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Editorial Office Address: P.O.Box: 16635-148, 5th Floor, No 9, Royan Institute Cell Therapy Center, East Shaghayegh Alley, Bani Hashem Sq, Bani Hashem St, Resalat Highway, Tehran, Iran
(Mohammad Hossein Nasr Esfahani, Ph.D.)
Tel & Fax: +9821-22510895
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Contents

Review Article

Coding and Non-Coding RNAs, as Male Fertility and Infertility Biomarkers

Fereshteh Aliakbari, Ph.D.[1], Nahal Eshghifar, Ph.D.[1, 2], Reza Mirfakhraie, Ph.D.[3], Parisa Pourghorban, M.Sc.[4], Faezeh Azizi, Ph.D.[5*]

1. Men's Health and Reproductive Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2. Department of Cellular and Molecular Sciences, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran
3. Department of Medical Genetics, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4. Department of Biology, Faculty of Biological Sciences, Sabzevar Branch, Islamic Azad University, Sabzevar, Iran
5. Non-Communicable Disease Control Department, Public Health Department, Ministry of Health and Medical Education, Tehran, Iran

Abstract

Semen analysis is usually the first step in the assessment of male fertility. Although analyzes provide valuable information about male fertility, success of cytoplasmic sperm injection using this method is not predictable. In the recent years, studies have shown that sperm quality assessment helps clinicians predict male fertility status based on the expression of biomarkers. To write this article, a comprehensive study was conducted on several RNA transcripts by searching related words on medical information databases by 2018. According to the literature, spermatogenesis based disorders in male infertility have a significant relationship with the expression level of some RNA molecules (like *DAZ* and *PRM1/PRM2* ratio) in semen and testicular tissue. Thus, they might be used as predictor biomarkers to evaluate success rate of testicular sperm extraction (TESE) procedure, but confirmation of this hypothesis requires more extensive research. By comparing the number of RNAs attributed to each fertility disorder in men, it is possible to trace the causes of disease or return fertility to some infertile patients by regulating the mentioned molecules. Further researches can provide a better understanding of the use of RNA expression profiles in the diagnosis and treatment of male infertility.

Keywords: Male Infertility, Semen, Spermatogenesis

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Introduction

Sperm RNA contains several coding and non-coding transcripts that represent a picture of past events, such as spermatogenesis and sperm maturation. This, they provide new insights for male fertility and infertility research. On the other hand, a new scope in infertility study is participation of sperm RNA in the epigenetic transgenerational inheritance of the altered phenotypic traits in the progeny associated with paternal exposure (1). One of the main problems in infertile men is reduction of normal sperm cell quantity. Currently, despite the importance of sperm movement in the reproductive process, limited information is available about the molecular mechanisms related to sperm motility. Nowadays, new strategies for treating spermatogenesis of infertility, such as intracytoplasmic sperm injection (ICSI), reduce sperm disorders and sometimes easily recover it. Despite utilizing these methods can help resolve the infertility problem, the risk of transferring genetic problems to the next generation still exist. The main emphasis of molecular evaluation and analysis of RNA sperm is the important role of male factors in idiopathic infertility and difficult testicular biopsy procedure. These cases can also be useful as predictors of male infertility. It is estimated that about 35% of cases in infertile male are caused by genetic factors (2). More than 30 years ago, presence of RNA in sperm had been the subject of argument. The concern in this issue has recently expanded, due to the development of modern molecular technologies and the need for designing non-invasive methods for studying and assessing testicular function. If it is possible to obtain useful information about molecular events of sperm, a semen analysis will be a non-invasive approach compared to testicular biopsy. Today, with remarkable advances in molecular medicine, study of the sperm RNA content is growing using techniques that simultaneously examine expression of large number of genes, such as RNAseq and microarray. In recent years, study of effective genes in the male infertility process has been considered, due to their important role in therapeutic planning and pre-

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***Corresponding Address: P.O.Box: 1467664961, Non-Communicable Disease Control Department, Public Health Department, Ministry of Health and Medical Education, Tehran, Iran**
Email: faezeh.azizi@sbmu.ac.ir

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implantation genetic diagnosis (3). On the other hand, if a gene is expressed in a particular stage of spermatogenesis, it will be possible to predict the progression of spermatogenesis through molecular methods and adapt it to histopathological findings. Therefore, study of these transcripts is important in the molecular identification of the spermatogenesis stage, oocyte fertilization and early stages of fetal development, as well as the association of genes with male infertility phenotype and its application in diagnostic procedures.

Literature search

This review study was conducted on over 95 articles published in the Google Scholar, PubMed, Scopus, IranMedex, MEDLIB, IranDoc and Scientific Information Database (SID) for the comprehensive information on the biomarkers introduced for male infertility. All articles were reviewed by the keywords of transcript, sperm, semen, testicular tissue and infertility, until September 2018 and among them, 74 related papers were included.

Spermatic transcripts

RNA evaluation in sperms is recommended because it may show a historical record of spermatogenesis. Additionally, it can be considered as genetic background as well as fingerprint of the individual. Therefore, some RNAs may be brought up as potential diagnostic tools for evaluating male infertility and they may also play an important role in the development of fetuses and zygotes.

Coding RNAs

Dynamic cellular diversity has been reported in the RNA profiles of fertile and infertile men, and therefore scientists refer to it as biomarker of infertility. Round spermatids contain numerous varieties of transcripts that are stored in the spermatid cytoplasm before expression of the related proteins. In the middle of spermatogenesis, chromatin remodeling results in genome transcriptional inactivation. Therefore, most RNA transcripts were transcribed before the inactivation process. Using techniques such as real-time PCR, presence of the transcripts in human spermatozoa was confirmed (4). Data evaluation, using microarray, showed that adult human sperm has about 5000 types of mRNA molecules, expression of which vary about 10% between different specimens (5). The semen mRNA content can provide valuable information about the condition of spermatogenesis in the patient's testis, which cannot be detected by the conventional histopathologic methods. Although their possible roles are not revealed, many hypotheses could be proposed to illustrate the presence of mRNAs in sperm. Most evidences suggest that transmission of these mRNAs to oocyte may be as important as transfer of the haploid genome. Moreover, it is suggested that some paternal traits are transmitted to the child through the contents of sperm transcripts. If these transcripts play a role in the early differentiation of the fetus, these findings could be useful in advancing the technology of somatic cell nuclear transfer in cloning and also identifying effective factors in the development of infertility. Recently, it has also been shown that the amounts of sperm mRNA are transferred to the egg during fertilization and where their related proteins are synthesized. Therefore, it seems that transcripts of sperm have vital role in fetal development of the early stages (6). Researchers analyzed the RNA profile of sperm and testis in normosperm patients. They suggested that RNA profile is valuable to be used, regarding that can be used as a genetic fingerprint in fertile and infertile individuals and reflect past events during spermatogenesis. Development of the new research methods such as microarray and RNAseq can be useful as additional diagnostic tools and prognosis, for fertility and pregnancy. So far, based on the role of genes in spermatogenesis, numerous gene expression analyses were carried out on different specimens to determine the associated genes (Table 1). In many studies, expression of specific testicular genes has been analyzed, some of which are described below.

Table 1: Gene expression analysis in sperm, testis tissue and semen of fertile and infertile men

Gene name	Function	Sample type	Association P value	Ref.
DNMT1, DNMT3A, and *DNMT3B*	Methylation of DNA	Semen	No	(7)
RXFP3	Peptide receptor	Spermatozoa	No	(8)
PLCζ	Phospholipase (testis-specific)	Semen	Yes	(9)
PLCζ	Phospholipase (testis-specific)	Sperm	Yes P≤0.05	(10)
PLCζ and *CAPZA3*	Phospholipase (testis-specific)/F-actin capping protein	Semen	Yes	(11)
PLCζ and *PAWP*	Phospholipase (testis-specific)/Meiotic resumption	Semen	Yes	(12)
PLCζ, *PAWP* and *TR-KIT*	Phospholipase (testis-specific)/Meiotic resumption/KIT proto-oncogene receptor tyrosine kinase	Semen	Yes	(13)
PAWP	Meiotic resumption	Semen	Yes P<0.05	(14)
TR-KIT	KIT proto-oncogene receptor tyrosine kinase	Semen	Yes P<0.01	(15)

Table 1: Continued

Gene name	Function	Sample type	Association P value	Ref.
JMJD1A	Demethylase	Testis tissue	Yes	(16)
PRM1, PRM2, YBX2 and *JHDM2A*	Compact sperm DNA (testis-specific)/DNA- RNA-binding protein (testis-specific)/Demethylase	Testis tissue	Yes/No for *JHDM2A*	(17)
YBX2 and *JHDM2A*	DNA- RNA-binding protein (testis-specific)/Demethylase	Testis tissue	Yes/No for *JHDM2A*	(18)
YBX2	DNA-RNA-binding protein (testis-specific)	Testis tissue	Yes P<0.0001	(19)
PRM1, PRM2 and *TNP2*	Compact DNA sperm (testis-specific)/Replacement of histones to protamine (testis-specific)	Semen	*PRM1*, *PRM2* decrease/*TNP2* increase	(20)
PRM1 and *PRM2*	Compact DNA sperm (testis-specific)	Testis tissue	Yes, for *PRM1* P<0.001	(21)
PRM2	Compact sperm DNA (testis-specific)	Semen	No	(22)
Casp 9 and *PRM2*	Apoptosis/Compact sperm DNA (testis-specific)	Semen	Yes, for *PRM2* P<0.05	(23)
KDM3A and *PRM1*	Demethylase/Compact sperm DNA (testis-specific)	Testis tissue	Decrease in NOA	(24)
DAZ, AKAP4, PRM1 and *PRM2*	RNA-binding protein/Regulatory subunit of protein kinase A/Compact DNA sperm (testis-specific)	Semen	Yes, for *DAZ* and PRM2	(5)
PRM1 PRM2 and *HILS1*	Compact DNA sperm (testis-specific)/Linker histone	Sperm	Yes, for *PRM1* and *PRM2* P<0.001	(25)
ZMYND15, TNP1, PRM1 and *SPEM1*	Transcriptional repressor/Replacement of histones to protamine (testis-specific)/Compact sperm DNA (testis-specific)/Spermatid maturation (testis-specific)	Testis tissue	Yes	(26)
PRM2, HSP90 and *WNT5A*	Compact sperm DNA (testis-specific)/Chaperone/Signaling proteins	Sperm	Yes P≤0.05	(27)
TNP1	Replacement of histones to protamine (testis-specific	Semen	Yes ---	(28)
HSPA2	Folding and transport	Semen	No	(29)
TGIFLX/Y	Transcription factor (testis-specific)	Testis tissue	Yes	(30)
SYCP3	Recombination	Testis tissue	Yes	(31)
Septin14	GTP-binding cytoskeletal proteins	Testis tissue	Yes	(32)
DAZ	RNA-binding protein	Testis tissue	Yes	(33)
TSGA10	Sperm tail fibrous sheath	Testis tissue	Yes	(34)
Clusterin	Chaperone	Testis tissue	Yes	(35)
hTSH2B	Histone	Testis tissue	Yes	(36)
BAX and BCL-2	Apoptotic regulators	Semen	No/Yes	(37)
ERα	Estrogen receptor	Sperm	Yes P≤0.05	(38)
		Semen	Yes	(39)
SREs	Sperm RNA elements	Sperm	P≤0.05	(40)

Sperm associated oocyte-activating factors genes

In about 1-3% of cases, failure of fertilization is due to the absence of sperm associated oocyte-activating factors (SAOAFs) in the posterior acrosomal region of the sperm head. During normal fertilization, when the sperm enters the egg, the egg is activated. This is associated with an increase in the concentration of calcium in the cytoplasm. Studies showed that increased intracellular calcium concentrations of oocytes are due to spermatozoa SAOAFs, including the *phospholipase C ζ (PLCζ)*, *postacrosomal sheath WW domain-binding protein (PAWP)* and *KIT proto-oncogene receptor tyrosine kinase* (*KIT-Tr* proteins), which initiate the cascade of oocyte activation signal. *PLCζ* gene in humans, located at 12p12.3, is a family of phospholipase C enzyme. *PLCζ* protein is a special sperm protein with catalytic and domains catalytic X, Y and the Y-X binding region. At present, researchers often identify *PLCζ* as the most likely candidate for SAOAFs (41).

In the research performed by Park et al. (42), it was revealed that low expression of *PLCζ* was related to the oxidation of DNA sperm in human. Heytens et al. (43) showed that expression of *PLCζ* in infertile cases is lower due to the reduced fertilization rates. It may be suggested that the cause of fertilization failure after ICSI, may be due to the decrease or absence of *PLCζ* protein in some infertile people; therefore, they introduced this protein

as a biomarker for fertilization failure. By studying this biomarker in infertility centers, an artificial oocyte activation (AOA) treatment method can be used to increase chance of improving fertilization rates in these individuals. Javadian-Elyaderani (11) demonstrated that due to the presence of a mutation in the vicinity of *PLCζ*, expression level of this gene was significantly reduced in infertile men with history of failed oocyte activation compared to normal men.

In addition, findings of Aghajanpour et al. (9) showed that expression of *PLCζ* was significantly lower in globozoospermic men or individuals with previously low or failed fertilization, in comparison with the control group. On this basis, they suggested that assessment of relative *PLCζ* expression may provide a useful marker for the ability of sperm to induce oocyte activation after ICSI. Unlike *PLCζ*, the exact molecular mechanism of the *PAWP* signal pathway is yet unknown. *PAWP* position in mammals was identified in the posterior acrosomal sheath of the sperm head. *PAWP* has no enzymatic activity, but it has hydrolytic activity on *PLCζ*. It is proposed that *PAWP* affects oocyte by interaction with other proteins. The results of these experiments showed that sperm injection with anti-*PAWP* antibody resulted in fertilization inhibition. Therefore, role of *PAWP* was considered as an oocyte activator. Abadi et al. (12) investigation showed that expressions of both *PLCζ* and *PAWP* were significantly reduced at RNA and protein levels of oligozoospermic men. They concluded that one of the reasons of fertilization failure after ICSI is due to the high percentage of sperm with small acrosomes and reduction of SOAFs might be associated with genetic abnormalities, such as mutations and gene deletions related to globozoospermia. The results of Tavalaee and Nasr-Esfahani (13) experiments were similar to those of the previous review. It was showed that expression profiles of *PLCζ* and *PAWP* were low in globozoospermic individuals.

On the other hand, in the study of Ghazavi-Khorasgani et al. (44), relative expression of *PAWP* was compared between varicoceles and fertile individuals at both mRNA and protein levels. Results showed that levels of *PAWP* mRNA and protein were decreased significantly in varicocele compared to fertile men. Therefore, one of the infertility etiologies in men with varicocele can be related to the decreased *PAWP* levels and inactivation of oocytes due to the effect of the increased testicle temperature on the expression of genes during spermatogenesis.

Compacting DNA sperm genes

Sperm transcripts play a dynamic role in reorganization of sperm chromatin. At the stage of spermatogenesis, somatic histones are replaced by transient proteins (TNP1 and TNP2) and then with protamine (*PRM1* and *PRM2*) (Fig.1). Protamine is one of the most prominent and smallest sperm nucleolar proteins that are conserved amongst different species. In dense and mature spermatids, protamine proteins are a substitute for transient proteins and they are associated with genomic DNA (45). In Iranian research, it was found that *PRM1/PRM2* mRNAs ratio differed significantly among azoospermic men and normal group. Based on similar researches, it was proposed that decrease in the expression of *PRM2* gene could lead to male infertility. In line with the mentioned study, a survey showed (17) that *PRM2* down-regulation occurred much more than *PRM1* in the sperm of infertile men. Although in Lambard et al. (46) study, increase of PRMT1 expression was reported in a low motile population. Due to the relation of protamin expressions with quality of sperm, they serve as biomarkers for diagnosis of male infertility. Results of several studies showed a significant relationship of sperm morphology with quantity of *PRM1, PRM2* and *TNP2* transcripts. Studies revealed significantly lower protamine transcript content in infertile fertile men (47).

Rogenhofer et al. (47) explained that *PRM1/PRM2* mRNA ratio in ejaculated spermatozoa could differentiate infertile from fertile groups. In terms of *TNP2*, Savadi-Shiraz et al. (20) reported a significant positive correlation between expression of *TNP2* gene and teratozoospermic samples, to compare with the control group ($P<0.001$) and sperm-head defects ($P<0.05$). Results of study performed by Liu et al. showed that normal development of sperm required microRNA-122 to control frequency of *TNP2* mRNA and its subsequent translation (48).

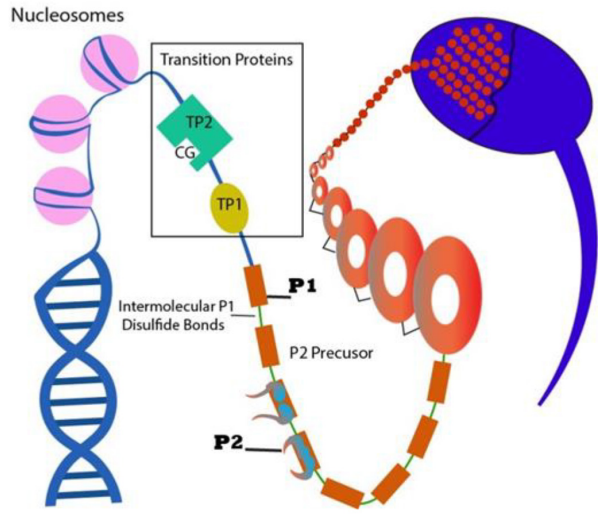

Fig.1: Schematic representation of the sperm epigenetics. Hyper-acetylation of histones and activation of topoisomerase, to induce double-strand DNA breaks, allow histones to be replaced with transition proteins 1 and 2 (TP1 and TP2). Transition proteins are subsequently replaced with phosphorylated protamine, *PRM1* and *PRM2*, which induce DNA compaction within the nucleus and form the nucleosome-bound chromatin. PRM1 is synthesized as a mature precursor, whereas the *PRM2* is generated by a partial processing of a single *PRM2* precursor (illustrated by the author).

Transcription factor genes (*TGIFL* and *YBX2*)

HOX genes, encoding transcription factors, play important roles in growth and development of mammals. Homeobox-containing genes (*TGIFLX/Y*) are members of this family and expressed in the testicles of mature males. However, their function is unknown and needs to be investigated (49). Aarabi et al. (30) evaluated the

expression of *TGIFLY* in 110 azoospermic men and found no significant relationship between the gene expressions and spermatogenesis progression. One of the reasons for this finding is variation of the *TGIFLY* gene expression in different spermatogenesis stages, causing genetic heterogeneity in male infertility screening.

Y-Box proteins are DNA and RNA-coupled proteins that play role in controlling gene expression. According to the animal studies, expression of the *pmr1* and *tnp2* genes containing *Y-box* in the promoter was controlled by this mechanism and null mice showed a significant reduction in YBX2 expression (50). Moghbelinejad et al. (51) evaluated association of low levels of *PRM* mRNA and *YBX2* gene expression in testicular tissues of azoospermia men. They showed a significant correlation between reduction of *YBX2* gene expression and low level of *PRM2* deficiency in testicular spermatozoa in infertile men. Hammoud et al. (52) explained that the loss of *YBX2* had no effect on transcription, splicing or intracellular mRNA transport, but instead it had a selective effect on the translation rate. With regards to Iranian population, results of Najafipour et al. (18) showed a significant reduction of *YBX2* mRNA level in samples with impaired spermatogenesis (P<0.001) compared to control group.

Non-coding RNAs

Duplication and unsuccessful differentiation of germ cells are the main causes of infertility and they are accomplished by regulating transcription of particular genes. Non-coding RNAs, such as microRNAs and long non-coding RNA (lncRNAs) are the main regulators of the expression of genes. The data obtained from deep-sequencing recently shows that lncRNAs are far more numerous than protein-coding RNAs, thus proving that the human genome is more active in terms of transcription compared to the previous view. Human testis tissue and immature sperm have 7% miRNAs and 17% piRNAs. These small RNAs regulate gene expression at the transcriptional, post-translational and chromatin levels. So far, it has been shown that one-third of human genes are regulated by miRNAs. In terms of numbers, more than 200 miRNAs have thus far been found in human sperm, which indicates the important role of these RNAs in morphogenesis and sperm maturation (53). Some non-coding RNAs associated with infertility in men are described below.

microRNAs

micro-RNAs (miRNAs) have been introduced as the key regulators of gene expression at translation level and control of post-translation changes. Several studies showed that these miRNAs interfere with spermatogenesis in controlling pathways that affect human reproduction, such as the survival of primordial germ cells and spermatogenesis. miRNAs are existed in various stages of spermatogenesis and they have great expression in spermatid and spermatocyte pachytene cells. They exist in the body fluid in combination with lipoproteins or they are enclosed in packages of double-layer membranes called exosomes. Focus on the role of microRNAs in male reproductive disorders can further explain the molecular mechanisms of male infertility and it can create a new pathway as an effective biomarker for treating infertility in men and for contraceptive pills (54). So far, extensive studies have been conducted (55) to determine level of different miRNA expressions and association of their polymorphisms with male infertility (Table 2).

For instance, by examining two miRNAs (miR-100 and let-7b) regulating the alpha estrogen receptor gene in oligospermia men, Abhari et al. (56) found that the expression level of both miRNAs were significantly increased (P=0.008 and P=0.009 respectively) leading to decrease in the expression of alpha estrogen, while estrogen plays a key role in spermatogenesis. They also found a significant expression change in miR-99, miR-196, miR-21 and miR-22 (39).

Table 2: Non-coding miRNAs expression in infertile men

Name	Sample size (case-control)	Sample type	Association	P value	Ref.
miR-21	43-43	Semen	Yes	P<0.0001	(39)
miR-22	43-43	Semen	Yes	P<0.0001	(39)
miR-100	43-43	Semen	Yes	P=0.008	(56)
let-7b	43-43	Semen	Yes	P=0.009	(56)
miR-34c	55 totally	Semen	Yes	----	(58)

Current investigations disclosed that let-7b has an inhibitory effect on cell proliferation (Fig.2) (56). The relationship of ER expressions with miR-7b, miR-21 and miR-22 were previously reported in other diseases (57). In another study, Rahbar et al. (58) showed statistically significant increased expression of miR-34c in moderate oligoasthenoteratozoospermic and non-obstructive azoospermia.

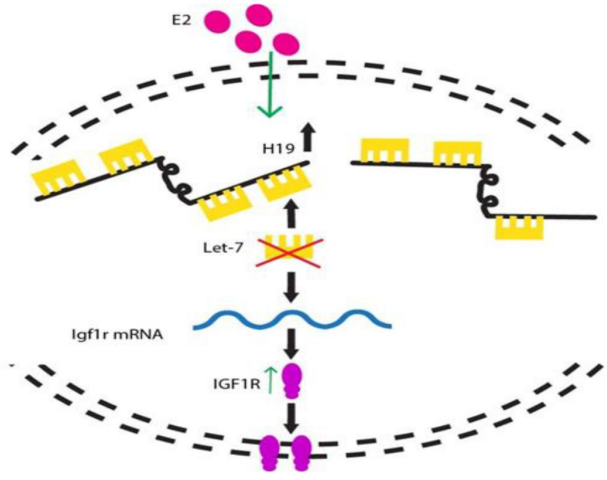

Fig.2: *H19* is a long noncoding RNA (lncRNA) that plays role in cell growth via the microRNA let-7. Decreased H19 expression leads to increased activity of let-7. Aberrations in the *H19*/let-7 regulatory pathway may represent one potential mechanism for male infertility. On the other hands, in the paternal allele of fertile men, *H19* leads to IGF2 expression. Repression of *H19* transcription increase IGF1R expression. Both *IGF2* and IGF1R transcripts are involved in sperm capacitation and embryo growth (illustrated by the author).

Bouhallier et al. (59) demonstrated that miR-34c was highly expressed in mouse germ cells and therefore, they introduced it as a promising candidate gene for test in male germ cell cancer and sterility.

piRNA

piRNA is a group of ncRNAs that act through their interaction with Piwi protein. It has 24-30 nucleotides and specific expression in the testicular tissue as well as the sex cells, while it has not been identified in mature sperm. This group of ncRNAs is often found among clusters of repetitive sequences in the genome and it is not translated into proteins (60). Some of these molecules include PRG-1, HIWI, MIWI2 and piRNA. PRG-2s are produced at the pachytene stage of spermatocytes. There are several reports suggesting that piRNAs protect germ cells from the retrotransposons. Similar to miRNA, piRNAs are the molecules that play important role in the regulation of the post-translation process of germ cells. They are expressed in spermatocytes. They play important role in inhibiting retrotransposition and regulation of gene expression after transcription during meiosis. It has also been shown that due to the role of piRNAs and miRNAs in the pathway of spermatogenesis, applying their inhibitors leads to disturbances in spermatogenesis, in addition to prevention of pregnancy (61). According to the literature, few investigations has been performed in Iran to determine role of piRNAs. In 2010 and 2017, researches on the Iranian and Chinese population disclosed cases and it can be proposed as a risk factor of male infertility (62). The association between *HIWI2* rs508485 (T>C) with non-obstructive azoospermia and *HIWI2* rs508485 (T>C) with non-obstructive azoospermia (male infertility in china) was previously confirmed in the Iranian and Chinese populations. Figure 3 summarized the most prominent study carried out on piRNAs and male infertility: moloney leukemia virus 10-like 1 (*MOV10L1* is a gene involved in piRNA biogenesis, playing a key role in primary and secondary function (63). *MOV10L1* may participate in the binding of primary piRNAs to the PIWI proteins. Several researches approved that many polymorphisms of *MOV10L1* caused a significant enhancement in men's infertility (64).

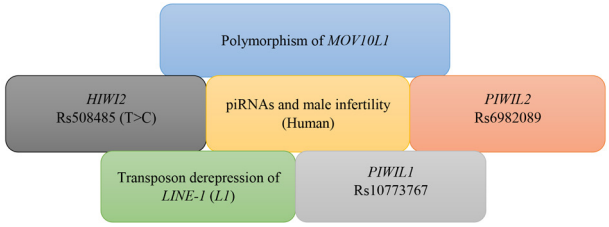

Fig.3: The genes associated with polymorphisms of piRNAs and male infertility. The latest researches on the relationship between piRNAs and human male infertility are showed in this figure (illustrated by the author).

lncRNAs

lncRNAs are dark matter of the genome. So far, a small number of lncRNAs have been studied in male infertility and function and expression pattern of many are yet unknown. Using next generation sequencing (NGS) technology, one of which is RNAseq, shed light on the molecular mechanism involved in infertility disorders, especially structural and functional defects in sexual cells. It is also believed that lncRNAs affect the proteins involved in sperm motility and its entry into the egg as well as the pathways and mechanisms involved in fertility. Therefore, by studying and recognizing these relationships as well as the manipulating and molecular interventions, new approaches and diagnostic-therapeutic kits can be designed. In the study performed by Zhang et al. (65), expression of *HOTAIR* in asthenozoospermic and oligoasthenozoospermic patients was significantly decreased in comparison with the control group. They also found that expression of *HOTAIR* was associated with sperm function parameters, including motility and vitality. On the other hand, Rinn et al. (66), demonstrated that lncRNA *HOTAIR* interacted with the polycomb complex PRC2, which methylates histone H3K27 to promote gene repression.

Discussion

Proper and complete spermatogenesis requires simultaneous expression of a very large number of coding and non-coding genes. So that stopping or disrupting expression of each one can lead to disruption of the spermatogenesis process. Identifying such genes and evaluating their performance provides valuable information about the role of these genes in adult sperm, process of spermatogenesis, their function in embryo fertilization and causes of idiopathic infertility. Genetic and epigenetic factors are elements contributed to this type of infertility.

Therefore, this aspect needs to be considered for the investigation of infertile men. The main emphasis for molecular evaluation and use of RNA sperm is the wide range contribution of male factor in infertility and testicular biopsy, which is a problem in the study of infertile men (67). Those who fail first sperm retrieval may be candidates for the second TESE, hoping to have their biological children. According to the success rate of 60% for TESE, a simple RNA analysis can help predict the relative success of sperm retrieval in biopsy and help in counseling and managing these cases. It may also help the surgeon predict the amount of necessary tissue for sperm retrieval for ICSI or diagnosis in future biopsies (68). Today, with remarkable advances in the field of molecular medicine, study of the RNA content in spermatids is possible by using techniques, such as RNAseq analysis and microarray, which reveals the combination of mRNA in adult sperm and relationship of the specific pattern of these transcripts with fertility and infertility in men. Clinical application of semen and germ cell RNAs is noticeable, due to the fact that sperm can provide the same information. Additionally, non-invasive sampling of the semen is better and more acceptable choice for the patient rather than biopsy (26).

In a study performed to evaluate expression of the specific genes, including *AKAP*, *PRM2* and *DAZ*, it was showed that presence of *DAZ* and *PRM2* genes can be used as a noninvasive molecular marker in seminal fluid of non-obstructive azoospermia patients to predict the presence or absence of sperm or mature spermatids (5). Recent studies showed that miRNAs and their transcripts in the seminal fluid were used to investigate spermatogenesis in infertile men. Although mature sperm is silent in transcription, it contains a set of transcripts of non-coding RNAs and mRNAs that play special role in the early stages of embryonic development as an epigenetic effect. New results from microarray, NGS and RNAseq techniques led to the discovery of new transcripts in sperm and clinical markers of male infertility. It seems that gene expression profile in sperm can help identify the required sperm factors in early embryonic growth. Although the amount of sperm RNA is negligible, it is important for the investigation and diagnosis of male infertility. Transcripts that are specifically expressed in germinal cells and present in adult sperm are suitable molecular markers for the diagnosis of cell lines in spermatogenesis and they can provide a generalized picture of spermatogenesis in the infertile testis instead of invasive testicular biopsy. Evaluation and quantification of more transcript content of normal human sperm could provide the essential biomarkers for assessment of male fertility in the future, while new qualifications and methods must provide for changes in diagnosis (40).

Conclusion

In the scope of infertility investigations, there are several available tests evaluating sperm quality and function. But, there is still a demand for better and more reliable procedures, considering that male factor infertility is involved in at least 45-50% of idiopathic cases. Use of sperm transcripts in molecular analysis of spermatogenesis and infertility treatment is also important, especially in patients with non-obstructive azoospermia. Studies showed that in these patients expression of genes in the pathway for sperm production is changed.

Today, the most common method for evaluating spermatogenesis in these individuals is testicular biopsy which is an invasive practice and recommended as an infertility study tool in the final stage. However, sperm may not be found due to regional spermatogenesis in the testicular tissue taken from the biopsy and need for multiple biopsies of the patient to find and extract the sperm. This procedure can cause tissue atrophy or infection. Thus, using sperm RNA content and preterm sexual cells can evaluate spermatogenesis molecular events. Clinical application of sperm RNA is very valuable because if sperm RNA can provide similar information compare to testis tissue, semen samples are a better and more acceptable choice for the patient than a biopsy. The existence of DAZ transcripts in the seminal fluid of non-obstructive azoospermia can be used as a noninvasive molecular marker to predict presence or absence of adult spermatozoa. From other futuristic studies, transformation of embryonic stem cells or adult stem cells into germinal cells can be a very valuable starting point for solving the infertility problem in an individual whose defect is related to the absence of stem cells in the testes. Research on the presence of RNAs in differentiated sperm from stem cells and normal sperm and their role in *in vitro* spermatogenesis can be suggested in the future.

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Authors' Contributions

F.A.; Designed analyzed data, co-wrote the manuscript and critically revised the manuscript for important intellectual content. F.AA.; Study concept and design, performed the searches and acquisition of data. N.E.; Acquisition of data, co-wrote the manuscript, and performed proof editing. R.M.; Analysis and interpretation of data, critically revised the manuscript for important intellectual content. P.P.; Co-wrote the manuscript, performed proof editing. All the authors provided their final approval for the completed manuscript.

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Original Article

Effects of Three-Dimensional Sodium Alginate Scaffold on Maturation and Developmental Gene Expressions in Fresh and Vitrified Preantral Follicles of Mice

Cyrus Jalili, Ph.D.[1], Fuzieh Khani Hemmatabadi, Ph.D.[1, 2*], Mehrdad Bakhtiyari, Ph.D.[2], Amir Abdolmaleki, Ph.D.[1], Fatemeh Moradi, Ph.D.[2]

1. Medical Biology Research Center, Health Technology Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran
2. Anatomy Department, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

Abstract

Background: Prior to chemotherapy interventions, *in vitro* maturation (IVM) of follicles through vitrification can be used to help young people conserve their fertility. The aim of study was to investigate effect of sodium alginat scaffold on follicles development and improvement of the culture medium.

Materials and Methods: This experimental study was conducted on immature female BALB/c mice (12-14 days). Follicles were gathered mechanically and placed in α-Minimal Essential Medium (α-MEM) containing 5% fetal bovine serum (FBS). Some pre-antral follicles were frozen. The fresh and vitrified follicles were cultured in different concentrations of sodium alginate (0.25%, 0.5%, and 1%) and two dimensional (2D) medium for 12 days. The samples were evaluated for viability percentage, the number of MII-phase oocytes and reactive oxygen specious (ROS) level. Additionally, *Gdf9, Bmp15, Bmp7, Bmp4, Gpx, mnSOD* and *Gcs* gene expressions were assessed in the samples.

Results: The highest and lowest percentages of follicle viability and maturation in the fresh and vitrified groups were respectively 0.5% concentration and 2D culture. There was no significant difference among the concentrations of 0.25% and 1%. Viability and maturation of follicles showed a significant increase in the fresh groups in comparison with the vitrified groups. ROS levels in the both fresh and vitrified groups with different concentrations of alginate showed a significant decrease compared to the control group. ROS levels in follicles showed a significant decrease in the fresh groups in comparison with the vitrified groups ($P \leq 0.0001$). The highest gene expression levels were observed in the 0.5% alginate ($P \leq 0.0001$). Moreover, the viability percentage, follicle maturation, and gene expression levels were higher in the fresh groups than the vitrified groups ($P \leq 0.0001$).

Conclusion: Alginate hydrogel at a proper concentration of 5%, not only helps follicle get mature, but also promotes the expression of developmental genes and reduces the level of intracellular ROS. Follicular vitrification decreases quality of the follicles, which are partially compensated using a three dimensional (3D) cell culture medium.

Keywords: Oocyte Maturation, Sodium Alginate, Vitrification

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Introduction

Beside the advances in the field of *in vitro* maturation (IVM) techniques, low applicable results are available. The IVM techniques can be used to help young people conserve their fertility throughout the vitrification of germ cells, before other therapeutic interventions like chemotherapy or radiotherapy. Over these procedures, the people lost their follicular reservoir partially or completely (1).

The modern technologies can help follicles grow in the culture medium and mature the oocyte for increasing fertility opportunity in patients under chemotherapy and radiotherapy procedures. These interventional methods cause full or partial destruction of the follicular reserve.

In ovulation induction during assisted reproductive technology (ART), more follicles could grow, while the patient is at risk of ovarian hyperstimulation syndrome (OHSS). Conservation of follicles has critical medicinal applications.

The follicle vitrification is considered as a reliable method conserving follicles in the early stages of maturation. Since no ovarian stimulation is required in this approach, thus it can be used as an appropriate method for follicle conservation. Due to the small size, immature follicles are ideal for freezing (2). Ovarian reserve procedures have been developed to conserve the fertility potential in cancerous patients, during gonadotoxic treatments like chemotherapy or radiotherapy (3). Primary follicles can be obtained from fresh and vitrified ovarian tissues. They usually have a small oocyte arrested in the prophase

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*Corresponding Address: P.O.Box: 6714869914, Medical Biology Research Center, Health Technology Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran
Email: fuziekhani@yahoo.com

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stage of miosis. One proper way for conservation of ovarian follicles is known as the slow follicular freezing (4). Formation of ice crystals is a challenging difficulty in freezing process. The vitrification is currently introduced to overcome this issue (5).

In addition to the oocyte physiological conditions, the culture medium as an impressive factor affects the quality of oocytes and embryos. Different types of culture systems have been invented, based on the follicle size and research purposes, to conserve and develop various degrees of follicular development and accompanied somatic cells (6). The low rate of viability and follicular maturation in two dimensional (2D) culture medium leads to application of three dimensional (3D) scaffold providing the proper follicular growth (7). The growth of follicle mainly depends on morphological changes of somatic and granulosa cells. Thus, any impairment in the morphology of follicular cells causes a delay in growth and incomplete development of preantral follicles, similar to those observed in the 2D culture system (8). The granulosa cells available in 2D culture of preantral follicles are scattered around the oocyte which disrupts the stimulation and feedback effects among the oocyte and granulosa cells. These cell communications have significant roles in transmission of both autocrine and/or paracrine signals (9). 3D porous polymer scaffolds as a substrate for cell growth and a provider for sufficient mechanical strength in order to cell survival maintenance are widely used in tissue engineering (10). According to the various studies, culture of preantral follicles with sodium alginate hydrogel can produce oocyte with a potential of high fertility rate (11).

Alginate, like other 3D culture matrices, supply mechanical support for tissues. Nevertheless, the cellular proteins show no reaction with alginate matrix. Alginate hydrogels in culture media also support *in vitro* tissue growth (12). Thus, 3D culture medium conserves follicles more effectively, than the cell culture in flat plates (2D). Alginate is an applicable substrate for microencapsulation due to its biocompatibility, high water dependency and gel formation ability in the presence of sodium ions. This material contains alternant mannuronic acid chains producing tensile strength of gel. Thus, application of follicles without ovulation stimulation and IVM therapy are known as appropriate methods to recruit immature cells and prevent the OHSS.

Application of IVM for immature oocytes is an appropriate procedure for patients, especially those with polycystic ovary syndrome. IVM can reduce ovulation induction and risk of OHSS as one of the clinical emergencies. Thus, these artificial conditions in IVM medium must compensate to the inappropriate conditions available in the patient group. These created artificial environments are similar to that of the normal follicular conditions, which stimulate and mature the oocytes to grow and develop, while prevent the adverse effects of culture medium on the oocyte (13).

Although in many studies the impacts of different alginate concentrations on follicle development have been investigated, there is no study evaluating the effects of culture medium alterations on developmental potential of follicles, the consequences of different concentrations of 3D sodium alginate scaffold (0.25%, 0.5% and 1%) and vitrification on genomic changes of follicles. In the present study we aimed to simulate the development of preantral follicles in 3D culture medium prepared by various concentrations of sodium alginate. One of the crucial challenges is improvement of the culture medium. The purpose of this study was to investigate effect of culture medium changes on developmental potential of follicles as well as effect of different concentrations of 3D sodium alginate scaffold on genomic alterations of follicles.

Medical treatment

Animal groups and ovary preparation

Eighty immature female BALB/c mice (12-14 days) with preantral follicles were grouped into eight (ten animals in each), including non-vitrified and warmed-vitrified groups. The mice were bred based on the 12 light/dark photo-cycle at 23°C and 44% humidity. The animals were sacrificed by cervical dislocation. Following a longitudinal abdominal incision, the ovaries were dissected, the follicles were obtained mechanically and placed in α-Minimal Essential Medium (α-MEM, Aldrich Chemical Co., USA) containing 5% fetal bovine serum (FBS, Aldrich Chemical Co., USA). In order to reach the complete isolation of follicles, the additional tissues attached to the ovary were removed using an insulin syringe needle. It was then washed and incubated in culture medium (2). They were examined at different time points. All materials were obtained from the Sigma-Aldrich company (Aldrich Chemical Co., USA). Animals were handled based on the ethical guidelines of the Iran University of Medical Sciences (Tehran, Iran; Ethical permission number: IR.IUMS.REC.1395.9221313207).

Isolation of preantral follicles

A G29 needle connecting to a 1 ml insulin syringe was used for follicles mechanical isolation by stereomicroscope. Normal preantral follicles with diameter of 100-150 μm were detected as a central oocyte with bilayer granulosa cells.

Preparation of sodium alginate hydrogel

All materials were provided by Sigma-Aldrich Company. To prepare alginate hydrogel, the sodium alginate at the concentrations of 0.25%, 0.5% and 1% was mixed with phosphate buffer saline (PBS). Then, 0.5 g of activated carbon was added to 1 g of sodium alginate powder to remove the alginate impurities. It was followed by filtration using a 0.26 μm Millipore Filter. Finally, it was kept at 4°C (14). Following washing the isolated follicles in culture medium, they were prepared for encapsulation in different concentrations of sodium alginate hydrogel.

Vitrification

The isolated follicles (100-150 μm in diameter) of vitrified-warmed group were washed in medium and transferred to an equilibration solution (pre-treatment) consisting of α-MEM medium, in addition 7.5% Ethylene Glycol (EG), 7.5% Dimethyl Sulfoxide (DMSO) and 10% FBS for 7 minutes. Then, they were transferred to a new vitrification solution, consisting of α-MEM medium with 15% DMSO, 15% EG, 0.5 M sucrose and 10% FBS for 3 minutes. All steps were performed at room temperature (22-24°C) using a stereomicroscope. An insulin syringe with a connector was also used to transfer the follicles into the vitrification straw as following 1 cm of vitrification solution (pulled into the syringe), immediately 0.5 cm of air, then 1 cm of vitrification solution containing follicles, followed ultimately by the air and vitrification solution. The vitrification straws were sealed using hematocrit sealant and immersed in a liquid nitrogen tank for a week (13).

Warming

The straws taken out of the nitrogen tank were left at room temperature (24°C) for 10 seconds. They were cut by scissors and connected to a pulled-end insulin syringe. Contents of the straws were evacuated in a clean plate and transferred immediately to sucrose solutions under stereomicroscopy. 100 μl drops of sucrose solutions were put in a four-well plate. The follicles were placed in various concentrations of sodium alginate 1, 0.5 and 0.25 each and temperature 24°C for 5 minutes. All thawing solutions consisted of α-MEM medium and 10% FBS with descending different concentrations of sucrose, 1 M sucrose (thawing solution 1), 0.5 M sucrose (thawing solution 2), 0.25 M sucrose (thawing solution 3). The follicles were then incubated for 30 minutes in MEM-α medium containing 10% FBS and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B) (13).

Encapsulation and 3D culture of preantral follicles

Initially, the preantral follicles were isolated from ovary and transferred separately into the 5 μl drop of sodium alginate with different concentrations. Then, they were transferred to a calcium bath (containing 140 mM of $CaCl_2$ and 50 mM of NaCl) using a micropipette tip to establish calcium bonds and formulate hydrogel droplets encapsulated follicles. After 2 minutes, alginate hydrogel drops were collected from the calcium bath and rinsed in culture medium. The encapsulated follicles were evaluated by a microscope and only those follicles located in the center of hydrogel were gathered for culturing. Cultivation of the isolated follicles is crucial due to the non-vascular structure of granulosa cells. In this method, the follicles could be cultivated independently and checked for probable changes during cultivation. Each encapsulated follicle was transferred into 40 μl droplets of culture medium beneath the oil in a 96-well plate. In the previous approach, the α-MEM medium which was used contained 1% insulin transferrin selenite (ITS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 IU/ml recombinant follicle stimulating hormone (rFSH), 5% FBS and 10 ng/ml recombinant epidermal growth factor (rEFF). Expire date of the culture medium was increased up to a week at 2-8°C in the refrigerator. The follicles were cultured in a humidified incubator for 12 days at 37°C and 5% CO_2. Half of the culture medium was changed every day (7). Trypan blue as vital stain was used to assess the viability of follicles and non-toxic effects of sodium alginate. For this purpose, several follicles in each replicate were selected randomly and stained with 0.3% trypan blue. The follicles were immersed in the dye for 30 seconds and then were observed under an inverted microscope following several washing processes. The cell membrane of dead cells changed into dark blue, but the living cells resisted against dye penetration and the cell membrane remained unstained.

Follicle retrieval

In order to perform RNA isolation and oocyte maturation, the encapsulated follicles with sodium alginate were removed from the hydrogel substrate on the day 12th. In 3D culture system, 5 mg of ethylene glycol tetraacetic acid (EGTA) was added to the culture medium to deplete the follicles from hydrogel. This process occurred for 5 minutes at 37°C in an incubator. For RNA isolation and other biochemical analyses, the obtained follicles were transferred into the -80°C refrigerator in sterile test tubes with a minimum amount of medium (7). The follicles required for maturation were transferred to a petri dish containing medium.

Ovulation of maturation induction and oocyte maturation

For induction of follicular ovulation, 10 ng/ml of epidermal growth factor (EGF) was mixed with medium containing 1.5 IU/ml human chorionic gonadotropin (hCG). They were suspended with 5% CO_2 and 37°C for 18 hours. Next, 0.1% of hyaluronidase was used to separate the cumulus cells around the oocyte (7). The oocytes were aspirated and counted using a glass pipette and evaluated for maturation, germinal vesicle (GV), germinal vesicle breakdown (GVBD) and metaphase II (MII) by the inverted microscope. Finally, the count and percentage of matured oocytes were assessed.

Evaluation of morphological changes and percentage of follicles viability

An inverted microscope was hired to assess the percentage of follicles viability. Thus, the follicles were examined morphologically at the end of day 12th of culture. The follicles with following features were considered as degenerated: arrest in proliferation of granulosa cells, cessation in follicular growth, early ovulation and dark follicles.

2D culture of follicles

For the control group, the preantral follicles were separately transferred into the α-MEM medium immediately

after removing ovarian tissue. This medium contained 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and growth factors similar to that of 3D culture.

Measurement of reactive oxygen specious in oocyte

Level of the biochemical reaction among H_2O_2 and DCFDA was considered as the level of intracellular free radical. Concentration of the required DCFDA for measurement of H_2O_2 in the oocyte was 10 µM. 50 µl of DCFDA solution (available on plates as droplets) were warmed up in incubator for 30 minutes. The oocytes were placed in droplets of DCFDA and incubated for 20 minutes at 37°C in a dark environment. Next, the oocytes were transferred to other plates for washing and removing the remaining DCFDA. Following the three times washing, the oocytes were transported on the slide with a minimum culture medium (7). The slides were examined using fluorescence microscopy. The reaction between H_2O_2 and DCFDA was visible inside the oocyte, reflecting green light at 460 nm (Olympus fluorescence microscope, Japan). Captured images of oocytes were examined using Image J (1.46r, USA) software. The background color was removed using software tools, but the color intensity of the oocytes also dropped to the same degree to avoid the error. By selecting the oocytes margin utilizing the software, amount of the obtained pixels was determined.

RNA isolation and real-time polymerase chain reaction

Following the IVM procedure, total RNA was extracted using TRIzol (TRI reagent, Sigma, UK). Optical density (OD) was determined using a spectrophotometer and RNA quantity of each sample was also analyzed. cDNA was constructed using Super-script II kit (Fermentase, Germany) in which random hexamer primers were hired to synthesize the cDNA. It was explicitly linked to mRNA as a template and provided the possibility of RNA transcription by a reverse transcriptase enzyme in the presence of dNTP. Real-time polymerase chain reaction (PCR) process was performed in triplicate using cDNA prepared from the oocyte of different groups. In addition, we used primers including ROX dye and SYBER Green Biosystem reagent (Applied Biosystems, USA) as passive control for signal intensity. Real-time PCR procedure was performed in the ABI Prism 7300 Sequence Detector (Applied Biosystems). Amplification carried 45 cycles and the optimal reaction conditions were included activation of polymerase enzyme at 95°C for 10 minutes, each denaturation cycle at 95°C for 15 seconds, annealing and elongation steps at 60°C depending on the temperature of the primer for 60 seconds (7). In addition to the primers designed in this study, *β-actin* primer was used as an internal control for standardizing (Table 1). Total reaction was performed in 45 cycles and three different technical replicates for each group.

Statistical analysis

In this study, all methods were performed in three biological replications. Data were analyzed using SPSS software (version 22). Changes in the viability percentage, MII oocyte, ROS level and gene expression levels were evaluated by One-Way Analysis of Variance (ANOVA) among the groups. Data were represented as mean ± standard deviation (SD) and the significant level was considered as less than 0.0001 ($P \leq 0.0001$).

Table 1: Primers used for real-time polymerase chain reaction

Gene (Mus musculus)	Primer sequence (5'-3')	Annealing temperature (°C)	Product size (bp)
Bmp15	F: CTGATTGAGACCAACGGGAG R: TGCCAGCTTTAACACAGTTTTC	60	181
Bmp4	F: GTAGTGCCATTCGGAGCG R: ATCAGCATTCGGTTACCAGG	58	114
Bmp7	F: CTCAACGCCATCTCTGTCC R: CATCGAAGATTTGGAAAGGTGTG	59	143
Gcs	F: GTACCTTGAACGAGTGGATGAG R: GGTGGGATTTTAAGCAGATGC	62	98
Gdf9	F: GTCACCTCTACAATACCGTCC R: CGATTTGAGCAAGTGTTCC	61	92
Gpx	F: AACCTGACATAGAAACCCTGC R: CAGTAATCACCAAGCCAATGC	59	130
mnSOD	F: GTGAACAACCTCAACGCCAC R: GCTGAAGAGCGACCTGAGTT	60	99
Actb	F: GATTACTGCTCTGGCTCCTAG R: GACTCATCGTACTCCTGCTTG	61	151

Results

According to the Figure 1, the number of the survived follicles in both groups of fresh (n=60, 68, 62, Fig.1A-E) and vitrification (n=58, 62, 59, Fig.1F-J) at different concentrations of sodium alginate was significantly increased, compared to the control group (n=54, 51, P≤0.0001). The concentration of 0.5% sodium alginate in comparison with the other concentrations was increased significantly, but there was no significant difference between the concentrations of 0.25% (n=60, 58) and 1% (n=62, 59). In the concentration of 0.5% (n=68), the survived follicles showed significant increase in the fresh groups in comparison with the vitrified groups (n=62), but this difference was not significant in the other groups (Table 2, Fig.2).

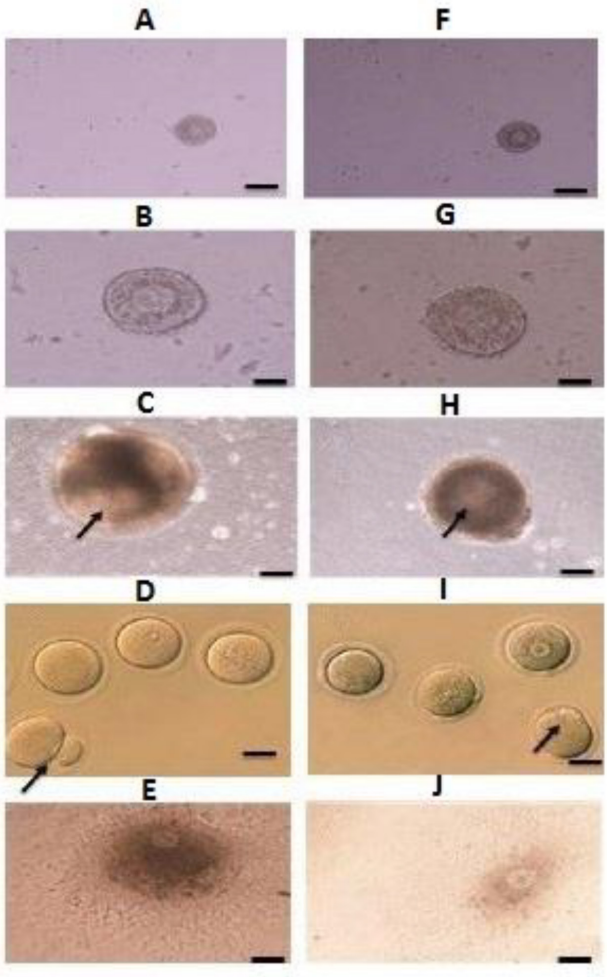

Fig.1: Fresh and vitrified follicles in 3D and 2D culture medium. **A-E.** Fresh, **F-J.** Vitrified follicles in **C, H.** 3D, and **E, J.** 2D culture medium. The arrows indicate in **C, H.** Antrum and **D, I.** Polar body. **A.** Day 1, **B.** Day 6, **C.** Day 12, **D.** MII- phase oocyte, **E.** Follicle in a 2D medium, **F.** Day 1, **G.** Day 6, **H.** Day 12, **I.** MII- phase oocyte and **J.** Follicle in a 2D culture medium (scale bar: 100 µm).

Comparison in follicles number of the fresh groups entered to the MII phase (Fig.2), indicating that the concentrations of 0.5% (n=48) and 1% (n=40) sodium alginate had a significant increase in comparison with the control group (n=32), but in the vitrified groups (Fig 2) only the concentrations of 0.5% (n=41) sodium alginate had a significant increase, in comparison with the control group (n=31, Fig.2); thus a significant increase in maturation level was observed in 3D culture medium (P≤0.0001). Moreover, comparison of maturation level at various concentrations of sodium alginate showed that the highest level of maturation was related to the concentration of 0.5% in the fresh (n=48) and vitrified (n=41) groups (Table 2). According to Figure 2, level of follicle maturation in all of concentrations of alginate as well as the control group was increased significantly (P≤0.0001) in the fresh groups compared to vitrified groups.

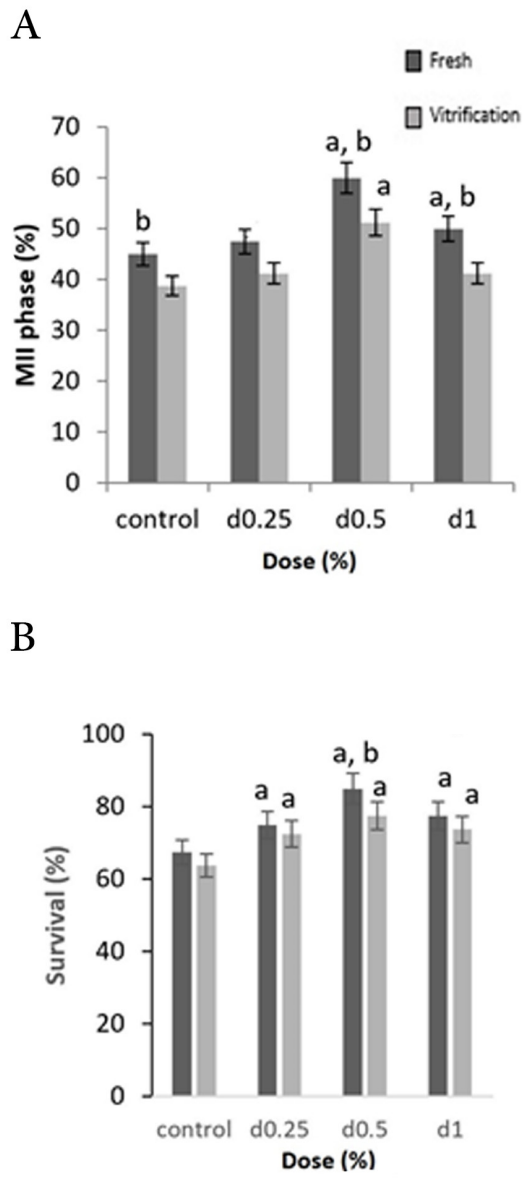

Fig.2: Various concentrations of 3D sodium alginate and 2D culture medium (control). **A.** Number of MII-phase oocytes and **B.** Viability percentage in the both fresh and vitrified groups. The highest viability percentage and MII-phase oocytes are related to the concentration of 0.5%. Number of MII-phase oocytes in the fresh groups is higher than the vitrified groups (P≤0.0001). a, b; Significant (P≤0.0001) difference than control and vitrified groups, respectively.

Table 2: Effects of various concentrations of 3D sodium alginate scaffolds (0.25%, 0.5% and 1%) and 2D culture medium (control) on survival, MII-phase oocytes, germinal vesicle breakdown (GVBD) and GV in the both fresh and vitrified groups

Groups	Alginate density (%)	Follicle (n)	Survival	MII	GVBD	GV
Fresh	0.25	80	60 (75)*	38 (47.5)	25 (31.25)	17 (21.25)
	0.5	80	68 (85)*	48 (60)*	22 (27.5)	10 (12.5)*
	1	80	62 (77.5)*	40 (50)*	26 (32.5)	14 (17.5)*
	Control	80	54 (67.5)	32 (45)	26 (32.5)	22 (27.5)
Vitrification	0.25	80	58 (72.5)*	33 (41.25)	22 (27.5)	15 (18.75)*
	0.5	80	62 (77.5)*	41 (51.25)*	25 (31.25)	14 (17.5)*
	1	80	59 (73.75)*	33 (41.25)	21 (26.25)	16 (20)*
	Control	80	51 (63.75)	31 (38.75)	20 (25)	29 (36.25)

Data are presented as n (%). The highest percentage of survival and MII-phase oocytes is related to the concentration of 0.5%. Number of MII-phase oocytes in the fresh groups is higher than in the vitrified groups. *; Significant difference in comparison with the control group (P≤0.0001).

The *Gdf9* gene expression level in the fresh groups was higher than that of the vitrified groups. In the both control and treatment groups, *Gdf9* gene expression level showed a significant increase in different concentrations of sodium alginate compared to the control group (P≤0.0001) and the highest expression level of this gene was related to the concentration of 0.5% (Fig.3).

In the both fresh and vitrified groups, *Bmp15* gene expression levels showed significant increase in all of the three sodium alginate concentrations, compared to the control group. In the fresh group, the highest *Bmp15* gene expression level was detected in the concentration of 0.5%. Expression level of this gene in 1% concentration was also higher than 0.25% (P≤0.0001). In the vitrified group, the highest expression level of Bmp15 gene was related to the concentration of 0.5% (P≤0.0001), but no significant difference was detected between the concentrations of 0.25% and 1%. In 0.5% and 1% of alginate, the expression level of this gene in the fresh group was higher than the vitrified group, but there was no significant difference between the fresh and vitrified groups at 0.25% concentration and control group (Fig.3).

The 3D sodium alginate scaffold showed positive and significant effects on Bmp7 expression in comparison with the control group (P≤0.0001). In the both fresh and vitrified groups, the lowest level of gene expression was related to the 2D culture medium and the highest expression level of this gene was observed in the concentration of 0.5%. *Bmp7* gene expression level in the fresh groups was higher than the vitrified group (Fig.3). In the both fresh and vitrified groups, *Bmp4* gene expression level was higher in all three concentrations of sodium alginate than in 2D culture medium. The highest expression level of this gene was observed in concentration of 0.5% (P≤0.001). Based on Figure 3, *Bmp4* gene expression level at different concentrations of sodium alginate was greater in the fresh group than the vitrified group (P≤0.0001). As it was shown in Figure 3, *Gpx* gene expression level showed a significant increase in all three concentrations of sodium alginate in the two fresh groups compared to the control group (P≤0.0001). The highest expression level of this gene was related to the concentration of 0.5%. *Gpx* gene expression level was higher in concentration of 0.1% than 0.25%. In accordance with Figure 3, *Gpx* gene expression level in all of the three concentrations of sodium alginate was higher in fresh groups than vitrified groups (P≤0.0001). In the both fresh and vitrified groups, the highest expression level of *mnSOD* gene was related to the concentration of 0.5% (P≤0.0001) and the lowest expression level of this gene was related to the 2D medium. The expression level of this gene was higher in the concentration of 0.1% rather than 0.25%. The expression level of this gene in the concentration of 0.5% in the fresh group was higher than that of the vitrified group, but in the other groups, no significant difference was found among the fresh and vitrified groups (Fig.3). *Gcs* gene expression level was significantly higher in the both fresh and vitrified groups with 0.5% concentration than the concentrations of 0.25% and 1% as well as the control groups. The lowest expression level of this gene was related to the control group. In the both fresh and vitrified groups, no significant difference was detected between the concentrations of 0.1% and 0.25%. Consistent with Figure 3, in all three concentrations of sodium alginate, *Gcs* gene expression level in the fresh group was significantly higher than that of the vitrified group (P≤0.0001).

According to the statistical results, level of free radicals in the fresh groups with 0.5% concentration showed a significant decrease compared to other concentrations and control group (P≤0.0001, Fig.4). The level of free radicals was decreased in both concentrations of 0.25% and 1%, compared to the control group, but there was no significant difference between the two concentrations of 0.25% and 1%. In the vitrified group, the highest free radical level was associated with the control group and the lowest level was related to 0.5% concentration. According to Figure 4, the level of free radicals in the concentration of 0.5% and 2D in the fresh group was lower than the vitrified group (P≤0.0001).

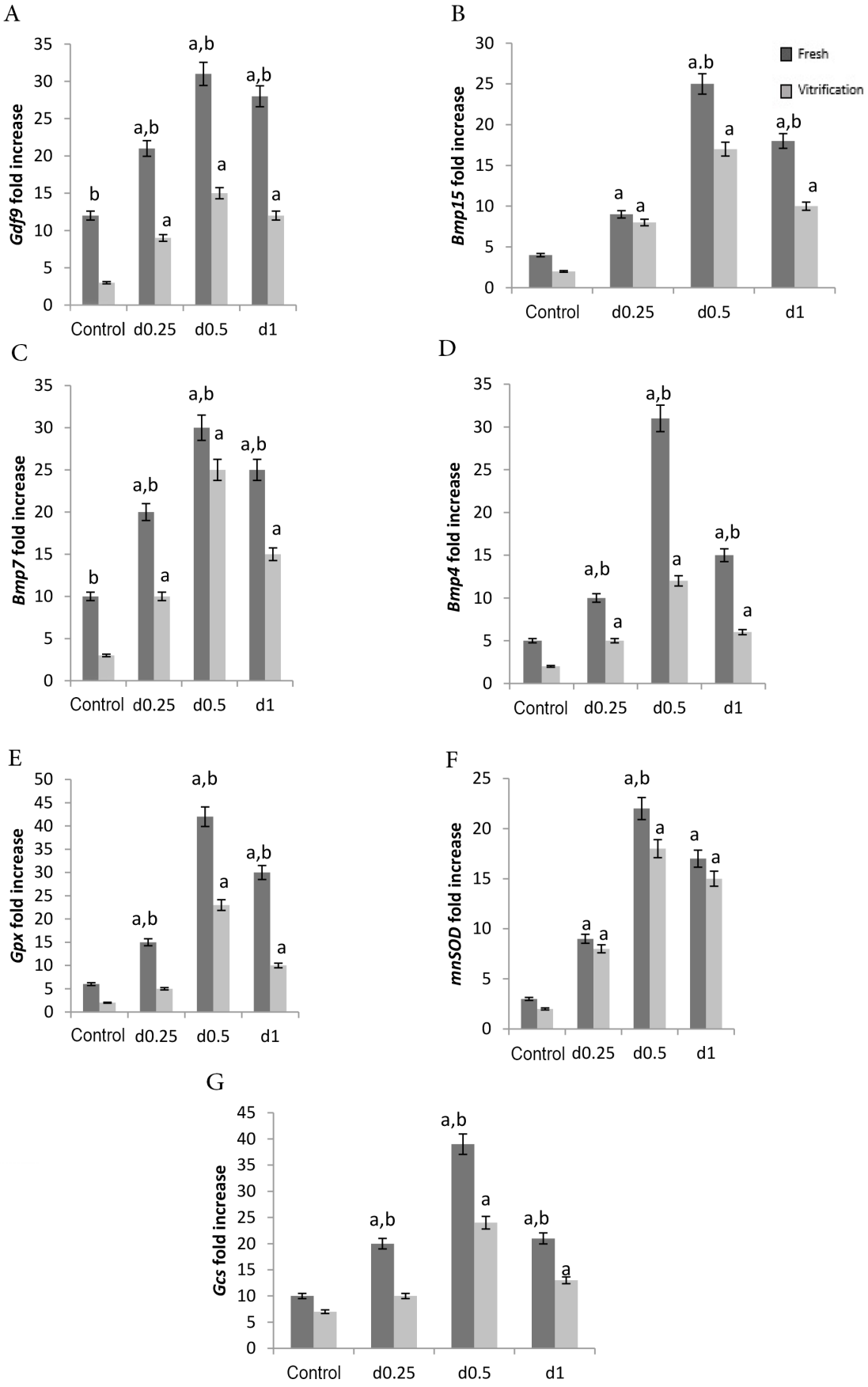

Fig.3: Different concentrations (0.25%, 0.5% and 1%) of 3D sodium alginate scaffolds and 2D culture medium (control) on genes expression. **A.** *Gdf9*, **B.** *Bmp15*, ***C.*** *Bmp7*, **D.** *Bmp4*, **E.** *Gpx*, **F.** *mnSOD,* and **G.** *Gcs*. Their expression level was more than the control group (P≤0.05). In both of the fresh and vitrified groups, the highest expression level of these genes was related to the concentration of 0.5% and the lowest was related to the control group. a; Significant difference than control and b; Significant difference than vitrified group (P≤0.0001).

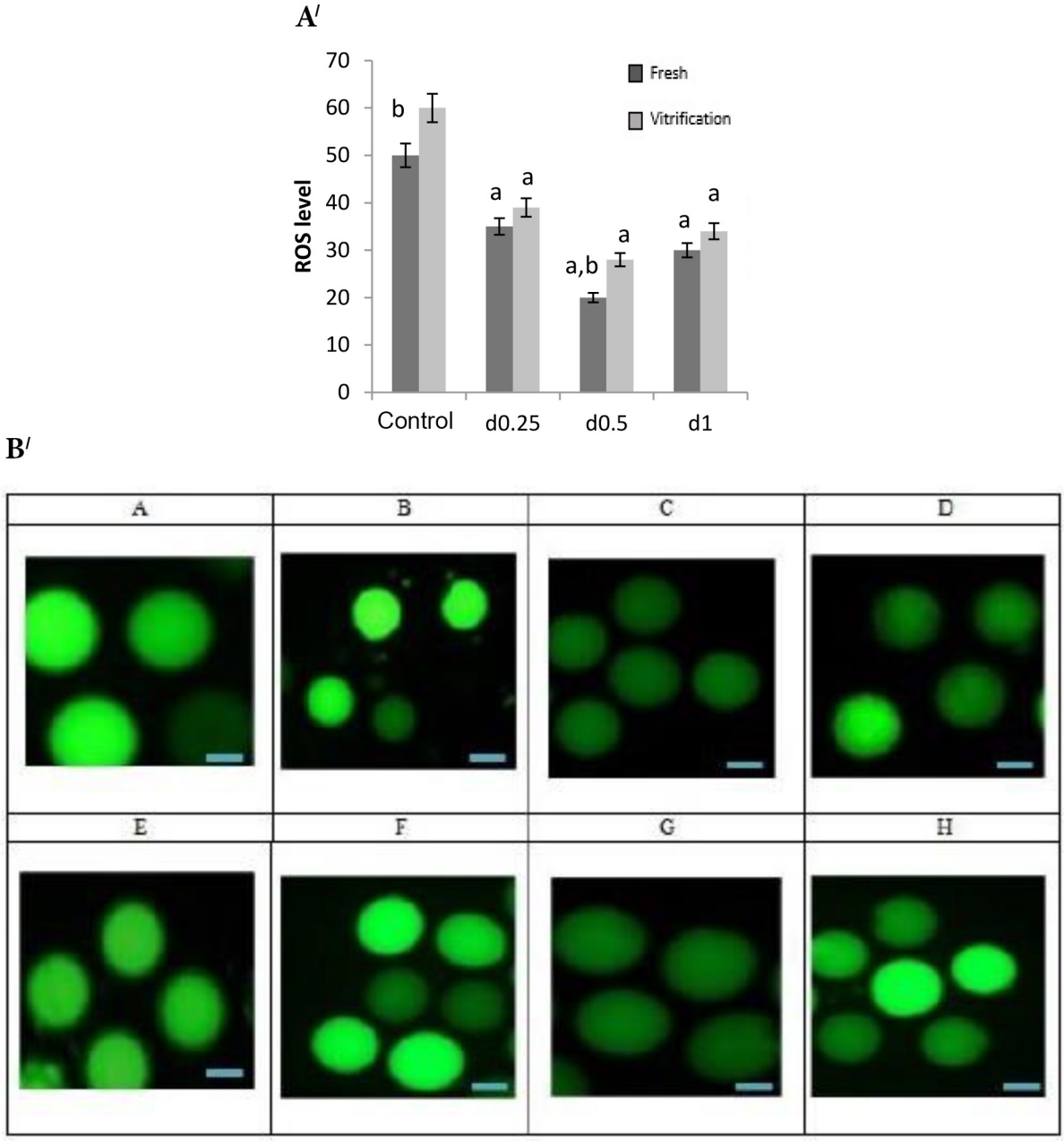

Fig.4: Effects of different concentrations of 2D and 3D culture medium on reactive oxygen specious (ROS) levels in both fresh and vitrified groups. **A/.** Different concentrations (0.25%, 0.5% and 1%) of 3D sodium alginate scaffolds and 2D culture medium (control) on ROS levels; lowest ROS level is related to 0.5% sodium alginate and the highest ROS level is seen in control group. ROS level in 0.5% sodium alginate and 2D culture medium was lower in fresh group than vitrified group. a; Significant difference than control group, and b; Significant difference than vitrified group (P≤0.0001). **B/.** ROS level in the fresh oocytes cultured in 3D sodium alginate medium; **A.** Control group, **B.** Concentration of 0.25%, **C.** Concentration of 0.5%, and **D.** Concentration of 1%. ROS level in vitrified oocytes cultured in 3D sodium alginate medium: **E.** Control group, **F.** Concentration of 0.25%, **G.** Concentration of 0.5%, and **H.** Concentration of 1%. The lowest ROS level was related to the concentration of 0.5% and the highest ROS level for control group (P≤0.0001, Scale bar: 100 µm).

Discussion

The results of present study approved a significant increase in the number of survived follicles in different concentrations of sodium alginate scaffold compared to the control group. According to the obtained results, both encapsulated and decapsulated follicles have not contributed to follicular damage and probably the encapsulated form of follicles can preserve the junctions between cells and the basement membrane of granulosa cells, leading to prevent follicular death (13). A significant increase was detected in the number of survived follicles encapsulated with 0.5% sodium alginate in the fresh groups compared to the vitrified group, but no significant alteration was found in the other concentrations between the other concentrations of sodium alginate in the fresh and vitrified groups. Filatov et al. (15) investigated the effects of vitrification and 0.1% alginate scaffold on follicle maturation. They concluded that 90% of the vitrified encapsulated follicles survived. This finding is in line with the results of our study containing 1% and 0.25% concentration groups as well as the control group.

The hydrated 3D alginate meshwork provides specific

conditions for cells to adhere, disperse, migrate and interact with the others. Thus, hydrogel is known as an excellent option for cell cultivation and differentiation in 3D culture medium. According to the findings of various studies, cultivation of preantral follicles in alginate hydrogel can provide survival of the high rate of in vitro follicles (16).

Follicular maturation is a process influenced by regulatory factors such as gonadotropin, secretory molecules, oocyte, surrounding granulosa cells and biological conditions of the oocyte itself (17). In IVM follicles, cytoplasmic and nuclear maturation occurs following the formation of metaphase II gametes. Significant advances have been made in developing *in vitro* gametogenesis (IVG) and maturation of follicles as well as the oocyte techniques in a wide range of mammalian species (18). Thus, sodium alginate is utilized as a 3D matrix for ovarian follicle encapsulation and maturation to produce the oocytes with fertility capability. This gel with unique biochemical properties is widely used in tissue engineering procedures and follicular culturing (2).

A comparison between the counts of MII phase follicles in the fresh group indicated that sodium alginate scaffold has significant effects on preantral follicle maturation compared to the 2D culture medium. Skory et al. (19) examined the growth of secondary follicles in alginate capsules with concentration of 0.5%. They concluded that 92% of follicles enter to MII phase. In the field of human follicles IVM, Lin et al. (4) concluded that the percentage of MII phase follicles in the 0.5% sodium alginate was decreased. This phenomenon implies that the follicles in humans and primates grow in the ovarian cortex, as an area with a high density of collagen. Thus, higher *in vitro* alginate concentration can provide an environment similar to that *in vivo* condition, resulting in increased levels of follicular maturation. However, due to the low collagen density in the ovary of mice, lower concentrations of alginate scaffold had more significant effects on follicle growth and gene expression. In murine follicles, reduction of alginate concentration in 3D scaffolds can improve the outcomes of IVM technique (4). Besides, the follicular maturation, in different concentrations of alginate, was significantly increased in the fresh groups compared to the vitrified groups. This outcome returns to the hydration-dehydration process during removal of antifreeze in preantral follicles exposed to vitrified-warmed process, which can induce changes in morphological features and survival rate of follicles. Long-term application of vitrification solution for follicles with leads to reduction of viability based on the toxic properties of antifreeze. In presence of the cells limited number, follicular and oocyte survival rates following vitrification is considered as an initial effect of vitrification.

IVM can change levels of gene expression, structure of mitotic spindles and metabolism of oocytes (20). The highest and lowest *Gdf9* gene expression level was related to the 0.5% concentration and 2D culture medium, respectively. Growth differentiation factor 9 (GDF9), through a direct effect on granulosa cells, induces rapid growth of follicles (21). Expression level of *Gdf9* gene in the fresh groups was higher than that of the vitrified groups. *Gdf9* is an ovulation agent strongly expressed in oocytes with a major effect on surrounding cells, especially granulosa, cumulus and theca cells. Paracrine interactions between the growing oocyte and surrounding cells are essential for both oocyte and follicular maturation (22). Song et al. (23) examined *Gdf9* gene expression level after 10 days follicular culture. They concluded that *in vitro* expression level of *Gdf9* gene was similar to the in vivo condition. In the both fresh and vitrified groups of the present study, *Bmp15* gene expression level showed a significant increase in all of the three concentrations of sodium alginate, compared to the control group and the highest *Bmp15* level was related to the concentration of 0.5% sodium alginate. BMP15 is a paracrine signaling molecule that interferes with growth of oocytes and the follicles. This protein may be involved in maturation, ovulation, follicular growth, regulation of the sensitivity of granulosa cells to follicle-stimulating hormone (FSH), determination of the number of ovulating oocytes, prevention of apoptosis in granulosa cells and acceleration of oocyte maturation (24). Both of *Gdf9* and *Bmp15* firstly affect function of granulosa cells and then the oocyte itself (25).

Parrish et al. (26) examined expression level of *Bmp15* and *Gdf9* genes *in vivo* and 0.25% alginate concentrated 3D culture medium. They reported that expression level of these two genes showed no significant difference in medium and 3D alginate culture in the stage of bilayered transition to multilayered secondary follicles. This finding confirmed effectiveness of 3D alginate scaffolds to stimulate physiological environment similar to that of the body to express developmental genes correctly. Expression level of *Bmp7* gene in different concentrations of sodium alginate in both of the fresh and vitrified groups showed that 3D sodium alginate scaffold has significantly positive effect on *Bmp7* expression in comparison with the control group. In the both fresh and vitrified groups, the lowest expression level of gene was related to the 2D culture medium and the highest level was seen in 0.5% concentration. *Bmp7* plays a vital role in the transition of primordial follicles to primary, preantral and antral follicles. *Bmp7* is secreted by theca and granulosa cells, which induces increasing and decreasing effects of FSH on estradiol and progesterone levels (27). In all of the three concentrations of sodium alginate, *Bmp4* gene expression level was more than 2D culture medium and the highest expression level of this gene was observed in concentration of 0.5%.

Bmp4, as a paracrine growth factor, is secreted by theca and granulosa cells. *Bmp4*, along with *Bmp7*, is responsible for follicular growth regulation and primordial follicles transition to the primary form (28). West-Farrell et al. (28) examined expression levels of Bmp4 and *Bmp7* genes in sheep follicles. They observed that these two genes were not expressed in sheep follicles, while they were significantly expressed in mice follicles caused follicular development. Levels of gene expression of *Bmp4*

and *Bmp7* were more in the fresh group compared to the vitrified group as well as the 3D culture compared to the 2D culture medium. Low expression of these genes in the follicle of 2D culture medium is different from the various concentrations of sodium alginate, because growth of the follicles depend on somatic and granulosa cells and any disorder of follicular cells causes a delay in preantral follicles development (29). In 2D culture of preantral follicles, granulosa cells disperse around the oocyte, resulting in a decreased relationship between oocyte and granulosa cells (30).

The ovulation process, which is associated with molecular, cellular and biochemical changes, can lead to increase in the level of ROS. Physiological levels of ROS are essential for ovulation, normal function and success in conventional assisted reproductive technologies (14). *mnSOD* gene expression level in all of the three concentrations of sodium alginate was significantly higher than the control group. Among the different concentrations of sodium alginate, the highest and lowest expression levels of *mnSOD* gene were observed in 0.5% and 0.25% concentrations, respectively. Activation of transcription factors to enhance the antioxidant gene expression is a defense mechanism against free radical activity. The antioxidant enzymes are considered as the first defense line to metabolize toxic substances into harmless products. The first step in neutralizing the free oxygen radicals in the presence of superoxide dismutase (mnSOD) occurs in the mitochondria. Under the influence of SOD, superoxide anion is converted to H_2O_2 with no radical activity but unfortunately it is changed rapidly to highly reactive hydroxyl radicals (31). Expression level of Gpx and Gcs genes in the all three concentrations of sodium alginate showed a significant increase compared to the control group. The highest expression level of this gene was related to the 0.5% concentration of sodium alginate. The first step in H_2O_2 removal is the presence of antioxidant enzymes like glutathione peroxidase (GPX) and glutamyl cysteine synthase (GCS) in cytosol and mitochondria. These enzymes convert H_2O_2 into $_2H_2O$. Combelles et al. (31) assessed the antioxidant genes in humans and mice. They found that only the Gcs gene was expressed in humans at the GV stage, but all of *Gpx*, *Gcs* and *mnSOD* genes were expressed in the MII phase. However, all of the mentioned genes were expressed in mice in both GV and MII phase.

Physical properties of the hydrogel are considered as the most critical factors affecting cell proliferation, growth factors, extracellular matrix and gene expression (32). As a result, the proper concentrations used in alginate hydrogel formation can provide the nutrient exchange, hormones and follicle expansion (33). In this study, the optimal concentration of alginate hydrogel for IVM was recognized at 0.5% concentration. The expression level of antioxidant genes in the fresh groups was higher than those of vitrified groups.

According to our results, level of the free radicals in both of the fresh and vitrified groups with 0.5% concentration showed significant decrease compared to the other concentrations and control group. Level of the free radicals in the fresh group with 0.5% concentration of sodium alginate and 2D culture medium was lower than the vitrified group. ROS has a dual function in culture medium; thus its certain level in IVM medium leads to the resumption of meiosis and maturation of the oocyte. Increased level of that is associated with a decreased level of follicle maturation and cessation of the cell cycle of egg cell. Thus, ROS in the IVM medium should be controlled in such a way to reduce the destructive effects (34). An inappropriate culture medium can reduce quality of the follicle. As it has been shown, developmental potential of the follicles is lower in the IVM medium than the follicle passing through the maturation stages *in vivo* conditions (35). In the culture medium, ROS production is also inevitable. Thus, the use of a culture medium with a concentration similar to the internal environment of the body can regulate the physiological levels of ROS (36, 37). According to the results of this study, the optimal concentration of sodium alginate to reduce the ROS level is found in 5%. This study showed that follicle maturation has several stages and the follicle vitrification with intrinsic potential property can damage the follicles.

Conclusion

The antifreeze agents used for the vitrification process could damage the cells by reacting with intracellular biomolecules and producing toxic substances. If these materials are used at high concentrations with enough time to contact with cells, their detrimental toxicity will appear causing cell destruction and death after the thawing step. But we used the method of incremental addition of the antifreeze to minimize its toxic effects and damages caused by increased cell quantity. Our results revealed that encapsulation of the follicles could conserve structure of the junctions between the cells and basement membrane of the granulosa cells, while preventing death of follicles.

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Authors' Contributions

F.Kh.H., C.J., M.B.; Contributed to conception and design of the study. F.Kh.H., A.A., F.M.; Carried out all experimental work, contributed to data and statistical analysis, as well as the interpretation of data and drafted the manuscript. C.J., F.Kh.H.; Were responsible for

overall supervision. A.A; Corrected the grammatical mistakes and improved English writing. All authors read and approved the final draft of the manuscript.

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Original Article

Influence of Autologous *In Vitro* Activation of Ovaries by Stem Cells and Growth Factors on Endocrine and Reproductive Function of Patients with Ovarian Insufficiency-A Clinical Trial Study

Suada Tinjić, M.D., M.Sc.[1*], Džihan Abazović, M.D.[2], Dušica Ljubić, B.Sc.[3], Danilo Vojvodić, Ph.D.[4], Tatjana Božanović, Ph.D.[5], Mirza Ibrišimović, Ph.D.[1, 6], Sergije Marković, Ph.D.[7], Aleksandar Ljubić, Ph.D.[8]

1. Department of Gynecology, Polyclinic, Korak do Života, Tuzla, Bosnia and Herzegovina
2. Department of Regenerative Medicine, Remedika Hospital, Belgrade, Serbia
3. Department of Gynecology, MediGroup Hospital, Belgrade, Serbia
4. Faculty of Medicine, Military Medical School, Belgrade, Serbia
5. Department of Gynecology and Obstetrics, Medical School, University of Belgrade, Belgrade, Serbia
6. Department of Medical Biology and Human Physiology, Sarajevo Medical School, University Sarajevo School of Science and Technology, Sarajevo, Bosnia and Herzegovina
7. Department of Histology, Medical School, University of Tuzla, Tuzla, Bosnia and Herzegovina
8. Department of Gynecology, Dubrovnik International University, Dubrovnik, Croatia

Abstract

Background: Premature ovarian failure (POF) can be found in 1% of women at the age of 35-40, mostly due to unknown causes. PI3K-Akt signaling is associated with both ovarian function and growth of primordial follicles. In this study, we examined the effects of autologous *in vitro* ovarian activation with stem cells and autologous growth factors on reproductive and endocrine function in patients with ovarian impairment.

Materials and Methods: The longitudinal prospective observational study included 50 patients (between 30 and 50 years) with a diagnosis of POF and infertility. This multicenter study was performed at Jevremova Special Hospital in Belgrade, Saint James Hospital (Malta), and Remedica Skoplje Hospital, between 2015 and 2018. All patients went through numerous laboratory testings, including hormonal status. The autologous bone marrow mesenchymal stem cells (BMSCs) and growth factors were used in combination for activation of ovarian tissue before its re-transplantation. The software package SPSS 20.0 was used for statistical analysis of the results.

Results: Differences in follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), and progesterone (PG) hormone concentrations before and after 3, 6, and 12 months post-transplantation were tested in correlation with the volume of transplanted ovarian tissue. A significant correlation (P=0.029) was found between the change in E2 level after 3 months and the volume of re-transplanted tissues. Also after re-transplantation, 64% of the patients had follicles resulting in aspiration of oocytes in 25% of positive women with follicles.

Conclusion: The SEGOVA method could potentially solve many human reproductive problems in the future due to the large number of patients diagnosed with POF, as well as the possibility of delaying menopause, thus improving the quality of life and general health (Registration number: NCT04009473).

Keywords: Growth Factors, Ovarian, Premature Ovarian Failure, Stem Cells

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Introduction

Female gametes are extremely sensitive cells in the human body, which decrease in number drastically from fetal period to the prepuberty when they count about 300 000. Their number continues to decrease in life, so that the residual pool in women over 35 years of age is about 25 000 oocytes (1). Early folliculogenesis begins autonomously and independently from follicle stimulating hormone (FSH). Inactive primordial follicles form an ovum reserve that is continually reduced by aging, genetic factors, drugs and surgery.

Follicles show initial growth but end with atresia. Those which continue to grow and develop further become preantral, and when they achieve 3-6 layers of granulosa cells, cell mobilization from the ovary stroma begins, and

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***Corresponding Address: Department of Gynaecology Polyclinic, Korak do Života, Tuzla, Bosnia and Herzegovina**
Email: suada.tinjic@gmail.com

the cells gradually group in the outer sides of the basal membrane. This event simply does not occur if the oocyte does not secrete biosignals, mainly the differentiating growth factor 9 (GDF-9) (2).

Peptide hormones of granulosa cells are important paracrine modulators of theca cells, and also affect the functions of gonadotropic pituitary cells. These effects in the middle of the follicular phase lead to the selection of a dominant follicle and formation of a preovulation follicle. Antimüllerian hormone (AMH), with its paracrine action, inhibits the FSH effect, and together with the pituitary gland, reaches its maximum production on the seventh day of the follicular phase, selectively inhibits the release of FSH and leads to atresia of the competitive follicle (3).

From 300,000 follicles at the beginning of puberty, 450 of them achieve the ovulation phase, while about 660 follicles are consumed for one ovulation. In each ovary we normally find 5-15 antral follicles called antral follicular count (AFC), which is correlated with both the level of AMH and the concentration of inhibitors B, in particular (4). In 1967, De Moraes Rueshen and Jones were the first to set the definition of primary premature insufficiency of the ovary (POF) as a non-physiological interruption of menstruation after puberty (1). POF is a syndrome recognized by hypergonadotropic amenorrhoea and hypoestrogeny. There are questions arising about possible existence of a common pathway that controls egg cell atresia or the possibility of its activation by different gene mutations (5, 6). The medical treatment of POF patients should include the following aspects: ovarian hormone replacement, fertility restoration, and improvement of the patient's psychological aspect as a prevention of complications arising from loss of ovarian function that affect the quality of life.

The SEGOVA is a group of procedures and methods designed by Ljubić et al. (7) to improve women's general and reproductive health by restoring ovarian function and improving the quality of life. This method offers minimally invasive approaches including ovarian cortex laparoscopic biopsia and retransplantation to the same site from which the tissue was taken for *in vitro* activation. The second laparascopic surgery is avoided together with chemical stimulation of genetic pathways due to application of autologous platelet rich plasma (PRP) growth factors. This novel approach involves application of bone marrow stem cells (BMSC) that can be readily obtained and amplified in large quantities *in vitro*. BMSCs are a good choice for transplantation in patients with POF. BMSCs migrate to the impaired ovary and together with cytokines that have antiapoptotic, antifibrotic, anti-inflammatory and immunoregulatory effects improve ovarian function (8, 9).

The results of this novel study indicate that the SEGOVA procedure can play a potentially important role in improving endocrine ovarian function in patients with impaired or lost ovarian function.

Materials and Methods

Study design and selection of respondents

In this longitudinal prospective observational study, we included 50 patients in the age of 30 to 50 years with a diagnosis of POF and infertility (Clinical Trials.gov Identifier: NCT04009473). The study was performed at Jevremova Special Hospital in Belgrade, Sant James Hospital in Malta, and Remedica Skoplje Hospital, between 2015 and 2018. The criteria for patient involvement included the following: signed consent form, women over 18 years of age, primary or secondary amenorrhea for at least 3 months, AMH hormone values <0.42 ng/ml and FSH >20 IU/L, and/or failure of previous attempts at assisted reproductive techniques due to limited ovarian response (less than 4 oocytes obtained), normal karyotype 46, XX, presence of at least one ovary, normal anti-microsomal antibodies, and proper thyroid function as evidenced by normal serum levels of thyroid stimulating hormone (TSH). The exclusion criteria included: currently pregnant or breastfeeding, history or evidence of existing gynecological malignancy, the presence of adnexal masses indicating the need for further evaluation, mental health disorder, active substance abuse or addiction, existence of contraindication to laparoscopic surgery and/or general anesthesia in the past two weeks, use of the following medicines: oral or systemic corticosteroids, hormones (estrogen, progestin, oral contraceptives), Danazol, anticoagulants, herbal or botanical supplements with possible hormonal effects, type I or type II diabetes mellitus or if they are receiving antidiabetic medicines, known significant anemia (hemoglobin <8 g/dL), deep vein thrombosis and/or pulmonary embolus, cerebrovascular disease, presence of heart and liver disease [defined as aspartate aminotransferase (ASt) or alanine aminotransferase (ALT)> 2 times normal, or total bilirubin> 2.5 mg/dL], and presence of kidney disease (defined as blood urea> 30 mg/dL or serum creatinine> 1.6 mg/dL). In addition, patients with positive history of cancer, in case of any positive tumor markers, or with structural changes on the ovaries, would be immediately excluded from the procedure. However, no such patients applied for this study.

Before the procedure, patients had to have balanced nutritional status, optimized trace elements and antioxidants through proper nutrition and supplements, as well as optimized physical activity (exercise plan).

Preparation for the SEGOVA procedure

All patients went through the following laboratory testing:

1. (Hormonal status, Ca-125, Ca-19, Ca-19,9:HE-4, ROMA index, Beta HCG, AFP, SE , CRP);
2. Karyotype;
3. Microbiological analysis of vaginal and cervical swabs with antibiogram, cervical swabs on Ureoplasm, Mycoplasma and Chlamydia) - not older than 3 months;
4. Kg Rh D factor, total blood cell count, blood glucose, urea, creatinine, K, Na, NaHCO, proteinogram, coagulogram;

5. Serological analysis: HIV, HBSAg, HCV;
6. Lung RTG, findings and opinion of internal medicine doctors for general anesthesia;
7. 3D ultrasound (presence of antral follicles and their number, ovarian volume), personal and family history, length of infertility, number of *in vitro* fertilization (IVF) cycles.

All patients answered questions about menstruation, parity, the prevalence of POF in the family, education, and possible pre-amenorrhea stress, and a quality of life questionnaire. In addition, all women diagnosed with POF included in this study had normal results for the following: blood count, glucose, and lipoprotein profile, prolactin, testosterone, androstenedione, DHEA sulfate, 17-OH progesterone, thyroxine, TSH, parathyroid hormone, adrenocorticotropic hormone and cortisol. All tested patients had negative test results for anti-ovarian antibodies, anti-cardiolipin antibodies, anti-thyroglobulin antibodies, anti-microsomal antibodies, and had a normal karyotype. Sonographic examination of the pelvis in all patients showed that there were no pathological changes in the ovarian or follicular activities. All women included in this study signed a written consent approved by the Ethic Committee of Jevremova Special Hospital for Gyneclogy in Belgrade, Serbia (number: 63/2015).

The SEGOVA method

The SEGOVA is a group of procedures and methods designed by Professor Aleksandar Ljubić to improve women's general and reproductive health by restoring ovarian function and improving the quality of life. The SEGOVA approach has several important advantages over other in vitro ovarian activation approaches

1. First, conservative minimally invasive surgery (ovarian cortex laparoscopic biopsy). This approach permits re-transplantation to the same site, from which the tissue was taken for *in vitro* activation.
2. Autologous platelet-rich plasma (PLRP) growth factors are used instead of chemical stimulation of genetic pathways.
3. Second laparoscopic surgery is avoided, *in vitro* activated ovarian tissue is re-transplanted through ultrasound control.

The procedure consists of several stages:

1. Laparoscopic ovary tissue biopsy (the isolated ovarian tissue was sent for pathohistological analysis and none of the samples were positive for malignancy);
2. Under laboratory conditions, micro-fragmentation inhibits a group of HIPPO genes that adversely affect follicular growth.
3. Autologous activation is then performed by the growth factors of the AKT group of genes that positively influence the growth and development of the follicles;
4. Bone marrow biopsy and MSC processing;
5. Transplantation of activated ovarian tissue under the control of 3D doppler ultrasound into the ovary;
6. Bone marrow stem cell implantation into ultrasound-controlled ovarian tissue;
7. Activated tissue has an effect on the cells, which triggers the function of hormone production, follicular growth, and the activation and differentiation of oocytes. The hormonal status of the patients were always assessed on days 2-5 of menstrual cycle.

Description of SEGOVA procedure and isolation of stem cells

The procedure of surgical resection of the ovarian cortex is performed using a modified "single port" laparoscopic technique with an entrance through the umbilicus. After an incision of about 2 cm in the umbilical zone, three 5 mm portals will be placed, establishing an intra-abdominal pressure between 10 and 12 mm Hg. A 5 mm diameter laparoscope is then introduced, as well as auxiliary trocars. In some cases, and as an aid to the technique, a uterine manipulator is introduced transvaginally. After visualization of the ovaries, the cortex is fixed with the help of adequate instruments and cut with another instrument (scissors). Hemostasis control, port removal and wound closure are performed subsequently.

Further, the obtained ovarian cortices are chopped by multiple cuttings with a scalpel no. 25 to fragments that are smaller than 1×1 mm. The tissue prepared in this way is weighed using an analytical balance and placed in a petri dish, where it is washed with gamete buffer (Cook). After washing, tissue samples are transferred to PRP medium and further activated by autologous thrombin medium (Sigma-Aldrich). The sample prepared in this way is incubated for 48 hours at a temperature of 37°C and in 5% CO_2. In addition, under ultrasound monitoring with a 3D color ultrasound (GE Voluson 730 Pro), a transvaginal puncture of both ovaries is performed under general anesthesia, with a 30 cm long 16 gauge needle. After incubation, fragmented ovarian tissue with PLRP is injected into the subcortical region of the right and left ovaries (2.3 ml and 2.1 ml, respectively).

Bone marrow cells are taken from the tibia or iliac bone. The biopsy is performed under general or local anesthesia. A small incision (7 mm) is made with a special needle to penetrate the periosteum. A volume of 100 to 150 ml of bone marrow is aspirated and centrifuged in a particularly automated Angel system-Arthrex under sterile conditions. After extraction, the samples were centrifuged to separate the acellular part and the erythrocytes which were discarded from the nucleated cells. Nucleated cells were further used for treatment. After treatment, 3-5 mL of bone marrow aspirate concentrate (BMAC) is obtained. Bone marrow concentrate contains a significant number of stem cells, leukocytes, platelets, erythrocytes, hematopoietic stem cells (HSCs), and MSCs. Each sample was analyzed by

flow cytometry using specific markers: CD34, CD146, CD90, CD45, CD105, CD73, CD133, Stro-1. Cell viability and the total nucleated cell count (TNCs), were assessed using 7AAD. The ovarian tissue is microfragmented, incubated and activated by autologous growth factors. After incubation, the prepared tissue is mixed with BMAC and re- transplanted via intraovarian injection.

Platelet rich plasma preparation

The platelet-rich plasma (PRP) procedure involves drawing blood from patients (104 ml) with a special double syringe, which is then left to be centrifuged at the appropriate number of revolutions over a period of time to separate plasma-rich growth factors. A separate syringe enriched with platelets is then drawn with another syringe and used for further treatment. The whole blood will be processed using the Angel separation system (Arthrek, USA). Separation of PRP from low-platelet plasma is performed in a closed system - in a fully automated machine designed for this purpose. This machine can process from 40 ml to 180 ml of blood taken from the patient. Under sterile conditions, using the Angel system, 18 times higher the platelet concentration in PRP than the baseline measured value in the patient can be obtained. In our case, the optimal concentration is between 6 and 8 times more concentrated than in patient's blood, which can be determined and adjusted on the machine. For the PRP procedure and laboratory analysis, a whole blood sample will be taken from the cubital vein and mixed with Anticoagulant Acid Citrate Dextrose (ACD) in a ratio of 7: 1. Two 60 mL syringes will be taken and filled with up to 60 mL blood, making a total volume of 104 mL whole blood and 16 mL ACD. The second phase, or laboratory phase, involves the application of complex technology, which uses special separators and systems, divides and filters certain cells, prepares them and activates the growth factors in them. At the end of this procedure, we get between 5-7 mL of active plasma containing a large amount of growth factors, including PDGF, TGF-β, VEGF, EGF, and many others. In the next step, a volume of PRP product (5 mL) is mixed with the prepared ovarian fragments. Immediately before administration, PRP is activated by autologous thrombin in a 10: 1 ratio. The maximum time interval from activation to the end of instillation in the ovary is 45 seconds. Activation is performed for each ovary separately.

Parameter monitoring

The changes in hormone levels in serum of the patients were evaluated 3, 6 and 12 months after the procedure. The effects of hormonal changes on ovarian reproductive function (follicle count, number and quality of aspirated cells, and number of embryos) were monitored using ultrasound and based on the outcome of IVF procedures.

Statistical analysis

Since the observed sample size of the included women was relatively small, and the data on the level of observed hormones, the volume of ovarian cortex autografted and the volume of the BMAC of the patients were not homogeneous, different methods of nonparametric statistics were used for data analysis, depending on the specific goals of the study. The data was processed in the statistical package SPSS 20.0. To investigate the changes in the levels of the hormones FSH, LH, progesterone (PG) and estradiol (E2), the amounts detected prior to the intervention, as well as 3, 6 and 12 months post-intervention, were compared using Wilcoxon's rank test. Namely, the following null hypotheses have been tested: median levels of a particular hormone detected before the intervention do not differ from the median hormone levels at 3 months post-intervention, 6 months post-intervention and 12 months post-intervention. The alternative hypothesis is that differences among hormone medians measured at different time points do exist. The hypotheses are being tested using Z statistics, based on the data difference directions, but also the relative strength of these differences. This approach represents one of the most useful non-parametric methods. The test statistic Z is approximately normally distributed with an arithmetic mean equal to zero and a variance equal to one, and has the form of:

$$Z = \frac{W - \mu_W}{\sigma_W},$$

where W is a statistic representing a smaller sum of rankings with one character

$$W = min(W_-, W_+);$$

μ_w is the arithmetic mean of the W statistics and is calculated according to the formula:

$$\mu_W = \frac{n(n+1)}{4};$$

a σ_w represents the standard deviation of the statistics W and is obtained according to the formula:

$$\sigma_W = \sqrt{\frac{n(n+1)(2n+1)}{24}}$$

In order to investigate the effects of the volume of re-transplanted activated ovarian tissue on changes in hormone values of FSH, LH, PG and E2, Spearman's coefficient of correlation of the rank was used. The Spearman's rank correlation coefficient r_s measures the dependence between the rankings of two data sets and is obtained according to the formula:

$$r_s = 1 - \frac{6\sum_{i=1}^{n} d_i^2}{n(n^2-1)},$$

where d_i is the difference between the rankings of two data sets. Certainly, after calculating the value of the Spearman's rank correlation coefficient, it is necessary to check the statistical significance. Testing is done by checking if the null hypothesis coefficient is not statistically significant (H_0:ρ_s=0) against the alternative hypothesis that it is statistically significant (H_0:$\rho_s \neq 0$). The test statistics formula used for this purpose is:

$$t = \frac{r_s - \rho_s}{\sqrt{\left(\frac{1-r_s^2}{n-2}\right)}}.$$

Results

Average hormonal levels over twelve months after the intervention

The follow-up of hormones FSH, LH, E2 and PG included evaluation of the changes in values of the observed hormones before the intervention and at three, six, and twelve months post-intervention.

When observing the pre-intervention period, our results showed that 50% of the women had a FSH hormone value ranging from 28.10 to 86. 50 IU/mL, while the median value of this hormone was 46.50 IU/mL. These values were obtained on the basis of a sample of 47 women.

Three months after re-transplantation, the median FSH level was raised (2.68%) compared to the pre-intervention period and amounted to 47.75 IU/mL, with 50% of the women ranging between 24.28 and 86.50 IU/mL. These values were obtained from 19 women at 3 months following the intervention.

Three months after re-transplantation, the median FSH level was raised (2.68%) compared to the pre-intervention period and amounted to 47.75 IU/mL, with 50% of the women ranging between 24.28 and 86.50 IU/mL. These values were obtained from 19 women at 3 months following the intervention.

For the period of 6 months after intervention, the median FSH hormone value was 34.49 IU/mL, and 50% of the women had a FSH hormone value between 15.80 and 63.50 IU/mL. These values were obtained from 23 women. At 6 months post-intervention, we noticed that both median values were decreased by 25.82% compared to this value prior to the intervention.

The last follow-up measurement was done at 12 months post-intervention, when the mean FSH hormone value was 35.36 IU/mL (23.95% decrease compared to the pre-intervention value), while 50% of the women had a FSH level ranging from 21.86 to 70.08 IU/mL. Due to the loss of the follow-up data in some of the patients at 12 months after the intervention, the results were obtained on a sample size of 14 women. The median values of the FSH hormone before intervention and at 3, 6 and 12 months after the procedure, are shown in Figure 1.

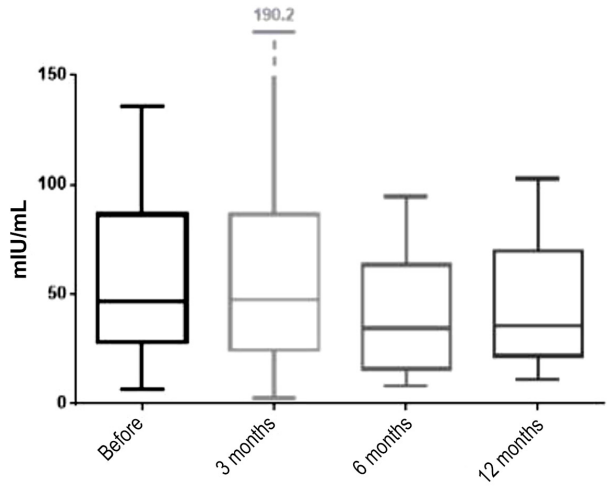

Fig.1: Medians, interquartile range and range of follicle stimulating hormone (FSH) before the procedure and 3, 6, and 12 months after procedure (line inside the box is median; the box is interquartile range; the lines outside the box are minimum and maximum values).

Before the intervention the median value of the hormone LH was 24.80 mIU/mL, based on the measurement of the hormone value in 45 women. The median LH value was slightly decreased (7.29%) after 3 months and was 22.99 mIU/mL. At 6 months post-intervention it further decreased to 18.60 mIU/mL median value (25% decrease compared to before the intervention). After twelve months the median value was 20.19 mIU/mL, which is 18.58% reduction compared to the value prior to the intervention. These values were obtained from sample sizes of 20, 21, and 16 at 3, 6 and 12 months, respectively. We can also observe the change in the borderline interval values of hormone LH, resulting from 50% of the women. Before the interventions the boundaries of this interval ranged from 15.22 to 46.56 mIU/mL, while 3 months after the intervention the boundaries were 9.23 to 41.73 mIU/mL, from which we can conclude that the interval of variation of these data has decreased. Six months post-intervention, the boundaries ranged from 11.00 to 32.29 mIU/mL, while at 12 months post-intervention, these values were 10.03 to 37.24 mIU/mL (Fig.2).

The third hormone observed in this study, E2, had a pre-intervention median value of 41.55 pg/mL, while 50% of the women had the value of this hormone in the range of 13.21 to 103.00 pg/mL. These values were obtained from a sample of 42 women who were evaluated for this hormone before intervention. From the observed 42 women, 24 were measured for E2 at 3 months post-intervention, when the median value of 52.38 pg/mL was

recorded (26.6% increase compared to pre-intervention). The range of E2 hormone value was from 21.34 to 151.60 pg/mL. At 6 months following the intervention, the median value of the observed hormone was decreased to 36.00 pg/mL (13.35% reduction compared to the pre-intervention value), from a sample of 22 women. The increase in the median value of E2 was recorded at 12 months post-intervention, when it was 45.10 pg/mL (8.54% increase compared to the pre-intervention value). This value was obtained from a sample of 19 women due to the loss of the follow-up measurements for this hormone (Fig.3).

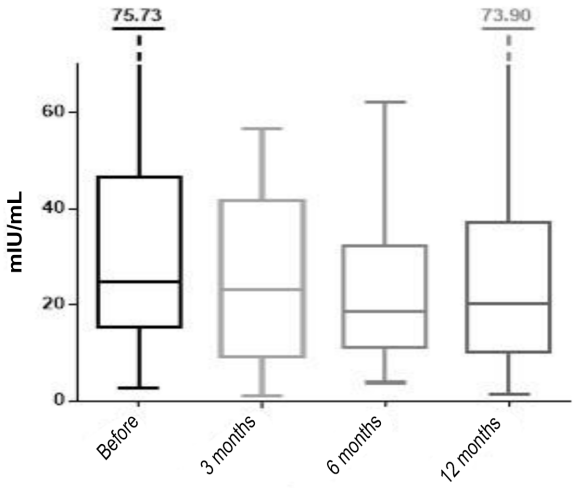

Fig.2: Medians, interquartile range and range of luteinizing hormone (LH) before the procedure and after 3, 6, and 12 months (line inside the box is median; the box is interquartile range; the lines outside the box are minimum and maximum values).

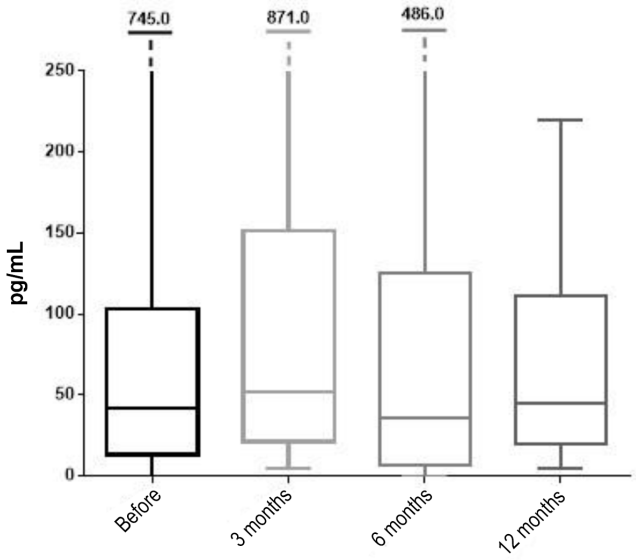

Fig.3: Medians, interquartile range and range of estradiol (E2) before the procedure and 3, 6 and 12 months after the procedure (line inside the box is median; the box is interquartile range; the lines outside the box are minimum and maximum values).

The hormone analysis also included follow-up measurements of PG levels in the subjects. Prior to the intervention, this hormone was measured in 32 women and its median value was 0.32 ng/mL. Half of the observed women had the value of this hormone in the interval from 0.09 to 0.89 ng/mL. After 3 months, the median PG value was 0.28 ng/mL (12.5% reduction compared to the value before intervention), while the intervals ranged from 0.14 to 0.73 ng/mL. These values were obtained from a sample of 12 women. At 6 months post-intervention, a decrease was observed in the median value of progesterone, being at 0.14 ng/mL (56.25% reduction compared to pre-intervention) based on the result of 13 women. The borderline intervals were, however, almost unchanged and ranged from 0.05 to 0.76 ng/mL. At 12 months post-intervention, based on the values measured from 11 women, the calculated PG median value was 0.28 ng/mL (12.5% reduction compared with the value obtained before intervention), with 50% of the women having the value of PG in the range from 0.05 to 0.58 ng/mL (Fig.4).

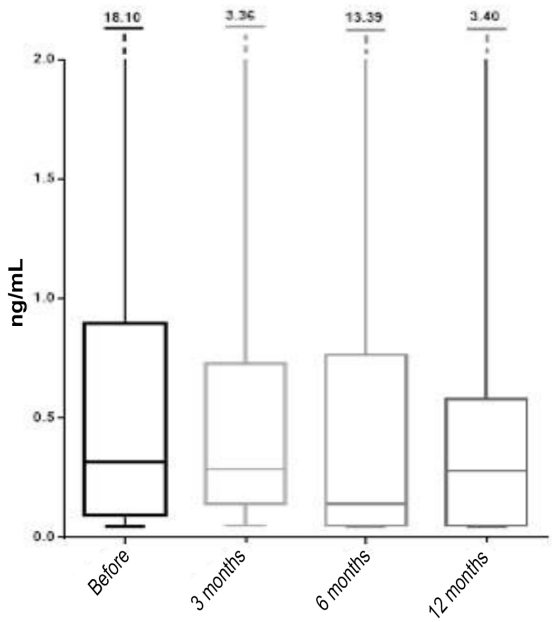

Fig.4: Medians, interquartile range and range of progesterone (PG) before the procedure and after 3, 6, and 12 months (line inside the box is median; the box is interquartile range; the lines outside the box are minimum and maximum values).

Changes in follicle stimulating hormone and luteinizing hormone hormonal levels before and after the intervention

The first aim of the study was to examine the differences in the levels of four different hormones and comparing their levels before and at 3, 6 and 12 months after ovarian intervention. Consequently, for each of the individual hormones, a null hypothesis has been tested that the hormone levels prior to and after the intervention are not different. This hypothesis was tested against the alternative hypothesis that differences in hormone levels do exist. Our results indicated that differences observed in FSH hormone levels in 19 women prior to the intervention compared to 3 months

post-intervention are not statistically significant, since the value of Z statistics is -0.283 with a corresponding P value of 0.777. However, when the FSH hormone values were analyzed in 21 women 6 months after the intervention, it was noted that there were statistically significant differences in hormone levels compared to the pre-intervention values, with Z statistics having a value of -2.091, and the corresponding P value as (0.037). At 12 months post-intervention the level of the hormone observed in a sample of 13 women showed an insignificant difference compared to the level of the hormone before the intervention, with the P value of 0.196 with Z statistics -1.293 (data not shown).

With regards to the hormone LH, the results show that in 18 women there is not a significant difference between hormone levels at 3 months post-intervention compared to the pre-intervention LH levels, since Z is equal to -1.285 with a correspondinge P value of 0.199. Subsequently, the level of this hormone was measured and compared at 6 and 12 months post-intervention. After 6 months, 20 women were evaluated for the level of hormone LH in the blood and the results showed that there were no statistically significant differences in relation to its pre-intervention levels (Z=-0.443 with a corresponding P value of 0.658). The results for 15 women who were assessed for the level of this hormone at 12 months post-intervention were Z=-1.590 with a P value of 0.112, which was not significantly different compared to the pre-intervention measurements, at the significance level $\alpha = 0.10$ (data not shown).

The blood levels of E2 at 3 and 6 months post-intervention were measured in a sample of 21 women, displaying no statistically significant differences in the levels of this hormone before and after the intervention. The Z value of the statistics for comparing the differences between E2 levels before and at 3 months after intervention -1,195 with the corresponding P value of 0,232, which clearly indicates that significant differences do not exist, confirming the null hypothesis. After 6 months, the result of statistical analysis is the same [Z=-0.408 (P=0.683)], showing no significant differences between the levels before and after the intervention. After 12 months, 15 women were re-evaluated for the E2 hormone and statistical analysis indicated that there were no significant differences in hormone levels before the intervention and 12 months after the intervention. The hormone PG was measured in a much smaller sample of women compared to the previously analyzed hormones; 8 women were evaluated at 3 months after the intervention, 10 women at 6 months after the intervention, and 7 women at 12 months post-intervention. It could be noticed that the differences in hormone levels prior to the intervention and 3 months after the intervention were not statistically significant, because the Z statistics was -0.845, and the corresponding P value was 0.398, therefore these findings suggested that the null hypothesis was correct. A null hypothesis was also adopted that there was no difference between the levels of hormone PG before and 6 months post-intervention, since the P value (0.735) that was corresponding to the Z statistics (-0.338) was greater than $\alpha=0.10$. When it comes to the differences in hormone levels of PG prior to and 12 months after the intervention, it is also concluded that these differences are not statistically significant due to a corresponding P value of 0.500, which is again greater than the level of significance of 10%.

Correlation between the re-transplanted tissue and hormonal excretion levels

There are several factors affecting graft function after ovarian re-transplantation. The ovarian reserve in most of the patients re-transplanted with ovarian tissue is usually low due to the limited primordial follicles, so most of the pregnancies are conceived within the first 12 months after auto-transplantation. However, the main disadvantage is the limited number of available tissue fragments to be transplanted, due to ovary size.

One of the aims of this study was to examine whether the volume of the autografted ovarian cortex affects the endocrine function of the ovary. The differences in the concentrations of the hormones FSH, LH, E2 and PG before and at 3, 6 and 12 months after transplantation were tested for correlation with the volume of the transplanted ovarian tissue (data not shown). Another parameter tested for a correlation between the differences in the concentrations of the hormones FSH, LH, E2 and PG before and at 3, 6 and 12 months post-transplantation, was the TNC from BMAC (Table 1). Our aim was to determine if a certain amount of stem cells are affecting the changes in the endocrine function of the ovarian tissue after re-transplantation. Multiple Spearman's correlation analysis showed that significant correlation was found between the changes in FSH at 3 months post-transplantation and the volume of BMAC (P<0.088). The volume of BMAC was also correlated significantly with changes in PG after 3 months (P<0.092). TNC also correlated significantly with the changes in E2 levels at 12 months post-transplantation (P<0.088).

Ultrasound examination and monitoring of the follicular development

Close monitoring of ovarian function was conducted after re-transplantation, including repeated measurements of FSH and E2, PG and LH levels at 3, 6 and 12 months post-intervention, and continuous sonographic evaluations during 12 months. AMH levels were not good predictors of graft function, so they were not measured after transplantation.

Repeated ultrasound examinations were performed in patients by a single physician to clearly monitor follicle development from each ovary separately. Table 2 presents a summary of the total results of ultrasonography for all 50 patients. During the 12 months period after the re-transplantation 64% of patients had presence of a follicles.

Table 1: Correlation analysis of the change in hormone levels (FSH, LH, E2 and PG) prior to and 3, 6 and 12 months post-transplantation, with the TNC from BMAC, and the volume of BMAC

Hormone	Time	Hormone change vs. BMAC volume P value	Hormone change vs. TNC P value
FSH	Change after 3 months	0.088	0.488
	Change after 6 months	0.843	0.641
	Change after 12 months	0.830	0.862
LH	Change after 3 months	0.314	0.229
	Change after 6 months	0.551	0.301
	Change after 12 months	0.665	0.713
E2	Change after 3 months	0.466	0.746
	Change after 6 months	0.183	0.599
	Change after 12 months	0.510	0.088
PG	Change after 3 months	0.092	0.391
	Change after 6 months	1.00	0.493
	Change after 12 months	1.00	0.670

These were tested by Spearman's correlation, and considered significant at α=0.1. Values in the table represent P values. FSH; Follicle stimulating hormone, LH; Luteinizing hormone, E2; Estradiol, PG; Progesterone, TNC; Total nucleated cell count, and BMAC; Bone marrow aspirate concentrate.

Table 2: Monitoring of follicular development. Percentages in the table were given out of total number of patients

Total number of patients with follow-up 50	Egg cells total number	Embryos total number	Embryo transfer total number	Vitrified embryos total number	Number of newborns
32 Women (64% out of total number of patients) had Follicles	24	15	9	10	4
	8 Women (16% out of total number of patients) had eggs	6 Women (12% out of total number of patients) had embryos	4 Women (8% out of total number of patients) had ET	3 Women (6% out of total number of patients) had freezed embryos	3 Woman were pregnant (6% out of total number of patients)
Successful rates	25% Follicle positive women had eggs	75% egg positive women had embryos	66.6% embryo positive women had embryo transfer	50% embryo positive women had vitrified embryos	75% women with embryo transfers resulted with successful pregnancies
Follicles total noumber					231

Attempts to perform oocyte retrieval resulted in aspirated oocytes in 25% of the follicle-positive women (16% of the total number of patients). Fertilization rate of the aspirated oocytes was 75%, resulting in embryos in 12% of the women out of the total number of patients. Embryo transfers were performed in 66.6% of embryo-positive women (8% of the total number of patients), while 50% of embryo-positive women had vitrified embryos (6% of the total number of patients). Two patients spontaneously conceived after transplantation, while one pregnancy was conceived with IVF, resulting in the birth of twin babies.

Discussion

In this study, we examined the effects of autologous *in vitro* ovarian activation using stem cells and autologous growth factors on reproductive and endocrine functions in patients with ovarian insufficiency. The results of the current study indicate that the SEGOVA procedure can play a potentially important role in addressing the problem of infertility in patients with impaired or lost ovarian function, as well as improving endocrine ovarian function, which affects a woman's overall health and quality of life. With regards to the recent published data in the literature, the results of our study make important contribution to today's scientific findings in the field of female reproduction, and open novel possibilities in treatment of female infertility.

The profound socioeconomic changes in our society have increasingly caused women to delay the decision to start a family. Today, this factor leads to a major problem in the reproductive field, since female age is a decisive cause of infertility, it is common knowledge that as a woman gets older, both the quality and the number of her eggs available decrease. Therefore, many centers around the world are trying to address this problem by using different strategies to preserve fertility at an earlier age by for instance vitrification of the oocyte or using different therapeutic alternatives, allowing the patients to enjoy motherhood that would have been impossible in the past with low follicular reserve.

The widespread use of oocyte donation is also a solution, but this is only a partial solution because many couples have serious difficulties in accepting such ideas. Therefore, preventive and therapeutic measures should be implemented. Preventive measures should address two relevant phenomena associated with ovarian aging:

decreased follicles and decreased quality of oocytes contained within these follicles. Accordingly, ovarian aging has become a key challenge for reproductive medicine, as the ovaries change chronologically before other organs, causing fertility decline in the thirties, leading to ovarian fibrosis and complete loss of ovarian function in the early fifties (9-30). Advanced age, which affects both the quantity and quality of oocytes, became a major determinant of fertility (9). In the case of ovarian insufficiency, such as poor ovarian response and reduced ovarian reserve or primary ovarian failure, there remains a need for methods to restore fertility in patients seeking reproductive success (10).

Due to the importance of aging in infertility, over the last 10 years, new research has emerged aimed at developing methods for rejuvenating oocytes by repairing genetic damage or introducing new sources of energy (11). Another source of healthy oocytes could be creation of gametes through the cell programing that could be expected in the future (12-14). The new potential egg source will pose a major challenge to the central dogma of reproductive biology, with which females of most mammalian species, including humans, lose their ability to create oocytes during fetal development. To date, recovery of ovarian function has been reported in preclinical models as well as clinical models using adult stem cells (12). MSCs ability to implant, survive, and reproduce in the ovaries was first evaluated by Liu et al. using mouse models after chemotherapy with damaged ovaries, where short-term fertility recovery and live births of healthy offspring have been reported (16, 17).

Cord blood, amniotic membrane, menstrual blood, adipose tissue and endometrial tissue are considered to be possible sources of MSCs with promising results for several degenerative diseases within and outside the reproductive system (16, 17, 21, 22). Bone marrow is also an important source for mesenchymal cells, but their clinical use still requires improvements in culturing techniques to obtain adequate numbers of therapeutic cells. Today *in vitro* cultures increase the risk of cells losing some of their specific regenerative properties or accumulating chromosomal aberrations, which is also one of the reasons that we transferred BMSCs to the ovaries on the third day of the procedure, shortly after they were isolated from the bone marrow. To circumvent this concern, it was proposed in our study (SEGOVA) to use minimally invasive protocols, where bone marrow stem cells are not incubated under in vitro conditions and are rather under the control of ultrasound. These cells are then applied to ovarian tissue together with *in vitro* activated and incubated ovarian tissue with autologous growth factors. Here we have been able to regenerate the ovary in women with impaired or lost ovarian function. Based on this, we sought to evaluate the effects of autologous in vitro activation of ovaries following the transfer of growth factors and stem cells (SEGOVA) to the ovarian reserve in women with very poor prognosis. This novel study showed that SEGOVA improves ovarian reserve biomarkers and reproduction results, leading to the development of more follicles and oocytes after ovarian stimulation. This technique allowed for spontaneous pregnancies in women with POF diagnosis. In short, ovarian rejuvenation is a difficult task because age is characterized by a number of significant changes, including genomic instability, telomeric shortening, mitochondrial dysfunction and epigenetic changes (28). Addressing one of these questions may not be enough, thus we are working hard in this area of research and we hope to be able to present new important data soon. The technique which researchers call "*in vitro* activation", or IVA, requires the ovary (or part of the ovary) to be laparoscopically removed and treated outside the body and then laparoscopically re-implanted near its fallopian tubes.

After *in vitro* ovarian activation and re-transplantation of the activated tissue, a woman undergoes ovulation stimulation and undergoes IVF procedures. Follicular growth was observed in eight women, all of whom had signs of retained follicles before transplantation. These eight patients underwent ovulation stimulation, with five women developing mature eggs for IVF. The oocytes were fertilized with sperm from a male partner, and the resulting embryos were frozen and then transferred to the uterus. During the study, one patient underwent embryo transfer of one embryo, but failed to become pregnant, for another patient successful embryo transfer and pregnancy were achieved, but that pregnancy ended with a miscarriage (missed ab). The third patient underwent embryo transfer of two embryos, and had a successful pregnancy that resulted in the birth of a healthy boy. The remaining two women were preparing for embryo transfer and undergoing additional egg collection cycles. Since some of the patients had unsuccessful IVF or intracytoplasmic sperm injection (ICSI) attempts in the past, and on the other hand, some of them who had ovarian insufficiency did not have any IVF attempts before the SEGOVA procedure, the results of our study are very promising. The main limitation of this prospective clinical study is that we are not able to have a control group of females since the laparoscopic treatment would be required for isolation of ovarian tissue and its placebo treatment. However, at the same time avoiding the second laparoscopy and performing simple re-implantation of the activated ovarian tissue by ovarian aspiration needle under control of transvaginal ultrasound is a less invasive method, as performed in the past. A future perspective would be in exploring ways to control Hippo and phosphatase and tensin homolog (PTEN) pathways with drugs, without *in vitro* ovarian activation.

Conclusion

SEGOVA ovarian rejuvenation procedure is unique because it uses the minimally invasive procedure of LPSC NOS surgery for taking a segment of the ovarian cortex for in vitro activation. Success criterion for this study was regaining the hormonal function in female subjects, activation of dormant follicles, promotion of antral follicle growth and development to mature oocytes. Another advantage of this method is that the transplantation of

the activated tissue is performed under ultrasound control and not through laparoscopy. In SEGOVA PRP process there are also special systems and machines for separating certain cell lines, allowing to increase the concentration of desired cells (growth factors derived from them) up to 18 times the initial concentration. This approach is different from most other PRP ovarian therapies. While autologous BMSC transplantation can have a positive effect on patients with POF, allogeneic BMSC transplantation in women with POF can cause transplant rejection with further complications and consequences. SEGOVA acts on the intracellular signaling system and BMSC transplantation is without previous culturing and incubation in a way to save the original stem cell niche. The main difficulty with stem cell therapy is to maintain cell viability, cell properties and cell function before and after implantation *in vivo*. When stem cells are isolated from native tissue and grown and incubated in substrates, they rapidly lose their role and function that they originally had. In addition, they may have a shorter lifespan due to overexpansion *in vitro*. Furthermore, cellular DNA becomes unstable during long-term culture. Such host-incorporated cells lead to low cell survival rates and poor outcomes in growth, localization, differentiation, and paracrine effects. SEGOVA program overcomes these problems by performing autologous stem cell therapy without incubation. Within the study population, we showed that the hormone levels were different 6 months after the intervention, and it was noted that there were statistically significant differences among participants with respect to the level of the same hormone before the intervention.

Acknowledgements

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Authors' Contributions

S.T., A.L., D.A.; Contributed to conception and design. S.T., A.L., D.A, D.L., D.V., T.B., M.I., S.M.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. A.L.; Was responsible for overall supervision. S.T., M.I.; Drafted the manuscript, which was revised by A.L. All authors read and approved the final manuscript.

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Original Article

Infertility Stigma: A Qualitative Study on Feelings and Experiences of Infertile Women

Mahboubeh Taebi, Ph.D.[1, 2], Nourossadat Kariman, Ph.D.[3*], Ali Montazeri Ph.D.[4], Hamid Alavi Majd, Ph.D.[5]

1. Department of Midwifery and Reproductive Health, School of Nursing and Midwifery, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2. Department of Midwifery and Reproductive Health, Isfahan University of Medical Sciences, Isfahan, Iran
3. Department of Midwifery and Reproductive Health, Midwifery and Reproductive Health Research Center, School of Nursing and Midwifery, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4. Health Metrics Research Centre, Iranian Institute for Health Sciences Research, ACECR, Tehran, Iran
5. Department of Biostatistics, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Abstract

Background: Infertility stigma is a phenomenon associated with various psychological and social tensions especially for women. The stigma is associated with a feeling of shame and secrecy. The present study was aimed to explore the concept of infertility stigma based on the experiences and perceptions of infertile women.

Materials and Methods: This qualitative conventional content analysis study was conducted in Isfahan Fertility and Infertility Center, Iran. Data were collected through in-depth interviews with 17 women who had primary infertility. All the interviews were recorded, transcribed and analyzed according to the steps suggested by Graneheim and Lundman. The Standards for Reporting Qualitative Research (SRQR) checklist was followed for this research.

Results: Eight hundred thirty-six initial codes were extracted from the interviews and divided into 25 sub-categories, 10 categories, and four themes. The themes included "stigma profile, self-stigma, defensive mechanism and balancing". Stigma profile was perceived in the form of verbal, social and same sex stigma. Self-stigma was experienced as negative feelings and devaluation. Defensive mechanism was formed from three categories of escaping from the stigma, acceptance and infertility behind the mask. Two categories; empowered women and pressure levers, created a balancing theme against the infertility stigma.

Conclusion: Infertile women face social and self-stigma which threatens their psychosocial wellbeing and self-esteem. They use defensive response mechanisms and social support to mitigate these effects. Education focused on coping strategies might be helpful against infertility stigma.

Keywords: Female Infertility, Infertility, Stigma, Qualitative Study

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Introduction

Infertility and subfertility affect a significant proportion of human beings (1). Infertility is defined as failure to achieve clinical pregnancy after 12 months of regular unprotected sexual intercourse. In general, 8 to 12% of couples of reproductive age suffer from infertility worldwide (2). According to a World Health Organization report, more than 10 percent of women are affected by infertility (1). In addition to the medical problems, infertility can cause numerous personal and social problems. It can be seen as a developmental crisis (3). Infertility can have damaging social and psychological consequences from exclusion and divorce to social stigma that leads to isolation and psychological distress (4).

Although infertility affects both sexes equally, it is women who are most frequently blamed (5). This causes infertile women to feel guilty and threatens their self-esteem. Thus, infertile women experience greater psychological stress than infertile men, and they are often stigmatized for being infertile and being childless (6). Many women experience infertility as a stigma. Although it seems that infertility stigma is likely to be greater in developing countries, infertility has been stigmatized in both developed and developing countries (7, 8).

Infertility stigma is associated with the feeling of shame and secrecy (9, 10). Stigma is defined as a negative feeling of being different compared to others in society and being contrary to social norms (11). If infertility is ex-

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*Corresponding Address: P.O.Box: 1996835119, Department of Midwifery and Reproductive Health, Midwifery and Reproductive Health Research Center, School of Nursing and Midwifery, Shahid Beheshti University of Medical Sciences, Tehran, Iran
Email: n_kariman@sbmu.ac.ir

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perienced as a stigma, it has the potential to deprive the infertile person of social support and cause depression, anxiety and stress (4, 12), feelings of guilt (13) and relationship problems (5). It may also cause psychological disturbance, decreased self-esteem and self-efficacy, and a tendency toward self-stigma (14). Infertility stigma and its related social pressures influence all the dimensions of women's lives and well-being. Qualitative studies can provide more in-depth understanding of infertility stigma and can help develop more effective interventional strategies. Due to the limited number of qualitative studies in this field, this study was conducted to explore the feelings and experiences of infertile women regarding infertility stigma.

Materials and Methods

Design and data collection

This study is a qualitative content analysis conducted in Isfahan Fertility and Infertility Center, Isfahan, Iran. Women with known infertility who were under infertility treatments participated in the study. The inclusion criteria consisted of having primary female infertility and absence of any psychological disorders. Participant's likelihood of withdrawing from the study was considered as the only exclusion criterion. Purposive sampling was carried out from 2019 to 2020 to ensure maximum variation in terms of age, education, occupation and infertility duration. The present article adheres to the EQUATOR guidelines of reporting research using the Standards for Reporting Qualitative Research (SRQR) checklist (15).

Twenty-one women were asked to participate in the study of which four refused because they were not interested in the subject or had a busy schedule.

A private and comfortable room was provided in the center and women were free to choose the place of the interview. All the participants preferred the private room in the center for their interviews. Semi-structured face-to-face interviews were conducted to assess the perceptions of women about infertility stigma. The researcher used interviewing skills to provide an intimate and comfortable atmosphere for the participants and helped them express their experiences of infertility stigma. All the interviews were conducted by the first author (M.T); a researcher in the field of infertility, and qualitative research. Two pilot interviews were conducted to improve the question guide. Interviews were organized based on the research question and the data from the literature review. The interviews began with open-ended questions such as "How did you feel about your infertility?", "How did infertility affect your life?", and "Did you experience any special treatment because of your infertility? Probing questions such as "How?", "What do you mean?" and "Please explain more on this issue" were asked to elicit further information. With the progress of the study, some direct questions were added to the interviews such as "Have you experienced labeling because of your fertility problem?" and "Do you feel any social pressure because of your fertility problem?"

In-depth interviews were continued until data saturation was reached; meaning that no new meaning unit was extracted from the interviews. The duration of the interviews varied between 30 to 45 minutes. All the interviews were voice recorded and then transcribed as soon as possible after the interview. The feelings and emotions of the participants during the interviews also were noted.

Data analysis and trustworthiness

Conventional content analysis using the Graneheim and Lundman method was applied throughout the data collection (16). Transcription, analysis and coding of each interview was done before the beginning of the next interview. The contents of the interviews were completely transcribed. Transcripts were read several times to gain understanding and identify initial categories of meaning and codes. Codes, sub-categories, categories and themes were derived from the transcripts. Combinations of related initial codes were labeled to form sub-categories and categories. Finally, the latent meaning of the text and the main themes were developed until consensus between the researchers was reached and the concept of stigma in infertile women was fully described.

Trustworthiness of the data was determined as suggested by Guba and Lincoln (16). To establish internal validity, transcripts were reviewed immediately after they were made. Adequate time was assigned to data collection, and the first author had prolonged engagement with the study subjects. The transcripts and codes were shared with two participants to ensure congruence between their experiences and the study findings (member check). For dependability of the data, external reviewers, who were not members of the research team and were familiar with qualitative studies, approved the units of meaning, codes, subcategories, categories, and themes and made suggestions that were considered in the final analysis. The external reviewer was asked to extract meaning units and initial codes of two interviews. Then the percentage of agreement between initial codes was calculated, which showed inter coder reliability (ICR) was more than 90% (17).

Finally, to establish the external validity that demonstrates transferability, the authors provided a detailed description of the participants and their experiences, and the research design. In addition, selected interviews, along with codes and categories, were shared with two infertile women other than the participants and they agreed that these codes represented their real experiences (18).

Ethical consideration

All participants were informed of the study purpose and assured of the confidentiality of their data and their voluntary participation. All the interviews were conducted in a private and comfortable room. Informed written consent was obtained from the participants that included consent to recording their interview. The Research Council

and Ethics Committee of the Shahid Beheshti University of Medical Sciences approved the study (Approval ID: IR.SBMU.RETECH.REC.1397.310).

Results

Seventeen infertile women participated in the study. Although data saturation was reached after 14 interviews, the authors conducted three more interviews to ensure saturation of the data. The mean age of the women was 32.88 years. The average duration of infertility was 4.25 years. The characteristics of the participants are shown in Table 1.

Table 1: The characteristics of the participants (n=17)

Characteristics of the participants	n (%)
Age (Y) [32.88 ± 4.82]*	
Less than 25	4 (23.5)
25-35	9 (53)
More than 35	4 (23.5)
Infertility duration (Y) [4.25 ± 3.71]*	
Less than 5	9 (53)
5-10	6 (35.3)
More than10	2 (11.7)
Education	
Less than diploma	3 (17.6)
Diploma	6 (35.3)
Academic	8 (47.1)
Employment status	
Housewife	12 (70.6)
Employed	5 (29.4)

*; Mean ± SD.

836 initial codes were extracted from the interviews and categorized into 25 sub-categories, 10 categories and four main themes. The four main themes that emerged during data collection were identified as: stigma profile, self-stigma, defensive mechanism and balancing (Table 2).

Theme 1: Stigma profile

The experiences of infertile women showed they have perceived infertility stigma. Stigma profile was experienced as verbal stigma, social stigma and same sex stigma.

Verbal stigma

One of the distressful behaviors mentioned by all the participants was verbal stigma in the form of sarcasm, humiliation, and use of offensive terms for infertility by acquaintances.

A 32-year-old participant, with secondary education, housewife, 10-year infertility duration said: *"The old people say that if someone doesn't have a child, their house is empty. They call them [OjaghKoor] (a humiliating word that means the couple's house is cold and spiritless).* Some say to me *"how incapable you are that you could not bring a child for your husband."*

Table 2: The theme, categories and subcategories of the infertility stigma concept

Themes	Categories	Sub-categories
Stigma profile	Verbal stigma	Sarcasm and humiliation
		Curiosity
	Social stigma	Discrimination
		Negative burden of infertility
	Same sex stigma	Women against women
		Sexism by women
Self-stigma	Negative feelings	Bitter feeling of infertility
		Sadness and regret
		Fear and concern
	Devaluation	Incomplete woman
		Transformation of values
		Low self-esteem
		Low self-efficacy
Defensive mechanism	Escaping from stigma	Looking for someone to blame
		Justifying the infertility
	Acceptance	Getting along with the problem
		Unchangeable fate
	Infertility behind the mask	Secrecy
		Silence
Balancing	Empowered women	Resilience
		Optimism
	Pressure levers	Supportive/Unsupportive husband
		Peer support
		Supportive family
		Pressure from husband's family

Most participants encountered a huge number of curious questions from their acquaintances such as *why haven't you had children yet? Do you have a problem or does your husband have any problems?* These questions were considered offensive and annoying in the eyes of the women.

Social stigma

The attitude of community members and their negative views toward infertility were pointed out by most participants.

"*From their type of look I can understand what they are thinking. Infertility does not bother me at all, but their looks do."* (34-year-old participant, with bachelor's degree, accountant, 5-year infertility duration)

"People think differently about you. It looks like you are different" (25-year-old participant, with primary school degree, housewife, 8-year infertility duration)

Most participants were reluctant to use the term infertility. They usually referred to it as "the issue", "the problem".

"I do not like the word of infertility at all. I do not think it is a good word at all." (35-year-old participant, with diploma degree, housewife, 9-year infertility duration)

Same sex stigma

Most participants complained about being labeled by other women.

"When my mother-in-law introduces me to others, she says: she is my daughter-in-law, she is in our family for 13 years but still has no children. Please pray for her. She wants to hurt me; she wants to say that the problem is from my side." (30-year-old participant, with middle school degree, housewife, 9-year infertility duration)

Some participants said that: *"They are women themselves, they should understand other women's problems, and they have daughters themselves."* (33-year-old participant, with doctoral degree, 1year infertility duration)

Some women experienced different types of sexism from other women. A participant said: *"The men in the family have more empathy with me than the women. My father-in-law is very kind and never asks a question to bother me, but women like their son in law more."* (32-year-old participant, with diploma degree, 1year infertility duration)

Theme 2: Self-stigma

Sometimes infertile women internalize the process of stigma. We could identify at least two elements that contributed to self-stigma: negative feelings and devaluation.

Negative feelings

The experiences of some of the participants indicated their suffering and sadness. Repeated questions from acquaintances would lead to psychological distress. The negative feelings that these infertile women experienced were expressed as bitterness, sadness and anxiety.

"I think that infertility is a disaster. The disease itself could be treated, but what happens in our society and the way that others treat you, it is really bad. The fact that everybody believes that it is your fault." (30-year-old participant, with middle school degree, housewife, 5-year infertility duration)

Infertility and the outcomes surrounding it, including the possibility of separation and remarriage of the husband, occupied the women's minds, and many of them, despite having the support of their husbands, were afraid that their marital lives would collapse. The idea that not having a child would make their husband bored with them and that they might look for someone else always bothered them.

Devaluation

Participants believed that infertility was the reason for their incompleteness and defect. Consequently, they had a feeling of inferiority.

"I always think that, because I cannot get pregnant, cannot have children, I am lower than others. This idea really bothers me." (34-year-old participant, with primary school degree, housewife, 10-year infertility duration)

Sometimes these feelings of inferiority made them transform their beliefs, and personal values and led to deterioration in their self-esteem.

"My cousin was divorced when she didn't get pregnant after 13 years. I supported her. I used to say that having a child is not the most important role of a woman. I did not know that I would have the same fate. "(26-year-old participant, with bachelor's degree, housewife, 2-year infertility duration)

"I'm not comfortable at parties at all. I don't have a good feeling. My self-esteem has really decreased. I don't want to be among others. I feel like I'm boring in comparison to them." (35-year-old participant, with diploma degree, housewife, 9-year infertility duration)

These negative emotions reduced women's self-efficacy, and they were not able to control their feelings and emotions.

"I became very sensitive. My brother's wife became pregnant. I did not want to see her during pregnancy at all." (37-year-old participant, with doctoral degree, 14 -year infertility duration).

Theme 3: Defensive mechanism

Infertile women unconsciously employed defensive response mechanisms when they encountered the stress of infertility stigma to protect themselves from psychosocial harm. Women used a combination of defensive response mechanisms, such as escaping from stigma; acceptance; and infertility behind a mask.

Escaping from stigma

Avoiding acceptance of their infertility, and irrational justifications for infertility were some of the mechanisms that participants used to escape from being labeled.

"Now that we are going to herbal therapy, it turns out that my husband is weak! I told my mother-in-law, now you see it was not my problem, but your son is weak. (29-year-old participant, with diploma degree, housewife, 2-year infertility duration).

Acceptance

Over time, as the duration of their infertility lengthened, some participants considered infertility undeniable and tried to face it rationally and accept it as their fate.

"It could not be denied. But it has become really normal to me and I am trying to get along with it. My grandma always used to say, the life is not always in our favor,

so be patient and satisfied by what you get" (37-year-old participant, with doctoral degree, 14-year infertility duration)

Infertility behind the mask

Most participants were hiding their infertility from their family and relatives, especially their husband's family. By remaining silent about their fertility problem, participants escaped the judgments and pitiful looks of others.

"I don't like anybody to know anything about this at all. I don't like to be looked on with pity. Whenever I'm asked when you're going to have children, I'd say I don't have time for children because I go to work. I come to the center for treatment, but I don't tell anybody" (42-year-old participant, with master's degree, consultant, 3-year infertility duration)

These participants always mentioned excuses such as working and being busy, studying or pretending to have decided not to have children when encountering curious questions from others.

Theme 4: Balancing

Infertile women used various factors to balance the psychological damage resulting from their perceived infertility stigma. This balancing was sub-divided into two categories; empowered woman and pressure levers.

Empowered woman

Women endured and managed stressful relationships using a sense of humor, modifying relationships, and ignoring the judgment of others to protect against the psychological pressure caused by infertility stigma.

"I turn it into fun, now. I say that my child doesn't like me to be his/her mom. He/she would come whenever he/she wants. I won't let them continue." (32-year-old participant, with diploma degree, housewife, 1year infertility duration)

By performing artistic, social, and athletic activities, women tried to avoid negative thoughts and eliminate the pressure of stigma, so they could bring balance into their lives.

"I always want to make others aware. I even have a page on Instagram and I give information anonymously. It is more for giving awareness to the society. These activities amuse me in a way and are also good for my spirit." (34 year-old participants, with bachelor's degree, accountant, 5-year infertility duration)

Pressure levers

There are factors in the lives of participants that act as positive or negative levers and modify the pressure of infertility stigma. Interviews showed that infertile women received emotional support from various sources including their husbands, families, peer groups, and, in a limited number of cases, their friends. According to most participants, husbands were the most important source of emotional support.

"My husband has said that the problem is with him, not me. He says all of this without putting any pressure on me." (32-year-old participant, with diploma degree, housewife, 1-year infertility duration)

"In response to others, my husband says that I know myself when is the right time to have a child. Right now, my life is good, I don't need children now." (26-year-old participants, with bachelor's degree, employee, 2-year infertility duration)

On the other hand, experiences of some participants showed that the behavior of their husband was not supportive, but, on the contrary, it was the source of tension for them.

"I said now that I have this problem, we can go and get a child from the orphanage, my husband objected, and he said I want a child of my own, even with another woman." (33-year-old participant, with diploma degree, housewife, 4-years infertility duration)

Some participants mentioned that it is hard for others to comprehend what infertile women are going through. They believed that only women with the same problem could understand them.

"I would like to talk with people who are similar to me. When I talked with this friend of mine, who had adopted a child, I felt really good. We could understand each other pretty well. I was very happy when I came home after meeting her. I did the house works; I liked to put on makeup." (34-year-old participant, with primary school degree, housewife, 10-years infertility duration)

Some participants identified their family as a source of support.

"My family comforts me a lot. They say do not have stress. Everything is going to be alright." (34-year-old participant, with diploma degree, housewife, 4-years infertility duration)

Most participants cited their husband's family as a source of tension and stigma. Spousal family pressure for remarriage or divorce was one of the concerns of the infertile women.

"My husband's sister tells him, think for yourself while you are young. Go get remarried." (25-year-old participant, with primary school degree, housewife, 8-year infertility duration)

"They say we want grandchildren. Why don't you do something? They ask which one of you is to blame for infertility?" (36-year-old participant, with diploma degree, housewife, 1-year infertility duration)

Discussion

The present study is one of the few studies that focuses on the perceptions and experiences of female infertility stigma. The research showed that the concept of infertility

stigma was perceived as verbal, social and same sex stigma. Self-stigma was experienced as negative feelings, and devaluation. In contrast, women used defensive mechanisms in the form of escaping from stigma, acceptance and infertility behind the mask. They try to make a balance between the sense of empowerment and pressure levers.

The participants stated that they had been verbally humiliated by their acquaintances, being called sterile, issueless and fruitless. Other studies have also mentioned verbal sarcasm and using terms such as hollow, fruitless tree, dried tree and barren land (9, 12, 19). Curious questions from acquaintances were one of the concerns of infertile women that could threaten their mental health and could be associated with a wide range of psychological damages such as anxiety, depression and low self-esteem (13, 20, 21).

Social stigma referred to a situation in which infertile women would face discrimination from others; a different and compassionate look which was torturous to them. Mumtaz. et al stated that women perceived more stigma than men and that being stigmatized was more painful than being infertile (22). Furthermore, most of the participants did not like the term "infertile". Psychologists believe that for such people, titles and labels should be used that do not imply a flaw; like using child free instead of childless (23).

Other women were the most considerable source of stigma. It seems that sometimes women are acting against women. A study in Niger showed that mostly women were the target of verbal and physical stigma from the women of their husband's family (24). In most societies, even advanced ones, having a child of your own is considered a great privilege (25). Motherhood and having children is the only way for women to raise their standing in the family and the society (26). In traditional societies motherhood is one of the important roles of women and those who are not capable of performing this role are powerless in the eyes of other women and would be humiliated (25).

According to interviews, women might internalize the stigma and see themselves lower than other women. These women usually lose their self-esteem and are suffering from social isolation. Feelings of shame and inferiority (27, 28), worthlessness and losing control, social isolation and decreased self-esteem (5, 29, 30) have been reported in other studies. Furthermore, women stated that infertility could threaten their marriage, this has been reported in other studies too (5, 27). Fear of divorce and separation has also been reported in Asian and African societies (5, 7, 9, 24, 31).

Goffman suggests that the individual sometimes initiates a process of stigmatization inside themselves - internal or self-stigmatization (11). Self-stigma refers to negative attitudes created in individuals by themselves due to the conditions they have been put through. One of the factors destabilizing individual identity is self-stigma which seems to affect their self-efficacy (32).

People do not react similarly to stigma. Women used defensive mechanisms against the tensions caused by infertility stigma. The most important of these were hiding the infertility and infertility behind the mask. Silence and hiding were reactions that have been reported in other studies too (33, 34). Goffman suggests that the first strategy for confronting stigma is hiding it. Thinking that the stigmatized person will not be accepted they try to reduce the intensity of the stigma by hiding the problem (11). However, it must be considered that, when individuals hide their problem, they end up facing the problem alone, which makes them more anxious. They may also use inefficient coping strategies. The infertile women's fear that their secret might be revealed is likely to increase tension, feelings of guilt and sadness, and leave them open to psychosocial pressures (5, 8, 35).

All the women, regardless of age, educational level or employment status, had experienced forms of stigma. However, empowered women, regardless of education and employment, were more successful in balancing the psychological outcomes of infertility stigma. Kabeer mentioned that self-respect, self-efficacy and psychological health could be improved by empowering women (36). Therefore, the care team should consider providing coping strategies to women suffering from infertility stigma.

Women mentioned some negative and positive sources that could help them to adjust to the pressures of infertility stigma. The most important source of support was their husbands. The husband played the most important role in defending his wife against the verbal and behavioral pressures of others, especially the in-laws. Results of a study in Australia also showed that a woman's husband and mother were the strongest, and the mother-in-law the weakest source of support for infertile women (35). In-laws were one of the pressure levers also mentioned in other studies (5, 6) and could be one of the main sources of stigma for infertile women.

One of the women's strategies for creating balance was communicating with other infertile women. Peer groups have been mentioned as an important source of support for women with fertility problems. Improving social relationships through the support of their peers could increase fertility-related quality of life (37). Peer support has a crucial role in therapeutic services, that should be considered by healthcare providers (38). This can complete the management of infertility and add mental health perspectives to formal treatments.

People make decisions about their problems according to their experiences (39), so interviewing women about their experiences of infertility stigma is valuable intself. The interviewer has a long history of working with women suffering from fertility problems as a faculty member of the midwifery and reproductive health department in the university. She introduced herself fully to the participants. The familiarity of the researcher with the subject of the study and cultural context might have helped participants

to express their experiences and feelings better. This could be a strength of the present study. The present study is one of the few qualitative studies that have undertaken an in-depth investigation of infertile women's experiences of infertility stigma.

Although the qualitative nature of the study means that its findings are relatively context dependent, they are likely to be generalizable to similar patient groups in similar settings. A limitation of the study is that the experiences of women who were infertile but had not been referred for treatment were not evaluated. This study presents a clear picture of infertility stigma and could be a springboard for further research related to infertility. It could also be used for developing protocols for psychological and counseling interventions appropriate for infertile women.

Conclusion

Infertile women confront different forms of stigma that can lead to devaluation and self-stigma. On the other hand, women use different defensive mechanisms and try to make a balance between a sense of empowerment and pressure levers. Health personnel who provide services to infertile women should be aware of the stigma experienced by these women and its influences on their well-being. Education focused on coping strategies might be helpful against stigma.

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Authors' Contributions

M.T., N.K., A.M., H.A.M.; Contributed to the concept and purpose of the study. M.T.; Participated in data collection and evaluation, drafting and data analysis. M.T., N.K., A.M.; Reviewed and were involved in the qualitative data analysis. M.T., N.K.; Reviewed the first draft of the manuscript. All authors edited the final version of the manuscript, participated in the finalization of the manuscript and approved the final draft for submission.

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Original Article

Follicular Fluid Zinc Level and Oocyte Maturity and Embryo Quality in Women with Polycystic Ovary Syndrome

Sima Janati, M.D.[1], Mohammad Amin Behmanesh, Ph.D.[2], Hosein Najafzadehvarzi, Ph.D.[3], Zahra Akhundzade, M.D.[4], Seyedeh Mahsa Poormoosavi, Ph.D.[5*]

1. Department of Obstetrics and Gynecology, School of Medicine, Research and Clinical Center for Infertility, Dezful University of Medical Sciences, Dezful, Iran
2. Department of Histology, School of Medicine, Dezful University of Medical Sciences, Dezful, Iran
3. Department of Pharmacology, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran
4. School of Medicine, Dezful University of Medical Sciences, Dezful, Iran
5. Department of Histology, School of Medicine, Research and Clinical Center for Infertility, Dezful University of Medical Sciences, Dezful, Iran

Abstract

Background: Polycystic ovary syndrome (PCOS) is considered to be one of the most common endocrine disorders in women of reproductive age. Zinc, a vital trace element in the body, plays a key role in maintaining health, especially due to its antioxidant role. On the other hand, lack of antioxidants and oxidative stress can adversely affect oocytes quality and consequently fertility rate. The available studies that report the effect of follicular fluid (FF) zinc in terms of the number and quality of the oocytes in infertile women with PCOS, are few and not consistent. We decided to investigate this issue.

Materials and Methods: In this cross-sectional study, from the women with PCOS referring to Omolbanin Hospital, Dezful, Iran (February to December 2019), a total of 90 samples (follicular fluid, oocytes, and embryos) were collected from those who had undergone *in vitro* fertilization (IVF). To measure zinc level in follicular fluid, high performance liquid chromatograpy (HPLC) was utilized. Also, oocytes maturity and embryos quality evaluation was performed using inverted optical microscopy. One-way ANOVA and Fisher's least significant difference (LSD) were used for data analysis.

Results: The amount of FF zinc was not associated with any significant differences in the number of oocytes and metaphase I (MI) and germinal vesicle (GV) oocytes, but a significant decrease was observed in the number of metaphase II (MII) oocytes at zinc values less than 35 µg/dL. The FF zinc levels less than 35 µg/dL were also significantly associated with decreased embryo quality.

Conclusion: A significant relationship was found between the level of FF zinc and the quality and the number of oocytes taken from the ovaries of infertile patients with PCOS history who were candidates for IVF treatment as well as the number of high quality embryos.

Keywords: Embryo, Oocyte, Polycystic Ovary Syndrome, Zinc

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Introduction

As one of the most common endocrinopathies, polycystic ovary syndrome (PCOS) is reported to affect 6-10% of reproductive-age women (1, 2); its prevalence rate is 5-10% in the general population and almost 30% among overweight women (3). This including hirsutism, amenorrhea, hyperinsulinemia, obesity, and hyperandrogenism. PCOS attributes to 3/4 of the ovulatory infertility cases (4). A high risk of endometrial and ovarian cancer has been shown in PCOS cases (5).

The oocytes obtained from PCOS patients who endure *in vitro* fertilization (IVF) often have low quality, leading to high rates of cancelation and low fertilization (6). Abnormal increased levels of androgen and/or insulin seem to be the main underlying pathophysiological mechanism for PCOS. Obesity worsens the condition of PCOS patients (7). Nonetheless, the complexity of PCOS, and how PCOS affects the oocytes development, are not fully understood and need further studies. The follicular fluid (FF) is produced by granulosa and theca cells in the growing antral follicles (8). It provides a micro-environment for the oocytes development and contains several factors including steroids, polysaccharides, proteins, antioxidant

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*Corresponding Address: P.O.Box: 65145-6461, Department of Histology, School of Medicine, Research and Clinical Center for Infertility, Dezful University of Medical Sciences, Dezful, Iran
Email: m.poormoosavi@ymail.com

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enzymes and trace elements which modulate the oocyte developmental capacity and ovulation. The FF also serves as a medium for the communication between follicular cells and oocytes during follicular development. The FF composition may reflect changes in ovarian cells secretory processes and changes in the plasma components due to pathological conditions (9).

The trace elements in human tissues are essential for cell growth, maturity, and physiological functions. For more than 300 proteins, enzymes, and transcriptional factors activities, Zinc is a main trace element present in the oocytes and FF(10) making it a structural, catalytic and regulatory ion (11). Hence, for maintaining homeostatic responses in the body including oxidative stress and several biological functions such as immune efficiency, zinc is crucial. zinc paucity in females may cause issues like reduced synthesis/secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH), estrous cycle disruption, extended gestation period, ovarian insufficiency, frequent abortion, teratogenicity, stillbirths, pre-eclampsia, toxemia, complexity in parturition, and lower infant birth weights (12).

Oocytes indicate the zinc transporters, metal regulatory transcription factors and metallothioneins. This highlights a substantial role for zinc, especially with possible association with the genome stability during early embryonic development (13). It is well known that IVF is one of the critical treatments for infertility, but the clinical pregnancy rate of IVF is affected by multiple factors including zinc level (14).

To date, however, there are only a few papers determining the trace elements levels in FF and serum of PCOS patients who undergo IVF, and the data are not consistent. Therefore, we aimed to measure zinc levels in the FF of PCOS patients who underwent IVF and find out the correlation between zinc level and oocytes and embryo quality.

Materials and Methods

This cross-sectional research studied 90 PCOS women (20-45 years old) who were selected from patients referring to the Omolbanin Infertility Center, Ganjavian Hospital, Dezful, Iran (October 2018-September 2019), using simple random sampling. The nominated subjects received assisted reproductive technology (ART) for infertility. The data was obtained via questions that investigated the age of men and women and the causes or duration of infertility, taking supplements comprising zinc in the past two months, body weight and height, and body mass index (in women).This research was approved by Dezful University Ethics Committee (IR.DUMS.REC.1397.005). Prior to initiation, the participants signed the informed consent forms. Controlled ovarian stimulation was performed for all the patients using the antagonist protocol (15). The participants received oral contraceptive pills (Ocp LD) in low-dose (0.3 mg norgestrel and 30 μg ethinyl estradiol, Aburaihan Pharmaceutical Co., Iran) which began on the 2nd day of the cycle then discontinued till menstruation occurred. Once menses began, gonadotropin stimulation was started by Gonal-F (Gonal- F, Serono, Italy) from the 2nd day of the menstrual cycle. The gonadotropin starting dose was 150-300 mIU/d, based on the patient's body weight and age. The patients were monitored on day 7 of stimulation and dose of gonadotropin was adjusted based on the serum estradiol (E2) concentrations and ovarian response as observed by ultrasound. Once the leading follicles reached 14 mm in diameter, Cetrorelix (Merck-Serono, Germany) was added subcutaneously (0.25 mg) and continued every day till the human chorionic gonadotropin (hCG) administration day. In all patients, 10,000 IU of hCG (Pregnyl, Daropakhsh, Iran) was administered intramuscularly (IM) when at least three follicles reached more than 18 mm in diameter. Then, 36 hours after hCG, the ovum was picked up in an ultrasound-guided manner. The puncturing was performed to remove oocytes and the FF for the aspiration by catheter. To avoid sample contamination from blood first tube of the FF was used. The samples were transferred to the test tubes. The oocytes were washed using G-MOPS medium (Vitrolife-Sweden), and then, incubated for two hours at 37ºC with 6% CO_2. After oocytes removal, the FF cells were pelleted by centrifugation at 3,000 rpm for 10 minutes. Then, blood-free supernatant was aliquoted and stored at -70°C until further evaluation (16). Using an inverted microscope (Olympus, Japan), the oocytes were classified into three categories. The categories were as follows: metaphase II (MII) (presence of the first polar body), metaphase I (MI) (absence of the first polar body and germinal vesicle breakdown), and germinal vesicle (GV) and degenerated oocytes (17). The FF sample (1 ml) was taken from each patient. Subsequently, the oocytes were inseminated. Pro-nucleolus (PN) score was noted 16-18 hours following insemination. The embryo quality (A, B or C) was evaluated before embryo transfer according to the reference (18).

Zn assays were performed via a colorimetric method using spectrophotometry at 560 nm, and dye (2,5-bromo-2-pyridylazo)-5- (N-propyl-N-sulpho-propylamino) phenol at a slightly acidic pH value. Trichloracetic acid was used for protein precipitation. Salicylaldoxime and dimethylglyoxime were included in the assay for zinc extraction and transition metal chelator, respectively.

Statistical analysis

To assess the differences among the sample groups to be significant we used Fisher's least significant difference (LSD) post-hoc test. Also, to do a comparison among the variances of the three sample groups, one-way analysis of variance (ANOVA) was conducted. To do the analysis, SPSS 22 (SPSS Inc., Chicago, Ill., USA) was used while the significance level was set at less than 0.05.

Results

Patients and oocytes

Totally, 740 oocyte samples were collected from 90 women enrolled in the current research. The mean oocyte count was 8.2. The mean counts of MII, MI, and GV along with degenerated oocytes were approximated at 7.67, 1.1, 0.94, respectively.

Table 1: Comparing the average number of oocytes considering different groups of variables

Variables	Oocyte number	MII	MI	GV, Degenerated
Women age (Y)				
25-30	12.12 ± 4.32[b]	4.17 ± 4.21[a]	5.46 ± 2.88[a]	2.36 ± 3.17[b]
30-35	11.35 ± 3.74[b]	4.36 ± 4.21[a]	5.82 ± 4.42[a]	2.7 ± 3.41[b]
35-40	11.27 ± 2.99[b]	2.71 ± 4.51[b]	5.31 ± 5.38[a]	3.16 ± 3.91[b]
40-45	12.64 ± 4.51[b]	2.4 ± 2.44 [b]	4.99 ± 5.12[a]	6.1 ± 4.27[a]
≥45	12.21 ± 1.83[b]	2.01 ± 4.12[b]	5.01 ± 3.49[a]	5.4 ± 4.28[a]
Women BMI (kg/m^2)				
<25	11.27 ± 3.51[a]	5.34 ± 2.68[a]	4.77 ± 3.42[a]	5.27 ± 2.37[a]
25-30	10.56 ± 3.98[a]	3.27 ± 3.81[b]	4.99 ± 48[a]	2.74 ± 4.71[b]
≥30	7.1 ± 4.11[b]	1.24 ± 4.52[c]	4.39 ± 4.74[a]	2.41 ± 2.73[b]
Cause of infertility				
Male factor	12.77 ± 6.42[a]	6.33 ± 4.97[a]	5.47 ± 3.71[a]	1.18 ± 3.07[a]
Female factor	8.21 ± 4.91[b]	2.27 ± 3.42[b]	4.77 ± 3.23[a]	2.1 ± 2.72[a]
Both	8.28 ± 3.91[b]	2.97 ± 3.342[b]	4.98 ± 4.18[a]	1.77 ± 3.71[a]
Infertility duration (Y)				
≤5	8.14 ± 3.61[b]	2.55 ± 5.12[a]	4.27 ± 3.91[a]	3.47 ± 2.35[a]
>5	11.44 ± 3.41[a]	2.98 ± 3.81[a]	5.32 ± 4.01[a]	3.22 ± 3.34[a]

Data are presented as mean ± SD. MII; Metaphase II, MI; Metaphase I, GV; Germinal vesicle, BMI; Body mass index, and [a], [b], [c]; Designate significant differences (P≤0.05).

Comparison of the studied variables in terms of oocyte maturity

Based on the results, the mean count of MII oocytes was significantly higher in the subjects aged less than 35 years old (P≤0.05), as well as those with lower body mass index (BMI) and male infertility factor (P≤0.05). However, in terms of infertility duration, no significant difference was observed in the mean count of MII oocytes among the study groups (P>0.05, Table 1).

The levels of zinc in the follicular fluid and its association with oocyte maturity

Between the zinc level in the FF and the counts of oocytes, MI oocytes and GV oocytes, no significant association was detected (P>0.05). The mean count of the MII oocytes in women with zinc levels less than 35 µg/ml, was significantly lower (P≤0.05). For MII oocytes, the highest mean (6.25 ± 3.6) and lowest mean (3.45 ± 3.6) was detected for the zinc levels of 35-45 µg/ml and 25-35 µg/ml in the FF, respectively (Table 2).

Patients and embryos

To tally, 450 embryos were collected from 82 women. There were 8 cases with no embryo. The mean number of embryos was 5.48 ± 3.98 with the range of 1 to 16. Most of the embryos (124 cases) were qualified as A, 193 cases as B and 133 cases as C.

Comparison of the studied variables in terms of embryo quality

Those with a BMI less than 25, age<35 years, and infertility duration of <5 years and couples with male infertility had embryos with significantly higher quality (P≤0.05). For cases with man's age>45 years, woman's BMI greater than 30 and woman's age >35 years, a greater percentage of obtained embryos showed significant C quality (P≤0.05, Table 3).

Table 2: Comparing the average distribution of oocytes among different levels of zinc in FF

Zinc (µg/dl)	Oocyte number	MII	MI	GV, Degenerated
15-25	11.74 ± 3.65[a]	3.95 ± 3.49[b]	6.35 ± 4.42[a]	1.28 ± 5.21[a]
25-35	12.21 ± 3.27[a]	3.45 ± 3.61[b]	7.41 ± 3.65[a]	2.96 ± 4.21[a]
35-45	13.49 ± 4.51[a]	6.25 ± 3.61[a]	6.28 ± 3.72[a]	1.55 ± 4.42[a]
45-55	13 ± 4.91[a]	6.1 ± 2.51[a]	6.1 ± 3.21[a]	1.3 ± 3.71[a]

Data are presented as mean ± SD. FF; Follicular fluid, MII; Metaphase II, MI; Metaphase I, GV; Germinal vesicle, and [a], [b], [c]; Designate significant differences (P≤0.05).

Zinc levels in the follicular fluid and its association with embryo quality

The mean count of embryos had grade A quality which was significantly lower in the women with zinc levels less than 45 µg/ml (P≤0.05). The mean count of embryos with B quality was significantly lower in women with zinc levels <35 µg/ml (P≤0.05, Table 4).

Table 3: Comparing the average number of embryos with A, B or C quality among different variable groups

Variables	Grade A	Grade B	Grade C
Women age (Y)			
25-30	8.34 ± 4.22[a]	7.66 ± 3.88[a]	1.41 ± 1.91[b]
30-35	8.45 ± 3.15[a]	6.97 ± 4.72[a]	1.36 ± 4.21[b]
35-40	4.21 ± 2.51[b]	3.31 ± 5.38[b]	3.71 ± 4.51[a]
40-45	2.25 ± 3.52[c]	3.99 ± 4.52[b]	3.42 ± 2.44[a]
≥45	2.3 ± 3.83[c]	1.71 ± 2.45[c]	4.09 ± 4.12[a]
Men age (Y)			
25-30	7.48 ± 4.22[a]	7.02 ± 4.28[a]	1.98 ± 3.31[b]
30-35	8.45 ± 3.15[a]	6.68 ± 5.82[a]	1.48 ± 3.47[b]
35-40	4.71 ± 5.26[b]	6.14 ± 5.38[a]	3.55 ± 4.28[b]
40-45	4.3 ± 4.28[b]	2.49 ± 4.51[b]	3.97 ± 2.29[a]
≥45	1.4 ± 6.43[c]	2.55 ± 4.28[b]	4.17 ± 3.71[a]
Women BMI (kg/m^2)			
<25	12.24 ± 4.31[a]	7.09 ± 4.74[a]	1.87 ± 3.31[b]
25-30	8.55 ± 4.61[b]	6.33 ± 4.81[a]	1.48 ± 3.25[b]
≥30	7.71 ± 3.15[b]	6.72 ± 3.22[a]	3.55 ± 4.32[a]
Cause of infertility			
Male factor	7.24 ± 3.71[a]	7.23 ± 2.91[a]	6.88 ± 5.34[a]
Female factor	5.37 ± 3.41[b]	5.27 ± 6.11[b]	8.78 ± 2.91[a]
Both	3.84 ± 3.71[a]	3.05 ± 4.42[a]	2.84 ± 5.51[a]
Infertility duration (Y)			
≤5	12.37 ± 4.52[a]	7.23 ± 5.61[a]	3.87 ± 3.71[a]
>5	6.58 ± 3.92[b]	6.98 ± 3.31[a]	4.32 ± 2.36[a]

Data are presented as mean ± SD. BMI; Body mass index, and [a], [b], [c]; Designate significant differences (P≤0.05).

Table 4: Comparison of the average number of embryos with A, B or C quality among different levels of zinc in FF

Zimc (µg/dl)	Grade A	Grade B	Grade C
15-25	3.58 ± 3.66[b]	2.64 ± 5.25[b]	2.3 ± 2.92[b]
25-35	4.23 ± 3.52[b]	3.18 ± 3.42[b]	1.54 ± 3.16[b]
35-45	3.44 ± 3.66[b]	6.35 ± 4.21[a]	2.1 ± 4.12[b]
45-55	7.25 ± 3.45[a]	6.14 ± 4.12[a]	4.5 ± 4.11[a]

Data are presented as mean ± SD. FF; Follicular fluid, and [a], [b], [c]; Designate significant differences (P≤0.05).

Discussion

PCOS is one of the most prevalent endocrine-metabolic ailments. It can be specified as a combination of anovulation (oligomenorrhea, infertility, and dysfunctional uterine bleeding) and hyperandrogenism (acneand hirsutism) along with polycystic ovaries. The effect of FF on the oocytes and embryos development was ratified; lack of several elements and nutrients may lead to a reduction in the possibility of successful natural fertility (19). In the current study, we determined the zinc level in the FF and assessed embryo quality in PCOS patients who underwent IVF.

Zn is present in all cells of the body, taking part in more than 200 enzymes formation. In fact, it performs important roles in proper function of different enzymes. According to the studies, zinc deficiency in women can lead to abnormalities in the production and secretion of FSH and LH, abnormal ovarian differentiation, recurrent miscarriage, etc. (20). In 2017 Sun et al. (21) observed a positive correlation between zinc in the FF and the number of oocytes in the patients undergoing IVF. They stated that low levels of zinc decrease the number of oocytes and their quality, which is in accordance with the present study results.

According to this research, there is a positive association between decreased oxidative stress and increased oocyte maturation in the PCOS and infertile women. It can be said that zinc has antioxidant properties and reduces oxidative stress in patients with PCOS during IVF. zinc deficiency significantly increases apoptosis induced by the cytokines, and oxidative stress in somatic cells (22). zinc deficiency-induced apoptosis can inhibit cumulus cells proliferation. The cumulus cells play important roles in oocytes maturation and they are needed for the cytoplasmic maturation and growth because they synthesize glutathione (GSH) and transmit it to the oocytes. Thus, poor growth and development of cumulus cells negatively affect oocyte maturation and quality (23). If zinc supplementation is done and its level reaches normal values, the number and quality of the oocytes will improve. Their results are consistent with the present study outcome. According to the findings of the present study, a reduction in zinc will decrease the number of better quality embryos. The findings of recent studies indicate that zinc is very important for the meiotic cell cycle regulation and ovulation (24). Nevertheless, Zn's role in promoting oocyte quality and growth potential, is not known yet. Research suggests that zinc deficiency in women just prior to ovulation, disrupts the epigenetic programming of the oocyte, including a decrease in DNA and histone protein methylation. These epigenetic deficiencies, along with meiotic defects, compromise fertilization and the embryo growth. Dietary deficiency of zinc reduces the potential for oocyte growth (25). The major part of the embryo cytoplasm originates from the oocyte. In fact, this is the oocyte that provides the required components to support fetal growth, such as mRNA and protein, to activate the fetal genome and maintain its growth. The quality of the oocyte largely determines the achievement of fertilization and the early development of the fetus. The ovary environment can determine the oocyte quality, but the mechanisms of optimal oocyte growth and maturation are not fully discovered yet. Diet and environment can impair the reproductive performance, including oogenesis at different stages. Recent findings have shown that zinc is an important factor in maintaining meiosis arrest before puberty (26). zinc is also required to complete meiosis I during laboratory puberty. Studies have shown that acute zinc deficiency reduces the oocytes quality (27). This finding is consistent with the results of the present

study. zinc is also required for the synthesis of vitamin A reductase and zinc deficiency may decrease serum vitamin A levels, possibly leading to oocyte failure (28).

Conclusion

According to the obtained results, there is a positive correlation between the level of FF zinc and the quality and maturation of oocytes taken from ovaries of infertile subjects with PCOS history. Also, among our participants, the embryos of subjects who underwent IVF and had higher FF zinc levels, had higher quality. There is not sufficient knowledge about the exact effect of zinc on the oocyte and embryonic quality, thus, further investigations on higher numbers of patients for further validation, are recommended.

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Authors' Contributions

S.M.P., S.J., M.A.B; Contributed to conception and design. S.M.P., M.A.B, S.J., Z.A.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. H.N., S.J., M.A.B.; Were responsible for overall supervision. Z.A., H.N.; Drafted the manuscript, which was revised by S.M.P., S.J, M.A.B., H.N. All authors read and approved the final manuscript.

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Original Article

Clinical and Molecular Effects of GnRH Agonist and Antagonist on The Cumulus Cells in The *In Vitro* Fertilization Cycle

Saeid Azizollahi, M.D., Ph.D.[1, 2], Maryam Bagheri, Ph.D.[1], Fedyeh Haghollahi, M.Sc.[1], Seyyede Momeneh Mohammadi, Ph.D.[3], Batool Hossein Rashidi, M.D.[1*]

1. Vali-e-Asr Reproductive Health Research Center, Tehran University of Medical Sciences, Tehran, Iran
2. Cell Therapy and Regenerative Medicine Comprehensive Center, Kerman University of Medical Sciences, Kerman, Iran
3. Department of Anatomical Sciences, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran

Abstract

Background: Gonadotropin-releasing hormone (GnRH) analogues have been extensively utilized in the ovarian stimulation cycle for suppression of endogenous rapid enhancement of luteinizing hormone (LH surge). Exclusive properties and functional mechanisms of GnRH analogues in *in vitro* fertilization (IVF) cycles are clearly described. This study was performed to evaluate clinical and molecular impacts of the GnRH agonist and antagonist protocols in IVF cycles. For this purpose, gene expression of cumulus cells (CCs) as well as clinical and embryological parameters were evaluated and compared between two groups (GnRH agonist and antagonist) during the IVF cycle.

Materials and Methods: Twenty-one infertile individuals were enrolled in this study. Subjects were selected from two groups of GnRH agonist (n=10) treated patients and GnRH antagonist (n=11) treated individuals. The defined clinical embryological parameters were compared between the two groups. Expression of *BAX, BCL-2, SURVIVIN, ALCAM,* and *VCAN* genes were assessed in the CCs of the participants using the real-time polymerase chain reaction (PCR) technique.

Results: The mean number of cumulus oocyte complex (COC), percentage of metaphase II (MII) oocytes, grade A embryo and clinical parameters did not show noticeable differences between the two groups. *BAX* gene expression in the CCs of the group treated with GnRH agonist was remarkably higher than those received GnRH antagonist treatment (P<0.001). The mRNA expression of *BCL-2* and *ALCM* genes were considerably greater in the CCs of patients who underwent antagonist protocol in comparison to the group that received agonist protocol (P<0.001).

Conclusion: Despite no considerable difference in the oocyte quality, embryo development, and clinical outcomes between the group treated with GnRH agonist and the one treated with antagonist protocol, the GnRH antagonist protocol was slightly more favorable. However, further clinical studies using molecular assessments are required to elucidate this controversial subject.

Keywords: Apoptosis, Cumulus Cells, GnRH Antagonist

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Introduction

The gonadotropin-releasing hormone (GnRH) agonist and antagonist protocols are extensively utilized in the ovarian stimulation cycle to inhibit the endogenous rapid increase in the luteinizing hormone (LH surge) levels. The unique properties and functional mechanisms of GnRH analogues in the *in vitro* fertilization (IVF) cycles are well defined (1).

GnRH agonist have a longer half-life and higher potential than native GnRH. They initially stimulate pituitary gonadotrophs and production of follicle-stimulating hormone (FSH) and LH hormones, thereby cause an expected response of gonads (2). In contrast, GnRH antagonist immediately suppress pituitary gonadotropin in the competition with the GnRH receptor, thereby prevent early excitatory phase of agonists. Recently, there have been an increasing interest in using GnRH antagonist in control ovarian hyperstimulation (COH). GnRH antagonist have beneficial effects compared to the GnRH agonist. Most notably they cause fewer follicles and lower daily usage of estradiol, and thus lower incidence of ovarian hyperstimulation syndrome (OHSS), a serious complication which eventually helps the reproductive treatment (3). However, it has been reported that GnRH antagonist administration is along with a reduced live birth rate and an increase in the risk of pregnancy loss,

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*Corresponding Address: P.O.Box: 1419733141, Vali-e-Asr Reproductive Health Research Center, Tehran University of Medical Sciences, Tehran, Iran
Email: bhrashidi@tums.ac.ir

which might be the result of impaired implantation and lower estradiol concentrations on the first day of COH (2). In addition to the pituitary, the role of GnRH in other tissues including ovary, uterus, and placenta have been demonstrated in previous studies. Although the mode of action of GnRH and its analogues are well determined on the pituitary level, its role in the extra pituitary tissues is still not fully understood (4).

GnRH receptors are present on the ovarian epithelial cells, granulosa cells (GCs), and cumulus cells (CCs). CCs are involved in the follicular development, maturity, and quality of the oocyte (5). There is a bidirectional paracrine communication between the CCs and oocytes during folliculogenesis (6). By secreting paracrine markers including growth differentiation factor 9 (GDF-9) and bone morphogenetic protein-15 (BMP-15), the oocyte induces CC gene expression to ensure its development and maturation (7). For this reason, optimal development and the quality of the oocyte can be evaluated by the CC gene expression as a non-invasive method (6). *Versican (VCAN)* and activated leukocyte cell adhesion molecule (*ALCAM*) are expressed in the CCs and contribute to the extracellular matrix (ECM) formation (8). *VCAN*, which is a proteoglycan, is expressed in the GCs after ovulation induction. *VCAN* is cleaved following LH surge by a precise molecular pathway and the cleaved *VCAN*, as the functional form, is observed in the COCs (9). Since important growth factor receptors are attached to this functional form, a change in the *VCAN* expression might also alter COC matrix properties during the oocyte maturation, ovulation, and fertilization (8). *ALCAM* is known as an ECM-related protein. Cell to cell and cell to matrix adhesion may be promoted by *ALCAM* in the reproductive tissues. *ALCAM* has been shown to be expressed in the epithelium and blastocysts and is involved in the implantation process (10). A significant association is reported between the expression of these genes and oocyte quality (11).

Moreover, it seems that apoptosis of CCs reduces the success rate in IVF (12) and the higher the incidence of apoptotic CCs, the lower the fertilization rate (13). The vital role of programmed cell death in different physiological events of reproduction is well established. For instance, during folliculogenesis, the number of follicles in a follicular cohort primarily diminishes due to the apoptosis of GCs (14). *SURVIVIN* is a member of inhibitors of apoptosis proteins (IAPs) and has an important caspase inhibitory function (15). Critical functions of survivin in folliculogenesis and follicular development are not limited to apoptosis inhibition, but also this protein participates in the regulation of the mitotic spindle checkpoint (16). Follicular development or atresia are regulated by different hormonal and microenvironmental factors (17). AMH, GnRH, androgens, and apoptotic (*BAX, P53, FOXO3*) and anti-apoptotic (*BCL-2, SURVIVIN*) genes are identified as the follicular atretogenic factors (18). The anti-apoptotic role of *BCL-2* against a variety of cell death-inducing factors has been proved in numerous studies. A correlation has been found between apoptosis acceleration and overexpression of *BAX*, as a pro-apoptotic agent (19).

Therefore, the present study was performed to examine the impact of GnRH agonist and antagonist on IVF cycles from clinical and molecular points of view. For this purpose, the oocyte quality, embryo development, CC gene expression, and pregnancy rate were compared between the two groups of patients who received GnRH agonist or antagonist throughout the IVF cycle.

Materials and Methods

Patients and study design

In this study, 21 eligible infertile women undergoing IVF cycle were chosen. This study was conducted in Vali-e-Asr Reproductive Health Research Center, Tehran University of Medical Sciences (Tehran, Iran) from December 2014 to February 2016. Study was approved by Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.VCR.REC.1396.2309). People who agreed to take part in this study signed a consent form. Participants were divided into two groups of subjects, who received either GnRH agonist or GnRH antagonist. Subjects had an equal chance of being in both groups.

The inclusion criteria for the subjects were age<40 years and body mass index (BMI) <30 kg/m^2. Furthermore, the cause of undergoing IVF was tubal factor infertility, and according to WHO criteria, partners had normal sperm parameters. The exclusion criteria were as follows: patients with ovarian dysfunction or other endocrinopathies, infertile couples with severe male factor infertility, poor responders, polycystic ovarian syndrome (PCOS), and endometriosis.

Study size

The choices of sample size and study duration were based on the primary outcome obtained from the study of Danhua Pu 2011. A sample size of 11 with 80% power was achieved by a Two-Sided One-Sample t test, which can detect an effect size (i.e. mean difference) of 1.9 between the null hypothesis with no mean difference and the alternative hypothesis mean=-1.9 with an assessed standard deviation of 2.4 and alpha= 0.05. The sample size estimation was conducted using PASS 15 software.

Stimulation protocols

Ten individuals were picked out from the GnRH agonist treated patients that received triptorelin 0.1 mg/day subcutaneously (Decapeptyl, Ipsen, Italy) in the luteal phase of their preceding cycles based on a standardized long protocol. Following gonadotrophin inhibition, which was confirmed by transvaginal ultrasound, the patients received 150-225 IU recombinant FSH (rFSH) (Gonal-F, Merck Serono Laboratories, Switzerland) on the 3rd day of their periods (20).

Eleven women in the GnRH antagonist group were treat-

ed with 150-225 IU/day rFSH subcutaneously beginning on the second day of their monthly periods followed by a single dose adjustment from day 5 of the cycle. Each patient received 0.25 mg/day of cetrorelix (Cetrotide, Serono) on the sixth day of COH according to a fixed protocol (20).

To assess the ovarian response to the stimulation protocol, prior to the injection of the human chorionic gonadotropin (HCG) hormone, the follicle sizes were measured and clinical tests such as serum estradiol and FSH concentration measurements and transvaginal ultrasounds were performed. Serum FSH and estradiol concentrations were measured using immunoassay kits (CALBIOTECH, USA) with an automated multi-analysis system.

A single dose of HCG (Gonasi HP 5000, AMSA, Italy) 10,000 IU was injected intramuscularly following the observation of at least three follicles with an optimal diameter of 18mm and serum estradiol ≥0.40 nmol. Oocytes were picked up 34-36 hours after HCG administration.

Evaluation of parameters

Embryological, clinical, and molecular variables were evaluated to compare the effects of GnRH antagonist and agonist. The pregnancy rate and the number of ovarian follicles were evaluated as clinical parameters.

For evaluation of oocyte competence, the percentage of metaphase II (MII) (Fig.1A), metaphase I (MI) (Fig.1B) and germinal vesicle (GV) oocytes (Fig.1C), were calculated. Furthermore, the percentage of 2 pronuclei (2PN, Fig.1D) from the total number of MII retrieved oocyteswere considered as fertilization rate.

The pregnancy rate was evaluated as the percentage of the subjects with positive βHCG test after receiving either the agonist or the antagonist protocol. The number of years that a woman was infertile was considered as the infertility duration. According to the constructor's instructions, serum prolactin concentrations were measured using an ELISA kit (Calbiotech, USA). The endometrial thickness and ovarian follicle count (number of follicles more than 18 mm) were measured using gynecological ultrasound.

The percentage of 7-cell embryos with less than 10% fragmentation (graded as A, Fig.1E) and the percentage of embryos with at least 7 cells having >10% fragmentation (graded as AB, Fig.1F) from the total number of fertilized oocytes on day 3 after insemination were assessed and compared between the two groups.

For the molecular investigation, expression of *VCAN*, *ALCAM*, *SURVIVIN*, *BAX* and *BCL-2* genes were evaluated in the CCs.

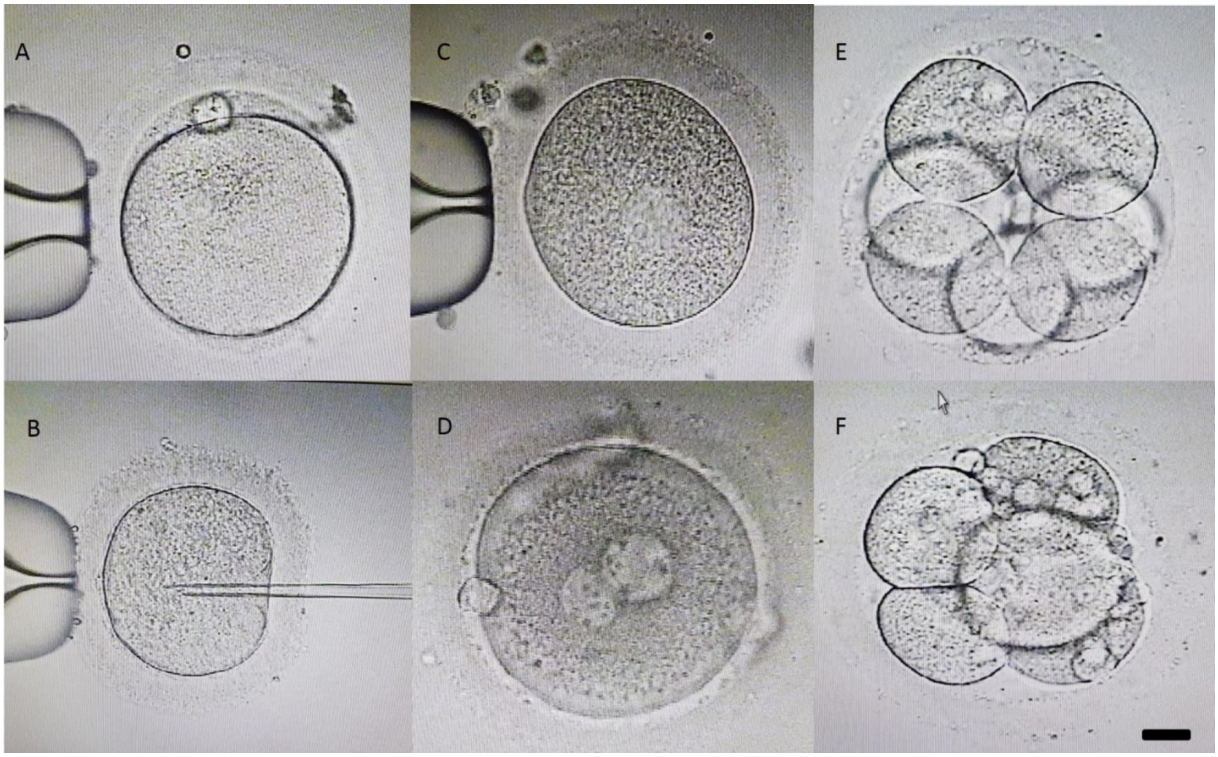

Fig.1: Different stage of oocyte and embryo development that was evaluated in the study. **A.** Metaphase II (MII), **B.** Metaphase I (MI), and **C.** Germinal vesicle (GV) oocyte. **D.** 2 pronuclei (2-PN), **E.** 7-cell embryos with less than 10% fragmentation (graded as A), and **F.** 7 cells with more than 10% fragmentation (scale bar: 20 μm).

Table 1: Primer sequences used in quantitative real time polymerase chai reaction

Gene	Primer sequence (5´-3´)	Annealing temperature (ºC)	DNA size (bp)
ALCAM	F: CTGGCAGTGGAAGCGTCATA R: CGTCTGCCTCATCGTGTTCT	55	189
VCAN	F: TCCTCGCAGAAACTGCATCA R: CCCAGGGCTTCTTGGTACTG	59	231
SURVIVIN	F: AGGACCACCGCATCTCTACA R: TTTCCTTTGCATGGGGTCGT	55	188
BAX	F: GTCTTTTTCCGAGTGGCAGC R: GGAGACAGGGACATCAGTCG	55	251
BCL-2	F:GGGAGGATTGTGGCCTTCTT R: ACTTGTGGCCCAGATAGGCA	59	286
B-ACTINE	F: GTCATTCCAAATATGAGATGCGT R: GCTATCACCTCCCCTGTGTG	60	121

Collection and isolation of cumulus cells

The retrieved COCs were washed several times in commercial human tubal fluid (HTF Lonza, Verviers, Belgium) in order to eliminate any blood cells, GCs, and debris contamination. Then, they were incubated in the fertilization medium for 5 minutes (Universal IVF medium, Medicul, Denmark). CCs samples were mechanically dissected less than 1 hour after OPU. Isolation of CCs was performed by washing these cells in the culture medium and centrifugation 10 minutes at 250 × g several times (21). Then, the cells were pooled and preserved by rapid freezing just after dissection and prior to the RNA extraction.

RNA extraction and real-time polymerase chain reaction

CC RNA extraction was performed using Arcturus Pico Pure RNA Isolation Kit (Applied Biosystems, USA) based on the manufacturer's instructions (from ~ 4 ng pooled oocyte to 100 ng of CCs, and 3 repetitions for this experiment). DNase I (Fermentas, St. Leon-Rot, Germany) was used three times to eliminate genomic DNA contaminations. The purified RNA was used for cDNA synthesis using oligo dT primers (Applied Biosystems, Foster City, CA) prior to real-time polymerase chain reaction (PCR) (22).

The primers were designed to the human sequence of *VCAN, ALCAM, SURVIVIN, BAX* and *BCL-2* genes using the Gene Runner (version 3) and Primer Express (version 3.05), and were blasted in http://www.ncbi.nlm.nih.gov/BLASt/. The primer characteristics are presented in Table 1.

Real-time PCR was accomplished with the SYBR Green Reagent (Applied Biosystems, USA) using ABI PRISM 7300 Analyzer (Applied Biosystems, USA). The PCR cycle was repeated for 45-55 cycles. The Q-PCR reaction was carried out at least three times using specific primers. The quantification of 5 genes was evaluated using the comparison with the housekeeping gene, beta-actin. Finally, $2^{-\Delta\Delta CT}$ technique was used for comparative quantification between the two groups.

Statistical analysis

Data were analysed using IBM SPSS Statistics software (version 25, IBM SPSS Statistics, Armonk, USA) and the graphs were drawn by GraphPad (Prism) (version 8, https://www.graphpad.com). Normality of the numeric variables was checked and confirmed by Kolmogorov-Smirnov test and measures of distribution including skewness and kurtosis were within ± 1.5 and ± 2, respectively. Data are presented as the mean (SD) and frequency (percent) for numeric normal and categorical variables, respectively. Comparisons of the variables between the groups were conducted by Independent Samples t test. The assumption of the homogeneity of the variances were assessed by Levene's test, and Welch correction was used when the assumption was not satisfied. For comparing the categorical variables between the two groups, Pearson Chi-square test with exact P value was utilized. In all analyses, a $P<0.05$ indicates statistically significant.

Results

Clinical characteristics of the woman in different groups are shown in Table 2. No significant difference was observed in the infertility duration, age, BMI and hormonal levels between the two groups. In addition, the number of the dominant ovarian follicles, endometrial thickness, and pregnancy rate were not different significantly between the groups.

Embryological assessments

In GnRH antagonist group, the mean number of obtained COCs was higher than the GnRH agonist group, which was not statistically significant (P=0.14, Table 3). In order to compare the oocyte nuclear maturity, the percentages of MII, MI, and GV oocytes were calculated and compared between the groups. As shown in Table 3, MII percentage is clinically higher in the GnRH antagonist group compared to the agonist group (84.8 ± 20 vs. 78.6 ± 27.6, P=0.57). No considerable difference was found in the percentage of MI and GV oocytes between the two groups (12.6 ± 17.8 vs. 9.2 ± 16.2, and 5.6 ± 9.6 vs 5.5 ± 8.9, P=0.65 and P=0.99, respectively). Moreover, a sta-

tistically significant difference was observed in the percentage of 2 PN between the GnRH agonist and GnRH antagonist groups (54.5 ± 19.2 vs. 72.5 ± 9.1, respectively, P<0.05). Finally, the percentage of grade A and AB embryos from the total number of fertilized oocytes were compared in each group. No significant difference was found in the percentage of type A (52.9 ± 34.3 vs. 55.5 ± 29.6%, P=0.24 and AB embryo (21.2 ± 20 vs. 22.9 ± 25%, P=0.87) between the GnRH agonist and GnRH antagonist groups.

Molecular evaluation

As shown in Figure 2, the relative *BAX* gene expression in the CCs of patients that received GnRH agonist was significantly higher than those with GnRH antagonist treatment (39.1 ± 2 vs. 27.01 ± 4.2, respectively, P<0.001). Furthermore, expression of *BCL-2* was higher in the CCs of the patients received GnRH antagonist against those who received GnRH agonist (61.4 ± 2.2 vs. 44.3 ± 4.2, P<0.001). The *ALCAM* expression was significantly different between the GnRH agonist and antagonist groups (16.8 ± 0.6 vs. 22.2 ± 1.3, respectively, P<0.001). No significant difference was seen in the expression of *VCAN* (40.5 ± 7.9 vs. 41.8 ± 6.7, P=0.789) and *SURVIVIN* (64.8 ± 6 vs. 66.9 ± 7.6, P=0.131) between the GnRH agonist and antagonist groups, respectively (Fig.2).

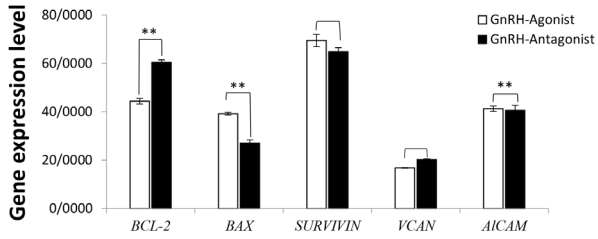

Fig.2: Relative gene expression of apoptotic and developmental gens by real time polymerase chain reaction (PCR). **; Show significant difference.

Table 2: Clinical characteristics and pregnancy outcomes of woman receiving GnRH agonist or GnRH antagonist

Evaluated parameter	COH type	Mean	SD	SE	Mean difference	95% CI lower	95% CI upper	P value#
Age (Y)	Agonist	30.90	4.65	1.47	0.70	-3.43	4.83	------
	Antagonist	30.20	4.13	1.31				
BMI (kg/m^2)	Agonist	25.63	3.03	1.36	-0.39	-4.26	3.47	-------
	Antagonist	26.03	3.25	1.08				
Infertility duration (Y)	Agonist	6.60	6.23	2.79	-38.70	-162.25	84.85	--------
	Antagonist	45.30	125.42	39.66				
Prolactin (U/L)	Agonist	10.71	7.45	4.30	-47.96	-181.94	86.03	-------
	Antagonist	58.67	102.17	32.31				
FSH (U/L)	Agonist	7.95	1.34	0.95	-64.85	-398.24	268.54	-------
	Antagonist	72.80	199.96	66.65				
Estradiol (ng/L)	Agonist	31.97	21.71	12.53	-76.22	-133.26	-19.19	--------
	Antagonist	108.19	71.51	22.61				
Follicular number	Agonist	12.50	8.66	4.33	1.70	-5.65	9.05	0.624
	Antagonist	10.80	4.29	1.36				
Endometrial thickness (mm)	Agonist	9.75	0.96	0.48	1.89	-0.68	4.46	0.135
	Antagonist	7.86	2.23	0.71				
					Agonist	Antagonist	Pearson Chi-Square (1)	Exact P value
Pregnancy	Positive	Count			0	3	3.938	0.114
		% within COH type			0.0%	75.0%		
	Negative	Count			3	1		
		% within COH type			100.0%	25.0%		

GnRH; Gonadotropin-releasing hormone, BMI; Body mass index, FSH; Follicle-stimulating hormone, COH; Control ovarian hyperstimulation, CI; Confidence interval, #; P value from the independent samples t test. In all variables, equal variances assumed based on the results from the Levene's Test for Equality of Variances (All P>0.05).

Table 3: Comparison of embryological parameters between GnRH agonist and GnRH antagonist groups

Evaluated parameter	COH type	Mean	SD	SE	Mean difference	95% CI lower	95% CI upper	P value[#]
COC number	Agonist	9.3	5.9	1.87	-5.30	-12.55	1.95	0.142
	Antagonist	14.6	9.1	2.90				
GV (%)	Agonist	5.6	9.6	3.07	0.01	-8.77	8.79	0.998
	Antagonist	5.5	8.9	2.84				
MI (%)	Agonist	12.6	17.8	5.64	3.46	-12.57	19.48	0.656
	Antagonist	9.2	16.2	5.14				
MII (%)	Agonist	78.6	27.6	8.79	-6.18	-28.98	16.62	0.576
	Antagonist	84.8	20.1	6.37				
PN (%)	Agonist	54.5	19.2	6.08	-18.08	-32.23	-3.93	0.015[*]
	Antagonist	72.5	9.1	2.90				
8-Cell (%)	Agonist	52.9	34.3	10.87	17.35	-12.80	47.50	0.242
A-quality	Antagonist	55.5	29.6	9.37				
8-Cell (%)	Agonist	21.2	20.8	6.59	-1.67	-23.37	20.03	0.873
AB-quality	Antagonist	22.9	25.1	7.96				

COC; Cumulus oophorus complex, GV; Germinal vesicle, MI; Metaphase I, MII; Metaphase II, PN; Pro nucleus, CI: Confidence interval, [#]; P value from the independent samples t test, and [*]; Significant P<0.05. The Levene's Test for Equality of Variances showed that the assumption was not satisfied for MI, MII, PN (all P<0.05).

Discussion

This study showed that *BAX* gene expression in the CCs of patients treated with GnRH agonist was higher than those treated with GnRH antagonist. Furthermore, mRNA expression of *BCL-2* and *ALCM* genes were considerably greater in the CCs of the antagonist group in comparison to the agonist group. The gene expression of CCs in the individuals treated with assisted reproductive technology (ART) have been evaluated in numerous previous studies (23). To best of our knowledge, this is the first study to investigate the effect of GnRH analogues on CC gene expression.

The correlation between apoptosis of CCs and ART outcome has been demonstrated in numerous studies (23). Clavero et al. (24) reported that the apoptosis rate of the GCs is not associated with the oocyte maturity, quality, and pregnancy outcomes. However, Lee et al. (25) found a strong correlation between the apoptosis of CCs and poor oocyte quality. Moreover, up-regulation of pro-apoptotic genes and downregulation of anti-apoptotic genes in the CCs of the non-early cleavage embryos have been previously described (26). It was shown that survivin plays an essential role in the function of GCs and the inhibition of apoptosis (15). In addition, a positive relationship has been observed between the *SURVIVIN* gene expression in the GCs and pregnancy rate (27). According to a study by Assou et al. (28), the overexpression of *BCL-2* is associated with pregnancy outcomes. The present study indicated that the relative expression of *BCL-2* is higher in the GnRH antagonist group as compared to the agonist group. Moreover, *BAX* was overexpressed in the GnRH agonist group as compared to the antagonist group. Furthermore, we found no positive correlation between the expression of apoptotic genes and oocyte quality, embryo development, and pregnancy outcome.

The effect of different protocols of GnRH analogues on the ART cycle is controversial (29). Similar results have been reported by Kara et al. (30) regarding the serum progesterone and estradiol levels and the pregnancy rate. Prapas et al. (31) reported positive effects of GnRH antagonist on the live birth rate as well as embryologic and clinical outcomes. Furthermore, higher quality of blastocysts have been noticed in the recurrent implantation failure (RIF) patients that received GnRH antagonist compared to those receiving agonist treatment (32). Contrary to the mentioned study, de Souza Jordão et al. (33) revealed a higher total oocyte number and quality, more embryo development, higher implantation rate, and better pregnancy outcomes following GnRH agonist administration. A recent meta-analysis showed an equal pregnancy rate, endometrial thickness, live birth rate, and cancelation rate after the use of GnRH agonist and antagonist in normal-responder patients (34). Although the clinical and embryological results of our study are not consistent with the aforementioned articles, the molecular findings are compatible. This contradiction may be due to different and incomparable sample sizes.

Two of the five genes that were found to be expressed during oocyte maturation were analyzed in the present study (*ALCAM, VCAN*) (8). A negative correlation was explained between *VCAN* expression level and the percentage of mature oocyte formation. Moreover, decreased *VCAN* expression was shown in the CCs of the subjects with mature oocytes (35). In our study, a relatively

lower expression of *VCAN*, which was not statistically significant, was observed in the GnRH antagonist group. *ALCAM* is known as an ECM-related protein. Cell to cell and cell to matrix adhesion may be promoted by *ALCAM* in the reproductive tissues. *ALCAM* has been shown to be expressed in the epithelium and blastocysts and it has an important role in the implantation process (36). A previous study stated expression of *ALCAM* in the CCs and GCs during the ovulatory response (37). Moreover, a positive correlation between the *ALCAM* expression and proper embryo cleavage has been indicated. It was also introduced as a promising new marker for non-invasive embryo selection (35). We also found a significantly higher *ALCAM* expression in the CCs of the GnRH antagonist-treated group in comparison to the agonist group.

Conclusion

Despite no considerable difference in the oocyte quality, embryo development, and clinical outcomes between GnRH agonist and antagonist, the GnRH antagonist protocol is more favorable considering our molecular findings. In fact, further molecular studies should be performed on this controversial subject to define the exact effect of GnRH analogues on the reproductive system and to identify any advantage or superiority between the GnRH agonist and antagonist protocols.

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Authors' Contributions

B.H.R., S.A.; Participated in the study design, data collection and evaluation, drafting and statistical analysis. S.A., M.B.; Performed Lab data preparation. M.B., S.M.M., F.H., B.H.R.; Contributed extensively in the interpretation of the data and the conclusion. S.A.; Conducted molecular experiments and RT-qPCR analysis. All authors performed editing and approving the final version of this manuscript for submission, also participated in the finalization of the manuscript and approved the final draft.

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Original Article

Protective Effect of *Aloe vera* Gel against Cisplatin-Induced Testicular Damage, Sperm Alteration and Oxidative Stress in Rats

Naeem Erfani Majd, Ph.D.[1, 2*], Mohammad Reza Tabandeh, Ph.D.[2, 3], Shima Hosseinifar, Ph.D.[1], Mahin Sadeghi, M.Sc.[1]

1. Department of Basic Sciences, Histology Section, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
2. Stem Cell and Transgenic Technology Research Center, Department of Basic Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
3. Department of Basic Sciences, Division of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

Abstract

Background: Cisplatin (CIS) is an effective antineoplastic drug that is used to treat various types of cancers. However, it causes side effects on the male reproductive system. The present study aimed to investigate the possible protective effects of *Aloe vera* (AL) gel (known as an antioxidant plant) on CIS-induced changes in rat sperm parameters, testicular structure, and oxidative stress markers.

Materials and Methods: In this experimental study, forty-eight adult male rats were divided into 6 groups including: control, CIS, AL, metformin (MET), CIS+AL, and CIS+MET. CIS was used intraperitoneally at a dose of 5 mg/kg on days 7, 14, 21, and 28 of the experiment. AL gel (400 mg/kg per day) and MET (200 mg/kg per day) were administered orally for 35 days (started one week before the beginning of the experiment). Testes weight and dimensions, and morphometrical and histological alterations, activities of antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase (GPx), serum testosterone concentration, lipid peroxidation level, and sperm parameters were examined.

Results: CIS caused a significant decrease ($P<0.05$) in relative weight and dimension of the testis, germinal epithelium thickness and diameter of seminiferous tubules, the numbers of testicular cells, and spermatogenesis indexes. The malondialdehyde (MDA) levels increased and antioxidant enzymes activities decreased in the CIS group compared to the control group ($P<0.05$). Additionally, sperm parameters (concentration, viability, motility, and normal morphology), and testosterone levels reduced significantly in CIS-treated rats ($P<0.05$). Also, CIS induced histopathological damages including disorganization, desquamation, atrophy, and vacuolation in the testis. However, administration of AL gel to CIS-treated rats attenuated the CIS-induced alterations, mitigated testicular oxidative stress and increased testosterone concentration.

Conclusion: The results suggest that AL as a potential antioxidant plant and due to free radicals scavenging activities, has a protective effect against CIS-induced testicular alterations.

Keywords: *Aloe vera*, Cisplatin, Oxidative Stress, Rat, Testis

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Introduction

Cisplatin [CIS-diaminedichloroplatinum (II)] is one of the most effective anticancer drugs which is used for treatment of a vast variety of human cancers. The anticancer activity of CIS is due to multiple mechanisms such as induction of DNA damage, oxidative stress, and programmed cell death (apoptosis) (1).

Despite the fact that CIS is a useful anticancer drug, it is very toxic and induces several side effects including reproductive toxicity, hepatotoxicity and nephrotoxicity (1, 2). Reproductive toxicity is one of the most common side effects of CIS in treated patients (2-5). CIS causes severe testicular damage which is characterized by apoptosis of germ cell, dysfunction of Leydig cell, testicular steroidogenic disorder and spermatogenic damage (3-6). The precise mechanism of reproductive toxicity induced by CIS is not fully established, however oxidative stress has been known as the major cause of CIS-related testicular dysfunction (5, 6). Hence, several investigators have used antioxidant compounds to reduce reproductive damages caused by CIS (2, 5, 6). For example, olive leaf extract which contains flavonoid and polyphenolic compounds ameliorated CIS-induced testicular oxidative stress in rats (5). Also, fenugreek seed extract reduced oxidative stress and testicular tissue damage induced by CIS and improved spermatogenesis in the rats (6).

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*Corresponding Address: P.O.BOX: 61357-831351, Department of Basic Sciences, Histology Section, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
Email: naeemalbo@yahoo.com

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Aloe barbadensis Miller or AL, is a perennial shrubby plant of the Liliaceae family. It considered an important medicinal herb because of its many medicinal activities including antitumor, antioxidant, anti-allergic, anti-viral, and anti-inflammatory properties. It has been proposed that the antioxidant activity of AL may be a major property of this plant used in the treatment of several diseases. The antioxidant property of AL is due to a large amount of antioxidants substances such as vitamins (A, C, B and E), flavonoids, phenolic compounds, and polysaccharides (7). Several researchers have provided experimental evidence for the reproprotective effect of AL in experimental animals (8-11). These studies have shown that AL can efficiently attenuate the testicular alteration induced by some drugs and heavy metals (8, 9). Other studies reported that AL due to its antioxidant compounds (especially vitamin E) can improve testicular weight, height of the germinal epithelium and diameter of seminiferous tubule, and ameliorate reductions in the number of testicular cells. Also, phenolic and flavonoids contents of AL can be effective in increasing the antioxidant enzymes activity and decreasing lipid peroxidation that can cause extensive damage to cell membranes lipids (8-11).

It has been found that oxidative stress plays a major role in the pathogenesis of reproductive toxicity induced by CIS. Because of the antioxidant property of AL gel, it was hypothesized that AL may attenuate CIS- mediated gonadotoxicity in rats. Therefore, this study was designed for the first time, to examine possible protective effects of AL gel on gonadotoxicity induced by CIS via evaluation of epididymal sperm parameters, alterations of testicular tissue, testosterone levels, and oxidative/antioxidant markers in the testis of rats.

Materials and Methods

Preparation of *A. vera* gel and analysis of its antioxidant properties

AL gel powder (A. barbadensis) was obtained from Barij Essence Pharmaceutical Co (Kashan, Iran). Total flavonoids content (TFC) was measured by aluminum chloride colorimetric assay (12). The catechin solutions (0-25 µg/mL) were prepared for flavonoid assessment. Aliquots (25 µL) of each AL gel (10 mg in 1 ml distilled water) and standard were mixed with 125 µL distilled water followed by adding 8 µL of 5% sodium nitrate. After 5 minutes, 0.15 ml of 10% aluminum chloride solution was added to 15 µL of that mixture. The absorbance was measured at 517 nm. TFC is expressed as the percentage of catechin equivalents (QE) per 100 g dry weight, and was determined from the standard calibration curve.

Total phenolic content (TPC) of AL gel was estimated using the Folin-Ciocalteu (FC) and aluminum chloride colorimetric assay as described by Im et al. (12). Contents are expressed as the percentage of gallic acid equivalents (GAE) per 100 g dry weight of AL gel.

Animals and experimental groups

In this experimental study, a total of forty-eight healthy male Wistar rats (180-200 g) were maintained under standard laboratory conditions (12-hour light: 12-hour dark at 22 ± 2 °C) and fed with commercial rat pellets (Pars Animal Feed Co, Tehran, Iran) and water. All experimental assays were approved by the Ethics Committee of Shahid Chamran University of Ahvaz for animal and human experiments (EE/99.3.02.15058/ssu.ac.ir).

After a quarantine period of 7 days, the rats were divided randomly into 6 groups (n=8) as follows: control group: rats fed with a standard diet and kept in normal conditions. CIS group (CIS): rats received CIS intraperitoneally (i.p) at a dose of 5 mg/kg on days 7, 14, 21, and 28 of the experiment. AL group (AL): AL gel powder was dissolved in distilled water and administered orally at a dose of 400 mg/kg/day for 35 days. MET group (MET): rats received MET (200 mg/kg/day, orally) for 35 days. CIS and AL group (CIS-AL): rats received CIS (i.p) at a dose of 5 mg/kg on days 7, 14, 21, and 28 of the experiment and AL (400 mg/kg/day, orally) for 35 days. CIS and MET group (CIS-MET): rats received CIS (i.p) at a dose of 5 mg/kg on days 7, 14, 21, and 28 of the experiment and MET (200 mg/kg/day, orally) for 35 days.

The experiment lasted for 35 days (13). The dose of CIS was selected based on a published report (14). The dosing regimen for AL and MET were selected based on reports by Behmanesh et al. (13) and Sahu et al. (15), respectively.

Sample collection

All rats were anesthetized using ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively), (Alfasan Chemical Co., Woerden-Netherlands) (16).

The blood samples were collected via cardiac puncture and centrifuged (at 3000 rpm for 10 minutes). Serum samples were separated and then stored at -20˚C for testosterone hormone analysis. Afterward, testes and epididymis were obtained from the abdominal cavity. The weight, dimensions (length and diameter) and volume of testes were measured using a digital scale, a caliper, and water displacement method, respectively (17). The left testis was fixed in a 10% buffered formalin solution for histological analyses and the right testes were stored at -20°C for oxidant/antioxidant assessment. The epididymis tissue samples were used for the analysis of sperm parameters.

The testicular index

The relative testis weight ratio (%) was calculated using the formula: (absolute weight of the testis/ total body weight)×100 (5).

Histological procedures

The formalin-fixed testes were embedded in paraffin blocks, then sectioned (5-µm thickness) by a microtome (Leica RM 2125, Leica Microsystems Nussloch GmbH, Germany). Sections were stained with hematoxylin and eosin (H&E).

Morphometrical analyses

For this purpose, 100 cross sections of seminiferous were chosen randomly in 5 non-serial sections per animal (10 tubules in the central zone and 10 tubules in the peripheral (sub-capsular) zone of each section). Then, the seminiferous tubule diameter and height of germinal epithelium were measured at ×10 magnification. Also, Sertoli, Leydig, spermatogonia, primary spermatocyte, early spermatid and late spermatid cells were counted in a marked scale (150 µm) at ×40 magnification (18, 19). All measurements were performed under a light microscope (Olympus Optical Co., Japan) using Dino-Lite digital lens (with Dino capture software, FDP2, Taiwan).

Spermiogenesis index (SI) and tubular differentiation index (TDI) were calculated for spermatogenesis assay. SI index was calculated using the following formula:

(Seminiferous tubules contained sperm/seminiferous tubules without sperm)×100.

For TDI index, the percentage of tubules that contained three or more differentiated spermatogenic cells from the type A spermatogonia (i.e. intermediate or type B spermatogonia, spermatocytes, or spermatids) were calculated (18).

Analysis of sperm parameters

The cauda epididymis was minced finely in (5 ml) Ham's F-10 medium and placed at 37°C for 15 minutes. Spermatozoa in the epididymis were counted by a standard hemocytometric method and motility of sperm (progressive, non- progressive, and immotile) was evaluated under a light microscope (Olympus Optical Co., Japan) at 3 consecutive estimates and reported as mean (20). Sperm viability and morphology were evaluated by the methods described by Turk et al. (21) and Adibmoradi et al. (18). Briefly, a 10-µL sperm suspension was slowly mixed with 40 µL eosin-nigrosin (1.67% eosin, 10% nigrosin and 0.1 M sodium citrate). Then, 10 µL of this mixture was transferred to a glass slide and spread slowly by another slide. After preparation of smears, viability and morphology of sperms were evaluated. Spermatozoa with red head were classified as dead sperm and spermatozoa with white head were classified as live sperm (18). Also, sperms were screened and classified into normal and abnormal types, and then the percentage of abnormality was determined for each group (21).

Tissue preparation for oxidant/antioxidant markers assay

Here, 100 mg of the right testicular tissue sample was homogenized in 500 µL RIPA lysis buffer (1 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 10 mM Tris-HCL; pH=8, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride) by a glass homogenizer (Heidolph, Germany). Homogenate was centrifuged at 10000 rpm for 15 minutes at 4°C (Centrifuge 5415 R; Eppendorf AG, Germany) and the supernatant was collected and stored at -70°C for subsequent analysis. The protein concentration of the supernatant was estimated using the Bradford method (22).

Analyses of lipid peroxidation levels and antioxidant enzymes activities

The content of malondialdehyde (MDA) in the testis was assessed as a lipid peroxidation marker using the thiobarbituric acid reactive substance (TBARS) assay with slight modifications (23). The MDA concentration was obtained based on MDA-TBARS complex optical density at 532 nm wavelength in comparison with the standard curve of MDA. The MDA results are expressed as nmol/mg of protein. Superoxide dismutase (SOD) activity was determined by the nitro blue tetrazolium (NBT) reduction assay, as described by Kakkar et al. (24). Finally, glutathione-peroxidase (GPx) activity was evaluated by a GPx detection kit according to the manufacturer's instructions (RANSEL, Randox Com, UK). Both SOD and GPx activities are expressed as units/mg protein.

Testosterone analysis

Testosterone concentration in the serum samples of the experimental groups was quantitatively assessed through enzyme-linked immunosorbent assay (ELISA) using the Diametra testosterone ELISA kit (Diametra Co, Italy), according to the manufacturer's protocol. Testosterone results are expressed as ng/dl.

Statistical analysis

Data are expressed as mean ± standard deviation and were analyzed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Differences among various groups were assessed by one-way analysis of variance (ANOVA) followed by the Tukey test. In all cases, $P<0.05$ was regarded as significant.

Results

Phytochemical content of *A. vera* gel

The results showed that concentrations of the total phenol and flavonoid contents in the AL gel were 49.81 µg GAE/mg and 56.42 µg QE /mg of gel powder, respectively.

Relative weight and dimensions of the testis

The results showed that CIS caused a significant ($P<0.05$) decrease in relative weight, length, diameter and volume of both the right and left testes compared to the control group. The co-administration of AL and CIS significantly increased relative weight, length and diameter of the testes (right and left), and volume of the right testis compared to the CIS group ($P<0.05$). Although, there was a numerical increase in the volume of the left testis in the CIS-AL group, it was not statistically significant. Treatment of CIS-treated rats with MET significantly attenuated the reduction of relative weight, length and volume of both the right and left testes and diameter of the left testis ($P<0.05$). Also, MET increased the diameter of the right testis, however this change was not significant compared to the CIS group (Table 1).

Table 1: Relative weight, volume and dimensions of testis in different groups

Groups testicular parameters	Control	CIS	AL	MET	CIS-AL	CIS-MET
Relative testicular weight (%)						
Right	0.60 ± 0.03	0.46 ± 0.06[a]	0.61 ± 0.03[b]	0.61 ± 0.04[b]	0.59 ± 0.07[b]	0.60 ± 0.03[b]
Left	0.59 ± 0.02	0.44 ± 0.06[a]	0.62 ± 0.03[b]	0.63 ± 0.06[b]	0.59 ± 0.01[b]	0.59 ± 0.02[b]
Length (mm)						
Right	19.00 ± 2.00	14.00 ± 1.58[a]	19.33 ± 2.08[b]	19.00 ± 1.87[b]	18.60 ± 2.19[b]	18.20 ± 2.86[b]
Left	19.33 ± 0.57	14.50 ± 0.50[a]	19.66 ± 2.08[b]	19.33 ± 1.57[b]	18.20 ± 0.43[b]	18.66 ± 1.15[b]
Diameter (mm)						
Right	8.20 ± 1.30	5.20 ± 0.83[a]	8.33 ± 0.57[b]	8.00 ± 1.00[b]	7.60 ± 1.51[b]	7.40 ± 1.34
Left	8.66 ± 0.57	6.83 ± 0.28[a]	8.66 ± 0.28[b]	8.50 ± 0.50[b]	8.16 ± 0.28[b]	8.33 ± 0.57[b]
Volume (ml)						
Right	1.66 ± 0.15	1.03 ± 0.20[a]	1.73 ± 0.15[b]	1.60 ± 0.10[b]	1.53 ± 0.15[b]	1.50 ± 0.10[b]
Left	1.53 ± 0.23	0.82 ± 0.10[a]	1.53 ± 0.12[b]	1.43 ± 0.15[b]	1.16 ± 0.05	1.23 ± 0.06[b]

Data were expressed as mean ± SD. Values with different superscripts are significantly different: [a]; Significant change from the control group at P<0.05, [b]; Significant change from the CIS group at P<0.05, Control; Control group, CIS; Cisplatin (5 mg/kg), AL; *A. vera* (400 mg/kg), and MET; Metformin (200 mg/kg).

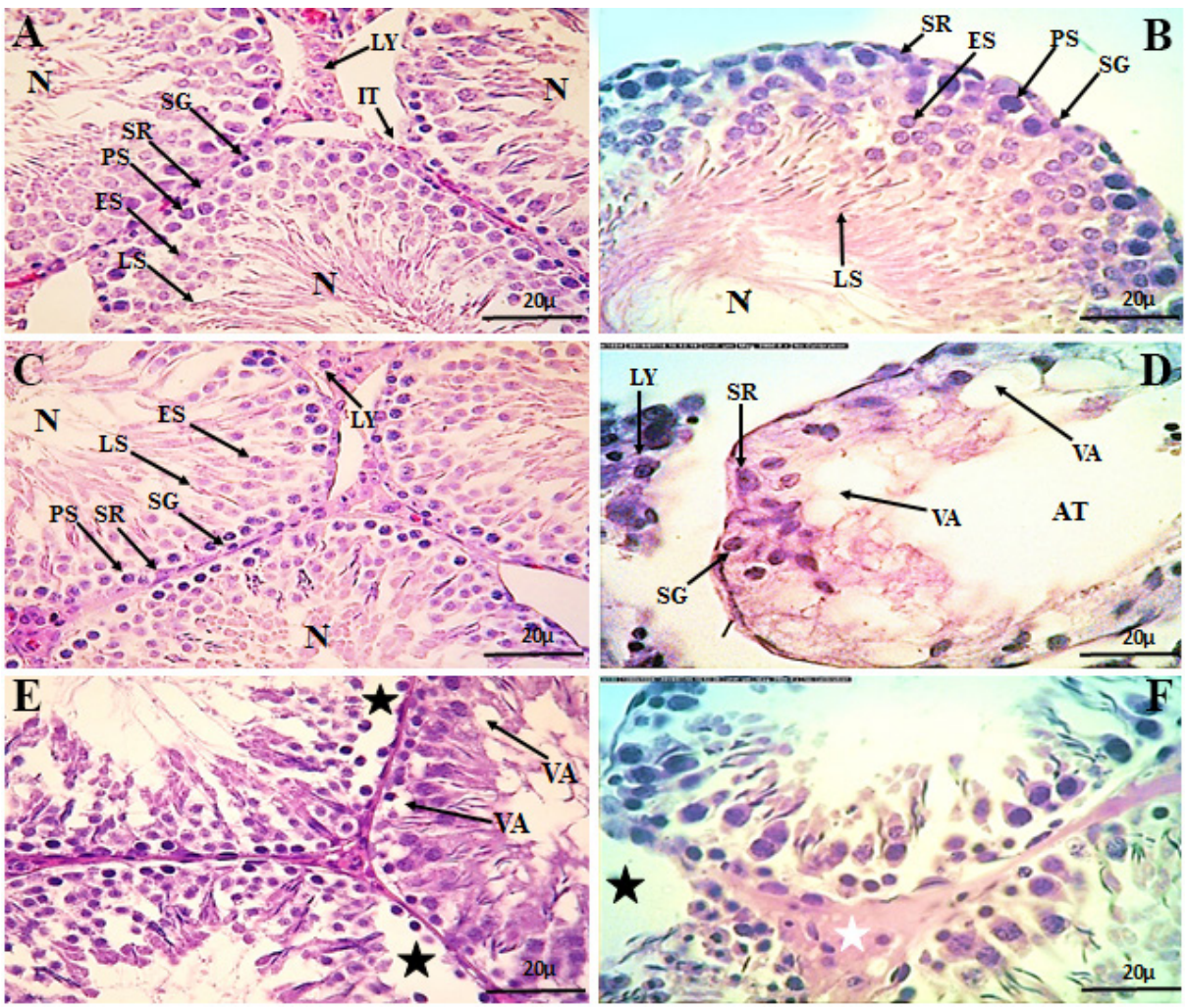


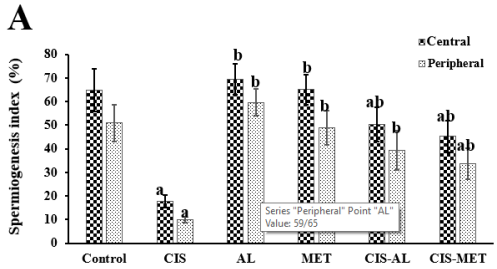
Fig.1: Testicular histopathology in different groups (H&E). **A-C.** The seminiferous tubules with normal germinal epithelium (N), interstitial tissue (IT) and active spermatogenesis in control, *A. vera* (400 mg/kg) and metformin (200 mg/kg) groups, respectively. **D.** Atrophy (AT), vacoulation (VA) and decreasing of spermatogenesisin cisplatin (5 mg/kg) group. **E, F.** A considerable improve in the seminiferous tubules observed in CIS-AL (400 mg/kg *A. vera*+5 mg/kg cispaltin) and CIS-MT (200 mg/kg metformin+5 mg/kg cispaltin) groups, but VA, desquamation (black stars) and interstitial edema (white stars) were seen still. LY; Leydig cell, SR; Sertoli cell, SG; Spermatogonia, PS; Primary spermatocyte, ES; Early spermatid, and LS; Late spermatids.

Histological findings

The testicular tissue of the control group composed of a high density of normal shape testicular tubules surrounded by interstitial connective tissues. Seminiferous tubules lined by a stratified germinal epithelium, showed features of active spermatogenesis. Spermatogonia cells with heterochromatin and rounded nuclei rested on the basal lamina. Primary spermatocytes were the largest spermatogenic cells in the germinal epithelium with different shapes of chromatin. Furthermore, early-stage spermatids with euchromatin and round nuclei and late-stage spermatids with heterochromatin and elongated nuclei, were attached to the membrane of Sertoli cells. Also, Sertoli cells rested on the basal lamina and had large, euchromatin nuclei with prominent nucleolus. The Leydig cells in interstitial connective tissues had eosinophilic cytoplasm with large and round nuclei (Fig.1A). In AL (Fig.1B, AL) and MET (Fig.1C, MET) treated groups, the seminiferous tubules showed normal cells associations without any structural changes compared to the control group (Fig.1B, C). CIS caused atypical morphological features such as disorganization, and desquamation in the seminiferous tubules. Also, widespread atrophy and loss of all germ cells and extensive vacuolation in the epithelium were observed in CIS-treated rats. In addition, maturation arrest and absence of spermatozoa in the lumen in a majority of seminiferous tubules were significant (Fig.1D). Co-administration of AL and CIS normalized these histological changes and amended spermatogenesis when compared with the CIS alone group; though a slight vacuolation was found, desquamation was still observed in the seminiferous tubules (Fig.1E). Likewise, MET attenuated the histological abnormalities induced by CIS, and protected the testicular tubules although it was less than that seen for AL (Fig.1F).

Morphometrical finding

The number of Sertoli, Leydig, spermatogonia, primary spermatocytes, early and late spermatids cells (Table 2), germinal epithelium thickness, diameter of seminiferous tubule and the spermatogenesis indexes (TDI and SI) decreased in the central and peripheral (sub-capsular) zones of the testis after the CIS treatment (P<0.05, Fig.2). But, administration of AL and MET along with CIS significantly restored these alterations (P<0.05, Table 2, Fig.2). The morphometrical parameters in control, AL, and MET groups were almost identical.



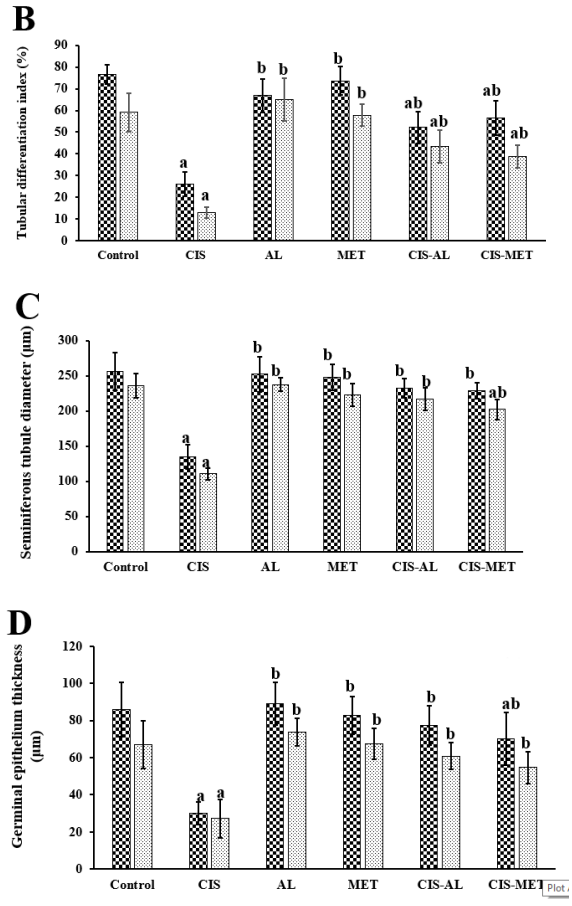


Fig.2: Comparison of the spermatogenesis indexes, germinal epithelium thickness and diameter of seminiferous tubules in different groups. **A.** Spermiogenesis index, **B.** Tubular differentiation index, **C.** Seminiferous tubule diameter, and **D.** Germinal epithelium thickness. Data were expressed as mean ± SD. Values with different superscripts are significantly different: [a]; Significant change from the control group at P<0.05, [b]; Significant change from the CIS group at P<0.05, Control; Control group, CIS; Cisplatin (5 mg/kg), AL; *A. vera* (400 mg/kg), and MET; Metformin (200 mg/kg).

Comparison of MDA level and antioxidant activities

Figure 3 shows the changes in MDA level and activities of antioxidant enzymes in testis tissues of different groups. There was a significant increase in MDA level along with a significant reduction of SOD and GPx activities in CIS-treated rats compared to the control group (P<0.05, Fig.3). Nevertheless, administration of AL or MET together with CIS significantly reduced the MDA level and elevated antioxidant enzymes activities in comparison to the CIS group (P<0.05). There was no significant difference in MDA level and antioxidant enzymes activities in the AL and MET groups compared to the control group (Fig.3).

Comparison of serum testosterone level

As showed in Figure 3, testosterone level was significantly lower in CIS group rats compared to the other groups (P<0.05, Fig.3). Treatment of CIS rats by AL and MET significantly ameliorated the reduction of testosterone level (P<0.05). AL and MET groups presented no significant difference in the serum testosterone level compared to the control group (P>0.05, Fig.3).

Table 2: Comparison of the number of testicular cells (Leydig, Sertoli, spermatogonia, primary spermatocyte, early and late spermatid) in different groups

Groups parameters	Control	CIS	AL	MET	CIS-AL	CIS-MET
Leydig cells						
Central	16.26 ± 1.85	7.66 ± 1.77[a]	16.01 ± 0.58[b]	15.66 ± 0.61b	12.20 ± 1.00	11.93 ± 1.51
Peripheral	11.26 ± 1.00	5.00 ± 2.16[a]	11.46 ± 0.94[b]	13.66 ± 0.64b	10.86 ± 0.98[b]	10.53 ± 1.36[b]
Sertoli cells						
Central	5.40 ± 0.60	2.33 ± 0.80[a]	5.20 ± 0.91[b]	5.06 ± 0.80b	4.20 ± 0.60	4.40 ± 0.52[b]
Peripheral	5.00 ± 0.91	1.86 ± 0.70[a]	4.60 ± 0.91[b]	5.13 ± 0.64b	3.53 ± 0.11[b]	3.13 ± 0.80
Spermatogonia						
Central	14.46 ± 2.40	6.40 ± 1.50[a]	14.60 ± 0.80[b]	15.33 ± 0.80b	10.80 ± 0.60[b]	10.20 ± 1.24[ab]
Peripheral	9.86 ± 2.10	5.86 ± 0.75[a]	10.06 ± 2.20[b]	9.93 ± 1.70b	7.40 ± 1.24[ab]	8.13 ± 0.70[ab]
Primary spermatocyte						
Central	15.60 ± 0.72	7.26 ± 0.61[a]	15.33 ± 1.40[b]	16.26 ± 1.00b	10.53 ± 0.83[ab]	11.00 ± 1.96 [ab]
sub-capsular	7.80 ± 0.60	4.80 ± 0.40[a]	7.33 ± 0.50[b]	7.86 ± 0.41b	6.80 ± 0.40[b]	5.86 ± 0.50[a]
Early spermatid						
Central	63.73 ± 10.21	14.06 ± 2.91[a]	54.63 ± 8.80[b]	58.46 ± 5.28b	38.60 ± 7.68[ab]	42.13 ± 3.55[ab]
Peripheral	27.13 ± 2.38	7.93±2.60[a]	26.73 ± 1.50[b]	25.40 ± 4.49b	15.26 ± 2.60[ab]	17.80 ± 1.40[ab]
Late spermatid						
Central	56.66 ± 9.16	19.20±6.39[a]	62.00 ± 8.19[b]	50.46 ± 2.66b	39.80 ± 2.82[b]	44.73 ± 8.76[b]
Peripheral	24.33 ± 3.62	5.66±2.93[a]	26.20 ± 4.72[b]	22.40 ± 2.90b	14.66 ± 3.84	16.06 ± 1.40[b]

Data were expressed as mean ± SD. Values with different superscripts are significantly different: [a]; Significant change from the control group at P<0.05, [b]; Significant change from the CIS group at P<0.05, Control; Control group, CIS; Cisplatin (5 mg/kg), AL; *A. vera* (400 mg/kg), MET; Metformin (200 mg/kg), CIS-AL; *A. vera* (400 mg/kg)+cisplatin (5 mg/kg), and CIS-MET; Metformin (200 mg/kg)+cisplatin (5 mg/kg).

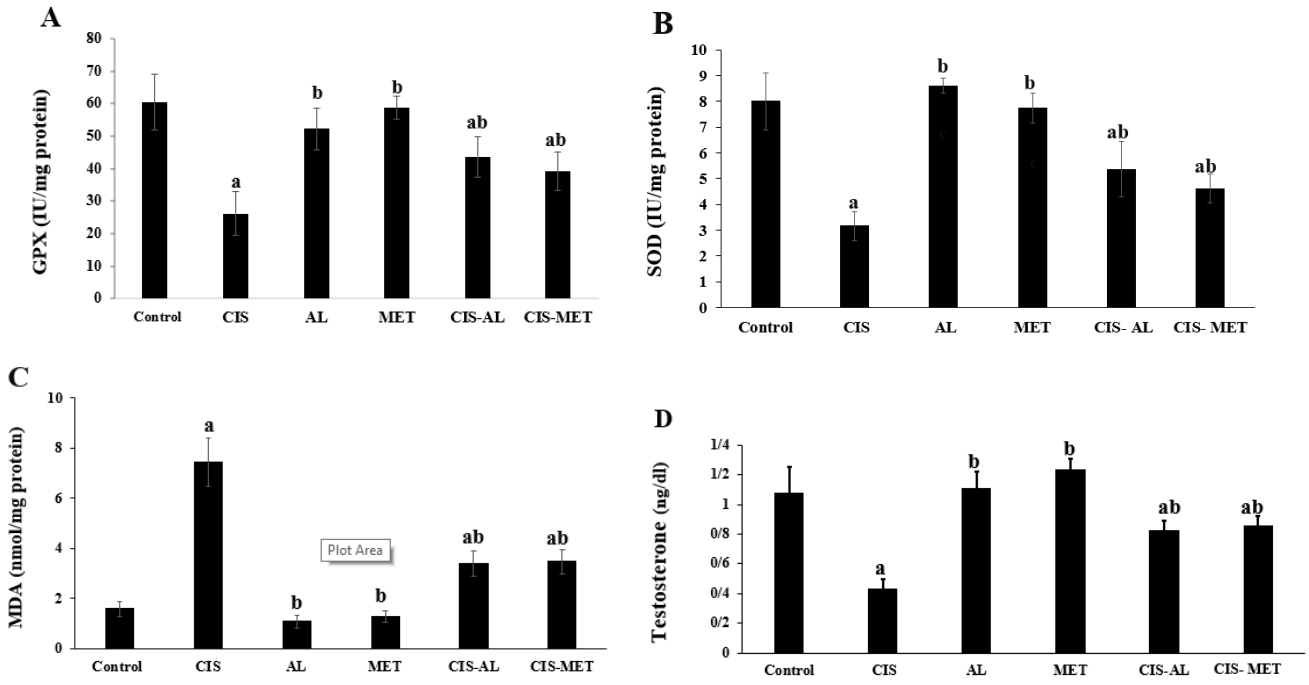


Fig.3: Comparison of antioxidant enzyme activities, malondialdehyde (MDA) levels and serum testosterone levels between groups. **A.** Glutathione peroxidase (GPx) activity, **B.** Superoxide dismutase (SOD) activity, **C.** Malondialdehyde levels, and **D.** Testosterone levels. Data were expressed as mean ± SD. Values with different superscripts are significantly different: [a]; Significant change from the control group at P<0.05, [b]; Significant change from the CIS group at P<0.05, Control; Control group, CIS; Cisplatin (5 mg/kg), AL; *A. vera* (400 mg/kg), and MET; Metformin (200 mg/kg).

Table 3: Comparison of sperm parameters in different groups

Groups Sperm parameters	Control	CIS	AL	MET	CIS-AL	CIS-MET
Viability (%)	86.26 ± 13.44	30.00 ± 11.33[a]	91.94 ± 11.41[b]	87.00 ± 16.00[b]	77.53 ± 13.66[b]	67.33 ± 9.01[b]
Concentration (10^6/mL)	120.55 ± 9.44	55.00 ± 7.12[a]	127.50 ± 10.68[b]	128.75 ± 8.75[b]	98.81 ± 13.68[b]	79.75 ± 12.28[ab]
Progressive motility (%)	78.17 ± 7.45	11.88 ± 4.16[a]	81.16 ± 7.56[b]	79.69 ± 3.59[b]	64.77 ± 11.78[b]	52.89 ± 17.18[ab]
Non- progressive motility (%)	5.76 ± 2.61	15.29 ± 3.90[a]	5.94 ± 3.16[b]	4.58 ± 1.39[b]	7.52 ± 3.72[b]	9.44 ± 3.77
Immotile sperm (%)	16.07 ± 4.99	72.83 ± 5.51[a]	12.90 ± 4.71[b]	15.73 ± 2.20[b]	27.71 ± 8.30[b]	37.67 ± 13.59[ab]
Abnormal sperm (%)	8.52 ± 2.40	33.93 ± 3.18[a]	9.65 ± 6.76[b]	11.56 ± 7.38[b]	18.67 ± 4.82[b]	21.64 ± 2.56[ab]

Data were expressed as mean ± SD. Values with different superscripts are significantly different: [a]; Significant change from the control group at P<0.05, [b]; Significant change from the CIS group at P<0.05, Control; Control group, CIS; Cisplatin (5 mg/kg), AL; *A. vera* (400 mg/kg), and MET; Metformin (200 mg/kg).

Comparison of sperm parameters

A comparison of the groups with regard to sperm parameters is presented in Table 3. The sperm concentration, viability, and progressive and non- progressive motility decreased significantly in the CIS group compared to the control group but the percentage of abnormal sperm morphology increased (P<0.05, Table 3). By contrast, administration of AL gel along with CIS could significantly improve the sperm parameters compared to the CIS group (P<0.05, Table 3). Also, treatment of CIS rats by MET significantly increased sperm concentration, viability and progressive motility and reduced abnormal morphology of sperm compared to the CIS group (P<0.05). Non- progressive motility increased in the CIS-MET group, but this change was not significant compared to the CIS group (Table 3).

No significant differences were observed in the sperm parameters between the AL and MET groups and the control group (Table 3).

Discussion

CIS-based chemotherapy induces gonadal toxicity and infertility by increasing oxidative stress (5, 6). Hence, administration of antioxidant agents may be a useful strategy in reducing CIS toxicity and preserve the fertilization capacity of patients receiving CIS.

The results of the present study showed that CIS decreased relative weight and dimensions of the testis, and reduced the germinal epithelium thickness, and the diameter of seminiferous tubules. Additionally, histopathological changes such as testicular atrophy, desquamation, vacuolation of germinal epithelium, and reduction of spermatogenesis activity were observed in CIS-treated rats.

Loss of testicular weight and dimension in CIS-treated rats could be due to the inhibition of spermatogenesis, atrophy of testicular tubules, reduction of spermatogenic cells, and other degenerative alterations caused by CIS (25). These histological damages may be explained by disruptions of the redox balance induced by CIS which result in DNA damage, lipid peroxidation, and inhibition of protein synthesis (4). Testis tissue is highly vulnerable to oxidative stress because it has a high metabolic activity and considerable amount of highly unsaturated fatty acids (26). Free radicals impair different parts of the testis especially testicular germinal cells and lead to atrophy in testicular tubules and reduction of sperm generation (20, 26, 27).

Data from the present study likely showed that CIS treatment impairs oxidant-antioxidant balance in testicular tissue so that it increased the levels of MDA and decreased antioxidant enzymes (SOD and GPx) activities, these results are in agreement with previous reports (27, 28). The peroxidation of lipids is one of the toxic effects of CIS in the testis and MDA is produced as the end-product of this process; thus, MDA content is the best marker for measuring oxidative stress and lipid peroxidation indirectly. Also, the increase in the MDA level may be related to DNA fragmentation as reported previously (29). The reductions of the antioxidant enzymes activities observed in this study, are probably due to either direct effects of CIS on these enzymes or enhanced consumption of antioxidant enzymes for detoxifying free radicals generated by CIS (30).

We found a CIS-mediated decrease in serum testosterone concentration which is fundamentally consistent with previous studies (27, 29). Saral et al. (27) reported that the reduction of testosterone level induced by CIS results from a decrease in the number of Leydig cells or their dysfunction. Another hypothesis is that CIS inhibits testosterone synthesis by depressing the cytochrome P-450-dependent 17-α-hydroxylase level and decreasing the numbers of luteinizing hormone (LH) receptors in Leydig cells.

CIS treatment reduced sperm concentration, motility and viability and increased abnormal sperm morphology, consistent with many reports that have indicated the side effects of CIS on sperm function (20, 28). The alteration in sperm parameters of the CIS group was probably caused by prolonged exposure of the testis to CIS-induced free radicals (20). Free radicals decrease the mitochondrial membrane potential in sperm cells which is associated with a decrease in adenosine triphosphate (ATP) production and inhibition of sperm motility (31). In addition, damage of the sperm cell membrane by CIS-induced free radicals may be the cause for the decrease in sperm viability and motility and the increase in the morphological defects (32).

In the present study, administration of AL gel at a dose of 400 mg/kg effectively inhibited the CIS-induced testicular oxidative stress by decreasing the MDA levels and increasing the antioxidant enzymes activities. Also, our results clearly showed that AL treatment attenuated adverse effects of CIS on relative testicular weight and dimension, sperm parameters, testosterone level, and histological changes of the testis.

These chemoprotective effects of AL a gainst CIS-induced toxicity may be related to the antioxidant effect of AL, as reported in previous studies (33, 34). Imaga et al. (33) reported that AL gel improves CIS-induced oxidative damages in the kidney and liver of experimental animals. Also, Chatterjee et al. (34) indicated that administration of AL along with CIS was associated with amelioration of antioxidant defense system and diminution of CIS-induced nephrotoxicity.

AL and especially its gel are highly spermatogenic and enhance male fertility by elevating sperm quality (10, 11). AL increases spermatogenesis process via affecting spermatogenic cells and stimulating cell division, and increases testosterone hormone by stimulating Leydig cells (10, 11, 35).

Estakhr and Javdan (10) reported that AL significantly increased testicular weight, testosterone hormone, and sperm concentration and motility and decreased sperm abnormalities. Also, AL increases cAMP responsive element modulator (CREM) gene expression that has a key role in the regulation of the expression of genes that control spermatogenesis (11).

AL contains a large number of antioxidant compounds including vitamins (A, C, B, E), flavonoids, phenolic compounds, and polysaccharides (7). Vitamin E has the highest antioxidant activity and plays a key role in the protection of plasma membrane against peroxidation by free radicals. Also, vitamin E improves testicular weight, germinal epithelium thickness, and diameter size of seminiferous tubule (36). Vitamin C in AL gel performs an important role in the integrity and fertility of semen and makes up to 65% of the total antioxidant capacity of seminal plasma. Also, vitamin C inhibits sperm agglutination and increases testosterone concentration (37). Furthermore, phenolic compounds and polysaccharides of AL have antioxidant capacity and prevent diseases induced by oxidative stress (38). Therefore, because of its antioxidant properties, AL can reduce CIS-induced oxidative damages in testis tissue and can support spermatogenesis and protect spermatozoa against free radicals.

Conclusion

Our findings demonstrated that oxidative stress can play a significant role in the pathogenesis of CIS-induced testicular and sperm injuries. Also, biochemical, hormonal, and histological results suggest that AL gel could be effective for prevention of gonadal toxicity induced by CIS in male Wistar rats. This study concluded that AL gel due to its potent antioxidant effect, can protect the testicular tissue from toxic damages caused by CIS.

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Authors' Contributions

N.E.M., M.R.T., S.H.; Contributed to conception and design. M.S.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. N.E.M., M.R.T.; Were responsible for overall supervision. N.E.M.; Drafted the manuscript, which was revised by M.R.T. and S.H. All authors read and approved the final manuscript.

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Original Article

Assessment of Organophosphate Pesticides Exposure in Men with Idiopathic Abnormal Semen Analysis: A Cross-Sectional Pilot Study

Induja Manikandan, M.D.[1], Sushmita Bora, M.Sc.[1], Prashant Shankarrao Adole, M.D.[1*], Chitra Thyagaraju, M.D.[2], Rajesh Nachiappa Ganesh, M.D., D.N.B.[3]

1. Department of Biochemistry, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry, India
2. Department of Obstetrics and Gynecology, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry, India
3. Department of Pathology, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry, India

Abstract

Background: Because of the widespread use of organophosphate (OP) pesticides in agriculture, they are major environmental contaminants in developing countries. OP pesticides decrease sperm concentration and affect its quality, viability, and motility. Studies have demonstrated the association between abnormal semen analysis and OP pesticides exposure among the high-risk population. As there is limited data on the percentage of OP pesticides exposure, the study aimed to determine the OP pesticides exposure in Southern Indian men with idiopathic abnormal semen analysis and find the possible source of their OP pesticides exposure.

Materials and Methods: In this cross-sectional pilot study, fifty men with idiopathic abnormal semen analysis as cases and fifty men with normal semen analysis as controls were recruited. Detailed history was taken and general and systemic examinations were carried out. OP pesticides exposure was determined by assessment of pseudocholinesterase and acetylcholinesterase levels and urinary OP pesticides metabolites dialkyl phosphate (DAP) consisting of dimethyl phosphate (DMP), diethyl thiophosphate (DETP), and diethyl dithiophosphate (DEDTP).

Results: Cases had statistically significantly lower levels of pseudocholinesterase (5792.07 ± 1969.89 vs. 10267.01 ± 3258.58 IU/L) (P=0.006) and acetylcholinesterase [102.90 (45.88-262.74) vs. 570.31 (200.24-975.30) IU/L] (P=0.001) as compared to controls. Cases had a statistically significantly higher percentage of urinary DAP positivity as compared to controls (80 vs. 38%, P<0.0001). Hence, cases had a significantly higher percentage of OP pesticides exposure as compared to controls (20 vs. 4%, P=0.015). OP-exposed cases had significantly higher urinary DETP and DEDTP levels as compared to OP non-exposed cases. Also, urinary DETP and DEDTP levels were significantly negatively associated with sperm concentration, motility, and normal morphology among OP-exposed cases.

Conclusion: Southern Indian men with idiopathic abnormal semen analysis had a significantly higher percentage of OP pesticides exposure as compared to men with a normal semen analysis.

Keywords: Acetylcholinesterase, Male Infertility, Pseudocholinesterase, Organophosphate Pesticides

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Introduction

Infertility as one of the major public health problems is defined as "a failure in achieving a clinical pregnancy after 12 or more months of regular unprotected sexual intercourse" as per the World Health Organization (WHO) (1). According to the WHO, 45-52.6 million married couples were suffering from infertility worldwide in 2010 (2). The prevalence of infertility among Indians was ranging from 3.9 to 16.8% as estimated by the WHO (3). As per the report of a multicentric study by the WHO, 20% of cases of infertility were due to male factors, 38% due to female factors, 27% due to both partners, and 15% cases of infertility were idiopathic. In India, nearly 50% of cases of infertility were due to the reproduction anomaly or disorders in males and in 25% of cases, no detectable causes were found and it was considered idiopathic (4). Male infertility is rising in society and its causes are multifactorial. Many studies have shown a declining trend in the semen quality and sperm count among the population (5, 6). A study conducted in the Indian population over the past 37 years has shown a decline in sperm count and motility, and altered sperm morphology with time (7). No

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*Corresponding Address: Department of Biochemistry, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry, India
Email: prashant.adole@gmail.com

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clear cause has been found for the decline of semen quality, but it might be due to environmental, dietary, or other unknown causes (5).

Organophosphate (OP) pesticides are synthetic chemicals used worldwide for controlling domestic and agricultural pests. Pesticides used to control pests and weed on crops are registered under the central insecticides board and registration committee (CIBRC), which comes under the Ministry of Agriculture and Farmer welfare. Section-3 of the Insecticides Act, 1968 has registered around 30 OP pesticides, which are in use in India (8). OP pesticides like monocrotophos, phorate, quinalphos, malathion, chlorpyrifos, diazinon, methyl parathion, ethion, and so on, used extensively in India, were already banned or severely restricted in the USA and Europe. The OP pesticides are associated with severe toxicity, contributing to more than 80% of pesticides-related hospitalization in India (9). OP pesticides cause phosphorylation of acetylcholinesterase resulting in acetylcholine accumulation in synapses. OP pesticides affect reproduction function by reducing acetylcholinesterase activity in the brain, and influencing gonads. OP pesticides like parathion and methyl parathion have a structure similar to hormones like estrogen, thus altering genes expression by interacting with hormone receptors. OP pesticides alter the hypothalamic-pituitary (HPO), pituitary-thyroid, and pituitary-adrenal axes and serum prolactin levels. OP pesticides affect spermatogenesis by damaging the Sertoli and Leydig cells and increasing their apoptosis (10). A toxicological study demonstrated that OP pesticides cause low sperm concentration by affecting germ cell proliferation and damaging the seminiferous epithelium (11). Also, OP pesticides disturb sperm motility by disturbing its tail assembly proteins or ATP synthesis (12). Concerning the association between semen parameters and OP pesticides exposure among agricultural workers, pesticide sprayers, and workers in pesticides manufacturing industries, several studies concluded that there was a decrease in sperm concentration, motility, viability, and normal morphology due to OP pesticides exposure (13-18). There has been contamination of agricultural soil, sediment, and water by various OP pesticides throughout India (9). Hence, subtle OP pesticides exposure is occurring among human beings through food, water, air, tainted breast milk, playing in the field, or skin contact.

Most of the studies were done on high-risk populations to find out potential associations between OP pesticides exposure and alteration in semen parameters. However, there is limited data available in the literature to say that environmental OP pesticides exposure associates with abnormal semen parameters among the general population. Therefore, the present study aimed to assess the environmental OP pesticides exposure among Southern Indian men from Pondicherry and surrounding districts of Tamil Nadu, like Tindivanam, Villianur, Chennai, and Villupuram with idiopathic abnormal semen analysis by measuring pseudocholinesterase and acetylcholinesterase levels and urinary OP pesticides metabolites. The objectives of the study were to compare environmental OP pesticides exposure between men with and without idiopathic abnormal semen analysis and to determine possible sources of OP pesticides exposure by comparing percentages of farmers, rural population, smokers, undergraduates, lower socioeconomic status, vegetarians, people using underground water source, and alcoholics.

Materials and Methods

Study design and population

This cross-sectional pilot study was conducted in the Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) hospital, Puducherry, 605 006 from January 2018 to July 2019 after obtaining the approvals from Institute Research Council and Institute Human Ethics Committee (JIP/IEC/2017/0351 dated November 27, 2017). All Southern Indian men, 25 to 45 years old, from Pondicherry and surrounding districts of Tamil Nadu, like Tindivanam, Villianur, Chennai, and Villupuram, who was attending the JIPMER Infertility Clinic, Pondicherry for the inability of their spouse to conceive after 1 year of unprotected sexual intercourse, were recruited. Written informed consent (both in English and Tamil) after explaining the purpose and the procedure of the study, was obtained from all the participants. After obtaining the consent, detailed history of the patient was taken including his occupation, location, source of water, history of any medication, or surgery, and other demographic factors. General and systemic examinations were carried out and the scheduled date for semen analysis was given. On the scheduled day, a semen sample, 10 ml of urine, and 5 ml of blood were collected under sterile conditions. Fifty men who had abnormal semen analysis (sperm count ≤15 million/ml, sperm motility ≤40% and sperm morphology ≤4% normal) as per WHO criteria 2010 (4) with no identifiable pathology, were recruited as cases. Cases with underlying pathology such as varicocele, history of diabetes, cardiovascular or thyroid disorders, tuberculosis, testicular carcinoma, obstruction or congenital bilateral absence of vas deferens, or use of lipid-lowering drugs, were excluded. Fifty men with normal semen analysis were recruited as controls. So, it was a pilot study with 50 cases and 50 controls, which was accepted by the institute research council.

Criteria for analysis of anthropometry, alcohol use, smoking, and socioeconomic status

The height, weight, body mass index (BMI), and waist circumference were measured by the same observer. The nearest half-kilogram for body weight and half-centimeter for height were recorded. The waist circumference was determined by measuring the shortest point below the lowermost rib cage margin and the iliac crest and was recorded to the nearest half-centimeter. BMI was calculated as weight (kg) divided by the square of height (m). Being alcoholic was defined by the consumption of at least two drinks per day. A standard drink was equal to either

10g/12.7 ml of pure alcohol, 330 ml of beer, 100 ml of wine, or 30 ml of straight spirits or liquor like gin, rum, vodka, or whiskey. Being smoker was defined as having a history of smoking over the past one year irrespective of the number of cigarettes per day (19). Being vegetarian was defined as eating animal products either never or rarely (less than once per month), consuming dairy products and eggs, but eating meat/ fish less than once per month or who ate fish more than once per month, but other meats less than once per month (20). The socioeconomic status was assessed based on the Kuppuswamy criteria (21).

Semen collection and analysis

Semen was collected in a sterile container by masturbation in a private room near the laboratory. Participants were asked to abstain from ejaculation for 3 days before the scheduled date of appointment. Semen volume was measured in a graduated cylinder. Sperm counts were determined on two separate drops of semen using a Neubauer haemocytometer. If the sperm counts determined in the two drops of semen differed by 10% or more, then the count was determined in the third drop of semen. In this case, the sperm count from the first two samples which was closest and within 10% of the third sample count was retained. Sperm count was calculated as the average of the two sperm counts. Sperm motility was determined microscopically in two 10-µl drops from the semen sample. Slides were prepared and observed for altered sperm morphology (22).

Estimation of pseudocholinesterase and acetylcholinesterase levels

Serum pseudocholinesterase levels were measured by sandwich enzyme-linked immunosorbent assay kit from LifeSpan Biosciences, Inc. according to the manufacturer's instructions. The intra-assay and inter-assay coefficient of variation (CV) for serum pseudocholinesterase was less than 10 and 12%, respectively. Serum acetylcholinesterase levels were measured by the Ellman method in which thiocholine, produced by acetylcholinesterase, reacted with 5,5-dithiobis (2-nitrobenzoic acid) to form a colorimetric (412 nm) product, proportional to the acetylcholinesterase activity present. One unit of acetylcholinesterase is the amount of enzyme that catalyzes the production of 1.0 mmole of thiocholine per minute at pH=7.5 at room temperature (23).

Sample preparation and gas chromatography-mass spectrometry

Ten milliliters of urine were collected and stored at -80°C until further analysis. All urine samples were thawed and mixed by a vortex. Cleaning of urine sample and derivatization of alkyl phosphate were done as per Hemakanthi De Alwis et al. (24). A Trace GC Ultra equipped with AI 3000 Auto-Injector (Rodano, Italy) and ITQ 900 mass spectrometer from Thermo Scientific (Austin, USA) was used for analysis with a constant flow rate of 1.2 ml/ minutes and Helium as a carrier gas. One microliter of a sample from the low volume insert was injected in splitless mode onto a Thermo Electron Corporation (Rodana, Italy) TR-5MS ([5%- phenyl]-polysilphenylene-siloxane) TRACE GC capillary column (30 m, 0.25 mm, 0.25 µm) using the autosampler. The GCMS protocol followed was as per Hemakanthi De Alwis et al. (24). All the standards for dimethyl phosphate (DMP), diethyl thiophosphate (DETP), diethyl dithiophosphate (DEDTP), Sulfotep, an internal standard and derivatization agent 2,3,4,5,6-Pentafluorobenzyl bromide were purchased from Sigma-Aldrich with a purity of ≥90%. The mass spectra of the pentafluorobenzyl esters of DMP, DETP, DEDTP, and Sulfotep was determined. The analysis was done on selective ion monitoring (SIM) mode. The retention time (RT), linearity, the limit of detection (LOD), and limit of quantification (LOQ) for DMP, DETP, DEDTP, and Sulfotep were detected. The data obtained were transferred to X-Calibur files and manually evaluated. The peaks of the samples processed were recognized using the RTs and confirmed by comparison with the analyte/Sulfotep ratio for the two ions of the analyte. Results were reported utilizing creatinine adjustment.

Organophosphate pesticides exposure criteria

Both the presence of DAP in urine and inhibition of pseudocholinesterase were mandatory for the patients to be labeled as OP pesticides exposed. Patients with DMP, DETP, and/or DEDTP detected in urine above the LOQ labeled as DAP positive. Proudfoot formula was used as a basis for the determination of inhibition of pseudocholinesterase. We considered 4621-11500 IU/L as normal level (≥ 50%), 2311-4620 IU/L as mild inhibition (20-50%), 460-2310 IU/L as moderate inhibition (10-20%) and less than 460 IU/L as severe inhibition (less than 10%) (14).

Statistical analysis

The normality of data was assessed by the Kolmogorov-Smirnov test. The distribution of categorical data such as socio-demographic status, occupation, being vegetarian, being smoker, being alcoholic, and people using underground water, are expressed as percentages. The continuous data such as semen parameters, pseudocholinesterase and acetylcholinesterase levels, and OP metabolites in urine are expressed as mean with standard deviation or median (interquartile range). Creatinine was analyzed in urine and all OP metabolites values were adjusted for creatinine. All OP metabolites concentrations were log-transformed for statistical analysis. Descriptive statistics for OP metabolites among exposed and non-exposed included the percent above the LOD, mean and standard deviation, geometric mean and standard deviation, ranges, and calculation of the 25^{th}, 75^{th}, and 90^{th} percentile. OP metabolites concentration below the LOD was assigned a value equal to the LOD/$\sqrt{2}$ (25). Binary logistic regression was done to estimate relative odd of urinary OP metabolites among exposed and non-

exposed groups after adjustment to age, the number of married years, height, weight, waist circumference, percentage of undergraduates, lower socioeconomic status, vegetarians, primary infertility and alcoholics. Spearman's or Pearson's correlation was assessed between semen parameters and urinary OP metabolites in the OP-exposed group. Normally distributed variables were compared using the student's t test. Non-parametric parameters were compared by the Kruskal-Wallis H test. Statistical analyses were done using SPSS 10 software at a significance level of 5% and P<0.05 was considered significant.

Results

General characteristics were compared between cases and controls (Table 1). Cases had a significantly higher percentage of farmers as compared to controls (44 vs. 18%, P=0.009). Similarly, cases had a significantly higher percentage of rural population as compared to controls (60 vs. 38%, P=0.045). Contradictorily, a high percentage of smokers was found among controls as compared to cases (28 vs. 10%, P=0.022). However, no significant difference was found between cases and controls in age, the number of married years, height, weight, waist circumference, percentage of undergraduates, lower socioeconomic status, being vegetarian, using underground water, primary infertility, and being alcoholic. Pseudocholinesterase levels (5792.07 ± 1969.89 vs. 10267.01 ± 3258.58 IU/L, P=0.006) and acetylcholinesterase levels [102.90 (45.88-262.74) vs. 570.31 (200.24-975.30) IU/L, P=0.001] were statistically significantly lower among cases as compared to controls.

Forty out of 50 cases had urinary DAP positivity. Pseudocholinesterase (4917.65 ± 900.54 vs. 5496.97 ± 1515.90 IU/L, P=0.007) and acetylcholinesterase [88.00 (45.62-249.47) vs. 197.64 (54.57-262.74) IU/L, P=0.001] levels were significantly lower among 40 cases with urinary DAP positivity as compared to 10 cases with urinary DAP negativity. Out of the 40 cases with urinary DAP positivity, 10 (25%) cases had mild inhibition (4417 ± 200 IU/L) and 30 (75%) cases had normal pseudocholinesterase levels. Nineteen out of 50 controls had urinary DAP positivity. Out of the 19 controls with urinary DAP positivity, 2 (10.6%) men had mild inhibition and 17 (89.4%) men had normal pseudocholinesterase levels. However, all controls with DAP negativity had normal pseudocholinesterase levels.

Ten out of 50 cases had both inhibitions of pseudocholinesterase and urinary DAP positivity, hence they were labeled as OP pesticides-exposed. Two out of 50 controls had both inhibitions of pseudocholinesterase and urinary DAP positivity, hence they were labeled as OP pesticides-exposed. Cases had a significantly higher percentage of OP pesticides exposure in comparison with controls (20 vs. 4%, P=0.015). Also, cases with OP pesticides exposure had significantly higher urinary DETP and DEDTP levels as compared to cases without OP pesticides exposure (Table 2). Binary logistics regression showed that OP-exposed cases had significantly higher urinary DETP (OR=1.12, 95% CI=1.01-1.26), DEDTP (OR=1.27, 95% CI=1.02-1.45) and DAP (OR=1.33, 95% CI=1.13-1.66) levels as compared to non-exposed cases after adjustment to age, the number of married years, height, weight, waist circumference, percentage of undergraduates, lower socioeconomic status, vegetarians, people using underground water, primary infertility and alcoholics (Table 3). Correlation analysis among OP-exposed cases showed that urinary DAP levels were significantly negatively associated with sperm concentration (P=0.001, r=-0.634), motility (P=0.001, r=-0.523), and normal morphology (P=0.001, r=-0.721).

Table 1: Comparison of general characteristics between cases and controls

Parameters	Men with idiopathic abnormal semen analysis, cases (n=50)	Men with normal semen analysis, controls (n=50)	P value*
Age (Y)	34.94 ± 5.23	34.20 ± 6.61	0.479
Duration of marriage (Y)	5.49 ± 3.74	5.04 ± 2.53	0.889
Height (cm)	169.64 ± 12.21	168.76 ± 4.74	0.636
BMI (Kg/m^2)	24.98 ± 4.44	24.65 ± 3.79	0.692
Waist circumference (cm)	92.28 ± 16.03	86.90 ± 17.44	0.112
Undergraduates (%)	41 (82)	37 (74)	0.334
Farmers (%)	22 (44)	9 (18)	0.009
Lower socioeconomic status (%)	17 (34)	8 (16)	0.068
Rural population (%)	30 (60)	19 (38)	0.045
Vegetarians (%)	7 (14)	7 (14)	1.000
Underground water source (%)	46 (92)	42 (84)	0.218
Primary infertility (%)	48 (96)	45 (90)	0.436
Smokers (%)	5 (10)	14 (28)	0.022
Alcoholics (%)	13 (26)	12 (24)	0.579
Semen volume (ml)	2.0 (1.5-2.5)	2.0 (1.5-2.5)	0.939
Sperm concentration (million/ml)	11.20 (3.00-46.25)	85.90 (65.45-121.90)	0.001
Sperm number (million/ejaculate)	26.95 (10.25-112.50)	127.75 (93.75-219.68)	0.001
Sperm motility (%)	8 (2-12.80)	53 (47-61)	0.001
Sperm morphology (%)	3 (2-4)	19.5 (17-23)	0.001
Pseudocholinesterase (IU/L)	5792.07 ± 1969.89	10267.01 ± 3258.58	0.006
Acetylcholinesterase (IU/L)	102.90 (45.88-262.74)	570.31 (200.24-975.30)	0.001
DAP positivity (%)	40 (80)	19 (38)	<0.0001

Data are presented as mean ± SD, median (interquartile range) or percentage (%). BMI; Body mass index, DAP; Dialkyl phosphate, and *; Independent sample t test/ Kruskal-Wallis H test.

Table 2: OP pesticides metabolites levels between OP exposed and non-exposed cases

OP metabolites (selected ions)	LOD*/LOQ	Mean (SD)	GM (GSD)	Median	25th per	75th per	90th per	Range
DMP* (306, 307)	10/33.4							
OP exposed		15.50 (11.43)	14.41 (89.66)	15.65	8.32	18.87	22.34	7.09-55.98
OP non-exposed		14.52 (6.87)	13.67 (76.67)	14.42	7.98	9.78	12.98	7.09-39.17
DETP* (274, 350)	0.14/0.457							
OP exposed		46.78 (39.78)	32.45 (7.98)	35.26	11.24	55.67	88.65	0.14-146.67
OP non-exposed		26.56 (22.34)	11.46 (6.54)	15.50	8.45	38.87	67.54	0.14-80.67
DEDTP* (366, 185)	3.06/10.20							
OP exposed		55.23 (46.32)	42.87 (9.45)	46.11	14.34	55.98	75.98	3.06-97.56
OP non-exposed		35.34 (30.21)	19.45 (8.67)	27.02	10.09	54.56	66.98	3.06-76.07

OP; Organophosphate, SD; Standard deviation, GM; Geometric mean, GSD; Geometric standard deviation, DMP; Dimethyl phosphate, DETP; Diethyl thiophosphate, DEDTP; Diethyl dithiophosphate, LOD; Limit of detection, LOQ; Limit of quantification, and *; Samples below the LOD were defined as LOD/√2.

Table 3: Relative odd of OP metabolites after adjustment among OP exposed and non-exposed cases

OP metabolites	OR	CI	P value
DMP	0.65	0.23-1.43	0.363
DETP	1.12	1.01-1.26	0.004
DEDTP	1.27	1.02-1.45	0.004
DAP	1.33	1.13-1.66	0.001

OP; Organophosphate, DAP; Dialkyl phosphate, DMP; Dimethyl phosphate, DETP; Diethyl thiophosphate, DEDTP; Diethyl dithiophosphate, OR; Odd ratio, and CI; Confidence interval.

To find out the possible source of OP pesticides exposure, the general characteristics between OP-exposed cases and non-exposed cases were compared. Percentages of farmers and residing in a rural area were significantly higher in OP-exposed cases as compared to non-exposed cases. However, there was no significant difference in age, BMI, or waist circumference as well as percentages of men with undergraduate education, lower socioeconomic status, being vegetarian, using underground water, being smokers, and being alcoholics among OP-exposed cases as compared to non-exposed cases.

Discussion

Our study reports that Southern Indian men with idiopathic abnormal semen analysis had a significantly higher percentage of OP pesticides exposure as compared to men with a normal semen analysis. Also, we found a significant correlation between urinary OP metabolites and semen parameters among OP-exposed cases.

As there is rampant use of OP pesticides in agriculture, their residues can be found in cooked meals, water, wine, fruit juices, refreshments, and so on. Also, washing and peeling cannot remove the OP residues completely (26, 27). Chronic, low-dose exposure to OP pesticides was found to be associated with neurodevelopmental problems in children, Parkinson's disease, metabolic syndrome, obesity, diabetes, reduced semen quality, reduced gestational age, reduced birth weight, and so on (28, 29). OP pesticides were found to affect the sperm quality directly or indirectly resulting in infertility and reproduction problems in the agricultural workers. OP pesticides act as endocrine-disrupting chemicals, alter the HPO axis, and impair spermatogenesis by damaging the Sertoli and Leydig cells (30). The general population is exposed to OP pesticides mainly through diet, inhalation of air, dermal absorption, and unintentional ingestion (31, 32).

Comparing general characteristics, we noticed that men with idiopathic abnormal semen analysis were mostly farmers and from the rural area as compared to men with a normal semen analysis. Our observations were consistent with those reported by Miranda-Contreras et al. (14) who concluded that sperm count, motility, and membrane integrity among Venezuelan farmworkers were affected by occupational pesticides exposure. Also, Katole and Saoji (33) have reported a lower prevalence of primary infertility among urban populations. Dutta and Bahadur (34) showed that pseudocholinesterase and acetylcholinesterase levels were decreased among occupationally-exposed tea garden workers of the Northern part of West Bengal, India, similar to our observations. Education has an important role in maintaining personal hygiene, prevention of sexually transmitted disease, and understanding the effect of alcohol and smoking on sperm count. Our study has not found any difference between cases and controls in education as the two groups have the same percentage of educated participants.

Many studies have used the measurement of urinary DAP as a tool for determining OP pesticides exposure (13-18). As Yucra et al. (16) showed that occupation exposure of OP pesticides cannot be decided solely by OP metabolites measurement in urine, we have included both the determination of DAP in urine and measurement of pseudocholinesterase levels for labeling patient as OP-exposed. Hence, we may conclude that men with idiopathic abnormal semen analysis had high baseline exposure to OP pesticides. Li and Kannan (35) established the baseline levels of exposure to OP and pyrethroid pesticides among the population of several Asian countries. They concluded that India has the second-highest sum concentration of 11 pesticides in urine, next to Vietnam. Also, they found that daily intake of chlorpyrifos and parathion was high among the Indian population as compared to the population from other Asian countries. We got higher urinary levels of

DEDTP and DETP in cases as compared to controls and these levels were significantly negatively associated with sperm concentrations, motility, and normal morphology. Perry et al. (10) concluded that men with lower semen quality had higher urinary DMP levels as compared to men with normal semen quality. Muñoz-Quezada et al. (36) concluded that urinary DAP levels were high in Chilean school children due to the presence of chlorpyrifos and phosmet residues in fruits.

There is a rising trend of male infertility among the population and for most of them, no detectable cause has been found. Hence, it has become the burning question and need of the hour to address what are the possible reasons for the decline in semen parameters? Because there is extensive use of OP pesticides in agriculture, its contamination in the food chain and its effect on sperm parameters, can sustain and a low dose of OP pesticides exposure be one of the causes for the decline of semen parameters among the Southern Indian population? In our study, we found that men with abnormal semen analysis had significantly higher OP pesticides exposure as compared to men with a normal semen analysis. OP-exposed men were farmers and from the rural population where they might be daily exposed to OP pesticides through food, water, and air, affecting their sperm parameters.

There were certain limitations in this study: i. We estimated acetylcholinesterase activity in serum instead of RBC. ii. There were six DAPs: DMP, DMTP, DMDTP, DEP, DETP, and DEDTP. Out of 6 metabolites, we estimated only 3 DAPs i.e. DMP, DETP, DEDTP due to lack of availability of remaining standards. iii. History of time of recent exposure was not known in our study. Hence, the impact of exposure on the spermatogenesis cycle was not estimated and there was not much information on the chemical insult window period in humans. iv. The seasonal variation of OP pesticides exposure was not considered in our study. v. Urinary DAP can be derived from pre-formed metabolites in the environment. vi. We have estimated semen analysis on one occasion. We were unable to repeat semen analysis hence characterization was not confirmed. vii. We didn't estimate the hormonal changes in our study population. viii. This observational study has various unmeasured confounders like an instrumental variable, design, and so on. Due to time constraints, we have not addressed these confounders.

Though this is a pilot study, it explained a strong association between unintentional OP exposure and semen parameters. Hence, OP exposure status can be included as one of the investigations during the workup of men with an abnormal semen analysis. However, a future study including a larger sample size, more DAP metabolites, collection of more detailed information on demographic and socioeconomic parameters will be required to support our claim.

Conclusion

As OP pesticides exposure can occur through inhalation, ingestion, and so on, their subtle and chronic exposure is affecting various organs of the human body. The current study showed the effect of OP pesticides on semen parameters and concluded that men with idiopathic abnormal semen analysis had significantly higher OP pesticides exposure as compared to men with normal semen analysis. OP-exposed cases had higher urinary OP metabolites levels and more inhibition of pseudocholinesterase and acetylcholinesterase as compared to non-exposed cases pointing towards a severe degree of OP pesticides exposure. A higher percentage of OP-exposed men were farmers and from the rural area.

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Authors' Contributions

I.M.; Sample collections, sample processing and analysis, data acquisition, preparation of Excel files, and statistical analysis. S.B.; Validation of method, sample processing and analysis, data acquisition, and statistical analysis. P.S.A.; Conceptualization, formal analysis, funding acquisition, project administration, resources, software, supervision, validation, drafting, and final approval of the manuscript. C.T., R.N.G.; Conceptualization, selection of cases and controls, project administration, supervision, and final approval of the manuscript. All authors read and approved the final manuscript.

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Original Article

Effect of Different High-Fat and Advanced Glycation End-Products Diets in Obesity and Diabetes-Prone C57BL/6 Mice on Sperm Function

Fahimeh Akbarian, M.Sc.[1#], Mohsen Rahmani, M.Sc.[1#], Marziyeh Tavalaee, Ph.D.[1], Navid Abedpoor, M.Sc.[2], Mozhdeh Taki, B.Sc.[1], Kamran Ghaedi, Ph.D.[2], Mohammad Hossein Nasr-Esfahani, Ph.D.[1*]

1. Department of Animal Biotechnology, Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran
2. Department of Animal Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran

Abstract

Background: We aimed to compare the effects of using high-fat (HF) and advanced glycation end-products (AGEs) containing diets to induce obesity and diabetes on sperm function in mice.

Materials and Methods: In this experimental study, twenty-five 4-week old C57BL/6 mice were divided into 5 groups and were fed with control, 45% HF, 60% HF, 45% AGEs-HF, or 60% AGEs-HF diet. After 28 weeks, fast blood sugar, glucose intolerance, insulin concentration, homeostatic model assessments (HOMA) for insulin resistance (IR) and HOMA for beta cells (HOMA beta) from systematic blood were assessed. In addition, body weight, morphometric characteristics of testes, sperm parameters, DNA damage (AO), protamine deficiency (CMAA3), and sperm membrane (DCFH-DA) and intracellular (BODIPY) lipid peroxidation were measured.

Results: Body mass and fasting blood sugar increased significantly in all experimental groups compared to the control group. Insulin concentration, glucose intolerance, HOMA IR, and HOMA beta were also increased significantly with higher levels of fat and AGEs in all four diets ($P<0.05$). The changes in the 60% HF-AGEs group, however, were more significant ($P<0.001$). Morphometric characteristics of the testis, sperm concentration, and sperm morphology in the diet groups did not significantly differ from the control group, while sperm motility and DNA damage in the 45%HF were significantly low. Although for protamine deficiency, both 60% HF-AGEs and 45% HF showed a significant increase compared to the control, the mean of sperm lipid in the 45% HF group and intracellular peroxidation in the 60% HF-AGEs group had the highest and the lowest increases, respectively.

Conclusion: Our results, interestingly, showed that is the negative effects of a diet containing AGEs on examined parameters are less than those in HF diets. One possible reason is detoxification through the activation of the protective glyoxalase pathway as the result of the chronic AGEs increase in the body.

Keywords: Advanced Glycosylation End Products, Diabetes Mellitus, High-Fat Diet, Reactive Oxygen Species, Sperm Parameters

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Introduction

Diabetes is a complex chronic condition that results in high levels of blood sugar and is one of the main causes of disability and death worldwide. It is mainly caused by either insulin deficiency due to the destruction of insulin-producing beta cells of the pancreas (Type 1 diabetes), or insensitivity of cells to insulin (Type 2 diabetes) (1). Developing type 2 diabetes is strongly associated with obesity, as 58% of global diabetes cases are attributed to body mass index higher than 25 kg/m^2 based on the World Health Organization (WHO) reports (2). As the global trends in diabetes are alarming, WHO has recommended the promotion of healthy diets and physical activity in societies as an attempt to reduce obesity and diabetes as well as to manage and lower their complications.

Mounting evidence highlights the close relationship between the adoption of western dietary pattern and the steady increase in worldwide obesity and diabetes over the past few decades (1). The western diet is characterized by high consumption of refined sugars and saturated fat, but insufficient amounts of fiber (3). This diet is rich in advanced glycosylation end-products (AGEs), which are highly reactive molecules produced by a non-enzymatic reaction known as the Maillard reaction between reducing sugars, such as glucose and other compounds such as proteins, nucleic acids, or lipids. Intra- and extracellular accumulation of AGEs interfere with various proteins and several cellular functions (4).

Although endogenous AGEs are constantly produced in the body during the glycation of various biomolecules, they can also be originated from exogenous sources in the process of modern methods of preparing precooked meals heated in

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*Corresponding Address: P.O.Box: 8165131378, Department of Animal Biotechnology, Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran
#The first two authors equally contributed to this work.
Email: mh.nasr-esfahani@royaninstitute.org

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high temperatures (3). In this context, it has been found that around 10% of exogenous AGEs that are taken up are eliminated by the body and the rest lead to increased AGE serum levels and elevated fat deposits in various tissues (4). Several conditions including hyperglycemia and insulin resistance (IR) accelerate the formation of AGEs so that the intracellular levels of these compounds could elevate 14-fold faster in a high glucose state (5). AGEs are considered as the main pathogenic factors in the development and progression of complications associated with diabetes (3). The production of reactive oxygen species (ROS) by AGEs is one of the biochemical mechanisms of diabetes pathology (4).

Physiological alterations driven in part by diabetes mellitus could affect the functions of male reproduction through several mechanisms and pathways as it disturbs the endocrine regulation of steroidogenesis, spermatogenesis, sperm maturation, as well as of penile erection and ejaculation. Proper glucose metabolism plays a pivotal role in spermatogenesis and fertilization capacity of mature sperm, as well as maintenance of basic cells (6). Fertility problems associated with obesity and diabetes mainly arise from unbalanced ROS and subsequent oxidative stress. ROS encompasses a vast number of detrimental effects on male reproductive functions. This is supported by the findings that showed antioxidant therapy to be beneficial for improving the sperm parameters in diabetic men (7).

Although the adverse consequences of diets rich in saturated fat and AGEs compounds on male fertility have been suggested in previous studies (4-7), there are some discrepancies in the literature and also there is no study that has simultaneously assessed the effects of high-fat (HF) and AGEs-containing diets on sperm function. In this context, the current study was conducted to compare the effects of obesity and diabetes in C57BL/6 male mice fed with diets containing different levels of saturated fat and AGEs compounds, to better understand their effects on infertility, by analyzing the sperm parameters, oxidative stress, and chromatin status, and also to investigate the mechanisms through which these diets could induce infertility.

Materials and Methods

Design of experiment

This experimental study was approved by the institutional review board from the Royan Institute (No: 97000269) and performed under the supervision of the animal Ethics Committee of Royan Institute. Twenty-five 4-weeks old healthy non-obese and non-diabetic C57BL/6 male mice were selected from Institute for Biotechnology (Isfahan, Iran) and housed under controlled conditions; temperature of 21 °C (± 2%), 65% humidity (± 5%), 12-hours light/12-hours dark cycles, and an ad libitum access to food and water. After one week of acclimatization in special cages, mice were randomly divided into five groups (control/ chow diet, 45%, and 60% HF diet groups, 45% and 60% AGEs diet groups; for each group, n=5 were considered based on the Kolmogorov-Smirnov test. The mice received the experimental diets when they were 5 weeks old. After 28 weeks of feeding special diets for inducing obesity and diabetes (8-10), body mass, fasting blood sugar, insulin concentration, glucose intolerance, homeostatic assessment of insulin resistance (HOMA IR) and HOMA for beta cells (HOMA beta) along with the weight and morphometric characteristics of testes (width, length, and thickness of the left and right testis), sperm parameters (concentration, motility, abnormality), and sperm function (protamine deficiency, DNA damages, membrane lipid and intracellular peroxidation) were measured in each group of mice.

Diets

The HF diet and AGE were obtained from Royan Biotechnologist immune-company (Iran, Tehran). Preparing the formulation of the diets was based on previous studies (11). Four types of diets were applied in this study. 45% HF and 60% HF groups, which 45% and 60% of the calories were provided from lipids respectively, as well as 45% HF- AGEs and 60% HF- AGEs, in which lipids and AGEs provide the 45% and 60% of the calories for each group. Notably, the fat ingredients of the diets are saturated. The details of the composition of the five diet groups are presented in Table 1. This study is the continuation of the study by Abedpoor et al. (unpublished data), and the results of several factors such as glucose tolerance test, fasting blood sugar, insulin concentration, HOMA-IR, and HOMA-beta are similar between these two studies.

Table 1: Characteristics of special mouse diets for each different studied group

Diet composition (% w/w)	Diet groups (n=5)				
	Normal diets	45% HF	60% HF	45% HF-AGEs	60% HF-AGEs
Protein	20.56	19.4	20	28	23
Fat	12.55	45	60	45	60
Carbohydrate	47.71	21.59	13.8	15.49	9.56
Fiber	3.8	2.26	1.2	3.35	0.96
Ash	10.38	7.85	0.9	7.18	5.9
Moisture	5	3.9	4.1	0.98	0.58
Calories (kcal/g)	3.8	5.6	6.7	5.8	6.7

HF; High-fat diet and AGEs; Advanced glycation end-products.

Fasting blood sugar and glucose tolerance test

Fasting blood sugar was measured from the tail vein by animal glucometer after 6 hours of fasting, following 28 weeks of keeping the mice on the special diets. Tolerance test was also performed after 28 weeks of keeping the mice on the special diets. After 6 hours of fasting, D-glucose (solution of 10 g/dL at a dose of 1 g/kg and volume load of 10 mL/g body mass, Sigma, Australia) was injected intraperitoneally (12, 13) and glucose level was measured at 15, 30, 60, and 90 minutes intervals from the tail vein. Blood glucose concentration was measured during daytime-fasting (14), using an animal glucometer (Alpha TRAK). The mice were sacrificed following these evaluations.

Insulin concentration

The mice were euthanized under combined administration of xylazine (10 mg/kg body mass per mouse) and ketamine (80 mg/kg body mass per mouse). For assessing the insulin concentration, heart blood was collected immediately after scarification. Following centrifugation of the collected blood at 4500 rpm for 5 minutes at 4°C, the insulin level in the serum (ng/dL) was quantified using Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem, USA) according to the manufacturer's instructions.

Homeostatic model assessment of insulin resistance

HOMA IR, a surrogate marker of IR was assessed using fasting glucose and insulin concentration according to the following formula (15):

$$\frac{\text{Fasting Glucose}\times\text{Insulin Concentration}}{405}$$ (glucose in mass units mg/dL)

Homeostatic model assessment of beta cells

HOMA-beta assesses the function of β-cells using insulin concentration according to the following formula (16):

$$\frac{360\times\text{Insulin Concentration}}{\text{Glucose}-63}$$ (glucose in mass units mg/dL)

Sperm collection

Following the sacrifice of mice, weight and morphometric measurements of the testes (width, length, and thickness of the left and right testis) were performed. The cauda segment was separated from the left epididymis and cut into pieces and incubated in 2 ml of sperm washing media+10% serum at 37°C for 30 minutes to retrieve spermatozoa. After analyzing the sperm parameters, the spermatozoa were washed with phosphate-buffered saline (PBS, Sigma, Australia) for further evaluation.

Assessment of sperm parameters

Sperm concentration (million/ml) and sperm motility (% motile) were measured using a sperm counting chamber (Sperm meter, Sperm Processor, India) under a light microscope. The collected sperm was stained by the Eosin-Nigrosin method as described previously for morphological evaluations (17). Abnormalities in the head, neck, and tail of the spermatozoa were assessed, and data were reported as the percentage of sperm abnormal morphology.

Assessment of sperm protamine deficiency

Histone replacement by protamine occurs during the late stages of spermatogenesis. We evaluated the protamine deficiency using the chromomycin A3 (CMA3) staining method as described previously (17). For this evaluation, 200 spermatozoa were assessed under an Olympus fluorescent microscope (BX51, Japan) and protamine-deficient spermatozoa (bright yellow or CMA3 positive) were distinguished from normal protamine content (dull yellow spermatozoa or CMA3 negative), and the percentage of protamine deficiency was reported for each sample.

Assessment of sperm DNA damage

The DNA damage was evaluated by acridine orange (AO) staining, as described previously (17). For this analysis, 200 spermatozoa were assessed by fluorescent microscope (BX51, Japan) and the percentages of spermatozoa with normal double-stranded DNA (green stained) and abnormal spermatozoa with denatured DNA (orange/red stained) were calculated for each sample.

Assessment of sperm membrane lipid peroxidation

Sperm membrane lipid peroxidation was assessed using the BODIPY probe as described previously (18). Briefly, BODIPY 581/591 C11 (D3861, Molecular Probes) with a concentration of 5 mM was added to 2×10^6 spermatozoa and incubated at 37°C for 30 minutes and the percentage of lipid peroxidation was assessed using the FACSCalibur flow cytometer (Becton Dickinson, USA). A positive control for each sample was obtained by adding H_2O_2 to sperm suspensions.

Assessment of sperm intracellular reactive oxygen species

DCFH-DA staining was used to detect cytosolic ROS and peroxidation as described previously (19). Briefly, 106 spermatozoa were incubated with 0.5 μM DCFH-DA at 37°C for 30 minutes. Intracellular ROS was assessed using the FACSCalibur flow cytometer (Becton Dickinson, USA). A positive control for each sample was obtained by adding H_2O_2 to sperm suspensions.

Statistical analysis

All data in the present study were analyzed by the Statistical Package for the Social Sciences for Windows, version 25 (SPSS, Inc., Chicago, IL, USA). All the parameters had a normal distribution, and a one-way analysis of variance (ANOVA) was used to compare the sperm pa-

rameters, lipid peroxidation, and chromatin status. Data were presented as mean ± standard error of the mean, and P<0.05 was considered significant.

Results

Effects of different diets on body mass, weight, and morphometric characteristics of testes

The initial and final mean body mass of the mice in the five studied groups is presented in Table 2. All the groups gained weight significantly compared to the control group after 28 weeks of being fed with the special diets. In addition, we assessed morphometric characteristics of testes (width, length, and thickness of the left and right testis) and found that none of them showed any significant differences in the mean values compared to their corresponding control group. On the other hand, unlike the 45% HF group (0.101 ± 0.001, P>0.05), the mean weight of the left testis in the 60% HF (0.107 ± 0.003, P<0.05), 45% HF-AGEs (0.116 ± 0.009, P=0.001), and 60% HF-AGEs (0.120 ± 0.003, P<0.001) groups significantly increased in comparison to the control group (0.084 ± 0.004). Additionally, the mean weight of the left testis in the 60% HF-AGEs group increased compared to the 45% HF group (P<0.05).

Table 2: Body weight of different studied groups at the beginning of the study and after 28 weeks of feeding special diets

Groups (n=5)	Body weight (g)		
	Baseline	After 28 weeks	Weight gain
Control	14 ± 0.02	26 ± 0.5	12 ± 0.3
45% HF	13 ± 1.5	50.5 ± 0.5*	37 ± 0.4*
60% HF	14.1 ± 1	37.8 ± 0.2*	23 ± 0.1*
45% HF-AGEs	12 ± 2	43 ± 1*	31 ± 0.8*
60% HF-AGEs	13.5 ± 1.5	62 ± 0.5*	48.5 ± 1*

Data are expressed as means ± standard error of the mean. HF; High-fat diet, and AGEs; Advanced glycation end-products. Significant difference is presented as *P<0.05.

Effects of different diets on glucose level and insulin status

Glucose tolerance was dropped along with the increase in fat and AGEs content in the diet of the different studied group, so that the 60% HF-AGEs was the most intolerant group to glucose compared to the control group (P<0.001). The results of fasting blood sugar showed a significantly increased level of this parameter in all four groups with a special diet in comparison to the control (P<0.05).

As shown in Table 3, the results of insulin concentration, HOMA-IR, and HOMA-beta were significantly higher in all groups with a special diet compared to the control group (P<0.05 for 45% HF, 60% HF, 45% HF-AGEs and P<0.001 for 60% HF-AGEs group). Fasting blood sugar insulin concentration and IR were higher in 60% HF-AGE compared to the other groups. Therefore, we considered 60% HF-AGE and 60% HF groups as type 2 diabetes, and pre-diabetes groups, respectively.

Table 3: Insulin concentration and homeostatic model assessment (HOMA) in different studied groups after 28 weeks of keeping C57/BL6 mice on special diets

Groups (n=5)	Insulin concentration (ng/mL)	HOMA-insulin resistance	HOMA-beta
Control	0.35 ± 0.05	0.09 ± 0.01	3.06 ± 0.25
45% HF	1.29 ± 0.04*	0.63 ± 0.02*	4.31 ± 0.21*
60% HF	0.82 ± 0.09*	0.31 ± 0.02*	3.15 ± 0.20
45% HF-AGEs	1.62 ± 0.07*	0.78 ± 0.01*	5.27 ± 0.27*
60% HF-AGEs	3.95 ± 0.19**	2.56 ± 0.13**	7.15 ± 0.28**

Data are expressed as means ± standard error of the mean. HF; High-fat diet, AGEs; Advanced glycation end-products. Significant difference is presented as *P<0.05 and **P<0.01.

Effects of different diets on conventional sperm parameters

Conventional sperm parameters are demonstrated as bar charts in Figure 1. The mean sperm concentration (10^6/ml), the mean percentage of sperms with total abnormal morphology as well as abnormal head and tail were not significantly affected by HF and HF-AGEs diets for 28 weeks. However, the mean motility of the sperms in the 45% HF group decreased compared to the control (P<0.05), whilst the mean motility of the sperms in the 45% HF-AGEs (P=0.004) and 60% HF-AGEs (P<0.05) groups increased compared to the 45% HF group.

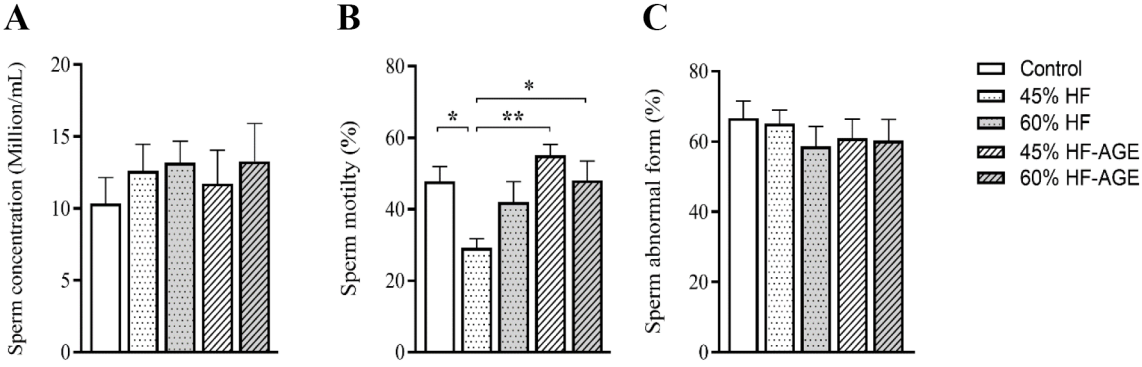

Fig.1: Sperm parameters of different studied groups after 28 weeks of feeding C57/BL6 mice with special diets. **A.** Sperm concentration (10^6/mL). **B.** Sperm motility (%). **C.** Total sperms with abnormal morphology (%). Data are expressed as means ± standard error of the mean. HF; High-fat diet, AGEs; Advanced glycation end-products, *; P<0.05, and **; P<0.01.

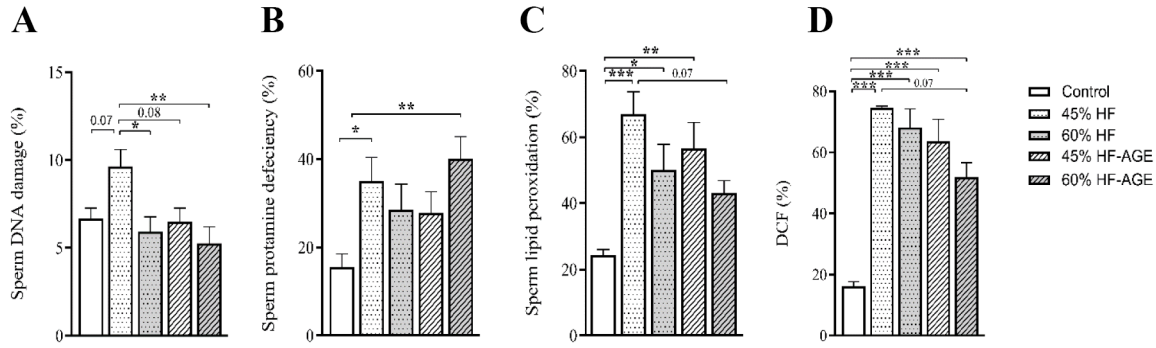

Fig.2: Comparison of sperm function tests within groups. **A.** Sperm DNA damages as shown by acridine orange staining (%) and **B.** Sperm protamine deficiency indicated by chromomycin A3 staining (%) in different study groups after 28 weeks of feeding special diets. Oxidative stress of sperms in different study groups after 28 weeks of feeding special diets studied by **C.** BODIPY probe (%) to analyze the sperm lipid peroxidation, and **D.** dichlorofluorescein diacetate (DCFH-DA) staining (%) to analyze intracellular ROS. Data are expressed as means ± standard error of the mean. HF; High-fat diet, AGEs; Advanced glycation end-products, *; P<0.05, **; P<0.01, and ***; P<0.001.

Effects of different diets on sperm DNA damage

As illustrated in Figure 2A, following 28 weeks of keeping the mouse groups on diabetes-inducing diets, the mean percentage of the sperms with damaged DNA was higher in the 45% HF group compared to the control (P=0.07), 60% HF (P=0.02), 60% HF-AGEs (P=0.004), and 45% HF-AGEs (P=0.08) groups.

Effects of different diets on sperm protamine deficiency

The results of protamine deficiency in different groups are depicted in Figure 2B. The mean of this parameter was significantly higher in the 45% HF (P=0.05) and 60% HF-AGEs (P=0.006) groups compared to the control group.

Effects of different diets on sperm membrane lipid peroxidation

According to Figure 2C, the mean percentage of lipid peroxidation of the sperm membrane in all the different diet groups experienced an increase in comparison to the control counterpart and the differences of 45% HF (P<0.001), 60% HF (P=0.02), and 45% HF-AGEs (P=0.004) groups was significant. In addition, the mean of this parameter was lower in the 60% HF-AGEs (P=0.07) group in comparison to the 45% HF group, although it was not statistically significant (P>0.05).

Effects of different diets on sperm intracellular reactive oxygen species

As can be seen in Figure 2D, the means percentage of intracellular ROS of the sperms in four out of five groups with HF and HF-AGEs diets were higher than that in the control group (P<0.001). In contrast, the mean of this parameter was lower in the 60% HF-AGEs group (P=0.07) compared to the 45% HF group, although the difference was not statistically significant.

Discussion

AGEs are produced through a non-enzymatic reaction known as glycation/Maillard, using reducing sugars (such as glucose and fructose) in combination with proteins, lipids, or nucleic acids. The AGEs content in the 45% HF and 60% HF diets in our study was not significantly different from the control diet. In contrast, the AGEs content in the 45% HF-AGEs and 60% HF-AGEs were higher than the standard rodent diet due to the high temperature heating process (3).

Fasting blood sugar analysis of the HF and HF-AGEs groups showed increased levels of blood glucose compared to the control group. Interestingly, de Assis et al. (20) reported that HF heat-treated diet has more negative impacts on glucose metabolism and also could induce type 2 diabetes more than the unheated HF diet (i.e. HF-AGE diet). The high glucose level is responsible for hyperglycemia, which is one of the early manifestations of diabetes and could have adverse impacts on semen quality. Hyperglycemia also accelerates AGEs formation, which results in pathophysiological damages in the male reproductive system (5).

In this research, there were no significant differences among the studied groups in terms of neither sperm concentration nor abnormal sperm morphology. Despite the pathologic conditions of diabetic men, numerous studies have reported that diabetes and obesity may have no direct effects on sperm parameters, but could indirectly impair sperm functions (6, 21-23).

Unlike the HF-AGEs diets, the obesity-inducing HF diets had significantly negative effects on sperm motility. The HF diet could result in an abnormal level of blood lipid known as dyslipidemia, which could trigger metabolic syndrome and have toxic effects on the reproductive system and semen quality (6, 24, 25). In addition, IR and increased level of glucose following HF diet consumption may alter the level of sperm energy (26). Consequently, both sperm glycolysis and oxidative phosphorylation pathways are disrupted, resulting in impairment of ATP synthesis and abnormal sperm motility. It is also reported that sperm metabolism is negatively influenced by the fatty acid composition of a diet, including high saturated fatty acids and low polyunsaturated fatty acids, leading to the hypothesis that HF diets could cause sperm lipotoxicity (24).

Based on the literature, more deleterious effects on sperm parameters and functions were initially expected with the concomitant rise of saturated fat and AGEs content in the

mouse diet. Ironically, instead of showing more reduction of sperm motility in the 60% HF or AGEs group, it was even restored to the control when compared to the 45% HF group. We believe that this discrepancy can be explained by the adaptation mechanism, which is explained in the below paragraphs, although, the literature on this topic is still controversial. An *in vitro* research by Portela et al. (27) suggested that a high concentration of glucose does not affect sperm motility and viability. It is also reported that the disrupted glycolytic process due to the hyperinsulinemia and hyperglycemia in diabetes leads to the decreased uptake of glucose by sperms, which is believed to be associated with an improvement in sperm motility (4).

Due to the fact that glucose and fructose are abundant in germ cells, and sperms are full of polyunsaturated fatty acids, they are prone to glycation reaction and AGEs formation. Chen et al. (28) believe that a diet that is rich in AGEs leads to testicular dysfunction through oxidative stress. It has been shown that AGEs induce ROS formation by inactivating copper, zinc superoxide dismutase (Cu-Zn-SOD), which attenuate cellular antioxidant capacity. AGEs are also very reactive since they act as electron donors and promote superoxide anions formation. On the other hand, oxidative stress is one of the contributory factors in metabolic disorders such as obesity and diabetes, which also accelerates AGEs formation in these physiological conditions. Therefore, there is a valid rationale to assume that there is a feedback loop between AGEs and ROS, which amplifies the formation and biological impacts of each other (4).

Lipid peroxidation is one of the major consequences of AGEs-induced ROS production in cells, which was significantly increased in both HF and HF-AGEs diets compared to the control group. A high level of polyunsaturated fatty acids in the sperm membrane is extremely vulnerable to excess ROS. Karimi et al. (7, 21) have reported that the lipid peroxidation in the semen of diabetic patients is markedly correlated with the high level of AGEs in the semen compared to the non-diabetic individuals. We also analyzed the intracellular ROS, which was significantly higher in the HF and HF-AGEs diet groups than in the control group. Additionally, multiple studies have referred to an inverse relationship between sperm motility and lipid peroxidation (17, 18).

Despite the above explanation on the toxic effects of HF and AGE, both lipid peroxidation and ROS production were high in the 45% HF group compared to the other groups, which were expected to have a more toxic effect. One of the reasons that the percentage of sperm lipid peroxidation, and ROS production were lower while percentage of sperm motility was higher in the 60% HF diet compared to the 45% HF diet could likely be related to the adaptation of mice to 60% diet. In the adaptation stage, the overall situation of blood glucose, insulin level, HOMA-IR, and HOMA beta function are more similar to the control group, therefore, it is not surprising to see better sperm motility, with reducing lipid peroxidation, intracellular ROS production, and reduced DNA damage in the 60% HF vs. 45% HF group.

Despite the results of earlier studies on diabetes that have linked the AGEs augmentation to complications like spermatogenesis impairment, our results showed that although the HF-AGEs diets had higher glucose concentrations and induced the diabetes complications more than the HF diets, they had significantly fewer negative effects on sperm motility, intracellular ROS and lipid peroxidation. Interestingly, Mallidis et al. (22) reported that although semen parameters of diabetic men were not affected, the amount of carboxymethyl-lysine, as the most prominent AGE in the sperm and semen of non-diabetic samples, were considerably higher compared to the diabetic individuals. These observations led to the hypothesis that the deglycation mechanisms would be initiated under the chronic diabetic state, thus eliminating the AGEs.

Methylglyoxal is known as a precursor of AGEs produced from fructose and glucose following a high sugar intake. Methylglyoxal level is increased in pre-diabetic and diabetic individuals and it leads to disruption of the insulin signaling pathway (29, 30). A recent *in vitro* study by Antognelli et al. (30) demonstrated that an increase in glycolytic flux by spermatogenesis in Sertoli cells leads to methylglyoxal formation as a toxic by-product of glycolysis and it could also elevate the dicarbonyl glycation. They reported that a super-physiological increase in AGEs-induced carbonyl stress, a detoxification mechanism named "glyoxalase pathway," is activated by Sertoli cells in order to protect the spermatogenesis (30).

In this context, the trend of decreased DNA damage, lipid peroxidation, intracellular ROS production with concomitant improved motility in the 60% HF, 45% HF-AGEs and 60% HG-AGEs diets compared to the 45% HF diet, suggest that an adaptation is taken place following the increased levels of glucose and insulin, which probably activated a detoxification mechanism or the glyoxalase pathway as an anti-glycation defense in the testes and epididymis. In this regard it has been shown that methylglyoxal is converted to D-Lactate, which is less toxic (31). Interestingly, our results also showed that the level of D-Lactate was increased in the latter groups compared to the 45% HF group, indicating that the detoxification mechanism has become activated in these mice. Another potential underlying cause of the milder effect of AGEs on lipid peroxidation is hypoxia. Rodrigues et al. (32) reported that the HF diet does not considerably affect the blood flow in adipose tissue. In contrast, adding methylglyoxal to the HF diet (i.e. HF-AGE diet) induces hypoxia by reducing the blood flow following the glycation in adipose tissue. In other words, with the expansion of adipose tissue and increased vasculature in this tissue, the blood flow to other parts or organs, including testes, is decreased, which results in a state of hypoxia. It is interesting to note that in a 45% HF diet the tissue expansion and weight gain is less than that in the other groups, therefore, the blood flow carrying toxic materials to testes in this group is not reduced compared to the other ones with reduced blood

flow (33, 34). Similarly, in our study, both tests showed that the HF-AGEs diets resulted in testicular hypertrophy and hyperplasia compared to the HF groups. Under hypoxia, less oxygen reaches the tissue, when oxygen is necessary for mitochondria to produce energy or ROS (35). Therefore, these mechanisms, in addition to adaptation, may account for reduced ROS and lipid peroxidation in the AGE groups.

Due to the limitations of DNA repair mechanisms in sperms, DNA damage could occur at any step of spermatogenesis, which is a common finding in diabetic patients (21, 36, 37). Although some reports have demonstrated that the AGEs diets exert more damage to DNA than the HF diets (20), our results were in agreement with several studies that concluded the accumulated AGEs in testis, epididymis, and sperm could trigger the protective mechanism of detoxification against AGEs-induced damages in pathological diabetic patients (4, 22, 23). Nevin et al. (4) showed that methylglyoxal, as the most dangerous AGEs in diabetes conditions, did not affect the sperm DNA damage, intracellular ROS, or sperm motility, as the glyoxalase pathway may be involved in the detoxification of AGEs. Additionally, it is reported that soluble AGE receptors (RAGE) is significantly higher in the semen of the infertile men compared to the fertile counterparts (38). Our results indicated the HF-AGEs diets had significantly fewer negative effects than the HF diets and there are moderate differences in DNA damage between the HF and control groups. Similarly, Hu et al. (39) reported that the consumption of the HF foods leads to an increased level of saturated fatty acids in the testes followed by DNA damage and apoptosis in the testes.

Surprisingly, our results indicated that the 60% HF-AGEs diet induces severe sperm protamine deficiency compared to the control group while it results in a minimum amount of DNA damage in comparison to the HF diets. However, AGEs exert their deleterious effects directly by modifying proteins, lipids, and DNA or indirectly by interacting with their specific receptors in cell surface known as RAGE. In this context, protein carbonylation is the worst consequence of AGEs. ROS also reacts strongly with amino acid residues rich in carbonyl groups like arginine, cysteine, and lysine. In humans, almost 85% of histones are exchanged with protamines during spermatozoa maturation, which is rich in amino acid residues like arginine and cysteine. Other studies have reported that such amino acids, especially arginine, are extremely vulnerable to glyoxal and methylglyoxal that are known as reactive glycating agents (40). Regarding previous findings and the competition of chromomycin A3 (CMA3) dye with protamine for binding to the same sites on DNA, it is possible to believe that toxic AGEs impair disulfide bonds; although their effect did not seem to be strong enough to damage DNA directly. As one of the limitations of our study, the long-term consumption of heated processed foods and the subsequent chronic elevated levels of AGEs in the body may result in adaptation and activation of the protective pathway. Therefore, it was worth investigating the short-term exposure to exogenous AGEs and to compare these results with the effects of longer AGEs intakes. We also did not measure the serum lipids, which should be considered in future research. Additionally, a more thorough understanding of the hidden negative effects of AGEs on DNA conditions requires looking into the genetics and epigenetics in the sperms (Fig.3).

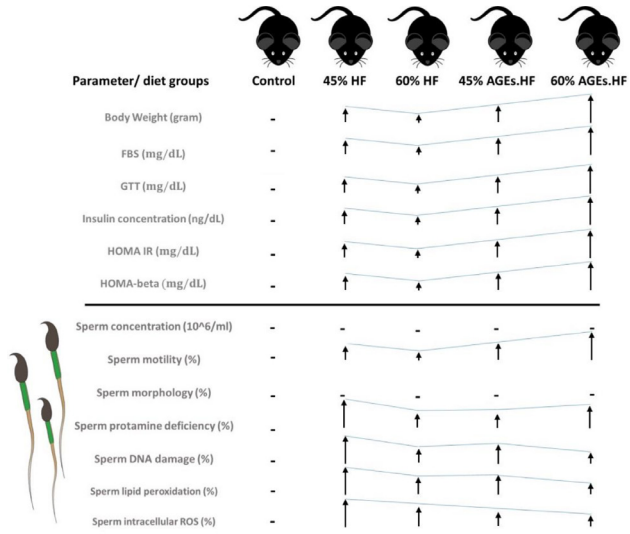

Fig.3: The schematic diagram of experimental results. All groups of diets show an increase in body weight more than the control group diet, although the 60% HF diet is less than the 45% HF, and AGE. HF diets (45% and 60%). Similarity, the assessment of metabolic tests (FBS, GTT, Insulin concentration, HOMA IR, HOMA beta) demonstrate similar results as body weight (g). Unlike sperm motility (%), sperm concentration (10^6/ml) and sperm morphology (%) do not show any significant difference among diet groups. While, the assessments of sperm DNA damage (%) showed an increase in 45% HF diet group compared to all the groups while percentage of sperm protamine deficiency demonstrate a highly negative effect in all diet groups compared to control diet group. Approximately, the assessments of sperm ROS [lipid peroxidation (%) and intracellular oxidation (%)] reveal an increase in all the groups compared to control group. HF; High-fat diet, AGE; Advanced glycation end-products, FBS; Fasting blood sugar, GTT; Glucose tolerance test, and HOMA IR; Homeostatic model assessment for insulin resistance.

Conclusion

To sum up, although the sperm concentration, morphology, and morphometric characteristics of the testes were not significantly affected in the C57BL/6 male mice fed with saturated fat- and AGEs-rich diets for 28 weeks, sperm motility, DNA fragmentation, and protamine deficiency as well as membrane and cytoplasmic peroxidation were negatively affected by the HF and HF-AGEs diets. A noteworthy finding in our results was that the adverse effects of the HF diets were more severe than those rich in AGEs, which could be the result of the activation of a protective glyoxalase pathway following the AGEs increase. However, the milder synergistic effect of obesity and diabetes in mice fed by an AGEs-rich diet could mislead our appreciation of the negative hidden effects of these compounds, which demands further studies on the mechanism of action of AGEs and detoxification through the glyoxalase pathway in the body.

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Authors' Contributions

F.A., M.R.; Preparation of samples and tests, collection and analysis of data, and manuscript writing. Ma.T.; Design, collection and/or assembly of data, data analysis, interpretation, and manuscript writing. Mo.T.; Preparation of tests. M.H.N.-E.; Conception, study design, data analysis, interpretation, manuscript writing and final approval of manuscript. N.A.; Preparation of tests, analysis of data, generating high-fat, and AGEs models. K.Gh.; Design, assistance in generating the study models. All authors read and approved the final manuscript.

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Original Article

Determinants of Birth Intervals Using Prentice-Williams-Peterson-Gap Time Model: Tehran Case Study

Arezoo Bagheri, Ph.D., Mahsa Saadati, Ph.D.*

National Institute for Population Research, Tehran, Iran

Abstract

Background: Total fertility rate (TFR) in Iran decreased from the year 2000 and recently Iran has experienced fertility rates below replacement level. Birth interval is one of the most important determinants of fertility levels and plays a vital role in population growth rate. Due to the importance of this subject, the aim of this study was analyzing three birth intervals using three Survival Recurrent Event (SRE) models.

Materials and Methods: In a 2017 cross-sectional fertility survey in Tehran, 610 married women, age 15-49 years, were selected by multi-stage stratified random sampling and interviewed using a structured questionnaire. The effects of selected covariates on first, second and third birth intervals were fitted to the data using the Prentice-Williams-Peterson-Gap Time (PWP-GT) SRE model in *SAS 9.4*.

Results: Calendar-period had a significant effect on all three birth intervals (P<0.01). The Hazard Rate (HR) for a short birth interval for women in the most recent calendar-period (2007-2017) was lower than for the other calendar-periods. Women's migration influenced second (P=0.044) and third birth intervals (P=0.031). The HR for both birth intervals in migrant women was 1.298 and 1.404 times shorter, respectively than non-migrant women. Women's employment (P=0.008) and place of residence (P<0.05) also had significant effects on second birth interval; employed women and those living in developed, completely-developed and semi-developed areas, compared to unemployed women and those living in developing regions, had longer second birth intervals. Older age at marriage age increased the HR for a short third birth interval (P<0.01).

Conclusion: The analysis of birth interval patterns using an appropriate statistical method provides important information for health policymakers. Based on the results of this study, younger women delayed their childbearing more than older women. Migrant women, unemployed women and women who live in developing regions gave birth to their second child sooner than non-migrant employed women, and women who lived in more developed regions. The implementation of policies which change the economic and social conditions of families could prevent increasing birth intervals and influence the fertility rate.

Keywords: Birth Interval, Fertility, Survival Analysis

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Introduction

Fertility influences population size and distribution, so analyses of fertility behavior provide important information for policy makers who plan population control and evaluate family planning programs (1). Family planning programs in Iran in the past two decades were aimed at fertility reduction and had reduced the total fertility rate (TFR) to 2.01 by 2016 (2, 3). In recent years, government and policy makers have applied new pronatalist policies to increase fertility. The success of such policies rely on understanding the determinants of low fertility.

Among the different indicators used to identify fertility patterns, such as number of children borne to each woman, birth interval is very important. The pattern of birth intervals not only denotes the pace of child bearing but also increases the chances of transition to higher parity (4). Many studies have shown that long birth intervals lead to a low fertility rate and decreased population growth (5). Since birth interval plays an important role in the health of mothers and children, it also merits special attention in public health. Birth interval has become one of the main strategies in health promotion programs for mothers and children in the last 20 years in Iran (6). Consequently, in recent years, many studies have examined the interval between marriage and first birth, and inter-birth intervals. Most of the research has focused on first birth interval (FBI) because of its advantages; women do not forget details of their first pregnancy, and the delay in the menstrual cycle that occurs after subsequent fertilizations is not observed. Furthermore, if FBI is short (<12

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*Corresponding Address: P.O.Box: 1531635711, National Institute for Population Research, Tehran, Iran
Email: mahsa.saadati@gmail.com

months) and occurs at a young age, subsequent pregnancies may happen faster and the fertility rate will be increased (7). Reduction of child mortality (8), increasing levels of education for women and their children (7), and balancing individual and family goals (9) are influential factors that affect first childbearing. Saadati et al. (10-12) showed that in Tehran and Semnan, calendar-period, place of residence, social insecurity, educational level, and employment had significant effects on women's FBI.

In addition to delayed childbearing, long inter-birth intervals (>75 months) can lead to a below-replacement level TFR (13-15). Many studies have considered determinants of long birth intervals; Soltanian et al. (16) showed that there were significant effects on birth intervals by women's age at first marriage, parental education, women's employment, use of contraceptives, and number of live births. Erfani et al. (5, 13-15) showed that several factors, such as woman's calendar-period, marriage age, contraceptive method, educational level, employment, place of residence and household income influenced women's first, second and third birth intervals in Tehran and Hamedan.

Due to its simplicity, the proportional hazards Cox model is used to analyze birth intervals in many studies in Iran and around the world (5, 6, 13, 14, 17, 18). Cox models can determine the relationship between the HR and covariates without specifying the baseline hazard function. The assumption underlying the validity of the Cox model is the proportionality of the hazards, or independence of event times, a fact often ignored in applications of this model. However, in most studies, including those on birth intervals, event times (births) are correlated. In these studies, using Cox models which ignore the correlations between birth intervals may lead to errors in estimating the standard deviation of the desired parameters and result in incorrect inferences (19). In such cases, SRE models, which allow for the given event (e.g. birth) to occur more than once for each individual and that include the correlations between events to be included in the model, should be used (19, 20). SRE models include Anderson-Gill (AG), Wei-Lin-Weissfeld (WLW), PWP-Total Time (TT), PWP-GT, and frailty models which should be selected for use based on the research objective, and the nature of the data (19).

According to the last census (2016), Tehran, Gilan and Mazandaran had the lowest TFRs in Iran; 1.38, 1.51, and 1.56, respectively (21), underlining the importance of studying the fertility behavior of women who live in Tehran. As birth interval is such an important determinant of women's fertility, the aim of the present study was to determine socio-demographic factors that affected women's first, second and third birth intervals in 2017 (22). In order to attain valid results the PWP-GT model was used to analyze the data. Data collection and statistical methods are described next, findings from the models fitted are illustrated in results, and some concluding remarks are given in the discussion and conclusion sections.

Materials and Methods

This study used data from a 2017cross-sectional survey "Effects of socio-economic rationality dimensions on childbearing behavior in Tehran" (22). All married women aged 15-49 years were eligible. The final sample included 610 women from Tehran province selected using multi-stage sampling (23). The structured questionnaire collected demographic data, fertility history and attitudinal factors related to childbearing. Based on the aims of this study, only demographic and fertility history questions were considered. 10 demographers and sociologists confirmed the validity of questionnaire, and its reliability was verified by a Cronbach's Alpha of at least 0.771.

Participants provided oral consent to participate in this study and the Ethical code was supplied by National Population Studies and Comprehensive Management Institute for the questionnaire (20/18627) (22). Birth intervals, defined as the length of time between two successive live births, were considered the response outcome of interest. Since very few women had more than 3 children, only three birth intervals, marriage to first, first to second, and second to third births were included in this survey. Data for nulliparous women and women with one or two children were considered as censored for the first, second, and third birth intervals, respectively (Table 1).

According to different studies devoted to the investigation of influential factors for birth intervals in Iran, the most important socio-demographic covariates, also analyzed in this study, are age at first marriage (5, 14, 24-26), educational level (9, 10, 25, 27, 28), couple's educational level (26, 28), employment (5, 26, 28), region of residence (14, 25), Internal migration (5, 14, 15), family expenditure (13, 15, 26), and calendar-period (5, 13-15, 29). Four calendar-periods were used in the present study, before May 1987, May 1987 - April 1997, May 1997 - April 2007, and May 2007 - April 2017, to cover the years during which the study participants would have given birth. These ten-year periods are assumed to measure to some extent the socio-economic changes and major policies that have taken place during these periods (13, 14).

To evaluate the influence of selected covariates on birth intervals accurately, PWP-GT SRE models were used to analyze the data in SAS 9.4.

Statistical methods

Recurrent event data refer to sequential events that occur more than once. As mentioned before, childbearing is an example of recurrent event data. Many studies have analyzed birth intervals based on conventional models which may provide misleading results. Conventional analysis of the FBI using a Cox model is described in Equation (1):

$$h_i(t)=h_0(t)\exp(\boldsymbol{\beta}X_i), i=1,\ldots,n$$

Where $h_i(t)$ denotes the hazard given the covariate values for the i^{th} subject and survival time (t). The term $h_0(t)$ is called the baseline hazard; it is the hazard for the re-

spective individual when the values of all the covariates are equal to zero. **β** is the vector of regression coefficients, and x_i is the vector of covariates for the i^{th} subject.

Table 1: Frequency distribution and median birth intervals in months (in parentheses) of women by selected covariates

Covariate	1st Birth	2nd Birth	3rd Birth
Calendar- period			
Before May 1987	5.1 (31)	7.0 (28)	10.8[a]
May 1987-Appril 1997	17.5 (38)	28.3 (57)	32.4[a]
May 1997-Appril 2007	33.7 (35)	42.6 (65)	36.5[a]
May 2007-Appril 2017	43.7 (40)	22.1 (41)	20.3[a]
Marriage age (Y)			
<16	9 (31)	14.8 (40)	25.7 (70)
17-19	19.9 (37)	27.7 (61)	33.8 (45)
20-24	41.8 (40)	36.3 (57)	27.0 (65)
25-29	22.5 (39)	16.4 (51)	10.8 (51)
30+	6.9 (38)	4.7 (43)	2.7 (31)
Educational level			
Primary and less	6.5 (31)	10.8 (36)	23.6 (70)
Secondary and high school	9.1 (31)	14.0 (41)	19.4 (57)
Diploma	45.0 (37)	50 (60)	48.6 (55)
B.Sc./Associate	30.2 (42)	20.4 (61)	6.9 (66)
M.Sc. and Ph.D.	9.1 (38)	4.8 (48)	1.4[a]
Couple's educational level			
Primary and less	6.3 (33)	9.6 (41)	20.8 (70)
Secondary and high School	14.8 (31)	20.0 (40)	26.3 (47)
Diploma	36.2 (37)	38.8 (58)	34.7 (58)
B.Sc./Associate	29.8 (42)	22.0 (64)	15.2 (60)
M.Sc. and Ph.D.	12.9 (37)	9.6 (48)	2.8 (30)
Woman's employment			
Unemployed	28.6 (37)	20.2 (63)	8.1[a]
Employed	71.4 (42)	79.8 (52)	91.9[a]
Migration			
Non-migrant	86.9 (38)	89.7[a]	93.0[a]
Migrant	13.1 (40)	10.3[a]	7.0[a]
Family expenditure (each month)			
Less than 2 million Tomans	56.6 (37)	63.5[a]	72.9[a]
2-3.5 million Tomans	32.2 (41)	27.3[a]	24.3[a]
More than 3.5 million Tomans	11.2 (38)	9.2 (48)	2.9[a]
Region of residence			
Developing	16 (41)	12.4 (63)	4.1 (31)
Semi-developed	15.4 (46)	10.5 (43)	6.8 (95)
Developed	44.1 (37)	46.9[a]	48.6 (60)
Completely-developed	24.5 (38)	30.2[a]	40.5 (55)
Total exposed to the birth interval (median birth interval)	610 (38)	469 (55)	258 (58)
Total experienced the birth (%)	469 (76.9)	258 (55.0)	74 (28.7)
Total censored (%)	141 (23.1)	211 (44.9)	184 (71.3)

[a]; **Medians were not computed, as the cumulative survival distribution did not go below 50% or less, which means more than half of women were pregnant but had not yet given birth.**

However, in this situation, the results of Cox model are misleading because the model does not take into account all the available data, and the correlation between recurrent event times. Ignoring this correlation leads to misleading results; in this case, confidence interval estimation could be artificially long, as a result the statistical power decreases. Consequently a statistical model that considers the correlations between the data must be applied in these situations (19).

Original Cox models have been extended to deal with recurrent event data. Examples include AG, PWP-TT, PWP-GT, WLW and frailty models (30).

The AG model assumes that the occurrence of the current event is not affected by the previous events, so each subject is at risk of all events over the entire follow-up period. Thus, the baseline hazard is common for all events. In this model risk intervals are considered as $(t_0, t_1]$, $(t_1, t_2]$ … $(t_m$, last follow-up time] for each subject and each recurrent event for the i^{th} subject is assumed to follow Equation (1). This a suitable model when correlations among events for each individual are induced by the measured covariates. Thus, dependence is captured by appropriate specification of the time-dependent covariates, such as number of previous events or some function thereof.

In the WLW model, time intervals are given as $(0, t_1]$, $(0, t_2]$ … (0, last follow-up time] for each subject, and is suitable for studies in which each subject is followed from study entry. In this model, all individuals are at risk of recurrence during the follow up, regardless of the occurrence of previous events, but different baseline hazards for each event are assumed in the model. The hazard function for the k^{th} event of the i^{th} subject is explained by Equation (2):

$$h_{ik}(t)=h_{0k}(t)\ exp(\beta_k X_{ik}),\ i=1,\ldots,n,k=1,\ldots,l \quad (2)$$

Where, "k" is the number of strata for each person at time t, X_{ik} denotes the predictor variable for i^{th} individual at time t, and β_k is the regression coefficient for k^{th} event (strata).

The PWP model analyses recurrent events by stratification, based on the prior number of events during the study. All subjects are at risk for the first event (stratum), but only those who experienced the previous event are at risk for the next event. PWP-TT models have the same outcome as the AG model and evaluate the effect of a covariate for the kth event since entry into the study. In PWP-GT models the outcome is defined as gap time, which is the time since the previous event. So, time intervals are given as (0, t1], (0, t2-t1] … (0, last follow-up time-previous time] for each subject. PWP-GT evaluates the effect of a covariate for the kth event since the time from the previous event.

In PWP-GT models, the hazard function for i^{th} subject, and k^{th} event is described in Equation (3):

$h_{ik}(t)=h_{0k}(t-t_{k-1})\ \exp(\beta_k X_{ik}), i=1,\ldots,n,k=1,\ldots,l$ (3) t-1 denotes the former occurrence time of the event.

Unlike the AG model, the effect of covariates may vary from event to event in the PWP models. If it is reasonable

to assume that the occurrence of the first event increases the likelihood of a recurrent event, then PWP would be the recommended model. PWP models (TT or GT) are also indicated when there is interest in estimating effects for each event separately. The PWP models assume that a subject can only be at risk for a given event after he/she has experienced the previous event.

When subject-specific random effects can explain the unmeasured heterogeneity in a model, a frailty model can be applied which leads to a person-specific interpretation of the parameter estimates. In this model production of consistent estimations depends on the number of events, number of subjects and the distribution of events/subject. The Frailty model is described in Equation (4):

$$h_{ik}(t)=h_{0k}(t)\,\omega_i\,exp(\beta_k X_{ik}),\ i=1,\ \ldots,\ n\ ,\ k=1,\ \ldots,l \quad (4)$$

Where, Frailty ω_i is the unobserved (random) factors for i^{th} subject.

Selection of the recurrent event models depends on many factors, including number of the events, relationships between subsequent events, effects varying or not across recurrences, biological process, and dependence structure. In this study only women who have already had one or two children can give birth to second and third children; so AG and WLW models are unsuitable for these data. Frailty models were not selected in this study because frailty variances were very low for second and third birth intervals (0.043 and 0.02, respectively). The PWP-GT model was selected instead of the PWP-TT model, because the distribution of children per women is small, and prediction of time to next birth was an outcome of interest (31).

Results

Mean age of the women in this study was 35.38±7.91years, and age of first marriage was 22.59 + 4.39 years. Most of women and their husbands had an academic level education (44.3%, 46.4, respectively), "less than 2 million Tomans" family expenditure (56.6%), were unemployed (68%), and lived in developed regions (44.1%). Only 15.7% of women had migrated in last 10 years. Among 610 married women, 21.2%, 34.7%, 31.3%, and 12.8% respectively had 0, 1, 2, and 3 children. Table 1 shows that half of the women had their first birth almost 3 years (38 months) after marriage but spaced their second birth by more than 4 years (55 months).

Median interval to first birth by educational level showed, as expected, that university-educated women had the longest interval to first birth. In employed women, immigrant women and women who had a family expenditure of 2 to 3.5 million Tomans childbearing was more delayed than among unemployed women, non-migrant women and women who lived in households with other expenditure profiles.

Survival curves based on Kaplan-Meir estimations for women's first, second, and third birth intervals are shown in Figure 1. As this figure displays, women gave birth to their first child sooner than the second and third one.

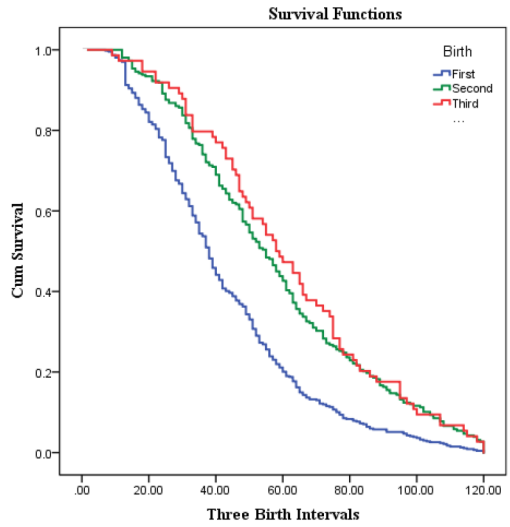

Fig.1: Survival curves for first, second, and third birth intervals

Table 2 shows the results of the PWP-GT model for first, second, and third birth intervals based on selected covariates.

The results of the PWP-GT model revealed that calendar-period had a significant effect on all three birth intervals (P<0.01). The largest gap from marriage to first, first to second, and second to third child was among women in the last calendar period. HRs for a short birth interval for first, second, and third children for women in last calendar-period were 0.479, 0.286, and 0.161 times lower than women in first calendar-period. In other words, the HR for short birth intervals decreased from the first to the last calendar-period. Women's employment and region of residence also affected the second birth interval. Employed mothers were at lower risk of a short interval between first and second child compared to unemployed women (HR=0.758, P=0.008). In other words, the likelihood hazard of having a second child for employed women was less than unemployed women. Women who lived in developed (HR=0.576, P<0.001), completely-developed (HR=0.705, P=0.015), and semi-developed (HR=0.819, P=0.041) regions were less likely to have a short second birth interval than women who lived in developing regions. So, women who lived in developing regions had a greater likelihood of having a second child than women who lived in other regions. HR of women's deduction in second and third birth intervals for migrant women was 1.298, and 1.404 times than non-migrant women, respectively. Therefore, the likelihood of having a second and third child was greater in migrant women than non-migrant women. Increasing age at marriage was associated with a higher HR for a shorter interval between the second and third birth (HR=1.047, P<0.001).

Recurrent event data structure and how to organize the data for each recurrent event model, and the SAS code for fitting these models are given in the Tables S1, 2 (See Supplementary Online Information at www.ijfs.ir), respectively.

Table 2: Estimated hazard rate from PWP-GT model assessing the impact of selected covariates on first, second and third birth intervals

Covariate		1st Birth			2nd Birth			3rd Birth		
		HR	SE	P value	HR	SE	P value	HR	SE	P value
Calendar-period	Before May 1987 (ref)									
	May 1987-Appril 1997	0.614	0.266	0.066	0.356	0.212	<.001	0.478	0.235	<.001
	May 1997-Appril 2007	0.755	0.268	0.296	0.308	0.210	<.001	0.190	0.239	<.001
	May 2007-Appril 2017	0.479	0.271	0.006	0.286	0.221	<.001	0.161	0.246	<.001
Marriage age (Y)		0.998	0.012	0.898	1.012	0.010	0.239	1.047	0.012	<.001
Educational level	Primary and less (ref)									
	Secondary and high school	1.287	0.267	0.344	1.078	0.193	0.697	1.215	0.195	0.318
	Diploma	0.812	0.255	0.415	0.855	0.179	0.382	0.981	0.194	0.923
	BS/Associate	0.684	0.277	0.17	0.872	0.207	0.509	1.025	0.243	0.919
	MS and PhD	0.615	0.344	0.158	0.762	0.274	0.322	1.424	0.331	0.285
Couple's educational level	Primary and less (ref)									
	Secondary and high school	1.214	0.249	0.436	1.074	0.182	0.693	1.109	0.188	0.581
	Diploma	1.017	0.255	0.949	0.896	0.183	0.549	0.924	0.202	0.696
	BS/Associate	0.816	0.269	0.449	0.907	0.195	0.615	0.841	0.225	0.441
Woman's employment	MS and PhD	1.012	0.319	0.97	0.955	0.243	0.848	0.882	0.288	0.662
	Unemployed (ref)									
Migration	Employed	0.969	0.128	0.804	0.758	0.104	0.008	0.879	0.131	0.325
	Non-migrant (ref)									
Family expenditure (each months)	Migrant	1.062	0.17	0.722	1.298	0.129	0.044	1.404	0.157	0.031
	Less than 2 million Tomans (ref)									
	2- 3.5 million Tomans	1.108	0.127	0.42	1.013	0.096	0.895	1.119	0.113	0.319
Regions of residence	More than 3.5 million Tomans	1.208	0.194	0.329	1.067	0.157	0.680	1.086	0.210	0.693
	Developing (ref)									
	Semi-developed	0.777	0.165	0.125	0.819	0.098	0.041	0.883	0.106	0.242
	Developed	0.65	0.225	0.056	0.576	0.146	0.000	0.768	0.185	0.152
	Completely-developed	0.922	0.199	0.684	0.705	0.143	0.015	0.734	0.189	0.102

ref; Reference group.

Discussion

According to various studies, birth interval is one of the factors affecting the number of children borne by a woman, with short birth intervals tending to lead to more children (2-5). For this reason the study of birth intervals has become important in Iran.

In most studies in which birth intervals have been analysed, each interval was modelled separately using Cox or parametric survival models regardless of the correlation between them. Rasekh and Momtaz (32) analyzed birth intervals using Cox models without considering correlation between the intervals. Soltani et al. (18) used Cox and Weibull parametric models to examine socio-economic factors affecting first and second birth intervals based on Demographic and Health Study (2000) data in Iran. Cox models assume that intervals are independent, when in fact a woman's birth intervals are correlated. Ignoring the interdependence of birth intervals cause a bias in estimating the variance of the model's parameters meaning results for the effects of covariates on the birth intervals are not valid.

In this article, the effect of selected covariates on first, second, and third birth intervals were determined using a PWP-GT SRE model. Based on the fitted model, calendar-period had significant effects on all three birth intervals. Women in the last calendar-period were least likely to give birth to children after a shorter interval than women in the other calendar-periods. While half of the women who were exposed to their first pregnancy before May 1987 gave birth to their first child 37 months after marriage, half of the women who were in last calendar-period (May 2007 to April 2017), delayed childbearing by up to 40 months. The HR for a short interval between 'first and second', and 'second and third' children decreased in recent calendar-periods. This finding is similar to the results obtained by Erfani et al. (5, 14).

Marriage to FBI has increased over the last three decades. Increasing age at first marriage is associated with an increased HR for a shorter interval between the second and third child. This means that with increasing age at marriage the interval between the birth of the second and third child decreased. This may be due to the shorter remaining fertile period and trying to reach the desired number of children. Many other studies have reported that birth interval decreases as marriage age increases (6, 33, 34).

The birth interval between first and second child for unemployed women was shorter than for employed women, as in other studies (6, 15, 16). Due to the time required to adapt to their new situation, migrant women are expected to have longer inter-birth intervals compared with non-migrants (15). In this study first birth intervals for migrant women were longer than non-migrant women. On the other hand, migrant women gave birth to their second and third child sooner than non-migrant women.

Region of residence had a significant effect on second birth interval. Women who lived in semi- developed, developed, and completely-developed regions gave birth to their second child later than those living in developing regions. Erfani (13) showed that women who lived in completely- developed regions in Tehran have their second child later than ones who lived in developing regions.

The main advantage of this study is the analysis of birth intervals using the PWP-GT model. In most studies these data are analyzed using Cox or parametric survival models which may lead to incorrect results. This study also has some limitations. Some fertility history factors such as contraceptive use, breast-feeding duration for previous birth, and survival status of previous children were unavailable. These questions will consider in the next survey which will be implemented in the near future.

Conclusion

Women in the 2007-2017 calendar-period delayed childbearing due to economic and social conditions in society and the current uncertainty. This finding also applied to second and third children. The longer interval between the first and second births of employed women indicates that they have a second child later than unemployed women, and as a result, may experience a lower fertility level. Policymakers can enable women to have children at shorter birth intervals by providing appropriate socio-economic conditions.

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Authors’ Contributions

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

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Name of author. Thesis title. Degree. City name. University. Publication date (year).
Example: Eftekhari Yazdi P. Comparison of fragment removal and co-culture with Vero cell monolayer's on development of human fragmented embryos. Presented for the Ph.D., Tehran. Tarbiyat Modarres University. 2004.
Internet references
Article:
Surname(s) and first letter of name &middle name(s) of author(s) .Manuscript title. Journal title (abbr). publication date (year); Volume (Issue): Page number. Available from: URL link. (Observation date).
Example: Jahanshahi A, Mirnajafi-Zadeh J, Javan M, Mohammad-Zadeh M, Rohani M. Effect of low-

frequency stimulation on adenosineA1 and A2A receptors gene expression in the dentate gyrus of perforant path kindled rats. Cell J. 2008; 10 (2): 87-92. Available from: http://www.celljournal.org. (20 Oct 2008).

Book:

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

Law:

Example: Embryo donation law. Iran Judicature, Official Gazette of the Islamic Republic of Iran. Available from: http://www.dastour.ir/Brows/?lid=245069.(20 Jul 2013).

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