about rubella immunity in pregnant women. Although this study showed that the level of immunity in pregnant Iranian women is acceptable, it is recommended to perform anti-rubella antibody screening for all women of childbearing age.

Acknowledgements

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Authors' Contributions

S.A., M.A.; Study concept and design. S.A., Z.J., G.B., A.S., M.A.; Acquisition of data, drafting the manuscript, critical revision of the manuscript for important intellectual content. G.B., S.A., M.A.; Quality evaluation of studies. M.A.; Analysis and interpretation of data. S.A.; Administrative, technical or material support. All authors read and approved the final manuscript.

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Original Article

Beneficial Effects of Oral Lactobacillus on Pain Severity in Women Suffering from Endometriosis: A Pilot Placebo-Controlled Randomized Clinical Trial

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Abstract

Background: This study assessed the effects of a lactobacillus-based medication on pain intensity scores in women with endometriosis.

Materials and Methods: The present randomized pilot placebo-controlled trial was done on eligible women who were surgically and pathologically diagnosed with endometriosis. Thirty-seven participants who had not received hormonal treatment in the last three months, were enrolled and randomized into LactoFem® and placebo groups. Lactobacillus capsules or placebo were administrated orally once a day for 8 weeks. Patients were assessed for pain severity using Visual Analogue Scale (VAS) scores for dysmenorrhea, dyspareunia and chronic pelvic pain at baseline and after 8 and 12 weeks post-intervention.

Results: Mean age of participants and mean body mass index (BMI) for the LactoFem® and control groups were comparable. All patients had stage 3 and 4 of the disease based on revised American fertility society (AFS) classification of endometriosis. Mean initial pain scores for dysmenorrhea, dyspareunia and chronic pelvic pain were 6.53 ± 2.88, 4.82 ± 3.76 and 4.19 ± 3.53, respectively in the LactoFem® group and 5.60 ± 2.06, 3.67 ± 2.64 and 2.88 ± 2.80, respectively for the control group; the two groups had comparable scores in this regard. There was more decrease in pain scores for both dysmenorrhea and the overall pain after 8 weeks of treatment in LactoFem® group compared to the control group. The scores for dysmenorrhea were 6.53 ± 2.88 and 5.60 ± 2.06 in the LactoFem® and control groups, respectively, before intervention but, after 8-week treatment, these values were 3.07 ± 2.49 and 4.47 ± 2.13 (P=0.018), respectively. The changes in overall pain score in the LactoFem® and control group during this period were 7.33 ± 7.00 and 4.11 ± 1.68, respectively (P=0.017).

Conclusion: This study showed some beneficial effects of lactobacillus administration on endometriosis-related pain (Registration number: IRCT20150819023684N5).

Keywords: Chronic Pelvic Pain, Dysmenorrhea, Dyspareunia, Endometriosis, Lactobacillus

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Introduction

Endometriosis, characterized by abnormal presence of endometrial tissue outside the uterus, is a major cause of discomfort in women (1, 2). This disease which occurs primarily in women of reproductive ages, seems to be an estrogen-dependent phenomenon (1-3). Although clinical symptoms are not seen in all women, the impact of endometriosis on physical, psychological and social performance is obvious in many other women (4). Endometriosis-associated pain includes dysmenorrhea, dyspareunia, dyschezia and dysuria, as well as chronic pelvic pain. Endometriosis patients at some time points endure debilitating pain which is worse than the pain experienced by women suffering from cancer (2). Moreover, ovarian endometriosis may have clinical and paraclinical manifestations of ovarian carcinoma (5). The mainstay of treatment of endometriosis consists of surgery accompanied by ovarian suppressive therapy (6, 7). Full consultation with patients and use of various types of analgesics, oral contraceptive pills, progestins or gonadotropin-releasing hormone agonists (GnRHa) are often required (8-12).

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There is sufficient evidence showing the efficacy of progestins and GnRHa against endometriosis-associated pain (13, 14), however, their side effects and patient tolerance, particularly in the long term, should not be overlooked (10, 13, 14). Based on molecular studies, changes in the function of immunologic cells like monocytes, macrophages, natural killer cells (NK), cytotoxic T cells and B cells have been detected in the peritoneal fluid of women with endometriosis. This alteration of immunologic defense which is not capable of removing the ectopic endometrial cells, leads to implantation of endometriosis lesions. Furthermore, the paramount role of NK cells was highlighted in many studies (15-20). According to Oosterlynck et al. (17), decreased activity of NK cells is remarkably associated with the severity of endometriosis. Previous studies led to the hypothesis that lack of ectopic endometrial clearance by NK cells in the peritoneal fluid contributes to the development of the disease. Therefore, any agent that stimulates the immune cells or increases the cytotoxicity of NK cells could be beneficial in treatment of endometriosis (21-24).

Sashihara et al. (21-23) showed that a kind of lactobacillus called Lactobacillus gasseri (OLL2809), which is of probiotic type, stimulates the production of interleukin 12 (IL-12) from murine spleen cells. IL-12, a cytokine secreted by antigen presenting cells, triggers the production of cytotoxic lymphocytes by activating NK cells and T cells (25, 26). Lactobacillus species including *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus fermentum* and *Lactobacillus gasseri,* constitute the predominant normal microbial flora of genitourinary and gastrointestinal (GI) tract of healthy individuals. The effectiveness of these probiotics in maintenance of the normal pH of vagina and prevention of genital infections has been well-studied (27). Host immunity modification and interference with colonization of external pathogens are considered their main mechanisms of action (28, 29). There is also evidence that alterations in the normal flora within the gastrointestinal (GI) tract caused by administration of probiotics, antibiotics or even transplantation of feces into the GI tract, could result in pain relief by affecting neurologic pathways (30). In this regard, some recent studies indicated the use of lactobacillus-mediated medications in the treatment of endometriosis-related lesions (31, 32). Considering the hypothesis that lactobacillus may have immunogenic properties, the present study was conducted to assess the efficacy of oral lactobacillus-based pills on pain relief in patients diagnosed with endometriosis.

Materials and Methods

This was a pilot randomized triple-blind placebo-controlled trial carried out in a referral center for endometriosis in a university-based hospital in Tehran, Iran from October 2016 to October 2017. Enrolled participants were women with endometriosis (diagnosed based on pathologic report) who had undergone laparoscopic surgery due to pain and were randomly allocated into one of the two groups at a 1:1 ratio. The study was approved by the Institutional Review Board of Iran University of Medical Sciences (IUMS) by the Ethical Committee number IR.IUMS.REC1395.9311290013. All participants were patients with stage 3 and 4 of endometriosis (according to the revised American fertility society (AFS) classification of endometriosis (33). Patients were between 18 to 45 years old with menstrual cycle ranging from 21 to 35 days, with initial overall pain score higher than 4 [based on the visual analogue scale (VAS) scoring system]. The overall pain score was defined as the sum of dysmenorrhea, dyspareunia, chronic pelvic VAS pain scores. A scale of 0 (without any pain) to 10 (most severe pain), by the use of a 10-cm ruler in the questionnaire filled by the physician at the initial visit and each follow up visit at 8 and 12 weeks post-treatment, was used in the VAS scoring. Patients had at least 3 months interval from surgery and in this period, they were not supposed to use hormonal treatment; also, the participants were asked not to take any pain-killer medications other than NSAIDs which have short-term effects and do not have interference with lactobacillus effects. Those with history of hormonal replacement after surgery, hepatic or renal disturbances, cancer, diarrhea after taking dairy products, or consuming any type of probiotic products were excluded. Written informed consent was obtained from all patients eligible for the trial. Data including demographic findings, medical history and medication use were recorded in questionnaires by a physician in the first visit and completed in the follow-up visits at 8 and 12 weeks post-treatment visits. The participants were asked to mention any kind of excessive GI upset, nausea, vomiting, or any other non-specific side effects.

Treatment protocol

The present study was a pilot placebo-controlled randomized clinical trial which recruited 20 patients for each arm (Fig.1). After exclusion of 3 patients, thirty-seven patients with endometriosis were randomly assigned (by simple randomization method using table of random numbers) to one of the two groups receiving either LactoFem®, Zist Takhmir Co. Tehran, Iran (one capsule per day) or placebo (as the control group). Each LactoFem® capsule contains 10^9 colony of four different lactobacillus strains (*Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus fermentum* and *Lactobacillus gasseri*). The lactobacilli contents and placebo contents were packed in the similar packing with 30 capsules in each pack by the manufacturer; two packs were given to each patient in the first visit, to be used during the 8 weeks. The lactobacilli packs and placebo packs were named A or B by the manufacturer. After completion of the analysis, the manufacturer revealed which one was lactobacilli or placebo.

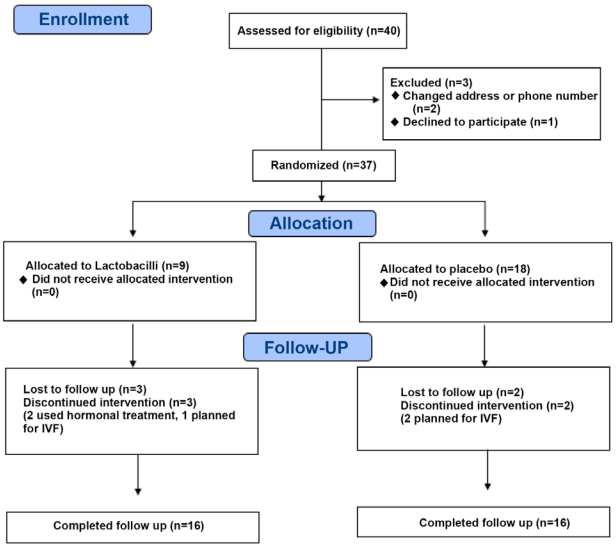

Fig.1: Flow diagram of the trial

All women had undergone complete laparoscopic removal of endometriosis lesions including deep infiltrating endometriosis (DIE). The procedures had been performed with similar extent of resection including ovarian cystectomy (endometrioma), salpingectomy, ureteral dissection, uterosacral ligament ablation or DIE removal. The interval between surgery and commencement of intervention was at least 3 months. At the beginning of the study, patients were evaluated for the intensity of pelvic pain, dysmenorrhea, and dyspareunia based on the VAS score rated from zero (no pain) to 10 (the most severe pain). Patients in the two groups continued taking medication for 8 weeks and then, the pain intensity was evaluated again 8 and 12 weeks following intervention by a follow-up visit or a phone call. During the time of follow-up, patients were allowed to use NSAIDs only as the rescue therapy. Patients who were not willing to continue the trial due to personal reasons were excluded from the study. This study was conducted as a triple-blind trial in which the researcher, the subjects, and the statistician were all unaware of the allocation of the two groups.

Statistical analysis

Results are presented as mean ± SD for quantitative variables and as absolute frequencies and percentages for categorical variables. Normal distribution of data was assessed using the Kolmogorov-Smirnoff test. Categorical variables were compared using chi-square test or Fisher's exact test. Quantitative variables were also compared using t test or Mann U test. ANOVA test was also used to analyze more than two means. For statistical analysis, the statistical software SPSS version 20 for windows (SPSS Inc., Chicago, IL) was used. $P \leq 0.05$ were considered statistically significant.

Outcomes

The main outcome of the study was the mean pain score (for dysmenorrhea, dyspareunia and pelvic pain) after 8 and 12 weeks of intervention as assessed by VAS scoring system. The secondary outcome was the change in VAS scores during the first 8 weeks of intervention and from 8 to 12 weeks post medication.

Results

The two groups were comparable regarding mean age (P=0.955), body mass index (BMI) (P=0.14), history of infertility (P=0.669), irregular menstrual cycle (P=0.264), underlying disorders (P=0.307), and history of medications (P=0.600). Demographic characteristics of the subjects are demonstrated in Table 1. All patients had undergone laparoscopy beforehand and endometriosis was pathologically diagnosed in all participants. According to revised American fertility society (AFS) classification of endometriosis (33), stage III was found in 25 and 0% and stage IV was observed in 75 and 100% of intervention and control groups, respectively (P=0.101).

Table 1: Baseline characteristics of the participants

Parameter	Lactobacillus group	Control group	P value
Age (Y)	33.81 ± 6.85	33.69 ± 5.63	0.955
BMI (Kg/m^2)	26.16 ± 5.46	23.64 ± 4.03	0.14
History of infertility	3 (18.8)	4 (25.0)	0.669
Irregular menses	7 (43.8)	4 (25.0)	0.264
Family history of endometriosis	3 (18.8)	1 (6.2)	0.600
Disease stage*			0.101
Stage III	4 (25.0)	0 (0.0)	
Stage IV	12 (75.0)	16 (100)	

Data are presented mean ± SD or n (%). BMI; Body mass index and *; Based on revised AFS classification.

As shown in Table 2, the mean pain scores at baseline as well as 8 and 12 weeks after intervention were not different between the groups. Using ANOVA analysis, the trend of the changes in pain intensity for dysmenorrhea, dyspareunia, and chronic pelvic pain during 12 weeks were evaluated. Concerning dysmenorrhea, the mean pain score decrease observed in the LactoFem® group was significantly larger than that of the control group during 8 weeks of treatment (3.46 ± 2.97 vs. 2.18 ± 1.06, P=0.018). The decreases in mean pain scores from week 0 to 12 and from week 8 to 12 were not however statistically significant (P=0.051 and 0.191 respectively). Concerning chronic pelvic pain, the mean pain score decrease from week 0 to 8 was 3.35 ± 2.18 for the LactoFem® group and 3.03 ± 0.37 for the placebo group (P=0.119). The decrease in chronic pelvic pain score from week 0 to 12 was not significant (P=0.458). The change in pain scores from week 8 to 12, however, was significantly larger in the control group (1.09 ± 1.00 vs. 1.34 ± 0.06, P=0.02). Concerning the overall pain scores, the mean pain score decreased significantly in the LactoFem® group during 8 weeks of intervention in comparison to the placebo group (7.33 ± 7.00 vs. 4.11 ± 1.68, P=0.017). Moreover, the change in pain scores between week 8 and 12 was statistically dif-

ferent between the groups (P=0.015). No serious side effects following ingestion of these capsules were reported.

Table 2: Pain scores (VAS) at 3 different time points

Parameter	Lactobacillus group	Control group	P value
Dyspareunia			
Week 0	4.82 ± 3.76	3.67 ± 2.64	0.402
Week 8	2.55 ± 2.77	3.25 ± 2.30	0.513
Week 12	3.09 ± 2.59	3.17 ± 2.08	0.939
Change between week 0-8	-3.55 ± 2.27	-2.02 ± 0.38	0.117
Change between week 0-12	-2.86 ± 1.72	-2.96 ± 0.46	0.301
Change between week 8-12	0.93 ± 0.54	-1.97 ± 0.07	0.350
Dysmenorrhea			
Week 0	6.53 ± 2.88	5.60 ± 2.06	0.316
Week 8	3.07 ± 2.49	4.47 ± 2.13	0.110
Week 12	3.80 ± 2.54	4.60 ± 1.92	0.339
Change between week 0-8	-3.46 ± 2.97	-2.18 ± 1.06	0.018
Change between week 0-12	-2.73 ± 2.68	-1.66 ± 1.06	0.051
Change between week 8-12	1.75 ± 0.73	1.95 ± 0.00	0.339
Chronic pelvic pain			
Week 0	4.19 ± 3.53	2.88 ± 2.80	0.253
Week 8	2.00 ± 1.93	2.50 ± 2.34	0.515
Week 12	3.00 ± 2.39	2.44 ± 2.13	0.448
Change between week 0-8	-3.35 ± 2.18	-3.03 ± 0.37	0.119
Change between week 0-12	-3.22 ± 1.18	-2.33 ± 0.43	0.458
Change between week 8-12	1.09 ± 1.00	-1.34 ± 0.06	0.02
Overall pain score			
Change between week 0-8	-7.33 ± 7.00	-4.11 ± 1.68	0.017
Change between week 0-12	-6.86 ± 4.93	-4.05 ± 1.81	0.127
Change between week 8-12	2.47 ± 2.06	2.27 ± 0.12	0.015

Data are presented mean ± SD.

Discussion

The aim of this study was to assess the therapeutic effects of oral lactobacillus on endometriosis-associated pain (including pain caused by dysmenorrhea, dyspareunia, and chronic pelvic pain). Few studies were conducted until now on the effects of lactobacilli on pain complaints related to endometriosis. A review of these few studies indicated the beneficial impact of lactobacilli on endometriosis (24, 31, 32). This possible effectiveness could result from increases in interleukin-12 levels and NK cells activity (15-18). Also, decrement of the activity of natural lethal cells seems to be related to the severity of endometriosis, and the inability to clear the ectopic endometrial lesions by the NK cells in the peritoneal space, contributes to development of disease (16-19, 22-24) which could be prevented by the use of probiotics. In a study done by Uchida and Kobayashi (32), lactobacillus therapeutic effect was evaluated in animal models following four weeks of treatment. It was finally observed that administration of lactobacillus was associated with a significant reduction in the volume of induced endometriosis in rats.

In another study (31), 33 patients with clinical diagnosis of endometriosis were given Lactobacillus gasseri capsules for 12 weeks. It was shown that 2 and 3 months post-treatment, use of lactobacillus was associated with significant improvements in pain intensity during menstruation in comparison with placebo. This finding was consistent with ours. The difference in pain scores during the first 8 weeks were apparently more in the mentioned study (31), and this was due to the lower initial pain scores post-surgical treatment in the present study. In both studies, no significant relief in non-menstrual pain was achieved. In our study, diagnosis of endometriosis was based on pathologic report and not just based on complaints of dysmenorrhea or other types of pain, which could be a strength of the present study. Furthermore, surgical staging was done based on the revised AFS classification. All the subjects had gone through laparoscopic surgery because of intolerable pain. An interval of at least 3 months was given to each patient before prescribing lactobacillus, to evaluate the effects of the surgical treatment. Lactobacillus-based medication used in our study consisted of four different strains of Lactobacilli including *Lactobacillus gasseri* used by Itoh et al. (31). Although the mean pain scores for two groups (according to VAS) after 8 weeks and 12 weeks were comparable, a larger decrease in dysmenorrhea intensity and the overall pain scores in the LactoFem® group was seen after 8 weeks of treatment. This improvement in pain after 8 weeks was not significant for chronic pelvic pain and dyspareunia comparing with dysmenorrhea and overall pain scores. Quite interestingly, during the four weeks following cessation of LactoFem® (i.e. from week 8 to 12), the mean pain scores related to chronic pelvic pain and the overall pain intensity increased significantly compared to the control group. This increase could be due to the withdrawal effects of the LactoFem® and the fact that the efficacy of the lactobacillus is limited to the treatment duration only. Our study was the first randomized trial using lactobacillus-based medication on stage 3 and 4 of endometriosis regarding three common pain types in such patients. Given the progressive nature of endometriosis and unbearable pain episodes related to this disorder, any intervention that could mitigate its symptoms, is certainly invaluable. Compared to other conventional medical therapies used for endometriosis-associated pain, LactoFem® capsules have no remarkable side effects such as weight gain, flushing or abnormal uterine bleeding and no serious side effects following ingestion of these capsules were reported in our experiment.

Furthermore, these capsules modify microbiota of urogenital and GI tract and prevent from infections by improving immune system function. LactoFem® capsules are readily available in our country at a reasonable price. The finding that the remedial outcome of LactoFem® was not as significant as expected could be due to the limitations of our study. The first limitation was the small sample size which was not large as many patients had received hormonal therapy during 3 month interval before initiat-

ing the study. Also, some patients were not able to refer to the clinic for participation in the study. Another limitation that should be mentioned was the lower initial pain scores of the patients, due to the surgical treatment, which could affect both the sample size and the results. This trial was designed as a pilot study and we believe that in a larger study population, more robust results could be achieved. The dosage of lactobacillus capsules administered could be another limitation. Maybe at higher doses, more declines in pain scores could have been resulted. Moreover, changes in microbiome caused by lactobacilli were not evaluated which could be another limitation of this study. It should also be mentioned that it was not possible to design a cross-over study because of the limited time that many of the patients agreed to participate in the study, since many of them planned for *in vitro* fertilization (IVF) or pregnancy in the near future. Also, there was no similar study conducted within a longer time of follow-up to be sure how long the effect of lactobacilli could remain on pain suppression, therefore to avoid a bias in this field, we preferred a non-cross over design.

Conclusion

It seems that lactobacilli have some beneficial effects regarding endometriosis-associated pain including dysmenorrhea and chronic pelvic pain. Regarding the dysmenorrhea, the best results happened after 8 weeks of the lactobacilli consumption, which also caused a significant decrease in the overall pain over the course of lactobacilli use in our study. The findings of our research may be used for sample size estimation for further randomized trials to better evaluate the impact of lactobacilli on endometriosis and its related symptoms.

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Authors' Contributions

S.Kh., S.N.; Study conception and design. L.M.; Acquisition and analysis of data. S.Kh., R.M.; Analysis and interpretation of data. S.N., F.Sh.; Literature research, pharmaceutical consultation, and manuscript edition. M.Kh, M.G.; Drafting and edition of manuscript, acquisition and analysis of data. S.Kh.; Critical revision. All authors read and approved the final manuscript.

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Original Article

Toxoplasma Serology Status and Risk of Miscarriage, A Case-Control Study among Women with A History of Spontaneous Abortion

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Abstract

Background: *Toxoplasma gondii* is one of the major causes of abortion in pregnant women. Most cases of abortion occur in the acute phase of infection and early pregnancy. The purpose of this study was to investigate the association between spontaneous abortion and seropositive status of toxoplasmosis in women with first-time spontaneous abortion.

Materials and Methods: This research is a case-control study on 240 serum samples from women experiencing spontaneous abortion for the first time as the case group, and 240 serum samples from women who had a normal delivery with no history of abortion as the control group. The level of anti-*Toxoplasma gondii* IgM and IgG antibodies were assessed in serum samples using ELISA. To separate the acute and chronic infections, all IgM-positive samples in both groups and IgG-positive samples of the case group were examined using IgG avidity.

Results: The *Toxoplasma* IgM antibody was detected in 3.3% (8/240) of the case group and 0.4% (1/240) of the control group, which was a statistically significant difference between the two groups [P=0.019, odds ratio (OR)=10.266]. Of all samples 47.5% and 46.3% of the case and control groups were positive for *Toxoplasma* IgG antibody, respectively. Seven out of 8 (87.5%) IgM-positive serum samples from the case group had low IgG avidity, indicating acute infections, whereas all IgG-positive sera and 1 IgM-positive serum, which was related to the control group, showed a high IgG avidity, indicating chronic infections.

Conclusion: Maternal acute toxoplasmosis during pregnancy is raised as one of the factors that increase the chance of spontaneous abortion. The necessary health training, especially on the parasite transmission ways to women before marriage, as well as the serological test in women before and during pregnancy is recommended. Polymerase chain reaction (PCR) and IgG avidity assays should be performed in the medical diagnostic laboratories for accurate distinguishing of the initial infection of toxoplasmosis in the pregnant women.

Keywords: Abortion, IgG Avidity, Pregnancy, Serology, Toxoplasmosis

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Introduction

Pregnancy is one of the most critical steps in women's lives, particularly those who want to become a mother for the first time. Abortion is a problem that any women might experience during pregnancy, and therefore suffer from psychological issues and medical expenses, which make it particularly important. One of the reasons for abortion is toxoplasmosis, which is due to an infection caused by *Toxoplasma gondii*, an obligate intracellular parasite, belonging to the phylum of Sporozoa, causing toxoplasmosis disease in humans and most of the warm-blooded animals around the world (1). This parasite, as one of the common human and animal pathogens, has accounted for numerous studies (2, 3). In humans, it is one of the most prevalent parasites, as in the serological studies it is estimated that nearly one-third of the human populations in Europe, South America, Africa, and Asia are infected with this parasite (4). Prevalence of *T. gondii* infection in pregnant women is investigated in different parts of the world, and estimated to be 14-77% (5).

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In general, a human typically becomes infected by three principal routes of transmission including drinking contaminated water or eating contaminated food, such as the tissue cyst in half-cooked contaminated meat or food that is contaminated with oocysts excreted from cat feces, and congenital transition, that means the transmission from an infected mother to her fetus (5). Toxoplasmosis infection might be acute or chronic with or without symptoms. The symptoms and complications of the disease mainly occur in the acute phase of infection. Following activation of the host immune system, the parasite proliferation is controlled and tissue cysts are formed in the host neuro-muscular tissues (3, 5). Although the acquired toxoplasmosis causes asymptomatic mild infections in people with a healthy immune system, it can cause severe clinical signs and even death in those with a weak or impaired immune systems. On the other hand, in people who are suffering from immune deficiency or are consuming immune-suppressive drugs, the chronic infection may be reactivated, causing severe and deadly complications such as encephalitis, myocarditis, and pneumonia (5). Transplacental transmission of *T. gondii* occurs mainly in the course of the first pregnancy (6). Congenital toxoplasmosis, which occurs during pregnancy, can cause spontaneous abortion, stillbirth, and some degrees of mental or physical retardation, hydrocephalus, blindness, and deafness (6, 7). Frequency and severity of the congenital toxoplasmosis are associated with the gestational age. The highest rate of congenital toxoplasmosis occurs in the third trimester of pregnancy, however, the highest infection severity is observed in the first and second trimesters, which can cause abortion or stillbirth (5-7).

The global estimated incidence rate of congenital toxoplasmosis is 190,100 cases annually, with an approximate incidence rate of 1.5 cases per 1000 live births [95% confidence interval (CI): 179,300-206,300] (8). The previous studies in Iran have shown that the seroprevalence rates of toxoplasmosis among childbearing age women are totally 39.9% among childbearing age women (9) and 39.3% (95% CI ¼ 33.0-45.7%) among the general population (10). Infection is more prevalent in hot and humid areas and relatively rare in cold and dry areas. The prevalence of infection is different among various ethnic groups, but the difference is more related to genetic differences, environmental health, and cooking habits (11).

One of the most popular medical concerns around the world is how to diagnose acute congenital infections in a pregnant woman that may lead to spontaneous abortion. This type of abortion is the disposal of pregnancy products before the twentieth week of gestation, without the use of medical and mechanical factors (12). Serological tests are the common diagnostic methods for congenital toxoplasmosis (13). Enzyme-linked immunosorbent assay (ELISA) test is currently the most widespread and most commonly used serological diagnosis method for toxoplasmosis (14). In recent years, efforts have been made to improve the ability to diagnose infections in pregnant women and congenital infections in the fetus and newborn. There are already a number of new methods to prove that there is great value for this purpose. For example, IgG avidity and polymerase chain reaction (PCR) applied on body fluids and tissue, as well as the western blot technique on the mother and infant serum samples, can be mentioned (15).

With regard to geographical and climatic differences in the prevalence of toxoplasmosis, and the lack of sufficient and precise data on the role of the parasite in abortion, in this study, the seroprevalence of anti-*T. gondii* IgM and IgG antibodies were investigated in women with first abortion experience in Khorramabad, Lorestan province, Western Iran. In order to determine the acute and chronic infections, all IgM- and IgG-positive serum samples were evaluated using IgG avidity.

Materials and Methods

Study region

Lorestan province is the thirteenth province in Iran in terms of population and is considered as one of the most populous provinces in Iran. The city of Khorramabad is the capital of the province. Lorestan province is located in Western Iran and placed between the latitudes 32º 30′ and 48º1′ N and longitudes 55º 17′ and 61º 15′E. The long-term annual mean temperature and precipitation are 17.07°C and 580 mm, respectively. The weather of this province is variable and is classified as a region with a semi-arid climatic condition (16).

Sample collection

This case-control study was performed on 240 serum samples from women with first spontaneous abortion referred to the only maternity hospital in Khorramabad city, during 2016, as the case group. The control group consisted of 240 serum samples from women who had a normal delivery and referred to the hospital for a checkup and had no history of abortion. All of the subjects in both the case and control groups had a history of at least one successful pregnancy, as those who did not have successful pregnancies were not included in the study. After obtaining the written consent from the participants in the study, a questionnaire based on age, education (Low literate, Diploma, Academic degree), occupation (Employee, Student, Housewife), place of residence (Urban, Rural), contact with cats, and consumption of raw/half-cocked meat was filled out by the participants. The blood sampling and serum isolation procedures were done under sterile conditions.

ELISA

The level of anti-T. gondii IgM and IgG antibodies were measured in serum samples using the commercial kit, de EIA de *Toxoplasma* IgG Foresight® ACON, according to the manufacturer's instructions (17). All specimens were run in duplicates. The results were considered positive when OD450 index was equal or higher than the cut off value. The cut-off values are estimated using known independent negative sera which are included in the titer-

plates amongst the unknown samples.

Avidity ELISA

To distinguish between the acute and chronic infections, all IgM- and IgG-positive samples of the case group were examined to evaluate IgG avidity by using the ELISA kit according to the manufacturer's instruction (ELISA: Euro immune Kit, Germany). The test result is expressed as relative index avidity (RIA). According to the kit manual, the values less than 40% were considered as negative while the value more than 60% were considered positive and the borderline ranged between 40-60% (18).

Statistical analysis

Statistical analysis was done using the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). The Logistic regression and chi-square tests were used to evaluate the association between the *T. gondii* seropositivity and potential risk factors. Differences were considered significant when the $P<0.05$.

Ethical statement

This study was approved by The Ethics Committee of Lorestan University of Medical Sciences (No. 200.93.11707). The written informed consent was obtained from all the participants before sampling.

Results

Serology status and demographic information

The results of the *Toxoplasma* serology status of participants in the study are shown in Table 1. The mean age was 27.01 ± 6.459 in the control group and 27 ± 6.499 in the case group. The mean of parity and gravidity of the control group, because of a lack of abortion history, were the same and it was 1.71 ± 0.86. The mean of parity and gravidity of the case group were 0.88 ± 0.99 and 1.88 ± 0.99, respectively. Our results, no significant association was seen between the maternal age and abortion (P=0.989). The results showed that the seropositivity rate for Toxoplasma IgM in the samples of the case group was 3.3% (8/240), while in the control group it was only 0.4% (1/240) of the samples, leading to a statistically significant difference between the two groups (P=0.019). The positive anti-*T.* gondii IgM antibodies had an odds ratio of 10.266, suggesting that the risk of abortion among women with positive IgM was about ten times higher than the other cases (P=0.019). Also, 47.5% (114/240) of the case group and 46.3% (111/240) of the control group were positive for anti-*T.* gondii IgG antibodies, but there was no statistically significant difference between two groups (P=0.784).

Additionally, there was no significant difference between the case and control groups in terms of the prevalence of abortion in relation to education level (P=0.645) or the place of residence (city versus rural areas) (P=0.404). Out of all participants, 75.8% (182/240) of the case group and 72.5% (58/240) of the control group were living in the city. The results also showed that most of the women who had an abortion (67.1%) were housewives, and most of the women in the control group (61.7%) were employees, indicating that there is a significant difference in the relationship between occupation status and abortion rate (P<0.001). Also, 15% (36/240) of the women in the case group and 13.8% (33/240) of the control group kept a cat at home, but there was no significant difference between the two groups with regards to living near a cat (P=0.39).

Table 1: Compare of seroprevalence of toxoplasmosis between women with first spontaneous abortion and control group

Variable	Case group n=240	Control group n=240	P value
Age (Y)	27 ± 6.499	27.01 ± 6.459	0.989
Level of education			0.645
Low literate	73 (30.4)	69 (28.8)	
Diploma	109 (45.4)	104 (43.3)	
Academic degree	58 (242)	67 (27.9)	
Occupation			<0.001
Employee	168 (70)	79 (32.9)	
Housewife	72 (30)	161 (67.1)	
Residence in the city			0.404
Urban	182 (75.8)	174 (72.5)	
Rural	58 (24.2)	66 (27.5)	
Contact with cats			0.696
Yes	36 (15)	33 (13.8)	
No	204 (85)	207 (86.2)	
Seropositivity rate for *Toxoplasma* IgM			0.019
Yes	8 (3.3)	1 (0.4)	
No	232 (96.7)	239 (99.6)	
Seropositivity rate for *Toxoplasma* IgG			0.784
Yes	114 (47.5)	111 (46.3)	
No	126 (52.5)	129 (53.7)	

Data are presented as mean ± SD or n (%).

Avidity ELISA

All samples, which were positive in terms of anti-Toxoplasma IgM in both groups (9 samples) and IgG in the case group (114 samples), were evaluated by IgG avidity. Seven out of 8 (87.5%) sera, which were related to the case group, had low avidity indicating acute infection, whereas all positive IgG sera (100%) and 1 positive IgM sample, which was related to the control group had high avidity indicating chronic infection.

Discussion

Maternal acute toxoplasmosis or congenital toxoplasmosis during pregnancy is one of the important factors that increase the chance of abortion. It was previously believed that the congenital toxoplasmosis is due to an initial infection that occurs during pregnancy (13), but not to the reactivation of a latent infection in pregnant women with an immune deficiency (19). In addition, some believe that latent toxoplasmosis can be reactivated to cause the congenital transmission of parasites to their fetus (20). Serological evidence suggests a high prevalence of toxoplasmosis worldwide (21), and in fact, based on several studies Iran is one of the countries with a considerable prevalence (9, 13, 22).

In this survey, we found that 8 out of 240 cases had the *T. gondii*-specific IgM antibodies, while in the control group there was only 1 woman with a positive result for anti-*T. gondii* IgM antibody. This observation may indicate a significant relationship between spontaneous abortion and acute toxoplasmosis. Also, our results showed that 47.5% of the case and 46.3% of the control group were positive in terms of anti-Toxoplasma IgG antibodies. There was no statistically significant difference between the two groups for *Toxoplasma*-specific IgG antibody, which is consistent with the results of a number of studies, yet, inconsistent with a few others. In a research project that was conducted in Bandar Abbas, Southern Iran, 124 women with an abortion history were studied for the frequency of anti-Toxoplasma IgG and IgM antibodies. The results showed that 79.03% and 15.32% of those women were positive for anti-*Toxoplasma* IgG and IgM antibodies, respectively (12). Also, a meta-analysis study on the relationship between toxoplasmosis and its outcomes showed that the infection rate in the abnormal-pregnancy group was significantly higher than the normal-pregnancy group (23).

With regards to other issues that may play as risk factors for abortion, our results showed that there was no significant relationship between the rate of abortion and urban or rural residence. Based on the results of this study with those of the present research, it can be concluded that health education and training classes for villagers has been effective in increasing the level of knowledge and personal care. Although there was no significant relationship between the level of education and prevalence of abortion, a significant correlation was observed between having a job and prevalence of abortion, since in the case group, 67.1% of the IgG-positive cases were housewives, and in the control group, 61.7% of the women were employees. The reason can be referred to as the lifestyle, which can affect the level of information. Furthermore, according to our results, there was no relationship between keeping a cat at home and the rate of abortion (24). In a study conducted in Egypt, the results showed that the seroprevalence of toxoplasmosis in high-risk pregnancy group was significantly more than a normal pregnancy group. Also, not consistent with our findings, in their study there was a significant difference between seropositivity and both living in a rural area, and undercooked meat consumption (25).

Due to the wide range of clinical signs of toxoplasmosis and the chance of getting confused with other diseases, it is necessary to use laboratory methods to confirm the clinical diagnosis. The serological assays have different sensitivities and specificities and are based on the affinity and avidity of antibodies. Detection of specific IgG antibodies is rarely a problem and has good sensitivity and specificity in different methods (26). In contrast, isolation and detection of IgM antibodies due to the less specificity of the methods used, long-term half-life or false IgM antibody resulted from other infections, leads to false-positive results, unnecessary treatments and even a false decision to terminate the pregnancy (27). Some IgM kits have lower reliability and credibility, which leads to an unacceptable increase in false-positive test results (28). For this purpose, in 1997, the Food and Drug Administration (FDA) advised physicians in the USA to clarify these limitations and advised laboratory staff and physicians to make sure of the quality of the kits prior to making decisions about clinical management of patients (29). For this purpose, the FDA has recommended that the positive IgMs should be approved by IgG avidity test, which is provided to discriminate between the old and new *Toxoplasma* infections (30), that is highly important in pregnant women and people with immune deficiencies (31). This method, primarily developed by Hedman et al. (32) in Finland, is now available as a kit throughout the world. The binding strength of IgG to *T. gondii* antigen shifts from low avidity to high within 5 months. This is the means by which it is possible to differentiate a recent infection from an old one in the first trimester of pregnancy in women with IgM or IgG (33).

In this study, IgG avidity test was used to evaluate IgM- and IgG-positive cases. The result showed that 8 cases of IgM-positive in the case group had low avidity, which indicated an acute phase of infection. On the other hand, 1 case of IgM-positive in the control group had high avidity, indicating a chronic phase of infection. Disagreement in the results of different studies may be due to the patients' varying health status, differences in consumption of raw or undercooked meat, and keeping cats as pets at home (34). In the current study, eating roast meat (locally called Kebab) was very common, as it is a traditional food with a high rate of consumption. As a result, this eating habit is one of the important risk factors for *Toxoplasma* infec-

tion. Consuming well-cooked Kebab could be considered as a way of reducing the risk factor and preventing *T. gondii* infection in our population. Interestingly, the eating habits of the people as well as the climate of this region has led to several research studies on the prevalence and treatment of other parasitic infections in Lorestan province (35-40).

Healthcare providers in women's hospitals should know that in the case of a pregnant woman the avidity test is not a validated and final test to be used solely for decision making, and that an equivocal IgG avidity result should not be considered in the diagnosis process.

Conclusion

According to the findings of this study, it is suggested that the necessary health information, especially on the *Toxoplasma* transmission routes to women before marriage, particularly for the seronegative women, be provided and easily available. Additionally, indicating the sensitivity of a woman to acute toxoplasmosis, as well as the serological assessment of toxoplasmosis, before and during pregnancy, is recommended. Although sometimes these assays do not lead to a definitive interpretation, more sensitive methods such as amniotic fluid studies using molecular techniques are also needed to decide on treatment or termination of a pregnancy. In addition, the PCR and anti-*T. gondii* IgG avidity assays should be performed in medical diagnostic laboratories for accurate identification of the initial infection of toxoplasmosis in pregnant women.

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Authors' Contributions

F.K., Sh.F., S.A.; Designed and supervised this study. M.J.T., B.E.; Developed an outline for the study and supervised the analysis process of the samples. F.K., A.K.R., P.H.; Wrote the original manuscript and Sh.F. revised it. S.J.S.T., M.J.T., B.E., A.K.R., P.H.; Contributed to data analysis and prepared the manuscript. All authors reviewed and approved the final version of the manuscript.

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Original Article

Effects of Body Mass Index and Biochemical Lipid Levels on Reproductive Outcomes during An Intracytoplasmic Sperm Injection: A Retrospective Study

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Abstract

Background: The aim of this study was to evaluate the impact of body mass index (BMI) and lipid profile on reproductive outcomes of women undergoing intracytoplasmic sperm injection (ICSI) cycles.

Materials and Methods: This retrospective observational study was conducted in the Center of Human Reproductive Physiopathology of University of Catania between April 2017 and March 2018 and enrolled 114 couples undergoing ICSI. Levels of total cholesterol, low-density lipoprotein-cholesterol (LDL-c), high-density lipoprotein-cholesterol (HDL-c) and triglycerides were determinate and, according to the BMI, samples were divided into the following groups: group A (BMI: 18.5-24.9 kg/m^2); group B (BMI: 25-29.9 kg/m^2); and group C (BMI >30 kg/m^2). BMI and lipid profile associations with the number of oocytes and embryos retrieved, the oocytes and embryo quality, the fertilization rate as well as the percentage of miscarriages and pregnancies, were assessed. The statistical analysis was performed using Shapiro-Wilk test, analysis of variance (ANOVA) and Kruskal -Wallis method.

Results: Fertilization and pregnancy rates were lower in women with BMI>30 than in women with BMI: 25-29.9 and BMI: 18.5-24.9, despite the not altered levels of lipoprotein.

Conclusion: Our results demonstrated that an excess of adipose tissue in women undergoing ICSI was not directly related with altered biochemical lipid values. However, overweight and obese patients showed poor fertilization and pregnancy rate despite the not altered values of lipoprotein.

Keywords: Body Mass Index, Infertility, *In vitro* Fertilization, Metabolic Diseases

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Introduction

Variations in lipid asset, body mass index (BMI) and abdominal adipose tissue affect the female reproductive system compromising ovulatory cycle, oocytes and embryo quality. Obesity is associated with hyperinsulinemia and insulin resistance causing an increased hormonal ovarian production and a reduced synthesis of sex hormone-binding globulin (SHBG), with subsequent hyperandrogenism. A peripheral conversion of ovarian and adrenal androgens determinates abnormal secretions of gonadotropin-releasing hormone (GnRH), a reduced peak of luteinizing hormone (LH) and metabolites of progesterone (1). These factors are in favour of development of anovulatory cycles, oligomenorrhea/amenorrhea, atypical follicular recruiting, poor oocytes quality, endometrial development failure and impaired corpus luteum function (2). The excess of abdominal adipose tissue induces the release of non-esterified acids causing a lipid increase in muscle and liver followed by dyslipidaemia (3). Dyslipidaemia is characterized by elevated plasma levels of triglycerides and low-density lipoprotein-cholesterol (LDL-c), small catabolism of apolipoprotein B (Apo-B) and increased degradation of high-density lipoprotein (HDL)-apoa-I (4). It was shown that obesity and its complications have negative consequences for oocyte quality, fertilization rate, embryo development and pregnancy rate (5).

Despite this fact, recent studies, did not report differences in oocyte maturity in function of BMI variation (6); the same cannot be said about the fertilization rate that seems to reduce proportionally with the increasing BMI; in particular, a 45% reduction in fertilization rate was identified in women with BMI>30 (7, 8). The effects of female obesity on embryo quality were also investigated

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during the last 10 years. Results indicated that embryo quality influences the number of discarded embryos, the number of frozen embryos and the rate of embryo used with a mean grade of embryos significantly lower in obese patients (9). Finally, it was shown that pregnancy and live birth rate drastically decrease when BMI increases; on the other hand, the pregnancy rate was not compromised in obese women receiving high quality heterologous oocytes suggesting that high BMI acted on multiple levels of reproductive process, including oocytes and embryos (10). Finally, no adverse effect of BMI on implantation and pregnancy rate was found in obese women receiving donor oocytes, supporting the hypothesis that BMI does not influence endometrium receptivity while oocytes and embryos of poor quality can produce poor reproductive results.

The aim of our study was to evaluate possible correlations between variations in BMI, triglycerides, HDL-c, LDL-c, and total cholesterol levels and ovarian response (in terms of oocytes and embryos retrieved, oocytes and embryo quality, fertilization rate and percentage of miscarriages and pregnancies) in women undergoing intracytoplasmic sperm injection (ICSI) cycles.

Materials and Methods

We conducted a retrospective observational study on 114 couples undergoing ICSI who referred to our Human Reproductive Physiopathology Centre between April 2017 and March 2018. Each couple, before entering the study, subscribed an informed consent, and anonymity was preserved. The study protocol conformed to the ethical guidelines of the Helsinki Declaration (as revised in Tokyo 2004) and was approved by the Local Research Ethics Committee. The enrolled couples underwent ICSI for idiopathic causes after a history of primary or secondary infertility from almost one year, and met the following criteria.

Inclusion criteria: i. Female age between 18-42 years, ii. Primary or secondary infertility for not less than 12 months, iii. ICSI techniques, iv. Negative results for Hepatitis B Australia Antigen (HbsAg), Hepatitis C Virus (HCV), Human Immunodeficiency Virus (HIV) for the male and the female, v. No exposure to toxic agents reported by the couples, vi. Female BMI<35, vii. Normal semen parameters, and viii. Follicle stimulating hormone (FSH) <15 IU/mL.

Exclusion criteria: i. Female age <18 or >42 years, ii. Primary or secondary infertility for less than 12 months, iii. Assisted reproductive techniques (ART) different from ICSI, iv. Positive results for HbsAg, HCV, HIV for the male and the female, v. Exposure to toxic agents reported by the couples, vii. Female BMI >35, vii. severe oligozoospermia, severe asthenozoospermia, and/or azoospermia, viii. FSH >15 IU/mL.

Following obtaining the signed informed consent, FSH, LH, estrogen (E2) and prolactin (PRL) blood test were done on the 3rd day of menstrual cycle. PRL samples were collected three to four hours after waking up in the morning by taking three samples with 20 minutes intervals after correcting the position of the vein catheter. Moreover, blood tests were performed in order to determinate plasma concentrations of total cholesterol, LDL-c, HDL-c and triglycerides. The blood samples were obtained before starting ovarian stimulation by using separated lipoproteins, by electrophoresis or precipitation method. Finally, weight and height of the patients were recorded. According to the BMI, the samples were divided into the following groups: group A: patients with BMI of 18.5-24.9 kg/m^2 (45.6%); group B: patients with BMI of 25-29.9 kg/m^2 (32.4%); and group C: patients with BMI greater than 30 kg/m^2 (22%). Woman who participated in the present study, had been treated with gonadotropin-releasing hormone (GnRH) agonist long protocol using leuprorelin acetate or triptorelin acetate and recombinant FSH was injected 14 days later. E2 level dosage and ultrasound control were performed three times a week after starting recombinant FSH therapy to monitor the follicular growth. When the E2 level and the follicular dimensions were adequate, human chorionic gonadotropin (hCG) triggering was performed using chorionic gonadotropin. All the pick-ups were performed 36-38 hours after the hCG triggering. Retrieved oocytes were inseminated by ICSI and thus, the zygotes were observed at their maturation by biologist. Embryos were transferred 48-72 hours after the pick-ups. Variable considered in our study were as follows: number of retrieved oocytes and obtained embryos as well as oocytes' and embryo quality using respectively "Pronuclear Scoring System" and "Day 3 Scoring".

The pronuclear scoring system was used with the aim of detecting the pronuclear stage. The evaluation began about 16-20 hours after ICSI. Oocytes were examined on the heated stage of an inverted microscope equipped with Hoffman modulation contrast (200 magnification). Normally fertilized oocytes presented two clearly distinct pronuclei (2PN) and two polar bodies. This pattern correlated with increased embryo competence. The presence of one pronucleus (1PN) might be a result of errors in the fertilization process due to the asynchrony in formation/fusion of pronucleus. The formation of three different pronuclei might be due to an altered fertilization process determining a triploid zygote. The "Day 3 Scoring" system evaluates the morphological appearance of embryo on day 3. Embryos were assessed using a scoring system graded 1 to 5 according to morphology, which took into account cell number, evenness of cell division and degree of fragmentation. In the following lines, the characteristics of each grade are explained. Grade 1: 8 cells, <10% fragmentation, good cell-cell contact, absence of multinucleated blastomers, grade 2: 8 cells, 10-20% fragmentation or lacking good cell-cell contact, absence of multinucleated blastomers, grade 3: 6-7 cells or 8 cells with 20% fragmentation or uneven blastomer size, absence of multinucleated blastomers, grade 4: >8 cells

or 4-6 cells or 8 cells with >20% fragmentation or uneven blastomer size or multinucleated blastomers, grade 5: <4 cells or grossly fragmented or with half of the blastomers being multinucleated.

Moreover, our study evaluated also the fertilization and rate and percentage of miscarriages as well as clinical pregnancies detected first by β-hCG blood test after 14 days from embryo transfer and then by transvaginal ultrasound visualization.

Statistical analysis

The primary endpoint was the establishment of a correlation between variations of BMI, triglycerides, HDL-c, LDL-c, and total cholesterol and ovarian response (in terms of oocytes and embryos retrieved, oocytes and embryo quality, fertilization rate and percentage of miscarriages and pregnancies) in ICSI cycles. The Shapiro-Wilk test was implemented to test the normally distribution of the variables. The level of significance of normality test for all variables was alfa=0.05. Analysis of variance (ANOVA) was applied to compare normally distributed variables. Variables with free distributions were analyzed using Kruskal-Wallis method. Normally distributed continuous data were presented as mean ± SD, while categorical data were presented as number (n) and percentage (%). Non-normally distributed data were presented as median (range).

Results

We demonstrated that normal weight patients (group A) and overweight patients (group B) had mean HDL-c levels, with parameters included within the normal range. Significantly high values were found for obese patients (group C) with a mean value of 45.3 mg/dl (SD: 21.68, P=0.0032) for HDL-c which was close to the inferior normal range and the mean value for triglycerides was 101.5 mg/dl (SD: 23.7, P=0.00001, Table 1). All the patients were treated with a long stimulation protocol. Also, we found a reduction in the mean value of retrieved oocytes (mean ± SD: 3.0 ± 3.16, P=0.00001) as well as fertilized oocytes (mean ± SD: 1.25 ± 1.50, P=0.00001) in group C respect in group A and B (Table 2). In group A, 106 embryos were obtained from a total of 122 fertilized oocytes (86.8%), with a mean value of 1.81 ± 1.15 (mean ± SD, P=0.25). In group B, 71 embryos were obtained from a total of 90 fertilized oocytes (78.8%), with a mean value of 1.94 ± 1.06 (mean ± SD, P=0.71). In group C, 18 embryos were obtained from a total of 25 fertilized oocytes (73%), with a mean value of 1.00 ± 1.15 (mean ± SD, P=0.0036, Table 2). The fertilization rate was 70.52% [confidence interval (CI): 32-82% for 122 fertilized oocytes] in group A, 70.9% (CI: 30-84.2% for 90 fertilized oocytes) in group B and 62.5% (CI: 24.3-73.2% for 25 fertilized oocytes) in group C, with similar value between normal-weight and overweight patients while it was slightly reduced in obese women. Data concerning oocyte maturation and zygotes pronuclear number for each group are reported in Table 3. Data referred to embryo grade of maturation descripted by Day 3 Scoring System are reported in Table 4. Fourteen days after embryo-transfer, blood levels of β-hCG were measured. Blood β-hCG was negative for 93 patients (80.9%, CI: 35-94.4%) and positive in 22 patients (19.1%, CI: 9.3-37.8%). Of these 22 patients, 17 were from group A, 3 were from group B, and the remaining 2 were from group C. We observed 3 biochemical miscarriages in group A, 6 clinical miscarriages equally distributed among group A, B and C, 12 pregnancies in group A and 1 pregnancy in group B and 0 pregnancy in group C (Table 5).

Table 1: Levels of blood lipids

Variables (mg/dl)	Group A		Group B		Group C	
	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
Total cholesterol	183 ± 33.43	0.23	178 ± 23.89	0.12	165 ± 21.79	0.071
LDL	110 ± 31.14	0.14	101 ± 25.71	0.13	101 ± 23.89	0.16
HDL	64.8 ± 17.49	0.16	60.4 ± 10.34	0.13	45.3 ± 21.68	0.0032
Triglycerides	68.1 ± 19.30	0.08	62.9 ± 21.08	0.24	101.5 ± 23.7	0.00001

P<0.05 were considered significant. LDL; Low-density lipoprotein and HDL; High-density lipoprotein.

Table 2: Number of retrived oocytes, fertilized oocytes and embryo obtained from each group

Variables	Group A		Group B		Group C	
	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
Retrived oocytes	7.88 ± 5.99	0.32	6.25 ± 4.15	0.13	3.0 ± 3.16	0.00001
Fertilized oocytes	2.97 ± 1.36	0.22	2.19 ± 1.32	0.003	1.25 ± 1.50	0.00001
Total embryos	1.81 ± 1.15	0.25	1.94 ± 1.06	0.71	1.00 ± 1.15	0.0036

P value are significant if <0.05.

Table 3: Number and percentage of oocytes in stage of maturation and zygotes pronuclear number

Oocytes/Zigotes	Median	Group A (%)	Group B (%)	Group C (%)
M1	18 (20-3)	11.5 (20/173)	14 (18/127)	7.5 (3/40)
M2	95 (136-34)	78.6 (136/173)	75 (95/127)	85 (34/40)
GV	12 (13-3)	7 (12/173)	10 (13/127)	7.5 (3/40)
DEG	1 (5-0)	2.9 (5/173)	1 (1/127)	0 (0/40)
PN1	5 (7-0)	4.1 (5/122)	7.7 (7/90)	0 (0/25)
PN2	83 (20-117)	95.9 (117/122)	92.3 (83/90)	80 (20/25)
PN3	0 (5-0)	0 (0/122)	0 (0/90)	25 (5/25)

Median (range) is used to indicate the middle value of zygotes in a determinate stage of maturation and with a determinate pronuclear number. Percentage is used to indicate the number of zygotes in a determinate stage of maturation and the percentage of zygote with a determinate pronuclear number in each group. M1; Immature retrieved oocytes in metaphase I, M2; Mature retrieved oocytes in metaphase II, GV; Germinal vesicular, DEG; Degenerated oocytes, and PN1-PN2-PN3; Zygotes with 1 pronucleus, 2 pronuclei or 3 pronuclei.

Table 4: Number and percentage of embryo descripted by day 3 scoring system

Embryos	Median	Group A (%)	Group B (%)	Group C (%)
G1	16 (42-0)	39.6 (42/106)	22.5 (16/71)	0 (0/18)
G2	25 (46-6)	43.4 (46/106)	35.0 (25/71)	33.3 (6/18)
G3	14 (16-5)	15.1 (16/106)	20.0 (14/71)	27.7 (5/18)
G4	2 (5-0)	1.9 (2/106)	7.0 (5/71)	0 (0/18)
G5	0 (2-0)	0 (0/106)	2.8 (2/71)	0 (0/18)
DEG	7 (9-0)	0 (0/106)	12.7 (9/71)	40 (7/18)

Median (range) is used to indicate the middle value of embryo in a determinate stage of maturation percentage is used to indicate the stage of maturation of embryo catalogued by day 3 scoring system in each group. G1; Grade 1 embryo according day 3 scoring system, G2; Grade 2 embryo according day 3 scoring system, G3; Grade 3 embryo according day 3 scoring system, G4; Grade 4 embryo according day 3 scoring system, G5; Grade 5 embryo according day 3 scoring system, and DEG; Degenerated embryo.

Table 5: Number and percentage of positive βhCG, biochemical miscarriages, clinical miscarriages and clinical pregnancies

Embryos	Median	Group A (%)	Group B (%)	Group C (%)
Positive βhCG	3 (17-2)	16 (17/106)	4.2 (3/71)	11.1 (2/18)
Biochemical miscarriages	0 (3-0)	1.8 (3/106)	0 (0/71)	0 (0/18)
Clinical miscarriages	2 (2-2)	1.8 (2/106)	2.8 (2/71)	11.1 (2/18)
Clinical pregnancies	1 (12-0)	1.9 (12/106)	1.4 (1/71)	0 (0/18)

βhCG; Beta human chorionic gonadotropin. Median (range) is used to indicate the middle value of positive βhCG, biochemical miscarriages, clinical miscarriages and clinical pregnancies. Percentage is used to indicate the percentage of positive βhCG, biochemical miscarriages, clinical miscarriages and clinical pregnancies for each group.

Discussion

The present study aimed to investigate the relationship between obesity and lipid variations and to determinate their association with ovarian response in terms of oocyte maturation, fertilization rate, embryo quality and pregnancy rate in women undergoing ICSI. Our literature survey demonstrated that obesity is strongly associated with metabolic syndrome (11), polycystic ovary syndrome (PCOS) (12) and dyslipidaemia (13). These conditions are characterized by hyperinsulinemia (14) and insulin resistance (13). High BMI causes ovulatory dysfunctions, anovulatory cycles, infertility (15) and hyperandrogenism by the reduction of SHBG as well as by hyperinsulinemia (16). Obesity and its complications were shown in both animal and human studies, to have negative consequences in oocyte quality, fertilization rate, embryo development and pregnancy rate (5). With respect to oocyte quality and maturation, our study showed a similar percentage of M2 oocytes in obese patients (85%) compared to overweight (75%) and normal-weight women (78.6%). This result is in accordance with the study of Shalom-Paz et al. (6) that did not report any BMI influence on *in vitro* maturation of oocytes in PCOS women. Despite the normal values of cholesterol in our samples, we found a lower fertilization rate in woman with BMI >30 compared to the other two groups, since the metabolic disorders related to obesity induced ovarian resistance towards the action of high-dose gonadotropins (17). The same result was obtained in the study of van Swieten et al. (7) that identified a 45% reduction in fertilization rate in women with BMI>30. On the other hand, Salha et al. (8) found that fertilization rates were 26.6% in patients with BMI ≥26 and 37.1% in patients within normal BMI range.

Considering the effect of obesity on embryo quality, Metwally et al. (9) after assessing the embryo quality on the 2nd day following the oocyte pick-ups, investigated the shape of blastomeres, the cytoplasm structure and the degree of fragmentation, and they demonstrated that the mean grade of embryo quality is significantly lower in obese patients. Our data confirmed that obesity alters

the quality and the number of embryos obtained with a mean value of 2 embryos in normal weight patients with normal lipid profile while averagely 1 embryo was obtained from patients with BMI>30 and plasma lipids concentrations near limits. We found that the percentage of embryos with morphological characteristics for pregnancy, decreased inversely proportional to BMI while the percentage of embryos with morphological anomalies increased proportional to BMI.

Finally, results of our study reported a rate of 19.1% for β-hCG positivity, with greater success among normal-weight, compared to overweight and obese patients. The pregnancy rate was 85.7% (12/14 pregnancies) in group A, and 14.3% (2/14 pregnancies) in group B, but no conception in group C. Consistent with our findings, as far as the pregnancy and live birth rate is concerned, Luke et al. (10) reported that pregnancy and live birth rate drastically decreased when BMI increased, while it seems likely that pregnancy rate was not influenced by obesity when women received high-quality heterologous oocytes. The correlation between obesity and pregnancy rate in ART was demonstrated in 3 large studies which revealed a reduced probability of pregnancy which was directly proportional to high BMI (18-20). Moreover, obesity causes a reduction of fertility in women undergoing ART cycles and in those who conceive spontaneously (21) demonstrating that high BMI adversely affects oocytes and embryo quality as reflected by a reduction of pregnancy rates (22).

Conclusion

Our study demonstrated that an excess of adipose tissue in women undergoing ICSI was not directly related with altered values of lipoprotein taken in consideration in our study. Overweight and obese patients (BMI: 25-34) showed poor fertilization and pregnancy rates despite the not altered levels of lipoprotein. Strengths of our work were the accurate collection of data on oocyte and embryo quality, as well as the careful processing of collected values. However, as our study was conducted in a small population, further research should be done to better understand the pathogenic mechanisms underlying poor reproductive outcomes in obese and overweight women. Finally, we believe that young women of reproductive age should be appropriately advised about the negative effects of obesity and insulin resistance on fertility, in order to perform some lifestyle modification.

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Authors' Contributions

L.M.D.G., E.Z., F.A.G.F., F.D.G.; Contributed to the design, implementation of the research and wrote the first draft of manuscript. L.M.D.G., E.Z., G.M., S.C.; Data collection and analysis manuscript. The revision process was entirely done by F.D.G. who improved the statistical analysis and the structure of the manuscript. All authors discussed the results and contributed to the final manuscript with the specific support of R.A. and M.P. that revised the final draft. They improved english, controlled the results and gave the approval for the final version.

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Original Article

Effects of Co-Administration of Bone Marrow Stromal Cells and L-Carnitine on The Recovery of Damaged Ovaries by Performing Chemotherapy Model in Rat

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Abstract

Background: L-carnitine (Lc) as a type of flavonoid antioxidants and bone marrow stromal cells (BMSCs) as a type of mesenchymal stem cells may recover damaged ovaries. It seems that Lc has favorable effects on differentiation, increasing lifespan and decreasing apoptosis in BMSCs. The aim of this study was to investigate effects of co-administration of BMSC+Lc on damaged ovaries after creating a chemotherapy model with cyclophosphamide in rats.

Materials and Methods: In this experimental study, cyclophosphamide was intraperitoneally (IP) injected to forty female wistar rats for 14 days, in terms of chemotherapy-induced ovarian destruction. The rats were then randomly divided into four groups: control, Lc, BMSCs and co-administration of BMSC+Lc. Injection of BMSCs into bilateral ovaries and intraperitoneal injection of Lc were performed individually and together. Four weeks later, levels of serum estradiol (E2) and follicle-stimulating hormone (FSH) using enzyme-linked immunosorbent assay (ELISA) kit, number of ovarian follicles at different stages using hematoxylin and eosin (H&E) staining and expression of ovarian Bcl-2 and Bax proteins using western blot were assessed.

Results: Co-administration of BMSC+Lc increased E2 and decreased FSH levels compared to the control group ($P<0.001$). The number of follicles was higher in the co-administrated group compared to the control group ($P<0.001$). Co-administration of BMSC+Lc increased Bcl-2 protein level, decreased Bax protein level and increased Bcl-2/Bax ratio ($P<0.001$).

Conclusion: The effect of co-administration of BMSC+Lc is probably more effective than the effect of their separate administration on the recovery of damaged ovaries by chemotherapy.

Keywords: Bone Marrow Stromal Cells, Carnitine, Chemotherapy, Ovary, Regeneration

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Introduction

Despite the great benefits of chemotherapy in treating cancer patients, it has some side effects on ovaries (1). Cytotoxic effects of chemotherapy damage the granulosa cells (GCs), so that folliculogenesis disruption may occur (2). Unfortunately, this issue is disappointing for girls and young women who receive chemotherapy. Cyclophosphamide is one of the most administrated chemotherapy drugs which directly affects ovaries (3). There are several methods to treat ovarian damage, including hormone therapy, freezing ovaries, stem cell therapy and applying antioxidants (4). Hormone therapy is not suitable for cancer patients, because it may increase the probability of the cancer recurrence (5). As disadvantages of ovarian cryopreservation, it requires surgical procedures for tissue harvesting and transferring, while probability of returning its function is low (6). Recently, it has been observed that transplantation of bone marrow stromal cells (BMSCs), a type of mesenchymal stem cells, may treat ovarian damage after chemotherapy (7, 8). BMSCs can produce some growth factors, differentiate into other cell lines and replace damaged cells (9, 10). On the other hand, it has been shown that some antioxidants such as L-carnitine (Lc) have beneficial effects on damaged ovaries (11). Lc is a flavonoid antioxidant that plays an essential role in fatty acid metabolism and is present in human serum and tissues (12, 13). However, the effect of Lc has not been assessed on damaged ovaries by chemotherapy.

Several reports have shown that Lc has favorable effects on mesenchymal stem cells, including suppression of apoptosis in BMSCs (14), modulating differentiation of adult mesenchymal stem cells (15) and improvement of the aged adipose tissue-derived human mesenchymal stem cells lifespan (16).

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Although the effects of individual BMSCs and Lc on the repair of damaged ovaries have been investigated, there is no report yet concerning the effect of simultaneous administration of them on the recovery of damaged ovaries. So, in this study, due to the beneficial effects of Lc on BMSCs, we evaluated for the first time the effect of co-administration of BMSC+Lc on ovarian function and structure after creating a chemotherapy model with cyclophosphamide in rats.

Materials and Methods

Animals

In this experimental study, forty female wistar rats (180-200 g) were used. They had free access to food and water under controlled temperature (25 ± 2˚C). Vaginal smear was daily obtained and only those showing at least two consecutive normal vaginal estrus cycles were used in the experiments. All procedures were approved by the Research Council of Semnan University of Medical Sciences (Semnan, Iran). The Ethical Code is IR.SEMUMS.REC.

Bone marrow stromal cell culture and characterization

After sacrificing an adult rat, femurs and tibias were dissected out. Bone marrow was ejected with 10 ml of Dulbecco's Modified Eagle Medium (DMEM) and cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Gibco, Germany), incubated at 37˚C, 95% humidity and 5% CO_2. After 48 hours, non-adherent cells were removed by replacing the medium. The cells were sub-cultured four times (17, 18).

To analyze expression of the stem cell surface markers, at least 100,000 cells were incubated with fluorescence-labeled monoclonal antibodies against CD29, CD34, CD44, CD45 and CD90 (Sigma, China). Following a 10 minutes wash in phosphate-buffered saline (PBS, Sigma, USA), the labeled cells were analyzed using a Becton Dikinson FACS Calibur Flow Cytometer (BD, USA) (7).

Creating the chemotherapy model

To destroy the ovaries, a model of chemotherapy was created. Cyclophosphamide (Sigma, China) diluted in normal saline was intraperitoneally (IP) injected at 50 mg/kg at the first day, followed by 13 days injection of 8 mg/kg daily cyclophosphamide (19).

The injection procedure in the groups

After creating the chemotherapy model, the rats were randomly divided into four groups (n=10 in each group): i. Control group, 25 µl of culture medium was directly injected into the bilateral ovaries, ii. BMSC group, 2×10^6 BMSCs suspended in 25 µl culture medium were directly injected into the bilateral ovaries (20), iii. Lc group, 200 mg/kg of Lc was injected IP, one day before beginning chemotherapy, until 7 days after chemotherapy (11), and iv. BMSC+Lc co-administrated group, combined BMSCs and Lc was injected.

Bone marrow stromal cell tracking in the ovaries

To track the transplanted BMSCs after four weeks in the ovaries, the cells were labeled with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate) (Sigma, China). Briefly, BMSCs were suspended in DMEM and 5 µl/ml DiI was added. After incubation for 20 minutes, the cells were centrifuged and washed with PBS, and then suspended again for transplantation. Four weeks after transplantation, prepared paraffin sections and the labeled cells were detected by fluorescence microscope (Motic, Spain) (21).

Hormonal evaluation

Four weeks after the end of chemotherapy, serum estradiol (E2) and follicle-stimulating hormone (FSH) levels of these groups were measured by enzyme-linked immunosorbent assay (ELISA) kits (East Bio-Pharm, China) for rat, according to the manufacturer's instruction (22).

Histological evaluation of the ovaries

Four weeks after the end of chemotherapy, the ovaries were collected and fixed in 4% paraformaldehyde, dehydrated, paraffin-embedded and serially sectioned at 5 µm thickness. Five representative sections from each ovary were randomly chosen and routine hematoxylin and eosin (H&E) staining was performed for histological examination with light microscopy. the number of primordial, primary, secondary and antral follicles were measured (1).

Western blot assays

Five ovaries in each group were lysed using RIPA buffer (Cell Signaling Technology, Netherlands) supplemented with protease inhibitor (Roche, Switzerland) on ice for 30 minutes. Then, the mixture was centrifuged at 13000 rpm for 20 minutes at 4°C. Equal value of proteins (80 µg) were loaded on sodium dodecyl sulfate (SDS, Sigma, Japan) polyacrylamide gel (Merck, Germany) and separated in a size manner by electrophoresis. The proteins were transferred to nitrocellulose membranes (Amersham Biosciences, USA). The membranes were blocked with 5% skim milk in tris buffered saline (TBS, pH=7.4). The membranes were incubated with primary antibodies for Bcl-2 (1:1000), Bax (1:1000) and β-Actin (1:1000, Abcam, USA) overnight at 4°C. After washing, the membranes were incubated with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP). All antibodies were diluted according to manufacturer's instructions. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, USA). X-ray films were scanned, and then the relative protein levels were semi-quantified by densitometric analysis using image j software. β-actin was tested as the internal control (23).

Statistical analyses

After verifying the normality of variance assumptions, data were analyzed by one-way analysis of variance

(ANOVA) followed by the Tukey Test. Obtained data are presented as the mean ± SE, and a level of P<0.05 was considered statistically significant.

Results

Cultivation and characterization of bone marrow stromal cells

BMSCs were cultured in the T25 flasks. After a few days, the cells appeared to be spindle-shaped. By repeating passages, the cells became morphologically homogeneous. Most of the cells expressed the mesenchymal stromal cell markers (CD29, CD44 and CD90) and did not express the hematopoietic cell markers: CD34 and CD45 (Fig.1).

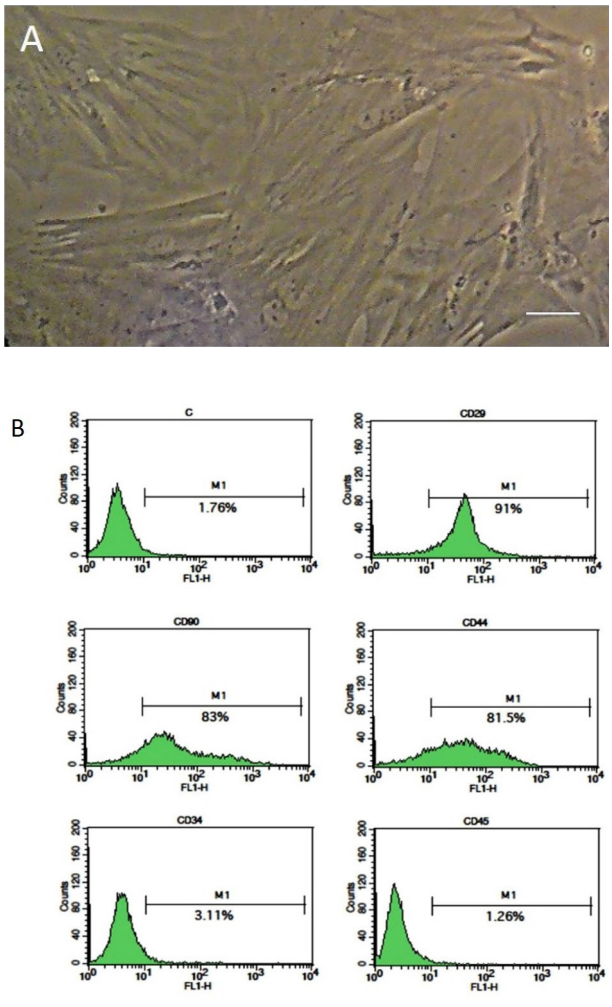


Fig.1: Isolation and identification of bone marrow stromal cells (BMSCs). **A.** Cultured BMSCs at passages 4 and **B.** The results of flow cytometry show that BMSCs are positive for CD29, CD44 and CD90, while it is negative for CD34 and CD45 (scale bar: 50 µm).

Bone marrow stromal cell tracking in the ovaries

The transplanted BMSCs were labeled with dii, as red spots in the sections of ovaries (Fig.2). The results confirmed presence of the transplanted cells in the ovaries four weeks after transplantation.

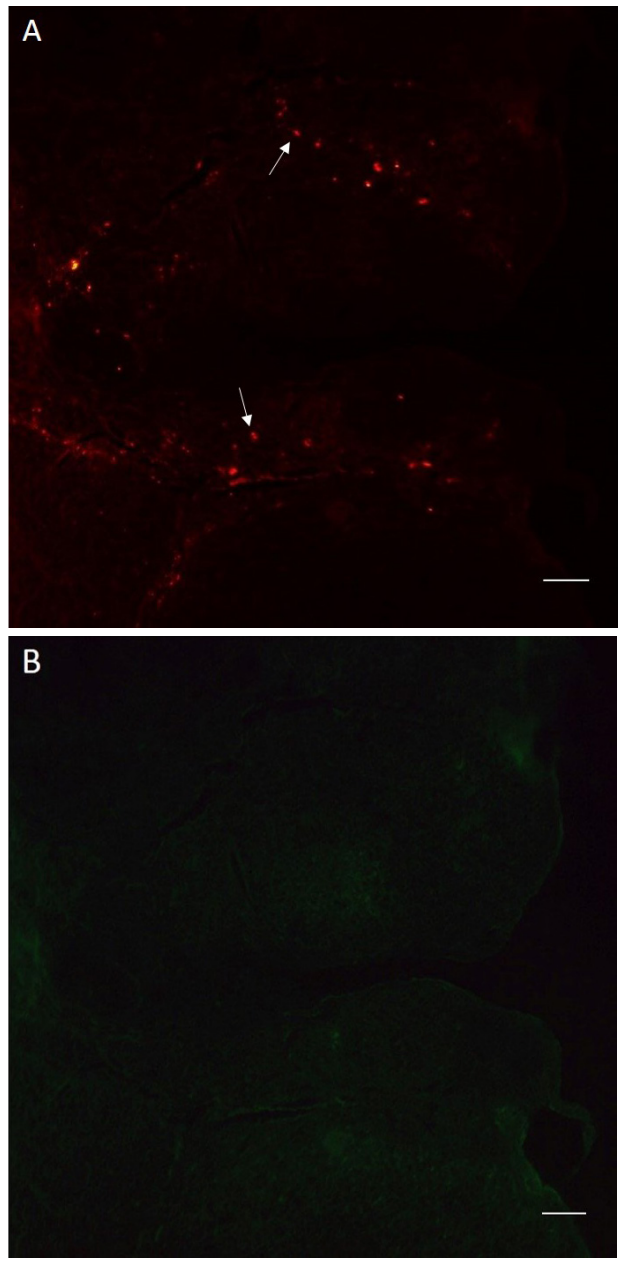


Fig.2: DiI labeled bone marrow stromal cells (BMSCs) in a section of ovary. **A.** The labeled BMSCs are visible as red spots and **B.** In the same section, the labeled BMSCs are not visible with green fluorescence (scale bars: 100 µm). Arrows show the labeled cells.

Levels of serum estradiol and follicle-stimulating hormone

Hormonal examination was performed, by determining levels of serum E2 and FSH, four weeks after treatment. The results showed that levels of serum E2 in the BMSC+Lc co-administrated group (P<0.001), BMSC group (P<0.001) and Lc group (P<0.01) were significantly higher than the control group. The results of BMSC+Lc group were significantly higher than BMSC group (P<0.05) and Lc group (P<0.001). The results of BMSC group were significantly higher than Lc group (P<0.001, Table 1, Fig.3A).

The levels of serum FSH in the BMSC+Lc co-administrated group (P<0.001), BMSC group (P<0.001) and

Lc group (P<0.01) were significantly lower than the control group. The results of BMSC+Lc group were significantly lower than BMSC group (P<0.05) and Lc group (P<0.001). The results of BMSC group were significantly lower than Lc group (P<0.01, Table 1, Fig.3B).

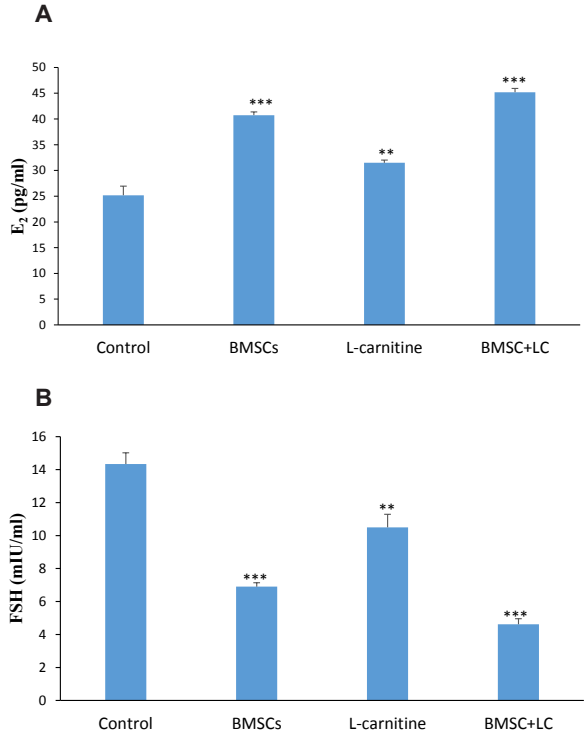


Fig.3: The levels of serum estradiol (E2) and follicle-stimulating hormone (FSH) in the experimental groups four weeks after treatment. **A.** The results of serum E2 level and **B.** The results of serum FSH level. **; P<0.01, ***; P<0.001 versus control group, and BMSC; Bone marrow stromal cells.

Histological evaluation of the ovaries

H&E staining demonstrated that the number of all follicles in different stages was significantly higher in BMSC+Lc group compared to BMSC (P<0.01), Lc (P<0.001) and control groups (P<0.001). Findings showed that the number of all follicles in BMSC group was significantly more than Lc group (P<0.05, Table 1, Fig.4).

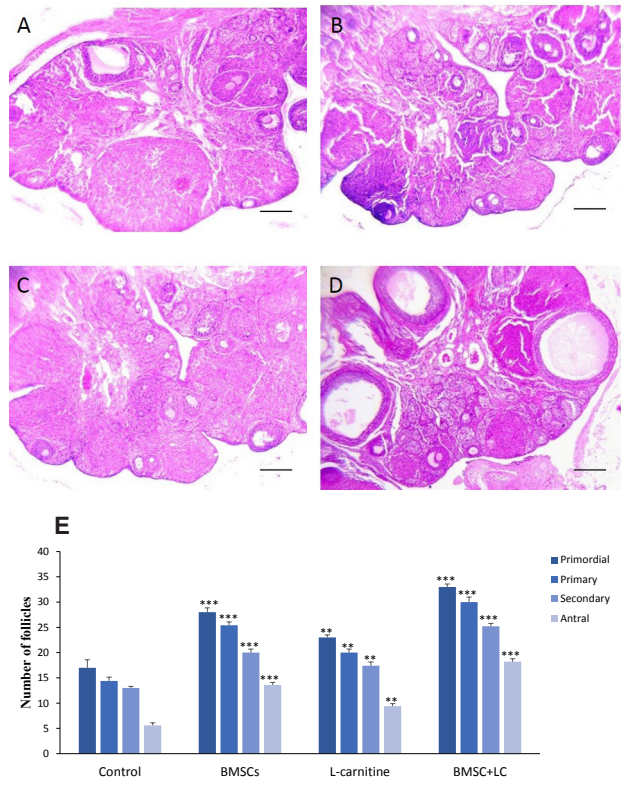


Fig.4: The number of follicles four weeks after treatment. H&E staining of ovaries in **A.** Control, **B.** BMSCs, **C.** L-carnitine, **D.** Co-administration of BMSC+Lc groups, and **E.** The number of follicles at different stages (scale bars: 200 µm). **; P<0.01, ***; P<0.001 versus control group, and BMSC; Bone marrow stromal cells.

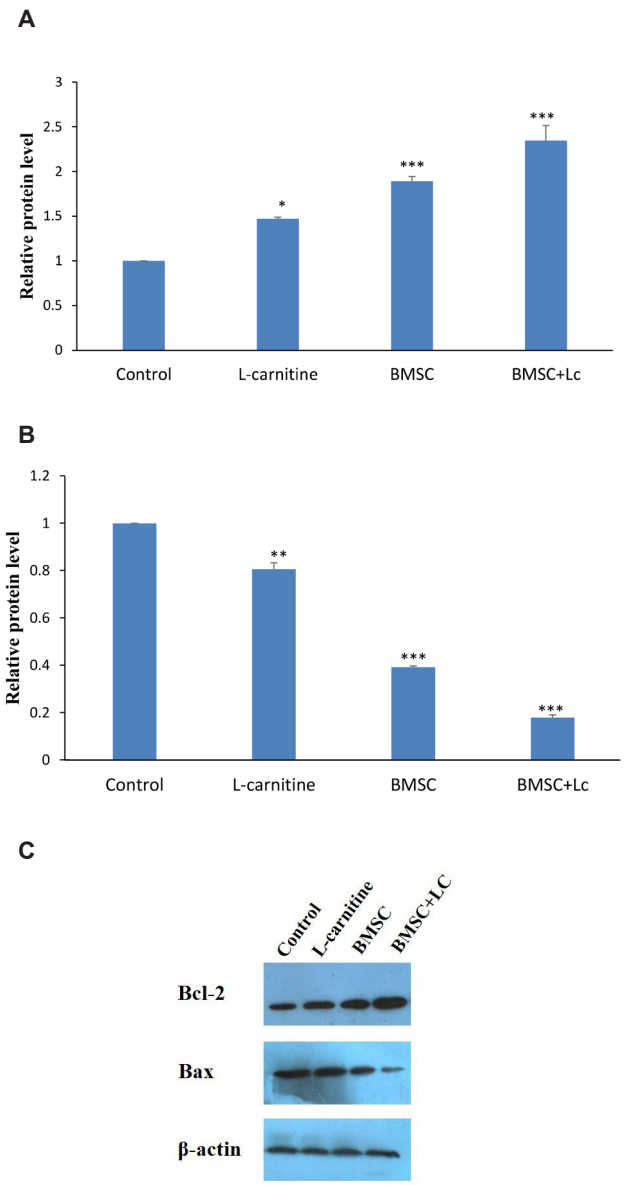
Table 1: Results of the hormonal, histological and expression of ovarian Bcl-2 and Bax proteins four weeks after treatment

Groups	Control	BMSCs	L-carnitine	BMSC+L-carnitine
E2 (pg/ml)	25.18 ± 1.769	40.74 ± 0.63***	31.48 ± 0.533**	45.2 ± 0.728***
FSH (mIU/ml)	14.34 ± 0.682	6.9 ± 0.24***	10.5 ± 0.791**	4.62 ± 0.338***
The number of ovarian follicles in different stages				
Primordial	17 ± 1.581	28.2 ± 0.86***	23.6 ± 0.51**	33.2 ± 0.583***
Primary	14.4 ± 0.748	25.4 ± 0.678***	20 ± 0.707**	30 ± 1.03***
Secondary	13 ± 0.316	20 ± 0.707***	17.4 ± 0.748**	25.2 ± 0.583***
Antral	5.6 ± 0.51	13.6 ± 0.51***	9.4 ± 0.61**	18.2 ± 0.583***
Expression of ovarian Bcl-2 protein	1.0 ± 0.0	1.473 ± 0.017***	1.89 ± 0.054*	2.347 ±0.167***
Expression of ovarian Bax protein	1.0 ± 0.0	0.806 ± 0.011***	0.392 ± 0.051**	0.179 ± 0.027***
Bcl-2/Bax ratio	0.723 ± 0.047	1.12 ± 0.026*	1.143 ± 0.046*	4.018 ± 0.127***

Data are presented as mean ± SE. E2; Estradiol, FSH; Follicle-stimulating hormone, *; P<0.05, **; P<0.01, and *; P<0.001 versus control group.**

Analysis of Bcl-2 and Bax in the ovaries

Expression of ovarian Bcl-2 and Bax proteins was determined by Western blot. The results showed that Bcl-2 expression in the co-administration of BMSC+Lc ($P<0.001$), BMSC ($P<0.001$) and Lc groups ($P<0.05$) were significantly higher than the control group; while it was significantly higher than BMSC ($P<0.05$) and Lc groups ($P<0.01$) in BMSC+Lc. In addition, it was significantly higher in the BMSC, compared to Lc group ($P<0.05$). Bax expression in the BMSC+Lc co-administered group ($P<0.001$), BMSC group ($P<0.001$) and Lc group ($P<0.001$) were significantly lower than the control. It was significantly lower in the BMSC+Lc compared to BMSC ($P<0.01$) and Lc groups ($P<0.001$). Additionally, it was significantly lower than Lc group, in the BMSC group ($P<0.05$). The Bcl-2/Bax ratio was significantly increased in BMSC+Lc co-administered group, in comparison with the control group ($P<0.001$), BMSC group ($P<0.001$) and Lc group ($P<0.001$, Table 1, Fig.5).



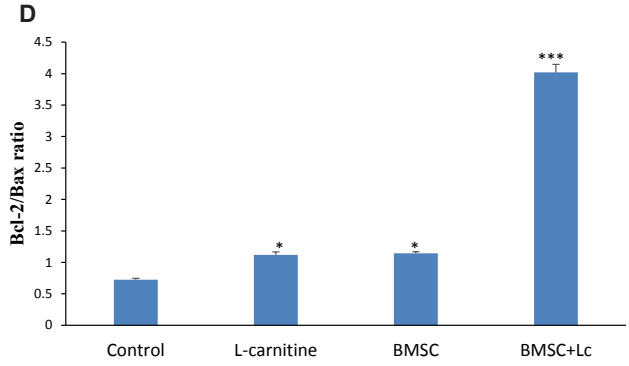


Fig.5: Analysis of Bcl-2 and Bax protein expressions by western blot assay four weeks after treatment. **A.** The expression of ovarian Bcl-2 protein, **B.** The expression of ovarian Bax protein, **C.** Immunoblot of Bcl-2, Bax and β-Actin proteins, and **D.** Bcl-2/Bax ratio in all groups. *; $P<0.05$, **; $P<0.01$, ***; $P<0.001$ versus control group.

Discussion

Chemotherapy may damage the ovaries of girls and women, however, there are some ways to prevent from happening this. In this study, for the first time, we evaluated the effect of co-administration of BMSC+Lc on damaged ovaries after creating a chemotherapy model with cyclophosphamide in rat. Overall, the results showed that levels of serum E2 and FSH, number of follicles in different stages and expression of Bcl-2 and Bax proteins in BMSC+Lc co-administrated group were significantly more favorable than the control, BMSC and Lc groups.

Some studies have shown that BMSC and Lc may individually improve damaged ovaries (7, 8, 11). However, the effect of BMSC+Lc co-administration has never been applied for the same purpose. Comparing the effect of BMSC+Lc co-administration with either of them alone may introduce a novel clinical approach to the recovery of damaged ovaries by chemotherapy.

BMSCs, as a mesenchymal stem cell type, are a suitable candidate for cell therapy in damaged ovaries. Liu et al. (24) have reported that mesenchymal stem cells improve tissue repair chiefly via differentiation and paracrine effects. Several studies have shown that BMSCs produce some growth factors preventing cell apoptosis and repair the ovaries. Some of these growth factors include vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF) (7, 8). VEGF is an angiogenic factor promoting formation of new capillary networks which provides nutrition for GCS (7, 8, 25). IGF-1 stimulates GC proliferation by regulating DNA replication of granulosa and theca cells. IGF-1 increases the function of gonadotropin hormones. Moreover, IGF-1 regulates aromatase activity, promotes follicular antrum formation and suppresses apoptosis in ovaries (7, 8). HGF promotes follicular maturation and inhibits apoptosis in ovarian follicles and GCS (7). Finally, bFGF works as a starter of folliculogenesis by inducing primordial follicle development (25). In this regard, Badawy et al. (26) showed that BMSCs could repair mouse ovarian insufficiency following cyclophosphamide induction, and Fu

et al. (27) showed that overexpression of *miR-21* in mesenchymal stem cells improved ovarian structure and function in rats with chemotherapy-induced ovarian damage. The results of our study are in agreement with these reports.

On the other hand, Lc as an antioxidant may also improve damaged ovaries. Zhang et al. (11) showed that Lc inhibits follicle apoptosis and increases the function of frozen-thawed ovaries in mice. However, the effect of Lc has not been assessed on rat ovaries damaged by a chemotherapy agent, cyclophosphamide. Some studies have shown that Lc has protective effects on other organs. For example, Aktoz et al. (28) showed that Lc has protective effects against testicular toxicity in rat, Mescka et al. (29) showed that Lc prevents oxidative stress in the brain of rats and Tousson et al. (30) showed that Lc has protective effects on rat cardiac injury.

Lc plays an important role in fatty acid transport and lipid catabolism of mitochondria. Lc produces ATP by increasing β-oxidation of fatty acid. Hence, it can provide energy for follicular growth. Lc may also suppress apoptosis by increasing β-oxidation of fatty acids and reduce fatty acid toxicity. Moreover, accumulation of reactive oxygen species (ROS) in follicles leads to evacuation of the ATP reservoir, which decreases follicle quality. Lc, as a ROS scavenger and an energy generation facilitator, can be responsible for useful effects on follicular survival and ovarian function (11, 12, 31). In relation to this issue, Giorgi et al. (32) showed that Lc prevents miotic oocyte damage induced by follicular fluid from infertile women with mild endometriosis and Xu et al. (33) showed that Lc, during *in vitro* maturation of buffalo oocytes, improves oocyte quality. The results of our study are in agreement with these reports.

In addition, several studies have shown that Lc has favorable effects on mesenchymal stem cells. Fujisawa et al. showed that Lc suppresses apoptosis in BMSCs, due to restoration of mitochondrial activity and suppression of senescence induction by blocking TGF-β, suggesting that Lc is involved in mitochondrial activation even in senescent cells (14). Lu et al. (15) showed that carnitine could affect differentiation rate of adult stem cells by regulating mitochondrial metabolism, and it may enhance tissue development. Farahzadi et al. (16) showed that Lc improves the lifespan of aged adipose tissue-derived human mesenchymal stem cells by overexpressing telomerase and lengthening telomeres.

Considering the beneficial effects of Lc on mesenchymal stem cells, in the present study, the combined effects of Lc and BMSCs were evaluated on the recovery of ovaries damaged by chemotherapy agent. We cultured BMSCs and transplanted them into the rat ovaries after creating the chemotherapy model. BMSCs expressed CD29, CD44 and CD90, but not CD34 and CD45. That was in agreement with other study (7). We labeled BMSCs with dii and transplanted them into the ovaries. It was shown that transplanted BMSCs could be present in the ovaries after four weeks. These results are in agreement with other report (21). To evaluate the ovarian function, levels of serum E2 and FSH were assessed by ELISA kit. To evaluate the ovarian structure, number of follicles at different stages was counted by HandE staining. Moreover, to evaluate apoptosis in the ovaries, expression of *Bcl-2* and *Bax* proteins was measured by western blot, since the protein products of Bcl-2 and Bax genes are respectively described as anti-apoptotic and pro-apoptotic factors (23). Findings obtained from these evaluations showed that the results of BMSC and Lc groups were significantly more favorable than the control group. These results are in agreement with the other studies (8, 11, 23).

Indeed, the results of hormonal, histological and expression of Bcl-2 and Bax proteins were in the same direction and confirmed each other. So that, these results in BMSC+Lc co-administrated group were significantly more favorable than BMSC, Lc and control groups. The reasons are probably due to the combination of useful properties of BMSCs and Lc with different mechanisms of action in the restoration of ovaries after chemotherapy. In addition, considering that Lc has favorable effects on differentiation, increasing lifespan and decreasing apoptosis in BMSCs, it may increase survival of the transplanted BMSCs in the ovaries.

The results of BMSC group were significantly more favorable than Lc group. In the present study, considering that BMSCs were injected into the ovaries, these cells might produce some growth factors or might replace damaged cells in the ovaries (7-9). In this regard, Liu et al. (24) compared local and systemic administration of mesenchymal stem cells and reported that local administration of stem cells is the most efficient route for cell homing and immediate generation. So, probably due to these reasons, the recovery of damaged ovaries after chemotherapy with in situ transplantation of BMSCs were more favorable than intraperitoneal injection of Lc.

This study has some limitations which should be considered. The number of samples was small, so a larger sample size is required. Additionally, more research is necessary to clarify the molecular mechanisms underlying the function of BMSC and Lc to repair damaged ovary after chemotherapy.

Conclusion

The results of this study suggest that the effect of BMSC+Lc co-administration is probably more effective than the effect of their administrations individually on the recovery of ovaries damaged by cyclophosphamide chemotherapy agent in rat.

Acknowledgements

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work. There is no conflict of interest in this study to declare.

Authors' Contributions

S.Z.; Gave the idea of the project and wrote the manuscript. R.S.; Contributed to cell transplantation and L-carnitine injection into the ovaries. H.R.S.; Supervised the histopathological works. B.Y., M.S.; Contributed to statistical analysis, and interpretation of data. N.Kh.; Contributed to process of bone marrow stromal cell culture. P.H.; Contributed to flow cytometer and western blot assays. All authors read and approved the final manuscript.

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Original Article

Stress, Depression, Sexual Function, and Alexithymia in Infertile Females with and without Polycystic Ovary Syndrome: A Case-Control Study

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Abstract

Background: Infertile females experience some types of distress such as social stress, depression, and sexual dysfunction that may be exacerbated by polycystic ovary syndrome (PCOS). The current study aimed at comparing psychological profile of infertile females with PCOS with that of women without PCOS with respect to four domains: infertility stress, depression, sexual dysfunction, and alexithymia.

Materials and Methods: The current case-control study was conducted on 240 infertile females (120 with PCOS and 120 without PCOS) in Fatemeh Azahra Infertility and Reproductive Health Research Center (Babol, Iran) from 2016 to 2017. The following questionnaires were used to collect data: the fertility problem inventory (FPI), the female sexual function index (FSFI), the Beck depression inventory-II (BDI-II), and the Toronto alexithymia scale (TAS-20).

Results: Females with PCOS had higher FPI total scores than the ones without PCOS (120.68 ± 29.42 vs. 112.83 ± 30.94). Of the subscales of infertility stress, the mean scores of social stress and rejection of a future life without a child were higher in females with PCOS than the ones without PCOS ($P<0.05$). Also, the mean total scores of alexithymia symptoms (TAS-20) in females with PCOS were significantly higher than those of the ones without PCOS (59.83 ± 11.36 vs. 55.69 ± 11.52). There was no significant difference between the two groups regarding the mean scores of depression symptoms and sexual function.

Conclusion: Infertile females with PCOS experienced higher levels of infertility stress and inability to distinguish and describe their feelings compared with the ones without PCOS. It is suggested that infertility care providers should provide more psychosocial support for infertile females with PCOS.

Keywords: Alexithymia, Depression, Infertilty, Polycystic Ovary Syndrome, Sexual Dysfunction

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Introduction

Polycystic ovarian syndrome (PCOS) is one of the most common etiological factors of infertility which is identified in up to 20% of infertile females (1). Studies emphasized that the prevalence of psychiatric disorders is high in patients with PCOS. A longitudinal study reported a prevalence of 40% for depression in patients with PCOS (2). A cohort study reported that PCOS can increase the risk of schizophrenia, bipolar disorder, personality disorders, and tics (3). Other psychiatric disorders such as anxiety, eating disorders, and sexual dysfunction disorders are common in patients with PCOS (4). In addition, females with PCOS reported lower body image satisfaction compared to the ones without PCOS (5). Clinical manifestations of PCOS including menstrual irregularity, hirsutism, and acne may exacerbate distress in the affected females (6).

Many females experience infertility as a feeling of distress and stigma (7). Infertile females experience some types of distress such as social stress, depression, sexual dysfunction, and marital dissatisfaction (8-10) that may be exacerbated by PCOS. Kitzinger and Willmott (11) introduced PCOS as a stigma, "the thief of womanhood". Infertile females with PCOS and infertility problems may experience being less feminine, due to excessive hair growth and absence of or irregular menstrual periods (12). Additionally, infertility management processes such as assisted reproductive techniques are more stressful in females with PCOS than the ones without it (13).

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Alexithymia is a personality construct with inability in normal affect regulation that is comprised of five characteristics including difficulty to identify and distinguish emotions from bodily sensations, difficulty to describe and verbalize emotions, externally oriented thinking style, poverty of fantasy life, and poor empathy (14). This personality construct is a risk factor for various physical and mental health problems including anxiety, depression, compulsive or addictive behaviors, physical symptoms, and potentially somatic diseases (15). Since a previous study showed that infertile females had higher rates of alexithymia than the fertile ones (16), it was assumed that the rate of alexithymia may differ among infertile females with different levels of stress. Therefore, infertile females with PCOS may have different levels of alexithymia compared with the ones without PCOS.

Although many previous studies indicated that psychiatric disorders are common in patients with PCOS (2-6), few researches reported psychiatric symptoms in females with PCOS and infertility. Diamond et al. (16) concluded that female sexual dysfunction does not vary between infertile females with PCOS and the ones with unexplained infertility. Another study reported that infertility did not appear to constitute a risk factor of psychological distress in females with PCOS (17). As differences in psychological profiles between infertile females with PCOS and those without PCOS are not clear yet, the current study aimed at comparing the psychological profile of these two groups. To the authors' best knowledge, it was the first study that compared psychological profiles of infertile females with and without PCOS in terms of four domains: infertility stress, depression, female sexual dysfunction, and alexithymia (i.e. the inability to distinguish and describe feelings and the absence of fantasies).

Materials and Methods

Participants

The current case-control study was conducted in Fatemeh Azahra Infertility and Reproductive Health Research Center (Babol, Iran) from May 2016 to December 2017 on 240 infertile females selected through census sampling method. The case group was composed of 120 females with a definite diagnosis of PCOS. The control group was comprised of 120 infertile females without PCOS based on Rotterdam diagnostic criteria. Besides, the control group was matched with the case group in terms of age, level of education, and duration of infertility.

Inclusion criteria for infertile females with and without PCOS were being 15-45 years old, completion of primary school as the minimum level of education, being married and having an active sex life, and lacking any problems in speaking or understanding the Persian language; also, a definite diagnosis of PCOS was an additional criterion for PCOS group. Definite diagnosis of PCOS was done based on two of the following Rotterdam diagnostic criteria: ultrasound scan of PCOS (presence of ≤12 follicles in one or both ovaries and/or increased ovarian volume >10 mL), clinical signs of hyperandrogenism (hirsutism or obvious acne), and/or an elevated plasma testosterone level, and/or irregular menstrual periods (interval between menstrual periods >35 days, amenorrhea defined as the absence of vaginal bleeding for ≥6 months, and/or variable menstruation) (18, 19).

Exclusion criteria for all participants (females with and without PCOS) were diagnosis of the husband with azoospermia or oligospermia, presence of other disorders that could mimic PCOS syndrome such as congenital adrenal hyperplasia, thyroid disease, or hyperprolactinemia.

Procedure

Four staff of the infertility center explained the study's objectives to the participants and accordingly, the subjects were required to sign the written informed consent forms. The staff interviewed the subjects and recorded their demographic characteristics, as well as their medical and gynecological history. Furthermore, the subjects were asked to complete five questionnaires of the study including the fertility problem inventory (FPI), the female sexual function index (FSFI), the Beck depression inventory-II (BDI-II), and the Toronto alexithymia scale (TAS-20). First, 258 females (129 with and 129 without PCOS) were enrolled of which 240 females with infertility (120 with and 120 without PCOS) completed the questionnaires.

Ethical considerations

The current study was approved by the Ethics Committee of Babol University of Medical Sciences (No.4834).

Measures

Demographic questionnaire

Demographic characteristics including age, educational level, infertility history, clinical information of PCOS, and assisted reproductive technology (ART) history were obtained from the subjects. In addition, weight and height were measured in order to obtain body mass index (BMI).

Infertility stress

Infertility stress was assessed using FPI developed by Newton in 1999. It is a multi-dimensional tool to detect stress and infertility problems. The FPI is comprised of 46 questions divided in five subscales: social concern, sexual concern, relationship concern, rejection of parenthood, and the need for parenthood. Each item is scored based on a six-point Likert scale, ranging from 1 (strongly disagree) to 6 (strongly agree). The total score

ranges from 46 to 276 with higher scores representing higher levels of stress (20). Validity and reliability of the Persian version of FPI were previously examined (21). In the current study, the Cronbach's alpha coefficient of the FPI was 0.898.

Sexual function

The FSFI was used to assess sexual function in subjects. The FSFI assesses sexual function over the past four weeks. It covers six domains: desire, arousal, lubrication, orgasm, satisfaction, and pain. The score for each domain ranges from 0 or 1 to 5 with higher scores representing better sexual function (22). It was previously shown that the Persian version of FSFI has high validity and reliability (23). In the current study, the Cronbach's alpha coefficient of the FSFI was 0.896.

Depression symptoms

Depression was measured by the BDI-II. It is a self-reported scale and a screening instrument for depression with 21 items, most of which assess depressive symptoms on a four-point Likert scale ranging from 0 to 3. Total scores range from 0 to 63. In clinical settings, the severity of depression based on BDI-II, is classified as follows: 0-13: minimal depression; 14-19: mild depression; 20-28: moderate depression; and 29-63: severe depression (24). A valid Persian version of the BID-II was used in the current study. The internal consistency (Cronbach's alpha=0.87) and test re-test reliability (r=0.74) of the BID-II Persian was high and acceptable (25). In the current study, the Cronbach's alpha coefficient of the BDI-II was 0.915.

Alexithymia

In the current study, alexithymia was assessed using TAS-20. It is one of the most common instruments to measure alexithymia that has 20 items in three subscales: difficulty to describe emotions, difficulty to identify feelings (DIF), and externally-oriented thinking. Items are scored based on a five-point Likert scale, ranging from 1 (strongly disagree) to 5 (strongly agree). The total alexithymia score ranges from 20 to 100 (26). A study conducted by Besharat supported the internal consistency, test-retest reliability, and concurrent validity of the Persian version of TAS-20 (27). In the current study, the Cronbach's alpha coefficient of the TAS-20 was 0.809.

Statistical analysis

All data were analyzed using SPSS for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA). To present characteristics of females with and without PCOS, continuous variables are expressed as mean ± SD and categorical variables as numbers (%). Chi-square test was employed to compare categorical variables such as educational attainment level, duration of infertility, regularity of menstruation, and BMI between the two groups. Also, independent samples t test was employed to compare the means of age and duration of marriage between the two groups. In addition, comparisons of the mean scores between females with PCOS and those without PCOS in all four questionnaires and their subscales including FPI, FSFI, TAS-20, and BDI-II, were done using independent t test. A P<0.0.5 was considered statistically significant.

Results

Table 1 provides the summarized demographic information of subjects in the two groups. There were no significant differences between the two groups regarding the subjects' age, husbands' age, educational level of the subjects, educational level of their husbands, and duration of infertility (P>0.05 in all cases). The frequency of irregular menstruation was significantly higher in females with PCOS than the ones without PCOS (P<0.001).

Table 1: Demographic characteristics of women with and without polycystic ovary syndrome (PCOS)

Variable	Yes (n=120)	No (n=120)	P value
Age (Y)	29.55 ± 5.17	29.33 ± 6.23	0.771
Education			0.278
≤12 years	51 (56.7)	39 (43.3)	
>12 years	63 (49.2)	65 (50.8)	
BMI			0.218
<25	41 (34.2)	29 (24.2)	
25-29.99	45 (37.5)	52 (43.3)	
≥30	34 (28.3)	39 (32.5)	
Duration of infertility (Y)			0.159
<5	66 (74.2)	56 (64.4)	
≥5	23 (25.8)	31 (35.64)	
Regular menstruation			<0.001
Regular	64 (53.3)	93 (77.5)	
Irregular	56 (47.7)	27 (22.5)	
Duration of marriage (Y)	5.9 ± 3.99(5)	6.04 ± 3.88(5)	0.587
Husband' age (Y)	33.06 ± 5.43	32.66 ± 4.82	0.554
Husbands' education*			0.504
≤12 years	54 (47.8)	45 (43.3)	
>12 years	59 (52.2)	59 (56.7)	

Data are presented as mean ± SD or n (%). BMI; Body mass index, *; There were some missing data; therefore, the sum of the frequencies for qualitative variables is not equal to 120.

Table 2 a comparison in the mean scores of FPI, FSFI, BDI-II, and TAS-20 between the two groups. The results of the t-test revealed that females with PCOS had higher total mean scores of infertility stress (FPI) than the ones without PCOS (120.68 ± 29.42 vs. 112.83 ± 30.94, P=0.046).

Table 2: Comparison of psychological profile of women with and without polycystic ovary syndrome (PCOS)

Variable	PCOS		P value
	Yes (n=120)	No (n=120)	
Infertility stress (FPI)			
Social concerns	24.20 ± 8.31	21.74 ± 8.39	0.024
Sexual concerns	17.53 ± 7.98	16.75 ± 7.81	0.455
Marital concerns	25.15 ± 7.25	24.30 ± 7.34	0.371
Acceptance of life without child	18.57 ± 7.28	16.27 ± 7.87	0.021
Need for parenthood	36.06 ± 9.56	35.15 ± 9.67	0.467
Total scores	120.68 ± 29.42	112.83 ± 30.94	0.046
Alexithymia (TAS-20)			
Difficulty in describing feelings	15.17 ± 4.08	13.94 ± 3.62	0.015
Difficulty in identifying feelings	22.62 ± 6.06	19.74 ± 6.03	<0.001
Externally-oriented thinking	22.04 ± 4.26	22.38 ± 4.03	0.532
Total scores	59.83 ± 11.36	55.69 ± 11.52	0.005
Sexual dysfunction (FSFI)			
Desire	3.94 ± 0.85	3.92 ± 0.84	0.835
Orgasm	3.5 ± 0.8	3.49 ± 0.84	0.975
Satisfaction	4.78 ± 1.19	4.92 ± 1.05	0.364
Pain	4.64 ± 1.13	4.80 ± 1.16	0.279
Arousal	3.92 ± 0.92	3.88 ± 0.91	0.752
Lubrication	4.41 ± 0.85	4.49 ± 0.73	0.476
Total scores	25.13 ± 3.95	25.35 ± 3.87	0.660
Depression symptoms (BDI-II)	18.06 ± 12.03	15.65±11.76	0.121
Severity of depression			0.114
Minimum	31 (26.1)	46 (39.0)	
Mild	35 (29.4)	27 (22.9)	
Moderate	33 (27.7)	33 (28.0)	
Severe	20 (16.8)	12 (10.1)	

Ranges scores; social concern (1-60), sexual concern (1-48), relationship concern (1-60), rejection of life without child (1-48), need for parenthood (1-60), total scores of infertility stress (46-276). Difficulty in describing emotions (1-25), Difficulty in identifying feeling (1-35), Externally-oriented thinking (1-40), total scores of alexithymia (20-100). Desire (6-0), arousal (6-0), lubrication (6-0), orgasm (6-0), satisfaction (6-0), pain (6-0), total scores of sexual dysfunction (36-0). Depression symptoms (0-63), Minimum (0-13), mild (14-19), moderate (20-28), severe (29-63). Data are presented as mean ± SD or n (%).

Of the subscales of infertility stress, the mean scores of social stress and rejection of life without child were higher in females with PCOS than those of the other group (P=0.024 and P=0.021, respectively). There were no significant differences in the mean scores of subscales of sexual stress, marital stress, and parental stress between the two groups. Also, females with PCOS had higher total mean scores of alexithymia symptoms (TAS-20) than the ones without PCOS (59.83 ± 11.36 vs. 55.69 ± 11.52, P=0.005). Of the subscales of TAS-20, DIF and difficulty to describe feeling were significantly higher in females with PCOS than the ones in the other group (P<0.001 and P=0.015, respectively). There was no significant difference between the two groups in the mean scores of depression symptoms. In addition, severity of depressive symptoms did not significantly differ between the two groups (18.06 ± 12.03 vs. 15.65 ± 11.76, P=0.121). Total scores of FSFI and all its six subscales did not significantly differ between the two groups (25.13 ± 3.95 vs. 25.35 ± 3.87, P=0.660).

Discussion

The current study aimed at comparing the psychological profiles of infertile females with PCOS with those of women without PCOS. The results showed that females with PCOS had higher total mean scores of infertility stress (FPI) than the ones without PCOS. Infertile females with PCOS had more social concerns than the ones in the other group. Also, infertile females with PCOS had more stress of rejection of life without child than the other group. To the authors' best knowledge, no published study has examined various aspects of infertility stress in infertile females with and without PCOS. However, some studies evaluated social relationships in patients with PCOS compared to the controls. Such studies reported that the social relationships of patients with PCOS was more impaired compared to the normal population (28, 29). A recent study on the development of specific measurements of quality of life in patients with PCOS emphasized on the negative effects of PCOS on family and friends and social

relationships (30). Consistently, another study reported that the majority of females with PCOS (76.1%) worried about their future life without any children (15).

Now, higher intensity of infertility stress observed in infertile females with PCOS compared to the ones without PCOS, should be explained. There are some hypotheses to explain this finding. First, the secondary analysis of the data showed that symptoms of PCOS such as obesity and hirsutism were related to infertility stress. Second, some previous studies confirmed that females with PCOS experienced social pressure due to hirsutism, especially excessive facial hair (31). A study showed that hirsutism score of females with PCOS was significantly correlated with mental health status (32). Infertile females with PCOS that had hirsutism felt "unfeminine" and "different" (33). Therefore, social concerns of infertile females with PCOS may be more than those of the ones without PCOS.

In contrast with the current study's expectation, the total scores of FSFI and all of its six subscales did not significantly differ between females with PCOS and those without PCOS. Results of some previous studies were consistent with the findings of the present study reporting that females with PCOS did not have more depression symptoms than the ones without PCOS (34, 35). However, a study reported that infertile females with PCOS had significantly higher depression scores compared to the ones without PCOS (36). A study reported that females with PCOS with ones with no desire for a child did not show significant differences in specific aspects of sexual satisfaction compared to the ones with no desire for a child (17). A recent study reported no significant difference in female sexual dysfunction disorders between infertile females with PCOS and those without PCOS (16).

The current study also aimed at comparing the alexithymia between infertile females with and those without PCOS. The results of the current study indicated that infertile females with PCOS had higher alexithymia scores than the ones without PCOS. Infertile females with PCOS had more difficulty to identify their feelings and describe their emotions compared to the ones without PCOS. To the authors' best knowledge, no previous study assessed the alexithymia in infertile females with PCOS. Although the current study did not have enough information about the reasons for higher alexithymia in females with PCOS compared to the ones without PCOS, several hypotheses could be proposed. First, there are associations between alexithymia and maladaptation to stress. A study investigated the association between alexithymia and fertility-related stress in females with infertility demonstrated that alexithymia was related to fertility-related stress. The authors concluded that alexithymia acted as a secondary coping strategy in females with infertility (37). Second, alexithymia is related to somatization disorders. A recent meta-analysis reported that females with PCOS were more likely to have higher somatization disorders compared to the ones without PCOS (38). Third, another study introduced alexithymia and somatization as the consequences of maladaptation to stress of infertility (39). Therefore, it is supposed that high somatization in females with PCOS and infertility was comorbid with alexithymia and higher infertility stress than ones without PCOS.

Due to some limitations of the current study, data should be interpreted with caution. First, the case-control nature of the current study prevents drawing any conclusions concerning possible relationships. Prospective cohort studies in the area using reliable approaches are required to describe the casual relationship between infertile females with PCOS and those without PCOS. Second, data was collected using self-report scales that may result in underreporting of the conditions. Future studies using more reliable methods such as interviewing, might give a better picture of the psychological profile of infertile females with PCOS. Third, all of the patients included in the current study were recruited from one hospital, rather than multiple centers, that could be a limitation of the current study. Fourth, the study sample was small and cannot be generalized to numerous phenotypes of PCOS. Further, multi-centered studies with larger sample sizes are recommended. Finally, since the study was the first work that showed higher alexithymia in infertile females with PCOS, more studies in the area should investigate the extent of the associations between alexithymia and PCOS in females with infertility. Additionally, future studies are required to clarify how alexithymia arises in infertile females with PCOS.

Conclusion

The current study results showed that infertile females with PCOS experience more infertility stress than the ones without PCOS. Also, infertile females with PCOS had higher means of alexithymia, especially with respect to the ability to distinguish and describe, compared to the ones without PCOS. The results of the current study indicated that infertility care providers should provide more psychosocial support for infertile females with PCOS. The current study was a step to present the profiles of infertile females with PCOS; thus, further longitudinal studies are required to follow the changes in psychological profiles of females with and without PCOS during infertility treatment.

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Authors' Contributions

Z.B., M.F.; Designed the study. S.A.F., T.M.; Wrote the protocol and collected the data. M.F., Z.B., S.E.; Wrote the protocol, and the first draft of the manuscript. Z.G.;

Performed analyses and designed the study. All authors read and approved the final manuscript.

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Original Article

Estimating The Annual Abortion Rate in Kerman, Iran: Comparison of Direct, Network Scale-Up, and Single Sample Count Methods

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Abstract

Background: Abortion is a sensitive issue surrounded by social, cultural and religious stigmata. Therefore, estimation of its prevalence involves methodological challenges. The aim of this manuscript is to estimate the abortion prevalence, stratified by type, using a direct and two indirect methods.

Materials and Methods: This cross-sectional study was done in 2016, we recruited 1020 women aging 18-49 years. Three methods were applied to estimate the abortion prevalence: direct question, network scale-up (NSU), and single sample count (SSC). In the direct method, to guarantee anonymity, data were collected by means of a self-administered questionnaire. In other methods, data were collected through gender-matched street-based interviews.

Results: The annual rate of abortion estimated by direct and NSU methods were respectively 29 (10 intentional, 4 therapeutic and 15 spontaneous) and 23 (9 intentional, 3 therapeutic, and 11 spontaneous) per 1000 women aging 18-49 years. The annual rate of intentional abortion estimated based on SSC method was higher (15 per 1000 women) than other methods.

Conclusion: The present estimates are higher than previously reported ones. The results of three methods more or less supported each other confirming the internal validity of our estimates.

Keywords: Abortion, Indirect, Network Scale-Up, Single Sample Count, Kerman

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Introduction

Abortion is an important contributing factor to women's health and it could even result in mother's death. Although in many societies, abortion has been associated with legal restrictions as well as social, cultural and religious stigmata (1-3), placing legal limitations has not reduced its prevalence. In contrast, legal restrictions have increased the number of women who seek for clandestine and unsafe abortion in illegal and underground abortion centers to terminate their unintended pregnancies (4). Almost all (i.e. 98%) unsafe abortions, which is regarded as the third cause of maternal death (5), occur in developing countries; nevertheless, in contrast to other causes of women's death, all complications and deaths caused by unsafe abortions, are preventable (6, 7).

Legal restrictions and stigmata around abortion make it invisible, as those who had abortion are not willing to disclose it. This in turn might lead to inaccurate data on the annual number of abortions (8, 9). This issue goes beyond the culture and law and even in communities with legal abortion policy, underreporting accounts for 70% of cases (10). A similar issue is likely to happen for spontaneous abortions in developing countries due to failure of regis-tration systems (11). Therefore, the available data might only present the tip of the iceberg (12). It should be also noted that policy makers need reliable data for appropri-ate decision making.

Due to the social stigma and legislations, direct estimation of abortion rate with face-to-face interviews

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might result in under-estimation of the true prevalence. In this context, indirect methods were proposed for estimation of the size of hidden and stigmatized subpopulations (13). Among indirect methods, network scale-up (NSU) is an appealing method that does not need direct contact with the target population (14-16). In NSU, a random sample of general population is recruited and interviewed to count the number of individuals with a given characteristics (for example those who had abortion) among their social network. The NSU method is based on the principle that the proportion of people that participants know from the target population, directly corresponds to the actual size of the community (17-19). This method preserves anonymity as respondents reply on behalf of their network rather than themselves. It was shown that NSU is a valid tool for estimation of the size of hidden groups (20).

Single sample count (SSC) technique is another efficient indirect method. In this method, a list of statements including several insensitive statements with certain distribution plus a sensitive one is given to the participants (21). Here, the participant is asked to determine how many of statements are true about herself /himself. In Iran, a country with Islamic State, which is located in the Middle East, intentional abortion is strictly prohibited. Hence, underreporting and misclassification of abortions is high in usual health reports. From 2012 onwards, the overall policy of the country has changed to increase fertility rate, which itself could impose further limitations on performing abortion.

Results of the studies which were designed to estimate intentional abortion rate in Iran are inconsistent, ranging from 1 to 20%. In a meta-analysis study, the annual prevalence was estimated to be 8.9 per 1000 women of fertile age (22). We conducted the present study in Kerman city located in south east of Iran, and aimed to estimate the abortion rate, and compare performance of direct and indirect methods.

Materials and Methods

Sampling process

This cross-sectional study was conducted in Kerman, south east of Iran. In this study, 1020 women aging 18-49 years were selected through street-based multistage sampling proportional to the age distribution of women in the 2011 census. At the first step, the city was classified into three categories based on socioeconomic status (SES): high, medium and low. To do so, we asked for the governor's office experts' opinion. Secondly, 5 regions were selected from each SES category. Finally, from each of 15 regions, 68 women were recruited through the convenience sampling method in streets. We adopted street-based sampling, as our previous experiences showed that in case of sensitive issues, other sampling schemes such as household or telephone-based methods do not work.

The eligible participants were women aging 18-49 years, who had been living in Kerman for the past five years and verbally consented to participate in the study. Data was collected through a structured, face-to-face interview both in the morning and evening times, performed by trained female interviewers. The proposal of this study was approved by the Ethical Committee of Kerman University of Medical Sciences (ir.kmu.rec.1394.223).

Network scale-up estimation

In the first section of the questionnaire, general explanations about the study and the aims were provided for participants. In the next section, NSU questions were asked "how many women do you know in the city of Kerman, who experi-enced an abortion within the last year?" In order to mini-mize the recall bias, this question was stratified. We asked participants to tell the number of such individuals they know among their relatives, husband relatives (in case she was married), and acquaintances (involving neighbor, friend, colleague, etc.). These questions were followed by questions on type of abortion (intentional, therapeutic, or spontaneous), and age of mother. The standard definition of 'know' was as follows: "you know them by name and face, and have had at least one contact through phone, mail, or meeting in person within the past two years, and are able to contact them at any time through one of the above-mentioned methods" (23). To help participants to distinguish different types of abortion, a short and simple description of the three types of abortions was provided in the questionnaire as follows: spontaneous abortion is an abortion which occurs without intervention. Therapeutic abortion is permitted due to fetal abnormalities or to protect the mother's life. Intentional abortion is elective termination of pregnancy without medical justification.

The first requirement of using NSU is knowing the network size (C) of the participants. In this study we needed the number of women at reproductive age known by residents of Kerman. This had been assessed previously (24) and it had been shown that, on average, women aging 18-49 years in Kerman know 111 women aging 18-49 years.

The following formulas were applied to estimate the crude size of abortion and its standard error (SE):

Formula (1): $e_j = \frac{\sum m_{ij}}{\sum c_i} t$

Formula (2): $se = \sqrt{\frac{e_j}{\sum c_i}} t$

Where i and j stand for respondent and hidden group (i.e. abortion), respectively, m is the number of abortions known by each respondent, c is the average network, and t is the total population of 18-49 year old females living in Kerman city, which was about 155,644 according to the latest Iranian census.

It is possible that those who had abortion, do not reveal it to their network members. This is known as visibility

bias. We already designed a study to measure visibility of different types of abortion. It was determined 11, 70, and 60%, for intentional, therapeutic, and spontaneous abortion, respectively (25). Crude estimations were divided by these visibility rates to provide the adjusted size estimates.

Single sample count estimation

In the SSC section, a five-statement list, including four insensitive questions plus a sensitive question on intentional abortion, was given to each participant. The prevalence of each of insensitive questions in the society was 50%. Then, the participant was required to determine how many statements were true for her case. We emphasized that it is not necessary to explain which ones, but simply declare the number of statements that apply to her. In this study, we only estimated the prevalence of intentional abortion. The five statements were as follows: i. My national ID card number is even, ii. My date of birth is in the first 15 days of the month, iii. The year of my birth is even, iv. I was born in the first six month of the year, and v. I had intentional abortion within the past year.

The probability of a 'yes' response to each of non-sensitive items was 50%. We assumed that each of them follows a binomial distribution with 50% probability of success. Therefore, the expected mean of replies to four insensitive questions was two. Therefore, any deviation from two can be attributed to the sensitive statements. The formulas used for calculating prevalence rate and its confidence interval are given below. Here, λ and n show the number of 'yes' replies and sample size, respectively.

Formula (3): $d=(\lambda/n)-2$

Formula (4): $\lambda \pm Z(0.95)^{*}\sqrt{(n^{*}(1+d^{*}(1-d)))}$

Direct estimation

Finally, at the last section, in the direct method, the participants were provided with a questionnaire about their own experience of abortion within the preceding year. This was conducted regardless of participants' marital status. Moreover, the questions were self-administrated and the completed questionnaires were collected through a ballot box to be more comfortable for the participants and increase the accuracy of responses. Data were analyzed using stata version 11 and Excel software.

Results

Among 1451 female who were residents of Kerman and aged 18-49 years and were invited to join the study, 1020 consented to participate, giving a response rate of 70.3%. The youngest and the oldest participants were 18, and 49 years old, respectively. The mean (SD) age of the participants was 30.84 year (8.57). About two-third of the participants were married, and nearly half of them had university educations (i.e. more than 12 years of education). Moreover, about 30% of them were employed. We asked married women to provide demographic characteristics of their husbands. Nearly one third of husbands had university educations, and more than half of them were self-employed (Table 1).

In total, 41.8% of participants did not know any woman who had an abortion in the past year. The mean (± SD) number of abortions known by respondents was 1.07 (± 1.55). Poisson regression model revealed that married and widowed subjects were respectively 39 and 12% more likely to reveal abortion than single participants. Those in age groups of 25-34 and 18-24 years, in comparison with those aged 35-49 years, were respectively 72 and 57% more likely to report abortion cases in their network. Employees and self-employed women were respectively 43 and 28% more likely to report abortion.

Table 1: Demographic characteristics of participants

Variable	Category	n (%)
Age (Y)	18-24	301 (29.5)
	25-34	391 (38.3)
	35-49	328 (32.2)
Marital status	Single	354 (34.7)
	Married	643 (63.0)
	Divorced/widowed	23 (2.3)
Job	Housewife	465 (45.6)
	Employee	159 (15.6)
	Student	195 (19.1)
	Self-employed	158 (15.5)
	Retired	7 (0.7)
	Unemployed	36 (3.5)
Education	≤9 years	136 (13.4)
	12 years	392 (38.4)
	12-16 years	403 (39.5)
	≥18	89 (8.7)
Husband's job	Employee	196 (30.5)
	Worker	54 (8.4)
	Self-employed	357 (55.5)
	Retired	26 (4)
	Un-employed	10 (1.6)
Husband's education	≤9 years	158 (24.6)
	12 years	271 (42.1)
	12-16 years	165 (25.7)
	≥18	49 (7.6)

As summarized in Table 2, NSU estimates for intentional, therapeutic, and spontaneous abortions were 9, 3, and 11 per 1000 women of reproductive ages. In SSC method, the average positive answers for five-item list were 2.015. This suggested an annual prevalence of intentional abortion at 15 per 1000 women of reproductive ages. The estimates of direct method for three types of abortion namely intentional, therapeutic, and spontaneous were 10, 4, and 15 abortions per 1000 women of reproductive ages, respectively.

Table 2: The annual abortion rate determined by the three methods

Type of abortion	Direct % (CI 95%)	NSU % (CI 95%)	SSC % (CI 95%)
Intentional	0.98 (0.38-1.58)	0.9 (0.73-1.1)	1. 5 (0-7.6)
Therapeutic	0.39 (0.006-0.77)	0.29 (0.25-0.33)	
Spontaneous	1.47 (0.73-2.21)	1.12 (1.04-1.2)	

NSU; Network Scale UP, SSC; Single Sample Count, and CI; Confidence Interval.

Discussion

In this study, using the NSU and direct method, the annual rate of abortion per 1000 women aging 18-49 years was calculated to be about 23 (9 intentional, 3 therapeutic, and 11 spontaneous abortion) and 29 (10 intentional, 4 therapeutic, and 15 spontaneous abortion), respectively. Also, using SSC method, intentional abortion was estimated to be 15 per 1000 women aging 18-49 years.

The results of direct and NSU methods were fairly close with overlap in their confidence intervals. The estimates of the direct method were slightly higher than those of the NSU method. This might be due to this issue that since for direct estimation, a self-administered questionnaire at the end of interview was submitted to the respondents and the forms were returned through a ballot box, the anonymity the response was maximized. This indicates that use of direct methods with consideration of methodological issues can provide useful statistics. It also implies the usefulness of NSU. In comparison with direct and item counts methods, the confidence intervals of NSU method were narrower. Within the NSU method, each person responds about the intended behavior of the whole network members rather than one individual. Therefore, the sample size required for NSU studies is much smaller than that of direct methods.

We applied SSC method only for intentional abortion. The SSC estimate was higher than those of the other methods and its confidence interval was wider, which might be due to the nature of this method (26).

Our estimate for intentional abortion (9-15 cases per 1000 women aging 18-49 years) was slightly higher than that reported by two national studies in Iran (12, 27). We should mention that comparison of our results with previous studies is not simple due to methodological differences. For example, face-to-face interview was the dominant data collection method in previous studies. Demographic and Health Survey data of 2000, estimated the annual rate of intentional abortion to be 7.5 per 1000 married women aging 15-49 years (2). Also, meta-analysis of manuscripts published by 2012, estimated an annual rate of 8.9 per 1000 married woman aging 15-44 years (22). The difference is even larger, as the denominator in our calculations included all women aging 18 to 50 years, while previous studies provided estimates just for married women of reproductive ages.

The denominator in our study included all women of reproductive ages, in order to make our results comparable with those of WHO and studies conducted in other countries (28). We should mention that the proportion of single cases in our sample was the same as that of the population.

Zare and Dastouri (29) recruited 550 married women aging 15 to 49 years, who referred to two governmental clinics in Shiraz, south of Iran. They reported a life time intentional abortion rate of 29 cases per 1000 married women aging 15-49 years. This value is higher than our estimate, as they provided life time not annual prevalence, and they considered a small population.

Nojomi et al. (30) carried out a study on 2470 ever-married women using the direct method and face-to-face interview. They estimated an annual intentional and spontaneous abortion rate of 27 per 1000 women aging 15-55 years in Tehran.

The worldwide estimate of intentional abortions per 1000 women aging 15-44 years is 35 (27 and 37 in developed and developing countries, respectively). This corresponded to 25% of pregnancies. Globally, married and single women account for 73 and 27% of abortions, respectively. The range of intentional abortion in Asia was reported to be 35-37, as well (28). These data suggested a lower prevalence of abortion in Iran than world statistics. Less pre-marital sexual relationships could be one of the reasons. Even pregnancy during "Aghd" period (when marriage contract is approved by the authorities but the couple does not yet share an accommodation) is against social norms. In addition to that, in some traditional Iranian families, being virgin on the wedding night, which is certified by a gynecologist, is an important custom. Even after marriage, the rate of intentional abortion is lower than that of other countries mainly due to religious believes, legal restrictions, punishments, and lack of access to standard health centers to provide services.

Based on the world statistics, more than half of the unplanned pregnancies (about 57%) ends in intentional abortion (4). We believe that, due to issues noted above, the corresponding figure in Iran is much lower. Results of a recent meta-analysis in 2012 suggested that the prevalence of unplanned pregnancy in Iran is about 28% (22). Based on another study unwanted birth accounts for 20% of all born children (31). This indicates that in Iran, most of unplanned pregnancies result in unwanted birth.

Although abortion is considered against social norms

and standard services are not available for that in Iran, we believe that its rate is still considerable. This is alarming and policy makers should be informed to explore for possibility of new legislations. Women need more assistance and guide from health care providers to make better decisions in their reproductive life. Moreover, providing enough resources for reproductive health services for them is vital (32).

One of the limitations of our study was that, due to ethical issues, we could not recruit those aged under 18. Moreover, street-based interviews does not guarantee access to a random sample. However, it was a trade-off between representativeness of the sample and accuracy of replies. On the other hand, our study had several strengths. It was the first study that compared performance of direct and indirect methods in estimation of abortion rate. We showed that even direct methods are applicable, if methodological issues are concerned and anonymity is preserved. We provided an updated figure for the abortion situation in Iran.

Conclusion

Estimates derived in our study are alarming and flashes the need for new legislations. The results of three methods are close confirming the internal validity of methods and methodologies. While direct method with methodological considerations might still provide an acceptable estimate, NSU method has practical appeal as it requires a much smaller sample size in sensitive issues with relatively low prevalence. In addition, it is possible to estimate size of several hidden groups in one study. Furthermore, these indirect methods might be useful and are suggested in estimating other sensitive issues through increasing the response and honesty rate. In addition, such methods enjoy from some advantages, like cost-effectiveness, quickness, and simplicity in performance and analysis, which make them an appropriate tool in low and middle income countries, where an accurate registration system is lacking.

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Authors' Contribution

M.Z.; Contributed to design the study, data collection, data analysis, data interpretation, drafting and revising the manuscript. F.Z.; Contributed to data interpretation, drafting and revising the manuscript. A.A.H.; Contributed to data interpretation, and revising the manuscript. M.R.B.; Contributed to primary design the study, data analysis, data interpretation, and revising the manuscript. All authors approved the final copy of the manuscript.

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Original Article

Development and Validation of A Decision-Making Donor Conception Questionnaire in Iranian Infertile Couples

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Abstract

Background: Despite the fact that many infertile couples have to decide about whether or not to choose donor conception, there is no predictive scale for evaluating the process of decision-making on donor conception and its determinants in such couples. The present study was conducted to develop a decision-making questionnaire for selecting donor conception and assess its psychometric properties in Iranian infertile couples.

Materials and Methods: This cross-sectional validation study was conducted based on the method developed by DeVellis (2012) in four steps at Milad Infertility Clinic, Mashhad, Iran. The dimensions of the concept of decision-making were determined in the first step based on the qualitative results obtained from 38 semi-structured in-depth interviews. Items that were appropriate for the questionnaire were developed in the second step using the qualitative data and a review of the literature. In the third step, the research team reviewed and eliminated some of the items. The fourth step evaluated the face, content and construct validity of the questionnaire through exploratory factor analysis on a sample of 220 infertile couples using convenience sampling and investigated its initial and final reliability.

Results: Based on the results of the qualitative study, a pool of 170 items was developed, 101 of which were eliminated after revision due to ambiguity, repetition or their poor face and content validity and initial reliability. The questionnaire was evaluated for its construct validity with 69 items. After the exploratory factor analysis, the decision-making donor conception questionnaire (DMDCQ) having 51 items and seven factors, was finalized. All the factors had Cronbach's alpha values of 0.75-0.87 and intra-class correlation coefficients (ICC) greater than 0.7.

Conclusion: This study led to development of a valid and reliable scale for examining infertile couples' decision-making about whether or not to use donor conception as well as the determinants of this decision.

Keywords: Decision-Making, Donor Conception, Infertility, Validation

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Introduction

Advances in assisted reproductive technology (ART) offer new methods of getting pregnant and make parenthood possible for people deprived of having children for various reasons (1). Although these technologies are a 'marriage saver' for those left without a child (2), give hope to millions of infertile couples (3, 4) and help them to realize their dream of raising a family (5), not all infertile couples use reproductive technologies (6) and the demand for these treatments is unexpectedly low (7). In fact, only half of infertile couples around the world seek treatments (7, 8). Deciding whether or not to use these technologies is definitely difficult (9), and many sociocultural, ethical, legal and religious challenges surrounding different aspects of ART, such as donor conception, can affect the practical use of these technologies (3, 4).

Deciding to use these technologies is influenced by people's perceptions and the society's expectations and attitudes toward their use (6). In other words, sociocultural beliefs affect couples' tendency toward using these methods (10, 11) and influence the rate of employment of these technologies by couples (12). Infertile couples who have a child born through donor conception, experience great prejudice not only by the society but also by their family, relatives and friends. In developing countries, the family's rejection and social pressures are among the factors affecting the decision about seeking a method of treatment and the choice of treatment is made under the heavy influence of family

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members (13). Many infertile couples suffer from the stigma of infertility and seeking treatment, and try to keep their condition a secret (14). They feel that they will be ethically judged for their infertility and their decision to use ART (15). The individual's beliefs and attitudes may be the most important determinant of his/her actions. Individuals with strong spiritual beliefs and specific sociocultural beliefs may adopt approaches and treatment methods that are different from those adopted by other infertile individuals, and their use of donor conception is also influenced by different factors, as they attribute different meanings to their condition and its treatment and interpret them differently (16). Some infertile couples for whom donor conception is the only way of becoming parents, they might prepare themselves for a childless life or accept to adopt a child and reject medical treatments. Some others, in contrast, try all the available treatments in different medical centers and greatly invest for this goal both in material and emotional terms (17). Sociocultural beliefs may also affect people's religious beliefs (18). In other words, cultural factors can reinforce or inhibit religious attitudes toward the use of ART. Religion also plays a major role in the use of ART, as it affects people's views and social norms. It is difficult to have access to ART in countries with religious dogmatism (2). The decision on the employment of ART is made according to the laws of the society (19). Laws have a significant effect on the access to ART (2). In some countries, donation is a process, while in others, there are limited rules. In New Zealand, embryo donation is a key process that is based on rules and policies (20), while in Australia, there are few rules about the donation process (21). Laws are largely based on the sociocultural state of the society and its ethical, spiritual and religious values (19, 22). The limited number of donors is also one of the main practical factors affecting most couples' decision about the selection of a donor (23). Economic issues also affect the access to ART (24).

Deciding about the use of donor conception services is therefore a complicated and difficult process for couples which challenges their values and beliefs. Making this decision is a complicated social and interactive process that is under the influence of various individual, social, economic, cultural, psychological and ethical factors and is affected by the couple's interactions with each other and with their family, friends, health workers, key people, etc. It is therefore necessary to develop a scale for identifying the determinants of infertile couples' decision about using donor conception to perform supportive interventions that improve the decision-making process and reduce the outcomes of the decision including regret. A review of the literature did not show any instruments developed for direct measurement of the subject in question. Given the complexity of the decision-making process about this issue and the absence of an instrument for its assessment, the present study was conducted to fill the gap, develop a decision-making for donor conception questionnaire (DMDCQ) and determine its psychometric properties in Iranian infertile couples.

The scale developed in this study measures the determinants of infertile couples' decision-making and can help specialists to understand the issues around infertile couples' decision making concerning the use of ART and design individual and public training programs and instructional decision-making packages for resolving the barriers and thus reducing the need for unnecessary interventions.

Materials and Methods

This cross-sectional validation study was performed using the method developed by DeVellis in 2012 (25) in four steps, after combining some of the stages:

First step: Performing a qualitative study and extracting the dimensions or constructs of the intended concept

In the first step, the concept under measurement (i.e. decision-making for donor conception) was theoretically defined. For the first step and in order to explain participants' experiences regarding the process of decision-making for donor conception, a qualitative study with a grounded theory approach was performed in 2014 in Mashhad, Iran, using individual interviews. A total of 38 participants including nine eligible infertile couples (four couples who were candidates for receiving egg donation, three couples candidates for receiving embryo donation, one couple candidate for receiving egg and uterus donation and one couple candidate for receiving uterus donation) and 14 eligible women (seven egg donor candidates, four embryo donor candidates, one egg and uterus donor candidate and two uterus donor candidates), were enrolled. The key people involved in decision-making for donor conception, including two gynecologists, two midwives and two clergymen, were also interviewed during the theoretical sampling, and this process was continued until the saturation of the categories without any restrictions on the number of participants and according to the theoretical requirements of the study.

The inclusion criteria were being married, Iranian, and infertile (either male or female infertility or both), having no biological or adopted children, nor other spouses, having the experience of using at least one ART in the past or being under treatment with ART or in the waiting list to receive ART, being willing to participate in the study and being able to communicate and express their experiences. The selected members of the infertility treatment team had at least one year of experience of working with infertile couples. The selected clergymen were experts in this field and were interested in participating in the study. The study was performed at Milad Infertility Clinic, Mashhad, Iran. The participants were selected through purposive

convenience sampling with maximum variation in terms of age, duration of infertility, duration of treatment, education and socioeconomic status. Sampling was continued until the saturation of the data. Data collection was mainly done through semi-structured in-depth interviews directed by the interview guide, that enabled the participants to freely discuss the matter. All interviews were done by one of the researchers. The interviews were conducted separately with the infertile men and women, but a couple interview was also held with both the husband and wife if there was an obvious difference in their answers. Each interview took 40-120 minutes and was held in one or more sessions. The interviews were recorded with participants' permission. Data were analyzed concurrently using MAXQDA-2007 and five dimensions ultimately emerged. The approval of the local Research Ethics Committee of Shahid Beheshti University of Medical Sciences was obtained along with the informed consent of all participants before beginning the study.

Second step: Producing an item pool using an inductive method

In the second step, an item pool was produced using an inductive method; for this purpose, items relevant to the main concepts of donor conception decision-making were developed based on the qualitative findings of the study (n=170). Participants' attitude toward each item was measured on a 5-point Likert scale from "quite agree" to "quite disagree".

Third step: Initial items reduction

In the third step, the initial items extracted from the qualitative study were reviewed by the research team and the repetitive and ambiguous items were removed. Eventually, 113 items were developed in five dimensions, including being offered to use donor conception (10 items), inner turmoil (4 items), attempts for coping with the current conditions (23 items), deciding to accept and use donor conception (54 items) and deciding to undergo treatment (22 items).

Fourth step: Validation of the questionnaire through assessing its face validity, content validity, initial reliability, construct validity and final reliability

The face validity of the questionnaire was evaluated both qualitatively and quantitatively in the fourth step. To perform the qualitative evaluation, face-to-face interviews were conducted with ten similar members of the target group (four infertile men and six infertile women who met the inclusion criteria) and difficulties in understanding the words and phrases, the degree of inappropriateness of the phrases or their irrelevance to the questionnaire dimensions, ambiguities causing misunderstanding of the phrases, or the words failing to convey a meaning, were examined. Once the items were modified according to the received feedback, the item impact was measured quantitatively. The objective in this step was to determine the item impact score in a sample that was similar to the target group. For this purpose, each item was scored on a 5-point Likert scale as follows: 5: "quite important", 4: "somewhat important", 3: "relatively important", 2: "slightly important", and 1: "not important at all". Ten individuals similar to the target group (four infertile men and six infertile women who met the inclusion criteria) were asked to determine the importance of each item based on their own experiences. The researcher calculated the impact score (IS) for each item separately based on the following equation (26):

Impact score=Frequency percentage×level of significance

Frequency percentage=The percentage of all the people who have reviewed each item

The items with an IS <1.5 were considered inappropriate and removed from the questionnaire (26).

The content validity of the questionnaire was evaluated both qualitatively and quantitatively. For the qualitative assessment of the content validity, the questionnaire was distributed among ten specialists (Ph.Ds in reproductive health or health education, and a number of gynecologists) and they were asked to give their feedback on the questionnaire. The content validity ratio (CVR) and content validity index (CVI) were used for the quantitative assessment of the content validity.

To determine the CVR, ten specialists were asked to review each item on a 3-point scale (3: necessary, 2: useful but not necessary, and 1: not necessary). The CVR was then calculated based on Lawshe's formula as follows (27-29).

$$CVR=(ne–N/2)/(N/2)$$

ne: The number of specialists who have selected the "necessary" response

Based on Lawshe's Table of minimum values, items with a CVR >0.62 as per the evaluation of the ten specialists, were deemed significant ($P<0.05$) and remained in the questionnaire (27-29).

The CVI for each item was examined based on the Waltz and Bausell CVI and the three criteria of simplicity, specificity (relevance) and clarity were separately measured on a 4-point Likert scale by the ten specialists. To calculate the CVI for each item, the total number of specialists who had given 3 and 4 points (i.e. the highest score) to that item was divided by the total number of specialists (n=10). The items with a CVI >0.79 were deemed acceptable (27-29). The items with a CVI of 0.7-0.79 were reviewed by the researcher and discussed again with the specialists. The items with a CVI <0.7 were eliminated from the questionnaire (30).

After determining the face and content validity, the initial reliability was calculated as the item analysis index. For this purpose, 30 infertile men and women visiting the infertility clinic were selected by convenience

sampling to complete the initial questionnaire, and the Cronbach's alpha was calculated to determine the internal consistency for each factor as well as the entire scale. Cronbach's alpha values of 0.7 were considered favorable in this study.

The construct validity was determined by exploratory factor analysis. For analysis of the data, the exploratory factor analysis was performed in seven steps: determining the sample size, examining the correlation between the items, deciding about the items being fit for the factor analysis, determining the number of initial factors extracted, rotating and extracting the final factors and naming the factors.

According to Tabachnick and Fidell (31), evaluation of the construct validity requires a sample size that is three to five times larger than the number of items in the scale. Given the number of items in the final questionnaire (i.e. 69) and the potential sample loss, 220 subjects were included in this study. The inclusion criteria consisted of being married, Iranian, infertile (with male and/or female infertility) and candidate for ART [intrauterine insemination (IUI), *in vitro* fertilization (IVF), gamete intrafallopian transfer (GIFT), and intracytoplasmic sperm injection (ICSI)], and having enough information about donor conception.

The correlation between each item and the other items was examined by principal component analysis (PCA), and the items that had correlation with the other items of <0.3, were eliminated from the analysis.

The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy was used to ensure the adequacy of the samples. If the KMO measure is >0.70, the set of data is deemed fit for factor analysis. Bartlett's test of sphericity was also used to examine the fit of the data for the factor analysis. If the P value is <0.05 in this test, factor analysis is considered an appropriate technique (32). The community statistic was used to detect inappropriate items whose variance was not used for explaining the variance of the main factor. In this study, the inflection point of 0.4 was taken as the minimum factor loading required for keeping each item in the factors extracted through the factor analysis. To extract the required number of factors, a scree plot (Fig.1) and eigenvalues were used and the percentage of variance of each factor was calculated. The factors with eigenvalues >2 remained in the study. The final factors were extracted by varimax rotation.

The reliability of the questionnaire was examined using the internal consistency and test-retest stability methods. To measure the internal consistency, 30 infertile men and women visiting Milad Infertility Clinic were selected by convenience sampling to complete the questionnaire, and Cronbach's alpha values were calculated for each factor and the entire questionnaire. Cronbach's alpha values of ≥0.7 were deemed acceptable. To determine the stability of the questionnaire, 20 infertile men and women completed the questionnaire within a two-week interval and the intraclass correlation coefficient (ICC) was then calculated. An ICC >0.70 was deemed acceptable (33).

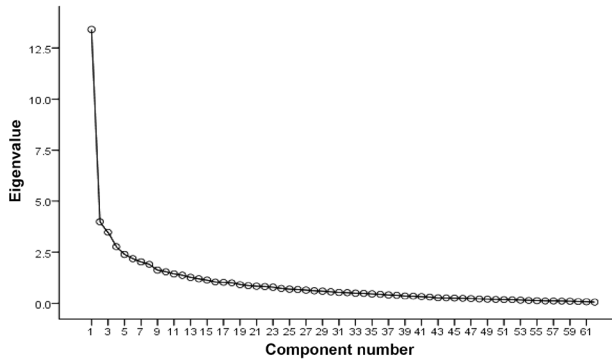

Fig.1: Scree plot.

Results

A total of 220 infertile men and women who met the inclusion criteria participated in this psychometric assessment. Table 1 presents the demographic and infertility characteristics of the participants.

Table 1: The demographic and infertility-related characteristics of the participants

Participants' characteristics n=220	n (%) or mean ± SD
Sex	
Male	55 (25)
Female	165 (75)
Education	
Below high school diploma	50 (22.7)
High school diploma	72 (32.8)
Associate or bachelor's degree	86 (39.1)
Master's degree or higher	6 (2.7)
No answers	6 (2.7)
Age (Y)	29.7 ± 5.08
Infertility duration (month)	63.4 ± 43.2
Treatment duration (month)	34 ± 32.5
Infertility cause	
Ovarian	64 (29.1)
Uterine	6 (2.7)
Ovarian and uterine	15 (6.8)
Tubal	12 (5.5)
Endometriosis	6 (2.7)
Male factor	56 (25.5)
Unknown	41 (18.6)
No answers	20 (9.1)
Family history of infertility	
Yes	79 (35.9)
No	137 (62.3)
No answers	4 (1.8)

SD; Standard deviation.

Based on the results of the qualitative content analysis, an item pool was composed of 170 items, and the ambiguous and repetitive items were removed after the revision done by the research team. Eventually, 113 items were developed in five dimensions or constructs, including being offered to use donor conception (ten items), inner turmoil (four items), attempts for coping with the current conditions (23 items), deciding to accept and use donor conception (54 items) and deciding to undergo treatment (22 items), which entered the psychometric assessment phase. The evaluation of face validity, which was performed qualitatively and quantitatively, led to the removal of eight items, and the questionnaire entered the content validity evaluation stage with 105 items. The content validity was also evaluated both qualitatively and quantitatively and 28 items were removed, leading to the existence of 77 items. In the stage of initial reliability evaluation, the Cronbach's alpha was calculated separately for each item, eight items were removed, and the remaining 69 items entered the construct validity evaluation stage. It should be noted that the questionnaire's reliability increased to over 0.7 once these items were removed, and according to the researcher, their removal did not destroy the basic information required. The initial Cronbach's alpha calculated for the entire scale was 0.82.

To determine the construct validity of the scale, 220 participants were selected through convenience sampling to complete the questionnaire. There was no sample dropout. The collected data were entered into SPSS-22. The PCA showed that the correlation between two of the items and the other items was <0.3; thus, both of these items were removed and the factor analysis was continued with 67 items. The KMO measure for the items was 0.768, which indicates the sampling adequacy. The Bartlett's test of sphericity showed the fit of the data for the factor analysis with $P<0.001$. The community statistic was >0.4 for most of the items in this study and the items were thus considered fit for factor analysis. Five items with a community statistic <0.4 were excluded from the study, and the factor analysis was continued with 62 items. Determining the number of factors constructing the questionnaire using the factor analysis of the items led to the identification of seven factors with eigenvalues >2 and explaining 48.796% of the total variance. The items were rotated and categorized in each factor using a varimax rotation. Of the 62 items that entered the factor analysis in this study, 51 items and seven factors remained.

The factors were named based on the meaning of their items, especially the meaning of the item with the maximum factor loading, and with regard to the correlation found between the items and the available theoretical knowledge. The researcher referred to the qualitative part of the study and the categories and subcategories forming each item, in order to name the factors (Table 2).

Table 2: The factor loading of the Desision-Making for Donor Conception Questionnaire items in Iranian infertile couples

	Factor						
	1	2	3	4	5	6	7
Factor 1: The role of social networks It is difficult for me to accept donor conception because of people's negative attitude toward this method.	0.638						
The treatment team's commitment to keep my information confidential is important to me to accept donor conception.	0.622						
The positive experiences of people who have used donor conception affect my decision to accept this method.	0.608						
The treatment team's honesty in explaining the cause of infertility affects my decision to accept this method.	0.577						
The infertility clinics' provision of clear information about the costs of donor conception affects my decision to accept this method.	0.559						
I need more time for making a decision to accept this method.	0.558						
I may have to accept donor conception in order to save my marriage.	0.554						
Clergymen's approval of donor conception helps me to decide about these methods more quickly.	0.552						
The existence of laws about donor conception affects my decision to accept this method.	0.542						
The society's familiarity with donor conception helps me to accept this method easier.	0.531						
Consulting sessions held before and during treatment with donor conception affect my decision-making.	0.469						
The failure to provide clear and proper information about donor conception affects my decision to accept this method.	0.459						
I may have to accept donor conception in order to free myself of other people's babble.	0.455						
Other people's refraining from interfering in our childbearing or way of childbearing affects my decision to accept this method.	0.427						

Table 2: Continued

	Factor						
	1	2	3	4	5	6	7
Factor 2: Coping strategies							
When offered to use donor conception, practices such as praying can make me calm and enable me to make a more rational decision		0.782					
The belief in God's will makes me peaceful and affects my decision about whether or not to accept this method		0.718					
When offered to use donor conception, changes in lifestyle, such as working more, make me think less about my problem and make a more rational decision.		0.539					
When offered to use donor conception, thinking about positive issues makes me calmer and enables me to make a more rational decision.		0.535					
Factor 3: The decision to disclose or conceal							
If I use donor conception, I won't inform others of my decision because I fear that my child may accidentally learn of the matter from them.			0.760				
The possibility of concealing the matter from others affects my decision about whether or not to accept donor conception.			0.714				
If I use donor conception, I won't inform others of my decision, because I fear their negative reaction (blaming, humiliation and ridicule) toward myself and my child			0.688				
If I decide to use donor conception, I will hide it from my child.			0.681				
If I decide to use donor conception, I may change my job or address			0.558				
If I decide to use surrogacy services, I will try to pretend to be pregnant.			0.526				
If I decide to use donor conception, I will inform my first-degree relatives (mother and sister) in order to get support from them.			0.422				
Factor 4: Interpersonal relationships							
I feel that if I decide to use donor conception, my emotional relationship with my husband might suffer				0.738			
I feel that if I decide to use donor conception, my sex life might suffer.				0.726			
If I decide to use donor conception, I reduce my relationships with others				0.575			
Factor 5: Religious quests							
If I decide to use donor conception, I won't inquire into the religious aspects of using these methods, because they are being performed in official infertility clinics in an Islamic country					0.564		
If I decide to use donor conception, I will ask people who have previously used these methods about its religious issues					0.464		
If I decide to use donor conception, I will seek the fatwa of other religious references in order to reach my goal of having a child, if my own religious reference opposes this method.					0.417		
Factor 6: Donor's characteristics							
If I decide to use donor conception, the donor's characteristics won't matter much to me; the only thing that will matter to me is to find the donor faster						0.802	
If I decide to use donor conception, I won't inquire much into the donor's background, because I have to accept her with any conditions due to the limited number of donors						0.731	
If I decide to use donor conception, I will prefer to use the services of a donation center in order to avoid future disturbances by the donor						0.696	
If I decide to use donor conception, I will try not to inquire much into the donor's background, because it may dishearten her and make her change her mind						0.695	
If the decision to use donor conception becomes certain, I will prefer a known donor because of her availability and the shorter waiting time						0.580	
If I decide to use donor conception, I will prefer to use donation centers because I can access the donor faster that way						0.576	
If the decision to use donor conception becomes certain, I will inquire greatly into the donor's background before selecting her						0.483	

Table 2: Continued

	Factor						
	1	2	3	4	5	6	7
If I decide to use donor conception, the donor's moral health will be the most important selection criterion for me						0.477	
If I decide to use donor conception, I will prefer an unknown donor because I fear others' learning of my decision						0.429	
Factor 7: Challenges in the process of treatment							
If I decide to use donor conception, the unavailability of a donor will be one of the main barriers							0.642
A better coordination between infertility clinics and the legal authorities shortens the duration of the legal procedures and accelerates the decision to use donor conception							0.567
If I decide to use donor conception, I will use a method that best fits my mental conditions							0.604
The lengthy and time-consuming stages of donor conception make me delay the decision to undergo this treatment							0.599
If I decide to use donor conception, I will choose a clinic that costs less							0.572
The support of others (including my spouse and family) accelerates my decision to use donor conception							0.565
If I decide to use donor conception, I will choose a clinic that has served longer and has more experienced personnel							0.551
The high cost of treatment is a barrier to my decision to use donor conception							0.638
If I decide to use donor conception, I will use a method that has the shortest waiting time							0.488
The availability of medical facilities at nearby infertility clinics accelerates my decision to use donor conception							0.481
If I decide to use donor conception, I will try to resolve the barriers with various solutions							0.449

Table 3 summarizes the number of items in each subscale and the range of scores for the entire DMDCQ and its subscales.

Table 3: The range of scores for the total and subscales of the DMDCQ

Subscale	Number of items	Range of scores
Role of social networks	14	14-70
Coping strategies	4	4-20
The decision to disclose or conceal	7	7-35
Interpersonal relationships	3	3-15
Religious quests	3	3-15
Donor's characteristics	9	9-45
Challenges in the process of treatment	11	11-55
Total	51	51-255

Table 4 summarizes the mean and standard deviation of the total and subscale scores of the DMDCQ in the entire sample of participants. When the total score of the questionnaire and the scores of its subscales are higher, higher numbers of individuals make positive decisions and the couple will be more inclined toward donor conception in the future.

Table 4: The mean and standard deviation (SD) of the total and subscale scores of the decision-making donor conception questionnaire (DMDCQ) in the entire sample (n=220)

Subscale	Mean ± SD	Min	Max
Role of social networks	53.57 ± 8.63	22	66
Coping strategies	17.75 ± 2.48	4	20
The decision to disclose or conceal	24.75 ± 5.16	11	35
Interpersonal relationships	8.41 ± 1.97	3	15
Religious quests	10.14 ± 2.76	3	15
Donor's characteristics	30.21 ± 5.26	9	45
Challenges in the process of treatment	43.20 ± 7.78	15	55
Total	188.50 ± 22.27	115	235

Min; Minimum and Max; Maximum.

The initial Cronbach's alpha was 0.82 for the entire scale and 0.75-0.87 for each subscale. The ICC was >0.7 for all the factors, which confirms the high reliability of the questionnaire (Table 5).

Table 5: The Cronbach's alpha and intraclass correlation coefficient (ICC) of subscales and the entire questionnaire

Subscales	Cronbach's alpha	ICC
Role of social networks	0.85	0.96
Coping strategies	0.79	0.80
The decision to disclose or conceal	0.83	0.91
Interpersonal relationships	0.75	0.78
Religious quests	0.76	0.84
Donor's characteristics	0.79	0.95
Challenges in the process of treatment	0.87	0.88
Total	0.82	0.86

Discussion

The questionnaire developed in this study is the first and only valid and reliable scale developed and psychometrically assessed in the world, concerning donor conception decision-making. The questionnaire consists of 51 items within seven factors, including the role of social networks, coping strategies, the decision to disclose or conceal, interpersonal relationships, religious quests, donor's characteristics and challenges in the process of treatment. These seven factors explained 48.796% of the total variance.

A review of the literature showed that no specific scale was developed for donor conception decision-making for infertile couples. Decision-making scales such as Flinders' decision-making questionnaire and the Melbourne decision-making questionnaire with different numbers of constructs, mostly address general issues.

Flinders' decision-making questionnaire was developed in 1982 by Mann, for the measurement of coping patterns and strategies for decision-making in conflict resolution and consists of 31 items and three constructs, namely vigilance, hyper vigilance and defensive avoidance (including procrastination, buck-passing and rationalization). Mann et al. (34) examined the construct validity (confirmatory) of Flinders' decision-making questionnaire in different cultural contexts (i.e. in the United States, Australia, Japan, Hong Kong, Taiwan and New Zealand). They eliminated the rationalization factor because it was not a good fit for the model and developed a new questionnaire called the Melbourne decision-making questionnaire, consisting of 22 items and four constructs, including vigilance, hyper vigilance and procrastination and buck-passing, and it replaced Flinders' decision-making questionnaire. Although the "rationalization" construct was eliminated from Flinders' decision-making questionnaire through the confirmatory factor analysis, coping strategies (including the use of rationalization and relaxation strategies) comprise an important factor of the DMDCQ, perhaps owing to the special nature of donor conception decision-making for infertile couples or because of the differences in the cultural contexts examined. A number of items from the Melbourne decision-making questionnaire was incorporated into the various items of the DMDCQ, such as the item "I may have to accept donor conception in order to free myself of other people's babble", which is similar to the item "I do not decide unless I really have to" in the Melbourne decision-making questionnaire.

Decision-making instruments about health issues include the decisional conflict scale (DCS), which measures decisional conflict in patients and contains 16 items and three subscales, including uncertainty in making a health-related decision, modifiable factors contributing to uncertainty and perceived effective decision making (35). This scale was translated into Dutch, French and Spanish and psychometrically assessed (36). Some of the items in the DCS have been incorporated into the various items of the DMDCQ, such as the item "The support of others (including my spouse and family) accelerates my decision to use donor conception", which is similar to the item "I have enough support from others to make a choice" in the DCS. A difference between the two scales is that one of the subscales in the DCS is about perceived effective decision-making, which indicates the user's degree of agreement about the informed decision, its compatibility with her personal values and her satisfaction with her decision. The scale developed in the present study, however, lacks a similar factor.

The decision-making scale for women with unplanned pregnancy is another decision-making scale in gynecology, which was developed by Nourizadeh et al. (37). This questionnaire consists of two scales that measure two important concepts of decision-making in women with unplanned pregnancy. The first scale measures the concept of perceived threats and is composed of 33 items within six factors, including fear of anomalies and violation of the norms, fear of difficulty and the aggravation of instability, fear of parental responsibility and commitments, fear of abortion and escape from abortion, role conflicts and social deprivations, and fear of negative physical-emotional consequences. The second scale measures decision-making style and strategies in women with unplanned pregnancy and consists of 27 items within four factors, including resistance against acceptance, avoidance-justification strategies, analytical strategies and confirmatory strategies (37). Coping strategies (the use of rationalization and relaxation) comprise an important factor of the DMDCQ that is similar to the decision-making scale for women with unplanned pregnancy, in which justification strategies (rationalizing to oneself and others) also comprise an important factor. Some of the items in the decision-making scale for women with unplanned pregnancy have been incorporated into the various items of the DMDCQ, such as the item "If I use donor conception, I won't inform others of my decision, because I fear their negative reaction (blaming, humiliation and ridicule) toward myself and my child", which is similar to the item "I have hidden my pregnancy from others because I am inclined toward abortion and fear others' objection or obstruction of abortion" in the decision-making scale for women with unplanned pregnancy. The review of items showed that both scales emphasize the role of social norms in decision-making in a way that the violation of norms is a barrier to decision-

making. Consequently, people who decide to use donor conception may try to conceal it in order to avoid others' blames. A difference observed between these two scales is that confirmatory strategies comprised one of the factors in the decision-making scale for women with unplanned pregnancy, which is concerned with others' approval and indicates counseling for the purpose of making a rational and acceptable decision. The instrument developed in the present study, however, does not include such constructs.

The general strengths of the questionnaire developed in this study include its specificity and its ease of completion. The average time taken to complete the questionnaire was 10-15 minutes depending on the respondent's literacy.

One of the limitations of this study was the limited number of samples applying for donor conception in the only governmental infertility center in Mashhad. Other limitations included sampling from the men, as some of their wives opposed to be interviewed. Also, due to the uniqueness of the study tool, it was not possible to compare the results with other countries or check the tool's empirical validity. Respondent bias was another limitation of this study.

Conclusion

The DMDCQ can contribute to the development of an instructional decision-making package and supportive interventions for improving processes of decision-making and reducing negative physical and psychological outcomes and regrets by informing caregivers and counsellors about the circumstances and procedures of decision-making by couples.

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Authors' Contribution

F.H.-T.; Was responsible for the study design and implementation, the analysis of the data and the drafting of the manuscript. R.L.R.; Supervised the study design and the analysis of the data and revised the manuscript. M.S.; Supervised the study design and the analysis of the data. H.E.; Supervised the analysis of the quantitative data and the steps of the psychometric assessment of the questionnaire. All authors read and approved the final manuscript.

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Original Article

Effects of Melatonin Administration on Chemical Pregnancy Rates of Polycystic Ovary Syndrome Patients Undergoing Intrauterine Insemination: A Randomized Clinical Trial

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Abstract

Background: Oxidative stress as a potential cause of poor oocyte quality can influence a female's reproductive system. This study aimed to investigate the effects of melatonin on chemical pregnancy rates of a significant number of polycystic ovary syndrome (PCOS) patients undergoing intrauterine insemination (IUI).

Materials and Methods: In this double-blinded randomized clinical trial (RCT) study, the samples included 198 PCOS patients fulfilling the inclusion criteria and undergoing the IUI treatment. On the third day of menstruation, a 3-mg melatonin tablet or its placebo was given to the patients according to the randomized study protocol; this prescription was continued until the day of human chorionic gonadotropin (hCG) administration. The current study attempted primarily to scrutinize the effect of melatonin administration on the rate of chemical pregnancy and mature follicles during the IUI treatment cycle, and secondarily to determine the endometrial thickness (ET) on the day of IUI.

Results: The mean age of the participants in the study was 28.9 ± 5.5 years. The chemical pregnancy rate in the group receiving melatonin was about 32%, when it was 18% in the control group (P=0.012). Furthermore, it was concluded that the addition of melatonin to the treatment cycle of PCOS individuals could significantly improve the ET after the treatment (P<0.001).

Conclusion: The results of this study demonstrated that the treatment of PCOS patients undergoing IUI with melatonin significantly improves the rate of chemical pregnancy (Registration number: IRCT2017021132489N1).

Keywords: Mature Follicle, Melatonin, Polycystic Ovary Syndrome, Pregnancy Rate

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Introduction

As one of the most common endocrine disorders, polycystic ovary syndrome (PCOS), has complex pathophysiological characteristics, which have not yet been understood completely. PCOS involves about 5-10% of women of reproductive age and it seems to be an important cause of infertility (1). It is worth mentioning that the quality of oocytes plays a pivotal role in the development of clinical pregnancies. It has been reported that in humans a cause of infertility in women and an essential obstacle to successful *in vitro* fertilization (IVF) is the poor quality of oocytes (2). In many inclusive investigations, numerous therapeutic strategies have been suggested for patients with repeated implantation failures, such as hysteroscopy, endometrial injury, stimulation protocol modification, blastocyst transfer, assisted hatching, pre-implantation genetic screening for aneuploidy, and the supplementation of vitamins and antioxidants (3-7). According to the present reports, the probability of achieving a live birth after an assisted reproductive technology (ART) cycle is approximately 30% (8). Moreover, it should be mentioned that quite a few strategies have been examined over time to improve this rate (6, 9, 10).

Oxidative stress as a potential cause of poor oocyte quality can influence female reproduction (11). Current investigations have discovered that melatonin acts as a free radical scavenger and stimulates antioxidant enzymes, so it protects cells from oxidative stress (12, 13). Therefore, melatonin supplementation can protect oocytes from oxidative stress leading to the unsuccessful reproductive outcomes of women undergoing ART (14). A number of clinical trials have depicted that melatonin supplementation with or without other treatments has been considered as a valuable approach to improve the quality of oocytes and the outcomes of IVF in both PCOS patients and normal women (15-17).

Consequently, the aim of the present study was to investigate the effect of melatonin on the rate of chemical pregnancy among PCOS patients undergoing intrauterine

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insemination (IUI).

Materials and Methods

Study design and sample size

This study was performed as a randomized, double-blinded clinical trial study (RCT) with a parallel-groups design. It was carried out using a 1:1 allocation ratio for the intervention group receiving melatonin and the controls receiving placebo at Yas Hospital in Tehran, Iran. The study population consisted of the PCOS-diagnosed patients who had been referred to the hospital due to infertility problems from March 2017 to September 2017. The sample of this study contained 198 patients with PCOS, meeting our inclusion criteria to participate in the study, and being recommended to undergo an IUI treatment by their physicians (Fig.1). The written informed consent was obtained from all individual participants included in the study. The proposal of this research has been approved in the Ethics Committee of Tehran University of Medical Sciences (Ethics committee code: 25667).

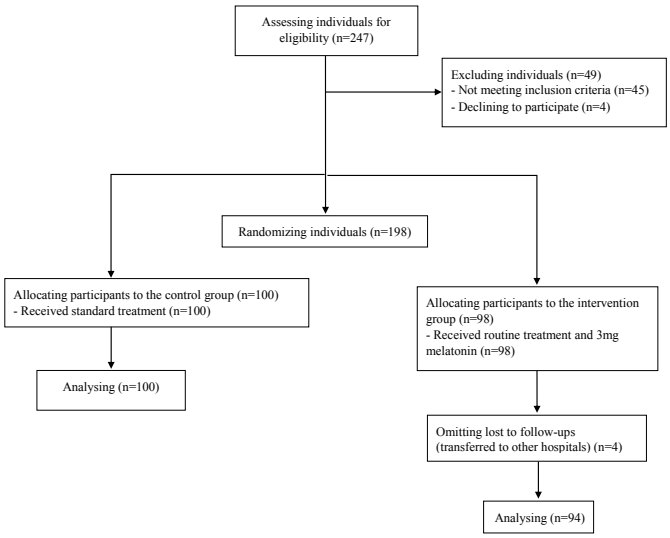

Fig.1: Flowchart of the study

Inclusion and exclusion criteria

The inclusion criteria were the following: i. Being aged between 20 to 40 years, ii. Having husbands with normal spermograms, iii. Having normal hysterosalpingography, iv. Having the Rotterdam diagnostic criteria for PCOS, v. Having no underlying endocrine diseases, and vi. Using no hormonal drugs within the past three months. Furthermore, the exclusion criteria included the following: i. Being deficient in an adequate ovarian response, ii. Suffering from ovarian hyperstimulation syndrome, and iii. No history of treatment for infertility.

Random allocation

In the present study, a random allocation was performed, and the participants were divided into an intervention group receiving melatonin and a control group receiving placebo using a balanced block randomization technique. Considering blocks of 4 in this study, the Stata software was used to generate random-number sequences from 1 to 6 until the desired sample size was achieved. Since the total number of modes to set two people in the blocks of 4 was 6 modes, if the generated number exceeded 6, the next number was regenerated regardless of the previous number. Preparing the random allocation sequences of the participants, putting them in sealed airtight envelopes, and numbering them with a five-digit serial number were all performed by a third person who was not involved in the study design. All the envelopes (n=188) having a random 5-digit serial number were opened immediately after the completion of basic information and examination of the participants. Then, the participants were assigned to the intervention or control groups.

Primary and secondary outcomes measured

In this study, the primary outcome was the determination of the rate of chemical pregnancies during the IUI treatment cycle and the secondary outcome was the determination of endometrial thickness (ET) on the day of IUI.

Sample size

Considering a type 1 error of 5%, a study power of 80%, and a difference of 20% between the chemical rates in the intervention and control groups, the sample size was estimated to be 93 patients in each group.

Treatment procedure

After initial evaluations by the physician, a basal vaginal ultrasound of the uterus and ovaries was performed during the days 1 to 3 of the menstrual cycle, using a Siemens ultrasound equipment (ACUSON X600, Germany), and the numbers of both antral follicles (AFC) and ET were recorded. Then, to induce ovulation two clomiphene citrate 50-mg tablets (Iran Hormone Company, Iran) were administered from cycle days 3 to 7. Furthermore, on the 3rd day of menstruation, a 3-mg melatonin tablet (Nature Made, USA) or its placebo (made by Faculty of Pharmacy, Tehran University of Medical Sciences) was given to the patients according to the randomized study protocol, and this prescription was continued until the day of hCG administration On the 10th day of menstrual cycle, another ultrasound was performed for both groups, and the thickness of the endometrium, AFC, and follicle sizes were recorded in millimeters. Subsequently, based on the information obtained at this stage, the need for 75 IU of human menopausal gonadotropin (HMG 75, Menogon, Germany) injection was estimated and it was injected intramuscularly by a trained nurse.

Vaginal ultrasound was performed periodically according to the needs of each patient by the physician until a suitable follicle size (i.e., greater than or equal to 18 mm) was seen. If the appropriate size of the follicle was observed, at the same time, estradiol was measured using Monobine kits and two hCG 500 ampoules (Choriomon, Swiss) were injected intramuscularly.

After the above steps, which took about 36-40 hours, the patients referred to the clinic and underwent IUI. Six days later, blood progesterone levels were measured by DRG

Progesterone ELISA Kit (Marburg, Germany). Then, 14 days after IUI, hCG-B blood test (GenWay Biotech. Inc, USA) was performed using an antibody kit to determine the rate of chemical pregnancy.

Statistical analysis

The normality assumption was assessed by the Kolmogorov-Smirnov test. Mean ± standard deviation (SD) and median (inter-quintile range) were used for presenting data with normal and non-normal distributions, respectively. Differences between the two groups of participants were assessed using independent Student's t tests. We used the Mann-Whitney test for continuous variables and chi-square tests for categorical variables. To evaluate the differences between the means of endometrial diameters in the intervention and control groups, an analysis of covariance with the adjustment of baseline scores was used. The Stata software (StataCorp LLC, version 13MP) was utilized to perform all the statistical analyses. Data with $P<0.05$ were considered statistically significant.

Results

In the present study, the information on the clinical infertility treatments of 198 infertile patients referring to Yas Hospital in Tehran, Iran, were used (Fig.1). The mean age of the participants in the study was 28.9 ± 5.5 years. In this study, 94 patients received melatonin as the intervention group and 100 patients received placebo as the control group. The results of comparing basic and clinical features of the participants in the study revealed that these two groups did not show any significant differences in the basic features at the time of entering the study. Table 1 summarizes their basic and clinical information.

Table 1: Basic and clinical features of the patients in the intervention and control groups

Variables	Intervention group (n=94)	Control group (n=100)	P value
Age (Y)	28.4 ± 5.5	29.3 ± 5.6	0.241
BMI (Kg/m^2)	27.6 ± 4.0	28.1 ± 3.7	0.056
Infertility duration (Month)	35.1 ± 21.7	43.5 ± 25.7	0.015
Primary endometrial thickness (mm)	4.6 ± 0.56	4.4 ± 0.52	0.093
Estradiol concentration (pg/ml)	1730 ± 281	1850 ± 534	0.001
IUI cycle duration (Day)	17.2 ± 2.6	16.8 ± 2.1	0.371
Total follicle count (n)	24.6 ± 4.7	23.9 ± 4.6	0.323
Infertility type			0.581
Primary infertility (%)	86 (91.5)	94 (94)	
Secondary infertility (%)	8 (8.5)	6 (6)	

Data are presented as mean ± SD or n (%)

Regarding the evaluation of the serum concentration of estradiol in both intervention and control groups, the results represented the mean of this hormone concentration in the control group as 1850 ± 534 pg/ml and in the intervention group as 1730 ± 281 pg/ml. An independent t test indicated that the two groups had significantly different levels of serum estradiol concentration (P=0.001).

The IUI cycle in the intervention group lasted for 17.2 ± 2.6 days and in the control group lasted for 16.8 ± 2.1 days. The result showed that there was no significant difference between the two groups (P=0.371). Furthermore, the results of Mann-Whitney test confirmed that there were no statistically significant differences between the rates of HMG doses used within the two groups (P=0.970).

In Table 2, the results of the ET, the number of mature follicles, and the chemical and clinical pregnancy rates are compared between the two groups. As demonstrated in Table 2, the chemical pregnancy rate in the group receiving melatonin was about 30%, while this value was 18% in the control group. The chi-square test indicated that the difference between these two values was statistically significant (P=0.011). Also, the addition of melatonin to the treatment cycles of PCOS individuals significantly improved the sizes of follicles during the IUI cycles (P=0.002). Regarding the mean ET of the patients, our covariance analysis showed that there was a significant difference between the intervention and control groups (P<0.001). The mean ET of the patients in the intervention group increased more than that in the controls.

Table 2: Comparison of IUI outcomes between the intervention and control groups

Variables	Intervention group (n=94)	Control group (n=100)	P value
Melatonin concentration (pg/ml)	190.7 ± 34.1	74.5 ± 17.1	<0.001[a]
Mature follicle (n)	2 (2-3)[b]	2 (1-3)	0.002[c]
Endometrial thickness after the treatment (mm)	9.2 ± 1.33	8.5 ± 0.87	<0.001[d]
Chemical pregnancy (%)	30 (32)	18 (18)	0.012[e]
Clinical pregnancy (%)	26 (27.6)	15 (15)	0.013[e]

Data are presented as mean ± SD or n (%). IUI; Intrauterine insemination, [a]; Independent sample t test, [b]; Median and IQR, [c]; Mann-Whitney test, [d]; ANCOVA test with adjusting baseline Endometrial thickness, and [e]; Chi-square test.

Discussion

The results of the present study suggest that following IUI, melatonin treatment has a favorable effect on mature follicles, ET, as well as chemical and clinical pregnancies in infertile PCOS women.

In recent studies, it has been shown that oxidative stress has an adverse effect on infertility treatments, so researchers are trying to find possible mechanisms of preventing these unfavorable effects (18). In this case, melatonin, an indoleamine synthesized from tryptophan, is a new oxygen scavenger, which can be used to improve pregnancy outcomes in infertile women (19, 20).

Studies at the molecular level have discovered that in pregnant rats, melatonin supplementation improves serum 17β-estradiol levels. Also, in rat uterine tissue, it enhances the expression of MT(1), MT(2) melatonin receptors, p53 receptor, and consequently may improve the uterine environment, thus playing an important role in embryo implantation at least in rats (21). However, it should be mentioned that based on Succu et al. (22), a high concentration of melatonin in embryo culture media could be harmful, as it

displays a degree of toxic activity on embryos.

In the present study, the results suggested that adding melatonin to the IUI treatment cycle could significantly improve the quality of follicles during the cycle in PCOS cases. Along with our findings, Eryilmaz et al. (23) in an un-blinded randomized controlled trial on IVF patients who were also suffering from sleeping disorders, observed a significant increase in the number of retrieved oocytes, metaphase II (MII) oocyte and grade 1 embryos after the prescription of 3-mg melatonin from days 3-5 until HCG injection. Batioglu et al. (24) revealed a higher percentage of MII oocytes and grade 1 embryos in a melatonin-treatment group. However, in their study, no significant difference was reported in the number of oocytes in women who underwent IVF cycles. Also a large number of studies have concluded that melatonin has a useful effect on retrieved oocytes, MII oocytes, and good quality embryos (17, 25, 26).

In a recent study, Jahromi et al. (27) showed that in women with diminished ovarian reserve, the mean of grade 1 embryos and mature MII oocytes were significantly higher in the melatonin-treatment group in comparison with the control group, but there was no significant difference in other ART outcomes, such as grade 2 embryos and metaphase I (MI) oocytes. It was also reported that oxidative stress had an adverse effect on oocyte maturation and melatonin supplementation, protecting oocytes against oxidative stress (28). Nikmard et al. (29), in a study on mouse models, concluded that melatonin could significantly improve nuclear maturation of PCOS oocytes.

The results of the current study also revealed that the mean of ET in the melatonin-treatment group increased more than that in the control group. Therefore, melatonin has a favorable effect on ET in infertile PCOS patients following IUI. Unfortunately, there are not many studies on the relationship between melatonin and ET, but based on an animal study on rats, it is suggested that melatonin can affect the endometrial morphology and increase embryo implantation (30).

In the present study, chemical and clinical pregnancy rates were significantly higher in the melatonin-treated group compared to the placebo group. In a systematic review and meta-analysis of five published randomized controlled trials conducted by Seko et al. (31), a pooled risk ratio of 1.21 for the clinical pregnancy rate in favor of melatonin was revealed and this pooled risk ratio turned out to be significant [95% confidence interval (CI) 0.98-1.50].

In a relatively large randomized controlled trial in PCOS infertile cases undergoing intracytoplasmic sperm injection, researchers compared outcomes in two groups including myo-inositol 4g, folic acid 400mcg and melatonin 3mg per day (n=178) and myo-inositol and folic acid alone (n=180). The results showed that patients in the first group had greater numbers of mature oocytes and grade 1 embryos. These results, therefore, support the positive effect of melatonin in the treatment of PCOS infertile women (32).

In the present study, which was an attempt to provide novel information on the effects of melatonin on PCOS patients of a certain age group, some limitations existed, including the difficulty of frequent ultrasound scanning for patients until the right size of follicle was seen, and also patients' poor cooperation with the treatment due to their unfamiliarity with melatonin prescription. Besides, the study was conducted on PCOS cases with the mean age of 28.9 years old and just IUI cycles were included in this study. Therefore, the obtained results are generalizable to infertile women suffering from PCOS in a relatively young age group. It is suggested to conduct a multi-center RCT to obtain more generalizable and valid results.

Conclusion

The results of this study demonstrated that the treatment of PCOS patients undergoing IUI with melatonin can significantly improve the quality of follicles and, as a consequence, the rate of chemical pregnancies.

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Authors' Contributions

O.A., F.A.A.; Conducted under the supervision. F.M.; Designed, executed, and analyzed the manuscript. O.A.; Drafted the manuscript. F.A.A., M.B., F.M., A.A.-H.; Interpreted the data, reviewed the manuscript critically, and revised it for a noteworthy intellectual content. F.M., M.B.; Analyzed the data, reviewed the manuscript critically, and revised it for an important intellectual content. All authors read and approved the final manuscript.

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Original Article

Association between Sexual Activity during Menstruation and Endometriosis: A Case-Control Study

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Abstract

Background: The prevalence of endometriosis in the general population is estimated at 7-10%. There are various risk factors for this disease, including early menarche age, prolonged menstruation or no history of pregnancy. It seems that sexual activity leading to orgasm during menstruation increases the retrograde menstruation, sending endometrial tissue to an abnormal sites and thus increasing the risk of endometriosis. The present study is aimed to determine the association between sexual activity during menstruation and endometriosis.

Materials and Methods: This case-control study, conducted in the year 2017, recruited 555 women who were visited at Alzahra Hospital in Tabriz, Northwest of Iran. The case group comprised 185 women of reproductive age with confirmed endometriosis. The control group comprised 370 women of reproductive age without endometriosis visiting the hospital for other issues. Data was collected using a researcher-made questionnaire based on previous studies. Bivariate analysis was performed by the chi-squared test and multivariate analysis was done using conditional logistic regression to control confounding variables.

Results: The sexual activity of the two groups during menstruation was significantly different. The occurrence of endometriosis in women who stated they had vaginal intercourse or non-coital sexual activities, leading to orgasm during menstruation, was significantly higher compared to those who stated they did not.

Conclusion: According to our findings, there is an association between sexual activities leading to orgasm during menstruation and endometriosis.

Keywords: Endometriosis, Menstruation, Orgasm, Sexual Activity

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Introduction

Endometriosis is a benign gynecologic disease (1) defined as the presence of uterine endometrial stroma and glands somewhere other than their natural location (i.e. uterine endometrial cavity). The most frequent places in the pelvic cavity include ovaries, uterosacral ligaments, and the recto-uterine pouch (2). Symptoms of endometriosis are dysmenorrhea, dyspareunia, chronic pelvic pain, irregular menstruation, and/or infertility (3). Although endometriosis is considered to be a disease of the 21st century, the first references and related symptoms were discovered in ancient Egypt in 1500 BC (4). The prevalence of endometriosis in the general population is estimated at 7-10% (1). Endometriosis is one of the causes of primary and secondary infertility in 30% of women (2).

Endometriosis has a complex and multifactorial etiology (5). The factors involved in the development of endometriosis include hormonal changes (6), genetic changes (7), and changes in the immune system (8). It has been well documented that endometriosis may be present for a long time before it is diagnosed (9). This is especially observed in European countries, as the overall delay in diagnosing the disease is ten years in Austria and Germany, eight years in Spain and UK, seven years in Norway, seven to ten years in Italy and four to five years in Ireland and Belgium (10-12).

Although no theory can cover all the manifestations of this disease, the retrograde menstruation is widely accepted to describe the dissemination of endometrial tissue to the peritoneal cavity through open fallopian tubes during menstruation (3, 5). Previous studies report that in 90% of healthy women with open fallopian tubes menstrual blood is present in the peritoneal cavity, as shown by laparoscopy (13). However, it is assumed that the level and volume of retrograde menstruation and the backward movement of endometrial cells have significant effects on the emergence

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and improvement of endometriosis (14). Studies on uterine pressure during menstruation have shown that myometrium and fallopian tube contraction significantly increase during menstruation and ovulation, respectively, supporting the theory of retrograde menstruation (15). Since this event is regarded as the major etiology for the improvement of endometriosis (9), it is essential to identify factors, which block menstrual blow flow and facilitate the retrograde movement of menstrual blood (16).

It is hypothesized that sexual activity leading to orgasm during menstruation may increase retrograde menstruation, seeding endometrial tissue in other locations, and thus increasing the risk of endometriosis. So far, few studies have examined the relationship between sexual activity during menstruation and endometriosis (16-18). Based on a case-control study in the Yale University School of Medicine, on the relationship between prevalence of endometriosis and sexual behaviors leading to orgasm during menstruation, the tendency to perform such activities during menstruation was lower in women with endometriosis compared to women without endometriosis (17). The results of another study at the University of Pennsylvania, Philadelphia, on the relationship among sexual activity during menstruation, endometriosis and pelvic inflammatory disease showed that endometriosis was higher in women who had sexual intercourse during menstruation compared to those who stated they did not (16). Based on the noted contradictory results and the need for further research, the aim of the present study is to answer the question of whether sexual activity leading to orgasm during menstruation can be a risk factor for occurrence of endometriosis.

Materials and Methods

Study type and participants

This case-control study, which was done in 2017, recruited women at reproductive age (20-50 years), with or without endometriosis. The participants in the case group were selected from women with endometriosis visiting Alzahra Hospital over the past two years, who had undergone laparoscopy and open surgery with a histological diagnosis of endometriosis. Participants in the control group were selected from the same age group of women visiting the same hospital for other reasons including vaginitis and an annual checkup. The absence of endometriosis in the control group was confirmed by a gynecologist colleague based on their signs and symptoms. The final selections were done based on our inclusion and exclusion criteria. The study inclusion criteria were: i. Age of 20-50 years, ii. Diagnosis of endometriosis by open surgery or laparoscopy and histologic diagnosis of endometriosis or the presence of endometrioma (case group), iii. Being married, iv. Being Iranian, v. Willingness to participate, vi. Absence of endometriosis (control group), vii. No history of tubectomy (control group), and viii. No history of infertility (control group).

The study exclusion criteria were: i. Being menopausal (amenorrhea for over a year), ii. Being suspected of endometriosis or endometrioma (control group), iii. Having endometriosis in the surgical site or involvement of remote organs, e.g. lungs or brain, iv. Having breast, ovarian, or endometrial cancer, v. Having polycystic ovarian syndrome (PCOS), vi. Having any other life-threatening disease, and vii. Suffering from chronic pelvic pain.

In this study, the sample size was determined according to the results of a pilot study on 150 participants and considering an odds ratio (odds of having sexual activity in the menstruation in the case group compared with the control group) to be about 1.8, was determined as n1=185 (case group), n2=370 (control group), and n=555 (total) (with the case-to-control ratio of 1:2).

Data collection

The present study was confirmed by the Ethics Council of Tabriz University of Medical Sciences (ethics code: 5/D1003687). Afterward, data collection was started in Alzahra Hospital, Tabriz, which is a referral Gynecology and Midwifery Hospital in Northwest of Iran. We reviewed pathological results in the medical files that were available at Alzahra Hospital and registered women patients with endometriosis, as confirmed by histological diagnosis through laparoscopy or open surgery, for our study. The addresses and phone numbers of all considering patients, who had been identified over the past two years, were extracted from their records. They were contacted by telephone, the research objectives and methods were briefly explained to them, the study inclusion and exclusion criteria were checked, and finally they were invited to participate in the study. For those who were willing to take part, questionnaires were filled in through the interview. For the patients' comfort, the interviewer and the patients were of the same gender. After sampling was done in the case group, the members of the control group were selected through purposive sampling from those visiting the gynecology clinic of the same center for other issues, such as vaginitis or an annual visit, and did not have endometriosis, as diagnosed based on symptoms by a gynecologist colleague. Research objectives and methods were first explained to them. For those who were willing to participate, inclusion and exclusion criteria were checked, and in case they met the criteria, they were recruited and questionnaires were completed by the researcher through interviews. Informed consent forms were obtained from all participants, and those in both case and control groups were matched for age ± 2 years.

Data collection tools

Data were collected by the researchers through interviews and using researcher-made questionnaires based on previous studies, highlighting sociodemographic and sexual activity characteristics. The sociodemographic characteristics questionnaire included questions on age; the level of education, employment, the level of income, smoking, alcohol use, the history of any diseases, allergies, and endometriosis in first-degree relatives (mother, sisters, and aunts). The sexual activity and reproductive and menstruation characteristics questionnaire included questions on vaginal or non-coital sexual activity (by touching other body parts by the person or her spouse to achieve sexual pleasure) and anal intercourse, leading to orgasm

during menstruation, cycle length, cycle intervals, number of pregnancies, menarche age, age at first pregnancy, oral contraceptive pill (OCP) user, intrauterine device (IUD) user, dysmenorrhea, dyspareunia, and recurrent vaginitis. Content and face validity were used to confirm the validity of the questionnaires, they were given to 10 faculty members and corrections were applied based on their opinions.

Data analysis

Data were analyzed in SPSS 21 software. Sociodemographic and sexual activity characteristics during menstruation were described using descriptive statistics including frequency (percentage). Sociodemographic characteristics were compared between the two groups using chi-squared test, chi-squared test for trend, independent samples t test, and Fisher's exact test. To determine the relationship between sexual activity during menstruation and endometriosis, chi-squared test was performed in the bivariate analysis. Conditional logistic regression was employed in the multivariate analysis to control confounding variables (level of education, level of income, occupation, cycle length, cycle interval, number of pregnancies, menarche age, age at first pregnancy, OCP user, IUD user, dysmenorrhea, dyspareunia and recurrent vaginitis). Because no woman in the control group reported a history of this disease in her first-degree relatives, the family history was not included in the multivariate regression as a confounding factor. In this analysis, the odds ratio and confidence interval was set at 95%, and P<0.05 was considered significant.

Results

In this study, 185 women with endometriosis and 370 women without endometriosis were analyzed. The participants' mean age was 35.21 years (SD: 7.09) in the case group and 35.28 years (SD: 7.03) in the control group. The two groups significantly were differed regarding the level of education; the percentage of participants with academic degrees in the case group was twice as high as those in the control group (P<0.001). Moreover, 32 (17.3%) women of the case group and 10 (2.7%) women of the control group were employed, again indicating a significant difference between the two groups (P<0.001). However, the two groups were similar regarding the sufficiency of monthly income (P=0.698). The two groups were compared in a history of diseases such as diabetes, hypothyroidism, hypertension, cardiovascular diseases, cerebrovascular diseases, seizures and asthma, and did not show any significant differences (P=0.860). The two groups were also similar regarding an autoimmune disease history, e.g. rheumatoid arthritis, multiple sclerosis, and lupus erythematosus (P=0.669). There were 38 (20.5%) women in the case group and 47 (12.7%) women in the control group with a history of allergies, indicating a significant difference between the two groups (P=0.016). Nevertheless, both groups were similar regarding the type of allergies (seasonal, food, drug, or skin) (P=0.946). In the case group 13 (7%) women reported a history of endometriosis in their mothers and sisters, and 7 (3.8%) women reported this in their aunts, while no woman in the control group reported a history of this disease in her first-degree relatives, demonstrating a significant difference between the groups (P<0.001). Only one woman in the control group had a history of smoking, and no one in either group had a history of alcohol use (Table 1).

Table 1: Comparison of sociodemographic characteristics in case and control groups

Social-demographic characteristic	Case n=185	Control n=370	P value
Age (Y)	35.21 (7.09)[§]	35.28 (7.03)[§]	0.909[**]
Education			<0.001[‡]
Illiterate/Primary	57 (30.8)	129 (34.9)	
Guidance/High school	37 (20.0)	82 (22.2)	
Diploma	39 (21.1)	107 (28.9)	
Academic	52 (28.1)	52 (14.1)	
Occupation			<0.001[*]
Housewife	153 (82.7)	360 (97.3)	
Employed	32 (17.3)	10 (2.7)	
Job type			1.000[†]
Doctor/University professor	7 (21.9)	2 (20.0)	
Employee	21 (65.6)	7 (70.0)	
Free	4 (12.5)	1 (10.0)	
Adequacy of monthly income			0.698[‡]
Weak	42 (22.7)	79 (21.4)	
Average	102 (55.1)	197 (53.2)	
Good/Very good	41 (22.2)	94 (25.4)	
Having a history of a disease	24 (13.0)	50 (13.5)	0.860[*]
Having a history of autoimmune disease	1 (0.5)	4 (1.1)	0.669[†]
Having a history of allergies	38 (20.5)	47 (12.7)	0.016[*]
The history of first-degree relatives			<0.001[†]
Yes	20 (10.8)	0	
No	165 (89.2)	370 (100.0)	

Data are presented n (%). [*]; Chi-squared test, [‡]; Chi-squared test for trend, [†]; Fisher's exact test, [§]; Mean ±SD, and [**]; Independent samples t test. Only one woman in the control group had a history of smoking, and no one in either group had a history of alcohol use.

Regarding vaginal intercourse during menstruation, the two groups were compared using multivariate logistic regression, while controlling the effects of possible confounding variables, such as the level of education, income, occupation, cycle length, cycle interval, number of pregnancies, menarche age, age at first pregnancy, OCP user, IUD user, dysmenorrhea, dyspareunia and recurrent vaginitis. The results showed that the risk of endometriosis approximately was five times higher in those women who stated they had vaginal intercourse during menstruation compared to those who stated they did not [(P<0.001), odds ratio (OR) (95% confidence interval (CI)=5.23 (2.16 to 12.66)]. Furthermore, 6 (20%) participants in the case group and 1 (3.6%) participant in the control group reported that they always had vaginal intercourse during menstruation, demonstrating a significant difference between the groups (P<0.001). Both groups were similar with regard to the days of vaginal intercourse (first three days, second 3 days, all days of menstruation) (P=0.111). Moreover, the risk of endometriosis

was approximately three times higher in those women who stated they had non-coital sexual activity during menstruation compared to those who stated they did not [(P=0.010), OR (95% CI)=2.90 (1.28 to 6.55)]. In addition, 9 (23.7%) participants in the case group and 6 (14.6%) participants in the control group reported that they always had non-coital sexual activity during menstruation, indicating no significant difference between the two groups based on a chi-squared test (P=0.141). Moreover, 2 (1.1%) participants in the case group and 15 (4.1%) participants in the control group stated that they have anal intercourse during menstruation, but there was no significant difference between the two groups [(P=0.130), OR (95% CI) = 0.08 (0.03 to 2.09)] (Tables 2, 3).

Table 2: Comparison of sexual activity during menstruation and reproductive and menstruation characteristics in case and control groups based on bivariate test

Sexual activity during menstruation and reproductive and menstruation characteristics	Case n=185	Control n=370	P value
Vaginal sex activity			0.002*
Yes	30 (16.2)	28 (7.6)	
No	155 (83.8)	342 (92.4)	
Sexual activity without vaginal penetration			0.003*
Yes	38 (20.5)	41 (11.1)	
No	147 (79.5)	329 (88.9)	
Anal sex activity			0.075†
Yes	2 (1.1)	15 (4.1)	
No	183 (98.9)	355 (95.9)	
Age at menarche			<0.001*
≤ 12	54 (29.2)	64 (17.3)	
>12	131 (70.8)	306 (82.7)	
Cycle interval			0.012*
≤ 28	109 (58.9)	176 (47.6)	
> 28	76 (41.1)	194 (52.4)	
Cycle length			<0.001*
≤ 7	136 (73.5)	357 (96.5)	
> 7	49 (26.5)	13 (3.5)	
Pregnancy number			<0.001*
0/1	102 (55.1)	80 (21.6)	
≥ 2	83 (44.9)	290 (78.4)	
OCP user			0.303*
Yes	75 (40.5)	167 (45.1)	
IUD user			0.017*
Yes	42 (22.7)	120 (32.4)	
Age at first pregnancy			0.037*
≤ 20	43 (30.1)	146 (40.0)	
> 20	100 (69.9)	219 (60.0)	
Dysmenorrhea			<0.001*
Yes	136 (73.5)	43 (11.6)	
Dyspareunia			<0.001*
Yes	82 (44.3)	7 (1.9)	
Recurrent vaginitis			<0.001*
Yes	50 (27.0)	18 (4.9)	

*; Chi-squared test and †; Fisher's exact test.

Table 3: Comparison of sexual activity during menstruation in case and control groups based on bivariate and multivariate logistic regression

Variable	Unadjusted		Adjusted	
	P value	OR (CI 95%)	P value	OR (CI 95%)
Vaginal sex activity	0.002	2.36 (1.36 to 4.09)	<0.001	5.23 (2.16 to 12.66)
Sexual activity without vaginal penetration	0.003	2.07 (1.28 to 3.36)	0.010	2.90 (1.28 to 6.55)
Anal sex activity	0.075	0.25 (0.05 to 1.14)	0.130	0.08 (0.03 to 2.09)

Conditional logistic regression was employed (P<0.1) in the multivariate analysis to control confounding variables: level of education, level of income, occupation, cycle length, cycle interval, pregnancy number, menarche age, age at first pregnancy, OCP user, IUD user, dysmenorrhea, dyspareunia, and recurrent vaginitis. CI; Confidence interval, OR; Odds ratio, OCP; Oral contraceptive pill, and IUD; Intrauterine device.

Discussion

The present study is the first study in Iran, which examined the association between sexual activity during menstruation and endometriosis. Our results revealed that vaginal intercourse and non-coital sexual activity leading to orgasm during menstruation increase the risk of endometriosis.

The case and control groups were significantly different regarding the level of education and occupation. Most recent epidemiological studies on risk factors for endometriosis have shown an increased incidence of the disease among women of high socioeconomic and occupational status (19). Results of the present study are consistent with the noted results. One possible justification for this relationship may be attributed to the diagnostic bias, because women of high socioeconomic status may have more awareness of their health-related issues (19-21). A strong evidence that shows the importance of a family history of endometriosis among women with the disease, is occurrence of endometriosis in twins (22, 23). In the present study, the participants in the case group reported a family history of endometriosis in their first-degree relatives, while no women in the control group reported a history of this disease in her first-degree relatives, therefore, family history was not employed into the model as a confounding variable.

There are few studies on the association between sexual activity during menstruation and endometriosis. For instance, Meaddough et al. (17) in the US explored the effect of sexual activity, orgasm, and health-related behaviors during menstruation on endometriosis. Results demonstrated that women with endometriosis were less willing to have repeated or occasional sexual activity during menstruation compared to those without endometriosis, a difference which turned out to be statistically significant. Moreover, in their study the case group reported less sexual activity leading to orgasm during menstruation than the control group, showing a significant difference between the two groups. Based on Meaddough's inference a possible explanation for these results could be the limitation of the research instrument or the presence of confounding variables such as dyspareunia, which was not included in the questionnaire. Dyspareunia may lead

to an unwillingness in women with endometriosis to have sex, thereby making them report a lower willingness than the control group. Furthermore, Meaddough concluded that sexual activity or orgasm during menstruation might facilitate the blood flow through the cervix (17). Other studies have shown that uterine contractions and pressure are increased during menstruation (16). Since sexual activity and orgasm during menstruation appear to increase uterine contractions as well, this type of activity may in fact lead to retrograde menstruation, which is the major etiology of endometriosis. In Meaddough's study, the number of women with and without endometriosis was determined based on self-report and not based on specialized criteria. Furthermore, questionnaires were sent and completed by the participants via email. Therefore, this study recommended further detailed studies on the effect of sexual activity during menstruation on the development of endometriosis (17). On the other hand, in the present study, women with endometriosis were selected based on histological diagnosis through laparoscopy, and those without endometriosis were selected based on signs and symptoms, inclusion and exclusion criteria, and confirmation of the absence of endometriosis by a gynecologist. Moreover, questionnaires were completed by the same interviewer for both groups, and all confounding variables were controlled as much as possible. To this end, the two groups were matched for age, and all possible confounding variables were controlled in statistical analyses.

Another study was conducted by Filer and Wu (16) in the University of Pennsylvania, Philadelphia to investigate the effect of sexual activity on endometriosis and pelvic inflammatory disease. The definite diagnosis of endometriosis and tubal factor infertility was done by laparoscopy or laparotomy. Subjects were asked about their history of sexual activity during menstruation and history of the pelvic inflammatory disease. The results showed that the prevalence of endometriosis was higher in women who tended to have repeated or occasional sexual activity during menstruation compared to those who did not. The prevalence of endometriosis was (17.5%) in women who had repeated or occasional sexual activity, and (10.9%) in those who rarely had sexual activity during menstruation, demonstrating a significant difference between the two groups. However, the groups were similar regarding pelvic inflammatory disease. Another study was conducted by Samir et al. (18) in Doha, Qatar, to examine sexual activity during menstruation as a predisposing factor for endometriosis. First, participants were asked about sexual activity during menstruation. A total of 78 participants were divided into two groups: the first group: 51 participants with a sexual activity history during menstruation and the second group: 27 women without a history of sexual activity during menstruation. Then, abdominal ultrasound, transvaginal ultrasound, or both were performed before laparoscopy or open surgery. Their results revealed that in the first group: 36 (66%) women tested positive for endometriosis and the second group: 9 (34%) were negative for endometriosis, showing a significant difference between the presence and absence of sexual activity leading to orgasm during menstruation and endometriosis (18). The results of the present study are consistent with the results of Samir's group. Based on our findings, the risk of endometriosis is approximately five times higher in women who stated they had vaginal intercourse leading to orgasm during menstruation and three times higher in those with non-coital sexual activity leading to orgasm during menstruation, compared to those who stated they did not.

In this study, there was no significant difference between the groups in terms of having anal intercourse leading to orgasm during menstruation. However, due to the limited number (only two women in the control group), a complete conclusion is not possible.

In this study, an attempt was made to select women with and without endometriosis based on precise medical diagnosis. Furthermore, the most important and relevant factors with endometriosis were examined while controlling confounding variables.

In this study, validity was only confirmed through face and content validity qualitatively and the quantitative indices such as content validity index (CVI) and content validity ratio (CVR) weren't calculated. Also, considering the criterion for the definite diagnosis of endometriosis is histologic diagnosis through laparoscopy or laparotomy, one of the other shortcoming of this study is that the control group wasn't selected based on histologic diagnosis. Thus, future studies should be conducted on selected case and control participants from women, in whom the presence or absence of endometriosis is confirmed by laparoscopy or laparotomy.

Conclusion

Based on the results of the present study, vaginal intercourse or non-coital sexual activity leading to orgasm during menstruation increases the risk of endometriosis in women during reproductive age. This study has raised interesting issues and requires further investigation to better understand the mechanism of occurrence of endometriosis in such cases.

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Authors' Contributions

S.M.; Study design and performance, data analysis, writing of the manuscript. B.S.O.; Corresponding author, monitoring and collaborating in study design, performance, analysis, and writing. M.K.; Monitoring and collaborating in study performance and writing of the manuscript. M.M.: Monitoring the study performance and the analysis of data, preparation of final manuscript, and

writing of the manuscript. N.A.; Design of methodology, monitoring the study performance and analysis of data. M.J.Sh.; Monitoring the sampling of study and checking inclusion/exclusion criteria and collaborated on completion of questionnaires. All authors read and approved the final manuscript.

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Original Article

Association of IL-1 and TNF-α Levels in Endometrial Secretion and Success of Embryo Transfer in IVF/ICSI Cycles

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Abstract

Background: In this work, we have determined the levels of interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), which function as cytokines in endometrial receptivity, through the endometrial secretion within the eligible individuals and thus studied their relationships with the success or failure of pregnancy in *in vitro* fertilization/intra cytoplasmic sperm injection (IVF/ICSI) cycles.

Materials and Methods: In this prospective study, 76 women were selected for their first IVF/ICSI and met the study inclusion criteria. All of the patients have undergone the endometrial secretion aspiration prior to performing the oocyte collection. The levels of IL-1and TNF-α were analyzed by the means of enzyme-linked immunosorbent assay method, using special standard kits. The patients were requested to undergo the serum human chorionic gonadotropin measurements and ultrasound evaluation for the purpose of detecting successful implantations and pregnancies.

Results: Among the 76 subjects of the study, 33 (43.4%) patients had a positive beta-human chorionic gonadotropin (β-hCG) and 44 (56.6%) resulted in a negative β-hCG. It should be also noted that through the patients with positive β-hCG, 23 (30.3%) of them displayed fetal heart rate in their transvaginal sonography (TVS). Compared to the group with failed pregnancies and their cytokine levels, we perceived a higher concentration of IL-1 in the group containing successful chemical pregnancies (P=0.00). However, there was no significant difference in terms of clinical pregnancy in the IL-1 levels between the two groups (P=0.06). In addition, there was not any notable difference in the levels of TNF-α between the two groups, neither in terms of chemical nor clinical pregnancy (P=0.8 and P=0.6, respectively).

Conclusion: The current study suggests that higher concentrations of IL-1 in endometrial secretions could be associated with improved endometrial receptivity and IVF outcome. With regards to TNF-α, no statistically significant difference was observed between the groups of with and without successful pregnancies.

Keywords: Embryo Transfer, Endometrium, IL-1/TNF-α, IVF/ICSI, Receptivity

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Introduction

Infertility is a common condition which can effect marital relationships, mental health and quality of life of couples (1-3). Recent advances in assisted reproductive techniques (ART), such as *in vitro* fertilization (IVF) and intra cytoplasmic sperm injection (ICSI), have resulted in development of effective methods for treating infertility. However, these methods are expensive and impose huge costs on families, while a significant number of IVF/ICSI procedures does not result in a live birth (2, 4).

The issue of endometrial preparation is largely overlooked since the infertility clinics often focused on the provision of appropriate quality embryos for transmission. The existing relationship between maternal immune system and embryonic tissues at the time of implantation is considered quiet vital for a successful implantation. This fact has been confirmed by one of the first letters of Betteridge (5). They discussed about the role of endometrial receptivity in the embryo transfer process, while indicating that the existence of an accommodation between the embryo and endometrium is necessary for pregnancy.

Several studies have been conducted to determine the effective factors that seemed to be responsible for the success of ARTs. One of these factors is the group of cytokines, produced by fetus and uterine mucosa. They are responsible for regulating the interaction between mother and fetus, ultimately causing the major influence on improvement of uterus receptivity (6).

Some of the certain known cytokines and growth factors that may contribute to increasing endometrial receptivity include interleukin-1 (IL-1), tumor necrosis factor-alpha

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(TNF-α), leukemia inhibitory factor (LIF), and transforming growth factor-beta (TGF-β) (7). For the first time, in 2003, a novel approach on the study of cytokines was presented by van der Gaast et al. (8), evaluating cytokines by analyzing the endometrial secretions. In fact, the endometrial tissue itself is not ideal for assessing biomarkers, due to the complexity of cell, its differentiation between different individuals and even different stages of the cycle. Furthermore, the uterine fluid collection, by either lavage or aspiration, is less invasive than tissue biopsy and ultimately the observed changes in this fluid can be sign of a true microenvironment for implantation. These discoveries have been discussed in detail through a study published by Salamonsen et al. (6).

Given the prevalence of infertility, high cost of fertility treatments and crucial role of cytokines in the success of these methods, the importance of identifying these cytokines and determining the best time for performing the process of embryo transfer is quiet evident. Therefore, in this study, we have determined the levels of IL-1 and TNF-α in endometrial secretion and assessed their roles in the success of embryo implantation.

Materials and Methods

Method of study

This prospective study has been conducted in the Infertility Center of Milad (Mashhad, Iran) from August to December 2017. Subsequent to performing sufficient explanation and signing the informed consent, 79 women enrolled the experiment, with the mean age of 32 years. They were candidates of obtaining their first IVF/ICSI due to tubal factor infertility caused by tubal obstruction (evidence of distal tubal obstruction in hysterosalpingography (HSG) was confirmed by laparoscopy).

The Research and Ethics Committee of Mashhad University of Medical Sciences (Iran) (IR.MUMS.fm.REC.1395.329) approved the study protocol. To determine the factors that are effective on the outcome of IVF, all subjects were provided with the following criteria for enrollment in this study: normal menstrual cycles between 21 and 35 days, less than 40 years of age, BMI less than 30, TSH values less than 10, FSH values less than 10 in the third day of the cycle and antral follicular count (AFC) with at least 5-7 in each ovary on the third day of cycle using the vaginal sonography, normal sperm analysis.

The exclusion criteria were also included: endocrine or metabolic disorders, history of previous pelvic or gynecological surgery (endometriosis, etc.). One or more than one occurrence of previous IVF failure, cigarette smoking, recurrent abortion, OHSS, inappropriate endometrium for embryo transfer (echogenic/non triple line and less than 7 mm), evidence of drosalpinx and uterine anomalies in HSG as well as transvaginal sonography (TVS) and other infertility causes.

In vitro fertilization/intra cytoplasmic sperm injection technique

To stimulate ovulation based on antagonist protocol, on the 3rd day of menstrual cycle, we have subcutaneously injected recombinant-follicle stimulating hormone (FSH) (Gonal-F) with a dosage of 150-225 IU/daily, depending on the AFC of each person. During serial vaginal sonography (using PHILIPS, Affinifi 70W device, Netherlands), after observing at least two follicles above 17 mm and follicular cohorts of 14-16 mm, 10000 U of urinary-human chorionic gonadotropin (hCG) were intramuscularly (IM) injected to induce the final oocyte maturation. Thirty six hours after hCG injection, we have performed the oocyte pick up process.

The luteal phase support was started from the day of pick-up by injection of 50 mg progesterone daily/IM. On the 4th day of progesterone, depending on the embryo grading, we transferred one to three cleavage embryo for each patient through the employment of one infertility specialist, utilizing cook catheter under transabdominal guide. The embryo grades were classified as follow: i. Embryo with the stage-specific cell size, <10% fragmentation and no multi-nucleation, ii. Embryo with stage-specific cell size for the majority of cells, 10-25% fragmentation and no evidence of multi-nucleation, and iii. Embryo with not stage- specific cell size, severe fragmentation (25%) and evidence of multi-nucleation (9) and the individuals conditions (such as the patients age).

The level of serum β-hCG was checked 14 days after performance of embryo transfer. Upon observing a positive result and an appropriate increase in the titer within 48 hours (confirmation of successful implantation), the patients were subjected to vaginal sonography between 4 and 5 weeks after embryo transfer, to confirm the clinical pregnancy by detecting the gestational sac and fetal heart rate.

Aspiration and endometrial secretion analysis

Before beginning the pick-up and after washing the perineum and vagina with normal saline, we exposed the cervix through the utilization of a speculum. Subsequent to washing the cervix with normal saline, a mannered catheter was employed to administer 2-3 ml of normal saline into the uterine cavity, using a 2-cc syringe. After 30 seconds of fluid infusion, the fluid was suctioned and transferred into a microtubule. The specimen was inscribed on the micro tube and stored in liquid nitrogen at a temperature of 80°C. This process was completely performed on all of the 76 samples. After collecting the 76 samples within five months, standard kits (IBL, USA) were used to measure IL-1 and TNF-α biomarkers by ELISA method. It should be noted that the safety of this method has been discussed and approved in previous studies.

Statistical analysis

The results of this study were collected in a coherent manner. After completing the statistical data of the in-

volved subjects, we performed the statistical analysis through application of SPSSII, version 23 and separately based on chemical pregnancy (positive serum β-hCG was checked 14 days after embryo transfer and successful implantation was confirmed by the appropriate increase) and clinical pregnancy (observing gestational sac and fetal heart rate (FHR) by TVS, 4-5 weeks after embryo transfer). In this experiment, $P<0.05$ was considered statistically significant.

Results

These 79 candidate women for their first IVF/ICSI were enrolled based on the provided criteria. Three cases were excluded, one due to the occurrence of OHSS while the other two contained inappropriate endometrium, followed by freezing their embryos. As the last step, the aspirated endometrial secretions of the enrolled candidates were evaluated for IL-1 and TNF-α mean levels by ELISA method. Findings showed among the 76 patients, 33 of them carried positive β-hCG (chemical pregnancy) and 43 candidates had negative β-hCG. Based on positive FHR in TVS (clinical pregnancy) throughout the 76 patients, 23 of them have shown FHR positive while 10 of the have resulted in FHR negative.

There was not any significant statistical difference between these two groups in demographic characteristics including age, body mass index (BMI), duration of infertility, AFC and 3rd day FSH as well as number and grade of transferred embryos, which have been mentioned in details in Table 1.

Table 1: Baseline and clinical characterization of pregnant and non-pregnant groups

Characteristic	β-hCG$^+$ n=33	β-hCG$^-$ n=43	P value
Age (Y)	33.3 ± 5.3	32.8 ± 5.3	0.7
Duration of infertility	6 ± 4.2	7.3 ± 4	0.1
Number of transferred embryos	1.8 ± 0.4	1.8 ± 5.4	0.7
Grade of transferred embryos			
A[a]	25 (75.7)	33 (76.7)	0.1
B[b]	8 (24.2)	10 (23.2)	0.1
BMI	24.6 ± 3.7	23.8 ± 3.8	0.1
Day 3 FSH (IU/L)	8.3 ± 5.4	7.8 ± 3.9	0.2
AFC	12 ± 6.2	11 ± 6.4	0.1

Data are presented as mean ± SD or n (%). [a]; Embryo with stage-specific cell size, <10% fragmentation and no multi-nucleation, [b]; Embryo with stage-specific cell size for the majority of cells, 10-25% fragmentation and no evidence of multi-nucleation, β-hCG; Beta-human chorionic gonadotropin, BMI; Body mass index, FSH; Follicular stimulating hormone, and AFC; Antral follicular count.

In terms of IL-1, chemical pregnancy (positive β-hCG) group significantly carrier higher level than that of the negative β-hCG group (P=0.000, Table 2). Although there were higher levels of IL-1 in FHR positive group in terms of clinical pregnancy (observing FHR in TVS), yet the difference has not been statistically notable (P=0.06, Table 3).

Table 2: Comparison of IL-1β and TNF-α levels in aspirated endometrial secretions in patients with positive and negative value of chemical pregnancy

Characteristic	β-hCG$^+$ Median (25-75)	β-hCG$^-$	P value
TNFα (ng/dL)	6 (3.6-7)	5.6 (3.6-7.6)	0.8
IL-1 (ng/dL)	11.4 (2.8-34.2)	2.4 (1-4)	0.000

IL-1β; Interleukin-1 beta, TNF-α; Tumor necrosis factor-alpha, and β-hCG; Beta-human chorionic gonadotropin.

Table 3: Comparison of IL-1 and TNF-α levels in aspirated endometrial secretions in patients with positive and negative value of clinical pregnancy

Characteristic	FHR$^+$ n=23 Median (25-75)	FHR$^-$ n=10	P value
TNF-α (ng/dL)	4.6 (3.3-7.1)	5.8 (3.6-7.4)	0.6
IL-1 (ng/dL)	5.3 (1.9-15.9)	2.6 (1.1-7)	0.06

IL-1β; Interleukin-1 beta, TNF-α; Tumor necrosis factor-alpha, and FHR; Fetal heart rate.

Discussion

Several studies have confirmed the role of interaction between embryo-endometrium and cytokines in implantation. To be stated as an example, in a study published by the Journal of Reproductive Biology in 2009, Haouzi et al. (10) reported that successful implantation of fetus is highly dependent on the fetus quality and endometrial reception.

Nieuwenhuizen et al. (11) and Lieberman et al. (12) mentioned in their reviews that cytokines, which are produced by fetuses and mucous membranes, are responsible for improving the endometrial receptivity. It has also been noted in a study by Zhou et al. (13), that IL-1 stands as an important factor through interaction between embryo and endometrium, as a functional factor during implantation in both maternal and fetal sites. This particular review was confirmed by other studies, such as Bourdiec and Akoum (14), pointing out the effective role of IL-1 through the success of embryo implantation process.

In accordance with the study done by Sequeira et al. (15) the levels of IL-1 in maternal serum levels and median culture of developing embryos were significantly higher in women with successful implantation. Moreover, a study by Loetscher et al. (16) has stated that TNF-α was at a very high level in people with a history of recurrent abortion and infertility.

Aside from this fact, Reid et al. (17) has also discovered that TNF-α is apparently associated with infertility and recurrent abortion, which can be considered as a confirmation of Clark's study. However, in contrast to Clark and Reid studies, a review performed by Boomsma et al. (18) from Netherlands in 2009, exhibited the existence of a positive correlation between successful pregnancy and higher levels of TNF-α and lower levels of IL-1 in endometrial secretions.

Similar to the present work, Rahiminejad et al. (1), assessed the levels of IL-1 and TNF-α in the endometrial fluid along with their effects on implantation

success. They concluded that lower levels of TNF-α in endometrial secretions results in the improvement of endometrial reception. However, no significant difference between IL-1 of the two groups was observed in terms of increasing chance of performing successful implantation.

At the end, next to the contradictions that are theoretically related to the relationship between the levels of these cytokines and successful outcome of pregnancy, various limitations such as technical differences in the aspiration-discharge procedure, aspiration scheduling, low sample size, the leading cause of tubal factor and the confounding effect of drugs could be the cause of the existing differences between the obtained results of different studies and the present investigation. In fact, evaluating the endometrial receptivity probably stands as one of the next steps that will be taken in infertility clinics. This particular test can provide clear information about the inferiority of endometrial receptivity as a primary cause of infertility and ultimately, contribute to the probable prediction of embryo transfer outcome in IVF/ICSI cycles.

The present study has concluded that there is not any significant statistical relationship between higher levels of IL-1 in endometrial secretion and successful implantation in IVF/ICSI cycles. Furthermore, with regards to the case of TNF-α, we have not discovered any statistical significant difference between the two groups with successful and unsuccessful implantation.

We believe that it would be quiet useful if researchers investigate some other categories, such as association between cytokine levels and ongoing pregnancy rate or even live birth rate, in addition to the relationship between cytokine levels and successful implantation with any specific infertility etiology.

Conclusion

This study suggests that higher level of IL-1 in endometrial secretions may associate with improved endometrial receptivity and subsequently, this can be related to the improved IVF/ICSI outcomes. In fact, this noninvasive method can enhance the understanding of immunological events, which are involved in the implantation process of fetus.

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Authors' Contributions

M.A., M.M.; Contributed to conception and design of all experimental work and interpretation of data. E.Z.; Participated in data collection and statistical analysis. A.A.; Conducted molecular experiments and analysis. N.Kh.; Was the supervisor of the study. M.A.; Was written the manuscript. All authors read and approved the final manuscript.

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Original Article

Efficacy of Intraoperative Mitomycin-C in Vasovasostomy Procedure: A Randomized Clinical Trial

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Abstract

Background: Two-six percentage of vasectomized men will ultimately seek vasectomy reversal, which late stricture and obstruction after operation are relatively common. To find a method for improving vasovasostomy outcomes, we used intra-operative local mitomycin-C (MMC) preventing possible fibrosis and stricture.

Materials and Methods: In this randomized clinical trial, 44 patients were assigned to two groups randomly during a one-year study and the data of 40 patients were analyzed. The patients were followed up for 6 months after surgery. The case group (n=19) was treated by vasovasostomy with intra-operative local MMC. The control group (n=21) underwent standard vasovasostomy.

Results: Mean sperm count in MMC group was significantly higher than the controls. The sperm count of more than 20 million/ml was respectively 53% and 14% in MMC and control groups. In a subgroup where the interval between vasectomy and reversal was 5-10 years, post-reversal azoospermia was absent in MMC group, but 50% of the controls were still azoospermic. In addition, 80% of MMC group had more than 20 million/ml sperms, but all of the controls had less than 20 million/ml sperms. No significant complication was seen.

Conclusion: Intra-operative local MMC in vasovasostomy can be regarded as a safe and efficient technique which has several advantages including lower cost. Increase of sperm count is the main effect of local MMC application that is more prominent when the interval between vasectomy and reversal is 5-10 years (Registration number: IRCT2015092324166N1).

Keywords: Clinical Trial, Mitomycin C, Sperm Count, Vasectomy Reversal, Vasovasostomy

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Introduction

Approximately 6-8% of married couples (about 42-60 million men), experience vasectomy as contraception (1). Surveys suggest that 2-6% of vasectomized men will ultimately seek for vasectomy reversal (2). The most common indications for vasectomy reversal are divorce, death of spouse or child and relief from post-vasectomy pain syndrome (3).

A meta-analysis on 32 studies about vasovasostomy with 6633 patients revealed that mean post-procedure patency and pregnancy rates were 89.4 and 73.0%, respectively, with the mean obstruction interval of 7.2 years. No statistically significant difference in vasovasostomy outcomes was seen in the comparison of single versus multilayer anastomosis. Obstructive interval less than 10 years was a predictor of higher patency and pregnancy rates (4). Other analyses and studies had less patency or pregnancy rates, 60-86% and 25-53%, respectively (5-7). The main predictors for success of the reversal procedure were the time between vasectomy and reversal, as well as female partner age (6, 8). History of conception with the current partner versus remarriage (7), average testicular volume (9), presence of a sperm granuloma, use of surgical clips instead of suture at vasectomy, presence and quality of vasal fluid and sperm in vasal fluid during surgical exploration, in addition to increased α-glucosidase in the postoperative semen also had a favorable impact on patency (5, 10). Some studies reported that smoking of the male or female partner and obstructive interval did not correlate with postoperative success (7, 11).

The most common early complication of vasovasostomy is hematoma. The hematomas are perivasal and very small, thus they usually require no surgical drainage. Wound infection is another possible early complication. Late complications include sperm granuloma at the anastomotic site (5%). Late stricture and obstruction are relatively common (12-

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18% in 12 months). With microsurgical techniques, patency can reach to 70-90% (12). Some newer techniques are introduced to obtain better results including laser tissue soldering (13), angled cutting for increasing vasal surface area, increasing neovascularity and decreasing fibrosis (14), using a double-ringed instrument designed to facilitate handling and dissecting vas away from perivasal tissue in an atraumatic fashion (15) and application of the fibrin glue (16).

Several surgeons have used mitomycin-C (MMC) as an antifibrotic adjunct to ab-externo trabeculectomy and Dacryocystorhinostomy (DCR). It seems that intra-operative local MMC with a controlled concentration is a safe agent for reducing fibrosis (17, 18). MMC is an antimitotic and cytotoxic agent that crosslinks DNA. This agent inhibits DNA synthesis, cellular RNA synthesis and nuclear division. MMC also induces apoptosis and inhibits protein synthesis by hampering synthesis of the collagen using fibroblasts (19-22). In animal models, studies on grafted tissue in mice have revealed that the differentiation of grafts was significantly inhibited by MMC (23). In human studies, fibroblasts showed a dramatic structural response to MMC, including intracellular edema, pleomorphic and vesicular mitochondria changes, dilated smooth and rough endoplasmic reticulum, as well as chromatin condensation (24).

Evidence for MMC-induced carcinogenicity is considered sufficient for animals, but inadequate for humans. As such, MMC is classified by International Agency for Research on Cancer (IARC) as possibly carcinogenic agent to humans (group 2B). A meta-analysis studied the effect of varying concentrations of MMC and treatment durations on cellular proliferation and viability of the fibroblasts. They found MMC at 0.4 mg/ml beyond the 5 minutes, and 0.5 mg/ml concentration at all time-points were lethal and caused extensive cell deaths, compared to controls. The minimum effective concentration appeared to be 0.2 mg/ml for 3 minutes (25). In a systematic review, it was found that intra-operative MMC adjunct in trabeculectomy appears to reduce the relative risk of failure, and no significant increase in permanent sight-threatening complications was detected. They reported that MMC was administered intra-operatively in concentrations of 0.1-0.5 mg/ml concentrations of saline for durations varying from 1-5 minutes (26). Local injection of MMC in the site of Internal Ureterotomy (IU) was also studied by several groups, reported that submucosal MMC injection reduced the stricture rate from 50% to 10%, after IU (27).

The important point is that all of the previous studies have examined MMC as an anti-fibrotic agent for ophthalmologic surgeries and internal urethrotomies. But intra-operative local MMC has not been studied in vasovasostomy yet. Therefore, our study is performed to determine the overall safety and efficacy of intra-operative local MMC as the anti-fibrotic agent in vasovasostomy.

Materials and Methods

In this randomized clinical trial, 58 patients, visited for vasectomy reversal in Shohada-e-Tajrish Hospital (Tehran, Iran) between January and October 2016, were enrolled.

Patient and public involvement statement

The main priority of these patients was to have the opportunity of becoming a father. It was indicated to the patients that this method may not improve the outcome of vasovasostomy procedure and they preferred to participate in this trial. All patients were fully informed about the method of trial and subsequently they were blindly sub-grouped. All recruited and conducted participants were informed about the trial results by email after data analysis.

In this randomized controlled trial (RCT) the burden of the intervention such as pain and surgical site infection, or hematoma were assessed by patients and also residents of urology in the outpatient clinic and they were then recorded in our database.

Inclusion criterion was ‘males who underwent vasectomy and wanted reversal of vasectomy. Exclusion criteria were testicular atrophy, history of urethral or bladder neck surgery, history of previous vasovasostomy, history of scrotal region radiotherapy, history of chemotherapy, age of partner out of fertility range and any situation suggesting the need for vasoepididymostomy.

Six patients had testicular atrophy, history of previous vasovasostomy and age of their partners was out of fertility range. Eight patients were candidates for vasoepididymostomy, because of previous scrotal surgery or manipulation like percutaneous sperm aspiration (PESA). Hence, all of them were excluded from the allocation.

Finally, 44 consecutive patients were allocated randomly into two groups: the case group (n=22) was candidate for vasovasostomy in addition to intra-operative local MMC. The control group (n=22) was allocated for standard vasovasostomy. Randomization was performed by a random number table and opaque envelopes were used for allocation.

The primary endpoints included presence of sperm in semen, sperm count more than 20 million/ml, sperm motility rate and normal morphology rate in sperms. The secondary endpoints include hematoma, inflammatory reaction, tissue necrosis and any sign of surgical site infection. As mentioned before, all patients were informed about the disease, method of study and treatment possibilities. They had been informed about the possible complications and other applicable managements. Then, an informed consent was taken from each patient.

The proposal of this study was approved by Shahid Beheshti Medical University (SBMU) Ethical Committee (IR.SBMU.MSP.REC.1395.100) and research board of Infertility and Reproductive Health Research Center (IRHRC). Ethical issues were respected based on Declaration of Helsinki. The RCT was approved and documented by IRCT (IRCT2015092324166N1).

Initial pre-operative evaluations included detailed medical history, complete physical examination and sperm analysis. In MMC group, pre-operation evaluation included laboratory tests and cardiovascular consultation. In the operating room, under spinal anesthesia, the procedure was carried out using bilateral high vertical incision of scrotum. After finding each vas deferens and preparing the site of anastomosis, two ends of vas deferens were floated in 0.2 mg/ml MMC solution for 5 minutes, and they were then washed by normal saline. Finally, anastomosis was performed microscopically (CARL ZEISS F170 T surgical microscope binoculars 10×/22B; Zeiss, Germany) using modified two-layered vasovasostomy. Two 5-0 poly-propylene sutures were placed at 5 and 7 o'clock positions in the sero-muscular layer to approximate two ends of the vas. Next, four 8-0 poly-propylene sutures were sequentially placed inside out in the mucosa of the vasal ends, at 3, 6, 9, and 12 o'clock positions and tied up. Two additional sero-muscular sutures were placed at 1 and 11 o'clock positions to complete the anastomosis. In the control group, vaso-vasostomy procedure was carried out as the MMC group, except for floatation in MMC solution. All surgeries were performed by the same surgical team.

Upon finishing the procedure, patients in both groups were in complete bed rest the day after operation. The second day after surgery, they were discharged providing the tests and general condition were normal. Patients were advised to have relative rest at home for two weeks, avoiding intercourse for one month and to have scrotal support for at least one week. The patients were informed about possible early and late complications, in addition to the time of next necessary following up visits. The patients were followed up at 1, 3, and 6 months after surgery by a complete history and a physical examination to monitor the complications (hematoma, inflammatory reaction, tissue necrosis and any sign of operation failure). Sperm analysis was also performed 1 and 6 months after surgery for measuring patency (presence of sperm in semen), sperm count, sperm morphology and motility.

These data were gathered and documented via checklists consisting demographical data which include the interval between vasectomy and vasovasostomy, intra-operative local MMC application, sperm analysis results and any complication related to the procedure. In MMC group, during the procedure, two patients were not compatible with the inclusion criteria, since they were candidate for vasoepididymostomy. So, they were omitted from the study and 20 patients received allocated intervention. In this group one patient lost the follow up. Finally, the data of 19 patients were analyzed. In the control group, all of the 22 patients received allocated intervention. During follow up, one patient immigrated to another city and he was out of reach. Therefore, the data of 21 patients were analyzed. Figure 1 shows the CONSORT flow-diagram of the data in this study. The data analysis method was per-protocol and performed by SPSS (version 23.0) software (SPSS, Chicago, USA). Fisher exact test, Independent t test, chi-square test and likelihood ratio chi square test were used to compare and analyze the data. P value significance level was defined as 0.05.

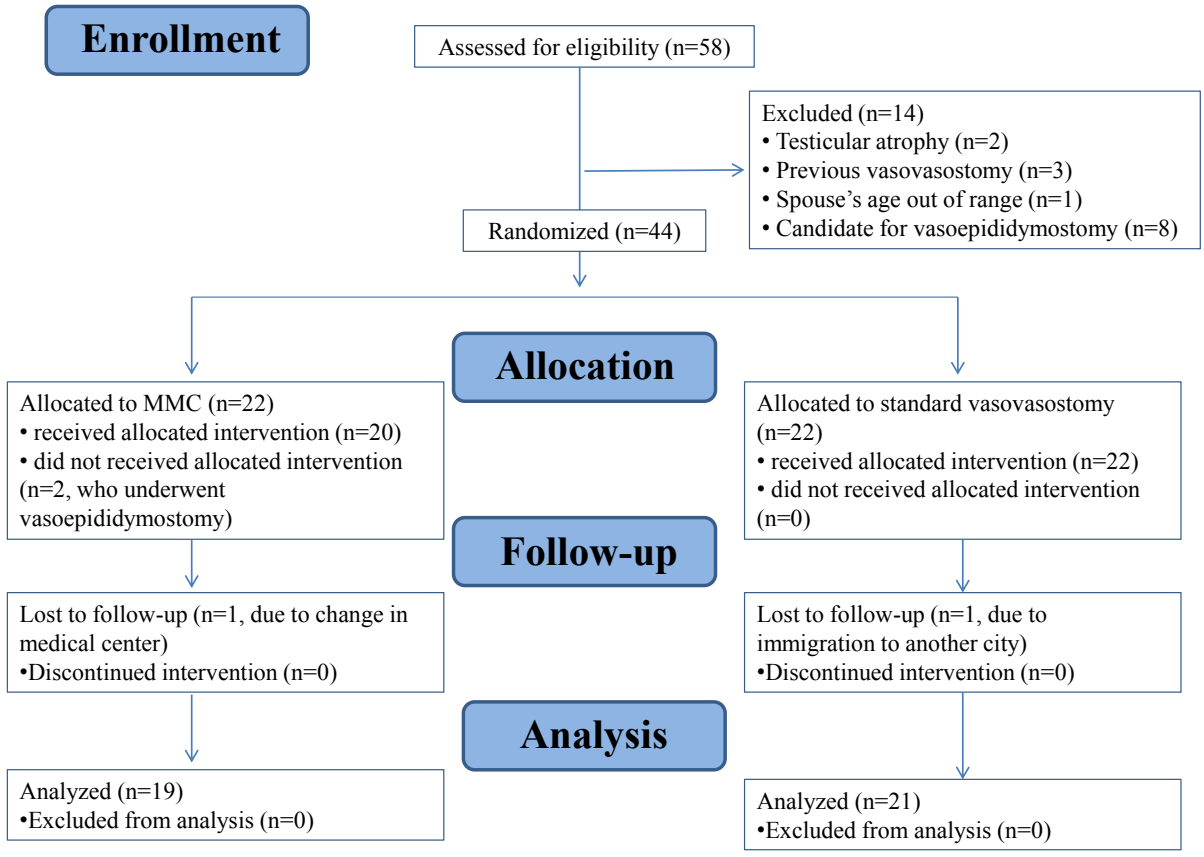

Fig.1: CONSORT 2010 flow-diagram.

Table 1: Primary data analysis

Group	Mean age (Y)	Normal morphology (%)	Motile sperms (%)	Sperm count		Mean sperm count (m/ml)	Patency	
				<20 M/ml	>20 M/ml		Azoospermia	Sperm present
MMC	39.95 ± 5.553	20.05 ± 14.69	27.05 ± 16.98	9 (47)	10 (53)	$(23.6 \pm 2.3)\times10^6$	4 (21)	15 (79)
Control	40.95 ± 6.659	17.05 ± 17	18.71 ± 15.96	18 (86)	3 (14)	$(9.4 \pm 1.4)\times10^6$	9 (43)	12 (57)
P value	0.609	0.559	0.118	0.017		0.023	0.186	

Data are presented as mean ± SD or n (%). MMC; Mitomycin-C.

Table 2: Data analysis based on post-vasectomy interval

Group	Patency		Sperm count	
	Sperm present	Azoospermia	>20 M/ml*	<20 M/ml
Interval<5 Y (n=7)				
MMC	2 (50)	2 (50)	0	4 (100)
Control	3 (100)	0	1 (33)	2 (67)
P value	0.092		0.166	
5 Y<interval<10 Y (n=18)				
MMC	10 (100)	0	8 (80)	2 (20)
Control	4 (50)	4 (50)	0	8 (100)
P value	0.005		0.0001	
Interval>10 Y (n=15)				
MMC	3 (60)	2 (40)	2 (40)	3 (60)
Control	5 (50)	5 (50)	2 (20)	8 (80)
P value	0.714		0.417	

Data are presented as n (%). *; Likelihood ratio chi square test, MMC; Mitomycin C, and Y; Year.

Results

Mean age in MMC group and control group was 39.95 (± 5.55) and 40.95 (± 6.65) years, respectively (P=0.609, Table 1). There was no early or late surgical complication in our allocated patients. Six months after surgery, mean sperm motility in MMC and the control group was identical (27.05 and 18.71% respectively, P=0.118). Normal morphology rate was also the same (20.05 and 17.05% respectively, P=0.559) (Table 1). Mean sperm count in MMC group was higher than the controls (23.5 and 9.4 million/ml) (P=0.023), and sperm count more than 20 million/ml in MMC and the control group was 53 and 14%, respectively (P=0.017). These differences were significant, but post reversal azoospermia in the two groups was not different (21% in MMC group and 43% in controls, P=0.186) (Table 1).

Then, we analyzed data in three subgroups based on the interval between vasectomy and reversal (less than 5, 5-10 and more than 10 years). In the first subgroup (less than 5 years interval), post reversal azoospermia (P=0.429) and sperm count more than 20 million/ml (P=0.429) in MMC and control groups were not statistically different. In the second subgroup (5-10 years interval), post reversal azoospermia was absent in MMC group, but 50% of the controls were still azoospermic (P=0.023). In addition, 80% of MMC group had more than 20 million/ml sperms, but all of the controls had less than 20 million/ml sperms (P=0.001). In the third subgroup (more than 10 years of interval), there was no statistical difference in post reversal azoospermia (P=1.000), and sperm count more than 20 million/ml (P=0.560) in the two groups (Table 2).

Discussion

Intra-operative MMC application is described for DCR, trabeculectomy, and some urological surgeries. All of these reports emphasized that MMC, as a local antifibrotic agent, is effective and safe. This trial, for the first time, demonstrates the effects of local intra-operative MMC in vasovasostomy. We cannot use previous trial estimate the best sample size. So we conducted a pilot study to find if any benefit exist using intra-operative MMC in vasectomy reversal. It seems that the increase of sperm count is the main effect of local intra-operative MMC in vasovasostomy, but it has no effect on sperm motility and morphology. This effect is more prominent in both patency and sperm count more than 20 million/ml; especially, in a subgroup with 5-10 years of interval between vasectomy and reversal. If the interval is less than 5 years or more than 10 years, MMC application has no benefit in the reversal outcomes. It is important that MMC application has lower cost in comparison with intracytoplasmic sperm injection (ICSI) or other new techniques described for vasovasostomy, and it has

no side effects if the concentration is controlled. It needs no special training and the time of surgery is relatively the same as standard vasovasostomy.

The main limitations of our study are small sample size, the use of very low concentration of MMC, relatively short follow up term and not enough follow up to study the pregnancy rate.

Conclusion

Intra-operative local MMC in vasovasostomy can be regarded as a safe and efficient technique which has several advantages including lower cost. Increase of sperm count is the main effect of local MMC application that is more prominent when the interval between vasectomy and reversal is 5-10 years. However, further studies should be conducted with larger sample sizes and different MMC dosage, longer durations, and multi-center sampling to attain more definite results.

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Authors' Contributions

F.A.; Proposed the idea of the project, and also designed and performed the analysis. H.Q.; Completed the study protocols and wrote the manuscript. H.M.; Edited the manuscript, J.H.; Supervised all steps of the project. All authors read and approved the final manuscript.

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Short Communication

Genetic Polymorphisms within The Intronless *ACTL7A* and *ACTL7B* Genes Encoding Spermatogenesis-Specific Actin-Like Proteins in Japanese Males

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Abstract

Actins play essential roles in cellular morphogenesis. In mice, the *T-actin1* and 2 genes, which encode actin-like proteins, are specifically expressed in haploid germ cells. Both *T-ACTIN1/ACTLB* and *T-ACTIN2/ACTL7A* have also been cloned and studied. The orthologous genes in humans are present on chromosome 9q31.3 as intronless genes. Defects of germ cell-specific genes can introduce infertility without somatic function impairment. We determined T-ACTIN1 and 2, specifically expressed in the testis using reverse-transcription polymerase chain reaction (RT-PCR). To examine whether genetic polymorphisms of the *T-ACTIN1* and 2 genes are associated with male infertility, we screened for *T-ACTIN1* and 2 polymorphisms by direct sequencing of DNA from 282 sterile and 89 fertile Japanese men. We identified five and six single nucleotide polymorphisms (SNPs) in the *T-ACTIN1* and 2 regions of the sterile and fertile subjects respectively. Among these genetic polymorphisms was a novel SNP that was not in the National Center for Biotechnology Information SNP database. Although we could not determine whether these SNPs cause infertility, the prevalence of these genetic polymorphisms may be useful for analyzing polymorphisms in future large-scale genetic analyses.

Keywords: Germ Cell, Infertility, Single Nucleotide Polymorphism, Sperm, Testis

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After meiosis, round spermatids undergo a dramatic change to develop the specific morphology of the mature sperm. Actin proteins play important functions in this process (1). We developed a mouse subtracted library including genes specifically expressed in spermatogenesis and cloned and characterized these genes (2). Among these genes were *T-actin1* and 2, which encode actin-like proteins and are specifically expressed in haploid germ cells. T-actin1 is located in the cytoplasm while T-actin2 is localized in the nuclei of testicular haploid germ cells and is present only in the heads and tails of sperm (3). In both the mouse and human genome, *T-ACTIN1* and 2 are positioned head-to-head and lack introns (4, 5). The resulting amino acid sequences, genomic construction, and cAMP response elements (CRE) consensus DNA sequence of the promoters of *T-actin1* and 2 are conserved in mice (4). These genes have been reported to cause infertility by inducing autoimmunity to sperm (6). Human T-ACTINs may play important roles in the specific morphogenesis of spermatozoa during spermiogenesis, as well as in sperm function.

We investigated genetic polymorphisms in the DNA sequences of germ cell-specific genes in infertile male patients and male volunteers with confirmed fertility (7-19) to identify polymorphisms potentially linked to male infertility (7, 8, 15, 19). In this study, we report our analysis of genetic polymorphisms in *T-ACTIN1/ACTLB* and *T-ACTIN2/ACTL7A* in Japanese men.

Defects in germ cell-specific genes may be a cause of idiopathic infertility. To detect the presence of small amounts of transcripts, we examined tissue-specific expression patterns of *T-ACTIN1* and 2 by reverse-transcription polymerase chain reaction (RT-PCR) using cDNA from various organs and a Rapid-Scan gene expression panel containing cDNA from different human tissues (OriGene Technologies, Rockville, MD, USA) (20). The specific primers:

TACT1-RTF:5'-ATGGCGACAAGGAACAGCCCCATG-3'
TACT1-RTR:5'-TCAGCACTTGCTGTAGATGGCCAC-3'
for *T-ACTIN1*
TACT2-RTF:5'-ATGTGGGCTCCACCAGCAGCAATC-3'
TACT2-RTR:5'-TCAGAAGCACCTTCTGTAGAGGAAG-3'
for *T-ACTIN2*

were designed to amplify fragments from the open reading frames. Polymerase chain reaction (PCR) was performed using Gflex Hot Start (Takara, Japan). The cycling conditions were 96°C for 2 minutes, followed by 35 cycles of denaturation at 96°C for 45 seconds, annealing at 58°C

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for 45 seconds, and extension at 68°C for 90 seconds. As a control, *β-actin* was also amplified using primers:

ACTBF: 5′-ACCGAGGCCCCCCTGAACCC-3′
ACTBR: 5′-TCCATCATGAAGTGTGACGT-3′

according to the manufacturer's protocol. *T-ACTINs* were specifically detected only in the testis (Fig.1).

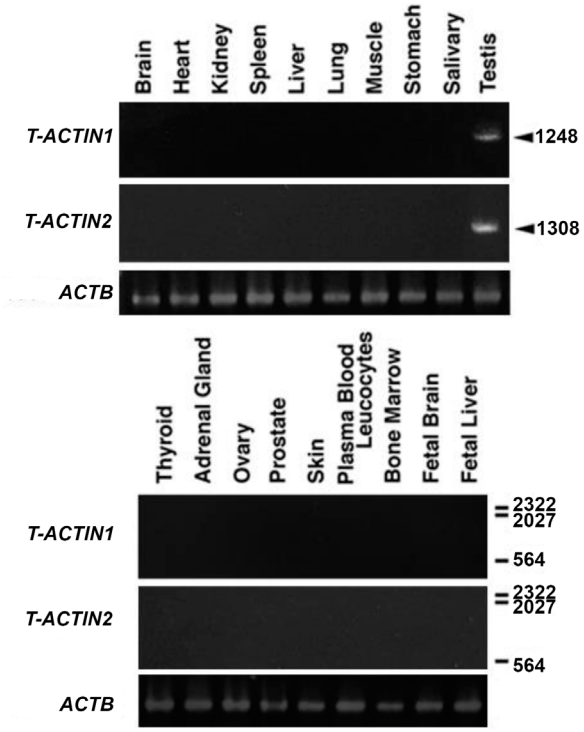

Fig.1: mRNA expression of *T-ACTIN1* and 2 in various human organs. Multiple human tissue cDNAs were subjected to polymerase chain reaction analysis. Fragments of *T-ACTIN1* and 2 were specifically detected in the testes. Numbers in the right-hand margin indicate the lengths of the amplified fragments and DNA ladder makers. The expression of actin mRNA was also examined as a control.

The entire coding sequences of *T-ACTIN1* and 2 (National Center for Biotechnology Information [NCBI] accession number: chromosome 9, NC_000009.12 (108862228..108863755), Fig.2) are intronless, similar to mouse T-actin1 and 2. As *T-ACTINs* are expressed at high levels in the human testis (Fig.1), we investigated whether genetic polymorphisms in *T-ACTINs* are associated with male infertility.

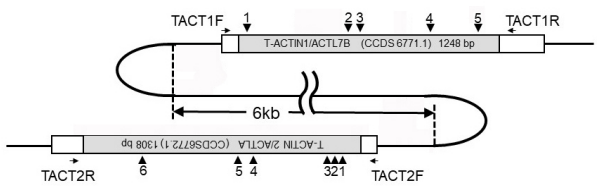

Fig.2: Schematic view of the *T-ACTIN1* and 2 genes. The *T-ACTIN1* and 2 intronless genes are located on chromosome 9 (NCBI accession number: NC_000009.12). The box indicates the transcribed region of the *T-ACTIN1* and 2 genes. The open reading frame is shaded. *T-ACTIN1* is transcribed to the right and *T-ACTIN2* to the left. The small horizontal arrows in the box indicate the locations of the polymerase chain reaction (PCR) and DNA-sequencing primers. The arrowheads indicate the positions of genetic polymorphisms. The NCBI accession numbers of *T-ACTIN1* and 2 are CCDS4295.1 and CCDS6772.1, respectively.

Infertile Japanese subjects (n=282) were divided into subgroups according to the degree of defective spermatogenesis: 192 patients (68%) had non-obstructive azoospermia, and 90 (32%) had severe oligospermia (<5×10^6 cells/mL), according to the criteria of the World Health Organization (Table 1). All patients had idiopathic infertility based on cytogenetic analysis and no history of other medical conditions, including cryptorchidism, recurrent infections, trauma, orchitis, varicocele, and others. The control group consisted of fertile males who had fathered children born at a maternity clinic (n=89). All donors were informed of the purpose of the study and gave permission for use of their blood for genomic DNA data. This study was approved by the institutional review board and independent ethics committee of Osaka University.

Table 1: Backgrounds of 371 Japanese men

Status	n (%)
Azoospermia	192 (68)
Severe oligospermia	90 (32)
Total infertile	282 (100)
Fertile control	89

Genomic DNA was isolated from blood samples by protease treatment and phenol extraction. *T-ACTIN1* and 2 sequences were amplified through PCR using the following primers:

*TACT1*F: 5'-GTGGATCCCTGGATGGTCCGCTGTGCGG-3'
*TACT1*R:5'-GGCCTGTGCCATCTGTGCTGGAGG-3'
for T-ACTIN1,
*TACT2*F: 5'-CTTTCAGGCCTTGAATCCAGTGGG-3'
*TACT2*R: 5'-GGTAGGCACTGCCAGTGCAGTGTC-3'
for *T-ACTIN* 2 (Fig.2).

PCR was performed using Ex *Taq* Hot Start (Takara, Japan) and consisted of 40 cycles of 96ºC for 45 seconds, 65ºC for 45 seconds, and 72ºC for 90 seconds. PCR-amplified fragments were purified using SUPREC PCR spin columns (Takara). The resulting DNA fragments were sequenced independently from both ends by the same PCR protocol using thermal cycle sequencing kits (Applied Biosystems, Foster City, CA, USA). Internal primers:

*TACT1*F2: 5'-GCCTGTGCCATCTGTGCTGG-3'
*TACT2*F2: 5'-TCTCAAGCTGGTTAACCCTCTGCG-3'
*TACT2*R2: 5'-AGGCACTGCCAGTGCAGTGT-3'

were used to confirm *T-ACTIN* genes with ambiguous identifications. The reaction products were analyzed using an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems). Differences in variables between the experimental and control conditions were compared using Fisher's exact test ($P<0.05$).

Nucleic acid base exchanges introducing one nonsense mutation and four silent mutations were found in the *T-ACTIN1* open reading frame (Table 2). Single nucleotide polymorphisms (SNPs) were found in three silent mutations (48C>T, 561C>T, 870C>T) as minor genotypes in the entire Japanese cohort. The minor 1137

C>T homozygous alleles on *T-ACTIN1* was not detected in the infertile group. One nonsense mutation was found in the volunteer group. The translated region of *T-ACTIN1* is 1248 bp long, and the nonsense mutation appears at base pair 1,171, near the C-terminus. This mutation thus has little influence on the function of the translated protein, making it unlikely to be a cause of infertility.

Table 2: Prevalence of single nucleotide polymorphisms (SNPs) in *T-ACTIN1* in infertile or proven fertile populations

	Position			Genotype	Number (%) of SNP		Reference	
	Nucleotide*	Amino acid			Infertile (%)	Proven fertile (%)	(NCBI dbSNP rs#)	
T-ACTIN1/ ACTL7B	48	16	D	C/C	161 (57.1)	54 (60.7)	rs3750468	
				C/T	102 (36.2)	28 (31.5)		
				T/T	19 (6.7)	7 (7.9)		P=0.74
	561	187	Y	C/C	161 (57.1)	54 (60.7)	rs11543179	
				C/T	102 (36.2)	28 (31.5)		
				T/T	19 (6.7)	7 (7.9)		P=0.74
	870	290	T	C/C	218 (77.7)	66 (74.2)	rs3750467	
				C/T	62 (22.0)	21 (23.6)		
				T/T	2 (0.7)	2 (2.2)		P=0.23
	1137	379	S	C/C	282 (100)	87 (97.8)	rs769443334	
				C/T	0 (0)	2 (2.2)		
				T/T	0 (0)	0 (0)		
	1171	391	Q	C/C	282 (100)	88 (98.9)	rs750564969	
			Q/Ter	C/T	0 (0)	1 (1.1)		
			Ter	T/T	0 (0)	0 (0)		
Total					282	89		

D; Aspartate, Y; Tyrosine, T; Threonine,S; Serine, Q; Glutamine, Ter; Termination, and *; The nucleotide positions relative to the first methionine.

Table 3: Prevalence of single nucleotide polymorphisms (SNPs) in *T-ACTIN1* in infertile or proven fertile populations

	Position			Genotype	Number (%) of SNP		Reference
	Nucleotide*	Amino acid			Infertile (%)	Proven fertile (%)	(NCBI dbSNP rs#)
T-ACTIN1/ ACTL7B	118	40	R	C/C	28 (99.6)	89 (100)	rs201549336
				C/A	1 (0.4)	0 (0)	
				A/A	0 (0)	0 (0)	
	133	45	R	C/C	282 (100)	88 (98.9)	rs368653764
			R/C	C/T	0 (0)	1 (1.1)	
			C	T/T	0 (0)	0 (0)	
	153	51	P	A/A	218 (99.6)	89 (100)	In present study
				A/	1 (0.4)	0 (0)	
				G/G	0 (0)	0 (0)	
	528	176	P	A/A	278 (98.6)	88 (98.9)	rs3739692
				A/T	4 (4.1)	1 (1.1)	
				T/T	0 (0)	0 (0)	
	657	219	V	G/G	261 (92.6)	82 (92.1)	rs3739693
				G/A	21 (7.4)	4 (4.5)	
				AA	0 (0)	3 (3.4)	
	1018	340	V	G/G	261 (92.6)	82 (92.1)	rs7872077
			V/M	G/A	21 (7.4)	4 (4.5)	
			M	A/A	0 (0)	3 (3.4)	
Total					282	89	

R; Arginine, C; Cysteine, P; Proline, V; Proline, M; Methionine, and *; The nucleotide positions relative to the first methionine.

Two nucleic acid base exchanges introducing amino acid substitutions and four silent mutations were found in the *T-ACTIN2* open reading frame (Table 3). The frequency of minor genotypes associated with *T-ACTIN2* nucleotide polymorphisms was low in Japanese males. One silent mutation, 153A>G, in *T-ACTIN2* was not registered in the NCBI SNP database (dbSNP), marking a novel discovery in our Japanese cohort.

Logistic regression modeling of the prevalence of haplotypes, including SNPs, revealed no significant differences between major and minor alleles lacking the 1,018 G>A on *T-ACTIN2* SNP in males proven to be fertile. The minor 1,018 G>A homozygous alleles on *T-ACTIN2* in males proven to be fertile is considered to be due to an error made by the sequencer.

The appearance of 48 C>T and 561 C>T in *T-ACTIN1* was linked; as was the appearance of 657 G>A and 1,018 G>A in *T-ACTIN2*. Thus, the SNPs in these two genes may have the same origin.

Although many SNPs have been registered in the NCBI dbSNP, we detected only 11 genetic polymorphisms in the open reading frames of the *T-ACTIN* genes among 371 Japanese men. Finally, z χ^2-test was used to compare genotype distributions between infertile males and proven fertile controls. There were no significant differences for the minor genotypes ($P>0.05$).

Our research group has focused on cloning and analyzing germ cell-specific genes. Chromosome mapping of these genes revealed that they are distributed across various chromosomes, and that many are intronless (21). *T-ACTINs* are among these intronless genes and are specifically expressed in the testis (Fig.2). The dysfunction of germ cell-specific genes does not affect ontogeny and may be a cause of unexplained male infertility. The dysfunction of these genes in mice has been shown to lead to infertility (22). Dominant-negative gene mutations are not passed on to the next generation, however other gene mutations can be inherited from a heterozygous male parent or from the female parent. More than 20% of married couples in Japan are affected by infertility and the male partner is responsible in two-thirds of these cases (23). We undertook an extensive analysis of genetic polymorphisms in germ cell-specific genes and of the relationship between gene polymorphisms and infertility (7-19). We found potential relationships between infertility in Japanese men and genetic polymorphisms or mutations in *PRM2*, *TP1*, *PGAM4*, and *SCOT-T* (7, 8, 15, 19). We analyzed SNPs in germ cell-specific genes and found that some included genetic polymorphisms with single amino acid substitutions, whereas other specific genes had few genetic polymorphisms. Most genes having several genetic polymorphisms encoded in proteins were involved in signal transduction or regulation, whereas those with few genetic polymorphisms were more likely to encode structural proteins (12). In this study, we discovered several different SNPs in *T-ACTIN1* and 2 in a cohort of Japanese men. The similar frequencies of these polymorphisms between the fertile and infertile groups in this study imply that these mutations are not associated with male infertility. However, the prevalence data for these genetic polymorphisms might be useful when analyzing the association of traits and genetic polymorphisms in further large-scale genetic analyses.

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Authors' Contributions

H.T.; Contributed to conception and design, all experimental work, data and statistical analysis, and interpretation of data, and wrote the manuscript. Y.M., A.T., M.W.; Contributed to materials and analyzed the data. All authors read and approved the final manuscript.

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

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

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

Thesis:

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

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

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

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

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