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Function of MicroRNAs in Normal and Abnormal Ovarian Activities: A Review Focus on MicroRNA-21

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Abstract

Some failures in ovary function, like folliculogenesis and oogenesis, can give rise to various infertility-associated problems, including polycystic ovary syndrome (PCOS) and premature ovarian insufficiency (POI). PCOS influences 8 to 20% of women; while POI occurs in at least 1% of all women. Regrettably, the current therapies for these diseases have not sufficiently been effective, and finding a suitable strategy is still a puzzle. One of the helpful strategies for managing and treating these disorders is understanding the contributing pathogenesis and mechanisms. Recently, it has been declared that abnormal expression of microRNAs (miRNAs), as a subset of non-coding RNAs, is involved in the pathogenesis of reproductive diseases. Among the miRNAs, the roles of miRNA-21 in the pathogenesis of PCOS and POI have been highlighted in some documents; hence, the purpose of this mini-review was to summarize the evidences in conjunction with the functions of this miRNA and other effective microRNAs in the normal or abnormal functions of the ovary (i.e., PCOS and POI) with a mechanistic insight.

Keywords: MicroRNA-21, Pathogenesis, Polycystic Ovarian Syndrome, Premature Ovarian Insufficiency

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Introduction

Dysfunctions in folliculogenesis and oogenesis, like failure in the formation of steroid hormones and maturation of oocytes, lead to various ovarian diseases related to infertility, such as polycystic ovarian syndrome (PCOS) and premature ovarian insufficiency (POI) (1-3). PCOS, as a prevalent endocrinopathy in the reproductive course of women, involves in 8 to 20% of women (4). This disease can be identified by detecting two of the three properties related to the Rotterdam criteria (i.e., hyperandrogenism, polycystic ovaries, and an- or oligo-ovulation) and excluding associated diseases (e.g., Cushing's syndrome, congenital adrenal hyperplasia, hyperprolactinemia, and thyroid disease) (5, 6). Additionally, POI [another name is premature ovarian failure (POF)] influences 1% of women at young ages and it is defined by decreased estradiol (E2) expression and follicular dysplasia, in addition to increased gonadotropin expression and follicle-stimulating hormone (FSH) (7, 8).

Unfortunately, against these disorders related to folliculogenesis and oogenesis, the current therapeutic approaches have not reflected enough effectiveness; thus, discovering a therapy with high efficiency and minimum side-effects is still a challenging issue (9, 10). One of the useful ways to improve remedies and the management of diseases is knowing the pathogenic mechanisms of illnesses (11). Accumulating evidences have implicated that abnormal expression of microRNAs was linked with pathological processes of different disorders, such as reproductive diseases, metabolic disorders, cancer, cardiovascular diseases, and neurological conditions (12-16). microRNAs (miRNAs/miRs) are a subset of non-coding and single-stranded RNAs. Their lengths are approximately 18-24 nucleotides, and they can down-regulate certain gene expression in a post-transcriptional way by binding to target messenger RNA [mRNA; 3′-untranslated region (UTR)] (17, 18). The published papers have indicated that microRNAs can be

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involved in ovarian functions, e.g., endocrine function, folliculogenesis and modulation of steroidogenesis, as well as apoptosis and proliferation of granulosa cells (19, 20). Recently, the role of dysregulation of miR-21 in the pathogenic occurrences of ovarian function-associated diseases (i.e., PCOS and POI) has been highlighted (21-23). Hence, in this mini-review, we aimed to discuss and review the role of this molecule and the other involved miRNAs in normal ovarian activities and pathogenic events of the mentioned diseases.

miR-21 is one of the most frequently present miRNAs in the ovary of different species, such as mouse, sheep, porcine, and bovine (24). miR-21 is divided into two types according to its strand, including miR-21 passenger strand (miR-21-3p) and miR-21 guide strand (miR-21-5p), the latter of which is the most frequent miRNA related to the RNA-induced silencing complex in granulosa cells (25, 26). It is approved that miR-21 (miR-21-5p) has a role in oocyte maturation as well as blastocyst and embryo development. It is considerably overexpressed at the time of transition from germinal vesicle to oocytes, arrested in the metaphase II (MII) stage of the meiotic division (27-29). In the research of Wright et al. (30), the results of reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR) analysis manifested that miR-21 is up-regulated about six times in oocytes and about 25 times in cumulus cells during *in vitro* maturation of pig oocytes. Based on the research of Han et al. (31) and Carletti et al. (32), oocyte-secreted factors (OSFs) promoted expression of miR-21 by targeting transforming growth factor-β (TGF-β) signaling, while miR-21 quenched apoptosis of cumulus and periovulatory granulosa cells by triggering the phosphatidylinositol 3-kinase (PI3K)/Akt signaling and decreasing cleaved caspase-3, respectively. The PI3K signaling pathway was described as a cell proliferation and survival regulator in the various cellular types triggered by basic fibroblast growth factor (bFGF). In addition, cleaved caspase-3 was considered a key actor of nuclear changes associated with apoptotic processes (33, 34). Furthermore, the results of Pan and Li (24) demonstrated that miR-21 enhanced porcine oocyte maturation and cumulus expansion by decreasing expression of tissue inhibitor of metalloproteinase-3 (TIMP3), as a matrix metalloproteinase inhibitor whose 3′-UTR sequence is targeted by this miRNA. Additionally, they proved a piece of evidence that the mentioned miRNA elevated levels of VERSICAN as well as the expressions of A disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1) and another gene associated with cumulus expansion, HAS2, in the time of COC maturation *in vitro* (24, 35). Indeed, the cleaved structure of VERSICAN, as a subset of aggregating chondroitin sulfate proteoglycans, by ADAMTS1 has a crucial role in the success of the matrix remodeling of cumulus cells (36, 37). Taken together, it seems that miR-21 can be involved in the normal activities of the ovary by affecting oocyte maturation, cumulus expansion, blastocyst and embryo development, and granulosa cell viability.

Based on growing evidence, miRNAs possess a striking role in ovarian activities. In this line, Timoneda et al. (38) explored expression levels of the porcine microRNAs by the RT-qPCR method and approved that miR-25, Let-7a, and miR-106a were expressed in ovarian tissues. McBride et al. (39) assessed the expression levels of some miRNAs at different phases of follicle development, comprising small follicles, medium follicles, pre-ovulatory follicles, early and late corpora lutea, and corpus albicans. Overall, let-7a, let-7b, miR-21, and miR-125b were the most frequently expressed miRNAs at different development phases. miR-31, miR-145, and miR-199a-3p showed a significant reduction in the follicular-luteal transition and a remarkable elevation at the follicular phase. In contrast, miR-21, miR-142-3p, and miR-503 represented a considerable increase in luteal tissues and they were normally expressed at lower levels during the follicular phases.

Different factors have function in follicle development, like TGF-β superfamily members, Smads, and activin receptor-like kinases (ALKs). Additionally, miRs can affect these agents (40-42). In this direction, it was stated that miR-224 potentiated granulosa cell proliferation by targeting Smad4, an important regulator related to follicular growth of the ovary (43). Moreover, a number of miRNAs (e.g., miR17-92 cluster and miR-183-96-182 cluster) have shown their roles in granulosa cell proliferation and differentiation as well as cell cycle transition by other mechanisms, including influencing BMPR2 and PTEN genes and FOXO1 transcription factor (44, 45). During folliculogenesis, above 99% of follicles experienced atresia, and activities of miRs in orchestrating follicle development and atresia have been illustrated (46, 47).

P-miR-1281, has-miR-936, hsa-miR-26b, hsa-miR-10b, mmu-miR-1224, P-miR-466 g-b, hsa-miR-574-5p, P-miR-1275, R-miR-26b, hsa-miR-1275, hsa-miR-149, and has-miR-99a are among overexpressed miRNAs during this degenerative process, whilst expression of has-let-7i, R-let-7a, hsa-miR-92b, P-miR-923, has-miR-92a, has-miR-1979, hsa-miR-1308, R-miR-739, hsa-miR-1826, ssc-miR-184, and P-miR-1826 were reduced during this occurrence (48-50). Folliculogenesis, as a highly dynamic occurrence, is linked with changes in circulating levels of ovarian hormones, and interestingly, the relationships between microRNAs and these hormones have also been scrutinized (51). As an example, Sirotkin et al. (52) indicated that 36 miRNAs (e.g., let-7b, let-7c, miR-17-3p, miR-15a, miR-92, miR-96, miR-108, miR-134, miR-133b, miR-146, and miR-135) suppressed progesterone secretion. In contrary, 16 miRNAs (i.e., miR-16, miR-18, miR-24, miR-25, miR-32, miR-103, miR-122, miR-125a, miR-143, miR-145, miR-147, miR-150, miR-152, miR-153, miR-182, and miR-191) enhanced release of progesterone hormone in granulosa cells. Regarding testosterone hormone, this study determined that let-7a, let-7b, let-7c, miR-17-3p, miR-16, miR-24, miR-26a, miR-25, miR-122, miR-108 repressed release of the aforementioned hormone. In addition, it was expressed that miR-378 influenced synthesis of estradiol hormone by binding 3′-

UTR of the aromatase coding sequence (53). miRNAs can also function in ovarian activities indirectly. Hasuwa and co-workers studied effects of miR-200b and miR-429 on infertility and anovulation in female mice, and finally they found that miR-200b and miR-429 abrogated zinc-finger E-box binding homeobox 1 (ZEB1) expression in the pituitary gland, whereby expression of these miRNAs were remarkably high. Plus, miR-200b and miR-429 inhibition suppressed luteinizing hormone (LH) biosynthesis, revealing that these miRNAs facilitated ovulation indirectly by affecting the hypothalamus-pituitary-ovarian axis (54). In summing up, it looks like the normal action of the ovary is regulated or mediated by the certain miRNAs.

It was addressed that miR-21 expression was elevated simultaneously with an increase in LH level after, during, and before ovulation (55). Interestingly, the increment LH level, causing disruption of ovarian folliculogenesis and change in production of steroid hormones, was commonly observed in PCOS women (56). miR-21 was also upregulated in granulosa cells, blood, and follicular fluid of patients with PCOS (57). Ovarian follicular fluid was produced from theca and granulosa cells. It is known as a necessary microenvironment for oocyte development and maturation (58). According to the work performed by Yu et al. (21), miR-21 can be involved in the inflammatory events of PCOS through regulation of cell proliferation and apoptosis of granulosa cells by affecting toll-like receptor 8 (TLR8). They found that expression levels of TLR8 and miR-21 were remarkably elevated in granulosa cells of PCOS cases, in comparison with the normal granulosa cells. In this regard, miR-21 elevated mRNA translation of TLR8 and consequently enhanced release of inflammatory agents, including interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). TLR8, as a subset of the family of TLRs, is dominantly expressed in myeloid dendritic cells, macrophages, and monocytes. It has a substantial role in inflammatory reactions (59, 60). Hilker et al. (26), in another scientific endeavor, inspected miR-21-5p function in porcine granulosa cells by RT-qPCR technique. They determined that this non-coding RNA was dramatically higher in granulosa cells obtained from large antral follicles, rather than those from small antral follicles. Moreover, they revealed that miR-21-5-p curbed Wilms tumor gene (WT1) expression (Fig.1), expressed by follicular cells, via binding to the 3′-UTR sequence of WT1 in granulosa cells to potentiate estradiol synthesis and aromatase expression (26, 61). Aromatase enzyme, a converter of androgens to estradiol, was significantly stimulated in human and animal cases of PCOS (62). However, Aldakheel et al. (63) and Ren et al. (64) declared that miR-21 can be an inhibitor factor for PCOS progression through suppression of proliferation of granulosa cells by affecting SNHG7, a subclass of SMAD protein family involved in cell apoptosis and proliferation adjustment. Additionally, in this new investigation, there was a reduction in the expression levels of miR-21 in ovarian tissue samples of PCOS subjects in comparison with the normal ovarian tissue samples (63). According to the majority of documents, miR-21 dysregulation may be linked with the pathogenic occurrences of PCOS; however, more *in vivo* and *in vitro* works are offered to be carried out to express its exact role in these conditions.

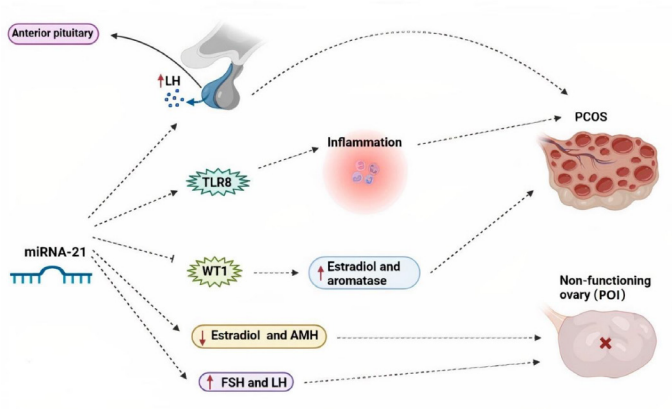

Fig.1: The possible pathogenic effects of miR-21 in the onset or progression of infertility-related disorders, like PCOS and POI. PCOS; Polycystic ovarian syndrome, POI; Premature ovarian insufficiency, FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, AMH; Anti-müllerian hormone, WT1; Wilms tumor gene, and TLR8; Toll-like receptor 8.

miR-21 dysregulation has been investigated in a few studies. In the study performed by Li et al. (65) on POI patients and animal model of the disease, caused by zona pellucida glycoprotein 3 (ZP3) antigen immunization, decreased expression of this single-stranded RNA was approved. Moreover, miR-21 had direct relationship with ovarian volume, uterus size, E2 and anti-Müllerian hormone (AMH). It also had an inverse relationship with LH and FSH levels, and a number of immune indices, including anti-endometrial antibody (EMAb), anti-ovarian tissue antibody (AOAb), anti-cardiolipin antibody (ACL), anti-double-stranded DNA antibody (ds-DNA), anti-adrenal cortical antibody (ACA), anti-nuclear antibody (ANA), immunoglobulin E (IgE), IgM, IgA, IgG, serum levels of complement 3 (C3) and complement 4 (C4). Finally, these results showed that this miRNA may be related to autoimmune POI pathogenesis. In another scientific project, effects of rat mesenchymal stem cells (MSCs) transfected by miR-21 on animal models of POI were studied. In this study, POI model was induced by intraperitoneal injection of a chemotherapeutic agent, cyclophosphamide. Unlike the previous study, this research team approved that transplanting the MSCs over-expressing miR-21 had reparative impacts on POI rats by upregulating expression of the miRNA in the ovary and subsequently reducing apoptosis of granulosa cells, elevating the follicle number, ovarian weight, and E2 levels, in addition to decreasing FSH levels (66). According to these studies, it is proposed that miR-21, based on its presence in different media, may play a positive or negative role against POI; however, more and large investigations are needed to demonstrate this theory.

Dysregulative and pathogenic roles of some miRNAs in ovarian function-associated infertility have been

documented. In a scientific effort, using TaqMan miRNA and Genome-wide deep sequencing assays, Sang et al. evaluated miRNA expression in human follicular fluid of PCOS cases. They observed that expression of miR-132 and miR-320 was reduced in the patient compared to the normal group. Other findings of this study implicated that miR-24, miR-132, miR-222, miR-320, and miR-520c-3p affected estradiol secretion, while miR-24, miR-483-5p, and miR-193b influenced progesterone release in PCOS patients (67). A preliminary study explored miRNA expression profile by TaqMan RT-qPCR method on 36 PCOS women and 16 normal subjects. It was shown that circulating levels of miR-26a-5p, miR-23a-3p, miR-21-5p were upregulated, whereas miR-222-3p, miR-19b-3p, miR-376a-3p, and miR-103a-3p were downregulated in PCOS subjects rather than normal individuals. Furthermore, miR-376a-3p, miR-21-5p, and miR-103a-3p were associated with total testosterone levels (68). The actions of some microRNAs have remained a challenging issue in ovarian disorders. For example, miR-483-5p is one of the challenging miRNAs in granulosa cells (69). A scientific work revealed upregulation of miR-483-5p in the granulosa cells obtained from PCOS cases, which may reflect its role in ectopic regulation of proliferation and apoptosis in these cells by targeting Notch-3 (Notch homolog 3) gene (70). On the other hand, the other authors observed reduced expression of this miRNA in the granulosa cells of PCOS subjects which may affect insulin-like growth factor-1 (IGF-1) and subsequently potentate granulosa cell proliferation (71, 72). Concerning POI, studies have been performed by notice to both ovarian tissue and plasma samples to determine miRNAs involved in POI development. Dang and colleagues inspected expression of plasma miRNAs based on the data of the microarray platform and RT-qPCR method between women with or without POI. Eventually, this study accentuated that miR-22-3p was a protective agent for this condition and it was negatively correlated with serum levels of the FSH hormone (73). Another research team identified 20 downregulated and 63 upregulated miRNAs in the samples of ovarian tissue from the rat POI model, caused by 4-vinylcyclohexene diepoxide (VCD), than the normal group. miR-144 and miR-29a, regulators of prostaglandin secretion via affecting phospholipase A2 group IVA (PLA2G4A), were downregulated in POI tissue samples; however, several miRNAs were upregulated, such as miR-672, miR-151, miR-190, and miR-27b.a, which play roles in apoptotic process (74). Another report also demonstrated that miR-23a was upregulated in the plasma samples of POI subjects, which in turn elevated apoptosis and diminished caspase-3 and X-linked inhibitor of apoptosis protein (XIAP) levels in human granulosa cells (75). These findings revealed that abnormal function of the ovary in PCOS and POI was along with the dysregulation of many miRNAs, influencing the ovarian structure and hormone secretion (Table 1).

Conclusion

It seems that miR-21 has a substantial role in processes leading to fertility, such as oocyte maturation, cumulus expansion, inhibition of cumulus and periovulatory granulosa cell apoptosis, transition from germinal vesicle to oocytes (arrested in the MII stage), blastocyst and embryo developments. However, their dysregulation may be involved in infertility-related conditions, like PCOS and POI, by different mechanisms. For example, miR-21 dysregulation by influencing TLR8, promoting secretion of inflammatory factors (e.g., IL-12, TNF-α, and IFN-γ), and inhibiting WT1 expression had a pathogenic role in PCOS. On the other hand, miR-21 malfunction exerted its negative role in POI by decreasing E2 and AMH and increasing LH and FSH levels. Thus, dysregulation of miR-21 can be associated with the pathogenic events of PCOS and POI. Despite these, multiple in vivo and in vitro investigations are required to determine pathogenic role(s) of miR-21 in these problems.

Table 1: Function of miR-21 in ovarian function-related disorders, including PCOS and POI

Ovarian function-related diseases	Expression	Targets	Mechanisms/influences/associations	Model (human/animal)	References
PCOS	Upregulation	TLR8	Potentiating IL-12, TNF-α, and IFN-γ levels and granulosa cell proliferation	Human	(21)
PCOS	Downregulation	SNHG7	Suppressing ovarian granulosa cell proliferation	Human	(63)
PCOS	Downregulation	-	Attenuating body weight and ameliorating energy expenditure	Animal	(76)
PCOS	Upregulation	-	Decreasing insulin resistance	Animal	(77)
POI	Downregulation		Positive associations with E2, AMH, ovarian volume and negative associations with LH, FSH and the number of positive immune parameters (AOAb, EMAb, ACL, ANA, dsDNA, ACA, IgG, IgA, IgM, IgE, C3, and C4)	Animal/human	(78)
POI	-	PTEN and PDCD4	Suppressing granulosa cell apoptosis	Animal	(66)

PCOS; Polycystic ovarian syndrome, POI; Premature ovarian insufficiency, IL-12; Interleukin 12, TNF-α; Tumor necrosis factor alpha, IFN-γ; Interferon gamma, E2; Estradiol, AMH; Anti-mullerian hormone, LH; Luteinizing hormone, and FSH; Follicle-stimulating hormone.

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Authors' Contributions

R.A., H.R.-Sh.; Have made substantial contributions to Conception and Design, Acquisition of data, Analysis and Interpretation of data. H.M., F.R.T.; Have made substantial contributions to Analysis of data. All authors have been involved in drafting the manuscript or revising it critically for important intellectual content. All authors read and approved the final manuscript.

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Association between Genetic Variants Linked to Premature Ovarian Insufficiency and Inflammatory Markers: A Cross-Sectional Study

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Abstract

Background: Premature menopause (PM) is the cessation of ovarian function before age 40. PM women are more likely to have cardiovascular diseases (CVDs), diabetes, and mental disorders. This is the first study that assessed the association of single nucleotide polymorphisms (SNPs) with anti-heat shock protein 27 (Hsp27), High-sensitivity C-reactive protein (hs-CRP), and PM and serum pro-oxidant-antioxidant balance (PAB), as putative risk factors for CVDs. We aimed to explore the association of oxidative stress markers with eight different SNPs shown to be related to premature menopause.

Materials and Methods: In this cross-sectional research, we included 183 healthy women and 117 premature menopausal women. We determined baseline characteristics for all participants and measured serum hs-CRP, anti-HSP-27 antibody titer, and PAB levels using the established methods. Genotyping for eight SNPs was done using the tetra amplification refractory mutation system polymerase chain reaction (Tetra-ARMS PCR) and allele-specific oligonucleotide PCR (ASO-PCR) methods.

Results: We found a significant difference between mean serum PAB levels and the genetic variant of rs16991615 (P=0.03). ANCOVA showed a significant effect of the genotypes rs4806660 and rs10183486 on hs-CRP serum levels in the case and control groups, respectively (P=0.04 and P=0.007). ANCOVA also showed an association between rs244715 genotypes and anti-hsp27 serum levels in the case group (P=0.02). There was a significant effect of the genotypes of rs451417 on the serum hs-CRP level in the control group (P=0.03).

Conclusion: There was a significant association of the genetic variants related to PM with oxidative stress and inflammatory markers (serum PAB, anti-hsp27 antibody, and hs-CRP). Accordingly, this seems to be an effective approach to predicting susceptible subjects for cardiovascular and mental disorders as well as various cancers.

Keywords: Genetic Variant, hs-CRP, Inflammatory Marker, Premature Ovarian Insufficiency

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Introduction

Premature menopause (PM), also known as primary ovarian insufficiency, is determined by amenorrhea before the age of 40 years (1). Fifteen to thirty percent of women with PM are familial, suggesting an essential role of genetic etiologies in the occurrence of PM (2). Multiple studies showed the effect of different genetic loci on age at natural menopause (3). Postmenopausal women are more susceptible to cardiovascular diseases (CVDs) and diabetes (4). Additionally, due to estrogen deficiency,

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PM women are prone to feel depression and anxiety (5). Early detection of PM women who are at higher risk of developing CVDs, diabetes, or depression via proper markers, will help us reduce the burden of these disorders.

Recent studies indicated that inflammation and oxidative stress predisposed individuals to CVDs and diabetes (6). The serum pro-oxidant-antioxidant balance (PAB) assay is a quick, cheap, and simple method for determining the pro and antioxidant activities (7). High serum PAB levels indicated enhanced production of reactive oxygen species (ROS) that may lead to oxidative stress (8). Recent studies also exhibited that serum PAB values were associated with the anti-Hsp27 antibody titers (9). Anti-Hsp27 antibody released in response to hsp-27 secretion could be taken into account for identifying oxidative events (10). Numerous investigations showed the association between PAB levels and risk factors for CVDs (11-13). Recently, anti-hsp27 levels have been suggested as a risk factor for CVDs (7). In addition, another study demonstrated the potential role of anti-hsp27 in predicting depression due to oxidative stress (14). However, high levels of hs-CRP as an inflammatory marker has shown a predictor nature for diabetes mellitus 2 occurrence (15). In the recent research, it was also shown that elevated hs-CRP levels were related to anxiety (13, 16).

As mentioned before, the role of genetic factors in PM has been well established. Extensive research has shown that mutations, such as SNPs, in a number of these genes were involved in PM (17, 18). Recently, a study showed significant association of eight single nucleotide polymorphisms (SNPs) (rs16991615 of *MCM8* gene, rs244715 of *ZNF346* gene, rs451417 of *MCM8* gene, rs1046089 of *PRRC2A* gene, rs7246479 of *TMEM150B* gene, rs4806660 of *TMEM150B* gene, rs10183486 of *TLK1* gene, rs2303369 of *FNDC4* gene) with PM in the northeastern population of Iran (19).

Genome wide association studies (GWAS) have recognized a polymorphism, (i.e. rs16991615) of mini-chromosome preservation 8 homologous recombination repair factor of *MCM8* gene that was involved in the age of natural menopause (20). Correspondingly, rs1046089 and rs4806660 polymorphisms were located on Proline Rich Coiled-Coil 2A (*PRRC2A*) and transmembrane (*TMEM*) genes, respectively. These are associated with the age at menopause (21). GWAS also identified several other variants that are associated with primary ovarian insufficiency (POI) (22). However, few research studies have been done on the association between this variants and PM risk. We selected the most common variants, including eight SNPs while their role in PM was recently established in the northeastern population of Iran (19). We aimed to evaluate association of the SNPs variants with PAB, hs-CRP, and anti-Hsp27.

Material and Methods

Study subjects

In this cross-sectional research, we included 183 healthy women as the controls and 117 patients with PM. All of them had originally participated in the Mashhad study, a cohort study that was accomplished over ten years with the joining of 9704 individuals (35-65 years old). The cases had a menopause history before 40 years old, twelve non-stop months without menstruation, and serum follicle-stimulating hormone (FSH) levels up to 40 IU/L (repeated at four-week intervals). Exclusion criteria for cases were the history of genetically confirmed diseases surgeries affecting menstruation, and drugs affecting menstruation. On the other hand, the controls were up to 40 years old without a history of menopause, disorders related to menstruation, and medication for conditions affecting menstruation or infertility. Using procedures endorsed by the Mashhad University of Medical Science Ethics Committee (IR.MUMS.MEDICAL.REC.1398.658), informed consent was acquired from all individuals. We determined baseline characteristics such as age, body mass index (BMI), smoking habits, and physical-activity-level (PAL). Moreover, we measured serum hs-CRP, anti-HSP-27 antibody titer, and PAB in all participants.

Physical-activity-level and body mass index measurements

We assessed PAL in the all participants by using the specific questionnaire, a modified version of the SHHS/MONICA questionnaire. Based on the subject PAL score, they were categorized into: extremely inactive (<1.40), sedentary active (1.40-1.69), moderator active (1.70-1.99), vigorously active (2-2.4), and extremely active (>2.40). In addition, we determined BMI by dividing the person's weight (kg) by the square of height (m^2).

Oxidative stress markers measurement

After 12-14 hours of fasting, blood sample was collected from the all participants in Vacutainer tubes. Blood samples were centrifuged at 5000 g, 4°C, for 15 minute to separate serum. It was followed by analyzing hs-CRP, anti-HSP-27, and PAB serum levels. Serum hs-CRP was measured using the Alycon analyzer (ABBOTT, USA) with a detection limit of 0.06 mg/l. As previously described, anti-HSP-27 antibody titers were assessed using the in-home enzyme-linked immune sorbent (ELISA) method (23, 24). PAB was measured using the method described by Alamdari et al. (25).

Genotyping

Extracting DNA

Genomic DNA extraction was performed from 200 µl blood using Parstous blood DNA extraction kit (Parstous, Iran) based on the manufacturer's protocol. To check quality of the extracted DNA, electrophoresis on 1% agarose gel was applied (Parstous, Iran). Quantification of extracted DNAs was also assessed by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, USA) with 260 and 280 nm of wavelengths.

Tetra-ARMS PCR

Tetra amplification refractory mutation system polymerase chain reaction (Tetra-ARMS PCR) was done in an overall volume of 15 µl, including 7.5 µl master mix (Parstous, Iran), 2 µl genomic DNA, 1.5 µl water, and 1 µl of each primer (4 µM). Primer 1 software was used to design the primers. Tetra-ARMS PCR was conducted as follows: an initial denaturation step at 95ºC for 5 minutes, then 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 58ºC for 30 seconds and extension at 72ºC for 40 seconds, followed by one extension cycle at 72ºC for 5 minutes.

ASO-PCR

We carried out allele-specific oligonucleotide PCR (ASO-PCR) in a total volume of 15 µl, containing 7.5 µl master mix (Parstous company), 2 µl genomic DNA, 1.5 µl water, and 1 µl of each primer (4 µM). Using the Primer 3 software, the primers were designed. ASO-PCR was performed as follows: an initial denaturation step at 95ºC for 7 minutes in one cycle, afterward 35 cycles of denaturation at 95ºC for 30 seconds, annealing for 30 seconds, extension at 72ºC for 30 seconds, followed by a final extension at 72°C for 7 minutes in one cycle.

Statistical analysis

SPSS software version 24 (SPSS Inc., USA) and SAS JMP Pro (SAS Institute Inc., USA) version 13 were used to analyze the data. All measurements were performed in triplicate. Kruskal-Wallis and Bonferroni correction tests were used to compare mean differences between different groups. Additionally, we used ANCOVA test to analyze SNP genotypes by removing the potential confounding variables. These factors (including PAL and age factors) influence oxidative stress marker levels. A P value below 0.05 was considered statistically significant ($P<0.05$). Multiple testing correction was applied using the Bonferroni correction. To describe the qualitative and quantitative variables, mean ± SD and frequency were reported, respectively. Chi-square and Fisher's exact tests were applied to measure association of qualitative variables. Moreover, mean of quantitative variables between the two groups was compared by an independent t test. Data mining techniques, including logistic regression (LR) and decision tree (DT) algorithms, were used to analyze data.

Logistic regression model

LR model is a prevalent statistical model to evaluate association between various predictor variables and binary outcomes in medicine, public health, etc (26).

Let Y_i denotes the response variable and takes the values of 0 or 1 depending on whether a response occurs or not. $\boldsymbol{X}$ is the vector of covariates associated with the response variable, $\boldsymbol{\beta}$ is the corresponding vector of regression coefficients. Subsequently, association between the covariates and binary response variable can be investigated as follows:

$$logit\{E(Y_i)\}=logit\{Pr(Y_i=1|\ \boldsymbol{X}, \boldsymbol{\beta})\} = \boldsymbol{\beta}^T \boldsymbol{X}.$$

The statistical model known as logistic regression, or LR, is used to model dichotomous targets and investigate how explanatory variables affect the dichotomous target variable. The likelihood of including each record in the target groups is also shown in LR (27). The LR ability to demonstrate a strong direct or inverse relationship between the target and inputs or explanatory variables is its primary advantage. Additionally, it is adaptable (28).

Decision tree model

One of the analyses of artificial intelligence that emerged toward the end of the 20th century is data mining (29). In other words, data mining is the process of finding hidden information within large data sets. Data classification is one of the important issues that researchers face during this process. Classification issues can be addressed using a variety of approaches (30). In the medical field, DT model can be used in a variety of ways (31). It is widely used and studied in these fields, due to its simplicity, clarity, and ability to extract straightforward rules (32). Components, nodes, and branches make up DT. Therefore, there are three kinds of nodes: i. A root node is the result of splitting all records into two or more distinct subsets, ii. The internal nodes are a possible connection point in the tree structure between the leaf nodes at the bottom and the root nodes at the top, iii. Leaf nodes that display the final results of the records obtained from division of tree target groups. Likelihood of placing records in target groups is indicated by the tree branches, originated from the root node and internal nodes (33). The Gini impurity index is used by the DT algorithm to choose the best variable.

$$Gini(D) = 1 - \sum_{i=1}^{m} P_i^2$$

In this calculation, P_i is probability of the record in D belonging to the class C_i and it is estimated by $|C_i, D|/|D$.

Results

Study population characteristics

We enrolled 117 women as the case and 183 women as the control group who were matched for BMI and smoking status. Participants in the case (PM) and control groups were 55.21 ± 5.56 and 54.62 ± 2.89 years old, respectively. Additionally, physical activity level of the case group was significantly higher than that of the control group [1.77 (0.27) vs. 1.68 (0.27); P=0.003]. Serum level PAB, hs-CRP, and anti-Hsp27 were not significantly different between the case and control groups (Table 1). As shown in Table 2, in this research, eight SNPs were examined, including rs16991615, rs244715, rs451417, rs1046089, rs7246479, rs4806660, rs10183486, and rs2303369.

Table 1: Demographic features and characteristics of the study population

Characteristics	PM cases (n=117)	Control (n=183)	P value
Age (Y)	55.21 ± 5.56*	54.62 ± 2.89*	0.4
Anti-hsp27	0.22 (0.34)**	0.23 (0.32)**	0.846
hs-CRP	1.78 (2.81)**	2.39 (4.52)**	0.443
PAB	81.90 (86.66)**	108.64 (94.24)**	0.111
PAL	1.77 (0.27)**	1.68 (0.27)**	0.003#
Smoking			0.392
Non-smoker	86 (37.1)***	146 (62.9)***	
Ex-smoker	10 (52.6)***	9 (47.4)***	
Current-smoker	19 (40.4)***	28 (59.6)***	
TC (mg/dl)	207.8 ± 35.8*	188.8 ± 33.1*	<0.001#
TG (mg/dl)	111.0 (82.0-162.5)	117.0 (80.0-159.0)	0.684
LDL-C (mg/dl)	128.39 ± 33.96*	110.47 ± 33.42*	<0.001#
HDL-C (mg/dl)	47.45 ± 9.33*	45.06 ± 11.34*	0.040#
FBG (mg/dl)	88.74 ± 20.06*	88.96 ± 31.43*	0.948
BMI (kg/m^2)	28.78 ± 5.06*	29.34 ± 4.22*	0.323

PM; Premature menopause, Anti-hSP; Anti-heat shock protein, hs-CRP; High sensitivity C reactive protein, PAB; Pro-oxidant-antioxidant balance, PAL; Physical activity level, TC; Total cholesterol, TG; Triglycerides, LDL-C; Low-density lipoproteins-cholesterol, HDL-C; High-density lipoproteins-cholesterol, FBG; Fasting blood glucose, BMI; Body mass index, SD; Standard deviation, IQR; Interquartile range, *; Mean ± SD, **; Median (IQR), *; n (%), and #; Significant at a level of 0.05.**

Association of single nucleotide polymorphisms and serum pro-oxidant-antioxidant balance levels

Serum PAB levels in the control group were significantly different (P=0.031) between the three genotypes of rs16991615: AA, GA, and GG. After adjusting confounders, including age and level of physical activity, there was no significant association between serum PAB levels in either group and the other selected SNP genotypes (Table 2).

Association of single nucleotide polymorphisms and serum hs-CRP levels

After adjusting for physical activity level and age in the both group, one-way ANCOVA was conducted to determine statistically significant difference between genotypes of the selected SNPs and hs-CRP levels s. Based on the obtained results, it was shown that the genotypes of rs4806660 significantly affected serum hs-CRP levels in the case group after adjusting for confounding variables (P=0.042). Additionally, the genotypes of rs10183486 significantly affected the hs-CRP level in the control group (P=0.007). On the other hand, in the control group, Kruskal-Wallis Test exhibited significant difference in serum hs-CRP levels among the each genotype of rs10183486 (TT, CT, and CC, P=0.014). The other results were not significant (Table 2).

Association of single nucleotide polymorphisms and anti-hsp27

The results showed significant effect of rs244715 genotypes on anti-hsp27 antibody titer in the case group after adjusting for confounding variables (P=0.023). Moreover, significant effect of the genotypes of rs451417 on the hs-CRP level was observed in the control group (P=0.031). The other results did not show any association between the genotypes of SNPs and anti-hsp27 antibody titer (Table 2).

Table 2: Association of genotypes related to PM with lipid profile in the studied patients (n=300)

SNPs	Markers	PM (n=117)					Control (n=183)				
rs16991615		AA	GA	GG	P1	P2	AA	GA	GG	P1	P2
	Anti- hsp27	0.28 (0.34)	0.21 (0.28)	0.23 (0.39)	0.51	0.48	0.28 (0.47)	0.19 (0.25)	0.25 (0.33)	0.15	0.27
	hs-CRP	2.44 (4.60)	1.30 (1.37)	1.88 (2.90)	0.23	0.34	1.62 (3.30)	2.87 (4.43)	1.89 (4.34)	0.28	0.88
	PAB	77.16 (65.34)	93.34 (66.98)	72.92 (103.01)	0.53	0.62	34.77 (-)[a]	117.01 (79.70)[b]	94.47 (124.92)[b]	**0.03***	0.08
rs244715		GG	AG	AA	P1	P2	GG	AG	AA	P1	P2
	Anti-hsp27	0.18 (0.35)[a]	0.28 (0.44)[b]	0.16 (0.26)	0.25	0.02	0.44 (-)	0.24 (0.34)	0.22 (0.27)	0.67	0.75
	hs-CRP	1.50 (4.08)	2.22 (3.43)	1.60 (2.62)	0.52	0.41	2.21 (5.54)	2.52 (4.91)	2.38 (4.35)	0.82	0.75
	PAB	81.91 (57.02)	71.53 (95.59)	89.74 (88.78)	0.56	0.56	-	112.52 (138.48)	108.64 (78.41)	0.40	0.39
rs451417		AA	CA	CC	P1	P2	AA	CA	CC	P1	P2
	Anti-hsp27	0.22 (0.38)	0.28 (0.24)	0.17 (0.39)	0.92	0.57	0.23 (0.16)	0.18 (0.25)[a]	0.24 (0.38)[b]	0.10	**0.03***
	hs-CRP	1.76 (2.90)	2.14 (3.86)	1.57 (2.30)	0.72	0.54	2.51 (5.45)	2.34 (4.32)	2.57 (4.63)	0.69	0.63
	PAB	77.16 (52.12)	15.02 (115.79)	91.54 (56.14)	0.38	0.46	137.26 (-)	88.29 (101.15)	108.64 (98.35)	0.83	0.99
rs1046089		AA	GA	GG	P1	P2	AA	GA	GG	P1	P2

Table 2: Continued.

SNPs	Markers	PM (n=117)					Control (n=183)				
rs7246479		AA	TA	TT	P1	P2	AA	TA	TT	P1	P2
	Anti-hsp27	0.16 (0.25)	0.19 (0.27)	0.30 (0.40)	0.19	0.16	0.31 (0.36)	0.23 (0.25)	0.23 (0.33)	0.47	0.58
	hs-CRP	1.49 (1.48)	1.68 (2.76)	2.08 (3.28)	0.34	0.54	6.58 (12.10)[a]	2.31 (3.75)[b]	2.32 (4.79)[b]	**0.03***	0.11
	PAB	88.19 (-)	64.86 (87.74)	90.13 (68.0)	0.56	0.73	-	94.47 (77.76)	121.27 (140.21)	0.18	0.15
rs4806660		CC	TC	TT	P1	P2	CC	TC	TT	P1	P2
	Anti-hsp27	0.25 (0.54)	0.22 (0.31)	0.21 (0.31)	0.37	0.54	0.38 (0.41)	0.24 (0.35)	0.22 (0.26)	0.24	0.11
	hs-CRP	2.20 (7.46)[a]	2.14 (3.16)	1.36 (2.30)[b]	0.23	0.04	2.52 (23.73)	2.33 (4.38)	2.42 (4.64)	0.49	0.27
	PAB	92.86 (-)	80.30 (86.66)	76.81 (84.60)	0.37	0.42	63.58 (-)	105.45 (121.63)	108.64 (103.96)	0.51	0.46
rs10183486		TT	CT	CC	P1	P2	TT	CT	CC	P1	P2
	Anti-hsp27	0.21 (0.17)	0.21 (0.34)	0.22 (0.42)	0.87	0.66	0.35 (-)	0.17 (0.32)	0.25 (0.32)	0.19	0.56
	hs-CRP	1.23 (6.05)	1.56 (2.67)	2.08 (2.85)	0.50	0.33	1.23 (19.81)	2.76 (4.97)[a]	1.82 (3.06)[b]	**0.01***	**0.007***
	PAB	147.12 (-)	85.82 (64.65)	76.81 (81.96)	0.15	0.12		64.07 (110.91)	114.48 (89.91)	0.72	0.49
rs2303369		TT	CT	CC	P1	P2	TT	CT	CC	P1	P2
	Anti-hsp27	0.21 (0.49)	0.22 (0.36)	0.22 (0.29)	0.65	0.33	0.31 (0.26)	0.23(0.34)	0.23 (0.32)	0.37	0.24
	hs-CRP	2.71 (6.00)	1.76 (2.84)	1.40 (2.47)	0.05	0.12	4.07 (7.15)	2.33 (4.54)	2.27 (3.95)	0.18	0.44
	PAB	91.30 (63.19)	74.66 (79.07)	81.90 (90.88)	0.88	0.91		73.55 (105.36)	112.82 (116.35)	0.78	0.95

Data are presented as level of Anti-hsp27, hs-CRP and PAB in serum of studied. P1; P value for the Kruskal-Wallis test, P2; P value for the analysis of covariance (ANCOVA) with age and physical activity level (PAL), as model covariates, *; Significant P<0.05. Anomalous letters indicate a significant difference. PM; Premature menopause, SNP; Single nucleotide polymorphism, Anti-HSP; Anti-heat shock protein, hs-CRP; High sensitivity C reactive protein, and PAB, Pro-oxidant-antioxidant balance.

Relationship of biochemical and SNPs predictors with PM, using LR and DT models

All variables were found to be significantly associated with PM (P<0.050) in the multiple LR model (Table 3). PAB, PAL, TG, rs10183486, rs451417, BMI, and rs244715 were found to be significantly associated with PM (P<0.05), after adjusting for the effect of other variables in the model. Additionally, rs10183486, PAB, PAL, and TG were the most significant variables (they have more LogWorth in Table 3).

Table 3: Parameters estimating of the LR model

Source	LogWorth	Importance	P value
PAB	145.178		<0.001
PAL	85.894		<0.001
TG	67.938		<0.001
rs10183486	39.144		<0.001
rs451417	15.160		<0.001
BMI	11.486		<0.001
rs244715	3.436		<0.001

LR; Logistic regression, PAB; Pro-oxidant-antioxidant balance, PAL; Physical activity level, TG; Triglycerides, and BMI; Body mass index

Figure 1 illustrated the outcomes of DT training for biochemical factors. DT algorithm determined various PM risk factors and categorized them into the four layers. According to the DT model, the first variable (root) is of the most significant classifying data, whereas the subsequent variables have different levels of significance (12). Figure 1 illustrated that age, followed by hs-CRP and anti-hsp27, had the most significant impact on the PM development risk.

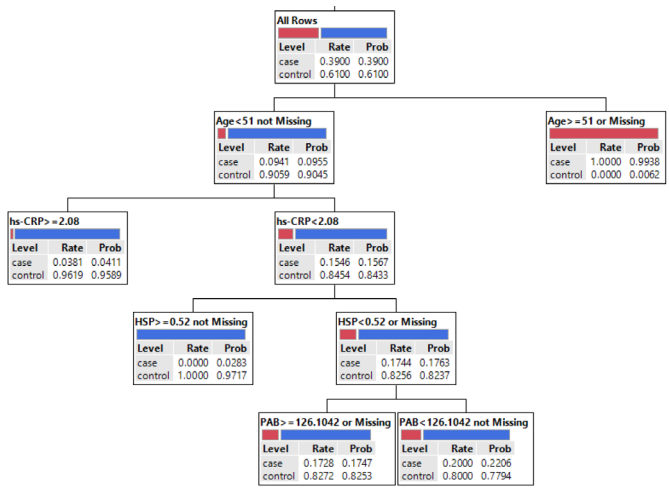

Fig.1: Decision tree for inflammatory risk factor of premature ovarian insufficiency diagnosis. hs-CRP; High sensitivity C reactive protein, HSP; Heat shock protein, and PAB; Pro-oxidant-antioxidant balance.

More participants with age<51 and hs-CRP≥2.08 were in the control group, according to the DT model, than those with age<51 and hs-CRP<2.08. Ninety-nine percent of the case group patients were ≥51 years old. Table 4 indicated specific PM developed by the DT model. Thus, age, hs-CRP, anti-hsp27, and PAB were determined as the most crucial variables in the DT model and diagnosis of PM. The receiver operating characteristics (ROC) curve for the DT model was performed to evaluate presentation of the model and comparisons (Fig.2).

Table 4: Detailed rules based on the DT model

Rules	Case (%)	Control (%)
R1: Age<51 and hs-CRP>2.08	4.11	95.89
R2: Age<51 and hs-CRP<2.08 and Anti-hsp27>0.52	2.83	97.17
R3: Age<51 and hs-CRP<2.08 and Anti-hsp27<0.52 and PAB>126.1042	17.47	82.53
R4: Age<51 and hs-CRP<2.08 and Anti-hsp27<0.52 and PAB<126.1042	22.06	77.94
R5: Age>51	99.38	0.62

DT; Decision tree, hs-CRP; High sensitivity C reactive protein, Anti-hSP; Anti-heat shock protein, and PAB; Pro-oxidant-antioxidant balance.

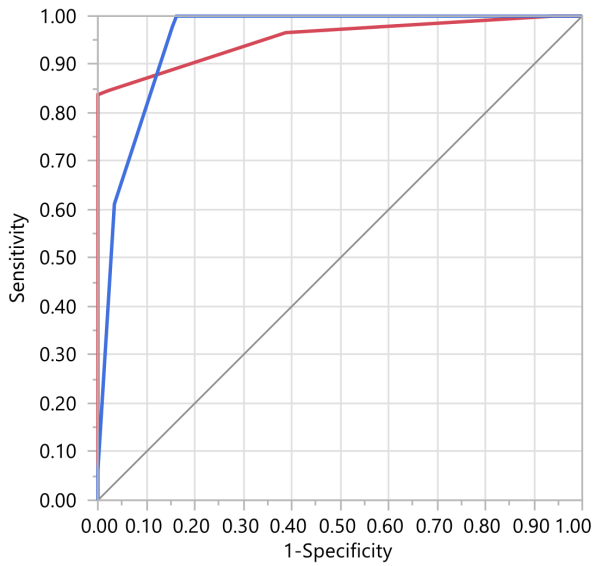

Fig.2: ROC curve of the DT model. ROC; Receiver operating characteristic and DT; Decision tree.

Discussion

Some recent studies investigated genetic determinants of PM (19). Women with PM were shown to develop disorders that are more prevalent or severe (33). CVDs, as an example, were found to be associated with oxidative stress markers (9). Interestingly, in the present study, for the first time, we aimed to investigate association of oxidative stress markers (including serum PAB, anti-hsp27 antibody, and hs-CRP) with eight different SNPs that were shown to be related to PM.

We found that rs244715 of the *ZNF346* gene and rs451417 of the *MCM8* gene were associated with anti-Hsp27 antibody titers. Thus, in the cases carrying rs244715 AG and rs451417 CC genotypes, anti-Hsp27 antibody levels were significantly greater than the controls. Both significant results were obtained after correcting for confounders, including PAL and age. As these SNPs were associated with PM (19), they may lead to CVDs, due to menopause and estrogen deficiency (34). An excess amount of Hsp27 to perform its antioxidant role may trigger the immune system to increase the corresponding antibody level (anti-hsp27 antibody), but this immune molecule may have pathological aspects (9, 27). PM was found to be significantly associated with an increased incidence of CVDs (33). Atherosclerosis progression was also shown to be related to the balance of serum Hsp27 and its antibody titers. Despite the protective role of Hsp27, anti-Hsp27 has a pathological role in atherosclerosis (35). Another study also showed that anti-Hsp27 antibody titers independently predicted depression, due to oxidative stress. Anti-Hsp27 antibody titers may exacerbate atherosclerosis. So Hsp27 and its antibody immune complex may be contributory, because of its pro-inflammatory property (9, 36). Recognizing PM women with AG genotypes of rs244715 and healthy women with CC genotype of rs451417 may screen women who are at risk of CVDs and mental disorders. Predicting and preventing these disorders may reduce the related socio-economic burdens.

We found that the rs4806660 and rs2303369 SNPs were associated with serum hs-CRP levels in PM women. Serum hs-CRP levels were significantly higher in the carriers of CC genotype of rs4806660 and TT genotype of rs2303369. In addition, we came to the conclusion that hs-CRP levels were significantly higher in the carrier individuals of AA genotype of rs7246479 and CT genotype of rs10183486, in our controls. Association of rs10183486 with hs-CRP levels was so strong, because it was significant either with or without removing confounders. Scientifics found a strong relationship between hs-CRP and serum PAB, due to the dysregulation of hs-CRP effects on the balance of pro-oxidants and antioxidants in the body. Menopause contributed to insulin resistance and the other cardiovascular risk factors (4, 37). hs-CRP has reportedly been associated with development of type 2 diabetes, because of endothelial adhesion molecule overproduction and insulin resistance of hs-CRP (38). In another study, it was shown that hs-CRP might predict diabetes mellitus type 2 (15). As a cardiovascular complication, in-stent restenosis (ISR) was demonstrated to be associated with higher hs-CRP levels (39). Attention to the CC genotype of rs4806660 and TT genotype of rs2303369 may help us identify PM women prone to cardiovascular and metabolic syndromes. Furthermore, in healthy women, the AA genotype of rs7246479 and CT genotype of rs10183486 may be helpful markers in predisposing to metabolic syndrome and mental disorders.

PAB levels were significantly higher in the women carrying GA genotype of rs16991615. Interestingly, there was a considerable difference between serum PAB levels in GA and AA genotypes of rs16991615. These significant associations were obtained without controlling confounders, including PAL and age. So, further investigations are needed to clarify it. Serum PAB was associated with the pathogenesis of CVDs. Risk factors of CVDs may elevate serum PAB, as a prognostic role in CVDs (9). Our findings indicated that many diseases, such as metabolic syndrome and various cancers, can be predicted. In other words, screening healthy women with GA and AA genotypes of rs16991615 may help us recognize people who are at high risk. It was found that oxidative stress might substantially increase the severity of Covid-19, as a global disaster (40). So, carriers of the

AA and GA genotypes of rs16991615 who have active menstrual cycles may develop more severe Covid-19.

Our study included some limitations. In this research, there was a small sample size. By using biochemical assays, we were unable to confirm premature menopause. Finally, according to our novel findings, further investigations are needed to validate our results. So, designing more studies is necessary for various regions with larger sample sizes.

Conclusion

Premature menopause SNPs may potentially influence oxidative stress/inflammatory markers. More precisely, there were significant effects of rs16991615 on serum PAB; rs244715 and rs451417 on the anti-hsp27 antibody titers, and four SNPs (rs7246479, rs4806660, rs10183486, and rs2303369) may affect hs-CRP levels. Accordingly, this seems to be helpful in predicting the susceptible subjects for several diseases, such as CVDs and mental disorders, as well as various cancers.

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Authors' Contributions

M.R.M., M.A., H.Gh.; Conception, Designed the experiments, and Drafting the manuscript. M.R.F.M., S.M., M.A.M., E.H.; Drafting the manuscript and Data analyzing. M.M.B., Sh.Y., A.R.E.; Designed the experiments and Revised the manuscript. M.M.Gh., A.E.D., E.A.; Performed the experiments and Drafting the manuscript. H.E., T.H.; Data analyzing and revising the manuscript. G.A.F.; Revising the manuscript and Conception. A.P., M.Gh.-M.; Conception and Corresponding author. All authors reviewed, considered, and approved the final manuscript.

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Evaluation of Oxidative and Nitrosative Stress Markers Related To Inflammation in The Cumulus Cells and Follicular Fluid of Women Undergoing Intracytoplasmic Sperm Injection: A Prospective Study

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Abstract

Background: Oxidative/nitrosative stress in the oocyte microenvironment could have an impact on intracytoplasmic sperm injection (ICSI) outcomes. The presence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can stimulate the secretion of pro-inflammatory cytokines, leading to chronic inflammation and potentially affecting embryo as well as oocyte quality. This study aimed to examine the relationship of lipid peroxidation [measured by the malondialdehyde (MDA) assay] with protein carbonyl [measured by the 2,4 dinitrophenylhydrazine (DNPH) assay] levels in cumulus cells (CCs), as well as nitric oxide (NO), peroxynitrite (ONOO-), and C-reactive protein (CRP) levels in follicular fluid (FF). The potential relationship of these levels with ICSI outcome was also evaluated.

Materials and Methods: In this prospective study, 63 FF samples and their corresponding CCs were collected for ICSI procedures. Spectrophotometry was used to assess levels of DNPH, MDA, NO, and $ONOO^-$. CRP levels were evaluated using an immunoturbidimetric assay.

Results: The patients under 37 years with normal ovarian reserve had significantly lower levels of MDA, DNPH, NO, $ONOO^-$, and CRP compared to those over 37 years. Additionally, we observed higher levels of MDA, DNPH, NO, $ONOO^-$, and CRP in the group with an oocyte maturity rate of less than 60%. No significant difference was observed between the DNPH levels and factors such as infertility duration, embryo quality, pregnancy, or the number of retrieved oocytes. A higher level of MDA, NO, $ONOO^-$, and CRP was found to be significantly related to the lower number of retrieved oocytes, longer periods of infertility, poor embryo quality, and negative pregnancy outcomes.

Conclusion: Oxidative/nitrosative stress, linking to inflammation in the oocyte microenvironment, can be considered as a potentially useful biomarker for assessing the development and competence of oocytes and embryos and predicting ICSI outcomes.

Keywords: Cumulus Cells, Follicular Fluid, Maternal Age, Oxidative/Nitrosative Stress, Pregnancy

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Introduction

During assisted reproductive technology (ART) procedures, assessment of oocyte quality is indeed crucial, as it directly affects the competence of embryonic development and rate of successful implantation. However, evaluation of oocyte and embryo quality mainly relies on morphological criteria, which have limitations in predicting successful pregnancy outcomes (1). Since quality of the oocyte and its microenvironment is important in early embryo development, several researchers are working to develop new non-invasive biomarkers by analyzing oocyte microenvironment components, such as follicular fluid (FF) and cumulus cells (CCs) to improve intracytoplasmic sperm injection (ICSI) outcomes (2, 3). Among the many reasons for ART failure, oxidative stress (OS) appears to be an important factor. OS refers to disruption of the balance between reactive oxygen/nitrogen species and the antioxidant system. In the female reproductive system, ROS plays physiological roles during oocyte maturation, embryo development, and pregnancy, while it may contribute in ART

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failure (4). Indeed, reactive nitrogen species (RNS), such as nitric oxide (NO), are involved in signaling molecules and control various aspects of reproductive physiology, including early embryonic development and implantation (5).

Recently, FF and CCs are considered as non-invasive biomarkers and they are used for prediction of *in vitro* fertilization (IVF) outcomes (3, 6). CCs are the specialized cells surrounding oocyte and they are connected to the oocyte cytoplasm. They form gap junctions, as channels that allow direct communication between the CCs and the oocyte (7). As a consequence of this close molecular dialogue, CCs play an important role in oocyte maturation and fertilization, as well as signaling and regulation of function. Moreover, high levels of ROS in the ovaries can negatively impact oocyte quality, leading to apoptosis in granulosa cells (GCs). This results in degeneration of the corpus luteum (6), deteriorating communications between oocytes and CCs, and disturbtion of preovulatory oocyte maturation (8). Oxidative damage occurs due to the spread of lipid peroxidation cascades, which can affect meiosis and ovulation. It can also contribute in the aging of ovaries (9). Indeed, FF components are either derived from plasma or secreted by GCs and include leukocytes and several mediators, such as growth factors, ROS, and antioxidants (3). We propose that FF biochemical characteristics are involved in oocyte quality and subsequently in fertilization, embryo development, and pregnancy. Furthermore, in our previous study, we showed that high level of OS could be one of the causes of ovarian aging, in FF (10). Several studies have shown that inflammation and oxidative stress are often associated with many diseases, while they can exacerbate each other. Indeed, ROS promotes pro-inflammatory cytokine secretion (11, 12). Additionally, several authors have reported that chronic inflammation could alter oocyte meiosis and reduce oocyte quality (13), it could also have an impact on ovarian aging (14).

In the field of ART, it is noted that approximately 85% of transferred embryos do not successfully implant, and only 20-25% of IVF attempts result in a live birth. As a result, various studies are being conducted to explore the potential of new biomarkers based on the analysis of oocyte microenvironment (2, 3, 6). The current study aimed to examine effect of the lipid peroxidation, as assessed by a malondialdehyde (MDA) assay, and protein carbonyl, as assessed by a dinitrophenylhydrazine (DNPH) assay, levels in CCs, as well as NO, peroxynitrite, and C-reactive protein (CRP) levels in FF, on the outcomes of ICSI. Additionally, the study aimed to assess status of the oxidative/nitrosative stress in patients of advanced age undergoing ICSI.

Material and Methods

Patients' characteristics

This prospective study included 63 women undergoing ICSI procedures, at the Fertilization Center IRIFIV in Casablanca, Morocco. All participants gave written permission for the utilization of FF and CC samples after being informed. The reasons for seeking consultation among the couples were female infertility in 28 cases, a combination of infertility factors in 12, and unexplained cause of infertility in 23 cases. Patients were disqualified from the study, if they have had any of the following conditions: i. Endocrine disorders or previous ovarian surgery that impacted the ovaries or the secretion of gonadotropins, ii. Undergoing hormone therapy, suffering from metabolic syndrome, having undergone pelvic surgery, having ovarian tumors, being morbidly obese, or having an autoimmune disease, and iii. Having polycystic ovary syndrome or endometriosis.

Ovarian stimulation procedure

All patients underwent stimulation through the use of follicle stimulating hormone (FSH, Orgalutran 0.25 IU and Gonal-F) according to the antagonist protocol. FSH (Gonal-F from Serono Laboratories, Saint Cloud, France) was administered daily through subcutaneous injections, with doses ranging from 150-225 IU/day or ¼ 300 IU/day, determined based on different factors, such as the patient ages, antral follicle count (AFC) in the early days of the cycle, and anti-müllerian hormone (AMH) concentration. The FSH dose was monitored and adjusted based on ultrasound results showing follicle growth (10, 15). On the 6th day of FSH administration, daily injections of the GnRH antagonist Ganirelix (Orgalutran VR, MSD Schering-Plough, France) were started. Injection of the human chorionic gonadotrophin (HCG, Gonadotrophins Chorioniques Ovitrelle VR, Merck Serono, Germany) was given when the triggering criteria were met, including presence of at least three follicles with 17 mm size (10).

Maturation rates of the oocytes were divided into two groups: "Group I" contained oocytes with a maturation rate of 60%, and "group II" consisted of FFs with a maturation rate of 60%. Level of AMH was evaluated for each patient on the third day of their menstrual cycle. AMH levels <1.1 ng/ml are thought to indicate a reduced ovarian reserve, whereas levels >1.1 ng/ml indicate a normal reserve. On the third-day of post-oocyte retrieval, quality of the embryos was classified into A-D subgroups based on morphological criteria, including number of the blastomeres, uniformity of the blastomeres, and fragmentation rate. An embryo was considered to be of the highest quality (A or B grade), if it have had 6-8 evenly sized blastomeres and a fragmentation rate of 25%.

Collection and preparation of the samples

Follicular fluid samples

FF samples was collected, and CCs were isolated for ICSI procedures. The FF was obtained from mature follicles, during the time of oocyte retrieval, and only clear

samples were utilized. These samples were purified using a Ficoll-based protocol (3 ml), as described by Ferrero et al. (16). The purified FF was then immediately stored at -20°C until it was assessed for nitric oxide, peroxynitrite, and CRP.

Cumulus cell samples

Following the oocyte pick-up procedure, the CCs were gently dislodged by aspirating them with a 100 micron pipette and then placed in a buffered culture medium from Gynemed company (Germany) with pH=7. The CC samples were transferred into a tube and disrupted with a lysis buffer with pH=7.5, consisting of 10 mM EDTA, 50 mM Tris, 1 mM PMSF, 1 mM glycerol, and 1 mM mercaptoethanol. The samples were stored at -20°C for the later analysis of lipid and protein oxidation.

Biochemical assay

The protein contents of CCs and FF were determined using the Bradford method (17) using bovine serum albumin (BSA, Sigma-Aldrich, Germany) as the standard. To evaluate lipid peroxidation, levels of MDA were measured, as a well-known end product of lipid peroxidation. This was performed using the thiobarbituric acid (TBA) assay, which is a commonly used method for determining MDA content (18). In this assay, high concentration of trichloroacetic acid (TCA) was added to release free MDA by treating it with TBA under acidic conditions and at high temperature approximately 100°C for 30 minutes. The reaction of two molecules of TBA with one MDA molecule generates a chromophore that absorbs light at 535 nm.

In short, 100 µl of the purified FF was mixed with 10% TCA and 0.67% TBA. The MDA concentration was expressed as micromole per microgram of protein, calculated using a molar extinction coefficient of $1.56\times105\ M^{-1}\,cm^{-1}$.

Protein oxidation was measured by quantifying carbonyl groups through the 2,4-dinitrophenylhydrazine (DNPH) method (19). To perform the measurement, 50 µl of the sample solution was mixed with10 mM DNPH in 0.5 M H_3PO_4, and after 10 minutes incubation, 400 µl of NaOH (6 M) was added. After a further 10 minutes of incubation at room temperature, absorbance was read at 450 nm. The results were expressed as µmol/µg protein of carbonyl groups and calculated using a molar extinction coefficient of 22,000 $M^{-1}\,cm^{-1}$.

Colorimetric assay of NO was carried out using the Griess reaction protocol as previously outlined by Arif et al. (20). This reaction involved mixing 0.02% naphthyl ethylenediamine dihydrochloride (NED), suspended in water, and 2% sulphanilamide (SA) in 5% phosphoric acid. In this procedure, nitrite is initially subjected to treat with the diazotizing agent SA in an acidic environment to generate a short-lived diazonium salt. The diazonium salt subsequently interacts with the coupling reagent NED to produce a robust azo compound, which exhibits a color ranging from pink to dark pink. The absorbance was measured at 540 nm, and quantity of nitrite was calculated based on µmoles/µg of protein using a reference $NaNO_2$ solution.

The procedure for measuring peroxynitrite ($ONOO^-$) levels involved the method described by Ben Anes and colleagues (21). The assay takes advantage of the ability of $ONOO^-$ to nitrate phenol, which leads to the formation of nitrophenol. To perform the assay, 100 µl of FF was placed in a glass test tube and combined with 5 mM phenol in a 50 mM sodium phosphate buffer. The mixture was then incubated for 2 hours at 37°C, followed by the addition of 100 µl of 0.1 N NaOH. Absorbance of the samples was then read at 412 nm, and $ONOO^-$ concentration was calculated by determining the yield of nitrophenol using a molar extinction coefficient of 4400 $M^{-1}\,cm^{-1}$.

The CRP level was assayed using a commercial kit (Cobas Tina-quant C - reactive protein IV, Switzerland) based on an immunoturbidimetric test on latex particles. Indeed, aggregation of the human CRP occurs when it is combined with latex particles coating with anti-CRP monoclonal antibodies. Particle clusters were measured by turbidimetry. CRP levels were expressed based on mg/l.

Statistical analysis

The results are reported as the mean ± the standard deviation (SD), and the differences between groups were analyzed through the Mann-Whitney U test, which was performed using the Statistical Package for the Social Sciences (SPSS, Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) software. A $P<0.05$ was considered to indicate a statistically significant difference.

Results

The study participants were 63 infertile women with ages ranging from 23 to 43 years, who received ICSI treatment. Assessment of MDA and DNPH levels were performed in CCs, while NO, $ONOO^-$ and CRP levels were assessed in FF. The outcomes of age, infertility length, oocyte maturity rate, embryo quality, number of oocytes, age combined with AMH, and pregnancies were separated into two groups: a lower group and a higher group, based on the statistical analysis.

As shown in Table 1, The levels of MDA and DNPH in CCs, as well as the levels of NO, peroxynitrite, and CRP in FF pools, were significantly lower in the patients younger than 37 years old compared to those who were 37 years old or older. Moreover, MDA, DNPH levels in CCs and NO, peroxynitrite, and CRP levels in FF pools were significantly lower in those with AMH >1.1 ng/ml than AMH <1.1 ng/ml. Statistical analysis did not show any significant difference between the older and younger women.

Table 1: Comparison of the oxidative and nitrosative stress markers, CRP levels, in human follicular fluid and cumulus cells with age and AMH combined to age

Subgroups/Biomarkers	Age (Y)		AMH (ng/ml)			
	<37 (n=34)	>37 (n=29)	<37		> 37	
			< 1.1 (n=14)	> 1.1 (n=14)	<1.1 (n=15)	<1.1 (n=15)
MDA (CCs) µmol/µg of proteins	0.24 ± 0.18	0.34 ± 0.25	0.29 ± 0.17	0.18 ± 0.14	0.36 ± 0.32	0.36 ± 0.32
P values	0.04*		0.01*		0.03*	
DNPH (CCs) µmol/µg of proteins	0.007 ± 0.007	0.002 ± 0.001	0.0028 ± 0.0018	0.0018 ± 0.0012	0.0029 ± 0.0021	0.0029 ± 0.0021
P values	0.03*		0.02*		0.04*	
NO (FF) µmol/µg of proteins	0.15 ± 0.14	0.27 ± 0.27	0.25 ± 0.25	0.18 ± 0.14	0.35 ± 0.26	0.35 ± 0.26
P values	0.02*		0.04*		0.02*	
Peroxynitrite (FF) µmol/µg of proteins	0.006 ± 0.005	0.037 ± 0.028	0.005 ± 0.005	0.002 ± 0.001	0.01 ± 0.01	0.01 ± 0.01
P values	0.02*		0.05*		0.03*	
CRP (FF) mg/l	1.43 ± 1.39	2.83 ± 2.78	0.92 ± 0.60	1.82 ± 1.82	1.65 ± 1.65	3.18 ± 2.38
P values	0.007*		0.04*		0.01*	

Values are in mean ± standard deviation. Statistical significance was defined as P<0.05 (*; Significant), analyzed with the Mann-Whitney U-test. CRP; C-reactive protein, AMH; Anti-müllerian hormone, MDA; Malondialdehyde, CCs; Cumulus cells, DNPH; 2,4 dinitrophenylhydrazine, FF; Follicular fluid, and NO; Nitric oxide.

According to the data presented in Table 2, we noticed that the levels of MDA in CCs and the levels of NO, peroxynitrite, and CRP in FF were significantly higher in the patients who had been attempting conception for more than five years compared to those who had tried for five years or less. In addition, the patients with a low number of retrieved oocytes had elevated levels of MDA, NO, peroxynitrite, and CRP, in their CCs and FF pools. Furthermore, we noted in the group with a maturity rate of <60% a higher level of MDA, DNPH in CCs and NO, peroxynitrite, and CRP levels in FF compared to the group with a maturity rate of >60%. Statistical analysis did not show significant difference between the DNPH levels and the duration of infertility, embryo quality, pregnancy, or number of oocytes. On the other hand, we noticed significantly higher levels of MDA, NO, peroxynitrite, and CRP in the samples associated with oocytes that produced poor-quality embryos (grades C and D) compared to the samples associated with high-quality embryos (grades A and B). Finally, MDA levels in CCs and NO, peroxynitrite, and CRP levels in FF samples were significantly higher in women who had not been pregnant compared to women who had been pregnant (Table 2).

Table 2: Comparison of the oxidative and nitrosative stress markers, CRP level in human follicular fluid and cumulus cells with infertility length, number of oocytes and ICSI outcomes

Subgroups/ Biomarkers	Infertility length		Number of oocytes		Maturity rates		Embryos quality		Pregnancy	
	<5 (n=30)	>5 (n=33)	>6 (n=33)	<6 (n=30)	>60% (n=43)	<60% (n=20)	A-B (n=42)	C-D (n=21)	Positive (n=18)	Negative (n=45)
MDA (CCs) µmol/µg of proteins	0.22 ± 0.18	0.31 ± 0.23	0.23 ± 0.21	0.30 ± 0.18	0.24 ± 0.19	0.35 ± 0.24	0.21±0.16	0.33 ± 0.30	0.20 ± 0.18	0.30 ± 0.23
Pvalue	0.05*		0.03*		0.05*		0.05*		0.02*	
DNPH (CCs) µmol/µg of proteins	0.002 ± 0.001	0.002 ± 0.002	0.002 ± 0.001	0.002 ± 0.001	0.0017 ± 0.001	0.0026 ± 0.001	0.002±0.001	0.002 ± 0.001	0.002 ± 0.002	0.002 ± 0.002
P value	0.13		0.10		0.02*		0.35		0.11	
NO (FF) µmol/µg of proteins	0.16 ± 0.11	0.27 ± 0.27	0.16 ± 0.14	0.27 ± 0.26	0.13 ± 0.11	0.19 ± 0.12	0.18±0.18	0.31 ±0.31	0.16 ± 0.08	0.26 ± 0.26
P values	0.02*		0.04*		0.03*		0.05*		0.02*	
Peroxynitrite (FF) µmol/µg of proteins	0.004 ± 0.002	0.01 ± 0.01	0.003 ± 0.002	0.014 ± 0.014	0.003 ± 0.002	0.004 ± 0.004	0.004±0.003	0.006 ± 0.005	0.004 ± 0.002	0.009 ± 0.002
P values	0.03*		0.05*		0.05*		0.03*		0.04*	
CRP (FF) mg/l	1.42 ± 1.27	2.25 ± 2.25	1.47 ± 1.40	2.33 ± 2.04	1.60 ± 1.47	2.68 ± 2.60	1.60 ± 1.60	2.99 ± 2.99	1.21 ± 0.95	2.13 ± 2.13
P values	0.04*		0.03*		0.05*		0.03*		0.01*	

Values are in mean ± standard deviation. Statistical significance was defined as P<0.05 (*; Significant) analysed with the Mann-Whitney U-test. CRP; C-reactive protein, ICSI; Intracytoplasmic sperm injection, MDA; Malondialdehyde, CCs; Cumulus cells, DNPH; 2,4 dinitrophenylhydrazine, NO; Nitric oxide, and FF; Follicular fluid.

Discussion

Inflammation is a response to any disturbance of tissue integrity, triggered to restore tissue balance by activating various repair mechanisms. It is important to regulate these mechanisms properly to prevent an excessive and uncontrolled inflammatory response, which can lead to development of various female reproductive disorders (22). Moreover, the redox reactions involved in cellular oxidative stress play a crucial role in the pathogenesis of inflammation. Excess levels of free radicals and inflammatory markers in FF can have toxic effects on germ cells, oocytes, and their early development (13, 14).

It is widely recognized that under conditions of antioxidant scarcity, ROS or RNS can oxidize membrane phospholipids, proteins, or DNA. Oxidative/nitrosative stress can cause damage through multiple mechanisms. The breakdown of peptide bonds, cross-linking, and modifications to amino acid side chains can cause changes to protein function and antigenicity, which can trigger the immune system and amplify harm caused by the inflammatory response (23, 24). This immune response, which is linked to oxidative stress, is associated with various pathological conditions affecting female fertility (25).

In addition, macrophages act as the first line of defense against invading pathogens or foreign substances and they generate NO, which can be harmful to ovulation, menstruation, and apoptosis, particularly under certain inflammatory circumstances (26). Combination of NO and superoxide anion leads to the creation of the highly toxic oxidant peroxynitrite. This typically occurs only when the concentration of NO surpasses toxic levels, causing it to compete with superoxide dismutase in eliminating superoxide. Indeed, in clinical practice, CRP is extensively used as a reliable marker of inflammation. CRP level is related to the prediction of reproductive outcomes (27). Therefore, in the current study, we measured lipid peroxidation (accessed by MDA assay) and protein carbonyl (accessed by DNPH alkaline assay) levels in CCs, as well as NO, peroxynitrite, and CRP levels in FF of women undergoing ICSI and these levels were compared to ICSI outcomes.

In our study, we also noted that MDA, DNPH levels in CCs as well as NO, peroxynitrite, and CRP levels in FF pools were significantly lower in the patients younger than 37 years old compared to those who were 37 years or older. This finding was consistent with another research noted that advanced age was marked by elevated levels of inflammatory markers, such as CRP (19), and higher production of ROS, leading to oxidative damage with age (10). While the body requires oxidative stress and inflammation to function properly, they can also speed up aging process and development of age-related diseases (11, 14). Furthermore, in the both younger and older women, levels of MDA and DNPH in the CCs, as well as levels of NO, peroxynitrite, and CRP in the FF pools were significantly lower in those with AMH >1.1 ng/ml compared to those with AMH <1.1 ng/ml. As far as we know, AMH is generated solely by the GCs of preantral and small antral follicles and it is a reliable measurement method for ovarian reserve. This result can be explained by the excessive OS and inflammation level affecting production of glycoprotein hormones such as AMH (28). Therefore, inflammation appears to impact ovarian reserve negatively, but the precise mechanism behind its effect on follicles is not clear yet (29).

We also noted that MDA, NO, peroxynitrite, and CRP levels weresignificantly elevated in the patients attempted conception for over five years, compared to those who tried for five years or less. Indeed, a long duration of infertility can result in elevated psychological stress levels among infertile couples. Various studies suggested that a prolonged period of psychological stress could be a contributing source of oxidative stress, leading to inflammation in follicular cells (30).

In addition, CCs and FF pools from patients with a low number of retrieved oocytes displayed elevated levels of MDA, NO, peroxynitrite, and CRP. These results agree with the various reports showing that ROS levels could influence number of the oocytes retrieved (5, 8). Moreover, ovarian stimulation promoted an increase in the number of leukocytes and lymphocytes. It also repaired some immune alterations in infertile patients. Thus, ovarian stimulation might affect integrity of systemic inflammatory hematologic parameters (31). Therefore, the rate of CRP can predict number of the oocytes retrieved.

Furthermore, our data showed that MDA, and DNPH levels in CCs and intrafollicular levels of NO, $ONOO^-$, and CRP were associated with poor oocyte maturity <60%. This finding was consistent with another research demonstrated a negative correlation between levels of ROS and oocyte maturation (5, 8). In the same line, there are various studies suggested that ONOO- played role in the activation of gene expression in response to cellular damage and it had an impact on pathways involved in oocyte maturation (32). Indeed, high levels of NO caused disruption in meiosis development along with a delay in the restart or resumption of meiosis (5). We hypothesized that OS activated the NF-κB pathway, which has a significant impact on triggering cytokine production and inducing an inflammatory response. There is evidence indicating that during the process of maturation from a germinal vesicle to a fully developed oocyte,activity of NF-κB is tightly controlled and suppressed. In support, impaired oocyte maturation was associated with elevated levels of inflammatory markers in FF (13, 29, 31).

We noticed that level of MDA in CCs as well as levels of NO, peroxynitrite, and CRP in FF were significantly higher in the samples associated with oocytes producing low-quality embryos (grades C and D) compared to those associated with high-quality embryos (grades A and B). Previous findings demonstrated excessive OS damages of oocyte membrane phospholipids and impairment of

cell signaling pathways, resulting in poor mitochondria function and altering embryonic development (33). Along with this, higher concentrations of NO can inhibit embryo development (34). In turn, presence of $ONOO^-$ can cause lipid peroxidation and cellular damage in the oocyte microenvironment, potentially lowering quality of the oocyte and embryo. It also hinder successful implantation (35). In addition, high level of OS could give rise to an inflammation process in poor embryo quality (36).

MDA, NO, $ONOO^-$ and CRP levels were significantly higher in the non-pregnant group compared to the pregnant group. These findings are consistent with various reports suggesting that high ROS levels could predict pregnancy failure by IVF (37-39). Moreover, in the physiological condition, pregnancy may also induce micro-inflammation and synthesis of inflammatory markers (37). Furthermore, higher levels of CRP are associated with women's infertility (40).

Conclusion

The study indicated that markers of oxidative and nitrosative stress as well as CRP levels in the oocyte microenvironment may be useful to assess developmental competence of oocytes and embryos. Deeper investigations of mechanism underlying the oxidative/nitrosative stress and CRP level in the human oocyte microenvironment help promote the clinical application of these non-invasive biomarkers in the future.

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Authors' Contributions

H.D., M.J., H.J., A.K., M.E., N.L., R.C.; Participated in the study design, Data collection and Evaluation, Drafting, and Statistical analysis. M.J., H.J., A.K., M.E.; Performed follicle collection and prepared oocytes for ICSI in this component of the study. H.D., R.C.; Contributed to the all experimental work, Data and statistical analysis, and Interpretation of data. N.L., R.C.; Were responsible for overall supervision. H.D.; Drafted the manuscript, which was revised by N.L., R.C. All authors read and approved the final manuscript.

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Association of Follicular Fluid Antioxidants Activity with Aging and *In Vitro* Fertilization Outcome: A Cross-Sectional Study

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Abstract

Background: This research was aimed at assessing the relationship between the follicular fluid (FF) antioxidants activity, aging and *in vitro* fertilization (IVF) outcome.

Materials and Methods: The present cross-sectional study was carried out on 65 women undergoing IVF/intracytoplasmic sperm injection (IVF/ICSI) cycles due to unexplained infertility. Ovarian stimulation was performed using the long gonadotropin-releasing hormone (GnRH) agonist protocol. After ovum pickup, FF was collected and processed to measure the level of superoxide dismutase (SOD), catalase (CAT) activity, total antioxidant capacity (TAC) and glutathione (GSH). Day 3 after ICSI, fresh embryos were transferred and later, possible pregnancy was assessed. Patients participating in this study were divided into four groups on the basis of age and pregnancy outcome.

Results: SOD activity was not significantly different between the groups (P=0.218). GSH in the group whose participants were aged ≤35 years and were pregnant was higher than that in other groups. CAT activity in groups with younger participants was higher compared to the other groups. The mean TAC was higher in groups with pregnant participants compared to the non-pregnant women. Correlation analysis showed that: GSH level had a significant negative correlation with age (P<0.001, R -0.55) and a significant positive correlation with pregnancy (P=0.015, R=0.30). CAT level also had a significant negative correlation with age (P<0.001, R=-0.42) and the level of TAC had a significant positive correlation with pregnancy (P<0.001, R=0.59).

Conclusion: According to our results, the levels of TAC, GSH and CAT in younger and pregnant women were higher compared with those undergoing ICSI cycles. Given the correlation of FF antioxidant activity with age and pregnancy, it is necessary to carry out more research on these compounds and the maintenance of pregnancy.

Keywords: Aging, Catalase, Follicular Fluid Antioxidants, Glutathione, Infertility

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Introduction

The frequency of age-related infertility has increased in recent years (1). In age-related infertility, in addition to the ovarian reserve factor, there is another important factor that is specific to the microenvironment of oocyte development, namely follicular fluid (FF). The content of this fluid that surrounds the oocyte has a vital role in the quality and fertility of the oocyte and subsequent embryo development (2, 3). This microenvironment contains growth factors, granulosa cells, and steroids hormones, as well as factors that produce reactive oxygen species (ROS), such as leukocytes, cytokines, and macrophages (4). ROS imbalance in ovarian FF has a negative impact on the development of oocytes and embryos, as well as sequent pregnancy outcomes (5).

Antioxidants are known as potentially useful factors that can keep the equilibrium between ROS production and clearance. Antioxidant pathways occur in all species, allowing them to cope with oxidative conditions and assisting cells to repair ROS-caused damage. They also play important roles in eliminating toxic oxygen products. These mechanisms are classified as nonenzymatic or enzymatic (6). FF samples taken from women after controlled ovarian stimulation contain antioxidants such as superoxide dismutase (SOD), glutathione oxidase

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(GPX), catalase (CAT), peroxiredoxins (Prx), glutathione transferase (GST), and glutathione reductase (GR) (7-9). Therefore, evaluation of FF in different types of infertility, which requires the use of assisted reproductive technology (ART), may reveal many predictive factors for improvement of outcome *in vitro* fertilization (IVF) (8). Lower levels of ROS and higher total antioxidant capacity (TAC) have been identified as pregnancy predictors for IVF cycles (10).

Identifying the changes in FF antioxidant levels and other related components, as well as their clinical potentials provide helpful means, by which physicians can decide on treatment strategies to increase fertility in infertile couples.

In certain previous investigations, increased SOD activity, reduced CAT and GST, and a modest rise in both GSH-Px and GSSG reductase were shown in the FF of reproductive-aged women (11). Also, it was shown that decreased FF homocysteine concentrations can significantly enhance the oocyte maturation and embryo quality (12).

The question that is posed at this juncture is whether with advanced age, the status of antioxidants in the FF is also affected. Also, it is not clear which antioxidant is most affected by aging. Therefore, this research aimed at assessing the relationship between the FF antioxidants (such as SOD, GSH, CAT and TAC) activity, aging, and IVF outcome.

Materials and Methods

Subject selection and ovarian stimulation

This cross-sectional study was carried out on women undergoing IVF/ICSI cycles. The FF samples were obtained from women referring to the ACECR Infertility Research and Treatment Center, who were under infertility treatment cycles due to unexplained infertility. The research excluded all cases of severe male factor infertility and azoospermia.

Ethical approval was achieved from the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1396.843). Participants were recruited to partake in the research and were given a study description as well as an informed consent.

A total of 65 patients participated in this research, and were categorized on the basis of age and clinical pregnancy outcome (yes/no):

Group 1: females aged > 35 years and pregnant (n=15).

Group 2: females aged ≤ 35 years and pregnant (n=20).

Group 3: females aged > 35 years and non-pregnant (n=15).

Group 4: females aged ≤ 35 years and non-pregnant (n=15).

In this study, women who did not become pregnant without contraception after one year and whose follicle-stimulating hormone (FSH) levels were less than 10 were recruited. Data were obtained using a questionnaire and were stored with the patients' clinical records. Before the start of ovulation induction, all patients had a transvaginal ultrasound (TVU) scan to check their ovaries and other pelvic tissues.

Women who were using antioxidant vitamins, hormonal contraception, or other hormonal preparations were not allowed to participate. Thus, patients with details on the etiology of infertility with tubular factors, endometriosis, cardiovascular problems, diabetes, nutritional diseases, depression, cancer, hypertension, hyperthyroidism, uterine fibroids, endometriosis, ovarian cyst, polycystic ovary syndrome, smoking history, hydrosalpinx and severe ovarian hyperstimulation (OHSS) (characterized by an egg count above 20 and clinical signs such as increased abdominal fluid, shortness of breath, pericardial effusion, pleural effusion, electrolyte disturbances and oliguria), and 19>body mass index (BMI)>30, were excluded from this study.

Ovarian stimulation protocol

The lengthy gonadotropin-releasing hormone (GnRH) agonist treatment was used for ovarian stimulation. On the 21st day of the menstrual cycle, a subcutaneous injection of GnRH agonist (Dipherelin triptorelin; Ipsen Pharma, Paris, France) was used to downregulate the hormone. Gonal F (Merck Serono, Germany) was introduced on the second day of the cycle. Gonadotropin injections were continued until at least two follicles reached 17-18 mm in diameter, at which point ovitrelle (250 µg, Merck Serono, Germany) was administered to induce the last oocyte maturation phase.

Collection and processing of follicular fluid

Under TVU guidance, the follicles were collected 34-36 hours following the ovitrelle injection. The FF from the first aspirated follicle was collected separately in a sterile tube without adding more culture medium. The remaining follicles were placed in regular culture media in preparation for the routine IVF process. According to the conventional classifications, the first aspirated oocytes were classified as germinal vesicle (GV), metaphase I (MI) stage, or metaphase II (MII) stage (13). MII oocytes were analyzed morphologically, and oocytes were divided into three groups based on the number of abnormalities: grade I: no abnormalities, grade II: one abnormality, and grade III: at least two abnormalities (14). Oocytes were implanted in conventional culture medium, and their development was tracked after intracytoplasmic sperm injection (ICSI) procedure using spermatozoa. After the removal of the oocytes, the FF was centrifuged at 2700 rpm for 5 minutes to remove cellular components. The supernatant was stored at -80°C for assessment of SOD, CAT, TAC, and GSH activities. Then, the day 3 embryo was transferred after morphological examination according to

the standard classifications (15). Only FF with minimal contamination and no blood or culture medium was used. After fresh embryo transfer, chemical pregnancy detected by checking beta-hCG in the serum.

Measurement of superoxide dismutase activity

SOD activity was measured using a Randox test combination (16). Superoxide radicals were created by combining 2-(4-iodophenyl)3-(4-nitrophenol)-5-phenyl tetrazolium chloride (INT) with xanthine and xanthine oxidase to give a red formazan dye. This reaction is inhibited by superoxide dismutase, which converts the superoxide radical to oxygen. SOD activity in cleaned erythrocyte hemolysates was measured at 505 nm using a CECIL 3000 SCANNING Spectrophotometer. The results were given in SOD U/ml.

Measurement of glutathione activity level

The GSH assay was carried out using the usual procedure (17). The standard curve was created with a 1 mM GSH solution and evaluated for GSH levels. In addition, 0.2 ml of FF or serum samples were mixed with 2.3 ml of potassium phosphate buffer (0.2 M, pH=7.6), followed by 0.5 ml of DTNB (0.001 M) in buffer. After 5 minutes, the absorbance of the reaction products was measured at 412 nm. Each sample's total protein concentration was determined, and GSH values were represented as nmol/mg protein.

Measurement of catalase activity

CAT activity was evaluated based on the method elucidated by Koroliuk et al. (18). In brief, this test is on the basis of the reduction rate of hydrogen peroxide per unit time due to the activity of CAT in the sample. Ammonium molybdate with hydrogen peroxide forms a yellowish complex that has a maximum light absorption at 510 nm. The enzyme CAT prevents this reaction by breaking down hydrogen peroxide. It has maximum light absorption. The enzyme CAT prevents this reaction by breaking down hydrogen peroxide. One U is the enzyme amount that breaks down one micromole of hydrogen peroxide in one minute. To measure the activity of Tris-HCl buffer CAT enzyme (0.05 mmol/l, PH=7.8), 15 mmol/l hydrogen peroxide was made and mixed with the sample and after 15 minutes 0.4 ammonium molybdate was added. Finally, the optical absorption of the samples and control against Buffer Tris-HCl at 510 nm were read by microplate spectrophotometer.

Measurement of catalase level

TAC in FF was determined in duplicate, first after defrosting and then again after 72 hours of storage at room temperature in the dark, using the ferric reducing/antioxidant power (FRAP) assay (14, 17, 18). TAC values obtained from this assay are proportional to those obtained from the Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) assay (19). In summary, 50 µl of FF was mixed with 1 ml of freshly made FRAP reagent, which contained 1 mM 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ), 2 mM $FeCl_3$, and 240 mM sodium acetate, at pH=3.6. Antioxidants in the sample cause the colorless Fe^{3+}–TPTZ complex to be reduced to an intense blue Fe^{2+}–TPTZ complex, which is detected using a spectrophotometer at 593 nm after incubation for 10 minutes at 25°C. A standard curve of 0-1 mM Fe^{2+}–TPTZ was prepared by diluting $FeSO_4.7H_2O$ (1 mM). TAC estimates are therefore given in mmol/l ferrous equivalents.

Statistical analysis

Statistical analyses were carried out with the help of the SPSS software version 23 (IBM Corp., Armonk, New York, USA). Since the data of the present study did not have a normal distribution (as attested by Kolmogorov-Smirnov test), non-parametric tests of Kruskal-Wallis and Spearman were used. Therefore, for pairwise comparison of the groups, Mann-Whitney U test was used. The data obtained from the present study were expressed as mean ± SD, and the significance level was set at $P<0.05$.

Results

In order to investigate the challenging relationship of antioxidant activity with age-related changes, fertilization rate, and pregnancy outcomes, we collected FF samples from women undergoing IVF/ICSI cycles due to unexplained infertility.

Comparison of physiological parameters in groups undergoing IVF/ICSI cycles

The mean age of the women in groups 1 to 4 were: 39 ± 2.29, 30.35 ± 2.73, 38.87 ± 2.10, and 31 ± 2.77, respectively.

The mean number of retrieved oocytes in the four mentioned groups was statistically significant (P=0.041). Groups 4 and 1 had the highest and lowest oocyte counts, respectively.

Statistical analysis showed that the mean number of MII oocytes in the four groups was not significantly different (P=0.260). Nonetheless, groups 1, 2 (1 and 2 were equal) and 4 had the lowest and highest MII oocyte counts, respectively.

Statistical analysis of the fertilization rate in each group showed a significant difference among the groups (P=0.042). The information of all four study groups is summarized in Table 1.

Comparison of oocyte and embryo morphological scores in groups undergoing IVF/ICSI cycles

There was no significant difference in oocyte morphological scores among the different groups (P=0.291). Embryo morphological scores also were not significantly different in the four study groups (P=0.188). The information of all study groups is summarized in Table 2.

Table 1: Comparison of physiological parameters in groups undergoing *in vitro* fertilization cycles

Groups/Variables	1[a] (n=15)	2[b] (n=20)	3[c] (n=15)	4[d] (n=15)	P value
Age (Y)	39 ± 2.29	30.35 ± 2.73	38.87 ± 2.10	31 ± 2.77	-
NO. Oocyte	8.07 ± 2.60	8.35 ± 2.23	9.13 ± 3.18	10.33 ± 2.09	0.041[abcd*] 0.532[ab] 0.270[ac] 0.017[ad*] 0.563[bc] 0.013[bd*] 0.103[cd]
	7 (5)	8 (3)	8 (3)	11 (4)	
NO. Oocyte MII	7 ± 2.07	7 ± 1.68	7.33 ± 3.65	8.27 ± 1.90	0.260
	6 (4)	7 (2)	7 (6)	8 (3)	
Proportion of MII oocytes (%)	88.03	85.02	78.12	81.46	0.656
Fertilization rate (%)	71.28	72.87	66.16	55.28	0.042[abcd*] 0.664[ab] 0.416[ac] 0.021[ad*] 0.299[bc] 0.007[bd*] 0.191[cd]

Data presented as n or mean ± SD or median (IQR). P<0.05 were considered significant. The values are compared by Kruskal-Wallis Test. *; Shows significant difference. [a]; Group 1: Females aged >35 years and pregnant, [b]; Group 2: Females aged ≤35 years and pregnant, [c]; Group 3: Females aged >35 years and non-pregnant, [d]; Group 4: Females aged ≤35 years and non-pregnant. MII rate; No. MII oocyte/all oocyte retrieved×100, Fertilization rate; No. oocyte with 2PN/No. MII oocyte injected ×100.

Table 2: Comparison of oocyte and embryo morphological score in groups undergoing *in vitro* fertilization cycles

Groups/Variables	Scoring	1 (n= 15)	2 (n=20)	3 (n=15)	4 (n=15)	P value
Oocyte morphological score	Grade I	3	7	7	3	0.291
	Grade II	11	9	4	5	
	Grade III	1	4	4	7	
Embryo morphological score	Good	9	10	5	4	0.188
	Fair	3	6	8	4	
	Poor	3	4	2	7	

Data presented as Number. The mean values are compared by Kruskal-Wallis Test. P<0.05 were considered significant. Group 1; Females aged >35 years and pregnant, Group 2; Females aged ≤35 years and pregnant, Group 3; Females aged >35 years and non-pregnant, and Group 4; Females aged ≤35 years and non-pregnant.

Levels of follicular fluid antioxidant activity in groups undergoing IVF/ICSI cycles

The levels of SOD activity in FF of all four groups were not significantly different (P=0.218).

However, the mean of GSH in FF was significantly different between the four groups (P<0.001).

Pairwise comparison of groups was performed to determine the differences in the level of GSH activity. The results showed that the level of activity of this enzyme in group 2 was significantly higher compared with group 3 (P<0.001). Also, the level of GSH activity in group 4 was significantly higher compared with groups 1 and 3 (P=0.016 and P<0.001, respectively).

Therefore, these results showed that the mean activity level of this enzyme is generally higher in younger women.

The mean of CAT activity in the four groups showed that there was a significant difference among them (P<0.001). Pairwise comparison of the groups was performed to determine the level of activity of CAT in each group. The results indicated that the mean of CAT activity in group 2 and group 4 was higher than that in the other groups. The activity level of this enzyme in group 3 was lower compared with all groups.

The four study groups were significantly different in terms of their mean TAC level (P<0.001). Pairwise comparison of the groups was performed to determine how different the level of TAC enzyme is among the study groups. The results showed that the mean TAC level in group 2 was significantly higher compared to groups 3 and 4 (P=0.004 and P<0.001, respectively). These results showed that the mean level of this enzyme was higher in pregnant women.

The results of FF antioxidant activity levels in the four study groups and their pairwise comparisons are presented in Table 3.

Table 3: Levels of FF antioxidant activity in groups undergoing *in vitro* fertilization cycles

Groups variables	1[a] (n=15)	2[b] (n=20)	3[c] (n=15)	4[d] (n=15)	P value
SOD activity (U/g)	0.23 ± 0.12	0.29 ± 0.26	0.22 ± 0.21	0.16 ± 0.11	0.218[abcd]
	0.17 (0.13)	0.25 (0.19)	0.17 (0.13)	0.11 (0.21)	
GSH (nmol/mg protein)	52.32 ± 12.54	66.29 ± 25.26	28.86 ± 3.45	64.71 ± 14.15	<0.001[abcd*] 0.064[ab] <0.001[ac*] 0.016[ad*] <0.001[bc*] 0.640[bd] <0.001[cd*]
	55.14 (13.14)	60.75 (24.75)	29.78 (5.07)	69.42 (19.50)	
CAT activity (mM/L)	1.44 ± 0.46	2.53 ± 1.55	0.96 ± 0.51	2.89 ± 1.55	<0.001[abcd*] 0.662[ab] 0.022[ac*] 0.003[ad*] 0.003[bc*] 0.473[bd] 0.001[cd*]
	1.44 (0.46)	2.52 (2.46)	0.79 (0.84)	3.10 (2.22)	
TAC activity (mMol/l)	0.69 ± 0.27	0.76 ± 1.30	0.58 ± 0.11	0.27 ± 0.11	<0.001[abcd*]
	0.70 (0.33)	0.75 (0.21)	0.64 (0.19)	0.29 (0.18)	0.250[ab] 0.198[ac] <0.001[ad*] 0.004[bc*] <0.001[bd*] <0.001[cd*]

Data presented as n or mean ± SD or median (IQR). The mean values are compared by Kruskal-Wallis Test. P<0.05 were considered significant. *; Shows Significant difference. [a]; Group 1: Females aged >35 years and pregnant, [b]; Group 2: Females aged ≤35 years and pregnant, [c]; Group 3: Females aged >35 years and non-pregnant, [d]; Group 4: Females aged ≤35 years and non-pregnant, FF; Follicular fluid, SOD; Superoxide dismutase, GSH; Glutathione, CAT; Catalase, and TAC; Total antioxidant capacity.

Correlation of age, pregnancy, and oocyte and embryo morphological score with the level of antioxidants activity Spearman correlation was used to investigate the relationship between age and pregnancy and the level of antioxidant activity. The results showed that GSH level has a significant inverse correlation with increasing age (P<0.001, r=-0.55), and a significant direct correlation with pregnancy (P=0.015, r=0.30). Therefore, it can be suggested that with increasing age, the level of GSH decreases, but this level increases during pregnancy. CAT level had a significant inverse correlation with increasing age (P<0.001, r=-0.42). Therefore, with increasing age, the level of this antioxidant decreases, and it is not correlated with positive pregnancy. The level of TAC, on the other hand, had no significant correlation with increasing age, but it had a significant direct correlation with positive pregnancy (P<0.001, r=0.59), as the level of this antioxidant increases with positive pregnancy. The data discussed in this section are summarized in Table 4.

Table 4: Correlations between age and pregnancy in relation to level of antioxidants activity

Variables	SOD (n=65)	GSH (n=65)	CAT (n=65)	TAC (n=65)
Age (Y)				
r	0.26	-0.55	-0.42	0.10
P value	0.835	<0.001*	<0.001*	0.417
Pregnancy				
r	0.19	0.30	0.08	0.59
P value	0.127	0.015*	0.498	<0.001*

Relationship between age, pregnancy and level of antioxidants were done by Spearman correlation. SOD; Superoxide dismutase, GSH; Glutathione, CAT; Catalase, TAC; Total antioxidant capacity, r; Correlation coefficient, *; Correlation is significant at the 0.01 level.

Spearman correlation was also used to investigate the correlation between oocyte morphological score and levels of antioxidants activity. The correlation between oocytes morphological score and levels of SOD, GSH, CAT, and TAC was not statistically significant (P=0.190, P=0.343. P=0.327 and P=0.190, respectively). Similarly, no statistically significant correlation was observed between embryo morphological score and the levels of SOD, GSH, CAT and TAC (P=0.315, P=0.852. P=0.853 and P=0.221, respectively).

Discussion

Considering the important effects of FF compounds on oocyte and embryo development, this research aimed to examine the impact of aging on the changes in FF antioxidant activity levels and their possible relationship with the outcome of IVF cycles. The main objective of the present study was to identify these changes and use them clinically to make decisions about treatment strategies in infertile couples.

According to our results, the proportion of MII oocytes (at ICSI) in the study groups was not significantly different. However, fertilization rates were significantly higher in pregnant women than in their non-pregnant counterparts.

The results of this study also show that the morphology scores of oocytes and embryos were not significantly different in all women undergoing IVF/ICSI cycles, while antioxidant levels and the outcomes of IVF cycles

were significantly different. These results may indicate the effects of antioxidants on non-morphological levels, including the molecular levels, which may affect the fertility potentials of oocytes and embryo implantation.

This investigation revealed that the SOD activity did not differ substantially in all of the studied infertile women. Nonetheless, mean SOD activity in younger and pregnant cases was higher than those undergoing IVF/ICSI cycles. Therefore, it could be argued that in the present study, changes in SOD activity were not age-related, as they were higher in groups with pregnancy outcomes. Human investigations have demonstrated that older women have lower levels of SOD and CAT in their FF compared with their younger counterparts, and that older women experience decreased fertilization rates and blastocyst development (20). A previous study showed that a statistically significant decrease was observed in the SOD activity of FF in polycystic ovary syndrome patients compared with the control group (21).

Our results also indicated that the mean level of GSH was higher in younger women. In addition, the mean SOD activity in younger and pregnant cases was more significantly different in comparison to other cases. Further, the level of GSH had a significant inverse association with age and a significant direct association with pregnancy. Indeed, both pregnancy and age contribute to changes in the GSH levels. The lower GSH content seen in endometriosis FF compared to the controls was also linked to low quality embryo (22). GSH plays a role in various biological activities, including cell proliferation, differentiation, and death (5). GSH, according to the literature, boosts gamete viability and fertilization (23). ROS are thought to be involved in the start of apoptosis, as ROS levels rise before any other signal associated with death in follicles. A statistically significant increase in atretic antral follicles was seen in rat ovaries after limiting GSH production with the inhibitor buthionine sulfoximine (BSO) (24). FSH is widely thought to prevent apoptosis in antral follicles, and surprisingly, FSH therapy enhances GSH production. The anti-apoptotic effect of FSH on granulosa cell death is significantly reduced by inhibiting GSH production with BSO in cultured follicles (25). A previous study on the effects of cyclophosphamide on the ovaries showed that this cancer drug causes follicular apoptosis as well as reduced GSH levels in the ovaries (24). Apoptosis in cultured preovulatory follicles is induced by oxidative stress, and the antioxidant GSH plays a role in regulating the anti-apoptotic impact of FSH on granulosa cells in preovulatory follicles (26). An investigation was conducted on blood samples and FF of the first-retrieved follicle from PCOS women, and the mean activity of GPx and GR, as well as GSH levels in the serum and FF were compared with the quality of the first follicle and resulting embryo. The mean GPx activity and GSH levels were considerably greater in the serum and FF of high-quality grade I embryos (27).

The present study showed that in younger women, regardless of pregnancy, the mean CAT activity was significantly higher compared to the older women. Moreover, a significant inverse association was detected between age and CAT activity. That is, as age increases, the activity of these antioxidants decreases significantly. In agreement with the present study, a decrease in CAT activity in FF was observed with advanced age in a previous study (20). In another study, select indicators such as CAT activity, TAC, and hydrogen peroxide (H_2O_2) were measured in FF samples derived from cow antral follicles. According to their results, although TAC rose dramatically, CAT activity and H_2O_2 dropped considerably as follicle size grew. Lower TAC and higher H_2O_2 levels in tiny follicles indicate an increase in ROS during the early stages of folliculogenesis. Because CAT levels are highest in the FF of small follicles in a low total TAC, CAT may serve as a major antioxidant defense in the early phases of folliculogenesis (28). In the present study, CAT activity in younger groups was significantly higher, which may be due to the fact that small-sized follicles (ovarian reservation) are more common in younger women. However, another study discovered that following FSH stimulation, CAT activity rose, and the degree of this rise was greater in large follicles than in medium or small follicles (29). According to some review studies, CAT plays a role in follicular formation, the estrous cycle, and steroidogenic events in the ovaries (5), and protects the DNA from oxidative damage (30). Furthermore, increased CAT activity was identified in obese and infertile women, revealing that the FF of obese women was associated with higher CAT activity, which indicates excessive oxidative stress (31). Results of the present study showed that with advanced age, the activity of this antioxidant decreases, and based on other studies CAT activity increases in pathologic cases. The distribution and oscillation of CAT during several ovarian cycles have been linked to gonadotropin regulation (32). As a result, CAT may be regarded as a protective factor neutralizing H_2O_2 and preserving ROS equilibrium. Measuring the activity of antioxidants may not be enough for a predictive marker alone. Therefore, future studies are recommended to assess the amount of balance between CAT activity and ROS associated with assisted reproductive technique (ART) outcomes.

Gonadotropin signaling modulates oocyte GSH levels throughout the preovulatory stage, according to both *in vivo* and *in vitro* investigations. FSH stimulation has been reported to increase ovarian GSH concentration *in vivo* (33). GSH, CAT, and SOD can also protect big antral follicles against apoptosis in rats (34). Reduced antioxidant systems have also been linked to age-related reproductive reduction (35, 36). Previous studies indicated that the ability of antioxidants to scavenge ROS is related to fertilization outcomes (5). According to the above explanations, it can be argued that high levels of CAT activity can create a level of confidence in the ROS-antioxidant balance. Increasing the ROS in different conditions leads to increased GSH and thus maintains the balance between ROS and antioxidants, consequently

reducing the destructive effects due to ROS. Given the association of age and gonadotropin with level of GSH and CAT activity, it is recommended to pay special attention to GSH and CAT in cases of age-related infertility.

The present study compared four different groups in terms of their mean TAC level, which was higher in the pregnant groups as opposed to the non-pregnant ones (regardless of the age). Also, pairwise comparisons and correlation analysis to investigate the changes in the level of TAC showed that pregnancy is more important than age as far as the changes in the level of this antioxidant are concerned. A previous study reported that TAC increased significantly as follicle size increased in estrous cycle (28). Another prospective cross-sectional study showed that FF TAC levels were higher in women with 'unexplained' (UE) or tubal factor (TF) infertility, while age did not affect FF TAC activity in general. Similarly, the results of the present study confirm this correlation between age and TAC activity. It has been demonstrated that low TAC is associated with fertilization incompetence, while high TAC is associated with embryo nonviability (optimum follicular TAC was ~0.68 mmol/l) (37). In the present study, TAC in younger and pregnant women was 0.76 mmol/l, which is higher compared with those undergoing IVF/ICSI cycles.

Since the antioxidant activity can be affected by different conditions, including increased FSH in menstrual cycle (33), age (10) and pregnancy, measuring the antioxidant activity, may not be enough for a predictive marker alone. Although the small sample size in this study is one of its limitations, based on data from the literature, it is suggested that the balance between antioxidants activity and the amount of ROS in FF and serum associated with the outcomes of IVF cycles, be addressed in future studies. Measurement of FF antioxidant activity/ROS ratio is necessary in women undergoing IVF cycles (related to aging and etiology of infertility). For this ratio, definition of a cutoff point could be predictive of the pregnancy outcome. Further studies on GSH and CAT activity are recommended to be performed with the aim of using these antioxidants for the prevention, diagnosis and treatment of infertility.

Conclusion

The present study represents the possible effects of FF antioxidants on fertility potentials of oocyte and embryo implantation at a molecular level. The level of the TAC was higher in pregnant women while the mean GSH and CAT levels were higher among younger women. The mean GSH and CAT levels decreased as age advanced. According to the results of this study, there is a correlation between GSH, age, and pregnancy, and it is necessary to carry out more research on FF antioxidants and their effects on maintenance of pregnancy.

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Authors' Contributions

M.A., R.N., M.H., P.D.; Participated in study design, Data collection, and Evaluation. R.N., M.A.; Collected the samples. M.A., M.H., E.Gh, S.Am., A.Z., S.Ad., M.M.; Analyzed antioxidants activity. P.D., M.A.; Carried out data analysis and Interpretation. P.D., R.N.; Wrote the first draft of the manuscript. All authors revised and approved the final version of the manuscript.

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Association between Myometrial Thickness and Assisted Reproductive Technologies Outcomes: A Prospective Cohort Study

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Abstract

Background: Myometrial thickness has been expected to be a prognosticator for lower uterine segment function. An abnormal function of the uterine muscle layer can cause common and important reproductive problems. This study aimed to evaluate the relationship between baseline myometrial thickness and assisted reproductive technologies (ART) outcomes.

Materials and Methods: In this prospective cohort study, 453 infertile women undergoing ART cycles without any obvious uterine pathology, participated in this prospective cohort study from February 2013 to May 2015. In order to measure the myometrial thickness in the anterior and posterior of the uterine, trans-vaginal ultrasounds were conducted on days 2-4 of the cycle (menstrual phase) preceding ovarian stimulation and the day of human chorionic gonadotropin (hCG) injection. We defined three groups based on the baseline myometrial thickness in the anterior and posterior, including (A) <25 mm, (B) 25-29.9 mm and (C) ≥30 mm. Ovarian stimulation, oocyte retrieval and luteal phase support were performed in accordance with the standard long protocol. Two weeks after embryo transfer, the patients underwent a pregnancy test by checking their serum β-hCG levels. The primary outcome measure was clinical pregnancy rate. Secondary outcome measures were, implantation rate, abortion rate and live birth rate.

Results: The clinical pregnancy (P=0.013) and implantation (P=0.003) rates were significantly lower in group A than in two other groups. Although the live birth rate was lower in group A than two other groups, this decrease was not statistically significant (P=0.058).

Conclusion: The findings may be a way for clinicians to draw focus on providing therapeutic strategies and a specific supportive care for women with a baseline myometrial thickness <25 mm in order to improve the reproductive outcome of *in vitro* fertilization/intracytoplasmic sperm injection (IVF-ICSI).

Keywords: Embryo Implantations, Myometrium, Pregnancy Rate, Ultrasonography

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Introduction

Uterine contractions are effective in varied reproductive processes of menstruation, gamete and embryo transport, implantation, pregnancy and to parturition (1, 2). An abnormal function of the uterine muscle layer can cause disorders including dysmenorrhea, infertility, implantation failure, spontaneous abortion or preterm delivery, that are common and important challenges of couples in fertility age (3-6). Based on the mathematical modeling, it has been proven that an uterine wall stress is an inversely proportional factor of the myometrium thickness (7). Some studies suggested that interactions between the innermost layer of the myometrium (junctional zone) and the endometrium, it seems it plays a significant role in the implantation process (8, 9).

An ultrasound imaging is a noninvasive method to evaluate an infertility and its treatment progress. Ultrasonography is an effective tool in improving the quality of services provided by the assisted reproductive technologies (ART) with facilitating timely diagnosis

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and appropriate management (10, 11). Transabdominal and transvaginal ultrasonography have been used as reliable methods to measure the myometrial thickness in predicting pregnancy outcome (12). In a first study by Lesny et al. (13), they observed that the myometrium thickness was significantly higher in the pregnant group in comparison with the non-pregnant group on the down regulation day, day 8 of ovulation induction and human chorionic gonadotropin (hCG) injection day during *in vitro* fertilization-embryo transfer (IVF-ET) cycles. However, Youm et al. (14) argues that a myometrial thickness more than 2.50 cm on trans-vaginal ultrasonography (TVUS) may predict adverse outcomes of IVF-ET for women with adenomyosis.

Apart from Lesny et al. (13), the effect of myometrial thickness on reproductive outcome has not been paying attention as much as it needed. To our knowledge, the present study is the first prospective cohort analysis was designed to assess the relationship between the thickness of the myometrium and outcomes of IVF/intracyto-plasmic sperm injection (ICSI) cycles.

Materials and Methods

Patients

This prospective cohort study was carried out in 453 infertile women undergoing IVF/ICSI cycles from February 2013 to May 2015 at the Infertility Center of Royan Institute, Tehran, Iran. The Institutional Review Board approved this study and the ethical clearance was issued by the Royan Institute Ethics Committee (EC/1020/91), Tehran, Iran, in compliance with the Helsinki Declaration. Also, a written informed consent was obtained upon their arrival at the clinic.

The inclusion criteria were as follows: no obvious pathology of uterine, being at their first IVF/ICSI cycle, having 2-3 excellent or good quality embryos, fertilization rate above 50%, and endometrial thickness of at least 7 mm on the day of the hCG injection. Patients were excluded from participation if either have obvious anomalies of the uterus, uterine myoma, uterine septum, or their husbands underwent testicular sperm extraction (TESE) or testicular sperm aspiration (TESA) or percutaneous epididymal sperm aspiration (PESA).

Sonographic procedure

All patients underwent TVUS to measure the myometrial thickness of anterior and posterior uterine on the days 2-4 of the menstrual phase preceding an ovarian stimulation. Measurements were performed in the mid-sagittal plane by one sonographist using with an Aloka-α10 ultrasound system (Alok, Japan) equipped with a 5-8 MHz transvaginal probe, from one endometrial-myometrial interface to the uterine serosa as end point where the area appears to be at its thickest in the fondus.

The summation of myometrial thickness in anterior and posterior was divided into three following groups: <25 mm (group A), 25-29.9 mm (group B) and ≥30 mm (group C).

Ovarian stimulation cycle

All patients were treated according to the standard long gonadotropin-releasing hormone (GnRH) agonist protocol (Buserelin acetate, Aventis Pharma Deutschland, Germany). Ovarian stimulation started with follicle stimulating hormone (FSH, Gonal F 75 IU, Merck Serono, Italy) with or without human menopausal gonadotrophin (hMG, Menogan 75 IU, Ferring, Germany or Menopur 75 I, Ferring, Germany) according to the ovarian response. The cycle monitoring was performed with sequential TVUS and measurement of the serum estradiol level. When at least one follicle was detected with a diameter of ≥18 mm and serum estradiol level reached to the 500-2000 pg/ml level, a dose of 10000 IU hCG (DarouPakhsh Co., Iran) was injected. The luteal phase was supported by a daily dose of the progesterone ampoule 50 mg (Aburaihan Co., Tehran, Iran) until 2 weeks after an ET. And 34-36 hours later, oocytes retrieval was performed under transvaginal ultrasound guidance to collect follicles. An embryo transfer was done after 48-72 hours. Pregnancy was detected by measuring the serum hCG level, two weeks after the embryo transfer.

Outcome definitions

The implantation rate was defined as the ratio of gestational sacs number per the ET number that were observed 4-6 weeks after ET. Clinical pregnancy was defined as the presence of at least one intrauterine gestational sac with the detectable fetal heart activity by TVUS 6-8 weeks after the embryo transfer. The abortion rate was defined as the total number of abortions obtained before 20 completed weeks gestation to the ET cycles.

The live birth rate was defined as the ratio of deliveries number that resulted in at least one live born baby to the ET cycles (15).

Statistical analysis and sample size calculation

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, SPSS Inc., Chicago, IL, USA) version 20. Continuous variables are presented as mean ± standard deviation (SD) and categorical variables are shown as number (percentage). Demographic and clinical characteristics were compared between groups using chi-square test and one-way analysis of variance (ANOVA). Logistic regression was used to examine the relationship between myometrial thickness and cycle outcome (clinical pregnancy). A $P<0.05$ was considered statistically significant.

Results

Totally, 453 couples participated in our study that divided in to three groups, including A (n=52), B (n=199) and C (n=202). Demographic and clinical characteristics of the three groups (A, B and C) are detailed in Table 1.

Table 1: Demographic and clinical characteristic of our participants

Parameters	Group			P value*
	A†(n=52)	B‡ (n=199)	C ‡† (n=202)	
Age (Y)	30.69 ± 5.06	30.86 ± 4.46	32.93 ± 4.67	<0.001
Body mass index (kg/m^2)	26.30 ± 3.86	25.93 ± 3.60	26.23 ± 4.37	0.698
Duration of infertility (Y)	6.50 ± 4.24	6.36 ± 4.16	6.47 ± 4.08	0.958
Cause of infertility				
Male factor	22 (42.3)	116 (58.3)	106 (52.5)	0.284
Tubal factor	3 (5.8)	7 (3.5)	14 (6.9)	
Anovulatory	8 (15.4)	28 (14.1)	32 (15.8)	
Recurrent abortion	1 (1.9)	2 (1.0)	4 (2.0)	
Unexplained factor	6 (11.5)	23 (11.6)	27 (13.4)	
Multi-factor	12 (23.1)	23 (11.6)	19 (9.4)	
Ovulation induction information				
No. of ampoules used	25.86 ± 8.07	26.70 ± 10.61	26.81 ± 9.73	0.896
Length of ovarian stimulation (days)	13.03 ± 3.34	13.02 ± 3.22	12.98 ± 3.23	0.990
Endometrial thickness on the day of hCG administration (mm)	9.18 ± 1.69	9.66 ± 1.85	10.31 ± 2.12	<0.001
No. of oocytes retrieved	7.93 ± 3.51	8.88 ± 3.98	8.10 ± 3.97	0.250
No. of injected oocytes	6.83 ± 3.45	7.09 ± 3.28	6.76 ± 3.64	0.761
Embryo's information				
No. of 2PN embryos	3.86 ± 2.51	4.50 ± 2.82	4.83 ± 2.91	0.244
No. of embryos transferred	2.57 ± 0.66	2.42 ± 0.75	2.52 ± 0.74	0.226
No. of excellent and good quality embryos	2.09 ± 0.91	2.02 ± 0.96	2.08 ± 0.91	0.729

Values are given as mean ± SD or n (%).†; Anterior-posterior myometrial diameter <25 mm, ‡; Anterior-posterior myometrial diameter between 25-29.9 mm, ‡†; Anterior-posterior myometrial diameter ≥30 mm, hCG; Human chorionic gonadotropin, 2PN; two-pronuclear, and *; Chi-square test for categorical variables and one-way analysis of variance for continuous variables.

The data showed that 94.7% patients had primary infertility and 5.3% had secondary infertility. Causes of infertility were as follows: male factor 53.9%, tubal factor 5.3%, anovulatory factor 15.0%, recurrent abortion 1.5%, mixed 11.9% and unexplained factor 12.4%.

Clinical characteristics of the three groups (A, B and C) are detailed in Table 1. Clinical characteristics including body mass index, duration of infertility, causes of infertility, number of ampoules used, length of ovarian stimulation, number of oocytes retrieved, number of injected oocytes, number of two-pronuclear embryos (2PN), number of embryos transferred, and excellent and good quality embryos number showed no significant differences among the three groups (Table 1).

Age and endometrial thickness on the day of hCG administration were significantly higher in group C as compared to groups A and B. The mean values of myometrial thickness in the anterior and posterior of the uterus were as follows: 14.30 ± 2.41 mm (range, 9.10-24.60 mm) and 15.47 ± 2.44 mm (range, 10.0-23.80 mm), respectively.

The overall rate of pregnancy based on the positive βhCG was 42.8% (194/453). Among the 194 pregnancies (based on the positive βhCG), 160 clinical pregnancies, 16 blighted ovums, 7 missed abortions and 11 biochemical pregnancies occurred. The overall rates of clinical pregnancy, implantation and live birth were 35.3, 18.7 and 32.3%, respectively. There were significant differences regarding the clinical pregnancy and implantation rates among three groups (P=0.013 and P=0.003, respectively). Loss to follow up after pregnancy was 5 (3.1%) in groups of B (n=3) and C (n=2) (Table 2). Although, the live birth rate was lower in group A than two other groups, this difference was not statistically significant (P=0.058). It is likely this lack of statistical significance is due to the small sample size.

Table 2: Cycle outcomes in our participants

Outcomes	Group			P value*
	A† (n=52)	B‡ (n=199)	C ‡† (n=202)	
Clinical pregnancy rate	9/52 (17.3)	78/199 (39.2)	73/202 (36.1)	0.013
Implantation rate	12/134 (9.0)	105/482 (21.8)	94/511 (18.4)	0.003
Abortion rate	0/9 (0)	5/78 (6.4)	3/73 (4.1)	0.631
Live birth rate	9/52 (17.3)	67/196 (34.2)	66/200 (33.0)	0.058

Values are given as number/n (%).†; Anterior-posterior myometrial diameter <25 mm, ‡; Anterior-posterior myometrial diameter between 25-29.9 mm, ‡†; Anterior-posterior myometrial diameter ≥30 mm, and *; Chi-square analysis.

In unadjusted analysis, the clinical pregnancy rate was significantly higher in the group B than group A (39.2 vs. 17.3%) with an odds ratio of 3.08 [95% confidence interval (CI): 1.42-6.67], while it changed only slightly after being adjusted for age and endometrial thickness on the hCG day to 3.10 (95% CI: 1.42-6.73). The group C had approximately the same results as the group B (Table 3).

Table 3: Crude and adjusted odds ratios for clinical pregnancy in our participants

Group	Clinical pregnancy rate, n (%)	Crud (unadjusted) OR		Adjusted OR[a]	
		OR (95% CI)	P value	OR (95% CI)	P value*
A (n=52)[b]	9 (17.3)	1	-	1	-
B (n=199)	78 (39.2)	3.08 (1.42-6.67)	0.004	3.10 (1.42-6.73)	0.004
C (n=202)	73 (36.1)	2.70 (1.25-5.86)	0.012	2.85 (1.29-6.31)	0.010

[a]; Adjusted for age and endometrial thickness on hCG day, [b]; Reference group, OR; Odds ratio, CI; Confidence interval, *; Logistic regression, and hCG; Human chorionic gonadotropin.

Discussion

According to the International Society of Ultrasound in Obstetrics and Gynecology (ISUOG), anterior-

posterior diameter is 2.00-5.00 cm in reproductive age women (16) that is compatible with our results. In our study, the findings showed that in ART cycles, the baseline myometrial thickness (anterior-posterior diameter) <25 mm is associated with a lower implantation and pregnancy rates in comparison with a myometrial thickness ≥25 mm.

Our results showed there was a significant reduction in the rates of clinical pregnancy and implantation and an insignificant reduction in the live birth rate of group A. Although, the reduction observed in the live birth rate was insignificant, this difference was clinically important; therefore, IVF-ET/ ICSI outcomes in the group A were lower than those of group B and group C. Youm et al. (14) findings showed the implantation and clinical pregnancy rates were considerably lower in patients with a myometrial thickness ≥2.50 cm in comparison with the patients with a myometrial thickness <2.00 cm and 2.00-2.49 cm. As it is clear, the study population in our study is different to those of Youm et al. (14). The patients in that study had adenomyosis, while in our study all had a normal uterine. So far as is known, the smooth muscle cells from normal myometrium differs ultrastructurally from adenomyosis cells (17). This is supported by the hypothesis that the adenomyosis tissue results from the invasion of endometrial tissue through the endomyometrial junctional zone (JZ) into the myometrium. Adenomyosis causes a variable degree of cellular hyperplasia and hypertrophy surrounding the heterotopic endometrial tissue (18), and myocytes in adenomyosis show differences in cytoplasmic organelles, nuclear structures, and intercellular junctions (17). A growing literature has proposed that this thickness and distortion of the myometrium can alter the coordinated peristaltic activity of the inner myometrium (19) which interfere with the sperm transportation and the embryo implantation, and can adversely affect the fertility potential (18).

Our finding is in agreement with Lesny et al. (13) study, in which they observed the thicker myometrium in the pregnant group in comparison with the non-pregnant group on the day of down regulation during IVF-ET cycles. However, there are the limited published and/or available evidences. Based on our results, we propose that a thin myometrium may be involved in the failure of implantation and pregnancy, but how to directly determine this relation is a question. It seems possible that these results are due to the association between myometrial thickness and uterine contraction. However, very little was found in the literature on this topic, too. Previous studies have demonstrated that each uterine contraction originates from a local contraction, which transiently increases the intrauterine pressure. Subsequently, a high intrauterine pressure increases the stress level throughout the uterine wall which causes contractions of more regions (20). Accordingly, Deyer et al. (7) showed that uterine wall stress (defined as the applied force per unit cross-sectional area of material) is inversely related to the myometrium thickness. Therefore, the thicker the myometrium, the lower the uterine wall stress or uterine contraction. Data suggest that there are no contractile fibers in the endometrium, so these contractions were produced in the myometrium (21). Compared to the outer myometrial layer, the JZ as the innermost layer of the myometrium consists of higher density of compacted myocytes (22). Lesny et al. (13) demonstrated significantly thicker JZ and lower JZ contractions related to the higher pregnancy rate in ART cycles. This was supported by significant temporal and dynamic variations at the time of the oocyte's retrieval and embryos transfer. It shows that the thinner myometrium provides further contraction (23).

In line with these observations, major roles of the uterine contractions in the process of implantation and pregnancy were reported (1, 24, 25). In menstrual cycles, the frequency and direction of uterine contractions differ during the menstruation. The frequency attains a peak immediately prior to the ovulation time (26) with a mainly cervico-fundal wave form (27) to enable an effective ascending migration of sperm to fallopian tubes (28). Subsequently, the contractile activity decreases, thus creating an ideal environment for implantation (29). Zhu et al. (26) confirmed that in both fresh and frozen embryo cycles, uterine contraction frequencies were significantly lower in women who conceived than non-conceived women. The combination of these findings can provide some support for the correlation between myometrial thickness and ART Outcomes. However, more research on this topic needs to be undertaken.

This present study has two limitations that should be noted when interpreting the results, including (1) it was a single center study, and (2) the complete report of live birth rate was not achieved. The information on live birth was not stated in 3.1% (5/160) of clinical pregnancies of this study, which might impact the statistical power of the test for the live birth parameter.

Conclusion

In conclusion, it seems that the myometrial thickness (anterior-posterior diameter) <25 mm measured on menstrual phase may have an adverse effect on IVF-ET/ ICSI outcomes. The findings may be a way for clinicians to draw focus on providing therapeutic strategies and specific supportive care in order to improve reproductive outcome of IVF/ICSI in these women.

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Authors' Contributions

T.M.; Conceptualization, Supervision, and Revised manuscript critically for important intellectual content.

Sh.I., E.M.; Data acquisition and Investigation. N.J., S.M.M.; Methodology, Validation, and Writing- review and editing. S.M.; Statistical analysis. F.A.; Supervision, Project administration, and Revised it critically for important intellectual content. All authors have critically reviewed and approved the final manuscript.

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The Effects of Thiamine Supplementation on General Health and Infertility Treatment Outcomes in Women with Polycystic Ovary Syndrome: A Triple-Blinded Randomized Placebo-Controlled Clinical Trial

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Abstract

Background: The aim of this study was to evaluate the effects of thiamine (vitamin B1) on general health and infertility treatment outcomes in women with polycystic ovary syndrome (PCOS).

Materials and Methods: The study is a triple-blinded, randomized, placebo-controlled clinical trial performed on 64 infertile women with PCOS referred to Sarem Hospital in Tehran, Iran. The primary outcomes of the study were general health and infertility treatment outcomes. Eligible women were randomly assigned to the vitamin B1 group (n=32, vitamin B1 tablet at a dose of 300 mg/day for 4 weeks) or the placebo group (n=32, placebo tablet daily for 4 weeks). A general health questionnaire was completed before and after the intervention by both groups, and treatment success was evaluated at the end of the study. Data were analyzed using SPSS software ver.16 $P<0.05$ was considered statistically significant.

Results: The mean age of participants in the vitamin B1 (VB1) group was 30.4 ± 3.27 years and in the placebo (Pl) group was 29.1 ± 2.66 years with the mean duration of marriage 12.7 ± 3.01 and 13.2 ± 2.97 years respectively. Our results showed that there were significant differences between the two groups in overall score ($P<0.001$) and scores for all domains of the general health questionnaire including somatic symptoms ($P<0.001$), anxiety and insomnia ($P<0.001$), social dysfunction ($P=0.028$), and severe depression ($P<0.001$) after the intervention. Four weeks consumption of vitamin B1 also resulted in higher numbers of positive pregnancy tests ($P=0.006$), although the number of fetuses was not significantly different between the two groups after the intervention.

Conclusion: The results of the current study support a possible favourable effect of vitamin B1 on improving general health, infertility treatment outcome, and retrieved follicle count without changing the number of fetuses in women with polycystic ovary syndrome (registration number: IRCT201510266917N3).

Keywords: Depression, Fetus, Polycystic Ovary Syndrome, Thiamine, Vitamin B1

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Introduction

Infertility is very important for couples (1) as it affects about 15% of couples of reproductive ages worldwide. Globally 48.5 million couples have trouble conceiving (2). In Iran, the prevalence of infertility has been reported to be between 10.3 and 24.9% (3, 4). Women's infertility is due to a variety of causes such as ovarian disorders, endometriosis, and uterus anomalies (5).

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder among reproductive-age

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women with an overall prevalence of 4-20%, and is a frequent cause of infertility (6). It is estimated that 46% of women with PCOS are infertile (7).

While infertility per se commonly causes distress, depression, anxiety, sexual dysfunction, and social discord, women with PCOS are more concerned about their fertility than other women. Indeed, women with PCOS face various socio-cultural and psychological pressures that lead to lower well-being and quality of life. These issues are due to adverse physical manifestations and endocrine disorders such as reproductive problems (irregular menstruation, infertility, hyperandrogenism), metabolic disorders (obesity, insulin resistance, cardiovascular diseases) and psychological changes (stress, depression, anxiety) (8, 9). Almeshari et al. (8), reported depressive and anxiety symptoms among women with PCOS to be 49 and 40% respectively. Other studies have also reported higher levels of general and psychiatric morbidity in women with PCOS (10).

Interventions in lifestyle, such as dietary modification, may improve fertility and reduce adverse effects of PCOS on mood and psychological well-being (11). Recent studies have shown that nutrition-associated signaling pathways are important in the regulation of ovarian function, meaning that nutritional modification with supplements may contribute to diminishing PCOS complications (12). There is some evidence that thiamine (vitamin B1) is associated with fertility function (12, 13). Vitamin B1 is a water-soluble vitamin associated with cellular energy metabolism. It seems that severe vitamin B1 deficiency can increase the number of abnormal oocytes (13) due to a reversible inhibition of the meiotic maturation of oocytes (14). DNA synthesis, which is the main stage of oocyte development in the reproductive cycle, is influenced by B vitamins (15). Studies also indicate that vitamin B1 plays an important role in the nervous system, can improve quality of life and decrease depression, fatigue, and anxiety (15, 16).

Although some studies of the effects of vitamin B1 on women's reproductive problems have shown positive results, surprisingly, to the best of our knowledge, there is insufficient evidence about the effects of vitamin B1 on infertility treatment outcomes and the general health of infertile women with PCOS. Despite an increase in the use of complementary treatments in infertility, little research on this has been done in Iran. For these reasons we conducted this study to determine whether oral vitamin B1, used as a complementary vitamin therapy in the infertility treatment process, would improve general health and infertility treatment outcomes in women with PCOS.

Materials and Methods

The present study was a triple-blinded randomized placebo-controlled clinical trial undertaken to investigate effects of vitamin B1 on mental health as the primary and infertility treatment outcomes as the secondary in infertile women with PCOS undergoing infertility treatment.

The study was conducted between September 2016 and July 2017, in Sarem Hospital, Tehran, Iran. Seventy infertile women (18-40 years) with PCOS referred to the Sarem infertility clinic for infertility treatment by *in vitro* fertilization (IVF) were randomly assigned to the intervention or control group (Fig.1).

The research protocol was approved by the Iran University of Medical Sciences Ethical Committee (IR.IUMS.REC.1394.26840). All participants were thoroughly informed about the study goals and methods and then signed an informed consent form. The trial protocol was registered in the Iran Registry of Clinical Trials (IRCT201510266917N3). The inclusion criteria for participation in this study were PCOS infertile women without any diagnosed psychological disorders. PCOS was diagnosed by a gynecologist based on the Rotterdam criteria (the presence of two out of three of the following: oligo-anovulation, hyperandrogenism and polycystic ovaries on sonography) (17). Eexclusion criteria included smoking, alcohol consumption, use of corticosteroids or psychological drugs, vitamin B1 or other vitamin consumption during the past month, chronic systematic disease (such as hypertension, diabetes, thyroid dysfunctions, autoimmune disease, inflammatory bowel disease), infertility treatment withdrawal for any reason, and vitamin B1 allergic reactions (such as fatigue, headache, dizziness, visual problems, nausea, and vomiting).

The sample size was determined using the appropriate formula for a two-mean comparison, according to the researcher's estimate and based on the general health as primary outcome. To achieve a study power of 80% with a 95% confidence interval, and accounting for at least a 10% sample loss due to follow-up or study dropouts, a sample size of 70 women (35 in each group) was determined. After women eligible for the study had been identified, they were randomly assigned to two study groups using a simple randomization method: the vitamin B1 group (VB1, n=35) given a tablet of vitamin B1 [1 tablet 300 mg/day (18), manufactured by Hakim Pharmaceutical Co., Tehran, Iran] and the placebo group (Pl, n=35) given placebo pills (1 tablet daily contains mannitol, magnesium stearate, and polyvinylpyrrolidone manufactured by Hakim Pharmaceutical Co., Tehran, Iran) for 4 consecutive weeks.

The intervention was started 4 weeks before the scheduled time of egg transfer. Simple randomization was performed by a person not involved in the research process. The vitamin and placebo pills were identical in appearance and packed in similar boxes named A or B by a person who was not affiliated to the research team. Accordingly, participants, researchers, and statisticians were blind to the groups (triple-blinded). The main outcomes were general health and infertility treatment outcomes including the number of retrieved follicles, fetuses, and a positive pregnancy [blood beta-human chorionic gonadotropin (β-hCG)] test.

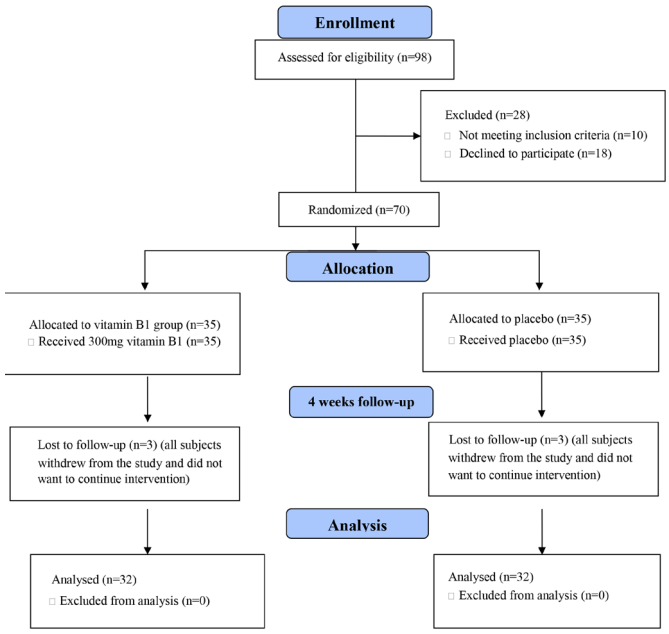

Fig.1: The CONSORT flow diagram.

After being assigned to the two groups the participants completed self-reported questionnaires. Data collection tools included: demographic information, the general health questionnaire 28 (GHQ-28), and the treatment success checklist. The GHQ-28 was introduced by Goldberg and Hillier (19) and is a suitable questionnaire for assessing the general health of infertile women. The questionnaire has four sub-scales, each consisting of 7 items as follows: physical symptoms (1-7), anxiety and insomnia (8-14), social function (14-20), and symptoms of depression (21-27). A likert scale of 0-3 is used for scoring each question making the score range for each domain and the total 0-21 and 0-84, respectively. Lower scores signify better general health (20, 21). Psychometric evaluation of the GHQ-28 has confirmed the questionnaire's reliability and validity in numerous studies (22). Shayan et al. (20) reported a 0.90 reliability coefficient. Scores higher than 23 for the total score and higher than 6 for each of the sub-scales were considered abnormal. The GHQ-28 was completed again by participants in the two study groups again after the intervention. The treatment success checklist, including data for the number of retrieved follicles and fetuses and the pregnancy test (blood β-hCG), was completed by researchers from hospital records two weeks after egg transfer. Finally, 64 women (32 women in each group) completed the study protocol and entered to data analysis (Fig.1). The quantitative data for the two groups were compared using independent and paired t-tests. Dichotomous variables were compared using the chi-square or Fisher's exact test. SPSS ver. 16 16 (Chicago, SPSS Inc; 2007) was used to perform the statistical analyses and$P<0.05$ was considered statistically significant.

Results

Mean age of participants in the vitamin B1 (VB1, n=32) group was 30.4 ± 3.27 years and in the placebo (Pl, n=32) group was 29.1 ± 2.66 years. Mean marriage duration in the VB1 group was 12.5 ± 3.01 years and in the Pl group was 13.2 ± 2.9 years. Infertility duration was 9.7 ± 3.00 years in VB1 group and 10.2 ± 2.97 years in the Pl group. Table 1 shows there were no statistically significant differences between the two groups regarding demographic and other characteristics.

Table 1: Characteristics of participants

Groups/Variables	Vitamin B1	Placebo	P value
Age (Y)			0.091**
≤25	2 (6.2)	5 (15.6)	
26-30	11 (34.4)	17 (53.1)	
≥30	19 (59.4)	10 (31.3)	
Husband age (Y)			0.051**
≤35	5 (15.6)	6 (18.7)	
35-39	20 (62.5)	24 (75)	
≥40	7 (21.9)	2 (6.3)	
Marital duration (Y)			0.533**
<10	4 (12.5)	4 (12.5)	
10-14	19 (59.4)	16 (50)	
15-19	9 (28.1)	12 (37.5)	
Educational duration (Y)			0.609*
≤12	8 (25)	5 (15.7)	
>12	24 (75)	27 (84.3)	
Employed			0.599**
No	10 (31.2)	12 (37.5)	
Yes	22 (68.8)	20 (62.5)	
Economic status			0.432**
Poor	5 (15.6)	2 (6.2)	
Relatively good	25 (78.1)	26 (81.3)	
Good	2 (6.3)	4 (12.5)	
Infertility duration (Y)			0.533**
5-7	8 (25)	6 (18.8)	
8-10	14 (43.8)	12 (37.4)	
>10	10 (31.2)	14 (43.8)	

Data are presented as n (%). *; Fisher's exact test and **; Chi-square test.

Results from the GHQ-28 and its domains are shown in Table 2. According to this table, the two groups did not show any significant differences in GHQ-28 total score or in scores for the body symptoms, anxiety and insomnia, social dysfunction, and severe depression domains before the intervention. After 4 weeks of intervention, the mean of GHQ-28 total score in the VB1 group was significantly lower than in the Pl group ($P<0.001$). Also, after the intervention, the mean scores for somaticsymptoms ($P<0.001$), anxiety and insomnia ($P<0.001$), social dysfunction ($P=0.028$), and severe depression ($P<0.001$) were significantly lower statistically in the VB1 groups compared with the Pl group.

Table 2: Comparison of genneral health status between the study groups before and after the intervention

General health		Vitamin B1 n (%)	Placebo n (%)	P value
Total score		Before intervention		
	Normal (score<24)	0 (0)	0 (0)	0.596*
	Distressed (score≥24)	32 (100)	32 (100)	
	Total	32 (100)	32 (100)	
	Mean ± SD	47.87 ± 10.43	46.5 ± 10.22	
		After intervention		<0.001*
	Normal (score<24)	9 (28.1)	1 (3.1)	
	Distressed (score≥24)	23 (71.9)	31 (96.9)	
	Total	32 (100)	32 (100)	
	Mean ± SD	30.68 ± 10.66	47.75 ± 10.09	
	Test result	P<0.001**	P=0.639**	
Somatic symptoms		Before intervention		0.574*
	Normal (score<6)	0 (0)	0 (0)	
	Distressed (score≥6)	32 (100)	32 (100)	
	Total	32 (100)	32 (100)	
	Mean ± SD	12.43 ± 3.93	11.91 ± 3.56	
		After intervention		<0.001*
	Normal (score<6)	10 (31.3)	0 (0)	
	Distressed (score≥6)	22 (68.7)	32 (100)	
	Total	32 (100)	32 (100)	
	Mean ± SD	7.25 ± 2.84	11.91 ± 3.96	
	Test result	P<0.001**	1.000**	
Anxiety and insomnia		Before intervention		0.722*
	Normal (score<6)	6 (18.8)	6 (18.8)	
	Distressed (score≥6)	26 (81.2)	26 (81.2)	
	Total	32 (100)	32 (100)	
	Mean ± SD	11.34 ± 5.05	10.91 ± 4.74	
		After intervention		<0.001*
	Normal (score<6)	20 (62.5)	3 (9.4)	
	Distressed (score≥6)	12 (37.5)	29 (90.6)	
	Total	32 (100)	32 (100)	
	Mean ± SD	6.09 ± 3.60	11.56 ± 5.07	
	Test result	P<0.001**	P=0.622**	
Social dysfunction		Before intervention		0.764*
	Normal (score<6)	13 (40.6)	11 (34.4)	
	Distressed (score≥6)	19 (59.4)	21 (65.6)	
	Total	32 (100)	32 (100)	
	Mean ± SD	7.62 ± 2.88	7.84 ± 2.93	
		After intervention		0.028*
	Normal (score<6)	10 (31.3)	11 (34.4)	
	Distressed (score≥6)	22 (68.7)	21 (65.6)	
	Total	32 (100)	32 (100)	
	Mean ± SD	6.78 ± 1.66	8.03 ± 2.66	
	Test result	P=0.176**	P=0.762**	

Table 2: Continued

Severe depression		Before intervention		0.637*
	Normal (score<6)	2 (6.3)	4 (12.5)	
	Distressed (score≥6)	30 (93.7)	28 (87.5)	
	Total	32 (100)	32 (100)	
	Mean ± SD	16.46 ± 4.98	15.84 ± 5.53	
		After intervention		<0.001*
	Normal (score<6)	6 (19.4)	2 (6.3)	
	Distressed (score≥6)	26 (80.6)	30 (93.7)	
	Total	32 (100)	32 (100)	
	Mean ± SD	10.96 ± 4.93	16.25 ± 4.87	
	Test result	P<0.001**	P=0.755**	

*; Independent t test and **; Paired t test.

Results for the infertility treatment outcomes (Table 3) showed that 4-weeks of vitamin B1 consumption was associated with a statistically significant increase in the number of retrieved follicles in the intervention group compared with the placebo group (P<0.001). There was also a significantly greater number of positive pregnancy tests (P=0.006) in the intervention group, despite no statistically significant differences between the two groups in the number of fetuses. No important harms or unintended effects were reported in VB1 group.

Table 3: Comparison of infertility treatment outcomes between the study groups after the intervention

Groups/Variable	Placebo	Vitamin B1	Test result
Number of retrieved follicles	7.15 ± 2.17	11.93 ± 2.13	P<0.001*
Number of fetuses	4.62 ± 0.97	4.65 ± 0.90	P=0.895*
β-hCG			P =0.006**
Positive	9 (28.1)	20 (62.5)	
Negative	23 (71.9)	12 (37.5)	
Total	32 (100)	32 (100)	

Data are presented as mean ± SD or n (%). β-hCG; Beta-human chorionic gonadotropin, *; Independent t test, and **; Chi-squared test.

Discussion

Our triple-blinded randomized placebo-controlled clinical trial showed that in infertile PCOS women undergoing infertility treatment a 4-week intake of vitamin B1 was associated with a statistically significant improvement in general health parameters measured using the GHQ-28. This improvement was seen in the total score and all its domains including body symptoms, anxiety and insomnia, social dysfunction, and severe depression. Vitamin B1 has been shown to have psychological effects in other studies. For example, Ghaleiha et al. (15) showed that in patients with major depressive disorder vitamin B1 alleviated symptoms of depression faster than placebo. Zhang et al. (16) showed an association between lower thiamine levels and symptoms of depression. Other research has shown the effects of thiamine on anxiety disorders, chronic fatigue, insomnia, aggression, diaphoresis, and headache (23). Regardless of the underlying cause of thiamine deficiency, it can have severe adverse effects on the nervous system (24) and positive changes have been shown to occur in energy, appetite, sleep patterns, and fatigue after thiamine supplementation (25). Vitamin B1 appears to be an essential micronutrient for neuronal cell function through its effect on oxidative metabolism and several coenzyme activities, and glucose utilization by nerve tissue (26). Vitamin B1 can also modulate cognitive performance and help people to fulfill work activities. However, Young et al. (27) noted that daily supplementation with B group vitamins reduced stress, but failed to reduce depressive symptoms and anxiety.

Other findings from the present study showed that 300 mg of oral vitamin B1 daily for 4 weeks could improve infertility treatment outcomes in PCOS women undergoing infertility treatment. It increased the number of retrieved oocytes and positive pregnancy tests (blood β-hCG) but had no effect on the number of fetuses. Tsuji et al. showed that there is a relationship between vitamin B1 nutrition and mice oocyte maturation (28). As the ART process does not occur under natural fertilization conditions, the development of reactive oxygen species (ROS) can adversely affect gamete or embryo development (29). In contrast, in the natural fertilization process, there is a physiological antioxidant system to protect gametes and embryos or repair free radical damage. This antioxidant system does not exist *in vitro* (30). Antioxidants can normally enhance oocyte maturation, fertilization, and embryo development by mitochondrial function enhancement, possibly improving ART outcomes (31). In women with PCOS, the conditions of follicular maturity are unfavorable because ROS can promote completion of the first meiotic division in the follicles rendering them immature (32). Consistent with our study, there is sufficient evidence that antioxidant supplementation significantly affects oocyte quality and pregnancy rate in women with PCOS (33, 34). Thiamine interacts with ROS, has desirable antioxidant properties, and can scavenge free radicals with high efficiency (35). Szczuko et al. (36) showed that women with PCOS have lower levels of thiamine in their serum. However this deficiency did not remain after lifestyle and dietary

changes Szczuko et al. (37).

We want to highlight our study limitations, which should be considered in interpreting our results. First, since our participants were in the treatment period, we could not continue our intervention for more than 4 weeks. Second, we did not measure our participants' vitamin B1 levels before the study started.

Conclusion

Results of the current intervention study in infertile women with polycystic ovaries support a possible favorable effect of thiamine (vitamin B1) on mental health, retrieved follicle count, and a positive pregnancy test after infertility treatment, despite no effect on the number of fetuses. We suggest future studies with larger sample sizes and longer study duration are required to clarify the potential effects of thiamine on mental health and pregnancy outcomes of infertile women with PCOS undergoing infertility treatment.

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Authors' Contributions

L.A.; Conceptualization., Methodology, Writing original draft, and Supervision. M.M.; Conceptualization, Methodology, and Sampling. H.H., M.R.N.; Formal analysis. H.H., M.R.N., M.M.; Writing-review and editing. All authors have read and agreed to the published this version of manuscript.

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Comparison *In Vitro* Fertilization Outcomes between DouStim and Minimal Stimulation Protocols in Poor Ovarian Responders: A Randomized Clinical Trial

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Abstract

Background: Various protocols have been approved to improve the response rate leading to successful fertilization in poor ovarian responders (PORs). The application of double ovarian stimulation (DuoStim) in the follicular and luteal phases of the same ovarian cycle has been shown as an intriguing option to achieve more oocyte retrievals in the shortest time. The aim of the current study is to compare the outcomes of different protocols, minimal stimulation (MS) and Duostim.

Materials and Methods: This randomized clinical trial was performed on 42 *in vitro* fertilization (IVF) candidates with POR diagnosis. Patients were classified into two equal groups and treated with the DuoStim protocol and MS protocol. The IVF outcomes, including retrieved follicles, oocytes, metaphase II (MII) oocytes and embryos, were compared between these groups.

Results: The patients' characteristics including age, anti-mullerian hormone (AMH), follicle stimulating hormone (FSH), luteinizing hormone (LH), and antral follicle count (AFC) were collected and compared. It showed there was no significant difference between the two groups baseline characteristics ($P>0.05$). We observed that the DuoStim protocol resulted in a significantly higher score in comparison with the MS protocols , including the number of follicles (6.23 ± 2.93 vs. 1.77 ± 1.66, $P<0.001$), retrieved oocytes (3.86 ± 2.57 vs. 1.68 ± 1.58, $P=0.002$), MII oocytes (3.36 ± 2.42 vs. 1.27 ± 1.27, $P=0.001$) and obtained embryos (2.04 ± 1.64 vs. 0.77 ± 0.86, $P=0.003$).

Conclusion: The DuoStim protocol is a favourable and time saving plan that is associated with more oocytes in a single stimulation cycle. The DuoStim protocol significantly can result in more frequent MII oocytes and embryos. We figured that the higher number of oocytes and embryos might have led to a higher rate of pregnancy (registration number: IRCT20200804048303N1).

Keywords: Clinical Protocol, *In Vitro* Fertilization, Oocyte Retrieval, Ovarian Follicle, Ovulation Induction

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Introduction

Please add at least two sentences that show why a reader must be attention to your "introduction" such as infertility and *in vitro* fertilization (IVF) and their challenges. A substantial increase in the daily dose of gonadotropins was presented for IVF for the first time in the 1980s. It seems that increasing the dose of gonadotropin leads to an increase in the number of oocytes in both groups of poor and good responders and also more embryos in number (1, 2). Studies have shown higher doses of gonadotropins results in the introduction of agonists and antagonists of gonadotropin-releasing hormone (GnRH) as luteinizing hormone (LH) suppressor markers (3, 4). Although, there is no doubt about the benefits of the conventional method for oocyte maturation triggering which lead to higher maturation response, but this approach has some limitations. First, this method is expensive. Second, it increases the incidence of multiple pregnancies when it is transferred to more than one embryo. Third, the danger of certain threats, such as ovarian hyperstimulation syndrome (OHSS), will increase in cases who human chorionic gonadotropin used to finalize the oocyte maturation (4-7). The usual method of long-term stimulation protocol using GnRH agonists prevents anterior pituitary inhibition, thereby preventing an increase in the LH (4).

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In many countries, long-term stimulation protocols by GnRH agonists are approved as a standard method. The GnRH agonist usually begins in the middle part of the luteal phase before the onset of the cycle, followed by high-dose gonadotropin stimulation, which results in multiple follicle production (8). However, the GnRH agonist protocol has potential side effects such as ovarian cysts and estrogen deprivation symptoms, such as mood changes and headaches (9). In addition, some of the side effects of the usual IVF procedure include the need for several daily injections that cause pain and local skin reactions in patients. These side effects have led to attention to minimally reintroduced protocols for better results and fewer potential complications (10-12). Minimally stimulation involves a mild and controlled final oocyte triggering that produces a maximum of 5 to 6 oocytes (13). The use of the mini-IVF method has caused eliminated the problems associated with conventional IVF.

Kuang et al. (14) developed Shanghai protocol, to retrieve more oocytes over time. Using letrozole or clomiphene citrate with human menopausal gonadotropin (hMG) or only GnRH antagonists to inhibit the ovarian LH elevation and stimulate GnRH agonists, ultimately leads to more embryos production. The protocol called dual ovarian stimulation, produced the maximum number of oocytes in the minimum time (15). In contrast to this study, Ubaldi et al. (16) used recumbent gonadotropins (FSH and LH), and reported an increase in embryo production rate ranging from 41.9 to 69.8%. After five days of oocyte recovery, they initiated stimulation of the luteal phase, similar to the previous stimulation; hence, DuoStim for IVF, was successfully used in patients with a time constraint from 2016.

Since poor responders represent more than a third of women undergoing assisted reproductive technology, it remains a notable challenge. Therefore, we designed the present study to compare outcomes between DouStim and Minimal Stimulation (MS) protocols in poor ovarian responders (PORs).

Materials and Methods

This study was registered in the Iranian Registry of Clinical Trials (IRCT20200804048303N1). The study protocol was approved by the Shahid Beheshti University of Medical Sciences' Local Medical Ethics Committee under the reference number IR.SBMU.RETECH.REC.1398.480.

We investigated the number of follicles > 14 mm and MII oocytes. Secondary outcome was the number of embryos obtained.

Study population

The samples were chosen among volunteers in Shahid Taleghani Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran. In this study, women with low functional ovarian reserve candidates for IVF with a history of poor ovarian response were included. A total of 42 women, who met our criteria, gave their informed consent and entered in our study.

The inclusion criteria were age ≥35 years, antral follicle count (AFC) level <5, and anti-mullerian hormone (AMH) level <1.2 ng/ml). The exclusion criteria include cycles with the only dominant follicle formation, it means produce one dominant follicle during menstrual cycle, uterus malformation and/or abnormalities , intrauterine adhesions, endometriosis, and history of tuberculosis or pelvic surgery.

In this study, all patients underwent a specific procedure called transvaginal ultrasonography. This procedure was used to measure and count the antral follicles on the second day of the menstrual cycle. Blood samples of the patients were collected in citrated or EDTA-containing tubes for the serum extraction by centrifugation at 3000 g for 20 minutes. The levels of LH and follicle-stimulating hormone (FSH) were measured by the immune radiometric assay (9800496 for LH and 9900196 for FSH, Pishtaz Teb, Iran). An Enzyme-linked Immunosorbent Assay (ELISA) kit (9900696, Pishtaz Teb, Iran) was used to measure the serum AMH concentrations.

Randomization and blinding

The statistician of the study prepared a computer-generated randomization schedule in blocks of four. A third party randomly assigned participants to one of two treatment arms. The clinician, embryologist, and data analyzer were blinded to the allocated treatments.

Interventions

Minimal stimulation protocol

Letrozole (Femati, AtiPharmed Pharmaceuticals, Iran), was given at a dose of 5.0 mg for five days, starting on the second day of the menstruation cycle. On the fourth day of treatment with Letrozole the Menotropins (Menopur, Ferring Pharmaceuticals, Copenhagen, Denmark), began with 150 units per day of gonadotropins (PDPreg, Pooyesh Darou Pharmaceuticals, Iran). Three days after Menopur initiation, the patients were evaluated with the conventional ultrasound sonography, and the Menopur dose was increased if the initial response rate was not satisfying and continued if response rate was good. By achieving the follicles with a size higher than 14 mm, a GnRH antagonist, Cetrotide, 0.25 mg) was administrated to prevent the LH level elevation. Then, 10,000 units of human chorionic gonadotropin (hCG) were prescribed to achieve at least one follicle with a size of 18 mm to stimulate the follicle final maturation. The gonadotropin dose in the MS group is 900 units for each patient during the study.

DuoStim protocol

The applying protocol for the DuoStim group was similar to the MS group, except that the final oocyte triggering was performed by using a GnRH agonist (triptorelin 0.2 mg daily SQ under Decapeptyl™ brand). The ovarian stimulation was repeated using the same protocol five days after

release the first oocyte. Continuing the second stimulation was similar to the first stimulation period with the onset of the GnRH antagonist by reaching a size of 14 mm follicles, re-stimulation with the GnRH agonist from three follicles with a minimum size of 16 mm. The number of retrieved oocytes, the number of metaphase II (MII) oocytes, and the embryo that was obtained from the final oocyte stimulation were then compared between the two groups. In the DouStim group, the gonadotropin dose for each patient was 900 units in the follicular phase and 750 units in the luteal phase during the entire study period.

Sample size

Assuming a reliability coefficient of 0.05 with a power of 90%, and considering a drop-out rate of 10%, the minimum sample size for each group was 21. This was calculated in accordance with previous studies based on our primary outcomes (17, 18).

Statistical analysis

Variables are represented as mean ± standard deviation (SD). Student's t test was applied to compare the groups using the statistical software SPSS version 28 (SPSS Inc., Chicago, IL) for the statistical analysis. A statistically significant level was considered to be less than 0.05 ($P<0.05$).

Results

Totally, 21 patients received the DuoStim protocol (DS group), while 21 patients were under our MS protocol (MS group) (Fig.1).

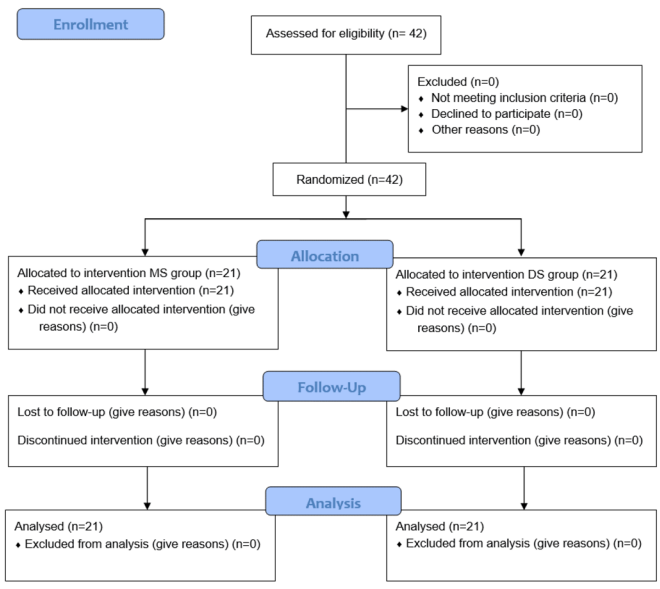

Fig.1: The flow diagram of the study. MS; Minimal stimulation and DuoStim (DS); Double ovarian stimulation.

We did not observe any significant differences between our group members in age and body mass index (BMI). There was also no difference in baseline hormone levels, including AMH, FSH, and LH (Table 1).

Table 1: Demographic data of our participants

Item	DuoStim group (n=21)	MS group (n=21)	P value
Age (Y)	39.19 ± 2.42	38.52 ± 3.03	0.44
BMI (kg/m^2)	26.41 ± 3.58	28.55 ± 4.95	0.12
AMH (ng/mL)	0.74 ± 0.39	0.65 ± 0.35	0.41
FSH (IU/L)	9.26 ± 1.84	10.32 ± 2.10	0.10
LH (IU/L)	7.48 ± 1.27	7.48 ± 2.09	0.51
AFC	5.36 ± 1.67	4.77 ± 1.77	0.26

Data are presented as mean ± SD. DuoStim; Double ovarian stimulation, MS; Minimal stimulation, BMI; Body mass index, AMH; Anti-mullerian hormone, FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, and AFC; Antral follicle count.

All findings of the DuoStim treated group were significantly higher than the MS treated group (Table 2).

Table 2: IVF outcome of our participants

Item	DuoStim group (n=21)		MS group (n=21)	P value
Number of follicles retrieved > 14 mm	6.23 ± 2.93	FP: 3.45 ± 1.71 LP: 2.77 ± 2.02	1.77 ± 1.66	<0.001
Number of oocytes retrieved	3.86 ± 2.57	FP: 1.91 ± 1.57 LP: 1.95 ± 1.84	1.68 ± 1.58	0.002
Number of MII oocytes retrieved	3.36 ± 2.42	FP: 1.63 ± 1.40 LP: 1.72 ± 1.72	1.27 ± 1.27	0.001
Number of embryos obtained	2.04 ± 1.64		0.77 ± 0.86	0.003

Data are presented as mean ± SD. DuoStim; Double ovarian stimulation, MS; Minimal stimulation, MII; Metaphase II, FP; Follicular phase, and LP; Luteal phase.

Discussion

Several protocols have been adopted to improve response rates leading to successful fertilization. This study aimed to compare IVF outcomes including retrieved MII oocytes and consequent embryos between DuoStim and MS protocols in PORs. The chance for a successful pregnancy is related to different baseline parameters, such as the woman's age, the number of aspirated oocytes, and the protocol employed (19). The included patients already had preliminary results with the MS protocol, and the opportunity to dual ovarian stimulation in the same cycle desiring to increase the number of oocytes and embryos was the debate for the new treatment plan.

The present study findings showed that the number of both MII oocytes and aspirated oocytes increased significantly following the DuoStim protocol, which resulted in embryos number. The number of obtained oocytes is one of the factors that impact the positive outcome rate of ART. Our study's critical and highlighted issue is a partially low fertilization rate following both DuoStim and MS protocols. It is not uncommon for an embryo quality and its inadequate response. Recently, it was reported that a total fertilization failure occurs in 5 to 10 % of IVF cycles (20). It usually does not make it past the blastocyst stage or only comes in small amounts to become a euploid embryo status, so cycles and transfers are canceled (21). In a study by Vaiarelli et al. (22), The use of DuoStim

increased the probability of obtaining at least one euploid blastocyst in a single ovarian cycle by 40 to 70 percent. Contrarily, Cecchino et al. (23) showed no difference in aspirated oocytes, MII oocytes and fertilization rate between the DuoStim and standard protocol (24). Cecchino et al. (23) also reported that higher doses of gonadotrophins would never balance the absence of follicles. No pharmacological co-treatments, such as growth hormone, Dehydroepiandrosterone (DHEA), or testosterone administration, have significantly improved the ovarian reserve. In the DuoStim protocol, co-treatment with maximal gonadotropins and GnRH antagonists was mainly considered to discourage ovulation in both follicular and luteal phases and improve the recruitment and development of the follicles (24). Administrating a dose of FSH and LH in an antagonist protocol instead of a minimized stimulation can reduce the likelihood of cycle cancelation and even shorten the time to pregnancy by increasing the number of oocytes per stimulation (25).

In several studies, the DuoStim protocol, which uses dual stimulation during the follicular and luteal phases of the same ovarian cycle, has been shown to be an intriguing method for retrieving two oocytes quickly (26, 27). However, its related advantages and limitations have been questioned, particularly compared to standard protocols, such as MS protocols. As revealed in our trial, the DuoStim protocol is superior to the MS protocol concerning the number of retrieved follicles, oocytes, and obtained embryos, therefore the DuoStim protocol is preferred to obtain a proper response in IVF. Also, more parameters and larger study groups are required for more generalize results.

Various alternative stimulation protocols and ovulation triggers have likewise been assessed and can be utilized to address patients' issues. Physicians should consider the patient's requirements while deciding the best treatment choices. Pregnancy outcomes may determine this study's validity; therefore, lost to follow-up with the patient who underwent embryo transfer is the most important limitations of this work. Furthermore, a future research model associated with male sub-fertility is recommended in order to consider probable differences of cure.

Conclusion

The most significant advantage of the DuoStim protocol is that it collects more oocytes in a single stimulation cycle, thereby reducing time required for its execution. The DuoStim protocol can lead to significantly more frequently MII oocytes and embryos in comparison with Duostim. We figured that the higher number of oocytes and embryos might have led to a higher rate of pregnancy due to two times ovulation induction in one menstrual cycle.

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Authors' Contributions

S.S., T.D., S.Sh.; Study conception and Design. T.D., L.N.; All experimental work, Statistical analysis, and Data interpretation. N.S., S.S.; Supervision, Conception, and Data analysis. T.D. L.N., S.H.; Drafted the manuscript, Data acquisition, and Data analysis. All authors read and approved the final manuscript.

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Investigating Ovulation Induction Outcomes in Patients with Decreased Ovarian Reserve Treated with Double Stimulation during The Follicular and Luteal Phases Compared to The Conventional Antagonist Cycle: A Randomized Clinical Trial

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Abstract

Background: It is difficult to obtain healthy oocytes in poor ovarian responders with conventional treatment methods. Thus, the need to investigate new methods is essential. This study aims to investigate ovulation induction outcomes in patients with decreased ovarian reserve (DOR) in two groups treated with double stimulation (DuoStim) during the follicular and luteal phases in comparison with the antagonist cycle.

Materials and Methods: This was a randomised clinical trial that enrolled the patients with reduced ovarian reserve. The patients referred for *in vitro* fertilization (IVF) at Molud Infertility Clinic, Ali Ebn Abitalib (AS) Hospital, Zahedan, Iran from 2020 to 2021. Participants were randomly divided into two groups, those who underwent treatment with DuoStim during the follicular and luteal phase (case group) and those who received the conventional antagonist cycle (control group).

Results: The mean number of metaphase II (MII) eggs was 7.7 ± 3.1 in the case group and 6.1 ± 3.9 in the control group (P=0.063). The mean total number of retrieved eggs in the case group was 9.2 ± 3.7 and in the control group, it was 6.9 ± 4.4 (P=0.023). The mean number of embryos obtained in the case group was 6.5 ± 3.9; in the control group, it was 4.7 ± 2.8 (P=0.016).

Conclusion: The DuoStim method can effectively play a role in increasing the total number of retrieved eggs and embryos (registration number: IRCT20120817010617N8).

Keywords: Antagonist, Double Stimulation, Follicular, *In Vitro* Fertilization, Luteal

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Introduction

The global prevalence of infertility in women is between 2.5 and 10.5% (1). Unfortunately, decreased ovarian reserve (DOR) occurs in 10-40% of these women, in which the ovary loses its normal reproductive potential, resulting in conception and menstrual cycle disorders (2). Some should undergo assisted reproductive techniques (ART) such as *in vitro* fertilisation (IVF). The poor prognosis group consists of people who are older and have a poor ovarian response (3, 4). In women with DOR, the quantity and quality of eggs produced by the ovaries are reduced, which leads to low-quality embryos. Ovarian stimulation improves the results of ART treatments by increasing the number of oocytes and embryos (5). Although various treatment regimens and many interventions have been performed to improve IVF results (3), there is no single protocol to treat people with poor ovarian response. Treatment of this population is based on the protocols

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of the treatment centre and the doctor's preference. In general, there are three common protocols: long-cycle agonist, short-term gonadotropin hormone-releasing hormone (GnRH) agonist with the flare-up method, and GnRH antagonist. Poor ovarian response to external gonadotropins is one problem of ART in 9-26% of cycles and can cause the cycle to stop, access fewer oocytes and embryos, and eventually reduce the pregnancy rate (6, 7). Factors related to poor ovarian response include advanced age, previous ovarian surgery, pelvic adhesions, and high body mass index (BMI); in some cases, a weak response is also observed in young women (8).

A new method called double stimulation (DuoStim) in one ovarian cycle has been proposed for controlled ovarian stimulation. This protocol is particularly suitable for women who have a poor prognosis and need to maximise ovarian reserve during a limited period of time. The double ovarian cycle method is performed by combining two stimulation methods in the follicular and luteal phases, and can be a valuable option for patients with a reduced ovarian reserve and for older women (9-11). For the first time, Kuang et al. (12) showed that DuoStim of the ovarian cycle in the combined method of follicular and luteal phases led to the development of eggs with appropriate growth ability. DuoStim during the follicular and luteal phases provides a promising alternative or a rescue approach for patients with poor ovarian response. The number of antral follicles (AFC) after first oocyte retrieval was similar to the counts in the early follicular phase, and this offers an exciting potential target for extending ovarian stimulation and additional oocyte retrieval (13). Liu et al. (14), in a retrospective case-control study, aimed to investigate the efficacy of double ovarian stimulation in older women. Their results showed that double ovarian stimulation could increase the chances of achieving pregnancy by accumulating more oocytes/embryos over a short time, and this might serve as a useful strategy for older women. Moreover, Li et al. (15) compared pregnancy outcomes between DuoStim and two consecutive mild stimulations in poor ovarian responders. They observed that the DuoStim protocol was inferior to the two consecutive mild stimulations protocol in terms of the number of frozen embryos, which mainly occurs in older patients. However, there was no difference in pregnancy outcomes between the two protocols. Vaiarelli et al. (16) concluded that during preimplantation-genetic-testing-for-aneuploidies (PGT-A) treatments in advanced-maternal-age and/or poor-ovarian-reserve (AMA/POR) women, DuoStim could be proposed to rescue poor blastocyst yields after conventional-stimulation.Another study by the same author indicated that DuoStim is a promising strategy to manage poor responder patients, especially to avoid discontinuation after a first failed attempt (17).

Poor ovarian response, having diversity in specific treatment protocols and regimens, and the use of regimens other than antagonists are among the current problems of infertility centres in different regions. For this reason, the current research aims to investigate the results of DuoStim during the follicular and luteal phases in comparison with the conventional antagonist cycle in patients with DOR.

Materials and Methods

Ethics statement

This research was approved by the Research Ethics Committee of Zahedan University of Medical Sciences, Zahedan, Iran (IR.ZAUMS.REC.1399.447) and the Iranian Registry of Clinical Trials (IRCT20120817010617N8). This study was conducted in accordance with the Declaration of Helsinki and its subsequent revisions.

Study design

This randomised clinical trial study evaluated all women diagnosed with reduced ovarian reserve who underwent IVF treatment at the Infertility Centre of Ali-Ebn-Abitaleb (AS) Hospital from 2020 to 2021. The inclusion criteria of the study comprised: presence of reduced ovarian reserve including anti-müllerian hormone (AMH) ≤1.2 ng/ml, antral follice counts (AFC) ≤6 on the third day of the menstrual cycle, less than five oocytes harvested in the previous cycle, serum FSH concentrations between 10 and 19 IU/L, and the absence of evidence of primary ovarian insufficiency, which included follicle-stimulating hormone (FSH)<20 IU/L. Also, the cases with endometriosis higher than grade 3, a contraindication for the use of gonadotropins, and couples without severe male factor infertility were excluded from the study. The number of metaphase II (MII) oocytes was used to estimate the sample size (12). The effect size (ε) was considered to be 1.3. We employed the superiority formula of the mean for sample size computations. Therefore, 54 patients were included in the study by using the convenience non-probability sampling method, according to the study of Kuang et al. (12) and by taking into consideration the sample size formula (18) and 20% possibility of exclusion of patients during the research. After enrolment, the patients were randomly assigned to either the case or control group (Fig.1).

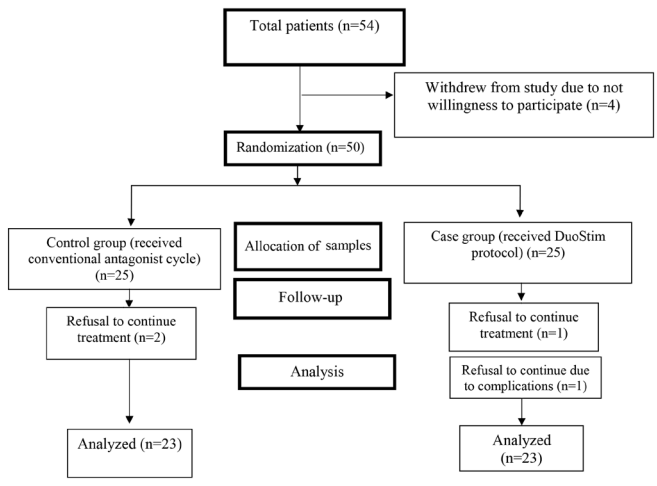

Fig.1: Flowchart for patient selection. DuoStim: Double stimulation

Procedure

The current research was performed in a randomised and single-blind method so that the patients were classified into the case (DuoStim) and control (conventional antagonist) groups using the permuted block stratified randomisation method (19). Initially, the objectives of the study were explained to the patients and written consent was obtained. The eligible patients were classified according to the order of entry, age, and BMI. Subsequently, they were assigned to one of the two groups based on blocks of four (consisting of two A and B groups and two repetitions for each) that were randomly selected from all the possible states of permutations (2). These blocks were created using statistical software R version 4.0.2. Finally, participants were assured that their information would remain confidential, and they were reminded that the research results would be provided to them if they wished.

The primary outcome was the total number of oocytes retrieved and the secondary outcomes were the number of MII oocytes and the number of embryos obtained.

Participants were assured that they could withdraw during any stage of the research if they did not want to continue. Two participants from each group withdrew from the study. In the case group, one patient refused to continue treatment and one was excluded due to complications; in the control group, two patients withdrew their consent to continue treatment.

Both groups of patients underwent transvaginal sonography (TVS) on menstrual cycle days 1-3. All patients daily received 225 units of Gonal-F (FSH, Merck, Serono, Italy) and 150 units of human menopausal gonadotropin (HMG, Karma Pharmatech, Germany). Patients had another TVS five to six days later to assess follicular growth, followed by TVS once every two days. When the cases had a dominant follicle greater than or equal to 14 mm, the GnRH antagonist (0.25 mg, Merck, Serono, Italy) was administered daily and continued until the presence of 2 to 3 follicles that were 18 mm in size, as observed by TVS. Then, in the DuoStim group, the final oocyte trigger was performed by two injections of Decapeptyl (0.1 mg); the control group in addition to Decapeptyl received 10,000 IU of human chorionic gonadotropin (hCG). Oocyte retrieval was done 36 hours after the injection, and all follicles above 12 mm were drained. In the DuoStim group, patients received a GnRH antagonist daily for 4 days from the day after the puncture, and five days after oocyte retrieval, regardless of the number of oocytes observed on ultrasound, similar to the previous cycle, ovarian stimulation was done using the GnRH antagonist protocol, and when at least two follicles reached 17-18 mm diameter, 10 000 IU HCG was administered. After 36 hours, oocyte retrieval was performed under TVS guidance.

The retrieved oocytes in both groups and each stage were incubated for 2-4 hours, after which the cumulus and corona rad iata cells were removed from the oocytes. The oocytes were subsequently evaluated and the MII oocytes were subjected to intracytoplasmic sperm injection. Then, the embryos were cultured in culture medium and placed in an incubator at 37°C with 6% CO_2 and 5% O_2. Three days later, the embryos were evaluated and scored, and finally, five days later, they were re-evaluated for blast formation. Subsequently, all of the embryos were frozen. The obtained data were recorded in information forms and analysed by SPSS software SPSS software (version 22, IBM Corp., Armonk, N.Y., USA).

Statistical analysis

The collected, raw data were entered into SPSS version 22 (IBM Corp., Armonk, N.Y., USA). The per-protocol approach was employed for data analysis. Frequency and percentage indicators were used to describe qualitative data. Common central indices (mean and median) and dispersion indices (standard deviation and interquartile range) were used to describe quantitative data. In order to compare the average variables between the two groups, the t test was used for two independent groups if the assumptions of the parametric tests were met. If the assumptions were not met we used alternative non-parametric tests, such as the Mann-Whitney U test. The relationship between categorical characteristics was assessed by the chi-square test. In all analyses, $P<0.05$ indicated statistical significance.

Results

A total of 57 infertile women with reduced ovarian reserve were selected for participation in the study that 4 patients were withdrawn due to not willingness to participate in the study; therefore 50 patients enrolled in this study and randomly assigned to DuoStim protocol (case group, n=25) or the conventional antagonist cycle (control group, n=25). After withdrawal of two participants from each group (case group: one patient refused to continue treatment and one patient had complications; control group: two patients refused to continue treatment), we assessed the treatment results in 46 patients (Fig.1). The participants had a mean age of 35 ± 4 years and a mean BMI of 26 ± 5 kg/m^2. There was no significant relationship between demographic factors and the duration of infertility in these women (P=0.508, Table 1).

Table 1: Comparison of demographic information and the duration of infertility in the two study groups

Group/Variable		Case (n=27)	Control (n=27)	Total (n=54)	P value*
Age (Y)	≤35	11 (40.7)	12 (42.9)	23 (41.8)	0.546
	>35	16 (59.3)	15 (57.1)	31 (58.2)	
BMI (kg/m^2)	≤25	10 (37)	9 (32.1)	19 (34.5)	0.461
	>25	17 (63)	18 (67.9)	35 (65.5)	
Duration of infertility		8.5 ± 1.3	7.4 ± 2.9	7.5 ± 6.1	0.508

Data are presented as n (%) or mean ± SD. BMI; Body mass index and *; Chi-square and t tests.

According to Table 2, the number of AFC and AMH were investigated in the two groups. No significant difference was found between the groups (P=0.335, P=0.973, respectively, Table 2).

Table 2: Comparison of the mean laboratory indices in the two study groups

Group/Variable	Case (n=27)	Control (n=27)	P value*
AFC	3.2 ± 9.4	4.3 ± 8.9	0.335
AMH	0.85 ± 0.6	1.3 ± 0.86	0.973

Data are presented as mean ± SD. AFC; Antral follicles, AMH; Anti-müllerian hormone, and *; t test and Mann-Whitney U test.

The mean number of MII oocytes in the case group was 7.7 ± 3.1; in the control group, it was 6.1 ± 3.9, which was not statistically significant (P=0.063, Table 3). Also, the mean total number of retrieved eggs in the case group was 9.2 ± 3.7 and in the control group, it was 6.9 ± 4.4 (P=0.023). The mean number of embryos obtained in the case group was 6.5 ± 3.9 and in the control group, it was 4.7 ± 2.8 (P=0.016). Also, the mean number of retrieved eggs and embryos obtained in the case group in the second round was significantly higher than in the first round (P=0.0001, Table 3).

Table 3: The frequency of MII eggs, retrieved eggs, and embryos obtained in the two studied groups

Group/Variable	Case (n=23)	Control (n=23)	P value*
MII oocytes	7.7 ± 3.1	6.1 ± 3.9	0.063
Retrieved oocytes			
Follicular phase	3.0 ± 1.5	6.9 ± 4.4	0.0001
Luteal phase	6.2 ± 3.3	-	-
Total	9.2 ± 3.8	6.9 ± 4.4	0.023
Total embryos			
Follicular phase	2.1 ± 1.4	4.7 ± 2.8	0.0001
Luteal phase	4.4 ± 2.5	-	-
Total	6.5 ± 3.9	4.7 ± 2.8	0.016

Data are presented as mean ± SD. MII; Metaphase II and *; t test and Mann-Whitney U test.

In this study, the number of days for medication administration and the number of doses used in the case group were higher than in the control group (P=0.001, Table 4).

Table 4: Frequency of the doses of medication in the two studied groups

Group/Variable	Case (n=23)	Control (n=23)	P value*
Medication administration (days)	19.4 ± 1.5	10.1 ± 5.9	0.001
HMG	3030.9 ± 14	1672.11 ± 5.1	0.001
Cetrotide	9.3 ± 4.3	6.4 ± 3.8	0.009
GONAL-F	3660.4 ± 25	1972.11 ± 5.8	0.001

Data are presented as mean ± SD. HMG; Human menopausal gonadotropin, GONAL-F; A brand name for a medication called gonadotropin, and *; t test and Mann-Whitney U test.

Discussion

Clinical knowledge and technological progress in recent years have greatly contributed to the success of ART methods, especially IVF. However, one of the most important success factors in this field is the number of oocytes produced by the ovaries following hormonal stimulation (20). Therefore, the main goal of the performed protocols is to stimulate the production of more oocytes and embryos, and to increase the probability of pregnancy (21). But this issue is more important in patients with risk factors that threaten their fertility over time. For example, cancer patients who need treatment with gonadotoxic drugs or surgery to remove their ovaries, or older people who have reduced ovarian reserves. Therefore, the implementation of methods that can induce good results over a shorter time is useful and satisfactory for many patients (22, 23).

Various studies, including a study by Kuang et al. (12), have shown that double ovarian stimulation in the same menstrual cycle provides more opportunities for egg retrieval in poor ovulatory responders. This stimulation can start in the luteal phase, and result in the retrieval of more oocytes in a short period of time. This is a new solution for women with a poor ovarian response who need to preserve their fertility. Therefore, in this study, the DuoStim method was used during the luteal and follicular phases in people with poor responses during IVF.

In the present study, the case and control groups were not statistically different in terms of participants' age, BMI, the number of AFC on the third day, and laboratory variables [AMH, FSH, thyroid stimulating hormone (TSH)]. The findings of the current study indicated that the mean numbers of retrieved oocytes and embryos obtained in the case group was higher than the control group. Also, the total number of oocytes and embryos obtained in the case group patients in the luteal phase was more than in the follicular phase. However, the total number of MII oocytes in the two groups did not show a statistically significant difference. The number of MII oocytes in the case group in the luteal phase showed a significantly better result than in the follicular phase.

DuoStim in one ovarian cycle is a new protocol developed for patients who undergo IVF that can maximise the number of retrieved oocytes in the shortest possible time. Unlike conventional IVF protocols in which patients undergo one round of stimulation with exogenous gonadotropins and egg retrieval in one menstrual cycle, patients who receive the DuoStim protocol undergo two rounds of gonadotropin treatment and two egg retrievals in the same menstrual cycle (1, 24, 25). Zhang et al. (26) showed that ovarian DuoStim in the luteal phase may be a promising protocol for the treatment of women with poor ovarian response, especially for patients who are not able to tolerate enough live embryos through follicular phase ovarian stimulation or other protocols. The results of their study were consistent with our study and it was observed that the percentage of eggs obtained was higher. Similarly, de Almeida Cardoso et al. (27) conducted a study on women who had a history of unsuccessful IVF and underwent DuoStim; they concluded that the number of eggs obtained increased from 6.7 to 11.7 compared to stimulation in the follicular phase. This

finding was consistent with our study. Vaiarelli et al. (28) reported that both stages of stimulation produced eggs of equal quality (based on fertilisation, blastocyst, euploidy rate, and clinical outcomes after euploid single embryo transfer). The second stimulation (luteal phase) considerably helped the patients who had at least one euploid blastocyst (from 42 to 65%). Finally, the DuoStim method was mentioned as the best method for fertility in patients with reduced ovarian reserve. However, de Almeida Cardoso et al. (27) conducted a study on 54 patients who underwent ovarian stimulation cycles, from which 13 patients underwent DuoStim. Although the results showed a higher number of extracted oocytes and mature oocytes in the patients that underwent DuoStim, there was no significant difference in terms of fertility and blastocysts (28). Similarly, Ubaldi et al. (9) reported no significant difference in the number of eggs and blastocytes or euploid blastocytes.

Therefore, according to the results of the conducted studies and the present study, it can be said that a greater number of MII oocytes are developed in this method in comparison with the conventional methods. In general, the data analysis showed that in patients with a weak ovarian response and the general infertile population, unconventional protocols such as DuoStim can be effective. This method can be a quick solution to recover more eggs and embryos in a shorter time, especially in older people who have reduced ovarian reserves or people with cancer. However, further studies with higher accuracy are needed to confirm these findings.

The efficacy of the DuoStim protocol was previously supported by the possibility to increase the oocyte yield and, more importantly, the number of euploid blastocysts. One of the limitations of the present study is that the clinical relevance of the finding in this study is limited by the lack of application of PGT-A. Another limitation is that since the study is a resident thesis and time was limited, so embryological and reproductive outcomes are missing. It is recommended that further studies in this field be performed in the future.

Conclusion

The findings of the current study indicate that the DuoStim method is significantly effective in increasing the total number of retrieved eggs and the number of embryos obtained in a short period of time. Therefore, this method can be useful in patients who, for various reasons such as increasing age or having underlying diseases, require a shorter treatment period and better results.

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Authors' Contributions

F.Gh.; Conceptualisation, Investigation, and Wrote the original draft. A.P.; Methodology, Validation, and Data collection. A.N.; Formal analysis and Software visualisation. M.Gh.; Review and editing, Resources, Supervision, Project administration, and Funding acquisition. A.J.; Data management and Data analysis. All authors read and approved the final manuscript.

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Does Culture of Post-Thawed Cleavage-Stage Embryos to Blastocysts Improve Infertility Treatment Outcomes of Frozen-Thawed Embryo Transfer Cycles? A Randomised Clinical Trial

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Abstract

Background: There is a definite shift in assisted reproductive centres from cleavage-stage embryo transfer (ET) to blastocyst transfer that is attributed to improvements in laboratory environments and advances in the development of embryo culture media. The aim of the study was to investigate the reproductive outcomes of thawed cleavage-stage ET versus blastocysts derived from an extended culture of these embryos.

Materials and Methods: This open-label, randomised, parallel group clinical trial study enrolled 182 women aged ≤37 years who underwent frozen-thawed ET from November 2015 to June 2020 at Royan Institute Research Centre, Tehran, Iran. The women were randomly assigned to either the thawed cleavage ET group (n=110) or the post-thaw extended culture blastocysts group (n=72). The primary outcome measure was the clinical pregnancy rate. Secondary outcome measures were implantation rate, live birth rate (LBR), and miscarriage rate. A P<0.05 indicated statistical significance.

Results: There were no significant differences between the two groups in terms of demographic characteristics. Both the mean numbers of embryos transferred and good quality embryos transferred were significantly lower in the post-thaw extended culture blastocysts group compared to thawed cleavage-stage ET cycles. However, the post-thaw extended culture blastocysts group had higher clinical pregnancy (56.94 vs. 40.91%, P=0.034), implantation (34.43 vs. 19.84%, P=0.001) and live birth (49.3 vs. 33.63%, P=0.036) rates compared to the thawed cleavage-stage ET group. Miscarriage and multiple gestations rates were comparable between the groups.

Conclusion: These results allow us to take a position in favour of post-thaw extended culture blastocysts; thus, it is important to improve the post-thawing extended culture technique (registration number: NCT02681029).

Keywords: Blastocyst, Cleavage-Stage, Cryopreservation, Culture, Embryo Transfer

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Introduction

Since the first live birth of a "test tube baby" in 1978 (1), assisted reproductive technology (ART) has been used worldwide for treatment of infertile couples (2) through conventional *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) with fresh embryo transfer (ET) or frozen-thawed ET. There are reports of negative effects of fresh ET cycles on early pregnancy with subsequent effects on perinatal outcomes in terms of hormone pre-treatment, including controlled FSH ovarian stimulation, anaesthesia and surgery for IVF oocyte retrieval (3). Frozen-thawed ET allows the extra embryos produced by IVF/ICSI to be stored and transferred later; therefore, it increases cumulative pregnancy rates and decreases the economic burden placed on the family and society (4).

Preventive measures should be considered by using a freeze-all strategy (5) with transfer in subsequent cycles in patients who undergo IVF/ICSI with a high risk of developing ovarian hyperstimulation syndrome (OHSS). This method does not pose a risk to patient safety and provides an opportunity for positive pregnancy outcomes (6). There has been a general shift in ART from cleavage-stage ET to blastocyst transfer attributed to improvements in laboratory environments and advances in the development

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of embryo culture media (7). Although blastocyst culture offers advantages such as self-selection and better growth potential for chromosomally normal embryos (8), in addition to improvements in live birth rate (LBR) (9, 10), recent systematic reviews and a meta-analysis (11, 12) show no additional benefits for blastocyst transfer compared with cleavage-stage ET in clinical practice.

A literature search of the freeze-all policy, which is commonly applied for young patients at risk of OHSS or higher progesterone levels on the day of triggering (13), indicates that evaluating the influence of diverse embryo stages on the reproductive results will be serious and important to advance the success of frozen-thawed ET. Selection of cleavage-stage ET versus blastocyst transfer for frozen-thawed ET cycles is a debatable topic (4). Many studies, systematic reviews, and meta-analyses (11, 14) have compared outcomes between blastocysts and cleavage-stage ET in ART in fresh cycles. However, the few studies that have examined the superiority of frozen-thawed blastocyst transfer in frozen-thawed ET cycles reported mixed results. To the best of our knowledge, only two studies (9, 15) focused on post-thaw extended culture blastocysts in frozen-thawed ET cycles. Therefore, the purpose of the current study is to examine reproductive outcomes of thawed cleavage-stage ET versus blastocysts derived from an extended culture of these embryos.

Materials and Methods

Participants

This open-label randomised, parallel group clinical trial was approved by the Institutional Review Board and Ethics Committee of Royan Institute, Tehran, Iran (IR.ACECR.ROYAN.1394.44), and conducted in compliance with the Declaration of Helsinki and its subsequent versions. All participants provided informed consent prior to enrolment. A total of 182 women aged ≤37 years who underwent frozen-thawed ET from November 2015 to June 2020 at Royan Institute Research Centre, Tehran, Iran enrolled in this study.

Eligibility criteria consisted of: primary type of infertility; age ≤37 years; enrolled in the gonadotropin hormone-releasing hormone (GnRH) agonist long protocol; and having at least four good quality frozen embryos. Patients were excluded if they had any of the following: surgical history on the uterus and ovaries; uterine factor infertility; severe male factor infertility (TESE, TESA, severe oligoteratozoospermia); history of recurrent abortion (≥2 abortions); and poor ovarian reserve.

The eligible women were randomly assigned (1:1) to two groups-a control group that received thawed cleavage embryos (n=110) or the study group that received post-thaw extended culture blastocysts (n=72). Block randomisation was conducted in equal block sizes of four. Randomisation was performed by a third party with the aid of computer-generated random numbers (SPSS version 18.0; IBM, Armonk, NY, USA) prepared by a statistician.

Sample size was calculated by PASS version 11 (NCSS, Kaysville, UT, USA) on the basis of our pilot study data (n=20). The sample size required for this study was estimated to be 97 patients in each group in order to detect a between-group difference of 0.2 (0.6-0.4) in clinical pregnancy, taking into consideration an alpha value of 0.05 and statistical power of 80% by the two-sided test. We enrolled 110 patients in each group, taking into account a dropout rate of 10%.

Stimulated cycles with IVF/ICSI

Controlled ovarian stimulation was performed with the standard long protocol using a gonadotropin-releasing hormone agonist (Suprefact; Hoechst, Frankfurt, Germany) and recombinant follicle-stimulating hormone (Gonal-F; Serono Laboratories Ltd., Geneva, Switzerland) or human menopausal gonadotropin (Menopur; Ferring GmbH, Kiel, Germany). An intramuscular injection of 10 000 IU human chorionic gonadotropin (Choriomon; IBSA, Lugano, Switzerland) was performed once at least one follicle reached 17-18 mm in diameter. Transvaginal oocyte retrieval was performed 34-36 hours later via vaginal ultrasound guidance; the oocytes were subsequently incubated for insemination or sperm injection.

Endometrial preparation and embryo transfer

For endometrial preparation, all patients received oral contraceptive pill-low dose before treatment beginning from the fifth day of their previous menstrual cycle in addition to a daily dose of GnRH agonist (0.5 mg/day, Suprefact; Hoechst, Frankfurt, Germany) from the 17th day of the cycle until pituitary down regulation, which was confirmed by serum estradiol (E2) <50 pg/mL, luteinizing hormone (LH)<5 IU/L, and basal ultrasonography. Then, 4 mg oral oestradiol valerate was started daily from the second day of the menstrual cycle; the dosage was adjusted according to the thickness of the endometrium. After ultrasound confirmation of an endometrial thickness of at least 7 mm, 100 mg of progesterone in oil (Aburaihan Pharmaceutical Co., Iran) was administered intramuscularly or 400 mg of vaginal progesterone (Cyclogest®, Actavis, Barnstaple, EX32 8NS, UK) twice a day. Luteal support was continued for two weeks. Serum β-hCG levels were measured on the 14th day after ET.

Patients were scheduled for thawed cleavage-stage ET or post-thaw extended culture blastocysts on the first day of progesterone administration based on the initial random allocation. ET was performed using a soft ET catheter (Labotect Labor-Technik, Göttingen GmbH, Germany) on day 3 or day 5 after initiation of progesterone.

Embryo vitrification-warming method and grading

All excellent and good quality embryos that were at the cleavage and blastocyst stages, as assessed by morphological scoring, were frozen by the vitrification method. The vitrification/warming method was performed according to a Royan protocol (16, 17). For this aim, the cryotop carrier system (Kitazato Biopharma Co., Ltd.,

Japan) was used for vitrification; 7.5% ethylene glycol and 7.5% dimethyl sulphoxide (equilibration solution) followed by 15% (v/v) ethylene glycol, 15% (v/v) dimethyl sulphoxide and 0.5 mol/l sucrose (vitrification solution) were used as the cryoprotectant. The 1.0, 0.5, and 0.0 mol/l sucrose solutions were used in warming the stepwise cryoprotectant dilution. With the exception of the first warming step, all steps were carried out at room temperature; the first warming step was carried out at 37°C.

Excellent and good quality cleavage stage embryos had four blastomeres on day 2 or six to eight cells on day 3, an equal blastomere size, less than 20% fragmentation, and absence of clear morphological abnormalities (18, 19). On day 5, blastocyst grading was performed according to the Gardner scoring system (20). Briefly, blastocysts were scored based on the level of cavitation or blastocoel expansion, and the number and quality of the inner cell mass and trophectoderm (TE).

Outcome measures

The primary outcome measure was clinical pregnancy rate. Secondary outcome measures were the implantation rate, LBR, and miscarriage rate. Clinical pregnancy was defined as the detection of at least one gestational sac by ultrasound examination over the number of ET cycles. Implantation rate was defined as the number of gestational sacs seen on transvaginal ultrasonography divided by the total number of embryos transferred. Live birth was defined as the number of deliveries that resulted in at least one live-born baby. Miscarriage rate was defined as a pregnancy loss before gestational week 20 per the total number of clinical pregnancies. Twin birth rate reflected the number of twin births per total number of clinical pregnancies. Blastocyst formation rate was defined as the total number of blastocysts formed in a cycle by the total number of thawed cleavage-stage embryos.

Statistical analysis

Data analysis was performed with SPSS version 22.0 (SPSS, Inc., Chicago, IL, USA). We used per-protocol analysis to test our hypothesis. Data for continuous variables are written as mean ± standard deviation (SD) and the Student's t test was used for comparison between groups. Categorical data are presented as frequencies and percentages; the chi-square or Fisher's exact tests were used for comparison between groups. $P<0.05$ indicated statistical significance.

Results

A total of 220 patients were randomised to each group, with 110 patients per group. Of the 110 participants in the blastocyst group, 38 women were excluded from the analysis. In two cases, the treatment cycle was cancelled before ET due to an inadequate endometrial thickness and menstruation, three cases had a change in their treatment protocols, and there were no blastocysts available for transfer in 33 cases. The number of cycles in each group was as follows: 110 cycles in the thawed cleavage-stage and 72 cycles in blastocysts from the thawed cleavage stage. Figure 1 summarises participants' recruitment, intervention allocation, follow-up, and analysis.

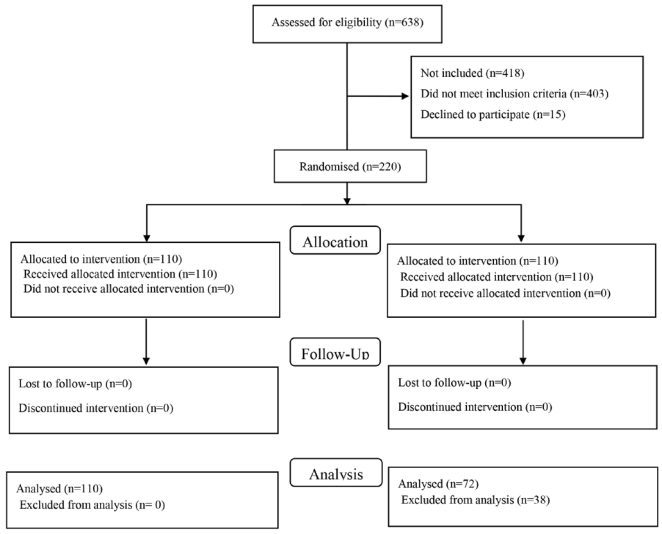

Fig.1: Flow chart of patient enrolment, allocation, follow-up, and analysis.

Table 1 provides baseline data on demographic and clinical characteristics between the thawed cleavage-stage ET and post-thaw extended culture blastocysts. There were no statistically significant differences between the two groups in regards to mean age, body mass index, infertility duration, and cause of infertility. The mean number of previous IVF/ICSI cycles, history of fresh ET, cause for freeze-all strategy, mean number of retrieved oocytes, mean number of MII oocytes, fertilisation rate, and mean number of retrieved embryos were similar in both groups.

Table 2 shows the clinical outcomes of patients in the frozen-thawed ET cycle. The two groups did not differ significantly in duration of cryostorage, basal hormone levels on day 3, maximum oestradiol dose, endometrial thickness on progesterone day injection, endometrial thickness on ET day, and difficulty of ET. However, there were statistically significant increases in the mean number of embryos transferred (2.38 ± 0.05 vs. 2.21 ± 0.05, P=0.016) and mean number of good quality embryos transferred (1.05 ± 0.09 vs. 0.71 ± 0.09, P=0.009) between the thawed cleavage-stage ET cycles compared to the post-thaw extended culture blastocysts cycles. The blastocyst formation rate in the post-thaw extended culture blastocysts cycles was 49.46% (138/279).

Reproductive outcomes of frozen-thawed ET cycles indicated that the post-thaw extended culture blastocysts group had significantly higher rates for clinical pregnancy (56.94 vs. 40.91%, P=0.034), implantation (34.43 vs. 19.84%, P=0.001), and live birth (49.29 vs. 33.63%, P=0.036) compared to the thawed cleavage-stage ET group. There were no significant differences in rates of miscarriage, twin birth, and other outcomes between both groups (P>0.05, Table 3). There were no reported birth defects in either group.

Table 1: Baseline characteristics and clinical history of ovarian stimulation cycle

Variables	Thawed cleavage-stage group (n=110)	Post-thaw extended culture blastocysts group (n=110)	P value
Age (Y)	29.96 ± 4.09	29.99 ± 4.09	0.961
Body mass index (kg/m^2)	25.28 ± 3.83	25.45 ± 4.08	0.755
Infertility duration (Y)	6.41 ± 3.78	6.19 ± 3.45	0.648
Cause of infertility			
Ovulatory	12 (10.91)	17 (15.45)	0.397
Tuboperitoneal	7 (6.36)	5 (4.55)	
Unexplained	16 (14.55)	23 (20.91)	
Male	67 (60.91)	61 (55.45)	
>1 factor	8 (7.27)	4 (3.64)	
No. of previous IVF/ICSI cycles	1.80 ± 0.96	1.96 ± 0.86	0.210
History of fresh embryo transfer			
Yes	36 (32.73)	27 (24.55)	0.180
No (freeze-all strategy)	74 (67.27)	83 (75.45)	
No. of retrieved oocytes	15.15 ± 5.97	16.02 ± 5.88	0.281
No. of MII oocytes	11.67 ± 0.48	12.55 ± 0.47	0.197
Fertilisation rate	1015/1540 (65.9)	1065/1616 (65.9)	0.997
No. of embryos	9.84 ± 3.67	10.28 ± 3.85	0.381

Data are written as mean ± SD or n (%). P value was obtained by the independent sample t test and chi square test. Statistically significant level was 0.05. IVF; *In vitro* fertilization and ICSI; Intracytoplasmic sperm injection.

Table 2: Clinical outcomes of patients in frozen-thawed ET cycles

Variables	Thawed cleavage-stage group (n=110)	Post-thaw extended culture blastocysts group (n=72)	P value
Duration of cryostorage (days)	316.85 ± 39.59	371.79 ± 43.69	0.352
Basal hormone levels on day 3			
LH (mIU/mL)	5.00 ± 0.23	5.75 ± 0.4	0.244
FSH (mIU/mL)	5.90 ± 0.2	5.95 ± 0.22	0.848
No. of embryos transferred	2.38 ± 0.05	2.21 ± 0.05	0.016
Quality of embryos transferred			
Excellent	1.08 ± 0.09	1.28 ± 0.08	0.126
Good	1.05 ± 0.09	0.71 ± 0.09	0.009
Fair	0.28 ± 0.05	0.21 ± 0.05	0.341
Assisted hatching			
Yes	23 (20.91)	54 (75.00)	<0.001
No	87(79.09)	18 (25.00)	
Maximum oestradiol dose			
4–8 mg	109 (99.09)	72 (100.00)	0.417
>8 mg	1 (0.91)	0 (0.0)	
Endometrial thickness: progesterone day injection (mm)	9.45 ± 1.27	9.42 ± 1.62	0.859
Endometrial thickness: ET day (mm)	9.74 ± 1.65	9.57 ± 1.52	0.569
Difficulty of ET			
Easy	104 (94.55)	70 (97.22)	0.506
Difficult	6 (5.45)	2 (2.78)	

Data are written as mean ± SD or n (%). P value was obtained by the independent sample t test and chi-square test or Fisher's exact test. Statistically significant level was 0.05. LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, and ET; Embryo transfer.

The results revealed that the laser-assisted hatching procedure was statistically higher in the post-thaw extended culture blastocyst cycles compared to the thawed cleavage-stage ET cycles (P<0.001, Table 2). Reproductive outcomes according to the laser-assisted hatching protocol are shown in Table 4. There were no significant differences in clinical pregnancy rates between those cycles with assisted hatching and unhatched cycles in the post-thaw extended culture blastocyst cycles and thawed cleavage-stage ET cycles (Table 4).

Table 3: Reproductive outcomes of frozen-thawed ET cycles

Variables	Thawed cleavage-stage group (n=110)	Post-thaw extended culture blastocysts group (n=72)	P value
Clinical pregnancies/ET cycle	45/110 (40.91)	41/72 (56.94)	0.034
Implantation/ET	52/262 (19.84)	52/151 (34.43)	0.001
Blighted	5/110 (4.54)	4/72 (5.55)	0.508
EP	0/110 (0)	1/72 (1.38)	0.396
Live birth delivery/ET cycle	37/110 (33.63)	35/72 (49.29)	0.043
Miscarriage/clinical pregnancy	3/110 (2.72)	1/72 (1.38)	<0.999
Twin birth/clinical pregnancy	7/110 (6.36)	10/72 (13.9)	0.088
Birth weight of first born	3012.08 ± 823.17	2964.57 ± 763.96	0.803
Birth weight of second born	1891.80 ± 554.18	2181.90 ± 594.15	0.379

Data are written as mean ± SD or n (%). P value obtained by the independent sample t test and chi square test. Statistically significant level was 0.05. ET; Embryo transfer and EP; Ectopic pregnancy.

Table 4: Clinical pregnancy and LBR of frozen-thawed ET cycle according to assisted hatching protocol

Outcomes	Thawed cleavage-stage group (n=110)		P value	Post-thaw extended culture blastocysts group (n=72)		P value
	Assisted hatching	No assisted hatching		Assisted hatching	No assisted hatching	
Clinical pregnancy rate	9/23 (39.13)	36/87 (41.37)	0.845	29/54 (53.7)	12/18 (66.6)	0.336
LBR	8/23 (34.78)	29/87 (33.33)	0.896	26/54 (48.15)	9/18 (50.00)	0.892

Data are presented as n (%). P value was obtained by the chi square test. Statistically significant level was 0.05. LBR; Live birth rate and ET; Embryo transfer.

Discussion

There is a recent, increasing trend towards a freeze-all approach. This trend will certainly impact decisions about the cleavage or blastocyst stages of embryo development for ET (7). Studies are being conducted to determine the key indicators that can affect the process of managing patients who benefit most from blastocyst culture. Approximately half of the trial results have shown higher success rates in the blastocyst transfer cycles. The trials can be separated into those that evaluated the superiority of blastocyst culture over standard cleavage-stage transfer in unselected patient populations or those that investigated the application of blastocyst culture in the clinical setting for enhanced success in specific patient subgroups (21). Here, we compared reproductive outcomes following transfer of post-thaw extended culture blastocysts and thawed cleavage-stage ET in patients who underwent frozen-thawed ET cycles.

We observed that the transfer of post-thaw extended culture blastocysts significantly improved rates for implantation, clinical pregnancy and live birth compared with thawed cleavage-stage ET. Enhanced pregnancy outcomes for blastocyst transfer compared with other stages of embryo development in frozen-thawed ET cycles have been reported (22, 23). To the best of our knowledge, only two studies (9, 15) assessed post-thaw extended culture blastocysts with conflicting results. A large retrospective population-based study (9) (n=150367) in Australia reported that the cycles with transfer of post-thaw extended culture blastocysts had significantly improved outcomes compared to thawed cleavage-stage ET cycles or thawed blastocyst transfer cycles. Remarkably, both LBR and a healthy baby delivery rate following transfer of post-thaw extended culture blastocysts were statistically comparable to those with fresh cleavage-stage transfer despite the physical damages to embryos from the freezing and thawing processes. The findings of this study indicate that the transfers of fresh blastocyst culture in fresh cycles and post-thaw extended culture blastocysts in frozen-thawed ET cycles improve the rate of healthy babies. The strong point of this retrospective cohort study was the use of population-based data extracted from all ET cycles performed in Australia during four years. In contrast, in a retrospective comparative study (15) that assessed the clinical outcomes of frozen-thawed ET with blastocysts found no benefit in pregnancy outcomes following frozen-thawed blastocyst transfer compared with frozen-thawed cleavage-stage or post-thaw extended culture blastocysts. They reported an extremely high multiple pregnancy rate (62.5%) in women who underwent post-thaw extended culture blastocyst transfer. A possible explanation for these results may be that the blastocysts in this study were cryopreserved by vitrification and the pronuclei were cryopreserved by the slow-freezing method, which might have impacted the results. An improved survival rate and similar or even better pregnancy rate for vitrification compared with the slow freezing method has been reported in numerous literature (24, 25). In line with these results, the data on transfer of post-thaw extended culture blastocysts is low; therefore, there is a need for further studies in this area.

Other studies sought to determine whether patients who underwent frozen-thawed ET cycles could benefit from the transfer of thawed blastocysts. The findings indicated significantly higher pregnancy rates (23) or

insignificantly higher cumulative ongoing pregnancy rates (26) in favour of thawed blastocyst transfer with fewer ETs and comparable multiple pregnancy rates as compared to thawed cleavage-stage ET.

The results of the present study show the benefits of transfer of post-thaw extended culture blastocysts. This higher success rate with blastocyst transfer might be attributed to the embryo selection process. It is reported that 59% of day 3 high-quality embryos are chromosomally abnormal, whilst only 35% of high-quality blastocysts have chromosomal anomalies (27). Even in cases where the blastocyst stage development has not been prevented by genetic abnormalities, the incidence of chromosomal abnormalities in blastocysts would be lower than those seen in cleavage-stage embryos. Blastocyst transfer would have a smaller risk of aneuploidy than embryo cleavage-stage embryos. This increases the chances for pregnancy (23). In addition, asynchrony between the developmental stage of transferred cleavage-stage embryos and the counterpart of the reproductive tract (28) may compromise embryo viability because the nutritional environments provided by the oviduct and uterus do not match with the developing embryo; thus, cleavage-stage ET might undergo metabolic stress (27, 29). However, blastocyst-stage embryos are better synchronised with the female reproductive tract during natural pregnancy and, therefore, are protected from this environmental stress (23).

Although our results indicated that the mean number of ETs was significantly lower in post-thaw extended culture blastocysts compared with cleavage-stage ET group, we observed a better outcome in the post-thaw extended culture blastocysts group. This finding was also reported in another large retrospective study. Those authors found a similar LBR with the transfer of frozen embryos on days 3 and 5 (30). An explanation for the fewer number of blastocysts-stage embryos than cleavage-stage embryos in different studies is the lack of options rather than policy, and the reason for the high rate of unsuccessful blastocyst transfer is mainly due to arrested embryonic development before the ET day (21). Some studies included patients that had transfer of developmentally delayed blastocyst stage embryos, whilst other studies were more selective and excluded those with transfer of embryos under stages of late morula or early blastocyst (7). The blastocyst formation rate is reported as 22.4 to 60% in different studies and may be associated with pregnancy rate per ET in each study (31, 32). This suggests that various formulations and brands of embryo culture media probably affect blastocyst formation rate and subsequent outcomes (21). We observed a blastocyst formation rate of 49.5% (138/279).

In our study, the percentage of treated post-thaw extended culture blastocysts with laser-assisted hatching was significantly higher than treated cleavage-stage embryos with assisted hatching (75 vs. 20.91%, $P<0.001$). Nonetheless, separate data analysis in thawed cleavage-stage ET cycles and post-thaw extended culture blastocysts showed no significant differences in clinical pregnancy rates between the assisted hatching and unhatched cycles. The evidence demonstrates that implantation rate of human embryos is associated with zona thickness (33), which might be related to zona pellucida hardening during vitrification (12). However, a considerable amount of meta-analysis and systematic reviews (34, 35) are uncertain about the effects of assisted hatching on LBR.

Although, there are some cost considerations associated with offering an extended culture to blastocyst stage for the patients including the cost of an additional incubator for culture, extra media costs, and increased weekend work for laboratory staff; for the patient, an increased probability of cancellation owing to the stricter selection process of the blastocyst culture might end to a lower treatment cost. The treatment cost is essential to be evaluated and compared to the chances of having a healthy baby (21).

A limitation of our study is the presence of factors that can affect pregnancy rate, such as the embryologist who performed the embryo grading, selection, and transfer.

Conclusion

The results strongly advocate in favour of using post-thaw extended culture blastocysts. It will be important to a improve the post-thawing extended culture technique before making the extra effort for transferring blastocysts in frozen-thawed ET cycles. A prospective randomised controlled trial that has a large sample size is suggested.

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Authors' Contributions

T.M.; Study conception and Design. N.J.; Methodology, Writing- review and Editing. A.Y., M.Z.; Data acquisition. S.V.; Statistical analysis. P.E.-Y.; Project development. All authors read and approved the final manuscript.

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Improvement of Mouse Preantral Follicle Survival and Development following Co-Culture with Ovarian Parenchyma Cell Suspension

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Abstract

Background: The parallel and continued improvements in both infertility treatment and the management of malignancy cases have brought to the forefront the potential for fertility preservation. Using ovarian follicular resources can effectively improve reproductive capacity and prevent infertility. The primary aim of this research was to try to generate an appropriate *in vivo* environment for the growth of the mouse follicles. Hence, the possible effects of the ovarian parenchyma cell suspension were explored on the growth and maturation of preantral follicles *in vitro*.

Materials and Methods: In this experimental study, ovarian parenchymal cells were mechanically dissociated from preantral follicles of 12-14 days-old NMRI mice and then divided into 5 experimental groups (G1: Control, G2: Fresh follicle with fresh parenchyma cell suspension, G3: Vitrified-warmed follicle with fresh parenchyma cell suspension, G4: Fresh follicle with frozen-thawed parenchyma cell suspension, and G5: Vitrified-warmed follicle with frozen-thawed parenchyma cell suspension). The diameter of the follicles and immature oocytes, viability, antrum formation, resumption of meiosis, *in vitro* fertilization (IVF), and *Gdf9*, *Bmp6*, and *Bmp15* gene expression were examined on different periods.

Results: The diameter of the follicles and the oocytes on days 4 and 8, as well as the survival rate of the follicles up to day 12, were significantly higher in G2 and G4 compared to the Ctrl group (G1: 73.66%, G2:87.99%, G3: 82.70%, G4: 94.37%, and G5: 78.59%). Expression of growth marker genes for G3, and G5 groups was significantly higher than other groups, which indicated the protective effects of parenchyma cell suspension on follicles damaged by vitrification solutions.

Conclusion: The growth, survival, and maturation of preantral follicles could be enhanced by co-culturing them with ovarian parenchyma cells. Further studies are needed to optimize the conditions for a successful parenchyma cell suspension-induced *in vitro* maturation (IVM) to occur in infertility clinics.

Keywords: Co-Culture, Fertility Preservation, *In vitro* Culture, Ovarian Tissue, Preantral Follicle

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Introduction

Female infertility may arise as a consequence of aging, various ovulation blocking diseases such as pelvic inflammatory disease, endometriosis, fibroids, and exposure to various treatments for oncological conditions. Hence, fertility preservation in women provides a realistic chance for potential future pregnancies. Improvements in both infertility and cancer treatments, allow for fertility preservation in cancer patients being treated with radio-chemo therapeutics. Therefore, there is an increasing demand to prevent or decrease the loss of fertility in young female cancer patients who are undergoing fertility-destroying, chemo or radiation therapy. Ovarian hyperstimulation and multiple oocyte collection, as per a full *in vitro* fertilization (IVF) cycle, offers cancer patients a chance of preserving their fertility. It's also well known that the number of ovarian reserves is reduced during life,

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so using ovarian follicular resources can effectively improve reproductive efficacy for a longer period of time and to prevent infertility (1-3).

In vitro ovarian follicle culture has been used to investigate ovarian pathology and to determine how ovaries or oocytes are affected by exposure to various toxic chemicals, with the purpose of obtaining fertilizable oocytes from primordial ovarian follicles. Numerous studies have shown that malignancy, chemotherapy, or radiation therapy in women, are likely causes of harm in their reproductive tissues and are often associated with premature ovarian failure/insufficiency (POF/POI) and infertility (4, 5). Due to the failure of the usual culture systems to satisfy the metabolic requirements of the growing follicles and the lack of paracrine connection with the surrounding stromal tissue, prolonged follicular cultures frequently result in atresia. The complex procedure of folliculogenesis is one of the primary reasons that small follicle development is not sustained or promoted under normal culture conditions (3); besides the endocrine, autocrine and paracrine glands, and complex cell-cell interactions facilitate this process. It is well known that folliculogenesis plays an essential role in normal ovarian functioning, because it allows for ovulation and the synthesis of the vital sex hormones including estradiol and progesterone. The mammalian ovary is composed of ovarian follicles, where each follicle has a single oocyte surrounded by granulosa cells, enclosed within a basement membrane. The opportunity to track and estimate the growth rate and follicle size during the culture phase is provided by the culture of intact ovarian follicles. Therefore, in vitro culture of follicles is considered as an excellent option to evaluate different aspects of follicular growth and development (2, 6). Moreover, it may also be regarded as an approach to examine fertility maintenance (3, 7).

Multiple efforts have been completed to create a healthy oocyte from individual follicles *in vitro*, leading to significant achievements and advances in preantral follicle culture from small mammals (8-10). Previous studies have shown that preantral follicles have been cultured with various cells, including mesenchymal stem cells, adipose-derived stem cells, and granulosa and theca cells (11-16). The present study uses ovarian parenchyma tissue in a two-dimensional culture medium to evaluate the survival and growth rates of preantral follicles. Embryonic stem cells with asymmetric divisions form the parenchymal tissue of the ovary, forming the ovarian cortex that comprises fibroblasts, lutein cells, granulosa and theca cells, and collagen connective tissue (17, 18). This work describes the co-culture of whole ovaries containing preantral follicles, with the ovarian parenchyma cell suspension, a method which can support the development of follicles, after which their oocytes may be able to undergo fertilization *in vitro*. On The other hand, it is well known that ovarian folliculogenesis and differentiation depend on coordinated interactions and expression of multiple growth marker genes. Thus, the maturity levels and development of cultured follicles were evaluated through expression of known growth marker genes such as the growth differentiation factor 9 (*Gdf9*), the bone morphogenetic protein 15 (*Bmp15*), and the bone morphogenetic protein 6 (*Bmp6*) (19, 20). This major objective of this study was to simulate an *in vivo* environment in the lab, and in order to do so, the effects of ovarian parenchyma cell suspension on the survival and development of preantral follicles were assessed.

Materials and Methods

Animals

This experimental study was approved by the Ethics Committee of Royan Institute (IR.ACECR.ROYAN.REC.1399.035). The NMRI mice used in this study were obtained from the laboratory animal science unit of Royan Research Institute and kept in suitable conditions with 12 hours of light and 12 hours of darkness at 20-25°C temperature and free access to food and water. At the initial steps of the study, female NMRI mice were sacrificed by cervical dislocation and their ovaries were removed, placed in modified minimum essential medium (α-MEM, 11900073, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, 10270106, Gibco, USA). Parenchyma cell suspension was prepared from the ovaries of adult 6 to 8 weeks (42-56 days) old female mice, while preantral follicles were isolated from ovaries of 2 weeks (12-14 days) old female mice.

Follicle isolation

First, 12-14 day-old mouse ovaries were removed and transferred into 100 µl droplets of α-MEM culture medium containing 10% FBS. 120 to 140 µm follicles were mechanically removed by sterile fine 29G syringe needle under stereo microscope. The isolated follicles were collected in a 30 µl droplet of α-MEM culture medium and individually transferred to experimental groups (21).

Vitrification and thawing of the follicles

To freeze the follicles, equilibrium and vitrified solutions were used. Briefly, the follicles were first placed in equilibrium solution at room temperature for 3 minutes. For preparation of the equilibrium solution, α-MEM culture medium was supplemented with 7.5% ethylene glycol (EG, 293237, Sigma-Aldrich, Germany), 7.5% dimethyl sulfoxide (DMSO, D2650, Sigma, USA) and 20% FBS.

The follicles were then vitrified at room temperature for one minute. To prepare the vitrification solution, 15% EG, 15% DMSO, 0.5 M sucrose (S7903, Sigma, USA), and 20% FBS were added to α-MEM medium. Afterwards, the follicles were removed with an appropriate pipette and loaded on the thin end of the Cryotop strip, which was immediately submerged into liquid nitrogen vertically with rapid horizontal movements to obtain the maximum cooling rate.

Thawing the follicles was performed by removing the samples from liquid nitrogen, and immersing them directly into the primary melt solution containing α-MEM medium supplemented with 20% FBS and 1 M sucrose. The samples were rinsed for 1 minute in the melt solution at 37°C. Subsequently, follicles were washed in the second thaw solution, which was α-MEM medium containing 20% FBS and 0.5 M sucrose. The second wash was done at room temperature for 3 minutes (14).

Isolation of ovarian parenchyma cell suspension

Adult (6-8 weeks old) female mice were sacrificed by cervical dislocation, and their ovaries were removed by creating a transverse abdominal incision. The ovaries were placed in 200 µl droplets of α-MEM culture medium containing 10% FBS, and cut into very small pieces using a 29G syringe needle under a stereomicroscope. The minced tissues were pipetted gently 5 to 10 times using 1000-µl, and 100-µl micropipettes and 2-ml syringes with 29G needles. After the ovarian tissues became uniformly shredded, they were centrifuged at 261 g for 5 minutes. The viable cells in the sediment were counted under an inverted microscope, and the samples with a specified number of 5000-10000 cells per drop were placed into the culture medium.

Freezing and thawing of ovarian parenchyma cell suspension

Parenchyma cell suspension was centrifugated at 261 g for 5 minute to separate the cells from the medium. The supernatant was removed, the remaining cell pallet was resuspended into a mixture of 90% FBS and 10% DMSO. The cell mixture was then transferred to cryovials and stored at -20°C for 1 hour to give the cell sample the opportunity to absorb DMSO. The long-term freezing process was initiated by transferring the cryovial to the -80°C freezer for 5 to 12 hours, followed by a final storage in a liquid nitrogen tank.

For thawing the cell suspension, the frozen parenchyma cells were removed from the nitrogen tank and left in bain-marie set to 37°C. The α-MEM medium was slowly added up to as much as 2 to 4 times the volume of the base culture medium solution to stabilize the suspension, and then it was centrifuged at 261 g for 5 minutes to completely separate the DMSO residues from the cell suspension (22).

Experimental groups

The preantral follicles were randomly divided into five experimental groups and cultured for 12 days:

G1: Untreated follicles that were cultured as controls (Ctrl).

G2: Fresh follicles co-cultured with fresh ovarian parenchyma cell suspension (FF+FPCS).

G3: Vitrified-warmed follicles co-cultured with fresh ovarian parenchyma cell suspension (VF+FPCS).

G4: Fresh follicles co-cultured with frozen-thawed ovarian parenchyma cell suspension (FF+FTPCS).

G5: Vitrified-warmed follicles co-cultured with frozen-thawed ovarian parenchyma cell suspension (VF+FTPCS).

In vitro culture of follicles

As much as 15 µl of fresh and frozen-thawed ovarian parenchyma cell suspension containing 5000-10000 cells were added to 45-µl droplets of α-MEM culture medium and placed in a petri dish covered with mineral oil and incubated at 37°C and 5% CO_2 for 3 to 5 hours. Then, fresh and vitrified-warmed follicles in 60-µl droplets of α-MEM culture medium supplemented with 10% FBS, 10 Iu/ml of human follicle-stimulating hormone (FSH, Gonal-f, Merck, Germany), and 1% insulin-transferrin-selenium (ITS 100X, 41400045, Gibco, USA) for the Ctrl group (23, 24), and the cell suspension with culture medium mentioned for the following groups were cultured for 12 days in an incubator at 37°C, 96% humidity and 5% CO_2. To feed the cells half of the culture medium was replaced with an equal volume of fresh medium every other day. Through the culture period, the progress of follicle growth, diameter, and morphological changes were evaluated.

Measuring the diameter of follicles and oocytes

Using an inverted microscope (Nikon, ECLIPSE TS100), the follicles were imaged after 3 hours of culture and also on days 4, 8, and 12 of the culture periods. Additionally, the diameter of the follicles and their oocytes were measured using the Image-J application during the aforementioned culture periods.

Assessment of morphological changes

The survival rate of the follicles was evaluated on culture days 0, 4, 8, and 12, and the dark follicles without oocytes were considered as degenerated follicles. Formation of clear spaces between the follicles' granulosa cells was considered the antrum cavity during the culturing period. Then, to evaluate the growth of the follicles and the healthy oocytes, the number of antral follicles, cumulus-oocyte complex (COC) formation, germinal vesicle breakdown (GVBD), and metaphase II (MII) were examined. Developed and enlarged antrum cavity was observed on day 12 of the culture period, at which time the culture medium was replaced with fresh medium supplemented with 1.5 Iu/ml human chorionic gonadotropin (hCG), for induction of ovulation (Pregnyl, Organon, USA). Subsequently, the ovulation rate was evaluated under an inverted microscope after 18 to 24 hours. Accordingly, the released oocytes were classified into MI and MII based on their detected morphologies, where MI represented when the germinal nucleus of the vesicle disappeared, and MII indicated when the first polar body (1PN) was developed (24).

In vitro fertilization

To evaluate the development competence of the oocytes, IVF was conducted on the developed oocytes. Thus, sperms collected from the 12-week-old male NMRI mice, were used for fertilization of the oocytes. First, the testis epididymal tail was removed and placed in a droplet containing T6 culture medium and 15% bovine serum albumin (BSA, a.3311, Sigma-Aldrich, Germany). Afterwards, drops containing sperm, were incubated in 5% CO_2 at 37°C for 1-2 hours. To conduct development, the fertilization process 6 or 8 MII oocytes were transferred to each fertilization drop, followed by the addition of 10-12 sperms for each oocyte and incubated for 4 hours. Oocytes that released their 2PN were successfully fertilized so they were transferred to the droplet containing 4% BSA+T6 to complete the development process (25).

Evaluation of expressed growth marker genes

The maturity levels and development of cultured follicles were evaluated through expression of known growth factor marker genes *Gdf9*, *Bmp15*, and *Bmp6*. Real-time polymerase ciain reaction (PCR) was employed to estimate the expression level of each gene in the cultured follicles. Initially, extraction of RNA was performed from the cultured follicles (20 healthy follicles/replicate for each time point) using RNeasy Micro Kit (Qiagen, Germany) according to the manufacturer's protocol. Three replicates were performed for each group to estimate the total RNA extraction. Total RNA from the cultured follicles was reverse transcribed into first-strand cDNA with cDNA Synthesis Kit (SMO Bio primer, Taiwan) and random hexamers based on the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an endogenous control, and primers were designed using PrimerExpress (Applied Biosystems, USA). Finally, to reach the final reaction volume (10000 µl), each PCR reaction consisted of 2500 µl Power SYBR ampliqon (realQ plus 2x master mix green), 2000 µl of cDNA, 1000 µl sense primer, 1000 µl antisense primer with a concentration of 5 pmol and 3500 µl dH2O. Analysis of expression level was performed by an ABI StepOne plus thermocycler and StepOne Software version 2.3 with the following protocol: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Each reaction was run in duplicates. Relative gene expression between all follicle groups was calculated by the ΔΔCT comparison method, in which -2ΔΔCT values were estimated for each expressed gene on both 3 hours and 4 days culture periods for the five experimental groups (13).

Statistical analysis

The results were presented as mean ± SEM, and a $P \leq 0.05$ was considered to be statistically significant. At least three separate repeats were performed for each experiment, and average values were used for data analysis. All data were analyzed by GraphPad Prism (Insightful Science, USA) and SPSS software version 26 (IBM, USA). The Kolmogorov-Smirnov test was used to determine the normality of all tests. ANOVA test was used for statistical assessment of the follicle and oocyte diameters and evaluation of oocyte and embryo development after IVF. The Kruskal-Wallis test was used to assess statistically significant differences in survival, antrum formation, and related gene expression results.

Results

In the present study, follicle growth rate and morphology were evaluated in 5 distinct experimental groups to identify the viable cells in each condition. We also assessed survival rate, percentage of antrum formation, resumption of meiosis, as well as COC compared to antral follicle for further growth analysis. The rate of oocyte maturation was examined in 7 replications, but oocyte fertilization and embryo development were performed in 4 replications.

For culture analysis, any naturally occurring, light-colored follicles containing healthy oocytes with clear zona pellucida were considered as healthy, whereas any changes, such as spontaneous release of an oocyte, termination of growth, or the darkening of the follicles, placed them in the category of degenerative follicles.

Evaluation of follicle and oocyte diameter during culture

The diameter of the follicles and oocytes were examined on days 0, 4, and 8 of culture. The results obtained for each of the experimental groups indicate a robust growth of fresh follicles in G2 and G4 groups, cultured from fresh and frozen-thawed ovarian parenchymal cell suspension, respectively, compared to the control (G1) group. Also, the growth rate in these groups was significantly different in comparison to the other two groups on day 8 of culture (Ctrl: 266.33 ± 20.59, G2: 373.49 ± 27.007, G3: 333.12 ± 15.63, G4: 409.55 ±16.45, G5: 356.87 ± 18.58 - P: G2: 0.0160- G4:0.020) (Fig.1A). Analysis of the oocyte diameters from follicles on different days among the groups, demonstrated that the G2 and G4 groups were significantly different from the control and G3 groups (Fig.1B) (day 8: Ctrl: 67.15 ± 1.18, G2: 73.02 ± 1.21, G3: 68.61 ± 1.05, G4: 72.75 ± 1.93, G5: 70.62 ± 1.89) (P: G2-Ctrl: 0.0004- G4-Ctrl:0.0006- G2-G3: 0.0002- G4-G3:0.0001). On the other hand, images taken on different days of culture showed that groups treated with fresh and frozen ovarian parenchyma cell suspension had higher growth rates (Fig.2).

Survival rate, formation of antral follicles and cumulus-oocyte complex

A noteworthy issue in assessing the survival rate of the follicles up to day 12 among the five experimental groups was that in the G2 and G4 groups, we found

significant differences between fresh preantral follicles co-cultured with fresh and frozen-thawed ovarian parenchyma cell suspension, respectively. [(survival rate %) Ctrl: 73.66 ± 4.56, G2:87.99 ± 3.39, G3: 82.70 ± 3.34, G4: 94.37 ± 2.80, and G5:78.59 ± 5.55] [P: (G2:0.006) (G4:0.0004)] (Table 1, Fig.3A). Although the experimental groups treated with fresh and frozen-thawed ovarian parenchyma cell suspension were better in the evaluation of antrum cavity formation, no significant differences were found among of the groups (P>0.05, Table 1, Fig.3B). The rate of COC formation in the experimental groups showed that fresh preantral follicles treated with fresh and frozen-thawed ovarian parenchyma cell suspension had a successful culture in COC formation compared to the other groups, especially the control and G3 groups (Ctrl: 81.66 ± 4.96, G2: 94.28 ± 2.97, G3: 80.95 ± 5.36, G4: 97.95 ± 2.04, and G5: 89.38 ± 5.80) [P: (G2:0.004) (G4:0.005)] (Fig.3C).

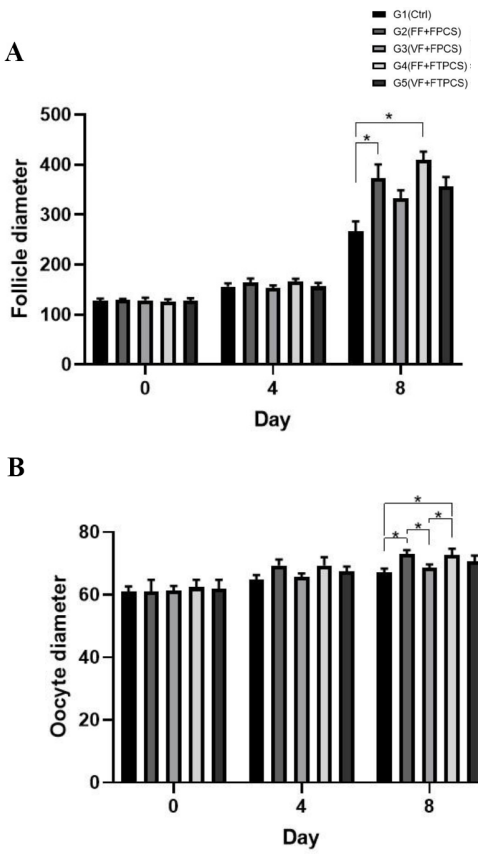

Fig.1: The effect of ovarian parenchyma cell suspension on follicle and oocyte diameter. **A.** Preantral follicle diameter increased notably in the presence of ovarian parenchyma cell suspension, and this increase was significantly higher when co-cultured with fresh follicles, especially at the end of the growth phase groups (P: G2: 0.016- G4:0.020). **B.** The diameter of the oocytes in the preantral follicles was examined on different days of culture and co-culture of fresh follicles with fresh and frozen-thawed ovarian parenchyma cell suspension was significantly higher than other groups (P: G2-Ctrl: 0.0004- G4-Ctrl:0.0006- G2-G3: 0.0002- G4-G3:0.0001. The Kolmogorov-Smirnov and ANOVA tests were used for statistical assessment, G1; Control, G2; Follicle with fresh parenchyma cell suspension, G3; Vitrified-warmed follicle with fresh parenchyma cell suspension, G4; Follicle with frozen-thawed parenchyma cell suspension, G5; Vitrified-warmed follicle with frozen-thawed parenchyma cell suspension, and *; Significant difference (P≤0.05).

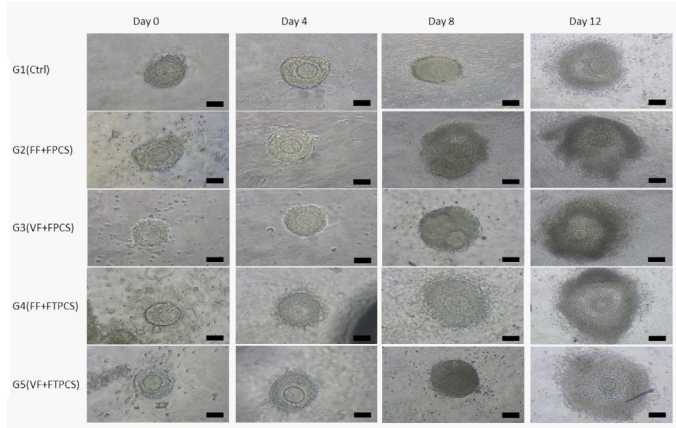

Fig.2: Images of preantral follicles on different days of culture (scale bar: day 0 and 4: 10 µm , day 8 and 12: 16 µm). G1; Control, G2; Follicle with fresh parenchyma cell suspension, G3; Vitrified-warmed follicle with fresh parenchyma cell suspension, G4; Follicle with frozen-thawed parenchyma cell suspension, and G5; Vitrified-warmed follicle with frozen-thawed parenchyma cell suspension.

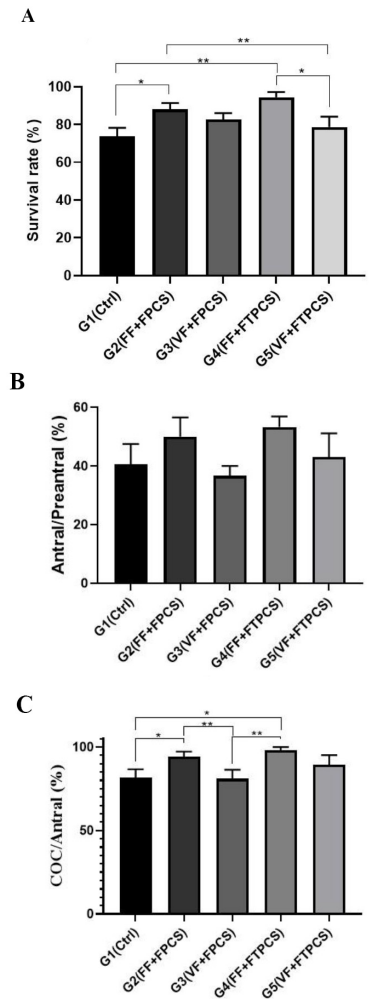

Fig.3: Graphs comparing the growth and survival rates of the follicles in 12 days of culture. A. Percentage of survival rate during in vitro culture. Data are expressed as mean ± SEM [P: (G2:0.006) (G4:0.0004)]. B. Percentage of antral/pre-antral follicles during in vitro culture. C. Percentage of antral follicles/COC during in vitro culture [P: (G2:0.004) (G4:0.005)]. The Kruskal-Wallis and Kolmogorov-Smirnov tests were used for statistical assessment. G1; Control, G2; Follicle with fresh parenchyma cell suspension, G3; Vitrified-warmed follicle with fresh parenchyma cell suspension, G4; Follicle with frozen-thawed parenchyma cell suspension, G5; Vitrified-warmed follicle with frozen-thawed parenchyma cell suspension, COC; Cumulus-oocyte complex, *, and **; Significant difference only compared to the G2 and G4 groups (P<0.05).

Table 1: The development of cultured mouse preantral follicles in five different experimental groups after 12 days of IVC in 7 replications (group A) and oocyte maturation and evaluation of embryo development after *in vitro* fertilization in 4 replications (group B)

Group A	Survival rate n (%)		Antrum formation n (%)	Developmental stage of oocyte n (%)	
				COC	GVBD+MII
G1(Ctrl)	70/95 (73.66)		39//95 (40.52)	31/39 (81.66)	24/31 (77.77)
G2(FF+FPCS)	89/100 (87.99)		50/100 (50)	47/50 (94.28)	40/47 (86.44)
G3(VF+FPCS)	61/74 (82.70)		27/74 (36.63)	22/27 (80.95)	15/22 (64.99)
G4(FF+FTPCS)	87/92 (94.37)		49/92 (53.29)	48/49 (97.95)	40/48 (83.69)
G5(VF+FTPCS)	52/68 (78.59)		28/68 (43.10)	24/28 (89.38)	17/24 (84.52)
Group B	**COCs (n)**	**MII (n)**	**2PN n (%)**	**2-Cell n (%)**	**4-Cell n (%)**
G1(Ctrl)	16	9/16	7/9 (79.16)	3/9 (58.33)	1/9 (8.33)
G2(FF+FPCS)	20	14/20	12/14 (81.25)	7/14 (48.33)	1/14 (5)
G3(VF+FPCS)	12	5/12	3/5 (66.66)	2/5 (66.66)	0
G4(FF+FTPCS)	23	14/23	12/14 (82.5)	7/14 (66.66)	3/14 (20.83)
G5(VF+FTPCS)	10	6/10	4/6 (83.33)	3/6 (55.55)	1/6 (11.11)

The Kolmogorov-Smirnov and Kruskal-Wallis tests were used for statistical assessment for group A. The Kolmogorov-Smirnov and ANOVA tests were used for statistical assessment for group B (mean ± SEM, P<0.05). G1; Control, G2; Follicle with fresh parenchyma cell suspension, G3; Vitrified-warmed follicle with fresh parenchyma cell suspension, G4; Follicle with frozen-thawed parenchyma cell suspension, G5; Vitrified-warmed follicle with frozen-thawed parenchyma cell suspension, COC; Cumulus-oocyte complex, GVBD; Germinal vesicle breakdown, MII; Metaphase II, and 2PN; Second polar body.

In vitro maturation

The present study we evaluated follicle maturation and fertilization up to the 4-cell stage. The G2 and G4 groups with cultured fresh follicles plus fresh and frozen-thawed ovarian parenchyma cell suspension showed a better maturation rate (Ctrl: 77.77 ± 3.92, G2: 86.44 ± 5.23, G3: 64.92 ± 8.45, G4: 83.69 ± 5.26, and G5: 84.52 ± 7.80) than the control and other groups. However, significant difference was not observed in maturation process (Table 1, Fig.4).

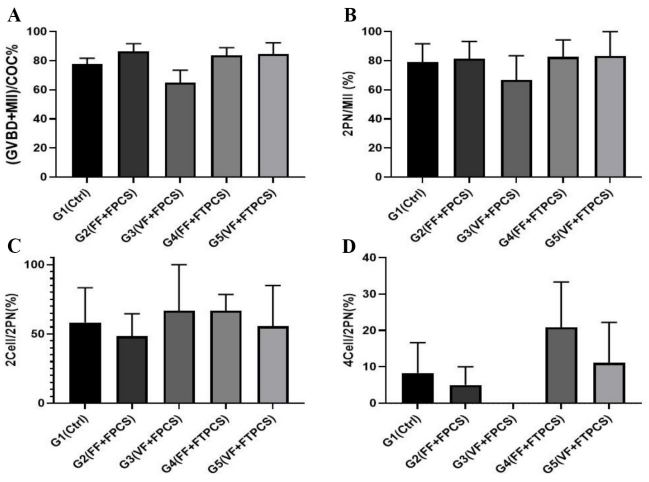

Fig.4: Evaluation of oocyte and embryo development after *in vitro* fertilization. Percentage of **A.** (GVBD+MII)/COC. **B.** 2PN/MII. **C.** 2Cell/2PN. **D.** 4Cell/2PN. No significant difference was observed in the above experiments (P<0.05). The Kolmogorov-Smirnov and One-way ANOVA tests were used for statistical assessment. G1; Control, G2; Follicle with fresh parenchyma cell suspension, G3; Vitrified-warmed follicle with fresh parenchyma cell suspension, G4; Follicle with frozen-thawed parenchyma cell suspension, and G5; Vitrified-warmed follicle with frozen-thawed parenchyma cell suspension, GVBD; Germinal vesicle breakdown, MII; Metaphase II, COC; Cumulus-oocyte complex, and 2PN; Second polar body.

Embryo development

Four experimental replications were done in the examination of 2PN, 2-cell, and 4-cell. Analysis of the 2PN formation rate (Ctrl: 79.16 ± 12.50, G2: 81.25 ± 11.96, G3: 66.66 ± 16.66, G4: 82.5 ± 11.81 and, G5: 83.33 ± 16.66) did not reach statistically significant difference. Also, the results for 2-cell and 4-cell did not reach statistically significant difference, despite the fact that Table 1 indicates that there are higher percentages of 4-cells in the G4 and G5 groups compared to the Ctrl group (P>0.05, Table 1 , Fig.4).

Expression pattern of *Gdf9*, *Bmp6*, *Bmp15* genes

The estimation of expression of maturity genes in the first 3 hours and on day 4 of culture in the 5 experimental groups showed that the highest expression in cultured groups occurred in the first few hours, but the level of expression was declined 4 days into the culture period.

In the present study, although the expression level of the *Gdf9* transcript was not significantly different in the first 3 hours of culture within the experimental groups, but it was significantly higher on day 4 of culture in the follicles of the G5 group compared to the other groups [P: (*Gdf9*: G5:0.01) (*Bmp15*: G5:0.019)].

The expression level of the *Bmp6* gene in the G2 group increased on day 4 compared to the first 3 hours of incubation in culture medium. Nevertheless, no significant difference was found among the experimental groups.

Furthermore, in the study of *Bmp15* gene transcript expression in the experimental groups during the first 3 hours of culture, the expression level in the G5 group increased compared to the other groups and was significantly different (Fig.5).

Expression of growth factor genes in cryopreserved groups was significantly higher than the other groups, which may indicate that the protective effects of parenchyma cell suspension on follicles is damaged by vitrification solutions.

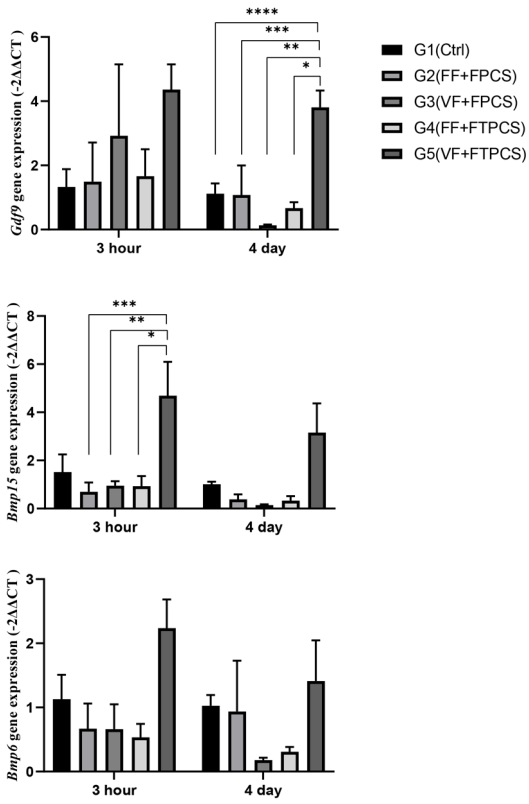

Fig.5: Relative expression of total mRNA for maturation genes in the follicles on different days of in vitro culture. Data are expressed as mean ± SEM. The Kolmogorov-Smirnov and Kruskal-Wallis were used for statistical assessment. Expression of *Gdf9* was higher in the G3 and G5 groups during the first 3 hours of culture than in the other groups. Expression of *Bmp6* and, *Bmp15* was higher in the G5 group during the first 3 hours of culture than in the other groups and in the fourth day of culture the expression level was decreased in all groups, but in the G3 and G5 group was higher than the other groups. This indicates the positive effects of cell suspension on follicles damaged by vitrification solutions (*Gdf9*: G5: P: 0.01) (*Bmp15*: G5: P: 0.019). *, **, ***, and ****; Significant difference only compared to the G5 group (P<0.05).

Discussion

Understanding follicle-oocyte interactions has important implications for identifying biological markers that influence the ability of an oocyte to be fertilized and develop into a healthy embryo in both natural pregnancy and pregnancy by assisted reproduction technology (ART). Ovarian follicles, the functional units of the ovary, comprise the ovarian parenchymal tissue that consists of embryonic stem cells and other cells including fibroblasts, lutein cells, granulosa and theca cells, as well as collagen connective tissue. Searching new ways for follicles to grow and develop efficiently may improve the pregnancy rate in ART.

Bi-directional communication between the oocyte and follicle somatic cells is the key element in regulating follicle and oocyte development. Follicle diameter as well as hormone levels are used as the primary markers for determining the maturity of the follicle and oocyte prior to oocyte retrieval in IVF (26). Prolonged culturing frequently results in atresia because the typical culture systems are unable to supply the metabolic needs of the developing follicle and because there is no paracrine communication with the stromal tissue around it. The complicated process of folliculogenesis is one of the main reasons why normal culture conditions are unable to support or stimulate the development of small follicles (3). One of the essential issues in ovarian follicle culture is the lack of complete knowledge of the influential growth factors with regard to the surrounding cells in parenchyma. Therefore, improving follicle culture systems and follicle cryopreservation is highly studied today. Based on the obtained information, no studies have been performed on the co-culture of ovarian parenchyma cells with follicles. In the present study, we investigated the effects of ovarian cell culture with preantral follicles on survival, follicle development, maturation, and fertilization of the resulting oocytes and the expression of their genes on different days *in vitro*. In terms of oocyte and follicle diameter, all treatment groups had better growth than the control group. However, G2 and G4 groups showed a significant follicle growth compared to the control and G3 groups, indicating that co-culturing with ovarian parenchyma cell suspension indeed stimulated the growth rate in the follicles. It has been demonstrated that growth and differentiation in a range of species' reproductive tissues are altered by fibroblast growth factors (FGFs). The effects of FGFs seem to be concentrated on the ovary in the female reproductive tract, and FGF2, which promotes granulosa cell proliferation and inhibits differentiation, has received the most attention. FGF2 acts on granulosa cells to promote cell proliferation and decrease apoptosis and steroidogenesis, which has been known for more than 20 years (27). In a 2014 study by Malekshah et al. (12) و the follicles that were co-cultured with fibroblast cells developed and survived better than the control group.

According to previous research on follicle survival, stress from mechanical separation, a high concentration of cryoprotectants, and abrupt temperature changes brought on by freezing are some of the main reasons of follicle mortality (23). In the present study, the survival rate of the follicles in both fresh and cryopreservation groups treated with ovarian parenchyma was higher than in the control group. However, statistically, G2 and G4 groups showed better survival rates than the control and G5 groups, in comparison to other studies conducted in the field of co-culturing with fibroblasts and enzyme-derived ovarian cells (11, 15). In a study conducted by Jamalzaei et al. (13, 16), the effects of different alginate concentrations along with enzyme-derived ovarian cells on the growth of preantral follicles was investigated. Their result has shown that the presence of ovarian cells in the culture medium may affect the survival of follicles and positively affect their structure.

On day 12 of the culture, the follicles that formed the

complete antrum cavity were examined. The number of follicles that formed antrum cavities was higher in both G2 and G4 groups than in the control group, with no significant difference among the groups. Studies conducted on fibroblasts and mesenchymal cells extracted from menstrual blood, by Kim et al. (11) and Rajabi et al. (14) illustrated that utilization of stem cells in culture with preantral follicles improve survival as well as development of antrum cavities. While only one cell line has been utilized in earlier investigations, all of the ovarian cortex cells were used in this study, and cell-cell interaction can be maintained to a large extent in this method. It has been proven that using FGFs, along with one cell line in the ovarian parenchymal tissue, are influential in the growth of follicles.

The transforming growth factor beta (TGF-β) superfamily has received additional attention due to its crucial role in the process of folliculogenesis, growth, and development of both follicles and oocytes. In the present study, the expression levels of its family members, including *Gdf9*, *Bmp15*, and *Bmp6* genes were determined to evaluate the growth and differentiation of the follicles. Many studies have been conducted on the mechanisms of the ovaries and their role in growth and development of follicles and oocytes. Moreover, it has been suggested that the rate of early follicular growth is a reliable factor for predicting pregnancy potential for both natural and ART-mediated pregnancies, with slow initial growth of the follicles being an indicative of negative pregnancy results. Although numerous studies indicated *Gdf9* and *Bmp15*, as essential maturation genes that are specifically expressed in oocytes, (28), but, in a previous study, Chen et al. (29) demonstrated that *Bmp15* is expressed in human cumulus cells in addition to oocytes. These two genes are also expressed in all follicular stages except the early one and play an important role in the proliferation of granulosa cells and subsequent growth of follicles, especially in the early stages of their development (30-32). *Bmp6* is other gene that expressed in the early stage of follicle development, different stages of oocyte development and granulosa cells (33, 34). Cook-Andersen et al. (35) and de-Castro et al. (28), studies on mice ovary illustrated that *Gdf9* stimulates the growth of primary follicles. Dong et al. (36) showed that follicles in mice with *Gdf9* mutations, did not grow after the primary stage. Therefore, the expression of *Gdf9* is essential for folliculogenesis and the fertility process and involved in the mitosis and steroidogenesis of granulosa cells and the proliferation of cumulus cells (37, 38).

So far, many studies have been performed on the structure, biological function, expression pattern, and how growth factors may affect the process of folliculogenesis. In the present study, the expression levels of *Gdf9*, *Bmp15*, and *Bmp6* genes showed a relatively similar pattern both after 3 hours and the fourth day of culture. In a study conducted by Jamalzaei et al. (13) on fresh and vitrified follicles, the highest expression of these genes was seen in frozen follicles 3 hours after culture; however, it decreased as the final days of the culture approached. These results showed that high mRNA levels are produced in the oocyte at the early stages of growth, but they gradually decreased as the growth continued. In the present study, the expression levels of the three growth factor genes were significantly higher in the groups with vitrified follicles compared to the other groups. This could indicate the protective effect of co-culture of preantral follicles with ovarian parenchyma cell suspension. As it was mentioned in previous studies, gene expression was specifically higher in the first 3 hours of culture and significantly decreased by the fourth day of the culture, with similar functions found in other studies.

Conclusion

Overall, this study provided evidence for the advantages of using ovarian parenchyma tissue for preantral follicle growth and development. The primary purpose of this research was to try to bring suitable *in vivo* environment for the effective growth of the follicles through a co-culture system that permits mice preantral follicles to develop into a 2D culture system. The co-culture of ovarian parenchyma cells with preantral follicles improved the growth, survival, and maturation rates of the follicles. In addition, higher expression of maturity genes in cryopreserved follicles indicates the compensation role and protective effects of ovarian parenchyma on the cryopreservation process. We demonstrated that parenchyma tissue could be used as a supportive tool for the growth and maturation of preantral follicles. This result may help clinical applications of the process in ART to improve fertility preservation and IVC-IVM approaches.

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Authors' Contributions

J.N.S.; Writing-Original Draft, Review & Editing, Investigation, Resources, Methodology, Conceptualization, Software, and analysis. H.E.; Project administration and Supervision. A.H.Sh., M.T.; Validation and Methodology. R.F., L.S.T.; Conceptualization and Methodology. S.A.M.; Conceptualization-Resources - Review & Editing. S.M.J.T.-M.; Methodology and Investigation. All authors read and approved the final manuscript.

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Effectiveness of Autologous Platelet-Rich Plasma Therapy in Women with Repeated Implantation Failure: A Randomized Clinical Trial

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Abstract

Background: Platelet-rich plasma (PRP) therapy has been shown to enhance tissue regeneration by expressing several cytokines and growth factors (GFs). This study investigated the effect of intrauterine infusion of PRP as a non-invasive autologous GF on pregnancy outcomes in women with repeated implantation failure.

Materials and Methods: This randomized clinical trial was conducted to compare the pregnancy rates between two groups of women who were candidates for the frozen-thawed embryo transfer with a history of two or more implantation failures. The PRP group (n=33) was treated with hormone replacement therapy+0.5 cc to 1 cc PRP infused into the uterine cavity two days before the embryo transfer. The control group (n=33) was only treated with hormone replacement therapy. The endometrial preparation process was done similarly in both groups. The chemical, clinical, and ongoing pregnancy, and implantation rates were compared between the two groups.

Results: Our results showed that the chemical pregnancy rate was not statistically higher in the PRP group in comparison with the control group (36.4 vs. 24.2%). In addition, the clinical pregnancy, ongoing pregnancy, and implantation rates were higher in the PRP group than the control group; however, the difference between the two groups was not statistically significant.

Conclusion: Administration of intrauterine PRP before embryo transfer in women with repeated implantation failure (RIF) does not affect assisted reproductive technology (ART) outcomes (registration number: IRCT2016090728950N3).

Keywords: Embryo Implantation, Platelet-Rich Plasma, Pregnancy Outcome

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Introduction

Despite many advances in the treatment of infertile couples in recent years, some couples still suffer from repeated implantation failure (RIF). RIF refers to the occurrence of unsuccessful embryo transfers after undergoing two to six cycles of *in vitro* fertilization (IVF). Many different factors, such as embryo quality, endometrial receptivity, and immunological factors, can be involved in this situation. Several methods have been proposed to manage RIF, including blastocyst transfer, assisted hatching, hysteroscopy, endometrial scratching, and immune therapy. However, a technique with the most impact is still discussed (1).

Previous studies have examined the immune system's role in the recurrent reproductive failure. Most of them focused on the role of peripheral blood markers more than the uterus environment. They showed the active role of local immune cells at the implantation site in the endometrial receptivity (2, 3). Our previous study showed that a dose of 0.5 cc (300 µg/ml) intrauterine injection of granulocyte colony-stimulating factor (GCSF) by the use of an IUI catheter immediately after ovarian puncture improved the pregnancy rate in women with a RIF history (4). While, the GCSF is naturally produced in the reproductive system and exacerbates the proliferation and differentiation of neutrophilic granulocytes, which acts on decidual cells macrophages and ultimately affects the implantation rate (5, 6). In addition, some studies showed differences in the effect of platelet-rich plasma (PRP) in women with thin endometrium undergoing IVF or intracytoplasmic injection (ICSI) with frozen embryo transfer cycles (7-11). Other studies have suggested PRP treatment as a low-cost and non-invasive approach that allows natural concentrations of autologous growth factors (GFs). This

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treatment has been widely tested in various medical fields to examine its potential for improving tissue regeneration (12, 13). PRP is defined as an autologous blood plasma fraction with a platelet concentration of about four-five times higher than the baseline. Intra-platelet chemical mediators, through the promotion of angiogenesis and the onset of cellular regeneration by mitotic mediators of mesenchymal cells, trigger the onset of regeneration (14).

PRP is prepared from fresh whole blood, and stored in acid citrate dextrose solution A (ACD-A) anticoagulant. After separating the various blood components, platelet concentrations are increased, and finally, by activating the platelets, the cytokines and GFs become bioactive and released 10 min after clotting (15). PRP can improve the regeneration of various tissues by expressing several cytokines and GFs. Studies have investigated the role of intrauterine injection of autologous PRP in suboptimal endometrium and have shown that PRP can improve embryo transfer (ET) and vascularization by releasing cytokines and GFs such as vascular endothelial GF, transforming GF, platelet-derived GF, and epidermal GF. These factors regulate cell migration, proliferation, and differentiation while increasing extracellular matrix accumulation (16).

The present study was designed to investigate the effect of intrauterine infusion of PRP as a non-invasive autologous GF on the pregnancy outcomes of women with a RIF history.

Materials and Methods

The study protocol was approved by the Yazd Research and Clinical Center for Infertility Ethics Committee, Yazd, Iran (IR.SSU.RSI.REC.1395.16) and the Iranian Registry of Clinical Trial (IRCT) (IRCT2016090728950N3). In addition, written informed consent was obtained from all couples before participating in this study. The present study was conducted at the Yazd Reproductive Sciences Institute, Yazd, Iran.

Primary outcomes: chemical and clinical pregnancy. Secondary outcomes: ongoing pregnancy and implantation rate.

Sample size

The sample size was estimated to be a minimum of 72 (36 in each group) by considering the significance level of 95%, the power of 80%, ongoing pregnancy of our center, and considering a 30% difference based on the study by Chang et al. (15), and used the following formula:

$$n = \frac{(Z_{1-\alpha/2} - Z_{1-\beta})^2 \times [P_1(1-P1) + (P_2(1-P2)]}{(P1-P2)^2}$$

In this randomized clinical trial, women aged 18-42 years with a history of two or more implantation failures candidates for a frozen-thawed embryo (s) transfer who were referred to the Research and Clinical Center for Infertility, Yazd Reproductive Sciences Institute, Yazd, Iran, between September 2016 and January 2017 were enrolled. The participants were randomized into two groups (PRP and control) with the use of a computer-generated randomization list. All women with immunological, hormonal, or hematological disorders, such as thrombocytopenia (platelet count<1050/ul) or Hb<10 g/dl, as well as congenital abnormalities and uterine abnormalities (congenital or acquired) were excluded. To conceal allocation, the interventions were sealed in serially numbered, opaque envelopes of equal appearance and weight and then distributed to participants.

All participants underwent hormone replacement therapy (HRT) for endometrial preparation, as described below. Each woman was orally prescribed 6 mg/day of Estradiol Valerate (Tablet 2 mg, Aburaihan Co., Tehran, Iran) from the second day of the menstrual cycle. From the 13th day of the menstrual cycle, the periodic vaginal ultrasonography was done to measure the endometrial thickness by an infertility fellowship. When the endometrial thickness reached 8 mm, 400 mg vaginal progesterone (Cyclogest; Actavis, UK Limited, England) was administered twice daily. Estradiol and progesterone were administrated in all women until two weeks after embryo transfer. In the case of positive chemical pregnancy, HRT continued until the 10th week of gestational age.

In the PRP group, 0.5-1cc of PRP was infused into the uterine cavity two days before embryo transfer. The control group was only treated with HRT. For PRP preparation, 8.5 ml of peripheral venous blood was taken into a syringe containing 1.5 ml of anticoagulant acid citrate A (ACD-A) solution (Aria Mobna kit, Iran) and centrifuged at 1600 g for 10 minutes at ambient temperature. After that, the plasma layer and buffy coat were transferred into another tube and centrifuged again at 3500 g for 5 minutes to obtain 1.5 ml PRP with a concentration of four to five times the platelet compared to expect. Then, 0.5-1 cc of PRP was infused into the uterine cavity in the PRP group, and the participants were asked to remain lying down for 10 minutes.

In all participants, three days after the progesterone administration, 1-2 cleavage embryos were transferred one day after thawing using a Labotect catheter (Labotect, Gotting, Germany). The serum beta human chorionic gonadotropin (βhCG)>50 IU/L, 12 days after embryo transfer, was considered a positive chemical pregnancy. The observation of fetal heart activity two weeks after the positive βhCG was considered a positive clinical pregnancy. Ongoing pregnancy was defined as a continuation of pregnancy after the 12th week of gestation, and the implantation rate as the number of gestational sacs per 100 embryos transferred.

Statistical analysis

Data were analyzed using the SPSS software (version 20.0, SPSS Inc., Chicago, Illinois, USA). The Chi-Square ($\chi 2$), One-way ANOVA, and Student's t tests were used to evaluate the relations between variables. $P<0.05$ was considered statistically significant.

Results

Initially, 72 women were eligible to enter the study. Six women were excluded due to dissatisfaction to continue participating in the study. Finally, 66 women with RIF history participated in this study and were randomized into two equal groups: the PRP group and the control group (Fig.1). No significant differences were observed between the two groups in age, etiology of infertility, and embryo transfer quality (Table 1).

Table 1: Demographic characteristics of participants in two study groups

Variables	PRP group (n = 33)	Control group (n = 33)	P value
Age (Y)	32.48 ± 4.95	33.45 ± 3.45	0.36*
Number of embryo transfer	1.96 ± 0.58	2.18 ± 0.46	0.10*
Etiology of infertility			0.66**
PCOS	6 (18.2)	7 (21.2)	
Male factor	6 (18.2)	8 (24.2)	
Unexplained	6 (18.2)	7 (21.2)	
Tubal factor	(12.1)	4 (12.1)	
Endometriosis	3 (9.1)	0 (0)	
Ovarian failure	3 (9.1)	4 (12.1)	
Combined	5 (15.2)	3 (9.1)	
Endometrial thickness (mm)	8.92 ± 1.00	9.25 ± 1.16	0.21*
Quality of embryo transfer			0.74**
A	5 (15.2)	6 (18.2)	
B	28 (84.8)	27 (81.8)	

PRP; Platelet-rich plasma, PCOS; Polycystic ovarian syndrome, *; Students' t test, and **; Chi-square test.

Our results showed that the rate of chemical pregnancy was higher in the PRP group than the control group (36.4 vs. 24.2%), but the difference was not significant (P=0.28). In addition, the rates of clinical pregnancy, ongoing pregnancy, and implantation were also non significantly higher in the PRP group than the control group (Table 2).

Table 2: Comparison of ART outcomes between the two study groups

ART outcomes	PRP group (n=33)	Control group (n=33)	P value
Chemical pregnancy rate	12 (36.4)	8 (24.2)	0.28
Clinical pregnancy rate	11 (33.3)	8 (24.2)	0.41
Ongoing pregnancy rate	8 (24.2)	6 (18.2)	0.54
Implantation rate (%)	8/65 (12.30)	12/72 (16.66)	0.47

All data presented as n (%). Chi-square test. PRP; Platelet-rich plasma and ART; Assisted reproductive technology.

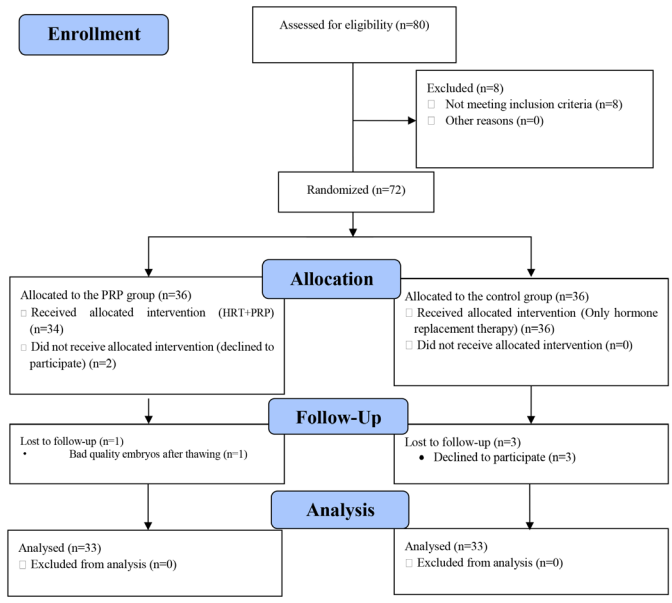

Fig.1: The study CONSORT flow diagram. PRP; Platelet-rich plasma, HRT; Hormone replacement therapy.

Discussion

In this study, we evaluated the endometrial receptivity after PRP administration in the RIF women according to the hypothesis that the local infusion of PRP due to the several GFs and cytokines may improve endometrial receptivity and implantation rate in the women with a history of RIF. Our present findings did not suggest that the PRP therapy is clinically effective for women receiving frozen-thawed embryo (s) transfer.

Tehraninejad et al. (17), in a study including 85 women with RIF and normal endometrial thickness, showed that the PRP is not a good option for treatment of women with a RIF history and normal endometrial thickness candidates for embryo transfer; similar to this study endometrial thickness was normal (more than 7 mm) in all of our participants. PRP was more effective in improving embryo transfer cycles in women with thin endometrium in our previous study. We showed PRP improved the endometrial thickness and pregnancy rates in frozen -thawed embryo (s) transfer with a thin endometrium (7)

In contrast to our findings, the results of a meta-analysis showed that an intrauterine administration of PRP increases the clinical pregnancy rate in women with a frozen-thawed embryo transfer. They introduced PRP as an alternative treatment strategy in patients with thin endometrium and RIF history (18).

The role of autologous PRP injection in the suboptimal endometrium has been evaluated in several studies. It is estimated that PRP, through the release of cytokines and GFs, the regulation of cell migration, adhesion, proliferation, and differentiation, as well as the enhancement of extracellular matrix accumulation, may effectively improve endometrial transfer and vascularity (16).

There are a few studies that reported the positive effect of PRP on the endometrial growth and pregnancy outcomes (15, 19, 20). Chang et al. (15) undertook for the first time a pilot study of PRP in 5 patients with thin endometrium. 48-72 hours after PRP infusion, they observed an increase of endometrial thickness in all the patients (>7 mm on the progesterone administration day), and all of them were pregnant. Garcia-Velasco et al. (21) reported that the use of autologous PRP improved the endometrial transfer in women with refractory endometrium. Also, we have previously evaluated the effect of an intrauterine administration of PRP in women with thin endometrium during a frozen embryo transfer cycle We observed that the of 0.5-1 cc PRP was associated with a significantly higher rate of implantation and clinical pregnancy rates in a 83 women (7).

Farimani et al. (22) reported a positive pregnancy in a 45-year-old woman with a primary infertility history after two IVF cycles failure candidate of the donor eggs. She was treated with an intrauterine injection of autologous PRP 24 hours before embryo transfer. Evaluating the effect of an intrauterine administration of PRP on the pregnancy rate, Nazari et al. (23) reported 90% pregnancy rate in a study of 20 women with a history of RIF, also 16 of them had clinical and ongoing pregnancies at the time of the study. They concluded that the use of PRP effectively improved pregnancy outcomes in RIF patients, that was inconsistent with Obidniak et al. (24) study. They concluded that this method should be considered perspective, safe, and cost-effective in treating these patients. Also, our previous study showed that intrauterine injection of 0.5 cc of GCSF before embryo transfer improved pregnancy outcomes in patients with a history of implantation failure (4). GCSF, as a hematopoietic lineage-specific cytokine, is naturally synthesized in the reproductive system and stimulates the proliferation and differentiation of hematopoietic cells of the neutrophilic granulocyte lineage, which affects the macrophages of the decidual cells, ultimately resulting in implantation (5, 6).

Further studies are recommended regarding the population under investigation, the time frame evaluated, and the comparative studies approach between drugs and autologous preparations to envisage effective therapeutic alternatives for RIF.

Conclusion

Administration of intrauterine PRP before embryo transfer in women with recurrent implantation failure does not affect asssisted reproductive technology (ART) outcomes.

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Authors' Contributions

M.E.; Study conception and Design. M.E., N.N., P.Kh.; Conducted the procedures and Data analysis. Further, all authors participated in the review of the literature and drafting the manuscript, approved the final version of the manuscript, and take responsibility for the integrity of the data.

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Fertility Rate and Sperm DNA Fragmentation Index following Varicocelectomy in Primary Infertile Men with Clinical Varicocele: A Prospective Longitudinal Study

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Abstract

Background: Varicocele is one of the most common treatable causes of male infertility, and its treatment may be beneficial for fertility. This study aimed to evaluate fertility rate and DNA fragmentation index (DFI) following varicocelectomy in primary infertile men with clinical varicocele.

Materials and Methods: This prospective longitudinal study was conducted on primary infertility men, in a tertiary center from December 2018 to December 2019 with one-year follow-up. Data of the semen parameters, DFI (%), and fertility rate were gathered before, as well as 4 and 12 months after undergoing varicocelectomy. For data analysis, SPSS software and analytical test were used.

Results: Out of 76 patients who were analyzed, 22 (29%) became fertile and 54 (71%) remained infertile. Semen parameters and DFI (%) were improved significantly following varicocelectomy (P<0.001). Smoking history, occupational heated exposure, body mass index (BMI), and infertility duration were determined as predictors associated with fertility status (P<0.05).

Conclusion: Although varicocele repair improved the DFI, the fertility rate was achieved in less than one-third of patients; it seems that the other parameters, such as the history of smoking, occupational heated exposure, overweight, and duration of infertility should be considered as predictors of fertility status, in primary infertile men who are a candidate for varicocelectomy.

Keywords: DNA Fragmentation, Infertility, Semen Analysis, Varicocele, Varicocelecto

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Introduction

Infertility is defined as an incapability to conceive after one year of regular sexual intercourse without using contraceptive utilities (1). It has been estimated that more than 180 million people suffer from infertility worldwide, most of them (about 50%) due to the male factor (2). Although the cause of infertility in men remains unknown in about 50% of people on early assessment (3, 4), the previous studies demonstrated that sperm DNA fragmentation (SDF) has an important role in etiology of this abnormality (5, 6). On the other hand, male infertility is significantly associated with excess reactive oxygen species (ROS) in semen (5), and SDF is a type of ROS-related damage that is mostly observed in infertile men's sperm (7).

Varicocele is defined as an abnormal enlargement pampiniform plexus veins. It is associated in about 15% of all adult men, 35% of people with primary, and more than 80% of secondary infertilities (8). This disease is associated with infertility in men by several non-exclusive mechanisms, such as increased ROS production which can cause deoxyribonucleic acid damage to sperm DNA (9). Excessive amounts of ROS accompanied by antioxidant deficiency can cause oxidative stress (OS), which can lead to sperm nuclear and mitochondrial DNA damages (5). This mechanism causes point mutations, chromosome deletion, chromosomal rearrangements, and breaks in sperm DNA (10). Excessive sperm DNA break, which is called SDF, mostly appears in the semen

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of individuals with varicocele and other fertility problems (11). Previous studies also demonstrated a higher rate of SDF in the semen of patients with varicocele and varicocele correlation with ruined sperm DNA integrity. It was shown that varicocelectomy notably increased sperm DNA quality (12, 13). Another study demonstrated that varicocelectomy resulted in better sperm parameters, regardless of its techniques type (12). Sperm DNA fragmentation index (DFI) is a key parameter in fertility indicating both sperm DNA integrity and injury (14). Previous studies demonstrated DFI as an indication for the varicocele surgical treatment. Most of these studies focused on infertility without distinguishing between primary and secondary types of varicocele. So, this study aimed to evaluate fertility rate and DFI following varicocelectomy in primary infertile men with clinical varicocele.

Materials and Methods

Study design and participants

This prospective longitudinal study was conducted on primary infertility men in a tertiary center, from December 2018 to December 2019 with one-year follow-up. Primary infertile men with clinical varicocele and abnormal sperm parameters, in two semen samples with three weeks intervals undergoing varicocelectomy, were selected by consecutive sampling. The inclusion criteria were abnormal sperm parameters, any grade of clinical varicocele, younger than 35 years old, and lack of infertility factors in men's sexual partners. Exclusion criteria included serum high level of follicle-stimulating hormone (FSH; more than 12.3 mIU/ml) (15), testicular atrophy due to the other causes such as trauma or previous surgery, and secondary infertility. Additionally, patients with a history of epididymitis, cryptorchidism, orchitis, previous inguinal surgery, acute urinary tract infections, leukospermia, any disease or malignancy which may affect spermatogenesis, and history of underlying disease and medication affecting spermatogenesis were excluded.

Patient management

According to the routine protocol, before the surgery, all patients were examined by a single urologist who conducted the varicocelectomy (Inguinal technique by conventional approach). Varicocele grading was determined according to the WHO criteria and Sarteschi criteria by urologist examination and color Doppler ultrasonography (CDU) (16). The semen and SDF level tests were done, and semen parameters and percentage of DFI were determined. The patient partners were also visited by a gynecologist, and they had normal fertility potential. All data were recorded in patients' clinical documents. Only fertile men with normal fertility potential spouses were included in the study. Patients were monitored during the 4th and 12th months after surgery. During this time, their semen was tested, and their DFI (%) and their partner's pregnancy testing were checked and recorded in checklists.

Demographic data

Demographic characteristics, including patient's age, partner's age, duration of the marriage and duration of primary infertility, as well as histories of smoking and occupational exposure were recorded into checklists using clinical patients' documents.

Hormone analysis

Serum FSH levels were evaluated in the all patients by blood sample early morning (before 10 a.m), and those with high FSH levels (>12.3 mIU/ml) were excluded (15).

Semen analysis

The semen analysis test was conducted within two to five days of abstinence before the varicocelectomy and again within four to 12 months after the surgery. The test was performed to evaluate semen parameters of the patients, including sperm count, motility, morphology, and DFI. Standard semen analysis was conducted within one hour after sample collection and semen parameters including semen volume, density, sperm morphology, total motility and progressive motility were compared to the normal levels according to World Health Organization (WHO) 2010 classification. Next, they were recorded in the checklists. According to this protocol, semen parameters must be in 1.5 ml volume, 15 million concentrations, 58% vitality, 32% progressive, 40% total motility, and 4% normal morphology (17).

DNA fragmentation index measurements

A semen sample was taken for the SDF measurement, like semen analysis and it was analyzed by sperm chromatin structure assay (SCSA) method. Abnormal sperm percentage (abnormal chromatin structure) was expressed as DFI (%), indicating ratio of single-stranded (denatured) DNA to the total DNA (18).

Fertility outcome

The occurrence of pregnancy during the 12 months follow-up was assessed by taking a detailed history of the patients and monitoring onset of pregnancy in the patients with delayed menstruation. The onset of pregnancy was monitored by measuring the beta-human chorionic gonadotropin (beta-hCG) levels in blood.

Data analysis

The data were analyzed using version 24 of SPSS (IBM Corp., USA) software. The results for quantitative data are reported as mean ± standard deviation (SD) for data with normally distribution or median (IQR) or as frequency (%) for not normally and qualitative data. The Shipro-wilk, Skewness and Kurtosis tests were used to verify the normality distribution. Chi-square, independent samples t test, and Mann-Whitney test were used to compare the

variable between fertile and infertile patients. Analytical tests, such as repeated measure ANOVA, Friedman, and LSD post hoc tests, were used to compare DFI (%) and semen parameters at times before, four months, and one year after surgery. Univariate and multivariate regression tests were used to explore correlation between fertility outcomes and related factors. The significance level was considered 0.05, and the two-tailed assay examined the significance.

Ethical considerations

The research followed the tenets of the Declaration of Helsinki. This study was carried out under the authority of the Guilan University of Medical Sciences, Ethical Research Committee (IR.GUMS.REC.1398.486). Accordingly, written informed consent was taken from all participants before any intervention. Besides, the authors ultimately observed ethical issues (including plagiarism, data fabrication, and double publication).

Results

Of the 83 cases, seven patients (8.5%) were excluded due to not completing the follow-up duration, and 76 (91.5%) were included in the final analysis. Out of 76 patients who were analyzed, 22 (29%) became fertile and 54 (71%) remained infertile. Demographic and clinical characteristics of the patients by fertility status (fertile/infertile) are summarized in Table 1.

Table 1: Demographic and clinical characteristics of the studied patients with fertility status

Variable	Sub-variable (n)	Fertility status		P value
		Fertile	Infertile	
Smoking history	No (33)	16 (48.5)	17 (51.5)	0.001*
	Yes (43)	6 (14)	37 (86)	
Occupational heated exposure	No (40)	17 (42.5)	23 (57.5)	0.006*
	Yes (36)	5 (13.9)	31 (86.1)	
Varicocele grade	Grade 1 (6)	1 (16.7)	5 (83.3)	0.426*
	Grade 2 (37)	9 (24.3)	28 (75.7)	
	Grade 1 (33)	12 (36.4)	21 (63.6)	
Patient age (Y)		34.63 ± 5.23	33.9 ± 4.83	0.582**
Spouse age (Y)		30.95 ± 5.84	30.33 ± 4.88	0.641**
BMI (kg/m^2)		23.18 ± 1.75	25.12 ± 2.17	<0.001**
Infertility duration (Y)		2.43 ± 1.10	3.90 ± 2.49	0.003***

Data are presented as n (%) and mean ± SD. BMI; Body mass index, n; Frequency, SD; Standard deviation, *; Chi-square, **; Independent samples t test, and *; Mann-Whitney**

Assessment of the correlation between demographic and clinical characteristics with fertility status showed that there was no statistical significance between varicocele grade, patient's age, and spouse's age with fertility status, while regarding the smoking history, occupational exposure history, body mass index (BMI), and infertility duration was significant (Table 1). When we adjusted variables, the results showed that all of the studied variables in multivariate regression analyses, smoking history, occupational heated exposure history, BMI, and infertility duration were determined as predictors associated with fertility status. Infertility duration with an odds ratio of 2.3 per one year increasing age and BMI with an odds ratio of 1.62 kg/m^2 increased the risk of infertility. Occupational exposure with an odds ratio of 4.7 compared to the patients without occupational exposure, and smoking compared to non-smoking patients with an odds ratio of 4.41 fold increased risk of infertility (Table 2).

Table 2: Determination of predictors associated with fertility status by using multivariate regression models

Variable	OR	Standard error	P value	95% CI	
				Lower	Upper
Smoking history	4.41	0.719	0.038	1.086	18.160
Occupational heated exposure	4.70	0.740	0.036	1.103	20.052
Infertility duration	2.30	0.307	0.006	1.265	4.214
BMI	1.62	0.179	0.007	1.145	2.312

OR; Odds ratio, CI; Confidence interval, and BMI; Body mass index.

In this study, we used DFI (%) to determine sperm DFI, following varicocelectomy at different times, such as before, four, and 12 months (s) after varicocelectomy. Results demonstrated that the percentage of DFI during 12 months of follow-up was significantly reduced. After varicocelectomy, there was a statistically significant reduction in the DFI at 12 months compared to the 4 months after the procedure as well as before it. Moreover, the reduction of DFI at 4 months after compared to the before varicocelectomy was significant (Table 3).

As indicated in Figure 1, the percentage of DFI during the 12 months follow-up after varicocelectomy was reduced.

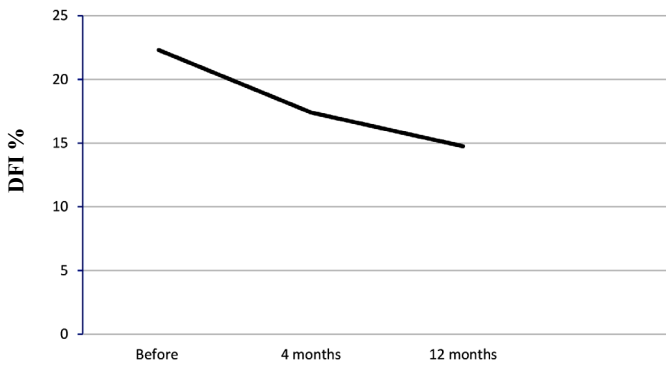

Fig.1: Comparison of DFI percentage during 12 months' follow-up after varicocelectomy. DFI; DNA fragmentation index.

The results of semen analysis, following the varicocelectomy, showed that the difference in normal morphology and total motility percentage during 12 months follow-up (before, four and 12 months after varicocelectomy) was not significant; while in terms of progressive motility percentage, sperm concentration and semen volume, it was significant. As time passed, progressive motility and sperm concentration were significantly increased. However, the semen volume was decreased after four months and then increased after 12 months (Table 4).

Table 3: Comparison of DFI (%), before, four and 12 months after varicocelectomy

Time	Before (mean ± SD)		4 months (mean ± SD)		12 months (mean ± SD)	P value
DFI (%)	22.31 ± 0.99		17.41 ± 0.79		14.76 ± 0.70	<0.001*
First time	Second time	Mean difference	Std. Error	P value	95% CI (lower)	95% CI (upper)
4 months	Before	- 4.91	0.831	<0.001**	- 6.565	- 3.256
12 months	Before	- 7.58	0.895	<0.001**	- 9.372	- 5.805
	4 months	- 2.67	0.609	<0.001**	- 3.891	- 1.464

DFI; DNA fragmentation index, CI; Confidence interval, *; Repeated measurement ANOVA, **; LSD post hoc test, and Std. Error; Standard error.

Table 4: Semen analysis, following varicocelectomy, during 12 months follow-up

Semen parameter	Before	4 months	12 months	P value
Semen volume (ml)	3.27 ± 1.48	2.88 ± 1.04	3.20 ± 1.35	0.025*
Sperm concentration (15×10^6 sperm/ml)	38.05 ± 22.42	45.82 ± 25.62	56.72 ± 27.28	<0.001**
Sperm morphology (%)	33.15 ± 34.87	38.97 ± 35.88	41.77 ± 33.94	0.096*
Total sperm motility (%)	53.48 ± 19.73	53.78 ± 20.79	55.95 ± 17.23	0.473*
Progressive sperm motility (%)	31.78 ± 14.54	39.29 ± 16.45	42.62 ± 17.42	<0.001*

Data are presents as mean ± SD. *; Friedman and **; Repeated measurement ANOVA.

Discussion

Varicocele is one of the known causes of infertility and the most common treatable disorder in infertile men (19). This treatable disease is found in about 40% and 80% of primary and secondary infertile men, respectively (20). Recent studies proposed association of elevated SDF levels with infertility in patients with varicocele (21). Additionally, the beneficial effect of varicocelectomy was found in patients with impaired sperm and clinical varicocele in the previous studies (20). In this study, during 12 months follow-up, fertility was achieved in 29% of 76 patients who underwent varicocelectomy. Previous studies reported different fertility rate, due to varicocelectomy. In a study performed by Sajadi et al. (22), 12.5% fertility rate was reported after varicocelectomy. Schlegel and Kaufmann (23), in a study on men with non-obstructive azoospermia, indicated that varicocelectomy resulted in 22% fertility. A rate of 31% fertility, following varicocelectomy, was achieved in a retrospective study on primary infertile men, by Ilktac et al. (24). Another study performed on primary infertile men by Mohamed al. (25) reported a fertility rate of 42.1%. It seems that, one of the most important mechanisms through which varicocelectomy can improve the fertility rate, is to increase perfusion of the testicular vessels and decrease temperature of the vessels, to remove the vessels that are the place of accumulation of venous blood with high temperature.

In this study, the fertility rate after varicocele treatment was achieved in less than one-third of the patients. Results showed that smoking, occupational exposure, BMI and infertility duration were the most predictors associated with infertility; however, the patient's age, spouses' age, and varicocele grade were not predictors. Consistent with our study Ren et al. observed a relationship between duration of infertility and incidence of pregnancy after varicocelectomy (26), and Bolat et al. (17) found no correlation between age and fertility rate. Studies also showed that smoking and presence of varicocele can cause more significant effect on semen analysis parameters and ROS via a synergistic effect (27). In contrast with our study, Choe and Seo (28) indicated that smoking was not associate with fertility status. Although in this study smoking had a negative effect on fertility, if duration of smoking and number of cigarettes smoked per day is evaluated, it may obtain more reliable results. On the other hand, low success rate in this study can be due to a shorter follow-up period, occupational exposure, high BMI or the surgical technique as with most of the previous studies.

Our results demonstrated that DFI during 12 months follow-up after varicocelectomy was significantly reduced. Furthermore, semen parameters were improved, following varicocelectomy, in some parameters, such as semen volume, progressive sperm motility and sperm concentration. This result was in line with the meta-analysis study performed by Birowo et al. (18), reporting that varicocele treatment reduced DFI, and this index can be an indication for varicocelectomy. In addition, they indicated that varicocele treatment can be beneficial in improving of sperm concentration, sperm progressive motility, and sperm morphology. Mohamed et al. (25) in their study found that semen parameters were improved after varicocele treatment in the both primary and secondary infertile men. Zampieri et al. (29) deduced that varicocelectomy improved rate of semen parameters. The other previous studies also showed similar outcomes, following varicocelectomy, on improving semen parameters (30). Alhathal et al. (31) demonstrated improvement of DFI and sperm progressive motility after varicocelectomy. Improvement of semen parameters and DFI, following varicocelectomy, in some other previous studies were approved (32-35).

The strengths of this study were the prospective, referral

center, and large sample size. Furthermore, a 12 months follow-up of semen parameters and SDF levels as well as a maximum follow-up of 12 months of pregnancy were performed, which were higher than similar studies. Precise registration of demographic information, history of smoking, BMI and occupational exposure history of the all participants were the other strengths of this study compared to the previous studies. However, there are some limitations in this study which should be noted. Firstly, Doppler Sonography was not performed after surgery to ensure the success of the procedure. Secondly, semen analysis was not performed in a common laboratory for the all patients, despite performing SDF assessment in a single laboratory. Thirdly, failure to use microscopic inguinal varicocelectomy as a gold standard surgical procedure for varicocele, due to limited equipment and facilities. Fourthly, lack of semen sample analysis with proper time in some patients. Finally, failure to study alcohol use, long-term use of mobile phones, as well as opium and drugs as factors affecting pregnancy and semen analysis parameters.

Conclusion

Although varicocele treatment improved DFI, the fertility rate was achieved in less than one-third of patients. It seems that the other parameters, such as the history of smoking, occupational exposure, overweight, and duration of infertility should be considered as predictors of fertility status, in primary infertile men who are a candidate for varicocelectomy.

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Authors' Contributions

A.H.M., Gh.M.; M.H.M., A.F.; Designed and directed the project, Data and statistical analysis, and Interpretation of data. E.K., N.R.H., M.P., R.R.; Contributed to sample preparation, Performed the experiments, and Wrote the paper. All authors read and approved the final manuscript.

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Cryopreservation of Limited Sperm Using A Combination of Sucrose and Taurine, Loaded on Two Different Devices, and Thawed at Two Different Temperatures

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Abstract

Background: Cryopreservation of sperm is essential for patients with low sperm counts and couples undergoing infertility treatment. The aim of this study was to compare the effects of Taurine (T) and Sucrose (S) in individual sperm cryopreservation utilizing cryotop and petri dish and thawing at 37 and 42°C.

Materials and Methods: In this experimental study, 17 normospermic semen samples were processed using the "Swim-up" procedure and progressively motile sperm were then isolated from these samples using an inverted microscope. Sperm were added to droplets of "sucrose medium" with 25 mM Taurine antioxidant (S+T) and the commercial cryoprotectant "Sperm Freeze" (CPA), loaded on a petri dish and cryotop. After rapid freezing of the samples, they were thawed at two different temperatures (37°C and 42°C), and the sperm classical parameters, viability, and DNA fragmentation were assessed.

Results: Statistical analysis displayed a significant increase in total and progressive motility in individual sperm freezing on cryotop with CPA and thawing at 42°C ($P<0.05$). Other parameters did not show any differences between the CPA and S+T groups and two thawing temperatures in either of the cryopreservation methods.

Conclusion: Although, both cryoprotectants (CPA and S+T) may preserve individual sperm effectively using cryotop, the CPA and thawing at 42°C showed a better effect on the motility percentage of the small number of sperm.

Keywords: Antioxidant, Cryotop, Sperm Cryopreservation, Taurine

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Introduction

Following the introduction of the sperm cryopreservation method in the 1960s, sperm cryopreservation approach has become an essential part of assisted reproductive technology (ART). These days, cryopreservation of sperms is essential in the treatment of infertile couples and in maintaining fertility in male cancer patients if sufficient sperm concentration is available (1). However, the traditional methods are associated with some significant challenges in scenarios where the sperm count is limited, such as in cases of azoospermia who undergo testicular sperm extraction (TESE) and severe oligospermia. The fact that the sperm sticks to the carrier vessel and demands centrifugation and thorough washing procedures, which may lower the sperm count when utilizing conventional sperm preservation techniques, makes the situation worse (2). Researchers and clinicians have considered carriers with small surface areas and high recovery ability, such as cryopiece (3), cell sleeper (4), cryoleaf (5), and culture dish (6), as potential solutions to the current problems. The cryotop is a carrier with a simple structure and easy handling that was used first time by Endo et al. (7) for the sperm freezing procedure. The cryotop carrier is the most up-to-date method for lowest volume vitrification. The vitrification technique by cryotop is intuitive to use. Anyone with basic knowledge of embryology may do it correctly after only a short amount of training. The procedure is straightforward and trustworthy, yielding the same outcomes with minimum variance among operators. After vitrification of human embryos and oocytes around the world, cryotop vitrification has led to the highest number of live births, and it is currently also successfully utilized in a number of areas of animal biotechnology (8).

Similar to traditional approaches, the cryopreservation of

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gametes poses significant challenges related to oxidative stress and osmotic damage caused by cryoprotectants (CPAs). In the case of sperm, which are a limited resource, the impact of such damage is particularly consequential. Various studies on the sperm cryopreservation have observed the utilization of antioxidants and the omission of cryoprotectants as two viable strategies (9-11). An essential natural antioxidant in the body, Taurine (T) is a sulfur-containing non-proteinogenic -amino acid that exhibits inhibitory effects on the reactive oxygen species (ROS) production and membrane stabilization via a redox balance maintenance linked to the control of different transcription factors (12). In recent years, there has been an increased consideration of the potential benefits of the T concerning male infertility and the mitigation of oxidative damage in the preservation of sperms (13). In this regard, previously we reported that the utilization of microdroplet and CPA-free method for the vitrification of a limited quantity sperm, coupled with the incorporation of T, has demonstrated the capacity to effectively safeguard the sperm parameters against cryo-damage, such as a reduction in motility, morphological changes, and the loss of acrosome integrity (14).

Moreover, the thawing rate is also an important factor in maintaining quality and motility of a sperm during the cryopreservation procedure. Traditionally, in most species, frozen sperm is thawed at 37°C (15). Mansilla et al. (16) showed that increasing the thawing temperature enhances the progressive motility of sperms following a vitrification of 5×10^6 sperms in a straw. They reported a significant difference in sperm motility at 38, 40, and 42°C after thawing. The motility of thawed sperm at 42 °C increased significantly in comparison with the two other temperatures.

As far as we know, there are no reports on the T utilization in the vitrification media for the limited sperm count. Nevertheless, in our previous study, the vitrification of sperm using microdroplets and T supplementation demonstrated the preservation of sperm parameters (14). Therefore, in the present study, we examined the effects of using a mixture of Sucrose (S) and T using two separate devices and two different thawing temperatures in the case of limited sperm cryopreservation.

Material and Methods

This experimental study was performed after the approval of the Royan Research Institute Ethics Committee, Tehran, Iran (IR.ACECR.ROYAN.REC.1395.112).

Semen sample collection

Seventeen semen samples according to previous studies (17) were collected after 3-4 days of sexual abstinence in a sterile container from men who had been referred to the Royan Institute (Tehran, Iran), with normal parameters according to World Health Organization (WHO) 2010 (18). All the experiments and stages used in this investigation are depicted in Figure 1. Following the semen liquefaction, macroscopic and microscopic examinations were performed. Using a Computer Assisted Sperm Analyzing system (CASA, SCATM motility module; Microptic, Version 4.2, Barcelona, Spain) with 10x magnification, the motility of sperms was determined. The Papanicolaou staining was used for assessment of sperm morphology at least 200 spermatozoa were checked under a light microscope 100x and immersion oil (18).

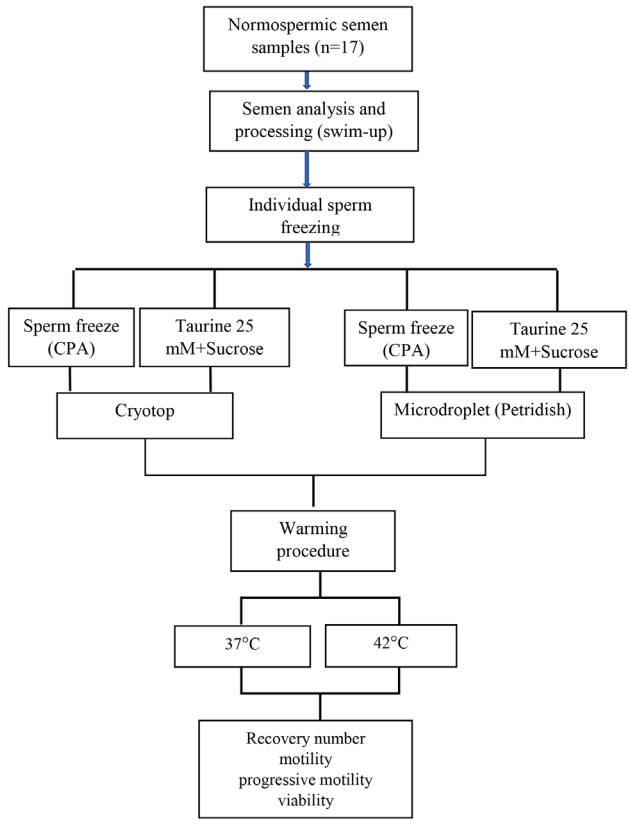

Fig.1: Flow chart of study design. CPA; Cryoprotectant.

Motile sperm isolation by swim-up method

The 1 ml of each semen sample was centrifuged at 3000 rpm for 5 minutes. The sediment was then removed and 1 ml of human tubal fluid (HTF) with 1% human serum albumin (HSA) (70024-90-7, Sigma-Aldrich, USA) that was made in the laboratory (Sodium Chloride 97.8 mM, Potassium Chloride 4.69 mM, Magnesium Sulfate 0.20 mM, Potassium Phosphate, Monobasic 0.37 mM, Calcium Chloride 2.04 mM, Sodium Bicarbonate 25.0 mM, Glucose 2.7 mM, Sodium Pyruvate 0.33 mM, Sodium Lactate 21.4 mM, Gentamicin 10 µg/mL, Phenol Red 5 mg/L) was added to the remaining and centrifuged for 3 minutes at 3000 rpm for the second time. Removing supernatant, 1.2 ml of HTF/HSA was added slowly to the pellet and then incubated for 30-45 minutes in a 5% CO_2 incubator at 37°C. After collection 600 µl of the medium above the pellet, concentration and motility of sperm were re-evaluated using the CASA software. Also, sperm morphology was examined using the Papanicolaou staining method. The swim-up sample was diluted at a 1:20 ratio with a HTF/HSA medium to

prepare for freezing procedure. Then, a limited number of sperm (3 to 10) were separated from the diluted medium by Pasteur pipettes (19). All experimental tests were performed individually on each semen sample, and the sample was divided into four experimental groups (CPA_{37}, CPA_{42}, $S+T_{37}$, $S+T_{42}$).

Cryopreservation and thawing of sperm by microdroplet method

After diluting the sample, a metal strainer was covered with a metal foil and placed in a foam box containing liquid nitrogen. We used two tools: a cryotop and a petri dish and two treatment groups were considered for freezing. The first group was frozen with the commercial cryoprotectant Sperm freeze media (CPA group), and the second group was frozen with a composition S (94474, Sigma, USA) and T antioxidant (107-35-7, Sigma, USA) (S+T). The S+T group was treated with a mixture of 90 µl HTF media+1% HSA, 90 µl of 0.2 M S and 20 µl of 50 mM T antioxidant (final concentration 25 mM) (14). The CPA group contained an equal ratio of the Sperm freeze (FertiPro Glycerol, HEPES, 4 g/Liter Human Serum Albumin, NV, Beernem, Belgium) and HTF.

After supplying the freezing medium, one µl of the frozen medium droplets was placed on the petri dish. A limited number of sperms (3-10) was transferred into each droplet. The droplets were covered with a 2.5 ml of mineral oil. The droplets inside the petri dish were examined by an inverted microscope (Nikon, USA) with 20x magnification to confirm the presence of transferred sperms. The petri dish was closed by a lid and covered with a plastic wrap and then was placed on a metal sheet in a foam box for 3 minutes to freeze its droplets mineral oil. The Petri dish was immersed in a liquid nitrogen and finally stored in a nitrogen tank for one week (Fig.S1, See Supplementary Online Information at ww.ijfs.ir). After removing the petri dish from liquid nitrogen, samples were placed quickly in the incubator at two different temperatures 37°C (for 10 minutes) and 42°C (for 5 minutes). The motility of sperms was evaluated immediately by an inverted microscope with 20x magnification. The sperms were transferred to 0.5 µl of wash drop (HTF with 1% HSA), and then the sperm parameters were re-evaluated (20, 21).

Rapid freezing by cryotop method and thawing samples

After diluting the swim-up samples, a metal strainer coated with a sheet of metal was placed one centimeter from the nitrogen surface in a foam box containing liquid nitrogen. Later, one microliter of the frozen medium droplets was placed on the cryotop strip. A limited number of sperm (3-10) were pipetted into the prepared freeze medium droplets on a cryotop strip. The cryotop was placed on the metal sheet in the foam box for 10 seconds to freeze the drop on the cryotop strip. The covered cryotop strip with a plastic cap was immersed in liquid nitrogen and finally stored in a nitrogen tank (Fig.S2, See Supplementary Online Information at ww.ijfs.ir).

For thawing, the washing medium (HTF with 1% HSA) was placed in an incubator for 20 minutes at the intended thawing temperature (37 and 42°C). Because of the bottom surface thickness, the petri dish was heated up two degrees higher than the intended thawing temperature, which this procedure gave our intended temperatures. Then, 1 ml droplets were placed on the petri dish and covered with the mineral oil and placed on a heating plate at the intended temperature for 30 minutes.

The cryotop strips were removed from the nitrogen and immediately transferred to the mineral oil-coated washing droplets. The droplets were examined under an inverted microscope at 20x magnification to measure the recovery and motility of the frozen samples.

Assessment of sperm viability

After thawing the frozen samples on cryotops and petri dishes in two different temperatures and investigating sperm retrieval and motility, the cells were transferred into 0.5 µl droplets of hypoosmotic solution (150 ± 5 mmHg) to equivalent to Ham's medium and sterile distilled water. These samples were incubated for 5 minutes at 37°C, and then their survival was examined according to WHO (2010) criteria by the Invert microscope (18).

Measurement of DNA fragmentation

To evaluate the sperm DNA fragmentation, the semen samples from three normospermic men were collected and after washing and dilution, a limited number of sperm were selected and frozen with two different cryopreservation medium as described above. The sperm transfer site was marked on the slide using a marker and at least 10 sperm were transferred to the slide. After drying the droplets (20 minutes), the dried droplets were covered using Carnoy solution as fixation and kept in a cool place for 2 - 24 hours. The slides were taken to a dark room, and acridine orange dye (65-61-2, Sigma, USA) was placed on the fixed droplets for 5 minutes, and then washed once with distilled water or phosphate buffered saline (PBS, MP Biomedicals, USA). After that, the marked sites on slides were examined immediately with a fluorescent microscope (AX70, Olympus Japan) with 100x magnification (22).

Statistical analysis

The present study used descriptive and inferential statistics to analyze the data. Descriptive statistics were used to prepare tables, draw graphs, and calculate statistical indicators. After confirming the normal distribution of values by using the Kolmogorov-Smirnov test, inferential statistics, including one-way ANOVA using Post-hocand Tukey range's test, were used to compare the four groups (CPA_{42}, CPA_{37}, $S+T_{37}$, $S+T_{42}$) in each method. Data were analyzed by the SPSS software version 16 (IBM company, USA), and the values of $P<0.05$ were considered significant.

Results

The study groups in the microdroplet on a petri dish and the cryotop method

The freezing groups included $(S+T)_{37}$ and $(S+T)_{42}$ freezing with S+T antioxidant, which was thawed at 37 and 42°C, and $(CPA)_{37}$ and $(CPA)_{42}$ included cryopreservation with CPA and thawed at 37 and 42°C, respectively. The results of statistical analysis after thawing at two different temperatures of 37 and 42°C have been reported in Tables 1 and 2.

Table 1: The mean of classical parameters studied after freeze/thawing by the microdroplet method

Studied groups	Parameters examined (%)	Mean ± SEM	Maximum	Minimum
$(S+T)_{37}$	Recovery number	100	100	100
	Total motility	11.9 ± 8.30	100	0
	Progressive motility	0	0	0
	Viability	22.60 ± 7.68	100	0
$(S+T)_{42}$	Recovery number	100	100	100
	Total motility	6.35 ± 4.97	60	0
	Progressive motility	1.67 ± 1.67	20	0
	Viability	34.74 ± 5.90	60	0
$(CPA)_{37}$	Recovery number	100	100	100
	Total motility	7.98 ± 3.09	25	0
	Progressive motility	0	0	0
	Viability	34.18 ± 4.97	50	11.11
$(CPA)_{42}$	Recovery number	100	100	100
	Total motility	16.77 ± 9.06	80	0
	Progressive motility	3.23 ± 2.32	20	0
	Viability	40.11 ± 9.09	81.82	0

SEM; Standard error of the mean, S+T; Sucrose+Taurine, and CPA; Cryoprotectant.

Sperm parameters of microdroplet on petri dish method

The recovery rate of frozen sperms in the microdroplet on the petri dish method was 100% and no difference was observed between the frozen groups. We did not observe significantly different of motility among our groups (Fig.2). Also, both cryopreservation groups thawing at 37°C, did not show any progressive motility, and this rate was zero. In contrast, there was not a significant increase in the progressive motility of $(CPA)_{42}$ group when compared to the group $(S+T)_{42}$ (Fig.2). Also, it was observed that there was no significant difference in the sperm viability rate among our groups (Fig.S3, See Supplementary Online Information at ww.ijfs.ir).

Table 2: The mean of classical parameters studied after freeze/thawing by cryotop

Studied groups	Parameters examined (%)	Mean ± SEM	Maximum	Minimum
$(S+T)_{37}$	Recovery number	99.60 ± 0.4	100	94.44
	Total motility	41.25 ± 6.79	100	0
	Progressive motility	27.69 ± 5.4	60	0
	Viability	62.49 ± 5.60	100	28.57
$(S+T)_{42}$	Recovery number	97.52 ± 1.45	100	83.33
	Total motility	44.55 ± 7.50	75	0
	Progressive motility	25.83 ± 6.6	64.29	0
	Viability	63.66 ± 6.64	100	20
$(CPA)_{37}$	Recovery number	98.05 ± 1.33	100	85.71
	Total motility	60.28 ± 6.58	100	25
	Progressive motility	38.38 ± 7.89	100	0
	Viability	68.42 ± 15.98	100	50
$(CPA)_{42}$	Recovery number	96.52 ± 3.02	100	66.67
	Total motility	75.50± 9.33	100	0
	Progressive motility	52.92 ± 8.13	100	0
	Viability	76.46 ± 9.41	100	0

SEM; Standard error of the mean, S+T; Sucrose+Taurine, and CPA; Cryoprotectant.

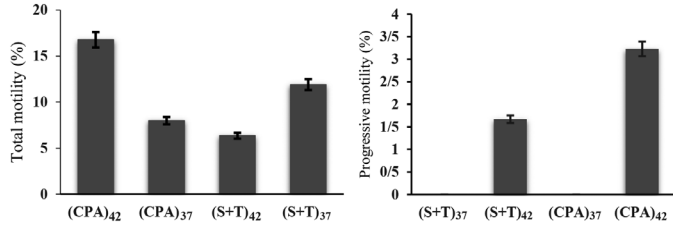

Fig.2: Comparison of total and progressive sperm motility in terms of mean percentage in frozen samples with two different cryoprotectants and by microdroplet method on the petri dish and thawing at two different temperatures (37°C and 42°C). Data were presented as mean values ± standard error of the mean using One Way-ANOVA (Post-hoc test: Tukey's range test). The significance level was considered as $P<0.05$. S+T; Sucrose+Taurine and CPA; Cryoprotectant.

Sperm parameters of cryotop method

Table 2 provides information on the classical sperm parameters in the presence of commercial cryoprotectant and S with the antioxidant T in the cryotop method.

We observed a more than 97% rate of recovery in all groups of the cryotop method, including, $(S+T)_{37}$ group: 106/107 cells (99.07%), $(S+T)_{42}$ group: 114/117 cells (97.44%), $(CPA)_{37}$ group: 104/106 cells (98.11%) and $(CPA)_{42}$ group :105/107 cells (98.13%). However, this rate of cell loss was not significantly different between groups. The average percentage of sperm recovery was not

significantly different among groups: $(S+T)_{37}$: 99.60%, $(S+T)_{42}$: 97.52%, $(CPA)_{37}$: 98.05%, and $(CPA)_{42}$: 96.52%.

Comparing the total motility of thawed sperms, although the average motility percentage in the $(S+T)_{42}$ group (44.55%) was higher than the $(S+T)_{37}$ group (41.25%), this increase was not statistically significant. Also, the mean percentage of motility in the $(CPA)_{42}$ group (75.50%) did not show a considerable increase in comparison with the $(CPA)_{37}$ group (60.28%). In the $(CPA)_{42}$ group, motile sperms (75.50%) were significantly higher than in the $(S+T)_{37}$ and $(S+T)_{42}$ groups (P =0.005, Fig.2). Also, the mean percentage of progressive sperm motility was not statistically significant among the groups, $(S+T)_{37}$ and $(CPA)_{37}$, $_{42}$ (Fig.3). The mean rate of progressive sperm motility in the $(CPA)_{37}$ group (38.38%) was not significantly higher than $(S+T)_{37}$ group (27.69%). While in the $(CPA)_{42}$ group progressive motility (52.92%) showed a significant increase in comparison with $(S+T)_{37}$ and $(S+T)_{42}$ groups (25.83 and 27.69%, respectively) (P=0.001, Fig.3). The rate of sperm viability in the $(S+T)_{42}$ group was more than the $(S+T)_{37}$ group, this difference was not statistically significant. Moreover, we observed an insignificant increase in the motility rate of the $(CPA)_{42}$ group in comparison with the $(CPA)_{37}$ group. The live sperm rate in the $(CPA)_{37}$ and $(CPA)_{42}$ groups, respectively, was not significantly higher than the $(S+T)_{37}$ and $(S+T)_{42}$ groups (Fig.S4, See Supplementary Online Information at ww.ijfs.ir).

Regarding DNA damage, statistical analysis revealed that both groups of sperm that were frozen with and without commercial cryoprotectant noticed an increase in DNA damage. The DNA fragmentation rate showed a non-significant decrease in the $(CPA)_{42}$ and $(S+T)_{42}$ groups in comparison with the $(S+T)_{37}$ and $(CPA)_{37}$ groups. There was also no significant difference in the percentage of DNA fragmentation in each temperature group.

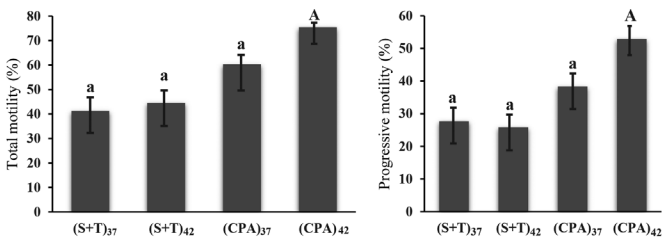

Fig.3: Comparison of the total and progressive motility of frozen sperms with two different cryoprotectants and cryotop methods and thawing at two different temperatures (37°C and 42°C) as a mean percentage in the semen sample. Data were presented as mean values ± standard error of the mean using One Way-ANOVA (Post-hoc test: Tukey's range test). Capital and small letters show significant differences among the evaluated groups (P<0.05). S+T; Sucrose+Taurine and CPA; Cryoprotectant.

Discussion

Sperm cryopreservation is an essential method for treating male infertility, especially in patients with severe oligospermia, obstructive and non-obstructive azoospermia. As is well known, intracytoplasmic sperm injection (ICSI) is the most impressive treatment for the serious cause of male infertility and sperm cryopreservation plays a significant role in maintaining male fertility throughout ART and ICSI strategy (2). However, the occurrence of injury during the freezing process significantly diminishes the probability of finding best motile sperm with higher morphology. The use of inanimate sperm during ICSI may result in complications about both fertilization and pregnancy (23). In this study, we compared the effect of a complex of S and T with the commercial CPA on the classical parameters during the rapid freezing of limited sperm by two different methods (microdroplet on the petri dish and cryotop).

Here, we found that freezing on a petri dish, brings a 100% sperm recovery rate in all our groups, while recovering sperms were easily visible after thawing that it seems because of their cryopreservation method. This outcome was in line with the Bouamama et al. study, that reported a complete recovery rate (100%) after thawing in a culture dish with motility around 50%. Also, they observed less than 2 sperms were recovered at thawing phase when used the straw classical technique to freeze 20 sperms (24).

The reduction rate of sperm parameters, including motility and viability is a common consequence of a freezing process, regardless of methods and materials. Microdroplet-based cryoprotectant freezing techniques are secure and advantageous in terms of both cost and safety. In these methods, sperm recovery rate, motility, and fertilization rate are highly significantly different from the results before sperm preparation. Methods currently used to freeze a sperm without adding any cryopreservation medium and usually use S (25). The Cryotop acts as a freezing vessel for the embryos and eggs with a 99% survival rate after thawing, and a good chance for small quantities sperms using. The cryotop technique used in this study was based on the Endo et al. (7) protocol. But we added T as an antioxidant, to try to increase the motility percentage in the free-cryoprotectant group (S+T). Therefore, we evaluated the effects of two freezing mediums . However, the motility rate was lower in the S in comparison with Sperm Freeze Medium. In the present study, a successful cryopreservation of a limited number of sperm, with an effective quick recovery rate was achieved. Moreover, it was determined that the cryotop approach was more appropriate for viral infections during nitrogen storage due to the convenience and speed of freezing-thawing and less infection than the petri dish method, as well as the lack of problems such as enough space and storage in a nitrogen tank (2).

Also, it was shown that the thawing at the 42°C maintains the survival and motility rates of all sperm better than 37°C. Similar results were obtained by Mansilla et al. (16) who reported a better sperm motility rate in the 42°C than the 37°C and 40°C, when thawed vitrified sperm (5×10^6/ml). The HOS test showed the

viability of motile sperm at 38, 40, and 42°C is 26.48 ± 8.4%, 56.6 ± 16.3%, and 65.4 ± 15%, respectively. The Plasma membrane integrity was supported better than at 42°C compared to other thawing temperatures (26). The results of another study (27) suggest that higher temperatures have beneficial effects on the metabolism restoring and membrane stability of sperms. It seems due to the thawing rate; the latter researchers observed that 60°C temperature for 5 sec resulted in a better sperm quality rate in comparison with the 37°C for 30 seconds conditions. The sperms motilities, total and progressive, were consistently and significantly better in the rapid thawing condition (60°C for 5 seconds). According to Malo et al., for thawing temperatures of 60°C, the overall motility rate at 0 hour and 1 hour was higher than 37°C (27). Also, El-Ahwany et al. (28) reported an increase in motility recovery rate in 42°C thawing condition in comparison with the 37°C temperature.

Studies drew attention to the possibility of induced sperm DNA fragmentations through the freezing/thawing procedure. It has shown that a cryopreservation procedure increases the ROS production level, impairs DNA repair enzymes, induces apoptotic changes in a sperm, and produces high frequencies of single- and double-stranded DNA fractures. Conversely, other studies have shown that cryopreservation does not affect the stability of a sperm DNA (23). In the present study, we did not encounter a significant increase in the DNA damage index in the groups subjected to thawing at 42°C, it is reasonable that a brief duration of thawing is less likely to induce DNA damages. Therefore, it can be concluded that 42°C thawing condition is suitable temperature than 37°C thawing. It is suggested that when sperm are incubated at lower temperatures, they become dormant, allowing them to retain their energy. As per the current research, it has been observed that upon entering the female reproductive system, sperm undergo a state of inactivity due to exposure to elevated temperatures. This hypothesis potentially elucidates the reduction in sperm motility observed at 37°C in contrast to lower temperatures (29, 30).

The creation of adequate space for preserving the culture dish and preventing nitrogen infiltration into this device was the main restriction, even though this study demonstrated the feasibility of using both a culture dish and a cryotop for the cryopreservation of limited sperm. Another difficulty we encountered was keeping the medium culture temperature constant while the sperm were freezing that be considered in next studies.

Conclusion

The combination of S and T showed a good ability to protect a frozen small number of sperms in both cryotop and petri dish procedures. The cryotop approach also features a safer thawing procedure and is quicker and easier to use than the microdroplet on a petri plate method. Limited sperm characteristics such total motility, progressive motility, and viability are better maintained at 42°C than at 37°C. Furthermore, there was no change in DNA damage between 37°C and 42°C when using the cryotop technique.

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Authors' Contributions

M.T., L.R.G.; Investigation, Data analysis, and Manuscript writing. A.D.; Methodology, Validation, Manuscript reviewing and Editing of manuscript. M.R.V.; Conception, Study design, and Project administration. All authors read and approved the final manuscript.

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Evaluation of The 1499T>C Variant in The *AKAP3* Gene of Infertile Men with Multiple Morphological Abnormalities of The Sperm Flagella Phenotype: A Case-Control Study

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Abstract

Background: Infertile men with multiple morphological abnormalities of the sperm flagella (MMAF) phenotype exhibit mosaic sperm flagella abnormalities such as short, bent, coiled, and irregular flagella or absent flagella. Sperm flagellum has an ultrastructurally axonemal structure that contains a large number of proteins. A-Kinase Anchoring Protein 3 (AKAP3) is expressed in spermatozoa. It may function as a regulator of motility and the acrosome reaction. This study aimed to compare genetic changes in infertile men suffering MMAF phenotype with the control group.

Materials and Methods: In this case-control study, genetic variants of the *AKAP3* gene were evaluated in 60 infertile men with MMAF phenotype and 40 fertile men, as control. As exon five of the *AKAP3* gene encodes the functional domain of this protein, its genetic variants were studied. Therefore, polymerase chain reaction (PCR)-sequencing was undertaken on the DNA extracted from control and patients' blood samples.

Results: Sixty infertile men with MMAF phenotype and 40 normozoospermic men, as control, were enrolled in this study. Four haplotype variants 1378T>C (rs10774251), 1391C>G (rs11063266), 1437T>C (rs11063265), and 1573G>A (rs1990312) were detected in all patients and controls. On the other hand, a missense mutation 1499T>C (rs12366671) was observed in four patients with the homozygous form while seven patients carried the heterozygous form. No mutation was identified in the controls (P=0.04). The difference between the variation allele frequencies was assessed in the patient and control groups by the Fisher Exact Test.

Conclusion: In the homozygous form, this mutation changed Isoleucine to Threonine. This alternation occurred inside the AKAP4 binding domain of the AKAP3 protein. The observed variants caused no significant deviation in the secondary structure of AKAP3 protein and probably its function in spermatozoa flagella. So, these variants cannot be considered as the causes of MMAF phenotype in the studied patients.

Keywords: AKAP3 Gene, Male Infertility, Sperm Flagella

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Introduction

Male factors are presented in roughly 40-50% of all infertility cases (1). Male infertility diagnosis is merely descriptive, as etiology of the sperm abnormalities, remains idiopathic in about 40% of infertile cases. One of the reasons leading to the lack of basic understanding is heterogeneity of the contributing factors (2). Although male infertility is a multifactorial defect with strong genetic rudiments, to date, only a few genes have been properly elucidated to be correlated with spermatozoa defects in humans (3). Multiple morphological abnormalities of the sperm flagella (MMAF) phenotype was mostly diagnosed by asthenozoospermia, due to the existence of different types of sperm flagella anomalies, such as bent, short, coiled, irregular, and absent flagella with a variable percentage among MMAF patients; however, all of these defects could be observed in healthy men but with extremely lower levels (4). Until now, 24

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MMAF-associated genes have been identified which are responsible for manifestation of the primary infertility without occurrence of any primary ciliary dyskinesia symptom, like left-right laterality disturbances and recurrent respiratory infections (5).

A healthy sperm flagellum has three main parts: the midpiece, principal piece, and end piece. The sperm flagellum contains an axonemal structure, along with accessory structures, such as fibrous sheath (FS), the outer dense fibers (ODFs), and mitochondrial sheath (MS) (6). In the midpiece part, a MS surrounds the axoneme structure (7, 8). Different components of sperm flagellum represents various ultra-structural and morphological defects in MMAF phenotype (4).

There are numerous proteins in different parts of the sperm flagellum, accountable for unique structural properties of spermatozoa. A-kinase anchoring protein 3 (AKAP3) and A-kinase anchoring protein 4 (AKAP4) are two essential constituents of the tail FS (9). AKAPs are a group of signal organizing scaffold proteins. Collaboration of AKAPs with kinase A-dependent CAMP causes phosphorylation of a fundamental group of proteins in flagella (10). AKAP3 is implicated to organize the fibrous sheath. This protein exists in the acrosome of the sperm head, so it is considered as a regulator of acrosome reaction and sperm motility (11).

AKAP3 protein comprises of two main domains. An RII binding domain exists at the N-terminal of the protein (12). This contains an amphipathic peptide structure and provides a binding site for the protein kinase A regulatory subunit (13). In mammals, impaired interaction between the protein kinase A regulatory subunit and AKAP3 protein could result in immotile spermatozoa (14). The C-terminal domain of AKAP3 protein constructs a binding site for AKAP4. Relationship of these two proteins can play an important role in the structure of sperm flagella's FS (9).

In this research, the aim of study is correlation of the genetic variants of the exon 5 of AKAP3 gene and male infertility, as a result of the MMAF phenotype in humans, was determined by DNA sequencing.

Materials and Methods

Participants

The current study is a case and control study which was approved by the Ethics Committee of Royan Institute (Tehran, Iran, EC/93/1045). Sixty infertile men with MMAF phenotype (P1-P60) and 40 normozoospermic men (C1-C40) as controls were enrolled and all the participants confirmed their consents. As this phenomenon is a rare sperm characteristic, it took near four years to collect the cases. Inclusion criteria for controls were according to the standard values for semen analysis according to World Health Organization (WHO) 2010 guideline.

Papanicolaou staining

Papanicolaou (PAP) staining was performed to provide valid staining of spermatozoa. This kind of staining is routinely used in andrology laboratories, as one of the most popular methods for identifying human sperm morphology (15).

DNA extraction, polymerase chain reaction and sequencing

DNA was extracted from peripheral blood leucocytes by using the salting-out method. Three primer pairs *(AKAP3-1*, *AKAP3-2*, and *AKAP3-3*) were used to amplify the exon 5 of the *AKAP3* gene. The exon 5 was studied in three coding regions: RII binding domain (*AKAP3-1*), the downstream RII binding domain till AKAP4 binding region (*AKAP3-2*) and AKAP4 binding region (*AKAP3-3*, Table 1). Polymerase chain reaction (PCR) amplification was performed in 50 µl mixture volume, containing 1.5 µM $MgCl_2$, 1 µM dNTP, 5 µl PCR buffer (1X), 0.8 unit/µl Taq DNA polymerase enzyme (all from Cinagen, Iran), 100 ng of the genomic DNA, and 1.5 µl for each 10 pmol/ul primer. The PCR cycle included an initial DNA denaturation at 95°C for 4 minutes, followed by 30 cycles of DNA denaturation at 94°C for 45 seconds, annealing at melting temperature (TM) set for 45 seconds, extension at 72°C for 45 seconds and ultimately one cycle of the final extension at 72°C for 7 minutes.

Table 1: The primers used for PCR and sequencing

Primers name	Sequence (5′-3′)	Product size	TM (°C)
AKAP3-1	F: AGACTATTACAACACCACCA	372	53
	R: TGTGATGATCCCGAGAC	372	53
AKAP3-2	F: ATCTCCACAGCGTCACAG	682	55
	R: GCTACAGGAGGTTCATTG	682	55
AKAP3-3	F: AGGAGGAGACTTGTGCGA	755	56
	R: CAACGAAGCATCACAGGA	755	56

PCR; Polymerase chain reaction and TM; Melting temperature.

PCR products were sequenced and the results were analyzed by FinchTV software version 1.4.0 and they were compared to the reference sequences from Ensembl release 103 databases.

Statistical analysis

The difference between the variations' allele frequencies in the patient and control groups was assessed by the Fisher Exact Test. GraphPad Prism version 7.04 (USA, GraphPad by Dotmatics) was used for statistical analyses. $P<0.05$ was considered statistically significant.

Results

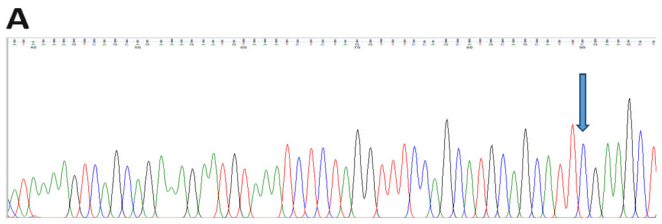
MMAF phenotype was identified in the patients using PAP staining. Short, coil, absent and bent flagellum defects were observed (Fig.1).

Fig.1: Papanicolaou staining shows morphological defects of flagella in spermatozoa of MMAF patients (scale bar: 10 µM). MMAF; Multiple morphological abnormalities of the sperm flagella.

Four haplotype variants were detected in all patients and controls. These variants were detected in all samples as homozygous genotype. The haplotype variants included ENSG00000111254.8, chromosome 12: 1378 T>C (rs10774251), 1391 C>G (rs11063266), 1437 T>C (rs11063265), and 1573 G>A (rs1990312, Fig.2). The other variant was ENSG00000111254.8, chromosome 12: 1499T>C (rs12366671) which was observed in four patients in the homozygous genotype and seven patients in the heterozygous form. Notably, this variant was not detected in the controls (Fig.3). In the homozygous form, Isoleucine was altered to Threonine.

With regard to 1499T>C variant, frequency of the genotype (P=0.04) and C alleles (P=0.0005) were significantly higher in the patients compared to the controls (Table 2).

Another variant was ENSG00000111254.8, chromosome 12: 1982T>C (rs953905145) which was only observed in three patients as heterozygote form. However, this variant was not significantly different between the patients and controls (Table 3).



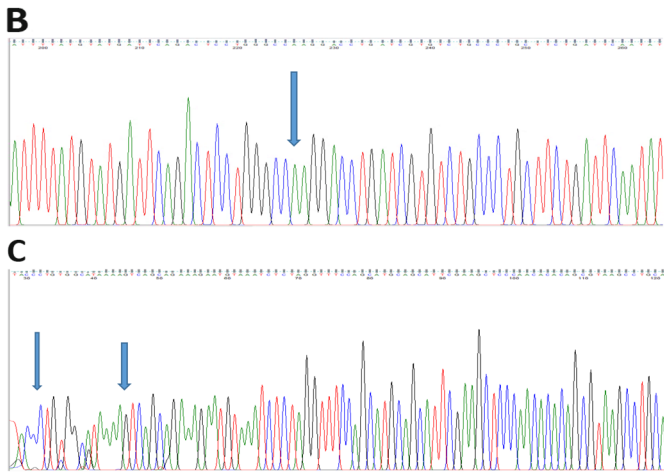


Fig.2: The sequencing results showing four different haplotype polymorphisms of the AKAP3 gene. **A.** 1437T>C, **B.** 1573G>A, and **C.** 1378T>C and 1391C>G polymorphisms observed in different patients and controls.

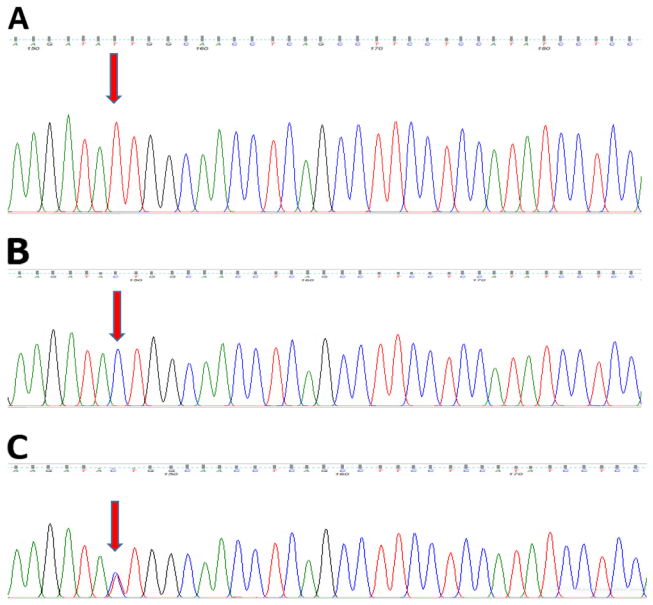


Fig.3: The different sequensing forms of 1499T>C (rs12366671) variant of the *AKAP3* gene. **A.** Wildtype (Homozygote (TT)], **B.** Mutant homozygote (CC), and **C.** Heterozygote forms (C/T).

Table 2: Genotype distribution and proportion of the genetic variants observed in the MMAF patients and controls

Variations	Case (total) 100%			Case (total) 100%			Significant P value
	WT	HETERO	HOMO	WT	HETERO	HOMO	
rs10774251 T>C	0	0	100	0	0	100	-
rs11063266 C>G	0	0	100	0	0	100	-
rs11063265 T>C	0	0	100	0	0	100	-
rs1990312 G>A	0	0	100	000	0	100	-
rs12366671 T>C	49	7	4	100	0	0	0.041*
rs953905145 T>C	57	3	0	100	0	0	0.053*

MMAF; Multiple morphological abnormalities of the sperm flagella and *; The significance difference is P≤0.05.

Table 3: Distributions of the (rs12366671 T>C) polymorphism allele frequencies in the studied groups

rs12366671 T>C Allels	Case alleles (total=120) 100%			Control alleles (total=80) 100%			Significant P value
	WT	HETERO	HOMO	WT	HETERO	HOMO	
C	49	7	4	100	0	0	0.041*
C	57	3	0	100	0	0	0.052*

*; The significance difference is P≤0.05.

Discussion

Male infertility is becoming an expanding multifactorial defect. Considerably, a large number of genes and genetic factors are correlated with spermatozoa defects including low sperm concentration, abnormal sperm morphology, and reduced motility (15).

In spite of many attempts to identify the responsible factors in the spermatogenesis, genetic factors involved in male infertility has not been completely discovered yet (16).

In our study 60 infertile men with MMAF phenotype were recruited and the *AKAP3* gene was evaluated as a gene with a causative role in creating MMAF phenotype. This phenomenon is a mosaic morphological sperm disorder, mainly affecting the sperm flagella. This commonly results in asthenozoospermia and male reduced fecundity. Consequently, four haplotype variants were detected in all samples of both patients and control groups, including 1378T>C (rs10774251), 1391C>G (rs11063266), 1437T>C (rs11063265), and 1573G>A (rs1990312). The previous reports performing in the East Asia population, indicated frequencies of 92% C allele and 8% G allele in 1391C>G variant.

Meanwhile, G allele was observed in 100% of present study cases. The difference observed in the mutant allele frequency could be due to ethnicity differences of the Asian population from our population.

1499T>C (rs12366671) variant was observed in four patients with homozygous genotype and seven patients in heterozygous form, whereas this variant was not identified in the control individuals. This genetic alternation changed Isoleucine (a hydrophobic amino acid) to Threonine (a polar amino acid) which occurs inside the AKAP4 binding domain of AKAP3 protein.

Baccetti et al. (17) reported some deletions in the coding regions of the AKAP3 and AKAP4 genes, however, these deletions were only characterized in one patient and more data is needed to confirm their role in male infertility.

Our new published experiment (18), which was based on structural modeling and in silico analysis of single nucleotide polymorphisms (SNPs), revealed that 1499C>T variant (and the other variants identified in the present study) caused no critical deviation in the secondary structure of AKAP3 protein and probably its function in sperm flagella. Therefore, according to our new findings, 1499C>T cannot be the cause of MMAF phenotype and male infertility. Additionally, our results are in line with the findings of the previous study performed in 2001. In this study, Turner et al. did not observe any evidence to hold up the hypothesis that *AKAP3* and *AKAP4* genes mutations lead to dysplasia of the fibrous sheath (DFS) in humans (9). The patients with 1499T>C variants were followed up, but unfortunately none of them requested for assisted reproductive technology (ART).

Conclusion

As no significant difference was observed in the four haplotype variants of the *AKAP3* gene [1378T>C (rs10774251), 1391C>G (rs11063266), 1437T>C (rs11063265), and 1573G>A (rs1990312)], therefore they cannot be considered as the causes of MMAF phenotype in the population of Iranian patients studied.

Acknowledgment

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Authors' Contributions

E.P.T.; Performed the experiments and collected some of the samples and drafted the manuscript. S.-H.H.; Drafted the manuscript and collected some of the samples and edited the article. H.G.; Provided some advice and guide during the project. M.S., A.M.M.; Developed the concept, supervised and interpreted the findings of the study. All authors read and approved the final manuscript.

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