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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
Web: www.ijfs.ir
Emails: ijfs@royaninstitute.org & info@ijfs.ir

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Contents

Review Article

Underestimated Aspects in Male Infertility: Epigenetics is A New Approach in Men with Obesity or Diabetes: A Review

Maryam Jazayeri, M.Sc.[1, 2], AliReza Alizadeh, Ph.D.[2], Mohammad Ali Sadighi Gilani, M.D.[3], Poopak Eftekhari-Yazdi, Ph.D.[2], Mohsen Sharafi, Ph.D.[2, 4], Abdolhossein Shahverdi, Ph.D.[2*]

1. Department of Reproductive Biology, Faculty of Basic Sciences and Advanced Medical Technologies, Royan Institute, ACECR, Tehran, Iran
2. Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
3. Department of Andrology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
4. Department of Poultry Sciences, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran

Abstract

Infertility is a complex multifactorial problem that affects about 7% of men and 15% of couples worldwide. Many molecular mechanisms involved in male infertility. Destructive effects of infertility on the next generations are not well understood. Approximately 60-75% of male infertility cases have idiopathic causes, and there is a need for additional investigations other than routine examinations. Molecular factors that surround DNA, which are mitotically stable and independently regulate genome activity of DNA sequences, are known as epigenetics. The known epigenetic mechanisms are DNA methylation, histone modifications and non-coding RNAs. Prevalence of metabolic diseases has been increased dramatically because of changes in lifestyle and the current levels of inactivity. Metabolic disorders, such as obesity and diabetes, are prevalent reasons for male infertility; despite the association between metabolic diseases and male infertility, few studies have been conducted on the effects of epigenetic alterations associated with these diseases and sperm abnormalities. Diabetes can affect the reproductive system and testicular function at multiple levels; however, there are very few molecular and epigenetic studies related to sperm from males with diabetes. On the other hand, obesity has similar conditions, while male obesity is linked to notable alterations in the sperm molecular architecture affecting both function and embryo quality. Therefore, in this review article, we presented new and developed technologies to study different patterns of epigenetic changes, and explained the exact mechanisms of epigenetic changes linked to metabolic diseases and their relationship with male infertility.

Keywords: Diabetes, DNA Methylation, Epigenetics, Male Infertility, Obesity

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Introduction

The concept of epigenetics was presented approximately about 30 years ago, after which researchers attributed cell inheritance to gene regulatory feedback loops, chromatin modifications (DNA methylation and histone modifications), and non-coding RNA (ncRNA) molecules, which are collectively referred to as "epigenomes" (1). Epigenetics is broadly defined as "molecular factors and processes that surround DNA, independently regulate genome activity of DNA sequence and they are mitotically stable" (2).

The term of "epigenetics" was first introduced by the English developmental biologist Conrad Hal Waddington in 1942. He published a research paper titled "The Genetic Assimilation of the Bithorax Phenotype" in 1956 and stated that environmental stimuli play important roles in the inheritance of an acquired trait in a population (3, 4). Gametes contain epigenetic information that plays a key role in embryonic development and any perturbation in the epigenome of gametes could alter the phenotype of the next generation offspring through epigenetic inheritance (5). Epigenetic mechanisms may be the main mediators in the occurrence and development of metabolic disorders and subsequent diseases (6). Study of the impact of epigenetic alterations on male infertility and complications in the next generation is an undeniable need and studies on this topic have recently been initiated.

Male infertility, as a complex multifactorial problem that affects about 7% of men and 15% of couples worldwide. It is commonly reported that both genetic and epigenetic factors play a role in infertility. Chronic diseases such as inflammations, obesity and infections as well as lifestyle choices and environmental factors play major roles in incidence and prevalence of infertility in men (7). Abnormal sperm epigenetic profiles are related to semen analysis parameters and reproductive function

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***Corresponding Address: P.O. Box: 16635-148, Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran**
Email: shahverdi@royaninstitute.org

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defined by embryo quality and abortion rate (8), In spermatozoa, DNA methylation and post-translational histone modifications are carriers of epigenetic signals. In addition, sperm-transmitted small RNA (sRNA) may also contribute to epigenetic inheritance (5). Abnormal epigenetic mechanisms play an important role in the pathology of infertility; therefore, new frontiers can be set in the search for infertility causes and their associated clinical manifestations. Here, we intended to review association of epigenetic alterations and metabolic diseases with male infertility.

Sperm genome and epigenome

Sperm nucleus has a very complex architecture. Sperm DNA methylation is substantially reduced in comparison with somatic cells. DNA methylation occurs at the cytosine residue of CpG dinucleotides in gene control regions, which are named CpG islands. These islands are frequently located at gene promoters. DNA methylation is an epigenetic landmark that results in regulation of gene transcription (9).

DNA is associated with histones and shapes the nucleosome structure in somatic cells; however, in mature sperm, only 5-10% of DNA wrap around histone octamers. In the rest, sperm DNA histones are replaced by protamines; thus, 90-95% of sperm DNA is bound to protamine. Only 5%-10% of the male genetic content is packed with retained paternal histones (10) and assist in sperm chromatin compression, which promotes motility, fertilization and sperm DNA protection. The remaining 5-10% of histones are essential and part of the sperm's epigenetic signatures (11, 12). These histones could be targeted by biochemical modifications. Their alterations have the potential to activate or suppress expressions of genes organized with these histones.

In addition, mature spermatozoa contain small RNAs. These RNAs are implicated in regulation of gene transcription. The exact mechanism by which small RNAs act as another part of the epigenetic profile and lead to the regulation of gene expression is not known (9).

Effects of epigenetics on male infertility

Studies over the past decade have shown that in addition to effective genetic differences, epigenetic alterations such as DNA methylation, histone modification and RNA Significantly contributed to male infertility and health problems of their offspring (13). In the following, we explained each epigenetic changes in greater detail. Table 1 presents an overview of these changes.

Table 1: The effect of some epigenetic changes on male fertility

Epigenetic changes	Alterations in infertile individuals	Impact on male fertility	Reference
DNA methylation	Hypermethylation of *MTHFR* promoter	Idiopathic infertility	(14)
	In repetitive sequences *LINE-1*, *Alu Yb8, NBL2, D4Z4*	Control of the functional capacity of germ cells	(15)
	Hypermethylation of the *RPS6KA2, APCS, JAM3/ NCAPD3* and *ANK2* genes	Oligospermia, abnormalities in some of the sperm chromosomes; reduced fertility	(16)
	Decreased methylation in *H19* gene and increased methylation in the *MEST* and *SNRPN* genes	Associated with male infertility	(17)
	Methylation level of *MEST, GNAS, LINE-1*	FSH and LH level	(18)
	Abnormal methylation of *IGF-2, KCNQ-1*	Sperm DNA damage and impaired fertility	(19)
	Hypermethylation of *SPATA4, SPATA5, SPATA6*	Oligozoospermia and infertility	(13)
	Hypomethylation of *H19*	Multiple sperm defects, Infertility biomarker	(20)
	Alterations in methylation of the *DEFB126, TPI1P3, PLCH2* and *DLGAP2* genes	Abnormal embryo growth	(21)
Histone modification	H3K4me2 activation; H3K27me3 suppressive changes	Fetal growth and formation	(22)
	H2A ubiquitination (ubH2A) and histone 3 K18 acetylation (H3AcK18); acetylation of four histones: H4 K5, K8, K12 and K16 (H4tetraAck)	Disruption of protamine 1 (Prm1) deposition in the testes	(23)
	testis specific histone H2B variant (TH2B)	Abnormal nucleus regeneration during spermiogenesis	(24)
Non coding RNAs	Reduction of *HOTTIP* expression	Promotes proliferation of testicular embryonal carcinoma cells	(25)
	high expression of lnc32058, lnc09522 and lnc98497	Immotile sperm	(26)

FSH; Follicle - stimulating hormone and LH; Luteinizing hormone.

DNA methylation

DNA methylation can regulate gene expression using different mechanisms. DNA methylation plays a key role in gene expression; therefore, coordination of the increase, maintenance and elimination of methylation between tissues must be carefully controlled. This regulation is mediated by function of the methylation enzymes, such as DNA methyltransferases (DNMT1, DNMT3A, DNMT3B and DNMT3L) and TET protein family (TET1, TET2 and TET3). TET protein family was recently discovered to mediate active demethylation processes, particularly after fertilization (27).

DNA methylation may potentially influence post-fertilization processes. Most signs of paternal methylation are actively corrected during epigenetic reprogramming after fertilization. However, some areas affected by incorrect methylation escape reprogramming and may pass this methylation status to the developing embryo (9, 28). Hypermethylation can suppress gene expression as methyl groups prevent recruitment of transcription factors and DNA polymerases. On the other hand, hypomethylation up-regulates gene expression (20). The results of a study showed that most epigenetic changes occurred at the pachytene stage of spermatocytes, which might lead to a large change in DNA methylation at this growth stage. Observations suggested that primary germ cells, pro-spermatogonia and spermatogonial stem cells had DNA methylation profiles correlating with the epigenetic programming cascade (29). Accordingly, inappropriate hypomethylation and hypermethylation can affect proper sperm function.

Genomic imprinting is an epigenetic modification process that allows gene to be expressed in a certain way by parents and it plays an essential role in normal growth and development. Expressions of imprinted genes are determined by the parents (30). Studies on DNA methylation in infertility have mostly concentrated on imprinted genes like *H19, IGF2, MEST, PEG3, LIT1, SNRPN* and *KCNQ1* in addition to some non-imprinted genes, like *MTHFR* and *DAZL*. These studies showed that alterations in DNA methylation of these genes are associated with abnormal semen parameters and spermatogenesis. Recently, upon analyzing methylation across the genome, a study found varying levels of methylation in a number of genes, including some of the *SPATA* family members (*SPATA4*, *SPATA5* and *SPATA6*). These researchers reported that hypermethylation of these genes is associated with infertility of oligozoospermia men (13, 17). In this context, the results of several studies have shown a close relationship between changes of cytosine methylation and *H19* gene expression in males with infertility. Therefore, *H19* hypomethylation is a proposed epigenomic infertility biomarker that could assess oligospermia in men with various sperm defects (20). Moreover, methylation of the *MEST*, *GNAS* and *LINE1* genes is significantly associated with sperm concentration, blood follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels (18). Thus, degree of methylation of the *MEST* and *GNAS* genes is significantly associated with increased levels of LH. Likewise, *LINE1* methylation is remarkably correlated by increased FSH levels (18). Abnormal methylation of the *IGF2* and *KCNQ1* genes is associated with DNA damage in sperm and consequent impaired fertility (19). Taken together, these studies suggested that abnormal methylation patterns in imprinted and non-imprinted genes or epimutation may play role in male infertility by causing abnormal spermatogenesis.

Histone modifications

Despite their relatively short history, histone modifications and histone methylation are one of the most important parts of epigenetic research, due to their important role in regulating transcription of gene expression in various organisms. During spermatogenesis, structure of chromatin changes due to histone modifications (31). Histones are sensitive to post-translational modification (PTM). These modifications include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and glycosylation among the others. Histone PTMs collectively act as "epigenetic codes" to activate transcription, suppression and coordination of chromatin structure in a more orderly fashion (10).

Environmental factors appear to alter histone modifications to some degree by directly regulating level and/or activity of histone-modifying enzymes. For example, hypoxia and nickel exposure increase H3K9me2 levels by inhibiting the histone demethylase JMJD1A (32).

Some cellular metabolites directly regulate expression of metabolic genes through histone modification. Lysine-specific demethylase-1-dependent flavin adenosine dinucleotide (LSD1-dependent FAD) is an enzyme which can degrade histones and regulate cellular energy levels by suppressing genes involved in mitochondrial respiration and energy consumption (33). In human spermatozoa, about 5-10% of histones are conserved and precise substitution of histone by protamine is critical for normal sperm production. The relationship between intracellular metabolites, histone signatures and their effect on gene transcription may play a role in disease progression. Therefore, some infertility and sterility problems may be attributed to abnormal histone modifications in spermatozoa.

Non-coding RNAs (ncRNAs)

Mammalian sperm RNAs is a source of paternal hereditary information beyond DNA. Environmental factors that include imbalanced diet, mental stress and exposure to toxins can alter sperm RNAs and cause phenotypes related to paternal environmental stress in offspring (34). Among the different types of RNA, messenger RNA (mRNA), transporter RNA (tRNA) and ribosomal RNA (rRNA) are the best-known RNAs in all organisms. In addition to these, RNAs can be broadly divided into coding RNA (cRNA) and ncRNA (35). Mature spermatozoa have several types of small ncRNAs that include silencing RNAs (siRNAs) and microRNAs (miRNAs), which vary in length and generally lack an open

reading frame. miRNAs are associated with molecular mechanisms regulating spermatogenesis, particularly endogenous genes in germline cells which regulate their complex process of renewal and/or differentiation. Recent studies identified another new class of siRNAs, PIWI-interacting RNAs (piRNAs), which are expressed in testes during spermatogenesis (36). Hypomethylation of repetitive elements in the male germline was associated with an increase in miR-29, proposed to reduce DNMT3a, a protein required for genomic methylation (37). In a study of male infertility, researchers identified 9879 long non-coding RNAs (lncRNAs) with differential expressions; only three (lnc32058, lnc09522 and lnc98497) showed high expressions in immotile spermatozoa compared to normal motile spermatozoa. Several lncRNAs (Mrhl, Drm, Spga-lncRNAs, NLC1-C, HongrES2, Tsx, lncRNA-tcam1, Tug1, Tesra, AK015322, Gm2044 and lncRNA033862) were confirmed to have functionally distinct roles in spermatogenesis (26). Recent evidence suggested that small RNAs (tsRNAs) derived from sperm tRNA, as a carrier of paternal epigenetic information, may mediate intergenerational inheritance (38). In the paternal high fat diet (HFD) mouse model, a subset of tsRNAs, with size range of 30-34 nucleotides, led to changes in the RNA expression profile and modifications. Injection of sperm tsRNA from HFD male mice into normal zygotes also caused metabolic disorders in F1 offspring and impaired the expressions of metabolic pathway genes in early embryos. Thus, sperm tsRNAs represent a paternal epigenetic factor that might cause intergenerational inheritance of dietary metabolic disorders (39). Based on these findings, it can be concluded that many lncRNAs have potential impacts on male spermatogenesis and infertility, but very few have thus far been identified and confirmed. Comparing the results of these studies may lead to recognition of important lncRNAs for spermatogenesis. This can also be used as markers for infertility in men with metabolic diseases. Figure 1 provides a summary of the epigenetics events in the testis.

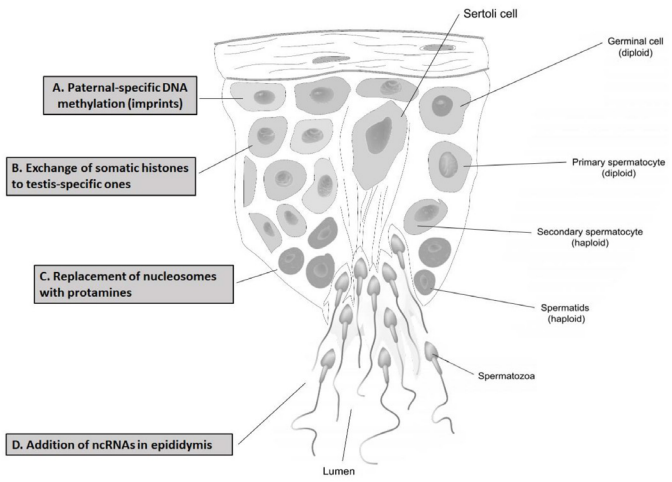

Fig.1: The seminiferous tubule regions of testis and possible epigenetics events in male fertility. Each process of epigenetics occurs in specific cells and regions of the testis: **A.** Paternal-specific DNA methylation in spermatogonia, **B.** Exchange of somatic histones to testis-specific ones in primary spermatocyte, **C.** Replacement of nucleosomes with protamines in spermatid, and **D.** Addition of non-coding RNAs in the epididymis.

Metabolic syndrome

Metabolic syndrome (MetS) is a complex pathophysiological state caused by an imbalance between calorie intake and consumption. Urbanization and lack of physical activity as well as an excessive and poor quality diet can lead to MetS. This metabolic syndrome is defined as a cluster of at least three out of five medical indicators: abdominal obesity, high blood pressure, high blood sugar, increased triglyceride levels and reduced high-density lipoprotein (HDL) level. MetS increases risk of stroke, cardiovascular disease and type 2 diabetes mellitus (40).

MetS is associated with disruption of hypothalamic-pituitary-testes (HPT) axis, as well as reduction of gonadotropins and steroidogenesis, specifically testosterone levels in men. In MetS, systemic chronic inflammation mediates hypogonadism, erectile dysfunction and poor spermatogenesis. Actually, oxidative stress cause inflammation in MetS. It finally leads to loss of DNA integrity, improper packaging and aberrant modification of sperm DNA. In other words, there is a change in MetS and sperm epigenetics of obese individuals. This change can be passed to the next generation. Thus, offspring will be susceptible to metabolic and reproductive problems (40, 41). The idea of paternal effects on offspring health is an exciting area for research and clinical studies.

Metabolic disorders and male infertility: epigenetics approaches

Study of the association of MetS with epigenetic aspects of male infertility is a spectacular, emerging topic for clinical and basic research. The main problem is multifactorial nature of these disorders. Metabolic disorders may be only one of the common causes of male infertility; however, infertile men suffering metabolic disorders during middle age. On the other hand, father lifestyle plays a key role in influencing spermatogenesis for future of child, as it has been proven that obesity, unhealthy diet, inactivity and pollution are factors that can alter sperm counts and sperm quality through epigenetics (42).

Previous studies supported the hypothesis that metabolic changes related to environmental factors can be passed from father to the offspring. These metabolic disorders acquired in offspring may be partly explained by potential genetic information carriers like epigenetic agents and their processes (38).

Diabetes

Association of type 2 diabetes with reproductive dysfunction is a serious challenge, due to the high prevalence in young people. Diabetes can affect reproductive function in the testes at multiple levels, including changes in sperm quality and spermatogenesis, and it may lower testosterone levels. Epigenetic alterations are important risk factors for diabetic complications (43, 44).

Glucose metabolism plays a vital role in spermatogenesis. Numerous studies in humans and animals have confirmed the effects of diabetes on sexual function, semen parameters, nuclear DNA and chromatin quality (45, 46). But there are few studies on the pattern of DNA methylation in spermatozoa from type 2 diabetic patients at the genome level (47). In 2019, Chen et al. (48) investigated the whole-genome DNA methylation profile of human spermatozoa by comparing eight individuals with type 2 diabetes and nine healthy individuals using the whole-genome bisulfite sequencing method. The results showed that ratio of methylated cytosine in the whole genome of type 2 diabetics was lower than the control group. They also identified differential methylation of 10 genes: *IRS1, PRKCE, FTO, PPARGC1A, KCNQ1, ATP10A, GHR, CREB1, PRKAR1A* and *HNF1B* in men with type 2 diabetes.

The results of previous studies have shown that abnormal levels of some lncRNAs can accelerate progression of diabetic retinopathy (49). Therefore, expression profiles of lncRNAs in the sperm of diabetic mice were studied to determine genes that might be related to reproduction and their association with the onset of diabetes. lncRNAs functions were assessed by subgroup analysis and their physical or functional relationships with the corresponding mRNAs. Expression profiles from six microarray evaluations of diabetic mice spermatozoa showed differential expressions of lncRNAs and mRNAs (4134 up-regulation and 3407 down-regulation of lncRNAs, 2590 up-regulation and 3507 down-regulation of coding-mRNAs, in sperm samples from mice diabetic mice group and control group, respectively). Genetics and pathway analysis revealed that function of mRNAs with differential expression was closely related to many processes associated with development of diabetes. In addition, study of lncRNAs and mRNAs identified potential nuclear genes that might play a substantial role in the pathogenesis of diabetes-related infertility (50).

Molecular studies that involved sperm of diabetic men are generally rare. Majority of men are unaware of their current status and develop diabetes later in life (47). Therefore, it is postulated that future of research on epigenetics and male infertility should focus on identifying the putative molecular mechanisms of effects of diabetes on epigenetic abnormalities during spermatogenesis.

Obesity

The interaction between obesity and diabetes is currently established; but more research has been conducted in the field of infertile obese men epigenetics rather than investigations on epigenetics of infertile diabetics. Male obesity is associated with notable alterations in sperm molecular composition, not only impairing the sperm function, but also posing risks to embryo (37). Many studies have shown abnormalities in sperm quality, particularly decreased sperm concentrations in over weight patients with high body mass index (BMI) of 25-29 Kg/m^2. Two recent meta-analyses, including 14 and 21 studies, reported an increased risk of azoospermia or oligozoospermia in obese men. In this regard, results of one study showed association of oxidative stress and sperm DNA fragmentation with sperm DNA methylation in men (51). In conclusion, it has been shown that spermatozoa epigenetic pattern changes in men with high BMI resulted in alteration of sperm DNA methylation. Thus, this abnormality is directly related to male infertility, low embryo quality and a decrease in fertility (52).

Obesity changes methylation status of DNA in other tissues. Soubry et al. compared sperm DNA methylation in 12 differentially methylated regions (DMRs) of 23 obese compared to 44 normal weight men. Percentage of DNA methylation was decreased in *MEG3*, *SNRPN* and *SGCE/PEG10,* while it was increased in MEG3-intergenic differentially methylated region (*MEG3-IG* DMR) and *H19* DMR in the sperm of the overweight/obese men (37, 53). Donkin et al. (54) focused on the regeneration of sperm methylation after weight loss by gastric bypass (GBP) in obese patients (mean BMI=31.8 Kg/m^2). The results showed that methylation status of 1509 unique genes was changed one week after surgery. In addition, 3910 unique genes had a different methylation status one year after GBP rather than before GBP. Additionally, sperm histone status was not changed in the lean and obese men, and sperm from the obese men altered small non-coding RNAs (sncRNAs) expression. These findings suggested that weight loss caused a gradual change in the sperm epigenome. Future efforts to determine epigenetic profile of human sperm exposed to other environmental factors, such as exercise or smoking, might reveal specific signatures which could influence metabolic health of future generations in positive or detrimental ways.

A recent study examined epigenetic changes of sperm, in relation to BMI. In this study, 144 samples (48.97%) were "normal weight" (BMI: 19.00-24.90) and 149 samples (50.68%) were "pre-obese/obese" (BMI: 25.00-40.30). Methylation levels of paternally imprinted genes (*H19-IG* DMR, *IGF2*-DMR0 and *MEG3-IG* DMR) and non-imprinted gene regions associating with obesity were reported. Regression analysis showed positive correlation between BMI and *MEG3-IG* DMR methylation in sperm DNA (55). These results suggested that obesity is related to sperm DNA methylation programming in humans and it may influence epigenome of the next generation.

Changes in histone of sperm DNA is another parameter that is affected by obesity. Expression of related genes to sperm motility, histone 3 and 4 modifications and post-translation global modification process in the testes of obese mice were reduced compared to the control group. In addition, the results of quantitative Western blot analysis showed a decrease in H3K23pr, H4K8cr, H3K122ac and H4K8ac in the testes of mice that consumed a HFD. These findings suggested that altered gene expression and PTM are associated with impaired reproductive function

in obese males (56).

In addition, abnormal lncRNA expression from HFD also causes epigenetic changes. There was a significant difference between lncRNAs and mRNA expressions in the sperm of obese mice fed HFD compared to mice that received normal diets. *Neat1* and *Malat1* expression levels were lower in obese mice compared to normal weight mice. *NEAT1* is expressed in human embryonic stem cells and plays role in spermatogenesis regulation. Decreased expression of *Neat1* is associated with reduced sperm quality and fertility, and its expression is negatively regulated by *Malat1* (57). *Malat1*, a long non-coding RNA, is present in active loci and it utilizes splicing factors. An increase in *Malat1* is recognized in all sperm samples and extra-nuclear areas. Enrichment of *Malat1* in sperm is due to the triple helix structure at its 3′ end. This RNA is associated with histone bound DNA of sperm and it plays role in chromatin remodeling (58).

Overall, male obesity may affect all aspects of sperm epigenetics including patterns of DNA methylation, histone modifications and ncRNA expression in spermatozoa. It ultimately leads to male infertility. Although the amount of RNA in spermatozoa is not significant, it has potential role in the clinical investigation and diagnosis of male infertility. It is also one of the factors specifically expressed in germ cells and present in mature spermatozoa. Therefore, RNAs are suitable molecular markers for detection of cell lineages in the spermatogenesis pathway. These markers can provide an overview of spermatogenesis status in infertile cases instead of invasive testicular biopsy.

Unexplained infertility

The molecular mechanisms involved in male infertility are not well understood yet and diagnosis of unexplained or idiopathic male infertility is made in cases where standard tests are unable to determine the cause. Such cases account for about 60-75% of male infertility. Uniquely, epigenetic changes such as DNA methylation and histone modifications have significant impact on this problem (14, 59).

Although different DNA methylation patterns of germ cells are associated with changes in sperm quality, few studies focused on the epigenetic investigation of infertile men with normal sperm parameters. For the first time in 2015, a genome-wide study examined sperm DNA methylation in patients with idiopathic infertility compared to fertile men. In this study, approximately 3000 CpG were detected which indicated defective methylation. These results suggested that these changes are precisely related to specific regions of sperm methylation, and it can be concluded that DNA methylation plays a role in controlling germ cell function (15). Results obtained from one study indicated that hypermethylation of *MTHFR* gene promoter is strongly associated with idiopathic infertility in males (14). Therefore, analysis of promoter methylation in specific genes may provide valuable biomarkers to identify men who are at high risk for infertility. Recently, a molecular experiment was conducted to identify male idiopathic infertility by using DNA methylation variations in sperm. The results of this study showed regions of DMRs in males with idiopathic infertility. In this study, researchers identified an epigenetic DMR signature of male infertility that can be used both as a diagnostic tool and to detect FSH response in the studied patient population (60). It is predicted that further development of this technology will improve diagnosis and management of infertile male patients, overall treatment options and development of therapies.

To date, much attention has been paid to the cellular and molecular mechanisms, in terms of infertility in men with asthenospermia (61, 62). Idiopathic asthenospermia (IAS) falls into this group, but its cause is unknown. IAS is one of the major causes of male infertility diagnosed by decreased sperm motility for which there is no effective treatment (63). Quantification of methylation specific polymerase chain reaction (MS-PCR) data has confirmed the significantly lower methylation level of *DAZ3* promoter in IAS patients compared to normozoospermic men (64). Moreover, by studying promoter area methylation changes of the other genes in men with idiopathic infertility, it was found that promoter methylation profiles of the *MLH1* and *MSH2* genes might be involved in sperm DNA packaging and sperm parameters, respectively (65). However, hypermethylation of *MTHFR* gene promoter in spermatozoa also appears to be associated with idiopathic male infertility (66). Although idiopathic infertility is associated with an increased risk of developing MetS, obesity, and an increased risk of subsequent cardiovascular disease, idiopathic infertility is controversial because the underlying mechanisms remain unknown (67).

Future perspective

Sperm epigenome is remarkably specific. It offers interesting opportunities for more research and can be assessed in clinical trials. Many attempts have been made to understand nature of this epigenetic perspective, in addition to role of sperm epigenetic patterns in normal sperm development and function, as well as in male fertility. Investigating these factors, along with epigenetic changes in sperm DNA, remains an intriguing target for dissemination of biomarkers that may be able to more accurately identify cause of male infertility.

The finding that "newly fertilized gametes and embryos are sensitive stages for epigenetic changes in the environment" has important implications as changes in lifestyle and reproductive system environment may have long-term consequences for child health, while they have not been fully explored yet. Understanding how and when metabolic diseases -such as obesity and diabetes- affect male fertility and finding ways to modify abnormal epigenetic signatures, in addition to determining the best time frame for reversing abnormal epigenetic symptoms may improve fertility in infertile men.

Currently, most environmental research on the sperm

epigenome focuses on DNA methylation. Recently, Abbasi et al. (46) opened a new horizon in terms of the stable, repeatable platform to measure changes in sperm DNA methylation. Uniquely, they suggested that Illumina Infinium platform is highly suitable for diagnostic use in a clinical setting. Progress in identifying other environmentally sensitive epigenetic mechanisms, including histone modifications, three-dimensional conformations and chromatin structure, as well as exosome loading and expression of ncRNA in seminal plasma could be important.

Conclusion

It is well-known that sperm is more than a cell that carries half of the genome and it has a unique epigenome. Any alteration in this epigenome can lead to infertility, abnormal fetal development, and increased risk of certain diseases in the next generation. *In vitro* fertilization (IVF) treatment cycles were repeatedly failed in some patients carrying obesity and diabetes while they may have normal sperm parameters. To date, new steps have been taken to understand methylation status in patients who refer to infertility centers. Future of this science likely depends on finding epigenetic codes along with epimutations in these individuals. It should be noted that finding epimutations alone is not sufficient and targeted treatment strategies should be sought. Perhaps these efforts can address large gap that exists in the treatment of infertile patients and prevent recurrent miscarriages.

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Authors' Contributions

M.J.; Designed the manuscript as a Ph.D. student, the main contributor and contributed to writing the manuscript's draft. A.R.A.; Designed the manuscript, contributed to the revision process, and performed manuscript editing. M.A.S.G.; Edited and approved the final manuscript. P.E.-Y.; Contributed to prepare the final manuscript. M.Sh.; Contributed to study conception and manuscript editing. A.Sh.; Supervised this project and contributed to approval of the final manuscript. All authors read and approved the final manuscript.

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Original Article

New Approaches to Define The Functional Competency of Human Sperm Subpopulations and Its Relationship to Semen Quality

Shannen Keyser, B.Sc. Hons, Gerhard van der Horst, Ph.D., Liana Maree, Ph.D.*

Department of Medical Bioscience, University of the Western Cape, Private Bag X17, Bellville, South Africa

Abstract

Background: This study aimed at comparing a comprehensive set of functional and structural sperm characteristics between sperm motility fractions and correlating results to the standard semen parameters. By grouping related variables, our objective was to establish the predictive power of semen parameters and whether they accurately reflect the functionality of sperm motility fractions or merely a small set of parameters within individual fractions.

Materials and Methods: In this non-invasive experimental study, donor semen samples (n=55) were separated via double density gradient centrifugation, isolating a high (HM) and low motile (LM) sperm fraction. Fractions were evaluated for percentage vitality, chromatin integrity, mature spermatozoa, motility and kinematic parameters, hyperactivation, positive reactive oxygen species, intact mitochondrial membrane potential (MMP) and acrosome reaction.

Results: HM fractions had significantly ($P<0.001$) enhanced percentages of induced acrosome reaction (HM, 55.6 ± 14.3%, LM, 25.0 ± 16.5%), motility and kinematic parameters, hyperactivation, vitality (HM, 70.4 ± 9.7%, LM, 47.9 ± 10.3%), mitochondrial membrane intactness (HM, 67.2 ± 10.4%, LM, 44.7 ± 15.0%) and mature spermatozoa (HM, 83.4 ± 10.0%, LM, 64.6 ± 8.2%) with intact chromatin (HM, 80.5 ± 8.1%, LM, 71.3 ± 8.0%). Various sperm morphology abnormalities correlated with LM fractions' grouped motility parameters (range, 0.46 to 0.51; range -0.4 to -0.75), whereas combined semen traits of total motility, progressive motility, viscosity and mucus penetration (MPT) correlated with HM fractions' grouped motility parameters (range, 0.44 to 0.84).

Conclusion: Collectively, total and progressive motility, viscosity and MPT may represent a reliable grouping of semen parameters for predicting the quality of HM sperm fractions. Separating the same donor semen samples into two significantly diverse motility sperm fractions could be a potential model in mimicking the qualities of fertile and sub-fertile males' sperm populations and used for future research on the improvement of sperm subpopulations from males with different fertility statuses.

Keywords: Computer-Assisted Sperm Analysis, Differential Gradient Centrifugation, Hyperactivation, Reactive Oxygen Species, Sperm Motility Subpopulations

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Introduction

The heterogeneous nature of human semen is well-known with distinct cell populations varying in degrees of maturation, functional quality and fertilizing ability (1). The exact physiological role of subpopulations remains unclear although correlations between the percentage of diverse sperm subpopulations in semen and sperm quality, fertility and the ability to resist cryopreservation damage have been reported (2). Many techniques are employed to separate semen into sperm subpopulations but ideally subpopulations representing spermatozoa of high sperm functionality relating to fertilization success should be identified and isolated - a challenge which still remains in modern andrology (3, 4).

Routine semen evaluations include small subsets of larger, heterogeneous number of spermatozoa from a single ejaculate, thereby inherently creating a large variability which may interfere with accurate evaluations of overall sperm quality (5). Thus, semen analysis may provide suitable information for preliminary evaluations of infertile males, but hardly represent true fertility or functional performance of spermatozoa (6). Complementary structural and functional tests with less inconsistencies relating to fertilization outcome such as evaluation of sperm DNA and chromatin integrity, reactive oxygen species (ROS), mitochondrial membrane potential (MMP), acrosome reaction (AR), cervical mucus penetration, motility parameters (particularly sub-populations such as rapid progressive sperm) and hyperactivation (HA) should therefore be utilized (3, 5, 6).

Various sperm selection techniques have been established based on the differentiation methods for sperm density, membrane surface charge, morphology, motility, membrane integrity and nuclear integrity (3,

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***Corresponding Address: Department of Medical Bioscience, University of the Western Cape, Private Bag X17, Bellville, South Africa**
Email: lmaree@uwc.ac.za

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7). Colloid centrifugation is such a technique, whereby a subpopulation of motile spermatozoa with good functional and structural integrity is isolated from the seminal plasma (3, 4, 8). Separated pellets generally comprise of higher numbers of motile and morphologically normal spermatozoa in comparison with lower density fractions (8-10). Additionally, recovered pellets should comprise of more spermatozoa with functional MMP, lower levels of ROS and less spermatozoa with apoptotic and necrotic markers (4, 8, 10). Nevertheless, discontinuous density gradient centrifugation (DGC) has been observed to result in high levels of DNA damage and ROS production (4).

With increased reports of male infertility, and an estimated 10 to 15% of men being affected by idiopathic male infertility at prime reproductive age, alternative approaches for fertility diagnosis including both functional and structural sperm tests are essential (11-13). In-depth, sperm assessment methodologies could assist to elucidate unknown factors affecting male fertility, provide individualized infertility treatments and getting valuable insights required for development of more relevant semen quality tests (11, 14). Additional sorting of ejaculates into subpopulations before evaluation may congruently detailed depth information on functional capabilities of entire ejaculates, further assist in the selection of recovered spermatozoa and seminal dose calculations in assisted reproductive technology (ART) (2, 12, 15). Furthermore, understanding and evaluating the biochemical and molecular mechanisms regulating human sperm functionality, especially in sperm subpopulations may assists clinicians in selecting the most appropriate ART treatment (12).

This study aimed to investigate and compare various functional and structural sperm characteristics between two sperm fractions [high motile (HM) and low motile (LM)] and correlate each fraction's results to the standard semen parameters. Despite differences between HM fractions and semen widely reported in literature (4), our approach was to assess a larger set of parameters for both HM and LM fractions and determine whether groupings of related parameters could refine potential relationships between neat semen and the functional quality of two individual motility fractions. Furthermore, since the same donor semen samples were used to produce the two sperm fractions, this approach could provide a good model for comparing sperm functionality in different sperm fractions, potentially mimicking the qualities in fertile and sub-fertile males' sperm populations.

Materials and Methods

Sample collection and standard semen analysis

In this non-invasive experimental study, 55 human semen samples were obtained from 39 healthy male donors as part of a donor program (Division of Medical Physiology, Department of Biomedical Sciences, Stellenbosch University). Samples were incubated permitting liquefaction (30 - 60 minutes at 37ºC in a 5% CO_2 regulated incubator) and processed as recommended by the World Health Organization (16). Semen volume, pH and viscosity were assessed in addition to several sperm parameters, including total motility, progressive motility, sperm concentration, total number of spermatozoa, mucus penetration (MPT), vitality and morphology (analysed with Sperm Class Analyser® (SCA®) computer-aided sperm analysis (CASA) system, version 6.2; Microptic S.L., Barcelona, Spain). As the study focused on investigating the functionality of two sperm motility fractions of different semen qualities, and determining if semen can accurately predict fertility, minimum cut-off points for percentage total sperm motility of 25% was used. Although this percentage is below the WHO lower reference limit, it was a non-biased reflection of the donors used, that otherwise had good semen parameters (16). This was a non-invasive *in vitro* study using semen from donors for research purposes only, and approved by the ethical boards of the University of the Western Cape (code 13/10/90) and Stellenbosch University (code N14/06/074). The Helsinki Declaration governing research on humans has been adhered to and each human donor provided written consent (17).

Preparation of sperm fractions

Semen samples were separated through DGC into two sperm motility fractions with AllGrad® 90/45% and AllGrad Wash® (Delfran, Johannesburg, South Africa). Semen aliquots (300 µl) were layered on top of equal volumes of the preheated (37ºC) density gradient 90 - 45%, and centrifuged at room temperature (RT) for 20 minutes at 500 *g*. Resultant top seminal plasma coats were discarded and remaining intermediate (less motile spermatozoa, LM fraction) and bottom (highly motile spermatozoa, HM fraction) pellets were separated into individual Eppendorfs. Separated fractions were re-suspended in 300 µl AllGrad Wash® and centrifuged at 500 *g* for 10 minutes. Washed pellets were re-suspended in non-capacitating human tubal fluid (HTF) to final sperm concentrations of 15 - 25×10^6/ml (18). For the purpose of this study, HTF was prepared without the supplementation of human serum albumin (HSA) in order to obtain an accurate functional representation of the two fractions without the interaction or stimulation of proteins.

Viscosity

Using the viscosity evaluation technique described by Rijnders et al. (19), 3 µl semen aliquots were loaded into preheated (37ºC) four-chamber, 20 µm-depth Leja slides (Leja Products B.V., Nieuw Vennep, The Netherlands) and the filling time recorded in seconds. Viscosity in centipoise (cP) was determined by the following equation:

$y=0.34x+1.34$

where y = viscosity in cP and x = filling time in seconds.

Sperm morphology

Semen aliquots (300 µL) were centrifuged in AllGrad

Wash® at RT for 20 minutes at 500 *g* and subsequent pellets re-suspended in HTF. Morphology smears were prepared (15 µL) and dried slides stained with SpermBlue® fixative and stain mixture (Microptic S.L., Barcelona, Spain) as described by Microptic (20). Coverslips were mounted with DPX mounting (Sigma Aldrich, Cape Town, South Africa) and 100 spermatozoa analysed with the Morphology module of the SCA® software using brightfield optics, a Basler ACA 1300-200uc camera, a blue filter and a 60x objective on a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa).

Sperm vitality

Following the BrightVit technique as described by Microptic (20) - semen samples and sperm fractions (n=35) were stained in suspension with BrightVit medium (Microptic S.L., Barcelona, Spain) for 10-15 minutes at 37ºC. Vitality smears (20 µl) were prepared and left to air dry before mounting with a coverslip using DPX mounting medium. Stained smears were viewed using the same equipment as described for sperm morphology.

Sperm motility, concentration and mucous penetration

Total sperm motility, progressive motility, concentration and MPT were assessed with the Motility module of the SCA® software and data captured with a Basler A312fc digital camera (Microptic S.L., Barcelona, Spain) attached to a Nikon Eclipse 50i microscope with a 10x positive phase contrast objective, a green filter and a heated stage. Preheated (37ºC) four or eight chamber, 20 µm-depth Leja slides were loaded with 2-3 µL of semen or prepared sperm fractions (n=35), and at least two fields with 200 motile spermatozoa analysed at 50 frames per second (f/s).

Percentages sperm motility assessed included total motility, progressive motility, rapid-, medium- and non-progressive motility as well as rapid-, medium- and slow-swimming spermatozoa. Kinematic parameters recorded for the average (overall fraction) and various progressiveness and swimming speed subpopulations included; curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). ALH was measured as half of the width the VCL track and not as the full VCL wave or doubling of riser values (risers' method) as described by Mortimer (21). Kinematic parameter cut-off values for MPT were VAP >25 µm/seconds, STR >80% and 7.5 µm< ALH >2.5 µm and DANCE was calculated as VCL×ALH.

Hyperactivation

Based on a previously established protocol for induced hyperactivation in human spermatozoa, both 2 mM procaine hydrochloride and 5 mM caffeine (Sigma Aldrich, Cape Town, South Africa) supplemented in HTF were used to induce sperm hyperactivation (22). HTF prepared as capacitating (CAP; HTF supplemented with 0.105 g $NaHCO_3$, 1.1915 g HEPES and 0.6 ml NaOH) and non-capacitating medium (HTF) were used as positive and negative controls respectively (18). Applying the flush technique described by Boshoff et al. (23), each chamber of a preheated (37ºC) four chamber Leja slide was loaded with 1 µl sperm preparation (HM or LM sperm fraction suspended in HTF, n=20) and flushed with 2 µl of each of the four preheated media mentioned above. Percentage hyperactivation [using cut-off values: VCL>150 µm/seconds; LIN<50%; ALH>7 µm (3.5 for SCA)] of at least 200 motile spermatozoa was assessed for each sperm fraction using the Motility module of SCA® and equipment as described for sperm motility,, after 5, 15, 30, 45 and 60 minutes of exposure to each medium (21).

Reactive oxygen species

Dihydroethidium (DHE, Molecular Probes, Eugene, OR, USA) was used to detect spermatozoa positive for ROS. Sperm fractions (n=20) were stained in the dark for 15 minutes in suspension (180 µL) with 20 µL of 20 µM DHE at 37ºC. Following incubation, 5-10 µL of suspension was placed on a clean slide with a coverslip, and immediately analysed using a 100x oil immersion objective and triband filter (excitation wavelengths: 457 nm=blue, 530 nm=green and 628 nm=red) on a Nikon Eclipse 50i fluorescence microscope (IMP, Cape Town, South Africa). Percentage of spermatozoa positive for ROS was calculated after manual assessment of at least 100 spermatozoa.

Mitochondrial membrane potential (ΔΨm)

The Mitochondria Staining Kit protocol (CS0390, Sigma Aldrich, Cape Town, South Africa) assessing MMP was optimized for this specific study. Fractions in HTF (n=20) were stained in suspension (1:1) with MMP staining solution (160 µl dH_2O, 40 µl JC-5 buffer and 1 µl frozen MMP 200x stock solution) and incubated in the dark at 37ºC for 20 minutes. Suspensions were subsequently centrifuged at 500 *g* for 5 minutes at 5-7˚C, pellets re-suspended in 200 µl JC-1 buffer (80 µl JC-5 buffer and 320 µl dH_2O) prepared and cooled on ice before use. Suspensions were centrifuged again as described above and pellets re-suspended in remaining 200 µl JC-1 buffer. Single drops of 5-10 µl suspension was placed on a clean slide with a coverslip, and immediately analysed as described for ROS. Percentage intact MMP was manually assessed for at least 100 spermatozoa for each fraction.

Acrosome reaction

Acrosome reaction was determined with the use of the FlouAcro protocol described by Microptic (20). Fractions (n=35) were divided into positive and negative controls and incubated at 37ºC for 3 hours in 1 ml of preheated (37ºC) capacitating media. Negative controls were treated with 10 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich, Cape Town, South Africa) and positive controls with 10 µl of 1 mM Ca-ionophore. Samples were left to incubate for 15 minutes, after which reactions were terminated with 100 µl of 70% ethanol. Two 5 µl drops of each suspension were

placed on clean slides and left to air dry before fixation in 95% ethanol (United Scientific, Cape Town, South Africa) at 4ºC for 30 minutes. Fixed spermatozoa were stained in a dark room for 30-40 minutes with 80 µl of fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Sigma-Aldrich, Cape Town, South Africa) on each drop. Slides were dipped twice in dH_2O to remove excess stain and subsequently counterstained for 7 minutes with 5 µl Hoechst (H33258, Sigma-Aldrich, Cape Town, South Africa), followed by destaining in dH_2O. Acrosome reaction of at least 100 spermatozoa per fraction were manually assessed as described in section 2.8.

Chromatin maturity and fragmentation

Chromatin maturity and fragmentation were determined following the aniline and toluidine blue protocols proposed by Erenpreisa et al. (24) and Erenpreiss et al. (25). For the assessment of chromatin maturity, dried smears were fixed at RT for 30 minutes in 4% formalin and rinsed in dH_2O. Fixed smears were stained for 5 minutes in 5% aniline blue, and excess stain rinsed off in dH_2O. Smears were subsequently stained in 0.5% eosin for 1 minutes, then rinsed in dH2O and left to air dry. For the assessment of chromatin fragmentation, dried slides were fixed in 96% ethanol-acetone (1:1) at 4ºC for 1 hour, then hydrolyzed in 0.1 N HCl at 4ºC for 5 minutes, and finally rinsed in dH_2O. Smears were subsequently stained for 5 minutes in 0.05% toluidine blue at RT, briefly rinsed in dH_2O and left to dry. After mounting with DPX mounting medium and coverslips, slides were viewed using the same equipment as described for sperm morphology. The percentages of spermatozoa with respectively immature chromatin and fragmented chromatin were manually assessed for at least 100 spermatozoa per fraction (n=20).

Statistical analysis

MedCalc statistical software version 14.8.1 (Mariakerke, Gent, Belgium) was used to calculate basic summary statistics and results were expressed as mean ± standard deviation in all the tables. The D'Agostino Pearson test was used to evaluate the distribution of the data, where after the Student's t test or the Mann-Whitney test was used to compare fractions. Where applicable, one-way analysis of variance (ANOVA) for parametric distributions or the Kruskal-Wallis test for non-parametric distributions was used to compare fractions, time points and treatments. Significance was determined at a level of $P<0.05$. Tables and radar plots were constructed with the use of Microsoft Office Excel™ 2016 (Microsoft Corporation, Redmond, Washington, United States). Additional analyses such as correlation coefficients and multivariate visualisations were performed with Statgraphics® Centurion XVII (Statgraphics Technologies, Inc.) to create Star glyphs and Andrews plots. Multivariate graphs provide additional tools for detecting patterns between cases when data sets are too large for standard scatterplots. The star glyph is a representation of each quantitative variable, and the direction and size of the polygon is accordingly scaled to the values of individual selected semen samples. Semen samples with similar characteristics will thus have star glyphs with a similar size and shape. Andrews plots assist in determining small differences between large data sets, thereby highlighting possible differences between cases with similar values. Multiple regression analysis and principal component analysis were further executed with the use of STATISTICA, version 10 (StatSoft Inc.).

Results

Standard semen analysis

Standard semen analysis parameters of the donor semen samples used in this investigation are displayed in Table S1 (See Supplementary Online Information at www.ijfs.ir). Average standard semen analysis parameter values predominantly fell above the lower reference limits as recommended by the WHO laboratory manual; however, with the exclusion of progressive motility (16).

Motility and kinematic parameters

Compared to LM fractions, values for recovered HM fractions were on average four to six times greater for percentage total motility, progressive motility, rapid progressive motility, medium progressive motility, rapid-, medium - and slow - swimming spermatozoa ($P<0.001$, Fig.1A). As seen in Figure 1B, HM fractions displayed higher values for average kinematic parameters in contrast to the LM fractions-with significant differences seen for VCL ($P<0.001$), VSL ($P=0.01$), VAP ($P<0.001$) and ALH ($P<0.001$). Furthermore, HM sperm fractions had significantly higher values for slow progressivity and medium speed group kinematic parameters (Table S2, See Supplementary Online Information at www.ijfs.ir), namely VCL ($P=0.001$), VAP ($P=0.002$) and ALH ($P=0.01$) of the medium speed group and for VAP ($P=0.01$), VSL ($P=0.03$), LIN ($P=0.03$) and WOB ($P=0.03$) of the slow progressivity group. In contrast, LM fractions obtained significantly higher values for medium speed STR ($P=0.01$) compared to the HM fraction.

Figure 2 illustrates star-glyphs of the two separated sperm fractions from individual semen samples. Each individual star-glyph was constructed using 12 kinematic parameters as indicated in the graph key below. While distinct differences between sperm fractions remain evident - star-glyph plots assist in visualizing similarities/differences between fractions of individual semen samples and within a single fraction group. HM sperm fractions illustrate similarities in star-glyph patterns amongst individual semen samples, whereas LM sperm fractions displayed a more heterogeneous pattern, indicating greater variability in kinematic parameter values within LM sperm fractions as compared to HM sperm fractions. Furthermore, Figure 2 displays the variability between fractions prepared from individual ejaculates. For example, substantial differences in the kinematic parameter values can be observed for the two fractions of semen samples 11 (S11), 14 (S14) and 24 (S24). In contrast, some semen samples illustrated kinematic parameter values that

were largely similar between the two sperm fractions, e.g. sample 3 (S3) where both had low values or sample 16 (S16) where both had high values.

Fig.1: Radar plot of the mean sperm motility and average kinematic parameter measurements for comparison of LM (red line) and HM (blue line) sperm fractions (n=35). **A.** Motility parameter comparison of LM (red line) and HM (blue line) sperm fractions. **B.** Comparison of sperm average motility kinematic measurements for LM (red line) and HM (blue line) sperm fractions. ALH; Amplitude of lateral head displacement, BCF; Beat cross frequency, HM; High motile, LIN; Linearity, LM; Low motile, STR; Straightness, VAP; Average path velocity, VCL; Curvilinear velocity, VSL; Straight-line velocity, WOB; Wobble. Values labelled with an asterisk were significantly different between the two sperm fractions for individual parameters (*; P<0.05 and **; P<0.001).

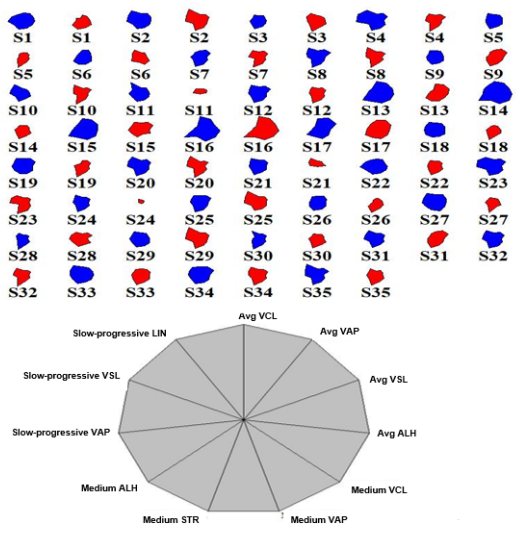

Fig.2: Star-glyph plots for comparison of sperm kinematic characteristics of two fractions separated from individual donor semen samples (n=35) for 12 input parameters (see key above). Data for each parameter was scaled by subtracting its minimum value amongst all the cases and dividing by the range. ALH; Amplitude of lateral head displacement, Avg; Average, LIN; Linearity, S#; Individual semen sample, STR; Straightness, VAP; Average path velocity, VCL; Curvilinear velocity, and VSL; Straight-line velocity.

Hyperactivation

After exposure to 5 mM caffeine, 2 mM procaine, capacitating HTF and non-capacitating HTF medium-significant differences between fractions were presented for each medium and time point, with HM fractions yielding significantly higher mean percentages compared to LM fractions (Fig.3A). No significant difference was apparent among the mediums at individual time points; however, significant differences in percentage sperm hyperactivation were observed when comparing different time intervals for individual mediums. Fractions generally exhibited significant reductions in percentage hyperactivation at 60 minutes compared to 5, 15 and 30 minutes for all the hyperactivation inducing mediums (5 mM caffeine, 2 mM procaine and capacitating HTF). A significant reduction was further observed at 45 minutes compared to 15 minutes for 2 mM procaine in the LM fraction (P=0.003) and 5 mM caffeine in both fractions (LM fraction: P=0.001, HM fraction: P=0.01). In contrast, sperm hyperactivation was significantly higher at 45 minutes compared to 60 minutes for capacitating HTF media in the LM fraction (P=0.002).

Pooled data of all mediums was used to determine the effect of time on percentage hyperactivation for each fraction as illustrated in Figure 3B. Fractions displayed a non-significant increase in percentage hyper activation between the first two time points followed by a steady decrease up to the 60 minutes. However, for HM fractions, the decrease in percentage hyper activation was significant for each time interval from 15 minutes to 60 minutes, whereas for LM fractions the decrease was more gradual. From these results, it is evident that sperm hyper activation should be measured after 15 minutes of exposure to the media.

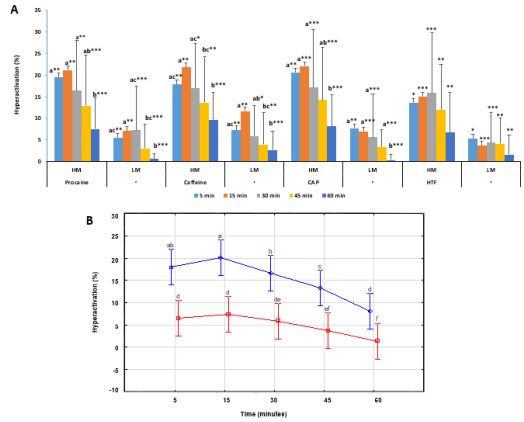

Fig.3: Comparisons of hyperactivation between the LM and HM sperm fractions at different time points and induced with different hyperactivating mediums (n=20). **A.** Bar graph illustrating the mean effect of different hyperactivating mediums (HTF, CAP, procaine and caffeine) on the HM and LM fractions at different time points (5, 15, 30, 45 and 60 minutes). **B.** The effect of time on mean percentage sperm hyperactivation for two sperm fractions separated from individual semen samples. Vertical bars denote SD in Figure 3A and 0.95 confidence intervals in Figure 3B. Time points labelled with different superscript letters (a, b, c, d, e and f) were significantly different (P<0.01) in Figure 3B, whereas time points labelled with different superscript letters (a, b and c) were significantly different (P<0.01) in individual fractions and mediums in Figure 3A. Corresponding bars labelled with an asterisk were significantly different between HM and LM fractions (*; P<0.05, **; P<0.01, ***; P<0.001). CAP; Non-capacitating HTF, HM; High motile, HTF; Human tubal fluid, and LM; Low motile.

Additional structural and functional characteristics

As illustrated in Figure 4A, HM sperm fractions contained significantly greater mean percentages of mature and viable spermatozoa with intact chromatin and MMP and responded significantly better to Ca-ionophore for induced AR (P<0.001). LM sperm fractions had significantly higher mean percentages of spermatozoa containing ROS and spontaneous acrosome reaction (AR-DMSO) - thereby resulting in significantly lower ARIC percentages compared to HM sperm fractions (P<0.001), however still remaining above the abnormal value (ARIC<15%) as recommended by WHO (16).

Figure 4B illustrates an Andrews plot constucted from data of the two fractions of individual semen samples. Each indiviudal line represents a sepecific fraction from a single sample, construscted from the data pertaining to the percentage vitality, ARIC, normal chromatin structure, mature spermatozoa, positive ROS and intact MMP (Table S3, See Supplementary Online Information at www.ijfs.ir). Distinct differences between the HM (blue lines) and LM sperm fractions (red lines) for each semen sample are confirmed in Figure 4B by clear separations of the blue and red lines (label A), thereby illustating the presence of the two distinct groups (subpopulations). In other areas of the plot (label B), the red and blue lines are dispersed from one another and lacking uniformity. This is an indication of how individual data for certain parameters varied among sample fractions; however, the LM sperm fraction (red lines) display larger variation in results compared to the HM sperm fraction (blue lines).

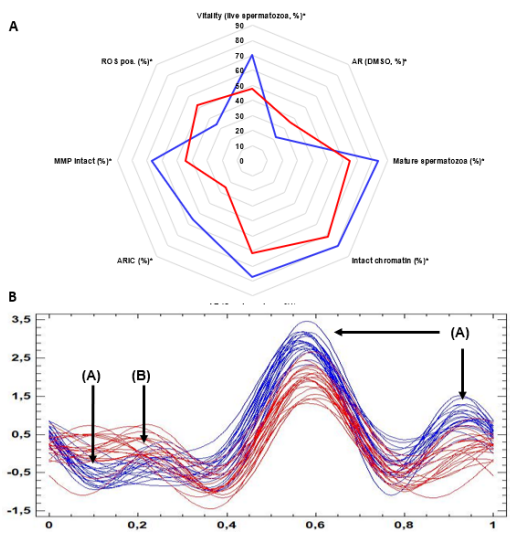

Fig.4: Comparison of the structural and functional characteristics between the HM (blue line) and LM (red line) sperm fractions. **A.** Radar plot comparing HM (blue line) and LM (red line) sperm fractions mean percentage for AR (n=35), viable (n=35) and mature spermatozoa (n=20), spermatozoa with normal chromatin (n=20), positive for ROS (n=20) and intact MMP (n=20). **B.** Andrews plot for comparison of sperm structural and functional characteristics of two fractions separated from individual donor semen samples and constructed using six input parameters (Table S3): percentage vitality, percentage AR, mature spermatozoa, normal chromatin, positive ROS and intact MMP. Data for each parameter was scaled by subtracting its minimum value amongst all the cases and dividing by the range. Values labelled with an asterisk were significantly different between the two sperm fractions for individual parameters (*; P<0.05). AR; Acrosome reaction, ARIC; Acrosome reaction to Ca-ionophore challenge, DMSO; Dimethyl sulfoxide, HM; High motile, LM; Low motile, MMP; Mitochondrial membrane potential, and ROS; Reactive oxygen species.

Correlation and multiple regression analysis of semen characteristics with two sperm motility fractions

Functional and structural parameters of the two sperm motility fractions were correlated with its initial semen parameters as shown in Table 1. Fraction parameters were arranged into two main groups based on the sperm motility and structure. Sub-groups for sperm motility comprised of the motility and kinematics for different speed groups (average, rapid, medium and slow) in addition to hyperactivation, whereas sperm structure comprised of percentage intact MMP, positive ROS, ARIC and immature spermatozoa. Kinematic parameters were further grouped into velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, DANCE, BCF and ALH) categories. Hyperactivation included both control (non-capacitating HTF) and induced (5 mM caffeine, 2 mM procaine and capacitating HTF) percentages. It should be noted that correlations were calculated for 20 semen parameters and 96 sperm fraction parameters, but that data presented in Table 1 only indicate significant (P<0.05) correlations where r≥0.40.

Semen parameters appeared to correlate more with the HM sperm fractions' grouped motility parameters, whereas correlations between the semen and LM sperm fraction parameters appeared to be equally dispersed in both motile and structural grouped parameters. Furthermore, majority of the sperm morphology parameters seemed to largely correlate (mostly negative) with the LM fractions' parameters, whereas semen viscosity appeared to correlate (majority positive) with majority of the HM fractions' parameters. Taking all correlations into account, it seems that a positive relationship exists among total sperm motility, viscosity of semen and fractionated sperm with high motility and swimming speeds in addition to hyperactivation capabilities. On the other hand, sperm morphology defects in semen samples were indicative of sperm fractions with less motile and immature sperm, less potential for hyper activation and acrosome reaction, lower MMP and more ROS.

Illustrated in Table 2 is the R-squared (R2) and beta coefficients (b^*) from the multiple regression analysis used to determine the relationship between four groups of semen parameters (20 predictor variables) and the HM sperm fraction's motility and structural parameters (96 dependent variables). Group I comprised of semen concentration and volume, group II of total motility, semen viscosity, MPT and progressive motility, group III of semen vitality, normal chromatin integrity and mature spermatozoa, and Group IV of semen morphology parameters which were further split into two sub-groups due to multicollinearity (inter-correlations amongst the independent variables which could result in a disturbance of the data) between parameters. Motility and structural parameters of the HM fraction were further grouped and categorised as previously mentioned in Table 1. Data from semen groups with significant (P<0.05) predictor variables and beta coefficients with $b^* \geq 0.40$ were presented in Table 2. Due to incomplete cases in results of the LM fraction, multiple regression analysis was only preformed on the HM sperm fraction.

Table 1: Significant (P<0.05) correlations (r≥0.40) between conventional standard semen parameters of whole semen and sperm structural and functional arameters of density-gradient centrifugation separated sperm fractions (high motile and low motile)

	Motility grouped parameters																		Structure grouped parameters		
	Motility sub-group					Kinematics sub-group									HA sub-group						
							Avg		Rapid			Medium			Slow						
Semen characteristics and fraction	Total	Progressive	Non-progressive	Rapid	Medium	Velocity	Linear	Vigour	Velocity	Vigour	Velocity	Linear	Vigour	Velocity	HTF	Induced	MMP	ROS	ARIC	Immature
Volume																				
HM																				
LM					-0.46															
pH																				
HM																			-0.49	
LM																				
Conc.																				
HM												0.41				-0.49				
LM																				
MPT																				
HM																-0.49				
LM				-0.43																
Visc																				
HM	0.47	0.69	-0.43	0.69		0.61		0.54	0.51	0.52	0.58	-0.45	0.55		0.53	0.75				
LM				0.43					0.62	0.57						0.48	0.61		-0.47	
Tot. mot																				
HM															0.53	0.50				-0.60
LM																			-0.42	
Prog mot																				
HM																				
LM										0.55										
Normal morph																				
HM																				
LM			-0.40			0.46	0.51							0.40			-0.46			
Normal acro																				
HM															0.45					
LM																0.50				
Head defects																				
HM																0.49				
LM														-0.46			0.52			
Midpiece defects																				
HM												0.41								
LM																				
Tail defects																				
HM																				
LM					0.44										-0.53					
Cytopl droplets										-0.64						-0.75			0.44	
HM																				
LM												0.41		-0.40						
TZI	-0.45			-0.40				-0.40								-0.52		0.52		
HM																				
LM																				
DI	-0.43	-0.45		-0.40		-0.42		-0.40							-0.63	-0.51				0.49
HM																				
LM																				
MAI							-0.40							-0.42						
HM																				
LM																-0.48				
							-0.41													

ARIC; Acrosome reaction to Ca-ionophore challenge, Avg; Average, Conc; Concentration, Cytopl droplets; Cytoplasmic droplets, DI; Deformity index, HA; Hyperactivation, HM; High motile fraction, HTF; Human tubal fluid, LM; Low motile fraction, MAI; Multiple abnormalities index, MMP; Intact mitochondrial membrane potential, MPT; Mucous penetration test, Normal acro; Normal acrosome, Normal morph; Normal morphology, Prog mot; Progressive motility, ROS; Reactive oxygen species, Tot. mot; Total motility, TZI; Tetrazoospermic index, VCL; Curvilinear velocity, Visc; Viscosity, and VSL; Sraight-line velocity.

Table 2: Significant (P <0.05 and P <0.01) beta coefficients (b*) ≤0.40 and R-squared (R2) values from the multiple linear regression analysis used to determine the relationship between four groups of semen parameter parameters (20 predictor variables) and the HM sperm fraction's motility and structural grouped parameters (96 dependent variables)

Basic semen parameter groups			Motility grouped parameters									Structure grouped parameters		
			Motility sub-group							HA sub-group				
			Total	Prog	Nprog	Rapid	Medium	Slow	Rprog	HTF	Induced	IM	Mature	MMP
Group I	R										0.53			
	R2										0.28			
	b*	Conc									-0.47			
		Vol												
Group II	R		0.60	0.65	0.51	0.74	0.60		0.62	0.68	0.83	0.71	0.75	
	R2		0.36**	0.43**	0.26	0.55**	0.36**		0.39**	0.46*	0.69**	0.5*	0.57**	
	b*	TM										-0.64	0.69	
		Visc	0.48	0.67	-0.52	0.76	-0.57		0.60	0.98	1.14			
		MP												
		PM								-0.62	-0.69			
Group IV (A)	R		0.62				0.68				0.85			0.74
	R2		0.38				0.46*				0.72*			0.54
	b*	Head def					0.42				0.97			
		Midpiece def					0.62							
		Tail def	-0.42				0.44							
		Cytopl droplets												
		MAI					-0.57							
		Micro												
		Macro												1.08
		Norm acro	-0.56								0.78			
Group IV (B)	R			0.54		0.53	0.67	0.58			0.82			
	R2			0.30		0.28	0.45*	0.33			0.68*			
	b*	Norm. morph					-0.76							
		Tail def						-0.52						
		Cytopl droplets									0.47			
		TZI		0.73		-0.54	0.51	0.69			-0.99			
		DI		-0.82			-0.69							
		Micro												
		Macro												
		Norm acro												

Table 2: Continued

Basic semen parameter groups			Kinematics sub-group															
			Avg			Rapid		Medium			Slow			Rap prog			Mprog	Sprog
			Velocity	Vigour	Velocity	Linear	Vigour	Velocity	Linear	Vigour	Velocity	Linear	Vigour	Velocity	Linear	Vigour	Vigour	Linear
Group I	R																	
	R2																	
	b*	Conc																
		Vol																
Group II	R		0.84	0.80	0.78	0.49	0.68	0.81	0.64	0.80	0.45	0.45	0.57	0.80		0.73		0.44
	R2		0.70**	0.65**	0.62**	0.24	0.46**	0.66**	0.41**	0.65**	0.20	0.20	0.33*	0.63**		0.53**		0.19
	b*	TM				-0.45												
		Visc	0.87	0.83	0.84		0.68	0.84	-0.51	0.80	-0.44	-0.42	-0.50	0.82		0.72		-0.43
		MP																
		PM																
Group IV (A)	R			0.44		0.55				0.57					0.51			
	R2			0.19		0.30				0.32					0.26			
	b*	Head def																
		Midpiece def								0.53								
		Tail def		-0.48														
		Cytopl droplets																
		MAI													0.78			
		Micro																
		Macro																
		Norm acro				0.48												
Group IV (B)	R			0.66		0.61	0.55										0.57	
	R2			0.43*		0.37	0.30										0.33	
	b*	Norm. morph		-0.60													-0.70	
		Tail def																
		Cytopl droplets																
		TZI																
		DI																
		Micro				-0.64												
		Macro				-0.74												
		Norm acro		0.45		0.54	0.48											

Semen parameter groups comprised of: (group I) - concentration and volume; (group II) - total motility, semen viscosity, mucus penetration and progressive motility and (group IV) - semen morphology parameters, which were further split into two sub-groups [groups IV (A) and (B)] due to multicollinearity between parameters. HM sperm fraction structural and functional sperm parameters are grouped and categorised into motility and structural groups. Motility groups include the different motility parameters, kinematics for different speed and progressivity groups (average, rapid, medium, slow and rapid -, medium - and slow-progressive) and hyperactivation as HTF (non-capacitating HTF) and induced (5 mM caffeine, 2 mM procaine and capacitating HTF). Kinematics are further categorized into velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, DANCE, BCF and ALH) groups and the structural group into the percentage intact MMP, vitality, immature and mature spermatozoa. Group (II) of the semen parameter groups had the best predictive value towards the HM sperm fractions structural and functional parameters, with semen viscosity contributing the most that model. Significant values indicated in bold as *P<0.05 and **P<0.01. ALH; Amplitude of lateral head displacement, Avg; Average, BCF; Beat cross frequency, Conc; Concentration, Cytopl droplets; Cytoplasmic droplets, DI; Deformity index, HA; Hyperactivation, Head def; Head defect, HM; High motile fraction, HTF; Human tubal fluid, IM; Immature, LIN; Linearity, LM; Low motile fraction, MAI; Multiple anomalies index, Mprog; Medium progressive, Midpiece def; Midpiece defect, MMP; Intact mitochondrial membrane potential, MP; Mucous penetration, Nprog; Non-progressive motility, Norm. acro; Normal acrosome, Norm. morph; Normal morphology, PM; Progressive motility, Prog; Progressive, Rprog; Rapid progressive, ROS; Reactive oxygen species, Sprog; Slow progressive, STR; Straightness, Tail def; Tail defect, TM; Total motility, TZI; Teratozoospermic index, VAP; Average path velocity, VCL; Curvilinear velocity, Visc; Viscosity, Vol; Volume, VSL; Straight-line velocity, WOB; Wobble, R2; R-squared, and b*; Beta coefficients.

Compared to semen groups I and IV (A) and (B), semen group II (total motility, semen viscosity, MPT and progressive motility) had majority R-squared values range above 0.50 for both structural and motility parameters of the HM fraction. However, significance was only seen for percentage rapid swimming spermatozoa, velocity and vigour kinematic parameters of average rapid, medium, and rapid progressive speeds, induced hyperactivation and immature spermatozoa. Consequently, the semen total motility, viscosity, MPT and progressive motility could therefore account for more than 50% of the variation seen in the above-mentioned parameters together. In terms of beta coefficients, semen viscosity had the most impact (positive) on the previously mentioned HM fraction parameters, whereas total motility had the most impact on immature spermatozoa in the HM fraction.

In groups IV (A) and IV (B), R-squared values for morphology parameters ranged above 0.5 for induced hyperactivation and intact MMP; however, significance was only seen for induced hyperactivation. Together, semen morphology parameters can therefore account for more than 50% of the variability seen in induced hyperactivation results of the HM fraction. Beta coefficients of the sperm head defects and normal acrosome in group IV (A), both appeared to have the most impact on induced hyperactivation, whereas in group IV (B), TZI and cytoplasmic droplets had the most impact.

Discussion

The present study evaluated an extensive set of functional and structural parameters of two sperm motility fractions (HM and LM) generated via double DGC of healthy donor semen samples. By collectively comparing a large number [96] of diverse characteristics between motility fractions, and using a new approach of grouping variables to correlate fraction's results to the standard semen analysis, the study provides possible insights into a select group of semen characteristics that could improve its predictive value on the quality of HM sperm subpopulations. Our results indicate significantly enhanced functional and structural characteristics in HM fractions compared to LM fractions. Grouping of various semen characteristics further revealed which of these characteristics relate to a specific fraction (HM or LM), as well as to a specific group of functional or structural variables in that fraction.

Greater values of viable, mature, motile, and morphologically normal spermatozoa have been isolated in HM fractions when compared to neat seam samples (4). Furthermore, several studies have compared the functionality of human sperm subpopulations but were limited in the number of functional and structural parameters investigated. Nonetheless, the latter studies reported higher motility and kinematic parameters in HM fractions compared to LM fractions for both human and bull semen, thereby agreeing with our motility and kinematic parameter findings (8-10). Although average total motility of the HM fraction falls below 90%, it is of note that donor semen was selected to have a non-biased reflection on the functionality of sperm subpopulations in various semen qualities (26). Despite having a large variation in values (range: 40.0-85.5%), total motility in the HM fraction remained than the LM fraction motility range (range: 1.1-3.5.4%). Additionally, we found significantly higher percentages of viable, mature spermatozoa with intact MMP, normal chromatin integrity and ARIC within the HM fraction compared to the LM fraction, which correspond to reports of sperm subpopulations with increased MMP containing more morphologically normal and motile sperm which respond better to induced AR (27-29).

The LM fraction presented with significantly higher levels of immature spermatozoa with positive ROS and abnormal chromatin integrity; all parameters thought to be key etiological causes in idiopathic male infertility (8, 30). Also, previous studies observed lower percentages of mature, motile, morphologically normal spermatozoa with intact chromatin integrity as well as more DNA damage and ROS in LM fractions compared to HM fractions of both donor and patient samples (8). High ROS levels, as documented in semen samples of infertile men, and are known to impair sperm viability and mitochondrial respiration, as well as increase DNA damage and lipid peroxidation (8, 30, 31). The resulting loss of sperm motility and membrane integrity, consequently impair the fusion with the oocyte (8, 30). Considering LM fractions consisted of significantly higher numbers of immature and ROS positive spermatozoa, it is likely such factors contributed to decreased motility, viability, MMP intactness and normal chromatin integrity that our study observed within this fraction. Separation and removal of sperm fractions with elevated levels of immature, ROS positive spermatozoa should thus improve sperm motility and help maintain membrane and chromatin integrity (30).

Higher concentrations of essential proteins involved in sperm functionality and spermatogenesis have been reported in HM fractions, whereas LM fractions consistently presented with alterations and gene expression profiles significantly associated with male infertility (28, 29, 32). These observations were also found to closely coincide with those seen in normospermic samples compared to asthenozoospermic samples (33). Considering the essential function of a selection of these proteins in permitting spermatozoa to undergo capacitation, it is conceivable that we observed significantly higher percentages of induced AR and hyperactivation within the HM fraction compared to the LM fraction (29). We admit that the hyperactivation values presented may be underestimated, as Mortimer et al. (34) have shown that by using VCL and the D-fractal, more accurate hyperactivation percentages can be obtained. However, using the flush technique in a Leja chamber seems to induce maximum hyperactivation of sperm in a more reasonable or accelerated time period to be employed in the clinical laboratory as compared to the traditional swim up technique (23). The LM fraction notably presented with similar structural and functional complications to what have been found in sub-fertile semen samples in terms of levels of immature spermatozoa, ROS, motility parameters hyperactivation and DNA fragmentation (30, 31). Additionally, normozoospermic samples tend to be more homogeneous compared to heterogeneous asthenozoospermic and sub-fertile samples, which closely corresponds to our observations that the HM fraction displayed more homogeneity in sperm functional and structural parameters compared to the LM fraction (32).

According to WHO guidelines (16), clinical human semen analysis is considered an important initial test in evaluating the quality of semen samples and is partly based on the assessment of sperm viability, morphology, concentration and motility (35). Sperm motility is frequently utilized for assessment of semen quality due to its general positive correlation with sperm MMP, concentration, viability and fertilising capacity, while progressive motility is suggested as the most important motility percentage to evaluate in ART programs (2, 3, 36). Nevertheless, it is suggested that more detailed analyses based on distinct sperm subpopulations may disclose other motility parameters and patterns, apart from progressive motility, as good predictors of male fertility (3, 37).

It is clear from our results that grouping of semen total motility, progressive motility, viscosity and MPT presents with higher predictive value towards HM fraction motility and structural grouped parameters in comparison to grouped semen morphology parameters. Interestingly, from this group-semen viscosity had the greatest predictive power towards the HM fraction's grouped motility parameters. Normal semen viscosity plays a critical role in sperm function by facilitating the entry of spermatozoa into cervical mucus, maintaining sperm swimming speed after MPT and preserving chromatin integrity of spermatozoa (38). However, whether the MPT could be a function of the viscosity of the semen, or alternatively a function of the spermatozoa remains difficult to explain as we used the centipoise Leja viscosity test and had unclear results from the correlations (19).

The intrinsic value of combining specific semen parameters into four groups, rather than considering individual semen parameters when assessing relationships with each fraction's sperm parameters should be highlighted. For example, on its own the percentage total motility of the standard semen analysis only had a positive correlation with the motility group of the HM fraction-more specifically the hyper activation subgroup. Interestingly, progressive motility from the basic semen analysis had a positive correlation with the LM fraction's rapid vigour kinematic parameters, but no correlation with the HM fraction. Furthermore; the various semen morphology abnormalities presented with numerous correlations to the LM fraction's lower motility and kinematic parameters, immature spermatozoa, positive ROS and reduced MMP. The strong correlations we found between the semen morphology abnormalities and the LM fraction, in addition to the heterogeneous trend of characteristics within this fraction are likely an attribute of the varying degrees of altered proteins which affect spermatogenesis and ultimately sperm structure and function (28, 29, 32).

Considering our results mentioned above as well as the strong correlations found between the percentage of diverse subpopulations and sperm quality and fertility, the predictive value of basic semen parameters should be re-evaluated (1, 2). For instance, if a high concentration of spermatozoa typical of the LM fraction exists in a semen sample, this can result in the standard semen analysis reflecting the quality of LM fraction, despite the presence of a significantly improved HM fraction that can be separated. Ultimately, such a scenario will result in a skewed semen analysis and subsequent fertility diagnosis. In contrast, if a semen sample contains a high concentration of HM-type spermatozoa, the semen morphology analysis will probably not bear much significance to the functional capabilities of the major sperm population. Similarly, Agarwal and colleagues showed that nine semen characteristics can be grouped and reduced to two scores (semen quality and relative semen quality scores) by principal component analysis, thereby providing a reliable alternative for the prediction of ART outcome in couples with male-factor or idiopathic infertility (39). It is important to note that despite isolating good quality spermatozoa for ART purposes, swim-up protocols result in recovery of motile spermatozoa, whereas DGC separation does not necessarily depend on motile spermatozoa and thus may be utilized for asthenozoospermic samples (40). These findings thereby substantiate the significant role that sperm subpopulations play in factors contributing to male infertility, indicating that even though a semen parameter may fall below the reference value as recommend by WHO (16), possibility of normal fertility should not be excluded as indicated by the different results of the both fractions' functional and structural parameters. As such, the evaluation of subpopulations present in an ejaculate can further assist in the selection of the most suitable treatment or management course to address fertility issues in individual patients or couples.

Conclusion

Our study confirms that spermatozoa of the HM fraction have enhanced functional and structural sperm parameters and display a more homogenous pattern in results amongst individual samples, thereby closely mimicking the functionality and quality of a potentially fertile semen samples. In contrast, separated LM fractions, marked by significantly lower sperm functionality, display more heterogeneous patterns amongst individual sample results and closely mimic functionality and quality of a sub-fertile semen samples. We therefore propose that neat semen samples be separated into sperm subpopulations for both clinical and research purposes. Quantification of functional and structural sperm characteristics for individual fractions may provide more accurate reflections of sperm and semen quality and improve the prediction and diagnosis of the fertilization potential of the whole ejaculate, especially in sub-fertile semen cases. Such sperm fractions can further be utilized as a potential research model of sperm physiology for investigating "fertile" and "sub-fertile" samples. In future, such a model should be utilized to investigate how spermatozoa from different subpopulations respond to various treatments and subsequently could provide insights on how to improve the functionality of sub-fertile semen samples to approximate that of fertile semen samples. Finally, when focusing on an individual semen trait as a possible predictor for male fertility, such as progressive motility, this may result in an over- or underestimated prediction. In contrast, using a combined group of related semen traits may elude more information into a specific group and even sub-group of the functional and structural variables of either sperm motility fraction relating to fertility. The grouped combinations of traits may compensate for an individual trait of poor quality, thereby producing more accurate estimations of overall functional quality of the spermatozoa.

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Authors' Contributions

L.M., S.K., G.v.d.H.; Conceptualization, design, methodology, and software. S.K., L.M.; Validation, visualization, project administration, and funding acquisition. S.K.; Formal analysis, investigation, data acquisition, interpretation, data curation, and writing-original draft preparation. L.M., G.v.d.H.; Resources, writing-review and editing, and supervision. All authors read and approved the final manuscript.

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Original Article

Unilateral Kidney Agenesis and other Kidney Anomalies in Infertile Men with Congenital Bilateral Absence of Vas deferens: A Cross-Sectional Study

Fattaneh Pahlavan, M.Sc.[1], Fatemeh Niknejad, B.Sc.[1], Hesamoddin Sajadi, M.D.[2], Ahmad Vosough Taghi Dizaj, M.D.[1*]

1. Department of Reproductive Imaging, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
2. Department of Andrology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

Abstract

Background: We aim to determine the prevalence of renal anomalies in patients with congenital vas deferens agenesis referred for infertility assessment.

Materials and Methods: This cross-sectional study was carried out on eligible infertile men from 2016 to 2019. Infertile men who were suspected of obstructive azoospermia were referred to the Ultrasound ward and they were examined by abdominal ultrasound for detecting the genital and kidney anomalies. An informed consent form was filled out by patients. Data was entered into SPSS software 21. Patients were divided into two groups in terms of congenital bilateral absence of vas deferens (CBAVD) or congenital unilateral absence of the vas deferens (CUAVD). Using the Chi-square test kidney anomalies between groups were compared. The $P<0.05$ was considered significant.

Results: The mean age of participants was 33.05 ± 6.35. The frequency of CBAVD was 66 and the frequency of left side VD and right side VD were 23 and 21, respectively. The percentage of other comorbidities was calculated. Out of 110 cases, 12 (11%) men had coexistence of vas deferens and kidney agenesis. Other studies are in agreement with our findings. Although the percentage of CBAVD and CUAVD were 9.1% and 1.8% respectively, the difference was not significant (P=0.07).

Conclusion: Considering the fact that kidney agenesis is a remarkable congenital anomaly that coexists with the majority of vas deferens agenesis cases and could not be detected by routine laboratory tests or transrectal ultrasound examination, it should be ruled out with transabdominal ultrasound examination after detection of vas deferens agenesis.

Keywords: Azoospermia, Congenital Absence of the Vas Deferens, Imaging, Kidney Anomalies

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Introduction

Infertility is said to affect 15% of the population of reproductive age who intends to become pregnant. In 50% of cases, infertility stems from male factors (1). Azoospermia which is responsible for 15% of all male infertility can be categorized into obstructive and non-obstructive azoospermia. In obstructive azoospermia, hormones and spermatogenesis are normal and the obstruction can be seen in different parts of the rete testis (2).

The vas deferens duct follows the epididymis and then shapes the ejaculatory duct (3). The vas deferens agenesis -congenital bilateral absence of the vas deferens (CBAVD) and congenital unilateral absence of the vas deferens (CUAVD) - is a rare anomaly that contributes to azoospermia and infertility (4).

Genital anomalies are seen along with urological anomalies (5). Congenital renal agenesis, for instance, is seen along with the vas deferens anomaly (6, 7). The direct effects, the side effects, and the repercussions of the urogenital anomalies have devastating impacts in the reproductive years, especially in men.

Different studies were carried out to estimate the prevalence of these comorbidities since their relationships and their prevalence are helpful for early detection, management, and timely treatment. No study has examined the prevalence of coexistence of the renal anomalies and vas deferens anomaly in Iran. Hence, in the present study, we aim to determine the prevalence of renal anomalies in patients with the congenital vas deferens agenesis referred for infertility assessment.

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***Corresponding Address: P.O. Box: 16635-148, Department of Reproductive Imaging, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran**
Email: vosough@royaninstitute.org

Materials and Methods

Participants

This was a cross-sectional study that was carried out from 2016 to 2019 on infertile men patients. To be eligible for this study, men patients had to be 18-45 years of age, infertile, and suspected of obstructive azoospermia. Exclusion criteria were insufficient medical history, incomplete file, and lack of incorporation of patients. Ultimately, a total of 125 patients who met the criteria were entered into the study.

Procedures

Main medical and demographic information and history of the patients were assessed by reviewing the patient's files. The physical examination was done in patients who were suspected of obstructive azoospermia. Ultrasound examination was performed and confirmed the vas deferens agenesis. Other genital anomalies and coexisted renal anomalies (e.g., renal agenesis) were assessed using abdominal ultrasound and recorded.

Ethics approval and consent to participate

All procedures performed in the study were in accordance with the ethical standards of the institutional committee (IR.ACECR.Royan.REC.1400.036). The written informed consent form was obtained from all individual participants.

Measures

A demographic questionnaire was used for describing the sample and basic variables (e.g., age, education etc.) and was filled out by the patients. A constructed form (Yes vs. No items) was used by the medical staff; containing presence, absence, or malformations of the vas deferens, seminal vesicles, kidney, or any other kinds of urogenital anomalies.

Statistical approach

Data was entered into SPSS software (version 24, SPSS, Inc., Chicago, IL, USA). The descriptive analysis was used for detecting the central tendencies and distributions. Frequencies of the main variable, the coexistence of the vas deferens agenesis, and other anomalies were calculated. Patients were divided into two groups in terms of CBAVD or CUAVD. Using Chi-square test renal agenesis between groups was compared. The $P<0.05$ was considered significant.

Results

All 125 cases were referred to the sonography ward after physical examination. They were suspected of vas deferens agenesis. The mean age of participants was 33.05 ± 6.35. The other basic evaluable variables of CBAVD and CUAVD groups are seen in Table 1.

Table 1: General characteristics of the patients

Characteristics	Total	CBAVD	CUAVD	P value*
Number	125	66 (52.8)	44 (35.2)	
Age (Y)	33.05 ± 6.35	32.77 ± 5.04	32.39 ± 6.9	0.74
Duration of Marriage (Y)	4.74 ± 3.06	4.72 ± 3.29	4.46 ± 2.68	0.67
BMI (kg/m^2)	26.05 ± 3.7	25.74 ± 4.14	26.54 ± 3.17	0.29
Education				
Intermediate	55	27 (24.5)	22 (20)	0.23
Upper intermediate	70	39 (35.5)	22 (20)	

Data are presents as mean ± SD or n (%). *; Obtained from t student/Mann-Whitney-U test for continuous variables and Chi-square test for Categorical variables, CBAVD; Congenital bilateral absence of the vas deferens, CUAVD; Congenital unilateral absence of the vas deferens, and BMI; Body mass index.

The frequency of CBAVD was 66, the frequency of left side VD Agenesis was 23 and the frequency of right side VD Agenesis was 21 (Table 2).

Table 2: VD Agenesis

Vas deferens anomalies	Frequency	Percent
VD agenesis	110	88
CBAVD	66	52.8
CUAVD	44	35.2
Left VD agenesis	23	18.4
Right VD agenesis	21	16.8

CBAVD; Congenital bilateral absence of the vas deferens and CUAVD; Congenital unilateral absence of the vas deferens.

The frequencies of Kidney Anomalies are shown in Table 3.

Table 3: Kidney anomalies

Kidney anomalies	Frequency	Percent
Kidney agenesis	20	16
Left kidney agenesis	9	7.2
Right kidney agenesis	11	8.8
Ectopic left kidney	7	5.6
Ectopic right kidney	7	5.6
Right side small ectopic kidney	1	0. 8
Left side small ectopic kidney	1	0. 8
Compensatory hypertrophic left kidney	11	8.8
Compensatory hypertrophic right kidney	4	3.2
Hypoplasic kidney	6	4.8
Hypoplasic left kidney	1	0.8
Hypoplasic right kidney	5	4
Ectopic hypoplasic right kidney	1	0.8
Horse shoe kidney	1	0.8

Out of 110 cases, 12 (11%) men had coexistence of vas deferens and kidney agenesis. Although the coexistence of vas deferens and kidney agenesis in the CBAVD and CUAVD groups were 9.1 and 1.8% respectively, the difference was not significant (P=0.07). The data of other comorbidities are figured in Table 4.

Table 4: Coexisted VD agenesis and kidney

Kidney anomalies	Total VD	CBAVD	CUAVD	P value*
Kidney agenesis	12 (11)	10 (9.1)	2 (1.8)	0.07
Left kidney agenesis	5 (4.5)	4 (3.6)	1 (0.9)	0.33
Right kidney agenesis	7 (6.4)	6 (5.5)	1 (0.9)	0.15
Ectopic left kidney	5 (4.5)	5 (4.5)	0 (0)	0.73
Ectopic right kidney	7 (6.4)	6 (5.5)	1 (0.9)	0.15
Right side small ectopic kidney	1 (0.9)	1 (0.9)	0 (0)	0.6
Left side small ectopic kidney	0 (0)	0 (0)	0 (0)	-
Compensatory hypertrophic left kidney	4 (3.6)	3 (2.7)	1 (0.9)	0.47
Compensatory hypertrophic right kidney	0 (0)	0 (0)	0 (0)	-
Hypoplasic left kidney	0 (0)	0 (0)	0 (0)	-
Hypoplasic right kidney	2 (1.8)	1 (0.9)	1 (0.9)	0.64
Ectopic hypoplasic right kidney	1 (0.9)	1 (0.9)	0 (0)	0.6
Horse shoe kidney	1 (0.9)	1 (0.9)	0 (0)	0.6

Data are presents as n (%). *; Obtained from Chi-square test, CBAVD; Congenital bilateral absence of the vas deferens, and CUAVD; Congenital unilateral absence of the vas deferens.

Discussion

Studies have revealed that the prevalence of CBAVD in infertile men is about 1-2% and it increases in patients who are diagnosed with azoospermia (4). The clinical sign of CBAVD is a bilateral absence of the vas deferens and distal portion of the epididymis (8). In such cases, the size of the testis is normal, however, the sperm analysis and its volume are abnormal due to the seminal vesicle agenesis. Considering the fact that the hormonal profile is normal in these patients, sonographic assessment is recommended for ruling out the vas deferens and seminal vesicle agenesis (9).

To the best of our knowledge, the exact prevalence of CUAVD is unclear since its clinical features vary and the reproductive status of those men with one vas deferens duct might be normal. These conditions might be detected during vasectomy procedures or other urologic assessments. Therefore, the CUAVD needs clinical examination and other follow-ups (4, 10).

In the present study, the frequency of the vas deferens agenesis is calculated 88% which is much higher than its prevalence in the infertile men population. It is worth bearing in mind that these patients were referred to the sonography ward after precise physical examination by an experienced urologist specialist.

Being the main purpose of our study, the percentage of coexistence of vas deferens and kidney agenesis was calculated at 11%. A recent study has revealed that the prevalence of kidney agenesis in patients with vas deferens agenesis is 11.8% which is in agreement with our finding (11). Although the fact that the percentages of the kidney anomalies in the CBAVD group were higher than the CUAVD group, the differences were not significant statistically. It might be due to the sample size.

The coexistence of urogenital anomalies stems from the embryogenic development period in 4-12 weeks of pregnancy. The urologic and genital organs originate from the mesonephric duct. Any distortion in the development of the mesonephric duct results in congenital urogenital anomalies. Therefore kidney agenesis occurs with genital anomalies such as vas deferens agenesis (4).

Generally, urologic anomalies account for approximately 33% of congenital anomalies. Additionally, the prevalence of kidney agenesis is between 0.05 and 0.025% (5). This kind of rare anomaly is higher in infertile patients with vas deferens agenesis.

The kidney agenesis is detectable simply using transabdominal sonography, hence, the transabdominal sonography is recommended after detection of the vas deferens agenesis in infertile men (9).

Moreover, some kidney anomalies are said to be due to a kind of compensatory mechanism. Kidney hypertrophy is a notable example in patients who deal with unilateral kidney agenesis. Studies have revealed different prevalence for hyperplastic kidneys considering the origins and the population structure. There is no general agreement on whether this phenomenon is useful or not. Further study and long-term follow-ups are needed until the puberty period (4).

Anyway, one kind of the other malformations such as ectopic kidney might be seen. Nevertheless, the hypoplastic or dysplastic kidney is probable. In patients with hypoplastic or dysplastic kidney, other causes such as radiotherapy and artery stenosis should be considered (12).

In the present study, the percent of the hypoplastic kidney was calculated at 4.8%. The hypoplastic kidney is the most common cause of chronic kidney disease. Developmental imperfection (e.g., genetic problems and prematurity) are said to be the most source of the hypoplastic kidney anomaly and there is no specific percentage for the coexistence of the hypoplastic kidney with genital malformations (13).

Moreover, the prevalence of the horse kidney is between 0.01 and 0.25% in the literature (5). In this study the percent of 0.8% notes its increasing prevalence in infertile men with genital malformations. These findings suggest that the patients who suffer from the vas deferens agenesis should be monitored and clinical follow-ups for the kidney anomalies, blood pressure, and serum creatinine should be performed in specific cases. These people have a normal quality of life and should only be prohibited from strenuous exercise (7).

Considering the fact that vas deferens agenesis is seen in cystic fibrosis disease, infertile men with the vas deferens agenesis should be assessed in three domains including infertility, urinary tract malformation, and cystic fibrosis disease (5).

According to recent studies, in patients with the CBAVD and normal urinary tract, it is more likely that

the CBAVD is due to cystic fibrosis. So the probability of cystic fibrosis disease should be assessed (7). Albeit, in some cases both kidney agenesis and cystic fibrosis disease were detected in the CBAVD patients; so cystic fibrosis disease should be ruled out in the CBAVD patients. These urogenital malformations stem from different developmental imperfections and genetic factors, (14) hence, further assessments and pre-implantation counseling are helpful for prevention of transition to a future generation.

Lack of following up of genetic counseling before the implantation accounts for the limitation of our study. The patients in the present study were not followed up inasmuch as it was a retrospective study.

The urogenital anomalies impose an extra and great burden on the patients, their families, and the societies. Considering those worthy preparations could be implemented in this regard, a much more comprehensive study containing genetic assessment is needed. Further research with a larger sample size concordant with laboratory analysis is recommended for cutting the chain of transition to the next generation.

Conclusion

Considering the fact that renal agenesis is a remarkable congenital anomaly that coexists with the majority of vas deferens agenesis cases and could not be detected by routine laboratory tests or transrectal ultrasound examination, it should be ruled out with transabdominal ultrasound examination after detection of vas deferens agenesis.

Moreover, since the assisted reproductive technology (ART) outcomes in these cases are satisfactory, genetic counseling before implantation is necessary and the fetus should be followed up in terms of urogenital anomalies during pregnancy.

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Authors' Contributions

A.V.T.D., F.P.; Participated in study design, data collection, and evaluation. A.V.T.D.; Performed ultrasound examinations. H.S; Performed the physical examination of patients, detected vas deference agenesia, and participated in follow up the outcomes of the patients. F.N.; Contributed extensively in the interpretation of the data and the conclusion. F.P; Wrote the manuscript. All authors read and approved the final version of the manuscript.

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Original Article

Evaluation of Azoospermic Patients to Distinguish Obstructive from Non-Obstructive Azoospermia, and Necessity of Diagnostic Testis Biopsy: A Retrospective Study

Iman Shamohammadi, M.D.[1, 2], Mohammad Ali Sadighi Gilani, M.D.[1, 3*], Seyed Mohammad Kazemeyni, M.D.[1], Tara Hasanzadeh, M.D.[2], Ahmad Vosough Taqi Dizaj, M.D.[3], Alireza Dizavi, M.Sc.[3]

1. Department of Urology, Shariati Hospital, Tehran University of Medical Science, Tehran, Iran
2. Department of Urology, Nemazee Hospital, Shiraz University of Medical Science, Shiraz, Iran
3. Department of Reproductive Imaging, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

Abstract

Background: Accurate etiology of azoospermia is required for optimal management of patients. The aim of this study was the determination of serum hormonal levels and testicular long axis cut off points to distinguish obstructive azoospermia (OA) from non-OA (NOA) in Iranian patients as well as the evaluation of the necessity of diagnostic testis biopsy in azoospermic patients.

Materials and Methods: In this retrospective study, data of 471 azoospermic patients such as history and physical examination, serum hormonal level, semen fluid parameter, and testicular long axis based on ultrasound were evaluated from 2016 to 2020. All patients were examined by a single urologist and underwent a diagnostic testis biopsy for a definite diagnosis. The diagnostic parameters were analyzed using Statistical Package for the Social Sciences (SPSS) version 16 with t test and chi-square test and receiver operating characteristic (ROC) curves to distinguish NOA from OA.

Results: A total of 127 patients with OA and 284 with NOA were included in this study. The mean serum testosterone level was significantly higher in OA than NOA (4.2 vs. 3.4 ng/ml), whereas the mean serum follicular stimulating hormone (FSH, 5.3 vs. 19.1 mIU/ml) and luteinizing hormone (LH, 5.3 vs. 11 mIU/ml) were lower in OA. ROC curve analysis showed that FSH and testicular long axis were the best diagnostic predictors. Using a combination of serum FSH (8.9 mIU/ml) and testicular long axis (39 mm), the positive predictive value for NOA was 97.02% and for OA was 78.8%.

Conclusion: Combination of serum FSH higher than 8.9 mIU/ml and testicular long axis lower than 39 mm were strong predictors to distinguish NOA from OA in Iranian participants in this study. In addition, diagnostic testicular biopsy seems to be necessary for patients with OA and NOA characteristics.

Keywords: Biopsy, Nonobstructive Azoospermia, Obstructive Azoospermia

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Introduction

Infertility is one of the major medical problems in the world (1). The prevalence of male infertility is unknown due to conflicting reports (2). One cause of male infertility is azoospermia, which is defined as a lack of sperm in semen analysis after centrifugation (3) and is confirmed by two consecutive semen tests (4). The prevalence of azoospermia is about 1 % in the general male population (5) and approximately 10% of infertile males (6). Azoospermia divides into two types, including obstructive azoospermia (OA) and non-OA (NOA) (7). About 40% of the azoospermic patient are in the OA group, which occurs secondary to a physical obstruction in sperm transfer from the testis to the urethra (8, 9). About 60% of azoospermic patients are in the NOA group, which occurs secondary to testicular failure in sperm production (10, 11). Accurate etiology of azoospermia is required for optimal management of patients (12). Sperm retrieval in NOA is done by microdissection testicular sperm extraction (mTESE) (13). OA is usually corrected by reconstructive microsurgery (14). There are several clinical and laboratory differences between OA and NOA that reduce the role of diagnostic testis biopsy

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***Corresponding Address: P.O.Box: 1411713135, Department of Urology, Shariati Hospital, Tehran University of Medical Science, Tehran, Iran**
Email: masadighi@gmail.com

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(15). According to studies, a combination of testicular long axis with a cutoff point of 4.6 cm and serum FSH with a cutoff point of 7.6 mIU/mL could predict the type of azoospermia with high sensitivity and specificity (16). However, those levels of the published data regarding the differentiation between OA and NOA weren't applicable in our population. Therefore, we decided to conduct a study with the aim of determining serum hormonal level and testicular long axis cut off points to distinguish OA from NOA in Iranian patients. The necessity of diagnostic testis biopsy azoospermic patients was also evaluated in this study.

Materials and Methods

Patients

In this retrospective study, from 2016 to 2020, a total of 471 azoospermic patients were evaluated. The present study is approved by the Ethics Committee of Tehran University of Medical Sciences with the code of ethics (IR.TUMS.MEDICINE.REC.1399.1005). All male participants in this study were examined by a single urologist and underwent a diagnostic testes biopsy as well as testis ultrasound by a single radiologist. 27 patients with a diagnosis of Klinefelter syndrome, 9 patients with a diagnosis of hypogonadotropic hypogonadism and 24 patients due to incomplete file information were excluded from this study. Finally, 411 azoospermic patients were evaluated, including 284 NOA and 127 OA. Data of patients include age, height, weight, history of herniorrhaphy, history of vasectomy, history of varicocelectomy, history of epididymo-orchitis or urinary tract infection, history of cryptorchidism and orchiopexy, testicular volume estimation with orchidometer, palpation of the vas, testicular long axis based on ultrasound, serum FSH level, serum LH level, serum testosterone level, semen fluid analysis report for fructose, pH and volume, final testicular pathology report and microscopic report during testis biopsy were extracted from the files and were entered into a checklist.

Statistical analysis

The collected data was analyzed using Statistical Package for the Social Sciences (SPSS, SPSS Inc., Chicago, IL, USA) version 16, with t-test and chi-square test. $P<0.05$ were considered statistically significant. The receiver operating characteristic (ROC) curve (17) was also used to determine the appropriate cutoff points for the hormonal profile and testicular long axis to distinguish NOA from OA. Accuracy was assessed by the area under the ROC curve (AUC), and results were considered to be excellent (AUC 0.9-1), good (AUC 0.8-0.9), fair (AUC 0.7-0.8), poor (AUC 0.6-0.7) and failed (AUC 0.5-0.6).

Results

In this study, 411 azoospermic patients were analyzed. All of them underwent diagnostic testis biopsy. 284 cases (69.1%) were NOA and 127 cases (30.9%) were OA. Demographic information, history, and physical examination, testicular long axis based on ultrasound, hormonal profile, and semen fluid analysis are compared between NOA and OA in Table 1.

Table 1: Comparison of demographic and testis ultrasound and laboratory data between OA and NOA

Baseline characteristics of patients	OA	NOA	P value*
Patients (%)	127 (30.9)	284 (69.1)	
Age (Y)	33.10 ± 6.22	31.91 ± 5.03	0.06
BMI (kg/m^2)	26.70 ± 3.80	26.17 ± 4.80	0.24
Genitourinary tract infection	1 (0.7)	4 (1.4)	0.17
Epididymitis history	5 (3.9)	3 (1.1)	0.051
Unilateral UDT and orchiopexy	8 (6.2)	20 (7)	0.53
Bilateral UDT and orchiopexy	5 (3.9)	12 (4.2)	0.57
Unilateral herniorrahphy	10 (7.9)	18 (6.3)	0.57
Bilateral herniorrahphy	4 (3.1)	6 (2.1)	0.53
Unilateral varicocelectomy	15 (11.8)	33 (11.6)	0.96
Bilateral varicocelectomy	7 (5.5)	11 (3.9)	0.45
Bilateral vasectomy	4 (3.1)	0 (0.0)	0.003
At least one palpable vas	103 (81.1)	283 (99.6)	0.0001
Testis volume in P/E (ml)	19.06 ± 4.50	10.50 ± 5.45	0.0001
Testis length in P/E (cm)	3.89 ± 0.38	2.78 ± 1.13	0.0001
Testis longitudinal axis in ultrasound (mm)	39.83 ± 3.36	33.05 ± 6.00	0.0001
Volume of semen fluid (ml)	1.66 ± 1.51	2.85 ± 1.53	0.0001
PH of semen fluid	7.08 ± 0.61	7.72 ± 0.23	0.0001
Fructose of semen fluid (mg/dl)	77.04 ± 114.80	202.02 ± 91.50	0.0001
Serum LH (mIU/ml)	5.33 ± 3.05	11.05 ± 6.16	0.0001
Serum FSH (mIU/ml)	5.30 ± 4.11	19.11 ± 10.61	0.0001
Serum testosterone (ng/ml)	4.26 ± 1.86	3.40 ± 1.75	0.0001

Data are presented as mean ± SD or n (%). OA; Obstructive azoospermia, NOA; Non-obstructive azoospermia, BMI; Body mass index, UDT; Undescended testis, P/E; Physical examination, LH; Luteinizing hormone, FSH; Follicular stimulating hormone, and *; Analysed with t test or chi-square.

OA patients had a mean age (33.1 years) and BMI (26.7). NOA patients had a mean age (31.9 years) and BMI (26.1). There were no significant differences between the two groups about age, BMI, history of urinary tract infections and epididymo-orchitis, history of UDT and orchiopexy, history of varicocelectomy, and herniorrhaphy ($P>0.05$). Only 4 out of 127 OA patients had a history of vasectomy. Clinical examination findings including at least one palpable vas, testis size, testis volume, and also the testicular long axis base on

ultrasound were significant differences between the two groups (P<0.05). OA patients had significantly greater testis size than NOA patients (P<0.05). The mean serum testosterone level in OA (4.26 ng/ml) was significantly more than NOA (3.40 ng/ml). The mean serum FSH level in OA (5.3 mIU/ml) was significantly lower than NOA (19.11 mIU/ml), and similarly, the mean serum LH level in OA was significantly lower than NOA (P<0.05). Semen fluid analysis findings including volume, pH and fructose in OA were significantly lower than NOA (P<0.05). ROC curve analysis was performed to determine cutoff points for differentiation between OA and NOA. Figure 1 shows the ROC curve for serum FSH, LH, testosterone level, and testicular long axis.

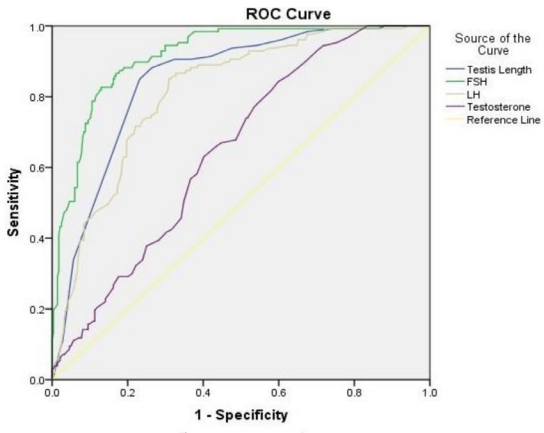

Fig.1: ROC curve for serum hormonal level and testicular long axis. Testis length (0.85), FSH (0.92), LH (0.81), and testosterone (0.65). ROC; Receiver operating characteristic, FSH; Follicular stimulating hormone, and LH; Luteinizing hormone.

According to Figure 1, serum FSH with a curved area (0.92), the testicular long axis with a curved area (0.85) and serum LH with a curved area (0.81) had excellent accuracy for differentiating OA from NOA, but serum testosterone with a curve area (0.64) had poor accuracy for differentiating OA from NOA. Based on the ROC curve by determining the cut off point 8.9 mIU/ml for serum FSH with the sensitivity of 85%, specificity of 77%, the positive predictive value of 92%, the negative predictive value of 62%, accuracy of 80%, and determining the cut off point 39 mm for testicular long axis with the sensitivity of 83%, specificity of 87%, positive predictive value 93%, the negative predictive value of 70%, accuracy of 84% can differentiate between OA and NOA. According to Table 2, by combination cut off points of serum FSH and testicular long axis with the highest accuracy, 69% of NOA patients had FSH ≥8.9 mIU/ml & testicular long axis <39 mm, and 76% of OA patients had FSH<8.9 mIU/ml & testicular long axis ≥39 mm. These differences were significant (P<0.05).

Based on the ROC curve, a combination of serum FSH level and testicular long axis had sensitivity (88.28%), specificity (94.17%), positive predictive value (97.02%), negative predictive value (78.86%) and accuracy (90.15%) for differentiating NOA from OA. According to Table 3, we can predict that azoospermic patients with serum FSH ≥8.9 mIU/ml & testicular long axis <39 mm are in the NOA group (positive predictive value 97.02%), and azoospermic patients with FSH <8.9 mIU/ml and testicular long axis ≥39 mm are in OA group (positive predictive value 78.86%).

Table 2: Combining testis longitudinal axis size and FSH for differentiation NOA from OA

FSH and testis length of patients	OA	NOA	P value*
FSH<8.9 and testis length≥39 mm	97 (76.4)	26 (9.2)	0.0001
FSH<8.9 and testis length<39 mm	11 (8.7)	40 (14.1)	0.12
FSH≥8.9 and testis length≥39 mm	13 (10.2)	22 (7.7)	0.40
FSH≥8.9 and testis length<39 mm	6 (4.7)	196 (69)	0.0001

Data are presented as n (%). FSH; Follicular stimulating hormone, NOA; Non-obstructive azoospermia, OA; Obstructive azoospermia, and *; Analysed with chi-square.

Table 3: Positive predicting value for NOA or OA using testicular long axis and FSH

FSH and testis length of patients	OA	NOA	PPV for NOA	PPV for OA
FSH<8.9 and testis length≥39	97 (76.4)	26 (9.2)	-	78.86
FSH≥8.9 and testis length<39	6 (4.7)	196 (69)	97.02	-

Data are presented as n (%) or %. NOA; Non obstructive azoospermia, OA; Obstructive azoospermia, FSH; Follicular stimulating hormone, and PPV; Positive predictive value.

The final pathological reports of azoospermic patients are shown in Table 4. Sertoli cell-only syndrome pattern was the most common pathology in azoospermic patients (37%).

According to Table 4, normal spermatogenesis pattern in the group of azoospermic patients with FSH <8.9 mIU/ml and testicular long axis ≥39 mm, and Sertoli cell only syndrome pattern in the group of azoospermic patients with FSH ≥8.9 mIU/ml and testicular long axis <39 mm were most common pathological reports (P<0.05). Photomicrographs describing pathological reports are shown in Figure 2.

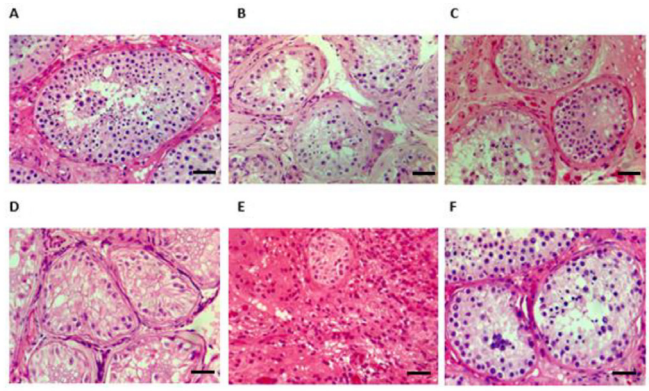

Fig.2: Photomicrographs pathological reports of testis biopsy. **A.** Normal spermatogenesis, **B.** Hypo spermatogenesis, **C.** Maturation arrest, **D.** Sertoli cell only syndrome, **E.** Lydig cell hyperplasia, and **F.** Mixed pattern (scale bar: 20 µm).

Table 4: Pathological report of testis biopsy specimen in azoospermic patients

Report of pathology	Azoospermic patients (n=411)	FSH<8.9 and testis length<39 (n=51)	FSH<8.9 and testis length≥39 (n=123)	FSH≥8.9 and testis length≥39 (n=35)	FSH≥8.9 and testis length<39 (n=202)
Normal spermatogenesis	127 (30.8)	11 (21.6)	97 (78.9)	13 (37.15)	6 (3)
Hypo spermatogenesis	29 (7.1)	5 (9.8)	4 (3.3)	6 (17.15)	14 (6.9)
Germ cell maturation arrest	62 (15.1)	9 (17.6)	15 (12.2)	7 (20)	31 (15.3)
Sertoli cell only syndrome	152 (37)	19 (37.3)	4 (3.3)	5 (14.3)	124 (61.4)
Lydig cell hyperplasia	12 (2.9)	0 (0.0)	0 (0.0)	0 (0.0)	12 (5.9)
Hypo spermatogenesis and maturation arrest (mixed)	29 (7.1)	7 (13.7)	3 (2.4)	4 (11.4)	15 (7.4)
P value*	0.0001	0.16	0.0001	0.015	0.0001

Data are presented as n (%). FSH; Follicular stimulating hormone and *; Analysed with chi-square.

Discussion

Azoospermia is one of the most common male infertility factors evaluated in various studies (18). Determination type of azoospermia helps urologists to treat patients appropriately. OA patients will undergo reconstructive surgery if possible. Otherwise, they will undergo sperm retrieval by testis biopsy for in vitro fertilization/ intracytoplasmic sperm injection (IVF/ICSI). NOA patients are candidates for sperm retrieval by testis biopsy (6, 19). If sperm was not found, they are candidates for sperm donation (20, 21). Several studies show that azoospermic patients can be correctly sorted into OA or NOA groups with multiple diagnostic parameters. Usually, testis volume in OA is normal, whereas in NOA is less than normal (7). The results of our study support this finding significantly. The average testis volume with an orchidometer in OA is 19.06 ml and in NOA is 10.5 ml. The average testicular long axis measured in ultrasound for OA is 39.83 mm and for NOA is 33.05 mm. A history of genital infections or epididymo-orchitis can obstruct an inflammatory process and the production of antibodies (22). However, in this study, a history of epididymo-orchitis or genitourinary system infections were no significant association with OA or NOA. The history of an undescended testis (UDT) and orchiopexy is a risk factor for azoospermia. In fact, UDT is associated with spermatogenic dysfunction (23), but findings in the present study show that the history of UDT and orchiopexy cannot differentiate OA from NOA. In the absence of bilateral vas deferens, evaluation of the CFTR panel for cystic fibrosis is recommended (24). Our finding similar to other studies show that lack of bilateral palpation of vas deferens is associated with OA significantly. In these patients spermatogenesis is normal and IVF/ICSI can be used for fertilization (25, 26). Usually, OA patients with ejaculatory ducts obstruction have semen analysis with features of low volume, low pH, and low fructose level. Trans-rectal ultrasound is helpful for the diagnosis of these patients (27). The results of this study also confirm that in OA patients compared to NOA, the mean semen parameters, including volume, pH, and fructose are significantly lower than normal. NOA includes primary testicular failure (high serum FSH with small testis size), secondary testicular failure (low serum FSH with small testis size), or incomplete features such as elevated FSH with normal testis, small testis with normal FSH, and normal testis with normal FSH. Maturation arrest histopathology is associated with normal serum FSH and normal testis size, while Sertoli cell only histopathology is associated with high serum FSH and small testis size (28). The results of our study also confirm this finding, since the most common pathology in NOA with normal serum FSH and normal testis size was maturation arrest and the most common pathology in NOA with high serum FSH and small testis size was Sertoli cell only.

According to Schoor et al. (16) study, differentiating with high accuracy between OA and NOA is possible by a combination of serum FSH and testis size. Their results showed that mean serum FSH, LH, and testosterone levels in NOA were significantly higher than OA. According to the results of this study, similar to Schoor's study data, the mean serum FSH and LH in NOA were significantly higher than OA, however, the mean serum testosterone in NOA was significantly lower than OA which is in contrast with Schoor's results. Based on the ROC curve in this study, similar to Schoor et al. (16) and Huang's study (29), serum FSH level and testis size have been identified as the best criteria for predicting the differentiation of OA from NOA. The best serum FSH level cut off points for differentiating OA from NOA was 7.6 mIU/ml (sensitivity 77%) in Schoor's study, 9.2 mIU/ml (sensitivity 89%) in Huang's study, and 8.9 mIU/ml (sensitivity 85%) in the present study. The best testicular long axis cut off point for differentiating OA from NOA were 46 mm (sensitivity 72%) in Schoor's study and 39 mm (sensitivity of 83%) in our study. This discrepancy between testicular long axis cut off points is likely due to ethnic and racial differences. For example, testis size in Asian races was significantly smaller than in Caucasians (30, 31). Azoospermic patients with testicular long axis lower than cut off point and serum FSH level higher than cut off point, based on Schoor's study (89% probability), Huang's study (99% probability), and our study (97% probability) are in NOA group. Azoospermic patients with testicular long axis greater than cut off point and serum FSH level lower than cut off point, based on Schoor's study (96% probability),

Huang's study (81% probability), and our study (78% probability) are in OA group.

The role of diagnostic testicular biopsy in these patients is questionable. According to Shoor's study in the Campbell-Walsh-Wein urology textbook (16, 32), patients with a high level of serum FSH and small testis size are NOA with 89% probability. These patients are candidates for mTESE and IVF/ICSI or sperm cryopreservation without diagnostic testicular biopsy. However, due to the impossibility of using sperm donation in some centers such as our center, a diagnostic testicular biopsy to find sperm for cryopreservation is recommended with our team. Then, if sperm are found, their female partners are candidates for IVF/ICSI. Therefore, inappropriate treatment costs and medical complications for the patient's female partners are reduced. Also, according to Shoor's study, patients who have low serum FSH and normal testis size are in the OA group with 96% probability. These patients are candidates for reconstructive surgery or testis biopsy and sperm extraction alone depending on their reproductive goals. According to our results, 23.6% of patients in the OA group did not have serum FSH levels less than 8.9 mIU/ml and a long testicular axis of more than 39 mm. If a diagnostic testicular biopsy is not performed in this group of OA patients, they may be misclassified in the NOA group and are candidates for mTESE with ICSI, which increases related risks of ovulation induction for patients' female partners and also wastes unnecessary treatment costs. On the other hand, 21.1% of patients with serum FSH levels less than 8.9 mIU/ml and a long testicular axis of more than 39 mm were in the NOA group. If a diagnostic testicular biopsy is not performed in this group of NOA patients, they may be misclassified in the OA group and are candidates for reconstructive microsurgery or are recommended for MESA or TESA with ICSI. Therefore, diagnostic testicular biopsy seems to be necessary for patients with OA and NOA characteristics.

This study has several limitations. First, we did not evaluate mTESE results of NOA patients with negative TESE. Second, since the spermatogenesis is not uniform in the testis and it could be patchy so the single testis biopsy cannot show the whole pattern of the spermatogenesis. Third, data of some patients were incomplete and we had to exclude these patients from the study.

Conclusion

In Iranian patients, azoospermic patients are predictable by the higher level of serum FSH than 8.9 mIU/ml as well as testicular long axis lower than 39 mm with 97% probability in NOA group. Azoospermic patients with serum FSH levels lower than 8.9 mIU/ml and testicular long axis greater than 39 mm with 78% probability in the OA group are also predictable. Findings in this study also emphasize the need for adjusting cut off points based on patients' ethnicity and race to improve diagnosis. In addition, diagnostic testicular biopsy seems to be necessary for patients with OA and NOA characteristics.

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Authors' Contributions

I.S.; Participated in study design, data collection, evaluation, and interpretation of data. S.M.K.; Participated in study design and interpretation of data. A.V.T.D.; Participated in testicular ultrasound and data collection related to testicular size. T.H.; Participated in the collection, interpretation of data, and drafting the manuscript. A.R.D.; Participated in the pathological data gathering and statistical analysis. M.A.S.G.; Participated in both study design and interpretation of data, and was responsible for overall supervision. All authors read and approved the final manuscript.

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Original Article

Comparison of Triggering Final Oocyte Maturation with Follicle Stimulating Hormone Plus Human Chorionic Gonadotropin, versus Human Chorionic Gonadotropin Alonein Normoresponder Women Undergoing Intracytoplasmic Sperm Injection: A Randomized Clinical Trial

Soheila Ansaripour, M.D.[1], Nayereh Tamizi, M.D.[1*], Mohammad Reza Sadeghi, Ph.D.[2], Azam Mohammad-Akbari, M.D.[1]

1. Nanobiotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
2. Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

Abstract

Background: Few studies have so far been done about the role of follicle stimulating hormone (FSH) in final oocyte maturation. However, none of these studies have been performed solely on normoresponder patients. This study aimed to determine whether oocyte maturation, as well as fertilization and pregnancy rates, could be improved in normoresponder women with concomitant FSH and human chorionic gonadotropin (hCG) trigger compared to those with the hCG trigger alone.

Materials and Methods: In this prospective randomized clinical trial, 117 normoresponder women, aged 19-40 years who were candidates for the gonadotropin-releasing hormone (GnRH) antagonist protocol at Avicenna Infertility treatment Center, were enrolled and claasified in two groups. Final oocyte maturation was triggered using 10000 IU of hCG plus 450 IU of FSH in the first group (59 subjects) and 10000 IU of hCG alone in the second group (58 subjects). The primary outcome was clinical pregnancy rate.

Results: Mean age of the patients was 33.21 ± 4.41 years. There was no difference in clinical pregnancy among the two groups (30.9% vs. 25.5%, P=0.525). There was no statistically significant difference in fertilization rate (80.0% vs. 74.1%, P=0.106), implantation rates (18.9% vs. 16.7%, P=0.352), and chemical pregnancy rates (38.2% vs. 32.7%, P=0.550). Oocyte maturation rate (84.2% vs. 73.6%, P<0.001), 2 pronuclei (2PNs) (6.53 ± 2.54 vs. 5.36 ± 2.85, P=0.021) and total embryos (5.85 ± 2.43 vs. 4.91 ± 2.58, P=0.046) were significantly higher in the first group.

Conclusion: Adding FSH to hCG for oocyte triggering, significantly improved oocyte maturation rates and total embryos. While there was no significant difference in the clinical and chemical pregnancy rates, between these two groups (registration number: IRCT20190108042285N1).

Keywords: Fertilization, Follicle Stimulating Hormone, Human Chorionic Gonadotropin, Pregnancy Rate, Triggering Oocyte Maturation

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Introduction

Ovarian and pituitary hormone changes during the midcycle period have been previously studied. Surge of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) precede normal ovulation (1). LH surge plays a crucial role in the last stages of oocyte maturation. The effect of different ovarian stimulation protocols has been reported in several studies (2-4). In practice, the gold standard method to trigger oocytes in the last stages of maturation is human chorionic gonadotropin (hCG), which has been routinely used for decades as a surrogate for LH surge (2, 3). However, ovarian hyperstimulation syndrome (OHSS), due to the prolonged luteotrophic effect of hCG, is an important and potentially fatal complication of hCG triggering (2-5).

Multiple randomized controlled trials (RCTs) have demonstrated the efficacy of gonadotropin-releasing hormone agonists (GnRHa) administration for the final phase of oocyte maturation as an alternative to the classic triggering by hCG (6, 7). GnRHa trigger has been suggested to be beneficial in GnRH antagonist protocols because it has a lower risk of OHSS than the conventional hCG trigger (4, 8). Nevertheless, several recent studies have shown that GnRHa triggering can be associated with corpus luteum dysfunction, decreased pregnancy rate and increased early miscarriage rate (4, 6, 9).

During the natural cycle, ovulation is induced by

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***Corresponding Address: P.O.Box: 1983969412, Nanobiotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran**
Email : dr.nayereh.tamizi@gmail.com

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simultaneous rises in LH and FSH (5). Effect of FSH surge in inducing ovulation has not been completely understood yet; however, its roles in stimulating LH receptors on luteinizing granulosa cells (4, 5, 10), resumption of oocyte meiosis (10, 11) and stimulation of plasminogen activator activity for follicular rupture (5) have been reported. In addition, a bolus of FSH can induce ovulation independently in rodents and macaques (5). FSH alone has been reported to induce oocyte maturation and ovulation in the monkey model (10). Similar findings were reported in humans who inadvertently administered a FSH bolus instead of hCG after undergoing *in vitro* fertilization (IVF) (12).

Few studies have been done on role of FSH in final oocyte maturation (5, 10, 13). However, none of the previous studies have been performed solely on normoresponder patients. "Normoresponder" refers to a group of patients who have neither decreased ovarian reserves nor predisposition to hyperstimulation (14). This study aimed to determine whether oocyte maturation, as well as fertilization and pregnancy rates, could be improved in normoresponder women with concomitant FSH and hCG trigger compared to those with the hCG trigger alone.

Materials and Methods

This prospective randomized controlled trial was performed between February 2019 and February 2020. The study was approved by the Ethics Committee of Avicenna Research Institute, Tehran, Iran (IR.ACECR.AVICENNA.REC.1397.016) and registered in the Iranian Registry of Clinical Trials (IRCT20190108042285N1).

This study evaluated women undergoing intracytoplasmic sperm injection (ICSI) treatment at Avicenna Infertility Treatment Center. Written informed consent was obtained from all couples participating in the trial.

Patient population

Women aged 19-40 years who were candidates for GnRH antagonist protocol at an academic centre, enrolled in this study.

Inclusion criteria were day-3 serum FSH levels <11 mIU/ml, two or fewer previous embryo transfer cycles, both ovaries present, absence of uterine abnormalities, antral follicle count (AFC) >5 on the third day of cycle, and anti-mullerian hormone (AMH) level >1 ng/ml. In this study, only normoresponding women were included, i.e. women who had 6-20 follicles >10 mm on the trigger day.

Exclusion criteria were estradiol (E2) level >3,500 pg/ml and <500 pg/ml on the day of hCG injection, severe male factor infertility, polycystic ovary syndrome (PCOS), undertaking a cycle involving preimplantation genetic diagnosis (PGD) or not having an embryo transfer, due to the freeze-all policy, donor or surrogate cycle, grade 3 and 4 endometriosis, contraindications to ovulation stimulation, important systemic diseases, such as hepatic failure and renal failure. Patients also were excluded if they were unable to give informed consent.

Ovarian stimulation

Ovarian stimulation was done using recombinant FSH (Cinnal-f, CinnaGen, Iran) from cycle day 3. The initial gonadotropin dose was based on the patient age, body mass index (BMI), AFC, and AMH level.

Transvaginal ultrasound was performed every 2 to 3 days from the sixth day of stimulation to measure the follicular diameter. The GnRH antagonist (Cetrotide, Serono International S.A., Switzerland) was administered (0.25 mg/day) when the dominant follicles reached ≥14 mm in diameter.

All patients were triggered when at least three follicles measured 18 mm or more in the transvaginal ultrasound. The patients were randomly classified in two groups using a computer-generated random number table with six blocks on the day of trigger. The random allocation and participants' assignment were done by an independent nurse who was not involved in the study or patient care.

All physicians, research coordinators and clinic personnel were blinded. The study coordinator who was not blinded prepared the appropriate study medication syringe, which IVF in-cycle nurses administered. The triggers administered were 10000 IU of hCG (Pregnyl, Netherlands) (10) plus 450 IU of FSH in the first group and 10000 IU of hCG alone in the second group. FSH and hCG were injected with two separate syringes, so participants and IVF in-cycle nurses were not blind.

Oocyte retrieval was done under ultrasound guidance, 36 hours after trigger administration for all subjects, using a Cook (Sydney, Australia) catheter. Retrieved oocytes were fertilized by ICSI. Oocyte maturation was evaluated after cumulus cell stripping, and 18 hours after aseessement of sperm injection fertilization. One to three good-quality embryos were transferred 72 hours after oocyte retrieval (15). Pregnancy was assessed by blood β-hCG test 14 days after transfer. Transvaginal ultrasound was performed 2-3 weeks after a positive β-hCG test to evaluate pregnancy sac and fetal heart rate.

Outcomes

The primary outcome was clinical pregnancy. Clinical pregnancy was defined as detection of a fetal heartbeat by transvaginal ultrasound scan. Secondary outcomes included oocyte maturation rate, ICSI fertilization rate, implantation rate and a chemical pregnancy.

Oocyte maturation rate was defined by the number of metaphase II (MII) oocytes divided by the number of oocytes retrieved. ICSI fertilization was defined as the proportion of injected oocytes with 2 pronuclei (2PN) the day after injection. Implantation rate was defined as the number of gestational sacs observed by transvaginal ultrasound divided by the total number of transferred embryos. Rate of good-quality embryos was defined as the ratio of embryos with good quality to the number of 2PNs. Good-quality cleavage stage embryos were defined as having a cell number between 7 and 10 with <10% of the volume of the embryo occupied

by cell fragmentation based on a modified Veeck's grading system. Chemical pregnancy was defined as positive hCG but an absence of gestational sac by ultrasound detection14 days after embryo transfer (5, 16, 17).

The patient age, BMI, duration of infertility (years), type and cause of infertility were collected. Serum E2, AMH, FSH and total FSH levels, as well as the number of total embryos and transferred embryos, in addition to number of follicles counted on trigger day were also recorded.

Statistical analysis

The sample size was calculated regarding the 95% confidence level, power of 85%, the least significant difference of 0.08 and the prevalence of 50% final oocyte maturation in the group receiving FSH plus hCG. Therefore, 50 patients were required in each group.

The SPSS (Ver. 20; SPSS Inc, Chicago. IL, USA) statistical software was used for data analysis. The Student's t test was performed to compare continuous variables. The Chi-square test was performed to compare categorical variables. Statistical significance was defined as a two-tailed P<0.05.

Results

A total of 156 infertile women were initially enrolled in this study. Of them, 39 women were excluded; finally, 117 subjects were included on the day of oocyte trigger and classified in two groups (Fig.1).

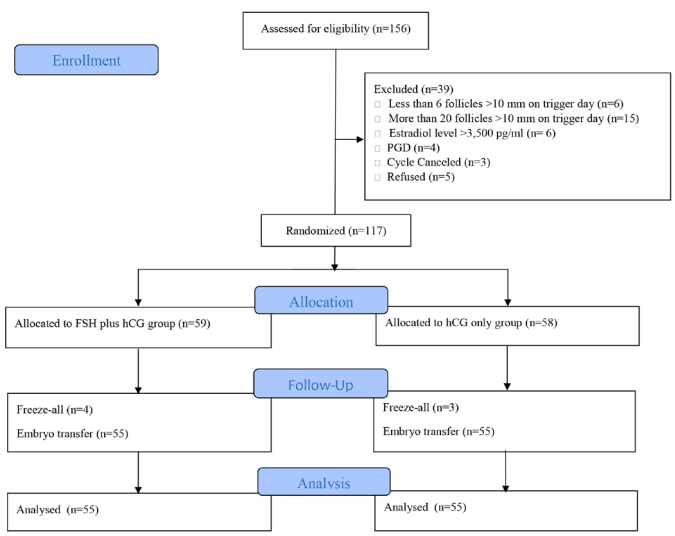

Fig.1: Consort flow diagram. PGD; Preimplantation genetic diagnosis, FSH; Follicle stimulating hormone, and hCG; Human chorionic gonadotropin

Among participants, 59 women received hCG plus FSH, and 58 women only received hCG. Four subjects in the FSH plus hCG group and three subjects in the hCG only group wanted to use the freeze-all procedure for future embryo transfer. Finally, fresh embryo transfer was done in 110 cases, and pregnancy results were compared between 55 women in the first group and 55 women in the second group. There was no loss follow-up evaluation.

The demographic characteristics of the both groups are shown in Table 1. There was no difference in baseline demographic characteristics among the two groups. Cycle characteristics are reported in Table 2. There was also no difference in cycle characteristics, such as total FSH dose, serum E2 on the trigger day, mean number of stimulation days, number of total follicles on the trigger day, number of embryos transferred and number of good-quality embryos. Nevertheless, the number of 2PNs and total embryos were significantly higher in the FSH plus hCG group compared to the other group.

Table 1: Demographics characteristics of both groups

Variables	hCG plus FSH (n=59)	hCG alone (n=58)	P value
Age (Y)	33.14 ± 4.85	33.28 ± 3.95	0.864
BMI (kg/m^2)	25.68 ± 4.63	25.66 ± 3.58	0.980
Type of infertility			0.752
Primary	37 (62.7)	38 (65.5)	
Secondary	22 (37.3)	20 (34.5)	
Cause of infertility	25 (42.4)	27 (46.6)	0.856
Male factors	6 (10.2)	6 (10.3)	
Ovulation dysfunction	5 (8.5)	7 (12.1)	
Tubal problems	12 (20.3)	11 (19.0)	
Mix Unexplained	11 (18.6)	7 (12.1)	
Duration of infertility (Y)	4.45 ± 3.37	4.49 ± 2.23	0.936
AMH (ng/ml)	2.52 ± 1.13	2.44 ± 0.92	0.644
Endometrial thickness (mm)	8.71 ± 1.48	8.61 ± 1.36	0.718
Number of prior ET	0.37 ± 0.67	0.33 ± 0.71	0.759

Data are presented as mean ± SD or n (%). Analyzed using Independent Samples Test and Chi-Square Test. BMI; Body mass index, AMH; Anti-mulerian hormone, ET; Embryo transfer, hCG; Human chorionic gonadotropin, and FSH; Follicle-stimulating hormone.

Table 2: Cycle characteristics

Variables	hCG plus FSH (n=59)	hCG alone (n=58)	P value
FSH on 3rd day	6.82 ± 1.94	6.76 ± 2.19	0.890
Serum estradiol on the day of trigger (pg/ml)	1628.36 ± 506.46	1582.55 ± 567.33	0.646
Number of days of stimulation	9.68 ± 1.32	9.62 ± 1.57	0.831
Total FSH dose (IU)	2065.25 ± 677.03	2046.05 ± 901.31	0.897
Follicles count on the day of trigger	11.88 ± 2.80	11.98 ± 3.16	0.855
Number of MII oocytes	8.32 ± 3.04	7.33 ± 3.15	0.085
Number of 2PNs	6.53 ± 2.54	5.36 ± 2.85	0.021
Number of total embryos	5.85 ± 2.43	4.91 ± 2.58	0.046
Number of embryos transferred*	2.15 ± 0.74	2.07 ± 0.77	0.550
Number of good quality transferred embryos*#	1.40 ± 0.89	1.29 ± 0.90	0.524
Excess count embryos for cryopreservation*	3.58 ± 2.23	2.60 ± 2.45	0.030
Fertilization rate (%)	80.8	74.1	0.106
Oocyte maturation rate (%)	84.2	73.6	<0.001

Data are presented as mean ± SD. Analyzed using Independent Samples Test and Chi-Square Test. *; There were 55 women in each group, #; Good quality embryos were defined as having a cell number between 7 and 10 with <10% of the volume of the embryo, hCG; Human chorionic gonadotropin, FSH; Follicle-stimulating hormone, MII; Metaphase II, and 2PN; 2 pronuclei.

The primary outcome was clinical pregnancy. There was no difference in clinical pregnancies among the two groups (0.31 vs. 0.26, P=0.53). Analysis of the secondary outcomes showed a fertilization rate of 80.8% versus 74.1% (P=0.11), implantation rates of 18.9% versus 16.7% (P=0.35), and chemical pregnancy rates of 38.2% versus 32.7% (P=0.55), in the "FSH plus hCG" and hCG-alone groups, respectively. Additionally, women in FSH plus hCG group had a significantly higher oocyte maturation rate than the hCG alone group (84.2% vs. 73.6%, P<0.001). Pregnancy outcomes are shown in Table 3.

Table 3: Pregnancy outcomes

Variables	hCG plus FSH	hCG alone	P value
Clinical pregnancy rate	17 (30.9)	14 (25.5)	0.525
Chemical pregnancy rate	21 (38.2)	18 (32.7)	0.550
Implantation rate	24 (18.9)	20 (16.7)	0.352

Data are presented as n (%). Analyzed using Chi-Square Test. hCG; Human chorionic gonadotropin and FSH; Follicle-stimulating hormone.

Discussion

The current study showed that co-administration of FSH and hCG for oocyte triggering improved the number of 2PNs, total embryos, excess count embryos for cryopreservation and oo-cyte maturation rate, in comparison with hCG trigger alone. To our knowledge, few randomized clinical trials have been done about the role of FSH in final oocyte maturation (5, 10, 13). However, in this study, only normoresponding women were included.

In a case report, 36 hours before oocyte retrieval, FSH bolus was administered instead of the standard 10,000 IU hCG. This was the first human report of FSH administration during oocyte trigger. The given random dose was more than four times (2100 IU) of what was administered in the present study (450 IU). In this case report, the maturity rate was 90% when recombinant human FSH was administered, and there were no adverse outcomes. By Italian legislation, only three oocytes were injected by ICSI, and all three underwent normal fertilization and cleavage, consequently giving rised to three good quality embryos (12).

The first randomized clinical trial to evaluate whether co-administration of FSH bolus at the time of hCG trigger could improve developmental competence of the oocyte was performed by Lamb et al. (10). Oocytes were triggered by injecting either 450 IU FSH or normal saline as a placebo at the time of hCG administration. They found that fertilization proportion (2PN/oocytes collected) and oocyte recovery rate were significantly improved in the FSH group compared to the placebo group. Similar to the current study, there was no statistically significant difference in clinical pregnancy rate, implantation rate and live birth or ongoing pregnancy rate. Although the oocyte maturation rate was not assessed, the IVF fertilization rate was significantly higher in the intervention group. Similarly, we found that FSH plus hCG women group had significantly higher oocyte maturation rate than the hCG alone group.

In another study performed by Qiu et al. (1), all patients received standard long GnRHa protocol for IVF/ICSI and hCG 6000-10 000 IU to trigger oocyte maturation. Then, subjects received a urinary FSH bolus (450 IU) or placebo, at the time of the hCG trigger, respectively. They did not find any statistically significant improvement in clinical pregnancy rate, good-quality embryos rate and the implantation rate in FSH co-trigger group. In another study, the experimental group subjects received 5000 IU hCG plus 450 IU FSH for final oocyte maturation and contril group subjects received 5000 IU hCG at the time of the oocyte triggering. Similar to our findings, MII oocyte, 2PNs and total embryos were significantly higher in the experimental group compared to the control group, respectively. Additionally, fertilization rate, implantation rate, clinical and chemical pregnancy rates were higher in the experimental group, while these differences were not statistically significant (13).

Juneau et al. (18) pursued to determine whether adding an FSH bolus (450 IU) administered at the time of hCG trigger could improve IVF cycle outcomes in a retrospective cohort. They included 874 cycles in the study, demonstrating no improvement in the number of oocytes retrieved or oocyte maturation, fertilization or blastulation rates with the administration of an FSH bolus at the time of hCG trigger.

The specific role of the FSH surge is not well understood yet; however, FSH has been shown to stimulate LH receptors on luteinizing granulosa cells (4, 5, 10). Recently, due to the availability of pure recombinant human FSH, studies have shown ability of FSH to supplement the midcycle LH surge. In general, FSH is known to promote oocyte cumulus expansion and oocyte nuclear maturation (10, 11, 19).

There are some limitations in this study. Firstly, the sample size was relatively small. Secondly, placebo was not used in the control group. Prominently, further studies are required to optimize this triggering strategy with regards to concentration, sample size, etc., to provide significantly higher pregnancy percentages.

Conclusion

Our results demonstrated that adding 450 IU FSH to 10000 IU hCG for oocyte triggering in normoresponder patients significantly improved oocyte maturation rates and the number of total embryos. Also, fertilization rate, implantation rate as well as clinical and chemical pregnancy rates were higher in the FSH plus hCG group, While there were no significant differences between the two groups.

Although further studies with different concentrations and larger sample sizes are needed to optimize this triggering strategy, these findings suggested that addition of the FSH trigger is an option to further improve assisted reproductive technology (ART) success.

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Authors' Contributions

S.A., N.T., M.R.S., A.M.A.; Contributed to conception and design. S.A., N.T.; Contributed to all experimental works, data and statistical analyses, as well as interpretation of data, and was responsible for overall supervision. N.T.; Drafted the manuscript, revised it by S.A., M.R.S., A.M.A. All authors performed editing and approved the final version of this manuscript for submission. They also participated in the finalization of the manuscript and approved the final draft.

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Original Article

Risk Factors for Anti Mullerian Hormone Decline after Laparoscopic Excision of Endometrioma: A Prospective Study

Maliheh Fakehi, M.D.[1], Fatemeh Davari Tanha, M.D.[2], Zahra Asgari, M.D.[3], Arash Mohazzab, M.D., Ph.D.[1], Marjan Ghaemi, M.D.[4*]

1. Shahid Akbarabadi Hospital, Iran University of Medical Sciences, Tehran, Iran
2. Yas Hospital, Tehran University of Medical Sciences, Tehran, Iran
3. Arash Hospital, Tehran University of Medical Sciences, Tehran, Iran
4. Vali-E-Asr Reproductive Health Research Center, Family Health Research Institute, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Background: Laparoscopic excision of ovarian endometrioma is believed to decrease the ovarian reserve, but the risk factors of declining ovarian reserve are not well studied. This study aimed to determine the risk factors of anti mullerian hormone (AMH) decline after laparoscopic surgery of endometrioma.

Materials and Methods: This prospective study was recruited in Yas and Arash Hospitals affiliated to Tehran University of Medical Sciences from 2020 to 2021. Women between 18-45 years with ovarian endometriomas with a diameter greater than 3 centimeters who were candidates for laparoscopy were included. AMH, luteinizing hormone (LH), and follicular stimulating hormone (FSH) as well as cancer antigen 125 (CA125) and cancer antigen 19-9 (CA19-9) were obtained and compared pre and postoperatively. Indeed, the relation of AMH decline rate and the demographic, symptoms and endometrioma characteristics were investigated either.

Results: In this study, 100 women were recruited. The mean ± SD age of the participants was 29.08 ± 4.6. AMH ($P<0.000$) and LH ($P=0.013$) declined significantly postoperatively. Whereas, no significant difference was observed between pre and postoperative FSH ($P=0.520$). AMH decline rate was 30.07 ± 2.30% and didn't have significant relation with the demographic characteristics, preoperative AMH, and the amount of CA125. Otherwise in the multivariate analysys, CA125 ($P=0.160$) and the grade of endometriosis ($P=0.05$) had significant correlation with AMH decline rate.

Conclusion: Ovarian reserve decline after laparoscopic excision of endometrioma. Otherwise, there may no specific risk factor to predict the degree of ovarian reserve decline. Therefore, the selection of patients for laparoscopic excision of endometrioma should be taken more cautiously as the ovarian reserve diminishes even in the patients with the lowest risks.

Keywords: Anti Mullerian Hormone, Endometrioma, Laparoscopy, Ovarian Reserve

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Introduction

Endometriosis is a common disease affecting about 10% of women of reproductive age. Ovarian endometriomas could be found in 17-44% of these patients which shows no symptoms in up to 50% of the cases (1-3). There is no accurate statistics in Iran but it seems near to 60% of the infertile couples in Iran had endometriosis (4). For many years, the first line therapeutic approach to these cysts was laparoscopic surgery (5, 6). Since endometriomas lack a true capsule separating the cyst from the ovarian tissue, it is inevitable to excise the cyst without cutting some of the normal tissue of the ovary. The point of question and worrisome in this approach is the damage to the ovarian reserve as a result of unintentionally excised normal ovarian tissue (7, 8).

Ovarian reserve is a potential predictor of a female's reproductive system and is based on the number and eventual quality of the ovum. In the last three decades, the level of anti mullerian hormone (AMH) (9), follicular stimulating hormone (FSH), estradiol (E2) and inhibin B as well as the ovarian volume and the antral follicular count (AFC) on transvaginal sonography have been accepted as reliable markers of ovarian reserve (10-12), among which AMH is the most attractive due to its ease of measurement and independency to the menstrual cycle (1, 13, 14).

Different studies have proved ovarian reserve decline after laparoscopic excision of endometriomas assessing all or some of the aforementioned markers. In some studies

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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: m_ghaemi@sina.tums.ac.ir

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cyst diameter, cyst bilateralism, preoperative AMH and patient's age were shown to be relative to the degree of ovarian reserve decline. Meanwhile, other studies proved wise versa (15-18).

This study designed to find any risk factors that make it possible to predict which patients may have higher declines in ovarian reserve postoperatively using ovarian decline rate as a marker showing the degree of the damage to the ovarian reserve and to give a more precise guide in cautious choice of patients for laparoscopy.

Materials and Methods

Population

This prospective study was conducted in Yas and Arash Hospitals affiliated to the Tehran University of Medical Sciences. All participants signed the written informed consent and were eager to participate in the study. Women with ovarian endometrioma between 18 to 44 years old who were planned for laparoscopic excision of the endometrioma between July 2020 and January 2021 were included. The exclusion criteria were a history of previous adnexal surgery, hormonal replacement therapy, and endocrine disorder, ovarian mass suspicious for malignancy, polycystic ovaries and endometriomas of less than 3 centimeters diameter.

This study was approved by the institutional review board Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1399.272). The protocol of the study was designed according to the ethical principles of the Declaration of Helsinki. All participants agreed to participate in the study and the written informed consent was obtained from all participants.

Sample size calculation

The sample size that was required with a power of 95 was 70 cases, of which 100 cases were considered for this study.

$$n = \frac{2(\left(z1 - \frac{\alpha}{2}\right) + (z1 - \beta))^2 p' q'}{(p_1 - p_2)^2}$$

p1: Preoperative AMH
p2: Postoperative AMH

$$z_1 - \alpha/2 = 1.96$$
$$z_1 - \beta = 0.84$$

Study conduct and objectives

The demographic (age, weight, height, age of menarche, marital state) and obstetrical data (gravid and para), as well as the clinical symptoms (dysmenorrhea, dyspareunia, persistent pelvic pain, dysphasia, fear of sex) and history of infertility for female cause were gathered via a questionnaire that was filled by a physician or a nursing staff. Also, ultrasound imaging was done for each patient to see the precise diameter of the cysts and their laterality status. Patients also underwent laboratory testing for the level of FSH, LH, AMH, CA125 and CA 19-9 pre and 3 months post operatively.

Also, a new variable was created as AMH decline rate calculated by the below formula. AMH decline rate is a new variable to assess any correlation between the severity of the damage to the ovarian reserve and any of the other independent variables hypothesized as possible risk factors.

$$\text{AMH Decline rate} = \frac{preoperative\ AMH - postoperative\ AMH}{Preoperative\ AMH} \times 100$$

Laparoscopic excision of ovarian endometrioma

All the procedures were performed by two expert laparoscopic surgeons with more than 15 years of experience in laparoscopic surgery, whose procedures were to excise the cyst by stripping, avoiding to damage ovarian normal tissue as much as possible.

Statistical analysis

All the data were analyzed by the software package for social sciences (SPSS, IBM, USA) for windows version 15.0. Quantitative values were presented as mean ± SD and qualitative values were presented as absolute and relative frequency. Chi-Square test was used to assessed statistical relations of qualitative variables. Comparison between the groups was performed using Mann-Whitney U test. Friedman M test was used to compare the differences of serum AMH concentrations between each sampling point and the changes of serum AMH levels For quantitative variables we used ANOVA and t test. Also for decreas the potential bias, multivariate analysis was performed to evaluate the AMH decline rate with other characteristics. $P<0.05$ was considered to be statically significant.

Results

One hundred patients were included in the study. The mean ± SD age of the patients and menarche were 29.08 ± 4.6 (with a range of 19-41) and 12.56 ± 1.35 years respectively. The characteristics of the patients with endometriomas preoperateivley are listed in Table 1. Also the frequency of the clinical symptoms and the mean of BMI and hormonal profiles in the participants are listed in Table 2. As seen, dysmenorrhea is the most prevalent symptom in these women.

AMH levels decreased significantly 3 months after surgery ($P<0.000$). The mean ± SD of AMH decline rate was calculated to be 30.07 ± 2.30% among all patients. LH levels also declined significantly ($P=0.013$) but there were no significant changes between the levels of FSH pre and postoperatively ($P=0.527$). There was no correlation between patients' charachteristics, preoperative AMH and CA125 with AMH decline rate (Table 3).

Table 1: Characteristics of the patients with endometriomas undergoing laparoscopy (n=100)

Charchteristics		n (%)	Mean ± SD	P value
Age (Y)	>35	8 (8)	28.92 ± 13.6	0.78
	≤35	92 (92)	30.22 ± 23.98	
Laterality of cysts	Unilateral	45 (45)	30.24 ± 23.97	0.948
	Bilateral	55 (55)	29.94 ± 22.45	
Cyst size (mm)	≤70	52 (52)	31.28 ± 22.17	0.602
	>70	48 (48)	28.87 ± 24.01	
Endometriosis	Stage III	58 (58)	24.27 ± 24.36	0.002
	Stage IV	42 (42)	38.09 ± 18.48	
Dysmenorrhea	Yes	88 (88)	30.08 ± 22.85	0.99
	No	12 (12)	30.00 ± 25.37	
Dyspareunia	Yes	43 (62.3)*	30.1 ± 24.74	0.63
	No	26 (37.7)	24.41 ± 20.61	
Persistent pelvic pain	Yes	34 (34)*	30.24 ± 23.71	0.96
	No	66 (66)	29.99 ± 22.85	
Dyschezia	Yes	29 (29)	26.96 ± 22.64	0.38
	No	71 (71)	31.35 ± 23.22	
Infertility	Yes	40 (59)	27.21 ± 24.8	0.434
		29 (42)	31.67 ± 20.81	

*; Calculated in married cases. SD; standard deviation

Table 2: Frequency of clinical symptoms and the mean of BMI and hormonal profiles in the participants

Charchteristics	n (%) or Mean ± SD
Dysmenorrhea	88 (88)*
Dyspareunia	43 (62.3)**
Persistent pelvic pain	34 (34)*
Dyschezia	29 (29) *
Fear of sex	16 (23.2)**
BMI (kg/m^2)	22.6 ± 2.0
FSH (IU/L)	6.66 ± 1.43
LH (IU/L)	7.43 ± 1.61
CA 125 (U/ml)	141.9 ± 104.6
CA 19-9 (U/ml)	44.15 ± 22.7

*; Percentage among 100 patients (married and unmarried), **; Percentage among 69 married patients, BMI; Body mass index, FSH; Follicle stimulating hormone, LH; Luteinizing hormone, CA 125; Cancer antigen 125, and CA 19-9; Cancer antigen 19-9.

Table 3: Correlation of the rate of AMH decline rate with patient's characteristics and lab data

Variable	Correlation	P value
Age (Y)	-0.017	0.873
Menarche (Y)	-0.045	0.654
BMI (kg/m^2)	-0.120	0.235
AMH (ng/ml) (preoperative)	0.163	0.106
CA125 (U/ml)	-0.099	0.329

BMI; Body mass index, AMH; Anti mullerian hormone, and CA 125; Cancer antigen 125.

In linear regression please the R and the relations of other factors with the outcome and their R quantity is listed in Table 4.

Table 4: The R square and adjusted R square for the laterality of the ovarian cyst and hormones

R square	Adjusted R square	Standard error of the estimate
0.209	0.101	20.59523

And at last, the multivariate analysis between the potential predictors and AMH decline rate is shown in Table 5. As seen just CA 125 (P=0.160) and the grade of endometriosis (P=0.05) had significant correlation with AMH decline rate.

Table 5: Multivariate analysis between the potential predictors and AMH decline rate

Model	Unstandardized coefficients		Standardized coefficients	T value	P value
	β*	Standard error	Beta		
Constant	-7.836	39.945		-0.196	0.845
Age (Y)	0.128	0.618	0.029	0.208	0.836
BMI (kg/m^2)	0.266	1.149	0.025	0.231	0.818
Menarch	0.148	1.650	0.009	0.090	0.929
Cyst size (cm)	0.095	0.125	0.087	0.756	0.452
AMH preop (ng/ml)	7.444	3.931	0.245	1.894	0.062
CA19-9 (U/ml)	0.112	0.104	0.118	1.075	0.286
CA125 (U/ml)	-0.057	0.023	-0.286	-2.477	0.016
FSH preop (IU/L)	-1.123	1.568	-0.077	-0.716	0.476
Grade of endometriosis	13.945	4.787	0.321	2.913	0.005
Cyst laterality	-1.142	4.696	-0.026	-0.243	0.809

β*; Unstandardized beta, BMI; Body mass index, AMH; Anti mullerian hormone, and FSH; Follicular stimulating hormone.

Discussion

Endometriosis is an obscure disease defined by extrauterine growth of endometrial tissue. Ovaries are one of the most prevalent sites for endometriosis to be found. Laparoscopic excision of endometriomas for long was accepted as the first line therapeutic approach in these cysts (6, 7), but the decreased number of the ovum's obtained through IVF cycles after endometrioma cyst excision, gave rise to some worrisome about this approach (19).

In the present study, a significant decline in AMH level as a marker of ovarian reserve was observed three months postoperatively. This indirectly addresses the inevitable damage to the ovarian reserve with the surgery of these cysts. Different characteristics of endometriomas can lead to ovarian reserve decrease. Indeed, the AMH level declined postoperatively especially large and bilateral endometriomas (20). In another study AMH decreased significantely at 1, 3 and 6 months after surgery, although, no difference was detected from preoperative and AMH values at 12 months (21). Otherwise, Sugita et al. detected no significant difference 12 months after surgery (22). Also the result of Goodman et al. study was interesting

that showed AMH levels have recovered in 12 months after a transient decrease (23).

FSH levels did not increase significantly according to the diminished AMH levels, this has also been explained in previous studies as FSH seems to be a less sensitive marker in determining the changes of the ovarian reserve and its level does not increase significantly until premenopausal years.

In another study, the only risk factor proved to be related to the severity of AMH decline rate was the patients' preoperative level of AMH, which was not proved in our study (24). Indeed, cyst diameter of greater than 4 centimeters was proved to be a predictor of higher AMH decline rates postoperatively (25) and finally cyst diameter≥7 centimeters, cyst bilateralism, preoperative AMH level and patients age were introduced to be effective and relative risk factors for greater decline in ovarian reserve (19). Otherwise, none of the mentioned items were proved in our study.

Regarding the importance of cyst size, in our study, we found no correlation with AMH decline. However, the literature is controversial. In one study the decline in AMH at 6 months after surgery was more evident in the patients with larger endometriomas (>5 cm) (26). Otherwise A meta-analysis showed the greater endometrioma may lead to the greater damage to ovarian reserves which leads to a decrease in serum AMH levels (27).

Regarding the importance of laterality we found no significant difference in post operative AMH levels in patients with bilateral compared to unilateral. This is in line with Suardi et al. study that serum AMH levels were not influenced by their laterality (28).

The limitation of our study is the short follow up duration. Therefore further studies with longer follow up and investigation of the effect of surgery on the fertility rate with a focus on AMH are recommended. Also the non-significant findings in the current study could also be caused by the small number of the participants.

Conclusion

Since the present study like other studies has shown a significant decline in AMH levels postoperatively, while none of the independent variables were found as a risk factor or predictor of the rate of this decline, we can conclude that probably surgical intervention even in patients with the lowest risks can result in diminished ovarian reserve. This conclusion makes it necessary to select patients for surgical intervention more cautiously at any age or with any clinical and paraclinical presentation.

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Authors' Contributions

All authors contributed to this work and were involved in designing the study, data collection, and writing the manuscript. All authore read and approved the final manuscript.

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Original Article

Early Postpartum Glucose Intolerance, Metabolic Syndrome and Gestational Diabetes Mellitus Determinants after Assisted Conception: A Prospective Cohort Study

Azam Kouhkan, M.D., Ph.D.[1, 2*], Roya Hosseini, M.D.[1, 3], Hamid Reza Baradaran, M.D., Ph.D.[4], Arezoo Arabipoor, M.Sc.[5*], Rezvaneh Cheraghi, M.Sc.[1], Ashraf Moini, M.D.[5, 6, 7], Farideh Malekzadeh, M.Sc.[5], Mohammad E. Khamseh, M.D.[4]

1. Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
2. Department of Regenerative Biomedicine, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
3. Department of Andrology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
4. Endocrine Research Center, Institute of Endocrinology and Metabolism, Iran University of Medical Sciences (IUMS), Tehran, Iran
5. Department of Endocrinology and Female Infertility, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
6. Department of Gynecology and Obstetrics, Arash Women's Hospital, Tehran University of Medical Sciences, Tehran, Iran
7. Breast Disease Research Center (BDRC), Tehran University of Medical Sciences, Tehran, Iran

Abstract

Background: This study aimed to determine the prevalence of postpartum metabolic syndrome (MetS), glucose intolerance, and the determinants, 6-12 weeks postpartum in women with assisted reproduction technology conception gestational diabetes mellitus diagnosis (ART-GDM) compared to women with spontaneous conception and GDM diagnosis (SC-GDM).

Materials and Methods: In this prospective cohort study, two groups consisting of 62 ART-GDM and 64 SC-GDM singleton pregnant women were followed 6-12 weeks after delivery for postpartum MetS. Fasting glucose, 75-g 2-h OGTT, and lipid profile were assessed. Waist and hip circumference, and systolic and diastolic blood pressures (BP) were measured at postpartum. Clinical, paraclinical, and obstetric data were recorded from registry offices. The prevalence of MetS and glucose intolerance were determined. Predictors of MetS and glucose intolerance were evaluated by logistic regression.

Results: The prevalence of postpartum MetS was 20.8% in ART-GDM women and 10.9% in SC-GDM (P=0.123). Mean postpartum BMI and systolic BP were significantly higher in the ART-GDM group (P=0.016 and P=0.027 respectively). Adverse pregnancy outcomes were significantly higher in the ART-GDM group. Postpartum glucose intolerance prevalence did not vary significantly between the groups. Family history of diabetes was a predictive factor for postpartum MetS and glucose intolerance 6-12 weeks after delivery.

Conclusion: Early postpartum MetS and glucose intolerance prevalence after assisted conception did not vary significantly; however, postpartum body mass index (BMI) and systolic BP were significantly higher in the ART-GDM group. Lifestyle modification programs and long-term health care of ART women with GDM diagnosis can be recommended. Further studies with larger sample size and longer follow-up are necessary to verify our findings.

Keywords: Assisted Reproduction Technology, Gestational Diabetes Mellitus, Glucose Intolerance, Metabolic Syndrome, Postpartum Period

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Introduction

Gestational diabetes mellitus (GDM) recognized as any degree of glucose intolerance with onset or first recognition during the second or third trimester of pregnancy, is one of the most prevalent metabolic disorders (1). The evident rise of GDM incidence could be due to advanced maternal age in pregnancy, application of the new diagnostic criteria with lower threshold and single abnormal value, or increasing trend of obesity and unhealthy diet in the general population (1, 2).

The metabolic perimeter of GDM in young women may persist in type 2 DM and the form of metabolic syn-

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*Corresponding Addresses:
P.O.Box: 16635-148, Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
P.O.Box: 16635-148, Department of Endocrinology and Female Infertility, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
Emails: akouhkan@royaninstitute.org, arezoo.arabipoor@gmail.com

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drome (MetS) (3). There is some conflicting evidence on the relationship between GDM and the early increased occurrence of MetS the in some mothers and their children (3-5). MetS and GDM have common clinical features (6) and the prevalence of MetS shows 3- to 4- fold increase in women with a history of GDM (7). Previous data has shown that MetS features such as glucose intolerance, low high-density lipoprotein cholesterol (HDL-C), hypertriglyceridemia, hypertension, and central obesity, are commonly observed in women post GDM (8). These conditions could be associated with hypertension, dyslipidemia, and cardiovascular disease (9, 10). Considering the increased risk of the above-mentioned complications in GDM and MetS and their interconnected nature, it is worth identifying risk factors of early postpartum MetS in women with GDM diagnosis.

Moreover, recent evidence suggested that the risk of GDM and the need for insulin therapy during pregnancy increases in pregnancy induced by assisted reproductive technology (ART) (11, 12). Some mechanisms that might explain this increased risk in the infertile population include higher rates of advanced maternal age and obesity, infertility etiology as well as the treatment procedures, drugs, and epigenetic modifications (13, 14). As a first study, it would be interesting to investigate whether the risk of early postpartum MetS increases in this population.

Based on the importance of the topic, this study was designed to determine the relationship between GDM and early postpartum MetS. The main goal of the study was to compare the prevalence of MetS and glucose intolerance at 6-12 weeks postpartum, between spontaneous and ART pregnancies. The secondary aims were to determine the contributing risk factors in both populations.

Materials and Methods

Study design and settings

In this prospective cohort study, the ART and spontaneous pregnancies populations including all singleton pregnant women diagnosed with GDM at 24-28 weeks of pregnancy, were followed at Royan Institute (Endocrinology and Female Infertility Clinic) and Arash women's Hospital (maternity teaching hospital in Tehran) respectively, between 2015 and 2017. This research project was approved by the institutional review board and Ethics Committee of Iran University of Medical Sciences, and Royan Institute, Iran (IR.ACECR.ROYAN.REC.1395.2). All participants signed an informed consent after ensuring confidentiality and that the data will be reported anonymously. The inclusion criteria were having GDM in the second or third trimester confirmed by 75-g OGTT (one-step glucose tolerance test) and availability of clinical and medical records.

Women with pre-gestational diabetes type 1 or 2, multiple pregnancies and chronic diseases such as hypertension, cardiovascular diseases, untreated thyroid disease, liver diseases, renal diseases, autoimmune diseases, and connective tissue disorders as well as those who were taking corticosteroids were excluded.

Two groups of GDM mothers (SC-GDM and ART-GDM) were followed during pregnancy, delivery and 6-12 weeks postpartum for assessment of adverse maternal, fetal and neonatal outcomes. In addition, the occurrence of MetS and glucose intolerance at postpartum was assessed.

Biochemical and clinical assessment during pregnancy

To distinguish pre-gestational diabetes, all pregnant women were evaluated during the first trimester by determination of fasting blood sugar (FBS) and the results were recorded in the hospital registry office. Gestational diabetes was approved by OGTT using 75 g oral glucose at 24-28 weeks of gestation, based on the American Diabetes Association/International Association of the Diabetes and Pregnancy Study Groups (ADA/IAPDSG) criteria (15).

After 8-12 hour fasting, blood samples were collected from all patients in the second and third trimesters of pregnancies. FBS (FBS 2nd trimester), insulin (insulin 2nd trimester), hemoglobin A1C (HbA1c 2nd trimester), and lipid profile were measured in the 2nd trimester of pregnancy. Homeostasis model assessment-insulin resistance (HOMA-IR) was calculated based on the formula. HOMA-IR was calculated using the following formula: Fasting insulin (U/mL)×fasting glucose (mg/dL)/405

FBS and HbA1c were rechecked in the 3rd trimester of pregnancy.

Data collection

A trained physician recorded socio-demographic characteristics, medical and obstetric history, potential risk factors of GDM and MetS, and details of GDM management and delivery, as well as maternal and neonatal outcomes. Postpartum questionnaires were also completed at 6-12 weeks postpartum. Clinical data were collected from hospital records.

Postpartum biochemical and clinical assessment

Postpartum FBS (PP FBS) determination, 75-g 2-h OGTT (PP GTT2 h), and lipid profile tests (in terms of PP cholesterol, PP triglycerides, PP LDL-cholesterol, PP HDL-cholesterol, and PP VLDL-cholesterol) were performed at 6-12 weeks after delivery. Postpartum prediabetes [impaired fasting glucose (IFG) and impaired glucose tolerance (IGT)], and diabetes were defined according to the ADA criteria (16). Weight (PP weight), height (PP height), waist circumference (PP waist), and blood pressure (PP systolic and diastolic BP) were measured at 6-12 weeks postpartum. The waist circumference was measured at the midpoint between the lower margin of the least palpable rib and the top of the iliac crest using a stretch-resistant tape that provides a constant 100 g tension. BP was checked two times at a 30 minutes interval. The height and weight of each subject were measured while wearing light clothing and barefoot to calculate

body mass index (BMI). The MetS was defined by two criteria including National Cholesterol Education Program Adult Treatment Panel (NCEP ATP III) and International Diabetes Federation (IDF). According to the NCEP ATP III criteria, MetS was diagnosed if any three of five of the following disorders was observed: waist circumference ≥88 cm, triglycerides ≥150 mg/dl, HDL-cholesterol <50 mg/dl, FPG ≥100 mg/ dl, or BP ≥130/85 mmHg (17). The MetS was recognized based on the IDF definition: central obesity (waist circumference >88 cm) plus any two of the four above-noted factors (18).

Statistical analysis

The primary aim was comparing early postpartum MetS and glucose intolerance prevalence between the study groups and the secondary aim was the evaluation of potential risk factors of MetS and glucose intolerance in the study population. Data were analyzed by the Stata software (Version 13.0, STATA Corp, College Station, Texas). The $P<0.05$ was considered a statistically significant level. The normal quantity variables are presented as mean ± standard deviation (SD). Chi-square test was applied for making a comparison with respect to the categorical variables between the groups. Student's t test and Mann-Whitney test were applied when appropriate. The multivariable logistic regression analysis was used to adjust for women's age and BMI for detection of predictive variables for early postpartum MetS and glucose intolerance in the whole study population (n=126). The covariate variables were FBS 2nd trimester, FBS 3rd trimester, HbA1c 3rd trimester, family history of DM, and prior GDM that were considered in the regression model.

The sample size estimation was made by using the NCSS software (Number Cruncher Statistical System software package 2007, Kaysville, UT, USA). According to the study by Vilmi-Kerälä et al. (19), the difference of 21.2% in the prevalence of MetS was considered between case and control subjects and a sample size of 64 subjects in each group would support us to evaluate early postpartum MetS prevalence in each group with a power of 80% and a type I error of 0.05.

Results

According to the determined sample size, 64 eligible patients in each study group were followed up prospectively in their pregnancy duration. Two patients in the ART-GDM group were lost to follow-up due to withdrawal the participation, this rate of losing subjects (1.5%) did not decrease the power of this study significantly. 126 women with GDM pregnancy including 62 ART (ART-GDM) and 64 spontaneous conceptions (SC-GDM) were followed for the incidences of postpartum glucose intolerance and MetS at 6-12 weeks after delivery (Fig.1).

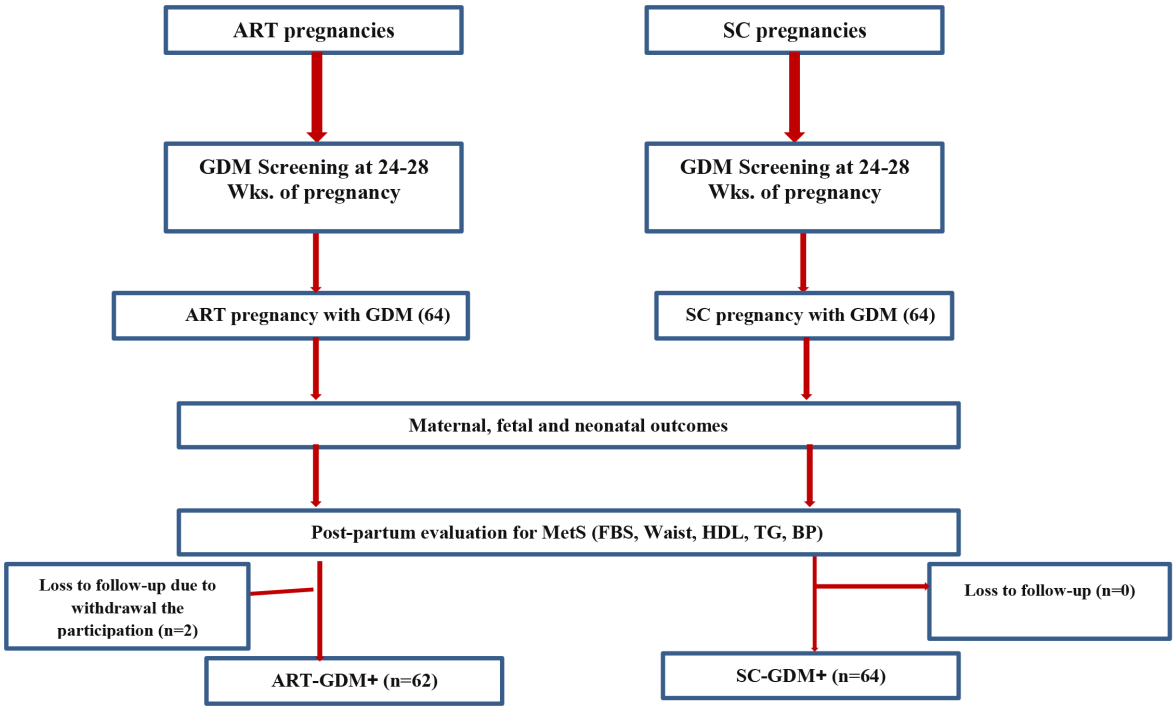

Fig.1: Flow diagram of the study, ending in follow up assessments at postpartum. ART; Assisted reproductive technology, GDM; Gestational diabetes mellitus, SC; Spontaneous conception, FBS; Fasting blood sugar, HDL; High-density lipoprotein, TG; Triglyceride, and BP; Blood pressure.

Table 1 summarizes the baseline characteristics of the women who participated in this study. There were no significant differences in the mean of maternal age, pre-pregnancy BMI, or systolic and diastolic BP between the two groups (P>0.05). In addition, the rates of first-degree family history of diabetes mellitus and hypertension, as well as most history of adverse outcomes in prior pregnancy, were not significantly different between the two groups. However, the rates of nulliparity and prior history of a macrocosmic infant were significantly different between the two groups (P=0.001 and P=0.025). Importantly, the results of biochemical assessments showed that the mean of second trimester insulin (P=0.041) and HOMA-IR (P=0.027), as well as third trimester FBS (P=0.008), were significantly higher in the ART-GDM group compared to the SC-GDM group.

Table 1: Comparison of clinical and biochemical characteristics before and during pregnancy between SC-GDM and ART-GDM groups referred for postpartum examination

Variables	ART-GDM+ (n=62)	SC-GDM+ (n=64)	P value
Clinical			
Maternal age (Y)	31.2 ± 4.9	32.3 ± 4.9	0.234
Parity (=0)	54 (87.1)	25 (39.1)	0.001
Pre-pregnancy BMI (kg/m^2)	27.3 ± 3.8	26.0 ± 4.7	0.112
Family history of DM	31 (50)	24 (37.5)	0.157
Family history of HTN	29 (46.8)	28 (43.8)	0.733
Systolic blood pressure (mmHg)	107.9 ± 11.4	106.7 ± 10.5	0.544
Diastolic blood pressure (mmHg)	66.9 ± 7.8	68.4 ± 8.6	0.334
Prior history of GDM	3 (4.8)	9 (14.1)	0.078
Prior history of pre-eclampsia,	2 (3.2)	1 (1.6)	0.540
Prior history of LBW	1 (1.6)	6 (9.4)	0.057
Prior history of preterm birth	2 (3.2)	5 (7.8)	0.261
Prior history of macrosomic infant	0 (0)	5 (7.8)	0.025
Prior history of multiple pregnancies	3 (4.8)	4 (6.3)	0.730
Prior history of abortion	42 (67.7)	47 (74.6)	0.397
Prior history of neonatal death	2 (3.2)	1 (1.56)	0.540
Prior history of oligohydramnious	0 (0)	2 (3.1)	0.161
Biochemical			
FBS 2nd trimester (mg/dl)*	87.9 ± 8.4	84.2 ± 12.2	0.053
HbA1c 2nd trimester (mg/dl)	5.0 ± 0.6	4.9 ± 0.6	0.296
TG 2nd trimester (mg/dl)*	191.4 ± 60.9	202.6 ± 54.0	0.294
Cholesterol 2nd trimester (mg/dl)	204.6 ± 39.3	225.4 ± 40.0	0.005
HDL 2nd trimester (mg/dl)	63.6 ± 13.2	65.8 ± 12.8	0.367
LDL 2nd trimester (mg/dl)	103.1 ± 35.4	119.1 ± 32.8	0.012
VLDL 2nd trimester (mg/dl)	37.8 ± 10.8	40.5 ± 10.8	0.177
Insulin 2nd trimester (mg/dl)*	14.4 ± 9.1	11.3 ± 5.7	0.041
HOMA-IR 2nd trimester	3.2 ± 2.1	2.4 ± 1.4	0.027
FBS 3rd trimester (mg/dl)*	88.5 ± 9.5	83.1 ± 10.8	0.008
HbA1c 3rd trimester (mg/dl)	5.1 ± 0.6	5.0 ± 0.7	0.863

The quantitative and qualitative variables are presented as mean ± SD or n (%), respectively. The normal quantitative variables and the qualitative variables were compared respectively by Student's t and chi-square tests between groups. *; These quantitative variables had non-normal distribution and compared between groups by Mann-Whitney test, ART; Assisted reproductive technology, GDM; Gestational diabetes mellitus, SC; Spontaneous conception, HTN; Hypertension, HDL; High-density lipoprotein, TG; Triglyceride, LDL; Low-density lipoprotein, VLDL; Very-low-density lipoprotein, HOMA-IR; Homeostatic model assessment of insulin resistance, FBS; Fasting blood sugar, HbA1C; Hemoglobin A1c, and LBW; Low birth weight.

The comparison of maternal, fetal, and neonatal outcomes between the SC-GDM and ART-GDM groups is presented in Table 2. The means of neonatal weight and gestational age at delivery were significantly lower in the ART-GDM group (P=0.003). Maternal outcomes showed that the rates of pregnancy-induced hypertension (PIH) and preeclampsia were significantly higher in the ART-GDM group (P=0.013 and P=0.031). In addition, the incidence of fetal and neonatal complications in terms of preterm birth, small for gestational age (SGA), neonatal intensive care unit (NICU) admission, and neonatal hypoglycemia, was significantly higher in the ART-GDM group (P=0.008, P=0.008, P=0.01, and P=0.04, respectively). Other adverse pregnancy outcomes did not show any significant difference between the two groups.

Table 2: Comparison of maternal, fetal and neonatal outcomes between SC-GDM and ART-GDM groups

Variables	ART-GDM+ (n=62)	SC-GDM+ (n=64)	P value
Neonatal sex, Male	25 (40.3)	36 (56.3)	0.074
Neonatal height (cm)	49.3 ± 3.0	50.1 ± 1.5	0.084
Neonatal weight (kg)	3096.9 ± 516.8	3339.4 ± 354.7	0.003
Neonatal head circumference (cm)	34.6 ± 1.7	35.0 ± 1.3	0.121
Neonatal chest (cm)	32.9 ± 2.0	33.5 ± 1.3	0.119
Gestational age at delivery (weeks)	37.8 ± 0.2	38.5 ± 0.1	0.003
Gestational weight gain	11.3 ± 4.9	11.5 ± 5.6	0.825
Delivery BMI (kg/m^2)	31.7 ± 4.1	30.5 ± 4.4	0.126
Maternal outcomes			
PIH	10 (16.1)	2 (3.1)	0.013
Preeclampsia	7 (11.3)	1 (1.6)	0.031
Antepartum hemorrhage	9 (14.5)	3 (4.7)	0.060
Emergency cesarean	25 (40.3)	16 (25.0)	0.066
PROM	6 (9.7)	1 (1.6)	0.060
Oligohydramnious	6 (9.7)	2 (3.1)	0.132
Polyhydramnious	3 (4.8)	3 (4.7)	0.968
Fetal death	1 (1.6)	0 (0)	0.308
Fetal and neonatal outcomes			
Preterm birth	9 (14.5)	1 (1.6)	0.008
IUGR	6 (9.7)	2 (3.1)	0.125
SGA	9 (14.5)	1 (1.6)	0.008
LGA	2 (3.2)	5 (7.8)	0.261
Macrosomia	1 (1.6)	4 (6.3)	0.189
LBW			
NICU admission	12 (19.7)	3 (4.7)	0.010
Respiratory distress	7 (11.5)	4 (6.3)	0.303
Neonatal hypoglycemia	8 (13.1)	2 (3.1)	0.040
Perinatal mortality	1 (1.6)	0 (0)	0.308
Apgar<7 at 5 minutes	2 (3.2)	1 (1.6)	0.559
Birth trauma	0 (0)	2 (3.1)	0.496

The quantitative and qualitative variables are presented as mean ± SD or n (%), respectively. All of the quantitative variables had normal distribution and compared between groups by student t test. The qualitative variables were compared between groups by chi-square test. ART; Assisted reproductive technology, BMI; Body mass index, GDM; Gestational diabetes mellitus, SC; Spontaneous conception, PIH; Pregnancy induced hypertension, PROM; Premature rupture of membranes, IUGR; Intrauterine growth restriction, SGA; Small for gestational age, LGA; Large for gestational age, LBW; Low birth weight, and NICU; Neonatal intensive care unit.

Table 3 compares the clinical and laboratory characteristics within 6-12 weeks after delivery between the SC-GDM and ART-GDM groups. The results of postpartum metabolic parameters revealed no significant differences between the two groups except for mean BMI and systolic BP which were higher in the ART-GDM group (P=0.016 and P=0.027). The parameters included in the diagnostic criteria of MetS, fasting plasma glucose, waist circumference, triglyceride, and HDL cholesterol were not significantly different between the ART-GDM and SC-GDM groups except for the systolic BP. Additionally, and the 2-hours glucose after 75-g GTT was not significantly different between the ART-GDM and SC-GDM women. The frequency of MetS using the NCEP ATP III criterion was 10.9 and 20.8% in the SC-GDM and ART-GDM groups, respectively. The univariate analysis presented that the odds ratio of postpartum MetS using NCEP ATP III Criteria did not vary significantly between ART and spontaneous GDM pregnancies after adjustment for age, and BMI [aOR; 1.88(0.68-5.22)]. These values for MetS obtained through the IDF criterion were 17.2 and 19.4, respectively. However, the rate of postpartum glucose abnormalities including pre-diabetes [IFG and IGT], and diabetes did not show any significant differences between the two groups.

Table 3: Comparison of postpartum parameters between SC-GDM and ART-GDM groups

Variables	ART-GDM+ (n=62)	SC-GDM+ (n=64)	P value
PP Weight (kg)	74.2 ± 13.1	69.6 ± 12.7	0.052
PP BMI (kg/m^2)	28.8 ± 4.4	26.7 ± 4.5	0.016
PP Waist (cm)	92.6 ± 9.8	92.2 ± 10.3	0.734
PP Hip (cm)	107.9 ± 1.3	105.5 ± 1.1	0.167
PP Systolic BP (mmHg)*	110.5 ± 10.2	104.9 ± 16.3	0.027
PP Diastolic BP (mmHg)	69.5 ± 8.1	68.9 ± 8.3	0.683
PP FBS (mg/dl)*	94.6 ± 10.7	92.2 ± 13.4	0.261
PP GTT2h (mg/dl)	104.4 ± 3.9	105.0 ± 3.2	0.902
PP TG (mg/dl)*	114.2 ± 91.9	116.6 ± 58.2	0.827
PP Cholesterol (mg/dl)	178.7 ± 31.4	186.7 ± 35.8	0.185
PP HDL (mg/dl)	55.1 ± 11.5	55.3 ± 10.8	0.946
PP LDL (mg/dl	100.9 ± 27.8	107.5 ± 26.8	0.179
PP VLDL (mg/dl)	21.6 ± 11.9	22.6 ± 10.9	0.657
PP metabolic syndrome	13 (20.8)	7 (10.9)	0.123
PP GTT 75 g result			0.718
Normal FBS	45 (73.8)	51 (79.7)	
Pre-diabetes (IGT or IFG)	14 (23.0)	11 (17.2)	
DM	2 (3.3)	2 (3.1)	
PP GTT 75 g result			0.433
Normal FBS	45 (73.8)	51 (79.7)	
Glucose intolerance (pre-diabetes+DM)	16 (26.2)	13 (20.3)	
MetS using NCEP ATP III criteria	13 (20.8)	7 (10.9)	0.123
MetS using IDF Criteria	12 (19.4)	11 (17.2)	0.753

The quantitative and qualitative variables are presented as mean ± SD or n (%), respectively. *; These quantitative variables had non-normal distribution and compared between groups by Mann-Whitney test. The normal quantitative variables and the qualitative variables were compared respectively by Student's t and chi-square tests between groups. ART; Assisted reproductive technology, BMI; Body mass index, BP; Blood pressure, FBS; Fasting blood sugar, GDM; Gestational diabetes mellitus, GTT; Glucose tolerance test, HDL; High-density lipoprotein, TG; Triglyceride, LDL; Low-density lipoprotein, VLDL; Very-low-density lipoprotein, DM; Diabetes mellitus, NCEP ATP III; The National Cholesterol Education Program Adult Treatment Panel III, and IDF; International diabetes federation.

Table 4 summarizes the results of the univariate analysis of the association between clinical and biochemical parameters with postpartum glucose intolerance and MetS in total population after adjusting for group. The results demonstrated a significant association between second trimester FBS (OR=1.06, 95% CI: 1.01-1.10, P=0.009), third trimester FBS (OR=1.10, 95% CI: 1.04-1.16, P=0.001), third trimester HbA1c (OR=3.04; 95% CI: 1.02-7.65, P=0.019), family history of diabetes in first relatives (OR=2.54; 95% CI: 1.07-6.01, P=0.034) and prior GDM (OR=4.60, 95% CI: 1.29-16.3, P=0.018) with postpartum glucose intolerance in GDM population. Also, there was a significant association between postpartum MetS with increased pre-pregnancy BMI (OR=1.20, 95% CI: 1.10-1.32, P=0.004), 2nd trimester FBS (OR=1.06; 95% CI: 1.01-1.11, P=0.011), insulin (OR=1.07; 95% CI: 1.01-1.14, P=0.040), and HOMA-IR (OR=1.40; 95% CI: 1.02-1.78, P=0.034), but decreased HDL cholesterol (OR=0.96; 95% CI: 0.92-0.99, P=0.040).

Table 4: Association of clinical and biochemical parameters and postpartum glucose intolerance and metabolic syndrome in GDM population

Variables	PP glucose intolerance (pre-diabetes/diabetes)		PP metabolic syndrome	
	OR* (CI 95%)	P value	OR* (CI 95%)	P value
Age (Y)	1.03 (0.95-1.12)	0.462	1.0 (0.9-1.1)	0.871
Pre-pregnancy BMI (kg/m^2)	1.04 (0.94-1.14)	0.448	1.20 (1.1-1.32)	0.004
2nd trimester systolic BP (mmHg)	1.00 (0.97-1.04)	0.839	1.0 (0.9-1.1)	0.174
2nd trimester diastolic BP (mmHg)	1.03 (0.98-1.09)	0.187	1.0 (0.9-1.1)	0.696
2nd trimester FBS (mg/dl)	1.06 (1.01-1.10)	0.009	1.06 (1.01-1.11)	0.011
2nd trimester HbA1c (mg/dl)	1.34 (0.69-2.57)	0.387	1.30 (0.6-2.8)	0.535
2nd trimester TG (mg/dl)	1.00 (0.99-1.01)	0.781	1.01 (1.001-1.02)	0.016
2nd trimester cholesterol (mg/dl)	0.99 (0.98-1.01)	0.097	1.0 (0.9-1.0)	0.167
2nd trimester HDL, (mg/dl)	0.98 (0.94-1.01)	0.213	0.96 (0.92-0.99)	0.040
2nd trimester LDL (mg/dl)	0.99 (0.97-1.00)	0.117	0.9 (0.8-1.1)	0.122
2nd VLDL (mg/dl)	1.00 (0.97-1.05)	0.662	1.04 (1.0-1.1)	0.074
2nd Insulin (mg/dl)	1.06 (0.99-1.15)	0.083	1.07 (1.01-1.14)	0.040
2nd trimester HOMA-IR	1.25 (0.96-1.62)	0.091	1.40 (1.02-1.78)	0.034
3rd trimester FBS (mg/dl)	1.10 (1.04-1.16)	0.001	1.04 (0.99-1.10)	0.105
3rd trimester HbA1c (mg/dl)	3.04 (1.02-7.65)	0.019	2.21 (0.81-5.94)	0.118
Family history of DM	2.54 (1.07-6.01)	0.034	2.19 (0.83-5.83)	0.113
Gravid	1.17 (0.48-2.80)	0.733	0.80 (0.33-2.41)	0.817
Parity	1.68 (0.61-4.61)	0.313	0.89 (0.20-2.22)	0.517
Prior GDM	4.60 (1.29-16.33)	0.018	0.40 (0.09-1.75)	0.223

***; These ORs were obtained by univariate, BMI; Body mass index, BP; Blood pressure, FBS; Fasting blood sugar, DM; Diabetes mellitus, HDL; High-density lipoprotein, TG; Triglyceride, LDL; Low-density lipoprotein, VLDL; Very-low-density lipoprotein, HbA1C; Hemoglobin A1c, PP; Postpartum, GDM; Gestational diabetes mellitus, OR; Odds ratio, and CI; Confidence interval.**

Multivariable logistic regression presented potential risk factors for MetS and glucose intolerance, 6-12 weeks postpartum (Table 5). The results showed family history of diabetes (OR=3.37, 95% CI: 1.10-10.30, P=0.033) as a predictive factor for early postpartum MetS. In addition, family history of diabetes (OR=2.69, 95% CI: 1.17-6.15, P=0.019) and second trimester FBS (OR=1.06, 95% CI: 1.02-1.11, P=0.004) were independent predictors of glucose intolerance, 6-12 weeks after delivery.

Table 5: Multivariable logistic regression for detection of risk factors of early postpartum metabolic syndrome and glucose intolerance in the study population

Variables	Model 1 for PP Metabolic syndrome OR (95% CI)	P value	Model 2 for PP Glucose intoleranse OR* (95% CI)	P value
Family history of DM	3.37 (1.10-10.30)	0.033	2.69 (1.17-6.15)	0.019
Prior GDM	2.01 (0.40-9.98)	0.393	1.78 (0.52-6.15)	0.363
2nd trimester FBS	1.03 (0.97-1.09)	0.354	1.06 (1.02-1.11)	0.004

*; In this model the women age and BMI were adjusted, GDM; Gestational diabetes mellitus, PP; Postpartum, OR; Odds ratio, CI; Confidence interval, FBS; Fasting blood sugar, and DM; Diabetes mellitus.

Discussion

Metabolic syndrome (MetS) is comprised of a cluster of glucose intolerance, hypertension, and dyslipidemia with abdominal adiposity. It is a well-known predisposing factor for insulin resistance, diabetes mellitus, and cardiovascular diseases and is rising rapidly worldwide particularly in Western and Asian countries (8). A recent meta-analysis reported an increased (approximately 4-fold) risk of MetS after GDM, especially in Caucasian and obese mothers (20).

The present study compared the delivery and postpartum outcomes of GDM between ART and spontaneous pregnancies, with regard to the early MetS and glucose intolerance and their components. Our results showed a higher prevalence of MetS according to the NCEP criteria in the ART-GDM (20.8%) compared to the SC-GDM (10.9%) group. The prevalence of MetS was 19.4% in the GDM-ART and 17.2% in the SC-GDM based on the IDF criteria. Moreover, there was an 88% increase in the risk of developing MetS following ART pregnancy; nevertheless, in our study, the overall differences between the two groups were not statistically significant. Numerous studies have demonstrated higher rates of postpartum MetS in GDM women compared to the control group within 1-11 years postpartum, with large variations according to the length of follow-up (7.5-60% in GDM and 4.6-26% in non-GDM women) (1). However, few investigations have reported MetS at 6-12 weeks after gestational diabetes and there is no evidence in GDM following assisted conception. Recently, Nouhjah et al. (2) observed that the frequency of early postpartum MetS was 18.2% in women with GDM and 11.6% in controls by the NCEP criteria, and 21% in women with gestational diabetes and 15.1% in controls by the IDF criteria.

Current findings showed that the incidence of postpartum glucose abnormalities including pre-diabetes (23 vs. 17.2%) and diabetes (3.3 vs. 3.1%) was not significantly different between GDM after ART and spontaneous conception. In a recent meta-analysis, the prevalence of pre-diabetes and diabetes was respectively 3.9-50.9% and 2.8-58% in Asian women with gestational diabetes within 4 weeks to 15 years postpartum based on the length of follow-up (21). Considerable evidence proposed that beta-cell dysfunction likely contributes to an increase in the risk of glucose intolerance in the first year postpartum in GDM women (22-24).

Based on our data from the univariate analyses, FBS, HbA1c, a family history of diabetes in first relatives, and prior GDM were risk factors for postpartum glucose intolerance in the GDM population. Furthermore, pre-pregnancy BMI, and second trimester levels of FBS, insulin, HDL, and insulin resistance, were risk factors for postpartum MetS in the GDM population. Multivariate analyses confirmed family history of diabetes as an independent predictor of both glucose intolerance and MetS 6 to 12 weeks postpartum, in the GDM population. Additionally, second trimester FBS was another predictive factor of glucose intolerance 6-12 weeks after delivery. Numerous studies have indicated several putative factors for postpartum glucose intolerance including a family history of diabetes, elevated glucose level 120 minutes after a 75-g OGTT, elevated HbA1c levels during GDM diagnosis, perinatal complications, history of GDM, obesity, systolic or diastolic BP, maternal age, parity, and insulin or metformin therapy (25-29). Additionally, a history of GDM, pre-pregnancy overweight or obesity, pregnancy systolic BP, or requiring insulin or metformin were reported as predisposing factors that predict postpartum MetS in the GDM population (1, 19).

Though there were no significant differences in baseline characteristics such as mean maternal age, pre-pregnancy BMI, systolic and diastolic BP between the two groups, interestingly, our findings demonstrated that mean postpartum BMI and systolic BP were significantly higher in the ART-GDM group. In addition, higher incidence of nulliparity, 2nd trimester insulin levels, insulin resistance and 3rd trimester FBS levels were observed in the ART-GDM group. These findings may be related to ART characteristics and drugs especially progesterone using assisted conception. Previous investigations have indicated the association between progesterone administrations, history of the polycystic ovarian syndrome (PCOS), previous ovarian hyper-stimulation syndrome (OHSS) risk with the risk of GDM following ART cycles (11, 13).

Several investigations have reported increased BP, dyslipidemia, and higher fasting glucose levels in ART-conceived children (30-32). The current study showed the impact of mode of conception on delivery and postpartum outcomes of GDM pregnancies, especially in mothers. The pathophysiological mechanisms underlying the MetS are under the debate, but insulin resistance and visceral obesity are considered major causes. The presence of at

least three of five criteria of MetS is linked to an increased risk of heart disease, stroke, and diabetes.

However, it is not clear whether early postpartum raised BMI and BP on future MetS in ART mothers. Moreover, ART mothers were suffering from anxiety and stress, hormonal and environmental alternations, ex vivo manipulations, inflammatory changes, endothelial dysfunction, metabolic disturbance, and medical procedures during their pregnancies (33, 34). These conditions may influence long-term women's health and predispose occurrence of MetS and its components.

According to the present data, the ART-GDM group had a higher risk of maternal complications including PIH and preeclampsia, and adverse fetal and neonatal outcomes such as preterm birth, SGA, NICU admission, and neonatal hypoglycemia, compared to the SC-GDM group. Also, a shorter duration of gestation and a lower mean neonatal weight were observed in the ART-GDM group. It seems that GDM following ART conception increased the risk of undesirable and adverse obstetric and perinatal outcomes compared with SC-GDM. Data from a systematic review and meta-analysis showed that ART singleton pregnancies are associated with higher risks of pregnancy-related complications and adverse obstetric outcomes (35). Previous investigations indicated higher risks of adverse maternal and neonatal outcomes in the ART-GDM group compared to the SC-GDM group (11, 14, 36).

By the way, our study had several limitations that need to be addressed. First, this study lacks information regarding subfertility and infertility treatments during pregnancy. Second, we did not evaluate postpartum MetS and glucose intolerance in the general population. Further prospective studies with a larger sample size, particularly with inclusion a new group (natural pregnancy without GDM) and long-term follow-up are required to verify our results.

Conclusion

The present study indicated a higher rate of MetS in ART women with GDM at 6-12 weeks postpartum compared to SC women with GDM; however, the difference was not statistically significant. Postpartum BMI and systolic BP were significantly higher in the ART-GDM group. Further investigations with larger sample-size and longer follow-up are necessary to verify our findings. Lifestyle modification and long-term health care of ART women with GDM can be recommended.

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Authors' Contributions

A.K., A.M.; The conception. A.K., H.R.B., M.E.Kh.; Design of the work. A.K., R.H., A.A., R.Ch.; The acquisition and analysis. A.K., A.A., F.M.; Interpretation of data. A.K., A.A., R.Ch.; Have drafted the manuscript and revised it. All authors have read and approved the manuscript.

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Original Article

H6PD Gene Polymorphisms (R453Q and D151A) and Polycystic Ovary Syndrome: A Case-Control Study in A Population of Iranian Kurdish Women

Rozita Naseri, M.D.[1], Yosra Alimoradi, M.D.[1], Maryam Sohrabi, M.Sc.[2], Mostafa Cheraghian Fard, M.Sc.[2], Elahe Barzingarosi, M.D.[1], Amir Abdolmaleki, Ph.D.[2], Cyrus Jalili, Ph.D.[2*]

1. Internal Medicine Department, Imam Reza Hospital, Kermanshah University of Medical Sciences, Kermanshah, Iran
2. Department of Anatomical Sciences, Medical School, Kermanshah University of Medical Sciences, Kermanshah, Iran

Abstract

Background: Polycystic ovary syndrome (PCOS) is known as the most common endocrine and metabolic disorder in the reproductive-age women. Due to the effects of PCOS on the physical and mental health, the investigation of the factors affecting the development of PCOS is crucial. Hexose-6-phosphate dehydrogenase (H6PD) is a microsomal enzyme that catalyzes the first two reactions of the oxidative chain of the pentose phosphate pathway. The present study examined the polymorphisms of the *H6PD* gene (R453Q and D151A) in PCOS patients of Iranian Kurdish women.

Materials and Methods: In this case-control study, a total, of 200 female volunteers in two equal groups participated in our study. The PCOS patients were selected based on the Rotterdam diagnostic criteria. The association of *H6PD* gene polymorphisms, D151A and R453Q, with the development of PCOS were investigated. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used for genotyping. Statistical analysis was applied by the SPSS (version 16) software.

Results: Statistically significant lower frequencies of AA+AG genotype (37% vs. 55%, P=0.01) and A allele (22.5% vs. 34%, P=0.01) of R453Q were observed in the patients compared to the controls. In the case of D151A, no significant differences were observed in the frequency of genotypes and alleles between the two groups.

Conclusion: The findings of this study suggest that variants of H6PD R453Q affect the risk of PCOS.

Keywords: Polycystic Ovary Syndrome, Polymerase Chain Reaction, Polymorphism

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Introduction

Polycystic ovary syndrome (PCOS) is known as an endocrine disorder with oligomenorrhea, chronic anovulation, hyperandrogenemia, and polycystic ovary morphology (1). Although the findings of a review study of twins and familial segregation patterns provide convincing evidence for genetic etiology, there is no obvious Mendelian inheritance pattern of PCOS (2); it is widely accepted that multiple genetic and environmental factors and their complex interaction are involved in the PCOS occurrence (3). Multiple disorganizations related to this endocrine disorder can lead to various pathological conditions such as irregular menstruation, hyperandrogenemia, high luteinizing hormone/follicle-stimulating hormone (LH/FSH) ratio, infertility, obesity, insulin resistance, and ovulatory dysfunction (4-6). The etiology of this complex heterogeneous disease is still unknown which has attracted the attention of many scientists (7).

In this complex disorder, PCOS, the genetic and environmental factors are closely related to the insulin resistance that leads to steroidogenesis dysregulation. On the other hand, PCOS is mimicked by steroidogenesis defects that exist in hyperandrogenemia like congenital adrenal hyperplasia and cortisone reductase deficiency (CRD) which possess a monogenic base. Also, CRD results in the 11β-hydroxysteroid dehydrogenase type 1 enzyme (11β-HSD1) regeneration failure (8). CRD is seen in hyperandrogenic signs, including oligo-amenorrhea, hirsutism, and female infertility, as well as premature puberty in males (9). The similarities between the CRD and the PCOS phenotypes are focused on the *HSD11B1* and *H6PD* genes to describe the overloaded androgen and adrenal hyperandrogenism which are common in the PCOS phenotypes (10).

Hexose-6-phosphate dehydrogenase (*H6PD*) gene is located on chromosome 1p36.22, spans 37 kb, including 5 exons. It encodes H6PD protein, a microsomal enzyme that catalyzes the first two reactions of the oxidative chain of the pentose phosphate pathway (11-13). H6PD influences the activity of the 11β-HSD enzyme, which is involved in cortisol metabolism. Also, H6PD through the 11β-HSD en-

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*Corresponding Address: P.O.Box: 1565, Department of Anatomical Sciences, Medical School, Kermanshah University of Medical Sciences, Kermanshah, Iran
Email: cjalili@yahoo.com

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zyme can modulate insulin sensitivity in the adipose tissue (10). It has been reported that mutation in the *H6PD* gene is associated with the PCOS phenotype (4, 8).

Gene polymorphisms are common DNA sequence variations among populations that are important in the development of several hereditary diseases. There are contradictions in the role of two polymorphisms in the *H6PD* gene, namely D151A (rs4603401) and R453Q (rs6688832) in the PCOS phenotype (8, 10, 14, 15). Therefore, a case-control study was designed to investigate the association of these two polymorphisms with PCOS in a population of Kurdish women in the West of Iran.

Materials and Methods

Subjects

In this case-control study, 200 Kurdish women participated from the West of Iran. They are divided into two equal groups, PCOS patients and healthy controls. The healthy control group was matched by demographic characteristics in the patient group. The inclusion and exclusion criteria were based on the revised Rotterdam diagnostic criteria (16): briefly, i. Oligo or anovulation, ii. Sign of hyperandrogenism, iii. Polycystic ovaries. The presence of two items of Rotterdam diagnostic criteria is necessary to consider a definite PCOS. According to the Rotterdam diagnostic criteria, members of each group were verified by a gynecologist. This study was approved by the Ethics Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran (IR.KUMS.REC.1397.260). And also, the informed consent form approved by the KUMS Ethics Committee has been signed by all participants.

DNA extraction

Using the salting-out method, DNA was extracted from peripheral blood samples (17) and stored at -20°C until use. The quality and quantity of isolated DNA samples were evaluated through agarose gel electrophoresis and Nanodrop spectrophotometer (Thermo, USA).

Genotyping

Polymerase chain reaction (PCR) amplification was used to assess the variants of H6PD (R453Q and D151A) followed by the restriction fragment length polymorphism (RFLP) analysis. PCR primers for amplification of the region spanning the selected polymorphisms were designed using NCBI (https://www.ncbi.nlm.nih.gov/) and Oligo 7 software (Table 1). PCR was performed in a total volume of 25 µl containing 2.5 µL of 10X PCR buffer, 20 pmol of each forward and reverse primers (SinaClon BioScience Co., Iran), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (SinaClon BioScience Co., Iran), 1 unit of Taq DNA polymerase (SinaClon BioScience Co., Iran) and about 100 ng of extracted DNA as a template. The thermal cycler program included an initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 40 seconds, annealing at 62°C (R453Q)/64°C (D151A) for 35 seconds, extension at 72°C for 40 seconds, and finally one cycle of extension at 72°C for 5 minutes. To perform RFLP, 10 µl of PCR products were digested with 2 units of MboII restriction enzyme (Thermo, USA) at 37°C for D151A and PstI restriction enzyme (Thermo, USA) at 37°C for R453Q, for 15 hours. Products of enzyme digestion were visualized after 2% agarose gel electrophoresis and staining with GelRed (Kawsarbiotech, Iran) under ultraviolet light (Figs.1, 2).

Table 1: The sequence of primers

NCBI rs#	SNP	Primer sequences (5´-3´)
rs4603401	D151A	F: agctgagccagtaccgccaac R: gctgatgctcacctgcttgccta
rs6688832	R453Q	F: actacgcctacagccctctgc R: ccaggaggccagcaagttctc

SNP; Single nucleotide polymorphism.

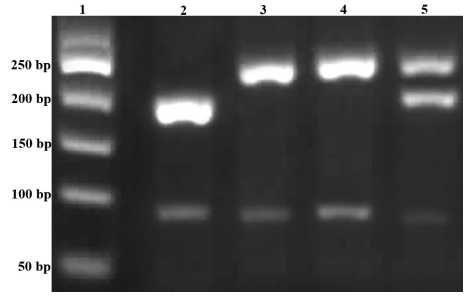

Fig.1: PCR-RFLP products of *H6PD* (D151A) on 2% agarose gel electrophoresis. 1; 50 bp DNA ladder, 2; Homozygote AA, 191 and 83 bp fragments, 3, 4; Homozygote CC, 223 and 83 bp fragments, 5; Heterozygote AC, 223, 191, and 83 bp fragments. PCR-RFLP; Polymerase chain reaction-restriction fragment length polymorphism.

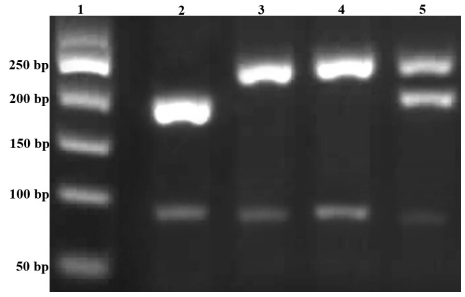

Fig.2: PCR-RFLP products of *H6PD* (R453Q) on 2% agarose gel electrophoresis. 1; 50 bp DNA ladder, 2, 4; Homozygote GG, 113 bp fragment, 3; Homozygote AA, 93 bp fragment, 5; Heterozygote AG, 113 and 93 bp fragments, and PCR-RFLP; Polymerase chain reaction-restriction fragment length polymorphism.

Statistical analysis

A statistical package for social sciences (IBM SPSS vs. 16, Chicago II, USA) was used for statistical analysis. Pearson's chi-square (χ^2) test analyzed genetic distribution variations between two groups. The odds ratios (OR), with 95% confidence intervals (95% CI), were measured with an unconditional logistic regression model.

Results

The mean age of the patient group was 27.09 ± 5.19 that in comparison with the control group (28.34 ± 4.58)

showed no significant difference (P=0.073). In terms of body mass index (BMI), a significant difference was observed, (29.14 ± 2.68 vs. 23.05 ± 2.48 for patient and control groups, respectively (P=0.001).

The frequency of *H6PD* genotypes in patients with PCOS was in Hardy-Weinberg equilibrium (P=0.27 for D151A, P=0.09 for R453Q).

The results of this study showed that the genotypic frequencies of the D151A polymorphism have no significant differences in the patient group in comparison with the control group (Table 2). Genotype frequencies of the R453Q polymorphism showed a significant difference between our groups. For R453Q polymorphism, the 'AG' genotype was associated with a 0.49-fold decreased risk of PCOS (P=0.023); Also, the A-allele compared to the G-allele reduced the risk of PCOS by approximately 0.56-fold (P=0.01, Table 2). We observed a 0.51-fold decrease in the risk of PCOS in the carriers of the genotypes 'AA' (for D151A), 'AA' (for R453Q), and also the carriers of the genotypes 'AA' (or D151A), 'AG' (for R453Q) (P=0.02). Also, there was no significant correlation among other combinations of the D151A and R453Q polymorphisms with PCOS risk (Table 3).

Table 2: Allele and genotype frequencies of *H6PD* D151A and R453Q

Genotypes\ Alleles	Controls n (%)	Patients n (%)	P value	OR (95% CI)
D151A				
AA	86 (86)	82 (82)		1
AC	13 (13)	16 (16)	0.53	1.29 (0.58-2.85)
CC	1 (1)	2 (2)	0.55	2.097 (0.186-23.57)
AC+CC	14 (14)	18(18)	0.44	1.35(0.63-2.89)
A	185 (92.5)	180 (90)		1
C	15 (7.5)	20 (10)	0.378	1.37 (0.68-2.76)
R453Q				
GG	45 (45)	63 (63)		1
AG	42 (42)	29 (29)	0.023	0.49 (0.27-0.91)
AA	13(13)	8 (8)	0.09	0.44 (0.17-1.15)
AA+AG	55 (55)	37 (37)	0.01	0.48 (0.27-0.85)
G	132 (66)	155 (77.5)		1
A	68 (34)	45 (22.5)	0.01	0.56 (0.36-0.88)

OR; Odds ratio, CI; Confidence interval, Pearson's chi-square (χ^2) test, (95% CI) with an unconditional logistic regression model.

Table 3: Calculation of the odds ratio between case and control groups for the combination of D151A and R453Q genotypes of *H6PD*

D151A	R453Q	OR (95% CI)	P value
AA	GG	1.63 (0.92-2.85)	0.089
AC+CC	GG	2.14 (0.77-5.94)	0.14
AA	AG+ AA	0.51 (0.28-0.9)	0.02*
AC+CC	AG+ AA	0.73 (0.24-2.19)	0.58

OR; Odds ratio, CI; Confidence Interval, Pearson's chi-square (χ^2) test, (95% CI) with an unconditional logistic regression model, and *; Statistically significant.

Discussion

Based on the importance of the single nucleotide polymorphisms in the development of the disease, such as PCOS and also, the important role of the *H6PD* gene in the human endocrine regulation (4), the *H6PD* (R453Q and D151A) variants were assessed in a population of Kurdish women from the West of Iran.

In the present study, we detected a significant difference in body mass index (BMI) between healthy controls and PCOS patients, although there were studies that reported a higher BMI and fat distribution could increase the PCOS risk (18-21).

Although the exact effect of H6PD on the PCOS phenotypes remains unknown, it seems that the H6PD has a specific role in influencing the function of the hypothalamic-pituitary-gonadal axis in humans (4). Some genetic studies reported that the *H6PD* variants are related to insulin resistance, obesity, and hyperandrogenism in PCOS patients (8, 22). However, the ovaries are the primary source of increased androgens in the syndrome, 20-30% of PCOS patients have adrenal androgen excess (23). In an animal study, the inactivation of the *H6PD* gene was associated with abnormalities of the hypothalamic-pituitary-adrenal axis, glucose homeostasis, and lipid metabolism (24). For the first time, in a population of Iranian women, we confirmed an association between the PCOS phenotype and the *H6PD* R453Q. In the present study, we observed that the 'A' allele of R453Q is less frequent in the patient group in comparison with the control group. Studies in different populations and ethnicities suggested that the H6PD R453Q polymorphism may be related the PCOS (4, 10, 14, 15). San Millan et al. (15) showed homozygosity for the 'G' allele of H6PD R453Q was more frequent in the patients with PCOS phenotype. In addition, they proposed that the variants of *H6PD* R453Q probably affect the adrenal activity involved in PCOS. Ju et al. (4) showed that the R453Q variant of the *H6PD* gene may affect the stability of the mRNA structure or its interaction with other macromolecules. *In vitro* study with H6PD as a bifunctional enzyme demonstrated that the R453Q preserved the two activities of this enzyme (25). However, *in vivo* consequences of this enzyme activity is not clear; thus the possibility can exist that the correlation of PCOS and R453Q may be due to variation in the activity of the H6PD enzyme or linkage imbalance with unknown variants of the *H6PD* gene (10). Despite several studies that confirm our results, several studies showed contradictory results. In the Ju et al. (4) study, the association between clinical features of PCOS and *H6PD* polymorphisms was investigated, and concluded that the FSH level and phenotype of hyperandrogenism of PCOS were associated with the 'AG' genotype of *H6PD* R453Q (rs6688832) in the PCOS patients. They also declared the 'GG' genotype and 'G' allele of R453Q likely have a protective role against PCOS. In another study, it was reported that the *H6PD* R453Q variant is associated with PCOS which has a modifying role in the phenotype of PCOS by adrenal hyperactivity improvement in the carriers of the 'A' allele (15).

A second genetic polymorphism in our study was *H6PD* D151A, which was not associated with the PCOS phenotype. Martínez-García et al. (10) reported that allele A151 was more frequent in obese women, particularly those with PCOS phenotype. So far, no functional studies reported the effects of D151A polymorphism on the activity of H6PD.

In addition, in our study, the interaction of the 'AA' genotype of D151A with the 'AA+ AG' genotypes of R453Q

significantly showed a reduction in reducing the PCOS phenotype development. It seems the D151 and R453 alleles, simultaneously, have a protective role against PCOS. Although, the interpretation of genetic studies should be done cautiously, and to confirm these results, studies in larger populations are necessary. San Millan et al. (15) declared that the compensatory hyperinsulinemia could elucidate the increased concentrations of androstenedione in the 'A' allele carriers (*H6PD*, D151A), whiles an increase in the local cortisol levels in the 'Q' allele carriers (*H6PD*, R453Q) would reduce the adrenal stimulation. This reduction in adrenal stimulation occurs by the protective effects of adrenocorticotropin against adrenal androgen excess and PCOS. The main limitation of the study lies in the fact that the BMI in the patient group was higher than in the control group. Although, the frequency of alleles between BMI groups was not significant (data were not shown).

Conclusion

The findings of this study suggest a protective effect of the R453 allele against the occurrence of PCOS. Due to the complexity of interaction in many genetic and environmental factors involved in this disorder, further studies are required to confirm these results in different ethnicity and larger sample size.

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Authors' Contributions

R.N., C.J.; Participated in conception, study design, and overall supervision. M.S., A.A.; Conducted molecular experiments (DNA extraction, PCR-RFLP analysis) and drafting. Y.A., M.C.F., E.B.; Contributed extensively to the statistical analysis, interpretation of the data, and the conclusion. All authors performed editing and approved the final version of this manuscript for submission, also participated in the finalization of the manuscript.

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Original Article

Therapeutic Effects of Eugenol in Polycystic Ovarian Rats Induced by Estradiol Valerate: A Histopathological and A Biochemical Study

Zahra Kokabiyan, M.Sc.[1], Parichehreh Yaghmaei, Ph.D.[1*], Seyed Behnamedin Jameie, Ph.D.[2], Zahra Hajebrahimi, Ph.D.[3]

1. Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
2. Department of Anatomy, Neuroscience Research Center, Iran University of Medical Sciences, Tehran, Iran
3. A&S Research Institute, Ministry of Science Research and Technology, Tehran, Iran

Abstract

Background: Polycystic ovary syndrome (PCOS) is a common type of endocrinopathy in women which is accompanied by androgens elevation, insulin resistance, and metabolic dysfunction. Eugenol is a phenolic component of clove oil that has an antioxidant, anti-inflammatory, and anti-diabetic activity. The present study aimed to evaluate the therapeutic effects of eugenol on the PCOS models of rats.

Materials and Methods: In this experimental study, thirty adults female Wistar rats weighing between 180 and 200 g were used. Estradiol valerate-induced PCOS rats (4 mg/rat) were treated with eugenol (12 and 24 mg/kg) for 28 days. The effects of eugenol were studied on levels of glucose, lipid profile, liver enzymes, reproductive hormones, oxidative stress, and the expression of cyclooxygenase-2 (*Cox-2*) and peroxisome proliferator-activated receptor alpha (*Ppar-α*) genes, using biochemical analysis of blood and histopathological evaluation of ovaries.

Results: Estradiol valerate-induced PCOS resulted in the formation of cystic follicles in the ovaries, hyperinsulinemia, hyperglycemia, hyperlipidemia, hyperandrogenism, and anovulation. It altered the *Cox-2* and *Ppar-α* gene expression and increased oxidative stress and activities of liver enzymes. Eugenol treatment improved the PCOS-associated endocrine and metabolic disorder and histopathological alterations, mostly through antioxidant, anti-diabetic, anti-hyperlipidemic, and anti-androgenic properties. It showed beneficial effects on serum glucose, serum insulin, fat profile, reproductive hormones, liver activity, oxidative stress, expression of *Cox-2* and *Ppar-α* genes, as well as restoration of normal ovulation in the PCOS animals.

Conclusion: Eugenol could represent a promising natural product to prevent PCOS or reduce its symptoms.

Keywords: *Cox-2*, Eugenol, Metabolic Dysfunction, Polycystic Ovary Syndrome, *Ppar-α*

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Introduction

Polycystic ovary syndrome (PCOS) is a highly frequent hormonal disease that affects women in their childbearing ages. The symptoms of PCOS vary with each woman; however, it is marked especially by amenorrhea, hirsutism, hyper androgens (male hormone), hyperinsulinemia, obesity, ovarian enlargement, ovulatory dysfunction, and infertility. PCOS is one of the important reasons for female infertility. In women with this syndrome, the irregularity of hormones interferes with the monthly growth and release of eggs from the ovarian tissues which is called ovulation (1).

The main reason for PCOS is not fully understood, although it usually begins with an increase in levels of reproductive hormones such as luteinizing hormone (LH), androgen, or estrogen. These conditions result in an abnormal cycle of gonadotropin secretion by the pituitary gland (1). There is evidence that resistance to insulin and an increase in the amount of insulin can stimulate ovarian/adrenal androgen secretion. Studies have shown that most women with PCOS suffer from insulin resistance and also show type 2 diabetes and cardiovascular disorder in comparison with healthy women. Obesity is a major cause of insulin resistance (2). There are also growing concerns about a link between PCOS and systemic inflammation as evidenced by a significant increase in inflammatory factors including C-reactive protein (CRP), interleukin-18, monocyte chemoattractant protein-1, white blood count, and increased oxidative stress. Elevated CRP is an independent prediction marker for the risk of cardiovascular disorder and type 2 diabetes that highlights the role of inflammation in PCOS (3). In addition, it has been proven that oxidative stress in women with PCOS increases, and the relation between oxidative stress and inflammation is undeniable (4). Therefore, this supports the association of PCOS with systemic inflammation.

Various therapeutic strategies are used for clinical management of PCOS such as lifestyle changes including weight loss, diet, and exercise for women's obesity and

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***Corresponding Address: P.O.Box: 14515-775, Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran**
Email: yaghmaei_p@srbiau.ac.ir

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anovulation; ovulation-inducing oral medications; and lowering insulin levels by taking diabetes medications such as metformin (5). In recent decades, considerable attention has been paid to herbal drugs in both developing and developed countries instead of the use of chemical drugs because of their minimal side effects, easy availability, and comfortable administration (6, 7). Eugenol (4-allyl-2-methoxy phenol) is a phenolic component of clove oil extracted from a variety of other plants such as Eugenia caryophyllus and Myristicafragrans. Anti-inflammatory and antioxidant activities of eugenol have been reported (8). Eugenol administration has been shown to suppress NF-κB activation in a rat model of gastric carcinogenesis (9) that has an essential role in inflammation (10).

Because of the importance of infertility treatment in women and the significant role of PCOS in anovulation, here we examined the therapeutic potential of eugenol on the ovulation improvement, liver activity, and cyclooxygenase-2 (*Cox-2*) and peroxisome proliferator-activated receptor alpha (*Ppar-α*) genes expression in a Wistar rat model of PCOS. COX-2 has cyclooxygenase and peroxidase functions that give rise to oxidative stress (11). In addition, studies have demonstrated that Cox-2 is expressed following the activation of NF-κB in inflammatory reactions (10). *Ppar-α*, also known as NR1C1 (nuclear receptor subfamily 1, group C, member 1), is a nuclear hormone receptor and a regulator of lipid and glucose metabolism and has anti-inflammatory activity. It has been shown that Ppar-α can decrease inflammation through the inhibition of the signaling pathway of NF-κB or reducing the level of activated NF-κB (12).

Materials and Methods

Experimental animals

In this experimental study, 30 adults female Wistar rats weighing between 180 and 200 g were purchased from Pasteur Institute of Tehran, Iran. Animals were kept in the animal house of Islamic Azad University, Science and Research Branch, Tehran, Iran, under standard laboratory conditions of constant temperature (22°C) and humidity with a 12H: 12H cycle of light and dark. They had free access to a normal diet and water. Animals were taken care of following the Guide for the Care and Use of Laboratory Animals (13). All procedures explained in this study were validated by the Animal Care and Use Committee of Islamic Azad University (IR.IAU.SRB.REC.1396.51). All animals were weighed weekly and body weight changes were recorded throughout the experiment. To familiarize the rats with the laboratory environment, animals were kept in the laboratory for 14 days without any intervention.

Intraperitoneal injection of a single dose of estradiol valerate (4 ml, 4 mg/rat, or 16 mg/kg; Aburaihan Pharmaceutical Co, Tehran, Iran) was used to induce PCOS during 28 days (14). To confirm PCOS induction, histological analysis of ovaries and hormonal and biochemical analysis of blood were performed on three rats.

Animals were randomly divided into 5 groups (each group consists of 6 rats) as follows:

i. The control group (C) receiving a normal diet. ii. The PCOS group receiving a normal diet. iii. Sham operation group: PCOS animals (as control of PCOS) which received intraperitoneal injection of the tween (80%) as eugenol solvent for 28 days. iv. Experimental group 12 (EXP-12): PCOS animals that received intraperitoneal injection of eugenol at a dose of 12 mg/kg body weight (Sigma Chemical Co, St. Louis, MO, USA) for 28 days. v. Experimental group 24 (EXP-24): PCOS animals that received intraperitoneal injection of eugenol at a dose of 24 mg/kg body weight for 28 days.

Hormonal and biochemical analysis

Ovary, liver tissues, and serum samples were collected at the end of the experiment as described previously (15). Briefly, anesthesia was performed using ketamine (80 mg/kg) and xylazine (10 mg/kg), and blood samples were obtained from the heart. After 2 hours of incubation at room temperature, centrifugation was done at 2500 × g for 5 minutes for serum separation. Serum samples were kept at -20°C until further analysis. The concentration of total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG), glucose, aspartate transaminase (AST/GOT) enzyme, alanine transaminase (ALT/GPT) enzyme, and alkaline phosphatase (ALP) enzyme were measured by quantitative photometric kits purchased from Parsazmun Company (Karaj, Iran) based on the manufacturer's recommendations. Also, a blood glucose meter (Accu-Chek, Germany) was used to determine the amount of whole blood sugar in the blood collected from the animal tail.

Serum levels of estradiol and progesterone were determined using rat ELISA kits (Monobind Co., USA) based on the manufacturer's instructions. Levels of insulin, testosterone, LH and follicle-stimulating hormone (FSH) were measured using rat/mouse ELISA test kits (Cosmo Bio Co. Ltd. Japan) as per the manufacturer's instructions.

To evaluate liver superoxide dismutase (SOD) and malondialdehyde (MDA), small fractions of liver tissue were removed and homogenized in ice-cold KCL (0.15 M, 3 ml/g liver tissue). The liver homogenate was centrifuged at 10000 rpm at 4ºC for 20 minutes and then the supernatant was separated and stored at -70ºC. Nasdox and Nalondic ELISA kits were used to evaluate the activities of SOD and MDA, respectively (Navand Salamat Co., Urmia, Iran) according to the manufacturer's instructions.

Histological analysis

Ovaries and pancreas were collected and examined for histopathological assays at the end of the procedure. Briefly, ovaries samples were removed and trimmed free of fat. Fixation was done using 10% paraformaldehyde for 24 hours. Then samples were dehydrated and cleared using alcohol and xylene solutions, placed in paraffin, sectioned at a thickness of 7 μm by a microtome, and then mounted on

glass slides. Finally, samples were stained with Hematoxylin and Eosin (H&E) and imaged under a conventional light microscope. The number of Primary, secondary (or antral follicle), Graafian follicles, Corpus luteum, and follicular cysts were assayed in all animal ovaries according to Erickson's classification (16). Images of islets were taken in pancreas samples using a light microscope.

Gene expression analysis by quantitative real-time polymerase chain reaction

Rats were sacrificed at the end of the experiment and ovarian tissue was removed and kept in liquid nitrogen and stored at -75°C until the time of use. Total RNA was extracted using a High Pure RNA Isolation kit (Roche, Germany) following the manufacturer's instructions.

The integrity of RNA was assessed by agarose gel electrophoresis and also measured by a Nanodrop (Thermo Scientific, USA) at 260 and 280 nm. One microgram of total RNA was used for the cDNA synthesis, using Prime ScriptTM 1st strand cDNA Synthesis kit (Takara, Japan) in a final volume of 20 µl and according to the manufacturer's recommendations. DNase I treatment was included in the cDNA synthesis process. The reaction was terminated by incubation for 5 minutes at 70ºC. The cDNA product was stored at -20ºC until use.

The mRNA levels of *Cox-2* and *Ppar-α* were studied using quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as a reference (internal control) gene to normalize *Cox-2* and *Ppar-α* expression levels.

Gene-specific primers were designed using Allele ID 6.0 software and then synthesized by Macrogen (South Korea). The specificity of primer sequences for the interesting genes was checked using BLAST search. The rat primers were:

Ppar-α-
F: 5'- GTTGAAAACAAGGAGGCAGAGG-3'
R: 5'-GAGGACAGCATGGTGAAGATGG-3'
Cox-2-
F: 5'- GGAGAGACGATCAAGATAGTGA-3'
R: 5'- GAGGATGGAGTTGTTGTAGAG-3'
Gapdh-
F: 5'- GGATAGTGAGAGCAAGAGAGAGG-3'
R: 5'- ATGGTATTGGAGAGAAGGGAGGG-3'

Real-time RT-PCR was run on a StepOnePlus Real-Time PCR (Applied Biosystems, USA) using the SYBR green PCR master mix (Takara, Japan). 10 µl of master mix, 0.8 µl of each primer, 0.4 µl of ROX reference dye II, 1 µl of cDNA template, and 7 µl of RNase free water were added. Real-time PCR was programmed into two steps: initialization at 94ºC for 30 seconds, amplification for 40 cycles with denaturation at 95ºC for 5 seconds, annealing at 60ºC for 30 seconds, and extension at 72ºC for 30 seconds. To ensure the specificity of amplification, dissociation curve analysis was performed for all genes. Change in the fold number was calculated by using the $2^{-\Delta\Delta Ct}$ method. RT-qPCR GraphPad Prism 6 software (GraphPad Software Inc., SanDiego, USA) was used to analyze the data.

Statistical analysis

SPSS statistical software (version 17; SPSS Inc., Chicago, USA) was used for the data analysis. All data are presented as the means ± SEM. Shapiro-Wilk and Levine tests were performed to examine the normality of data distribution and homogeneity of variances. One-way analysis of variance (One-Way ANOVA) with t test and duncan test was used for analyzing the data and comparison of the different group means. $P<0.05$ was considered as significant.

Results

Measurement of weight, biochemical characteristics, hormones, and enzymes

The serum levels of glucose, insulin, TG, TC, and LDL were significantly higher in PCOS and sham operation groups as compared to the control group. Administration of eugenol at a dose of 24 mg/kg body weight decreased their serum value (EXP-24 group). Administration of estradiol valerate decreased the serum amount of HDL in PCOS and sham rats in comparison to control ones. However, its level nearly increased to the normal value following eugenol administration in the Exp-24 group. In contrast to EXP-24, eugenol treatment at a concentration of 12 mg/kg (EXP-12 group) only improved the serum levels of TG and HDL and did not affect the value of glucose, insulin, TC, and LDL (Table 1).

Table 1: Serum levels of glucose, TG, TC, LDL, HDL, and insulin in PCOS and non-PCOS rats

Group	Glucose (mg/dl)	TG (mg/dl)	TC (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	Insulin (µIU/ml)
Control	93.75 ± 2.75	53.67 ± 4.10	68.00 ± 2.74	34.40 ± 1.55	31.00 ± 0.58	0.146 ± 0.008
PCOS	129.60 ± 6.36**	76.00 ± 2.31**	82.75 ± 2.43**	42.50 ± 0.69**	22.68 ± 1.03**	0.204 ± 0.013**
Sham	125.00 ± 6.45**	73.75 ± 2.25**	86.33 ± 3.18**	42.63 ± 0.71**	22.50 ± 1.44**	0.199 ± 0.018*
EXP-12	115.67 ± 3.18	57.67 ± 1.20##	78.00 ± 1.00	40.40 ± 0.92*	28.00 ± 1.41#	0.183 ± 0.014
EXP-24	100.67 ± 4.78##	54.00 ± 2.89##	69.00 ± 2.35#	35.60 ± 0.60#	29.67 ± 1.20#	0.144 ± 0.004##

Values are presented as mean ± SEM from 5 rats in each group. TG; Triglyceride, TC; Total cholesterol, LDL; Low-density lipoprotein, HDL; High-density lipoprotein, PCOS; Polycystic ovary syndrome, EXP-12; Experimental group 12, EXP-24; Experimental group 24, *; Statistically different from the control rats, #; Statistically different from the PCOS rats, *, #; P≤0.05, and **, ##; P≤0.01.

The serum amounts of LH, FSH, testosterone, progesterone and estradiol hormones are presented in Table 2. We observed that injection of estradiol valerate for 28 days decreased the serum level of FSH and progesterone and increased the levels of LH, testosterone, and estradiol in PCOS and sham rats in comparison to control rats. However, their levels nearly reached the normal value following eugenol administration inEXP-12 and Exp-24 groups (except for the LH hormone in the EXP-12 group).

The content of SOD decreased, while the MDA, AST, ALT, and ALP significantly increased in the PCOS and sham rats in comparison to the control rats (Table 3). However, their amount improved to near normal levels following eugenol administration in EXP-12 and EXP-24 groups (except for the AST and ALT enzyme in EXP-12 rats).

Gene expression analysis by real-time polymerase chain reaction

The relative expression of *Cox-2* and *Ppar-α* genes was studied using quantitative real-time RT-PCR.

The expression of *Cox-2* increased, while the expression of the *Ppar-α* gene significantly decreased in the PCOS and sham rats in comparison to the control rats (Fig.1). Eugenol treatment at a concentration of 24 mg/kg improved the expression of *Cox-2* and Ppar-α genes in the EXP-24 group. The expression of *Cox-2* decreased, while the expression of *Ppar-α* significantly increased in the EXP-24 group after eugenol administration. In contrast to EXP-24, eugenol treatment at a concentration of 12 mg/kg (EXP-12 group) had no effect on the expression levels of *Cox-2* and *Ppar-α* gene in PCOS rats.

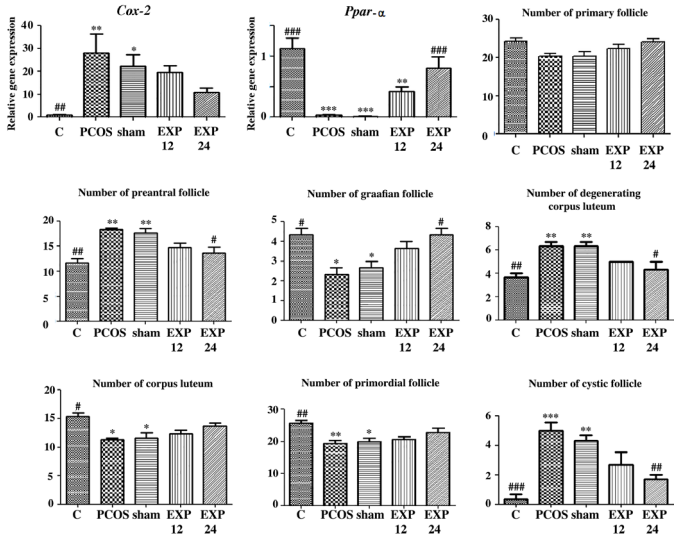

Fig.1: The expression of *Cox-2* and *Ppar-α* genes, and the number of primary follicles, preantral follicles, Graafian follicles, degenerating corpus luteum, corpus luteum, primordial follicles, and follicular cysts in all animal groups. C; Control, PCOS; Estradiol valerate-induced polycystic ovarian rat with a normal diet, Sham; Control PCOS rat with IP injection of tween as eugenol solvent, EXP-12; PCOS rats with IP injection of eugenol (12 mg/kg body weight), EXP-24; PCOS rats with IP injection of eugenol (24 mg/kg body weight), *; Statistically different from the control rats, $^{\#}$; Statistically different from the PCOS rats, *, $^{\#}$; P≤0.05, **, $^{\#\#}$; P≤0.01, and ***, $^{\#\#\#}$; P≤0.001.

Table 2: Serum levels of LH, FSH, testosterone, progesterone, and estradiol in PCOS and non-PCOS rats

Group	LH (mIU/ml)	FSH (mIU/ml)	Testosterone (ng/ml)	Progesterone (ng/ml)	Estradiol (pg/ml)
Control	0.36 ± 0.005	0.49 ± 0.002	0.17 ± 0.035	3.23 ± 0.273	2026.0 ± 16.74
PCOS	0.47 ± 0.003***	0.34 ± 0.003***	3.24 ± 0.074***	2.16 ± 0.130**	2413.5 ± 40.74***
Sham	0.45 ± 0.006***	0.34 ± 0.005***	3.33 ± 0.118***	2.17 ± 0.189**	2469.7 ± 28.58***
EXP-12	0.42 ± 0.015$^{**, \#}$	0.40 ± 0.007$^{***, \#\#\#}$	0.31 ± 0.045$^{\#\#\#}$	2.98 ± 0.107$^{\#}$	2142.7 ± 44.05$^{\#\#}$
EXP-24	0.38 ± 0.005$^{\#\#\#, +}$	0.49 ± 0.002$^{\#\#\#, +++}$	0.58 ± 0.086$^{*, \#\#\#}$	3.18 ± 0.028$^{\#\#}$	2033.0 ± 11.00$^{\#\#\#}$

Values are presented as mean ± SEM from 5 rats in each group. LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, PCOS; Polycystic ovary syndrome, EXP-12; Experimental group 12, EXP-24; Experimental group 24, *; Statistically different from the control rats, $^{\#}$; Statistically different from the PCOS rats, $^{+}$; Statistically different from the EXP_12 rats. *, $^{\#}$, $^{+}$; P≤0.05, **, $^{\#\#}$; P≤0.01, and ***, $^{\#\#\#}$, $^{+++}$; P≤0.001.

Table 3: SOD and MDA contents in liver tissue and serum levels of AST, ALT, and ALP in PCOS and non-PCOS rats

Group	SOD (U/ml)	MDA (µL)	AST (U/L)	ALT (U/L)	ALP (U/L)
Control	0.49 ± 0.034	3.46 ± 0.075	149.50 ± 5.14	143.00 ± 5.80	971.7 ± 40.45
PCOS	0.27 ± 0.006***	5.02 ± 0.277***	232.40 ± 8.72**	215.20 ± 10.91***	1229.0 ± 28.29**
Sham	0.28 ± 0.021***	4.94 ± 0.113***	223.33 ± 14.53*	210.00 ± 10.00**	1212.5 ± 31.46**
EXP-12	0.42 ± 0.003$^{\#\#}$	3.53 ± 0.084$^{\#\#\#}$	189.00 ± 14.74	186.00 ± 8.88*	1022.7 ± 15.84$^{\#}$
EXP-24	0.49 ± 0.008$^{\#\#\#}$	3.74 ± 0.174$^{\#\#}$	159.33 ± 18.55$^{\#\#}$	149.50 ± 7.41$^{\#\#}$	975.7 ± 55.64$^{\#\#}$

Values are presented as mean ± SEM from 5 rats in each group. SOD; Superoxide dismutase, MDA; Malondialdehyde, AST; Aspartate transaminase, ALT; Alanine transaminase, ALP; Alkaline phosphatase, PCOS; Polycystic ovary syndrome, EXP-12; Experimental group 12, EXP-24; Experimental group 24, *; Statistically different from the control rats, $^{\#}$; Statistically different from the PCOS rats, *, $^{\#}$; P≤0.05, **, $^{\#\#}$; P≤0.01, and ***, $^{\#\#\#}$; P≤0.001.

Histological observation

Morphological studies of the ovaries were done after 28-day administration of eugenol. A comparison of ovarian samples from different groups showed distinct morphological alterations in the ovaries. Injection of estradiol valerate during 28 days induced the formation of cystic follicles in PCOS and sham operation groups (Figs.1, 2). In comparison with the control group, counting the number of follicles in different stages in PCOS and sham operation animals specified that the injection of estradiol valerate decreased the number of primordial follicles, Graafian follicles, and corpus luteum; however, it increased the number of preantral follicles and degenerating corpus luteum. The administration of eugenol improved the histological features of the ovaries. Treatment with eugenol (12 or 24 mg/kg), markedly decreased the number of follicular cysts in EXP-12 and EX-24 groups. Furthermore, the administration of eugenol significantly increased the number of primordial follicles, Graafian follicles, and corpus luteum; however, it decreased the number of preantral follicles and the number of degenerating corpus luteum in the treated animals. Administration of estradiol valerate or eugenol had no effect on the number of primary follicles.

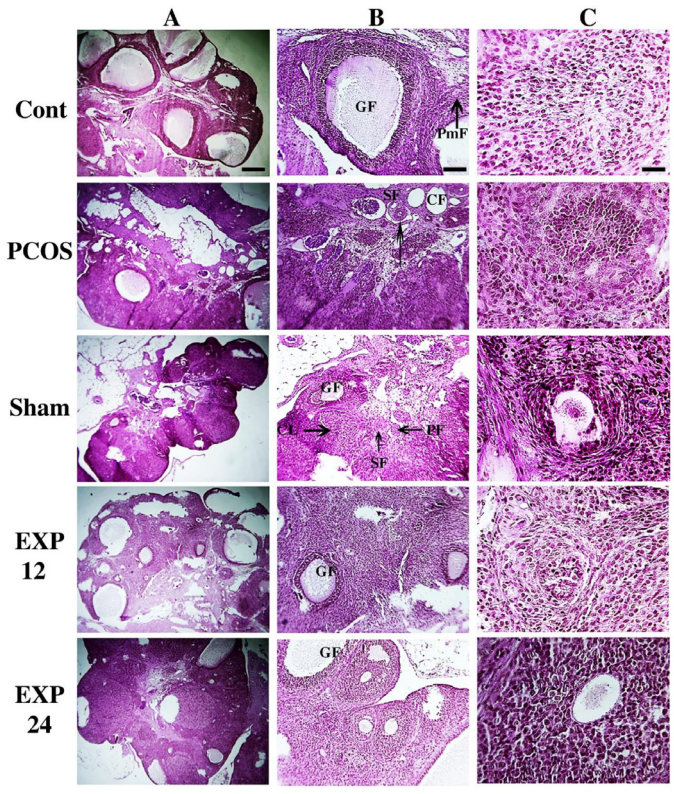

Fig.2: Photomicrograph of a section in ovarian tissues in all animal groups using hematoxylin and eosin staining. **A.** 200 µm scale bar, **B.** 100 µm scale bar, and **C.** 50 µm scale bar. Cont; Control, PCOS; Estradiol valerate-induced polycystic ovarian rat with a normal diet, Sham; Control PCOS rat with IP injection of tween as eugenol solvent, EXP-12; PCOS rats with IP injection of eugenol (12 mg/kg body weight), EXP-24; PCOS rats with IP injection of eugenol (24 mg/kg body weight), PmF; Primordial follicle, PF; Primary follicle, SF; Secondary follicle, GF; Graafian follicle, CF; Cystic follicle, and CL; Corpus luteum.

Discussion

This study reported the potential therapeutic effects of eugenol on the improvement of the physiological changes of PCOS rats against ovarian toxicity induced by estradiol valerate. In the present work, treating rats with estradiol valerate resulted in significant histological and biochemical changes such as the formation of cystic follicles in the ovaries, hyperinsulinemia, hyperglycemia, hyperlipidemia, hyperandrogenism, anovulation, and increased oxidative stress and activities of liver enzymes indicating the development of PCOS. Previous studies of rodent models of PCOS have also shown hormonal changes, histological alterations of ovaries, and metabolic disorders (15, 17-19).

Many studies have used estradiol valerate to model PCOS in rodents (15, 17-19). Estradiol valerate is a long-acting estrogen that disrupts the secretion of hypothalamic gonadotrophin-releasing hormone (GnRH). This hormone is responsible for the secretion of FSH and LH from the anterior pituitary. Studies have reported impaired secretion of GnRH disrupts the release and storage of LH (17). In this study, administration of estradiol valerate caused a markedly increase in LH, estradiol, and testosterone and a significantly decrease in FSH and progesterone hormones similar to the characteristic of hormonal alterations in women with PCOS disorder (1). Decreased progesterone production indicates anovulation that similar to the human PCOS (1) and other rat models of estradiol valerate-induced PCOS (17-19). Hyperandrogenism (high level of testosterone) is the most typical hormonal change of PCOS that has an important role in the pathology of PCOS. Many studies of rats with estradiol valerate-induced PCOS model also have shown changes in sex hormonal secretion (18, 19).

The histological alterations in ovaries may be the result of the hormonal dysregulation caused by estradiol valerate. It is well demonstrated that pituitary gonadotropins (FSH and LH) are the main regulators of ovarian follicle evolution, oocyte maturation, and ovulation (20, 21). FSH stimulates the proliferation of granulosa cells and induces the production of estrogen hormone. It is also involved in follicle evolution from primary follicle to preantral follicle (20). LH is required for androgen synthesis and induces the onset of meiosis in oocytes, as well as the development of the corpus luteum and ovulation (21). In the present study, it has been observed that estradiol valerate-induced hormonal changes led to abnormal follicular development and an increase in cystic follicles. The number of primordial follicles, Graafian follicles, and corpus luteum, decreased markedly in PCOS animals, suggesting anovulation. This was while the number of preantral follicles increased significantly in PCOS rats, as compared to the control ones. Therefore, based on the analysis of ovaries, injection of estradiol valerate resulted in the formation of cystic follicles and a decrease in ovulation and the number of corpus luteum, probably due to hormonal alterations. In line with this data, previous

reports have revealed that continuous treatment of rats with steroid hormones causes anovulation (22).

Due to symptoms such as hyperinsulinemia, dysglycemia, and dyslipidemia, PCOS has been suggested to be a metabolic disorder (2). Data from the present study suggest that levels of blood glucose and insulin are markedly higher in PCOS than in the control animals, which is the property of insulin resistance. The cause of decreased insulin sensitivity in PCOS is not yet well understood, but factors that compensate for insulin resistance often have a therapeutic effect on the outcomes of the disease. A study by Holmäng et al. (23) demonstrated that continuous exposure to androgens leads to insulin resistance due to effects on the transportation of glucose. In line with the present study, elevated testosterone levels may be partly involved in insulin resistance.

Insulin resistance may be the result of oxidative stress, too. There is supportive evidence that oxidative stress plays a critical role in the pathology of diabetes and the development of insulin resistance. Reactive oxygen species (ROS) can decrease insulin sensitivity by affecting insulin signal transduction and decreasing the expression of GLUT4 transporter (glucose transporter type 4) on the cell surface (24). On the other hand, oxidative stress could play a role in the development of PCOS, too. There are many reports on the role of oxidative stress in the pathology of PCOS (25). In this study, we showed that the induction of PCOS using estradiol valerate generated oxidative stress. Our data revealed that the serum levels of MDA significantly increased in the PCOS rats, while the levels of SOD decreased. MDA is a widely known biomarker for oxidative stress. It is the final product of the peroxidation of polyunsaturated fatty acids in the membrane of cells when the free radicals and ROS generate. Therefore, its increase in PCOS rats indicates the creation of oxidative stress. SOD is the mainline defense antioxidant with a primary function of dismutation of the superoxide anions into molecular oxygen and hydrogen peroxide (26). The reduction of this enzyme in the present study again supports the development of oxidative stress in PCOS rats. Therefore, insulin resistance in PCOS rats may be due to oxidative stress.

Androgens also affect the metabolism of lipoproteins in different ways. Hyperandrogenemia can affect the profile of lipids. In fact, one of the features of PCOS is a disturbance in the profile of lipids that can be caused by hyperandrogenemia (27). In the present work, estradiol valerate had adverse effects on serum lipid profile and markedly increased TG, TC, and LDL in PCOS animals. In addition, it markedly decreased HDL in PCOS animals in comparison to controls. In line with this study, Karateke et al. (28) have also shown a decrease in HDL and an increase in TG, TC, and LDL in PCOS rats.

The liver is the largest organ inside the body, responsible for almost all metabolic processes including fat metabolism, maintaining of blood glucose levels, and insulin metabolism that is all associated with PCOS (29). Because of the central role of the liver in the homeostasis of glucose and metabolism of lipids, dysglycemia and dyslipidemia may affect the liver and leads to hepatic injury. In fact, Zhang et al. (30) have shown that an increase of androgen levels and resistance to insulin contribute to hepatic steatosis and can alter the metabolism of lipids in the liver, liver response to inflammation, and cellular function. In the present study, our data demonstrated that the level of AST, ALT, and ALP was significantly high in estradiol valerate-induced PCOS rats suggesting the dysregulation of liver function in PCOS. AST, ALT, and ALP are enzymes that are mostly present in the liver. High levels of them in the serum can indicate a liver injury, due to the release from damaged hepatocytes into the extracellular space (31). Results of this study confirmed the previous finding by Osman and co-workers. They showed an increase in AST, ALT, and ALP along with dyslipidemia and insulin resistance in estradiol valerate-induced PCOS rats. They also found that injection of estradiol valerate to female rats provokes oxidative stress, as also shown in the present study (32). Liver injury in PCOS subjects may be the result of increased oxidative stress. Parenchymal cells, hepatic stellate cells, Kupffer cells, and endothelial cells are the most sensitive cells to free radicals and oxidative stress and damage in the liver (33). Therefore, an increased level of AST, ALT, and ALP in the present study can confirm oxidative stress and above data in PCOS animals. These data led us to evaluate the expression of the *Cox-2* and *Ppar-α* genes in PCOS animals. *Cox-2* has cyclooxygenase and peroxidase functions that can be induced by ROS and give rise to oxidative stress (11). *Ppar-α*, also known as NR1C1 (nuclear receptor subfamily 1, group C, member 1), is a nuclear hormone receptor and a hepatic regulator of lipid and glucose metabolism. It is expressed in organs with more oxidative activity and has a central role in fatty acid oxidation, inflammatory responses, and oxidative stress (34). As mentioned above, the expression of *Cox-2* increased, while the expression of the Ppar-α gene significantly decreased in the estradiol valerate model of PCOS rats that can indicate oxidative stress and dyslipidemia. Decrease of *Ppar-α* suggests that the dysregulation of lipids profile in PCOS subjects may be the result of *PPAR-α* inhibition. Karimzadeh et al. (35) reported similar results of increased Cox-2 expression in PCOS rats. Wang et al. (36) induced the PCOS model in rats by DHEA and evaluated the *Ppar-γ's* expression in adipose tissue. They found that both protein and mRNA levels of *Ppar-γ* are reduced in the DHEA model of PCOS rats.

In this study, we searched the therapeutic potential of eugenol on ovulation improvement, liver activity, and *Cox-2* and *Ppar-α* genes expression in an estradiol valerate model of PCOS rats. Based on our data, administration of eugenol could attenuate symptoms of PCOS and return normal ovulation in PCOS animals. Eugenol is a phenolic component of clove oil with some potential therapeutic effects including anti-inflammatory, antioxidant, and neuroprotective activity (8). Our data revealed that after

using eugenol, the levels of FSH, LH, progesterone, testosterone, and estradiol improved to near the control value. These results are in line with some data reported previously by others. Moghimian et al. (37) have shown that clove extract could improve reproductive hormonal changes induced by morphine in rats. They observed a considerable increase in FSH levels following treatment with clove extract. Another study by Poli and Challa (38) demonstrated that eugenol could enhance the serum level of progesterone in female albino rats. A study by Barghi et al. (39) has shown that eugenol could decrease the serum level of testosterone in adult female rats after ovarian torsion/detorsion. Therefore, it seems that eugenol has beneficial effects on reproductive hormones and can improve hormonal changes in PCOS rats. We also observed that eugenol improved the histological features of the ovaries. Treatment with eugenol decreased the number of cysts in treated animals. Furthermore, we observed that eugenol can improve the number of primordial follicles, Graafian follicles, corpus luteum, preantral follicles, and decrease the number of degenerating corpus luteum in treated animals. These results may be due to the improvement of hormonal disorders following treatment with eugenol. Therefore, it may suggest that eugenol has a direct effect on the hypothalamic-pituitary-gonadal axis.

The improvement of hormonal levels and ovaries histological parameters in the EXP-1 and EXP-2 groups may be due to a decrease in oxidative stress, too. Some previous studies also have demonstrated the antioxidant activity of eugenol (8, 37, 38). As mentioned before injection of estradiol valerate produced oxidative stress in PCOS rats. Treatment with eugenol improved the levels of oxidative stress markers. The amount of SOD and MDA improved to near normal levels following eugenol administration in EXP-12 and EXP-24 groups. It increased the level of SOD and decreased the level of MDA in subjected animals. These data suggest that eugenol is beneficial as a protective agent against oxidative stress in PCOS individuals.

Benefit effects of eugenol were observed on liver function and metabolic characteristics of PCOS, too. Eugenol at a dose of 24 mg/kg body weight decreased the levels of glucose, insulin, TG, TC, and LDL; and increased the serum level of HDL in PCOS and sham rats in comparison to control ones, suggesting anti-diabetics and anti-hyperlipidemic effects of eugenol. Therefore, it seems that eugenol can prevent insulin resistance and dyslipidemia in the PCOS model of estradiol valerate. Some previous studies have demonstrated anti-hyperlipidemic and anti-diabetic effects of eugenol in the diabetic animal model (40).

As mentioned above, androgens and oxidative stress can affect the metabolism of lipoproteins and develop insulin resistance and dysglycemia. Therefore, it may be concluded that the anti-diabetic and anti-hyperlipidemic properties of eugenol are the result of a decrease in testosterone.

AST, ALT, and ALP act as biomarkers of liver function and their increase indicates hepatocyte injury observed in PCOS rats. Eugenol (at a dose of 24 mg/kg) decreased serum levels of AST, ALT, and ALP in EXP-1 and EXP-2 groups. Because of the central role of the liver in the homeostasis of glucose and metabolism of lipids, dysglycemia and dyslipidemia may affect the liver and leads to hepatic injury. Therefore, these results may confirm the anti-diabetic and anti-hyperlipidemic properties of eugenol.

Eugenol treatment at a concentration of 24 mg/kg improved the expression of *Cox-2* and *Ppar-α* gene in the EXP-24 group, too. The expression of *Cox-2* decreased, while the expression of *Ppar-α* significantly increased in the EXP-24 group after eugenol administration, which can confirm again a decrease of oxidative stress and prevention of dyslipidemia.

Conclusion

The present study focused on the effects of eugenol on some adverse features of estradiol valerate-induced PCOS in Wistar rats. The study provided supportive evidence that eugenol possesses potent antioxidant, anti-diabetic, anti-hyperlipidemic, and anti-androgenic properties. It showed beneficial effects against serum glucose, serum insulin, fat profile, reproductive hormones, liver activity, and oxidative stress, as well as restored normal ovulation in the PCOS animals. Eugenol could represent a promising natural product to reduce PCOS symptoms. A challenge for future studies will be to elucidate the mechanisms.

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Authors' Contribution

Z.K.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. Z.H.; Drafted the manuscript, and contributed to data and statistical analysis, and interpretation of data. P.Y.; Contributed to conception and supervised, directed, designed, and managed the study. S.B.J.; Contributed to conception and designed the experiments. All authors read and approved the final manuscript.

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Original Article

The Protective Effects of Trans-Anethole against Polycystic Ovary Syndrome Induced Histopathological and Metabolic Changes in Rat

Faezeh Moradi Negahdari, M.Sc.[1, 2], Mousa-Al-Reza Hadjzadeh, M.D., Ph.D.[1, 2*], Zahra Gholamnezhad, Ph.D.[1, 3*], Farzaneh Sohrabi, Ph.D.[2], Zahra Samadi Noshahr, Ph.D.[1]

1. Department of Physiology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
2. Division of Neurocognitive Sciences, Psychiatry and Behavioral Sciences Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
3. Applied Biomedical Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

Abstract

Background: Aim of the study was to evaluate the protective effects of trans-anethole, against polycystic ovary syndrome (PCOS) induced histopathological and biochemical changes in female Wister rats.

Materials and Methods: In this experimental study, forty-eight animals were randomly assigned into 6 groups: control; PCOS; PCOS+trans-anethole (20, 40, 80 mg/kg); and PCOS+metformin (300 mg/kg). Testosterone (1 mg/kg/day) was injected intraperitoneally for 35 days to induce PCOS. After PCOS induction, animals were treated by trans-anethole and metformin (30 days oral gavage). Finally, serum oxidative stress and insulin levels as well as histological changes in ovaries, kidneys and liver were evaluated.

Results: In PCOS group, the serum level of malondialdehyde (MDA) was 1.391 ± 0.18 mmol/L and significantly increased (P=0.000) compared to the control group with the MDA level of 0.35 ± 0.08. Meanwhile the activity of superoxide dismutase (SOD) and catalase (CAT), and total thiol levels were significantly decreased (P=0.000 for all groups), compared to the control group. In the trans-anethole (80 mg/kg) treated group, insulin (P=0.000) and MDA (P=0.000) levels were significantly decreased while total thiol (P=0.001) and activity of SOD (P=0.000) and CAT (P=0.007) were significantly increased compared to the PCOS group. In the metformin treated group the insulin level (P=0.03) decreased compared to the PCOS group. Histological evaluation showed multiple cysts in the ovarian tissue, an increase in inflammatory cells in the liver, and a loss of order in the structure of the tubules and glomeruli of the kidney in the PCOS group. Tissue damage was reduced in the trans-anethole treated group.

Conclusion: Tarns-anethole at a dose of 80 mg/kg improved metabolic status, oxidative stress, liver and kidney damage as well as the cystic mass of ovarian tissue. To understand the exact protective effects of trans-anethole in PCOS, more experimental or clinical studies are suggested.

Keywords: Histopathology, Metformin, Oxidative Stress, Polycystic Ovarian Syndrome, Trans-Anethole

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Introduction

Polycystic ovary syndrome (PCOS) is considered as an endocrine-metabolic disease, which highly increases the risk of infertility and metabolic disorders including obesity, insulin resistance, and type2 diabetes (1). Hyperandrogenism signs or symptoms such as alopecia, acne, and hirsutism are common in most women with PCOS. A major feature of PCOS is the increase of androgen production and insulin levels that result in suppressing the production of sex hormone binding globulin (SHBG) and its release from the liver. As a result, the level of free testosterone would increase in the PCOS patients (2). Moreover, PCOS is associated with elevation of free radicals and decrease in activities of antioxidant enzymes and serum levels of antioxidants. However, the role of oxidative stress markers in the pathogenesis of PCOS needs to be completely determined. It was suggested that oxidative stress might alter ovarian steroid synthesis, which leads to increased androgen levels, disturbance of follicular development, and infertility in PCOS patients (3).

Induction of insulin resistance and hyperglycemia in PCOS have also been identified as factors in promoting oxidative stress (4). Insulin resistance might result in ovarian cysts by increasing tumor necrosis factor-α production (5). Lipid peroxidation and reactive oxygen species (ROS) formation have been shown to cause

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***Corresponding Address: P.O.Box: 9177948564, Department of Physiology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran**
Email: gholamnezhadz@mums.ac.ir

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oxidative stress damage due to the conversion of the glutathione reduced form (GSH) to the Glutathione disulfide (GSSG) oxidized form (6). Factors contributing to oxidative stress, including chronic inflammation and infection, obesity, and insulin resistance have been shown to be related to an excess of oxidative stress markers in women with PCOS (2). Different types of anti-PCOS drugs are prescribed for reducing these complications, but these drugs have no definitive therapeutic effects, and in most PCOS patients these drugs have undesirable side effects (7). Nowadays, the tendency to use herbal medicines and natural products for the treatment of disease has been increased. *Foeniculum Vulgare (F. Vulgare)* is a functional food plant widely used to treat hormonal disorders and regulation of menstrual cycles for its estrogenic properties (8). *F. Vulgare* contains protein, calcium, phosphorus, iron, potassium, vitamins A and C, and has antimicrobial and antioxidant properties (9). Trans-anethole is one of the most important active ingredients of *F. Vulgare*, with many pharmacological properties (10).

Trans-anethole has antioxidant, anti-inflammatory and estrogenic properties (11). In a previous study, 35 days testosterone injection in rats increased the serum levels of testosterone, dehydroepiandrosterone and lipids which are markers of PCOS induction (12). However, to the best of our knowledge the effects of trans-anethole against metabolic and histological changes of PCOS induced by testosterone had not been evaluated. Moreover, metformin is one of the most common medications prescribed to PCOS patients which can modulate oxidative stress and decreases serum androgen levels in PCOS patients (13). Therefore, in the present study, our aim was to examine the effects of trans-anethole and metformin on the histological changes in the ovaries, liver and kidneys as well as serum biochemical markers of testosterone-induced PCOS rats.

Materials and Methods

Drugs and materials were obtained as follows: Testosterone enanthate (IM) (Caspian, Iran), trans-anethole (Sigma, China), metformin (Sigma, India), and olive oil (Farabi, Iran). Enzyme immunoassay kits were used for measurement of insulin (Cayman Chemical, USA) by ELIZA (14) and materials for measurement of oxidative stress marker were obtained from Merck (Germany). All chemicals and reagents for histological assessments were purchased from Farzan Azma (Iran).

Animals

In this experimental study, forty-eight female Wister rats (8 weeks, 180-210 g) were obtained from the animal care facility of the Faculty of Medicine, Mashhad University of Medical Sciences, Iran. During the experiment, animals were kept under standard conditions (22 ± 2°C with 12 hour light-dark cycles) with free access to water and food. The study protocol was performed in accordance with ethical policies and principles approved by the Committee on Animal Research of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1399.075).

Experimental design

Animals were randomly divided into six groups (n=8) as: control; PCOS non-treated; PCOS treated group with metformin (300 mg/kg) which is represented as metformin; PCOS treated groups with 3 doses of trans-anethole (20, 40, 80 mg/kg) (12, 15); which are represented as trans20, trans40 and trans80. To induce the PCOS model, dissolved testosterone in olive oil (1 mg/kg/day) was intraperitoneally injected for 35 days (12). After PCOS induction, treatments were given orally by gavage for the next 30 days. At the end of the study, for measurement of serum biochemical and oxidative stress parameters, rats were fasted overnight and blood samples were collected, serum was isolated and kept at -20°C. The serum levels of insulin, MDA, total thiol content, and activities of CAT, and SOD were assayed. In addition, for histological examination, the liver, ovary, and kidney tissues were dissected and isolated.

Measurement of superoxide dismutase activity

SOD activity was measured by the Madesh and Balasurbamanian colorimetric methods, using 96-well microtiter plates (16). Briefly, the appropriate amount of serum, MTT [3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide] and pyrogallol were poured into the wells and incubated for 5 minutes at room temperature and in a dark environment. After adding the dimethyl sulfoxide inhibitor, its absorption was read at 570 nm, and the results were reported in u/ml.

Measurement of catalase activity

The CAT activity was assayed according to the Abei colorimetric method. The decomposition of hydrogen peroxide (30 mM) micromoles per milligram of protein sample was considered as one unit of CAT activity. The reduction was measured using a spectrophotometer at 240 nm (17).

Measurement of malondialdehyde

MDA Measurement was performed to determine serum lipid peroxidation. The method was done as previously described (18).

Measurement of total thiol content

Total thiol content (mM) was assayed using the method of Ellman (19).

Histology

For histology, after removing the organs, (kidneys, ovaries, liver) they were placed in 10% formalin solution. After 2-3 hours, the tissues were divided in half and placed in formalin for another 48-72 hours, then the tissues were washed with running water and distilled water and numbered. The samples were dehydrated using alcohol with ascending degrees of 70, 80, 90, 95, and 100%. The time of placing the samples in each of these concentrations

of alcohol was two hours. After dewatering, the samples were clarified with xylene, then molding was done using paraffin by TBS 88 machine; 5-micron sections were prepared by a Lietz 1512 microtome machine. Next, the slides were stained with hematoxylin-eosin (H&E). The prepared slides were checked to examine the tissue damage by considering necrosis, inflammation, number of cysts, size of the ovarian follicles, cell swelling, loss of tissue integrity, increased glomerular space in the kidney, and dilation of the veins in the liver. Then the samples were photographed using a Olympus BX51 microscope (20).

Statistical analysis

Experimental data were analyzed by SPSS 22 software (SPSS, Inc., Chicago, IL, USA). The method of Kolmogorov and Smirnov was used for evaluation of data distribution, which was not normal. Therefore, a non-parametric Kruskal Wallis test was used for data analysis and was expressed as mean ± SEM. The significant level was considered as $P<0.05$.

Results

Effects on serum insulin level

The serum insulin levels of the PCOS animals were higher than those of the control animals, but the difference was not significant. One month treatment of animals with trans-anethole (80 mg/kg) or metformin significantly reduced the insulin levels compared to the PCOS animals (P=0.000 and P=0.03 respectively). The insulin levels in all treated groups were nearly the same as the control group (Table 1).

Effects on oxidative stress parameters

Total thiol

Serum total thiol levels were significantly decreased in the PCOS and trans-anethole 40 groups compared to the control group (P=0.000 and P=0.015, respectively). PCOS animals treated group with trans-anethole 80 showed a significant increase in total thiol levels compared to the PCOS group (P=0.001). There was no significant difference between total thiol levels in PCOS animals treated with trans-anethole (20, 40 and 80 mg/kg) and metformin (Fig.1A).

Malondialdehyde

Serum MDA levels were significantly increased in the PCOS group compared to the control group (P=0.000). MDA level in the trans-anethole 40 and 80 groups were significantly decreased in comparison to the PCOS group (P=0.023 and P=0.000, respectively). There was no significant difference between the MDA levels in all treated groups (Fig.1B).

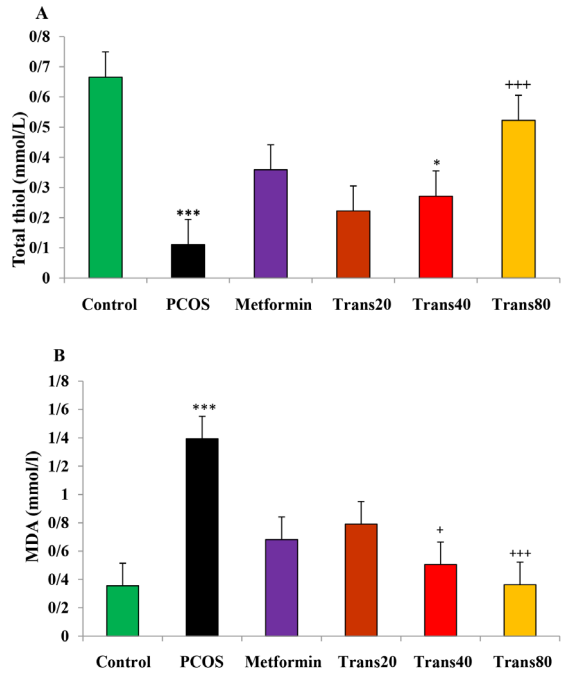

Fig.1: Effect of trans-anethole and metformin on serum levels of total thiol and malondialdehyde (MDA). **A.** Total thiol, and **B.** MDA levels in control, polycystic ovary syndrome (PCOS), PCOS+metformin (metformin) and PCOS+trans-anatole (trans20, 40, and 80) groups. Values are expressed as mean ± SEM. *; $P<0.05$, ***; $P<0.001$, as compared to control group, $^{+}$; $P<0.05$, and $^{+++}$; $P<0.001$, compared to PCOS group (n=8).

Activity of superoxide dismutase

SOD activity was significantly decreased in the PCOS, trans-anethole 20 and 40 groups compared to the control (P=0.000, P=0.003, and P=0.04, respectively). The SOD activity in PCOS animals receiving trans-anethole 80 was significantly increased in comparison to the PCOS group (P=0.000). In addition, SOD activity was significantly higher in the trans-anethole 80 treated group than in the trans-anethole 20 (P=0.017, Fig.2A).

Activity of catalase

CAT activity was significantly decreased in the PCOS group compared to the control (P=0.000). In the group receiving trans-anethole 80 CAT activity was significantly increased in comparison to the PCOS group (P=0.007, Fig.2B).

Table 1: Serum insulin levels

Groups	Control	PCOS	Metformin	Trans20	Trans40	Trans80
Insulin (µU/mL)	6.33 ± 1.50	24.67 ± 4.20	6.98 ± 2.6^{+}	10.35 ± 3.09	8.27 ± 2.09	2.96 ± 1.46^{+++}

Values are expressed as mean ± SEM. Kruskal Wallis test was used for data analysis. $^{+}$; $P<0.05$, $^{+++}$; $P<0.001$, compared to PCOS group (n=8), and PCOS; Polycystic ovary syndrome, trans20, 40, 80, PCOS+trans-anethole (20, 40, 80 mg/kg).

Histological results

Ovarian tissue examination

The results showed that in the control group, the morphology of the ovaries, follicle numbers, and the follicle cell layers of theca and granulosa cells were normal (Fig.3A). In the PCOS group the number of immature follicles increased, granulosa cell destruction (atresia) and cystic follicles were seen (Fig.3B). In PCOS treated group with trans-anethole 80 mg/kg, the number of follicles and cystic follicles compared to the PCOS group was decreased and had the greatest effect in comparison between the 3 treated groups (Fig.3C-F). In groups receiving trans-anethole 20 and 40 mg/kg, and metformin, the number of follicles was decreased, but irregularities in the theca and granulosa cells were seen (Fig. 3C, D, F).

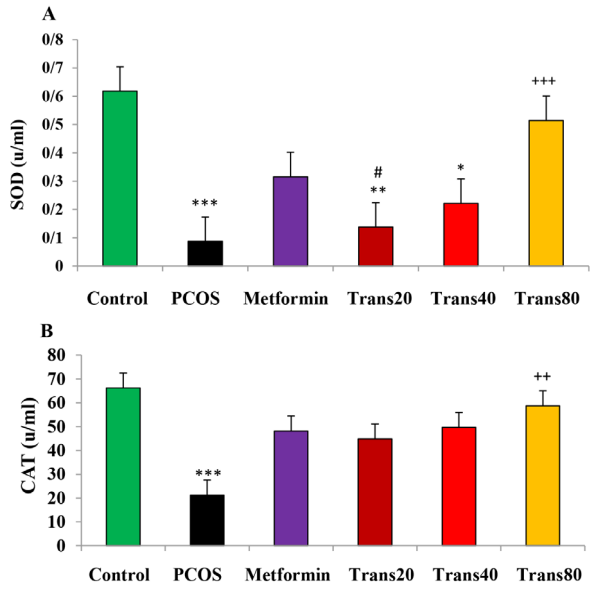

Fig.2: Effect of trans-anethole and metformin on serum activity of superoxide dismutase (SOD) and catalase (CAT). **A.** SOD and **B.** CAT activity in control, polycystic ovary syndrome (PCOS), PCOS+metformin (metformin) and PCOS+trans-anethole (trans20, 40, and 80) groups. Values are expressed as mean ± SEM. *; P<0.05, **; P<0.01, ***; P<0.001, compared to the control group, $^{++}$; P<0.01, $^{+++}$; P<0.001 as compared to PCOS group, and #; P<0.05, compared to trans-anethole 80 (n=8).

Liver tissue examination

Histological evaluation of the liver showed that in the control group, veins and sinusoids were healthy with a regular structure (Fig.4A). In the PCOS group, tissue structure was irregular, veins were dilated, inflammatory cells infiltration, coalesced vacuoles, and lipid droplets were present, which indicate progress towards fatty liver (Fig.4B). In the PCOS treated with doses of 20 and 40 mg/kg trans-anethole inflammatory cells and dilated sinusoids were still visible (Fig.4C, D). In the PCOS group which received trans-anethole 80 mg/kg, inflammatory cells infiltration and the distance between the sinusoids were decreased, and in general, the structure of the liver became more regular and very close to the control group (Fig.4E). In the PCOS group treated with metformin, the sinusoids and veins became more regular, but inflammation and irregular structure were still present (Fig.4F).

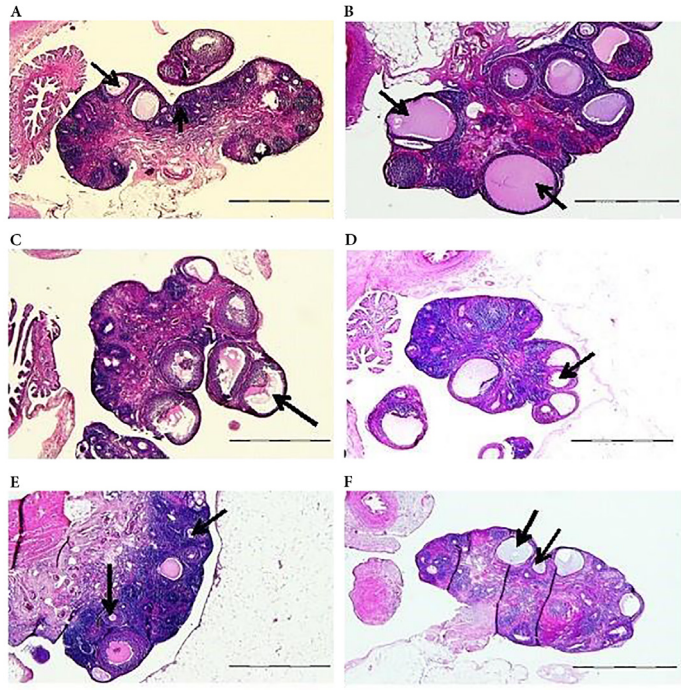

Fig.3: Photomicrograph of ovarian tissue section with a scale of 1000 µm and H&E staining. **A.** Light microscopic examination of a healthy control group showed the regular structure in parenteral and antral follicles. **B.** In PCOS group, increased number of follicles, cystic follicles, and irregular ovarian structures were observed. **C.** PCOS treated group with trans-anethole 20 mg/kg, **D.** PCOS treated group with trans-anethole 40 mg/kg. **E.** PCOS treated group with trans-anethole 80 mg/kg, demonstrates a decrease in the number of follicles, and **F.** PCOS treated group with metformin 300 mg/kg. Black arrow; Cystic follicles.

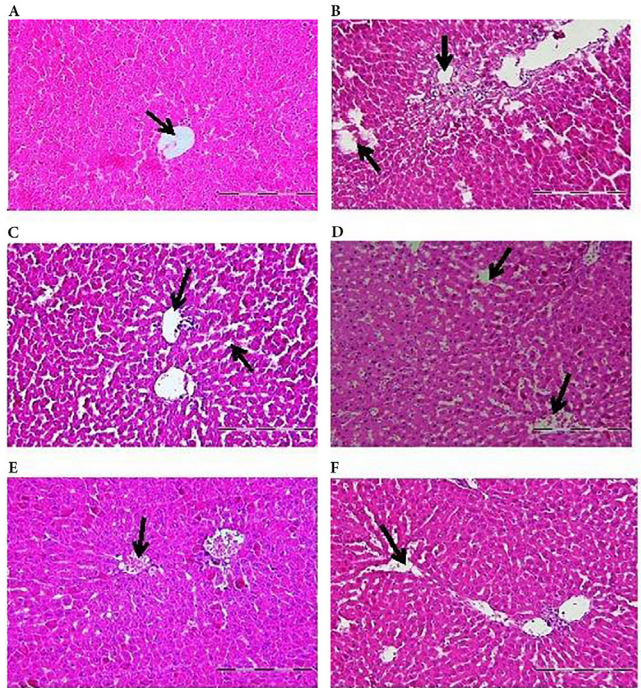

Fig.4: Photomicrograph of liver tissue section with the scale of 200 µm and H&E staining. **A.** Light microscopic evaluation of the control group, veins (arrowed) and sinusoids with a regular structure. **B.** In the PCOS group, areas of inflammatory cells infiltration, coalesced vacuoles, dilated vein (arrowed) and irregular structure were seen, **C.** PCOS treated group with trans-anethole 20 mg/kg, **D.** PCOS treated group with trans-anethole 40 mg/kg; **E.** In PCOS treated group with trans-anethole 80 mg/kg, less inflammatory-cell infiltration, coalesced vacuoles, dilated vein (arrowed) and more regular structures were seen, and **F.** PCOS treated group with metformin 300 mg/kg.

Kidney tissue examination

Histological examination of the kidneys showed that in the control group the tubules, glomeruli, and brush border were normal, the number of cells were normal and the distal and proximal tubes were separable (Fig.5A). In the PCOS group, glomeruli were damaged, the Bowman spaces were increased, inflammatory cells were seen, and the distal and proximal tubules were indistinguishable (Fig.5B). In all treated groups, the Bowman spaces were reduced, but inflammatory cells and tubular destruction were still observed. Among the treatment groups, the highest improvement was observed with a dose of 80 mg/kg trans-anethole, which reduced the damage (Fig.5C-F).

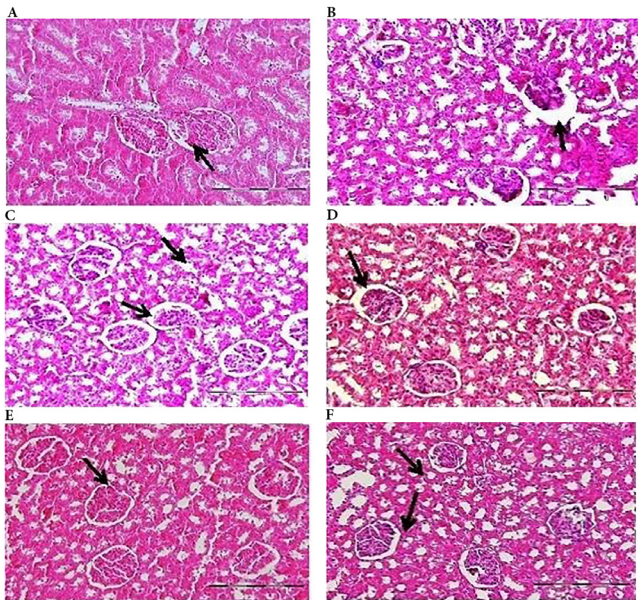

Fig.5: Photomicrograph of kidney tissue section with a scale of 200 µm and H&E staining. **A.** In light microscopic analysis of healthy controls, the number of cells were normal, and generally regular structure was seen. **B.** In the polycystic ovary syndrome (PCOS) group, tubules and glomeruli were damaged and irregular kidney structure was seen. **C.** PCOS treated group with trans-anethole 20 mg/kg, **D.** PCOS treated group with trans-anethole 40 mg/kg. **E.** PCOS treated group with trans-anethole 80 mg/kg which decreased injury rate, and **F.** PCOS treated group with metformin 300 mg/kg. Black arrow; Damaged glomeruli and irregular kidney structure.

Discussion

Elevated testosterone and high insulin levels due to increased insulin resistance, hirsutism, ovarian cysts, irregular menstruation, and lack of ovulation are hallmarks of PCOS in patients (21). In this study, PCOS induction in animals resulted in a significant increase in insulin levels, and the polycystic feature of the ovaries as well as pathological changes in the liver and kidneys which demonstrated a metabolic disturbance similar to non-alcoholic fatty hepatic disease. A significant increase in insulin and testosterone levels in PCOS animals suggested an association between increased androgens and hyperinsulinemia. Insulin resistance plays a pivotal role in anovulation and metabolic disorders in PCOS disease. Insulin promotes PCOS metabolic distribution via mitogen-activated protein kinase and phosphoinositide 3-kinases (PI3K) signaling pathways. Insulin induces inhibition of PI3K in the follicular cells of polycystic ovaries and reduces 17α-hydroxylase, proposing that insulin might enhance steroid synthesis through the PI3K pathway, which enhances hyperandrogenism (22).

In addition, imbalanced serum oxidative stress markers in the PCOS group might support the hypothesis that there is an early association between insulin resistance and impaired oxidative metabolism. It has been indicated that oxidative stress induced by ROS overproduction might have a role in the development of hyperandrogenism and insulin resistance in PCOS patients (23). Elevation of the MDA level, and reduction of thiol content and activity of antioxidant enzymes (SOD and CAT) in PCOS rats had been reported previously and might explain some features of tissue damage and metabolic disturbance of PCOS patients. Abdominal adiposity and hyperlipidemia in PCOS patients might contribute to the development of local and systemic oxidative stress. In PCOS patients and animal models blood lipids and weight usually increase, which could induce a vicious cycle contributing to oxidative stress and insulin resistance and play an important role in PCOS pathogenesis. However, there is no effective treatment for these complications (24).

In recent decades the therapeutic effects of medicinal plants, among them *F. vulgare* has been shown in PCOS patients. *F. Vulgare* oil has antioxidant capacities and the effectiveness of the plant in many gynecological diseases including premenstrual syndrome, heavy menstrual bleeding, menopause, vaginal atrophy, amenorrhea, hirsutism, infertility, and PCOS have been demonstrated. The main therapeutic constituent of *F. Vulgare* is trans-anethole. Moreover, the therapeutic effects of *F. Vulgare* on the genitals and mammary glands have been attributed to the estrogenic properties of trans-anethole (25). In this study, treating PCOS rats with trans-anethole and metformin, returned the elevated serum insulin levels to normal; these results are in line with the study of Salehi et al. (26), who found that treatment with trans-anethole significantly reduced plasma insulin in PCOS rats. The metabolic effect of trans-anethole against insulin resistance might be related to its antihyperlipidemic, hepatoprotective, estrogenic and antioxidant properties.

In this study MDA decreased significantly after treatment with trans-anethole and metformin which indicates that both trans-anethole and metformin can suppress the oxidative stress induced in PCOS. In addition, SOD activity was significantly increased in animals

receiving trans-anethole 80mg/kg, which indicates that trans-anethole improves oxidative stress in PCOS rats in a dose dependent manner. All doses of trans-anethole resulted in a significant increase in CAT activity while the serum level of thiol was only increased in the group receiving trans-anethole 80 mg/kg. The antioxidant and protective effects of trans-anethole in PCOS patients have been attributed to estrogen and phytoestrogen compounds (26). Metformin might prevent oxidative stress induced damage in diabetic patients and have a potent antioxidant activity (27). As can be seen in this study, in general, trans-anethole increased antioxidant factors and decreased oxidative factors. The effects of trans-anethole on oxidative stress might be related to the IL-6 signaling pathway. The modulatory activity of trans-anethole on the IL-6 inflammatory pathways which is an important factor in the pathological changes and ovulation processes of PCOS patients might decrease androgens and improve ovulation processes (28). In the present study, the number of cystic follicles with destroyed granulosa cell layers was increased in the ovaries of PCOS rats, while trans-anethole, especially at a dose of 80 mg/kg, reduced the number of cystic follicles and improved those histological features. Our findings are in agreement with a study conducted by Yavangi et al. (28).

Ovarian histological changes in the PCOS group support other current and previous study results such as high serum testosterone and insulin levels, accumulation of glycogen and lipids in hepatocytes, and irregularly shaped and dilated veins in the liver, which are markers of progression to fatty liver. However, trans-anethole especially at a dose of 80 mg/kg decreased the number of inflammatory cells very close to the control group. In general, rats treated with trans-anethole showed significant restorative changes in the tissue structure of the liver. Based on these findings it may be suggested that trans-anethole which is a component of the essential oils of F. Vulgare, has protective effects on the liver (29). Insulin resistance, abdominal obesity and hyperlipidemia are the most typical endocrinopathies in both nonalcoholic fatty liver disease and PCOS patients.

In PCOS patients, hyperandrogenism resulted in hyperinsulinemia and insulin resistance, which in turn might induce steatohepatitis, abdominal adiposity and dyslipidemia (30). Moreover, metabolic disturbance, chronic low-grade inflammation, and cardiovascular events (hypertension) in PCOS patients have shown to be associated with renal injury markers such as decreased glomerular filtration rate and microalbuminuria and increase in kidney dysfunction (31). A definitive relationship between PCOS and kidney injury have been demonstrated previously. In PCOS patients, clinical analyses have showed that the urine albumin to creatinine ratio and urinary protein excretion levels is higher and is correlated to serum testosterone, which are reflective of injury in the kidney tubules (32). In kidney tissue, mesangial glomerular cells, primary tubules, and cortical collecting ducts contain androgen receptors, so the kidneys can be affected by androgens (33). Therefore, hyperandrogenism and its related metabolic consequences have been proposed as a main factor in inducing immature cystic follicles, oligoanovulation, and pathological injury in the kidney and liver (2, 33). In the present study, in the PCOS group, glomeruli were damaged and inflammatory cells were seen due to high serum testosterone levels. Trans-anethole had a positive effect on glomeruli and tubules and reduced the inflammatory features in the kidney of treated groups. These findings indicate the protective effect of trans-anethole against the destructive activity of high testosterone on kidney tissue. In line with our finding, a study by Sadrefozalayi et al. showed that *F. Vulgare* improves kidney structure and kidney function in PCOS female rats (25).

In PCOS patient metformin was showed to prevent metabolic and endocrine disturbances which are the contributors to liver and kidney injury by decreasing serum androgen levels, oxidative stress and low grade inflammation (13) and also having insulin-sensitizing and hypolipidemic effects (34). In the present study, although treatment of PCOS rats with metformin decreased the insulin level and the number of cystic follicles in the ovary, and improved kidney and liver histopathology, there was not significant improvement in oxidative stress markers. However, a between groups comparison indicated that, both pathological changes in the ovaries, liver and kidneys, and the levels of serum insulin and oxidative stress markers were significantly improved in the trans-anethole 80 group. These findings showed the higher effectiveness of trans-anethole 80 in comparison to metformin. In this study and the previous one, trans-anethole showed ameliorating effects against PCOS induced hormonal and metabolic disturbance by reducing insulin resistance, hyperlipidemia, and excess androgen and ROS production (12). Therefore, it might be safer than hormone replacement therapy such as estrogen-progestin contraceptives, which is prescribed in combination with metformin, and spironolactone for PCOS patients (35). Although the safety of trans-anethole at a dose of 80 mg/kg was confirmed in this and previous studies (12, 15), more studies are needed to determine the potency of the estrogenic effects of this compound.

Novel insights: Tarns-anethole (especially at a dose of 80 mg/kg) improved metabolic status, oxidative stress, liver and kidney damage as well as the cystic mass of ovarian tissue.

Established facts: Elevated testosterone and high insulin levels due to increased insulin resistance,

hirsutism, ovarian cysts, irregular menstruation, and lack of ovulation are present in PCOS patients.

Conclusion

These results indicated that trans-anethole especially in higher doses (80 mg/kg) has a therapeutic effect on PCOS induced histological changes and metabolic complications. More clinical studies are necessary to uncover the beneficial effects of trans-anethole in PCOS patients, and further experimental findings are needed to reveal the mechanism of its hepato and renal protective activity.

Acknowledgments

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Authors' Contributions

F.M.N.; Participated in study design, data collection, evaluation, drafting, and statistical analysis. M-A-R.H.; Contributed to conception of study design, manuscript drafting and was responsible for overall supervision. Z.G.; Contributed extensively in interpretation of the data and the conclusion, and revising the manuscript. F.S.; Drafted the manuscript and contributed to statistical analysis. Z.S.N.; Participated in study design, data collection and evaluation, and drafting. All authors read and approved the final manuscript.

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Original Article

Dietary Total Antioxidant Capacity and Risk of Polycystic Ovary Syndrome: A Case-Control Study

Nargeskhatoon Shoaibinobarian, M.Sc.[1], Ghazaleh Eslamian, Ph.D.[2*], Morvarid Noormohammadi, M.Sc.[3], Shirin Malek, M.Sc.[4], Shayesteh Rouhani, B.Sc.[1], Seyedeh Nooshan Mirmohammadali, B.Sc.[1]

1. Department of Nutrition, School of Medical Sciences and Technologies, Islamic Azad University, Science and Research Branch, Tehran, Iran
2. Department of Cellular and Molecular Nutrition, Faculty of Nutrition and Food Technology, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran
3. Student Research Committee, Department of Nutrition, School of Public Health, Iran University of Medical Sciences, Tehran, Iran
4. Department of Nutrition and Food Science, California State University, Chico, CA 95929, USA

Abstract

Background: Among multiple factors that affect the etiology of polycystic ovary syndrome (PCOS), diet has an important contribution. Chronic oxidative stress has also been implicated in the development of PCOS. The present study is an attempt to evaluate dietary total antioxidant capacity (TAC) and its relationship with odds of PCOS in Iran.

Materials and Methods: The study was carried out as a case-control study in hospital outpatient clinics, Tehran, Iran. Totally, 310 female participants with a history of PCOS and 602 age-matched controls took part in this study between June 2015 and December 2018. A reproducible and valid 168-item semi-quantitative food frequency inventory was utilized to determine the entire antioxidants of the usual diet in order to calculate dietary TAC. The relationship of dietary TAC with odds of PCOS were assessed adjusting for potential confounders through an estimation of two multivariable conditional regression models. The first tertile was presented as a reference category.

Results: In a fully adjusted model, the highest tertile of dietary TAC was associated with a reduced odds of PCOS [odds ratio (OR): 0.81, 95% confidence interval (95% CI): 0.59, 0.96, P for trend: 0.038). In addition, PCOS odds decreased in the highest tertile of α-tocopherol intake (OR: 0.73, 95% CI: 0.56, 0.88, P for trend: 0.023). The adjusted ORs in the highest tertile of vitamin C, β-carotene and magnesium were 0.79 (95% CI: 0.83-0.97), 0.81 (95% CI: 0.67-0.98) and 0.91 (95% CI: 0.55-0.98) respectively, with a significant trend.

Conclusion: Our results provide evidence that there was a relationship between high TAC diets and lower odds of PCOS.

Keywords: Antioxidant, Diet, Oxidative Stress, Polycystic Ovarian Syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is a common gynecological endocrinopathy that affects 8-13% of women in the reproductive age (1). Although, the cause of PCOS is still not fully understood, there are several possibilities. Main of them included: i. Hypothalamic/pituitary dysregulation contributes to a rise in the ovarian androgen production and ii. Hyperandrogenism leads to insulin resistance that results to PCOS (2). Due to insulin resistance, hyperinsulinemia happens and speeds up the ovarian androgen production, which can lead to the PCOS occurrence (3, 4). Recent studies have demonstrated that oxidative stress contributes to the development of PCOS, infertility and hyperandrogenism (5, 6). Important factors that increase the oxidative stress in PCOS are insulin resistance and hyperglycemia (7).

Oxidative stress is clarified as a lack of balance among oxidants and antioxidants in the alive biological systems (8). The oxidative stress decrease is positively related to further matured oocytes in the infertile PCOS women (9). Oxidative stress causes widespread atherosclerosis lesions in the ovarian arteries (10, 11). Thus, the use of anti-oxidative agents has become an increasingly popular method in the PCOS management (12). Research works have shown that there is a relationship between PCOS and dietary intakes (13-15). The dietary antioxidants with a negative relationship with PCOS are vitamin C, vitamin E, selenium, zinc

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*Corresponding Address: P.O.Box: 19395-4741, Department of Cellular and Molecular Nutrition, Faculty of Nutrition and Food Technology, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran
Email: gh.eslamian@sbmu.ac.ir

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and beta-carotene (13). A recent study showed that plant-based dietary pattern is associated with lower odds ratio (OR) of PCOS and antioxidant-rich foods may protect the body against oxidative damage in these patients (14). Furthermore, adherence to an antioxidants-rich diet may be inversely associated with PCOS (15). There are emerging evidences that total antioxidant capacity (TAC) of dietary can be used as a measurement index that covers combined actions of dietary total antioxidants. This assay gives better predictions of dietary intake relation with chronic diseases (16). Studies demonstrate that higher dietary TAC is associated with lower weight and abdominal fat gain (17). Furthermore, higher dietary TAC is related to greater improvement in the heart disease risk factors and reduced risk of pancreatic cancer (18, 19).

Unfortunately, there is a shortage of data, especially on dietary TAC and PCOS relationship. Thus, this finding encouraged us to design a case-control study to examine the relationship of dietary TAC and the odds of PCOS.

Materials and Methods

Study design and population

All volunteers signed informed consent before the onset of the study. The Ethics Committee of Shahid Beheshti University of Medical Sciences approved the study (IR.SBMU.RETECH.REC.1398.750). The whole procedure was conducted as per the Helsinki Declaration of 1975, as revised in 2013 and the ethical standards of the responsible committee on human experimentation.

A total of 310 patients with a PCOS history between the ages of 20-35 years and 602 age-matched healthy volunteers participated in this case-control study. This study was performed in the various hospital endocrinology clinics of Tehran, Iran. Our case group inclusion criteria comprised women with a diagnosis or a history of PCOS. Based on the international Rotterdam Criteria 2003, PCOS patients diagnosed in the past three months had to have two out of three symptoms at least (20). They were diagnosed, follow of clinical or biochemical signs of hyper-androgenism, oligo-ovulation or anovulation and polycystic ovaries by ultrasonography. Also, control group was invited from age-matched referred patients to other clinics such as orthopedic, ear, nose, throat clinics or elective surgeries were referred to the same clinics that were not suffered of ongoing or previous PCOS. They had regular menstrual cycles (26-33 day cycles) without any ovulatory abnormalities, polycystic ovary and hyper-androgenism. The rest of the inclusion criteria for the control group were similar to the case group. On the third day of their menstrual cycle, endocrine hormones serum level and full pelvic ultrasound assessment were performed. All participants, cases and controls, were free of cardiovascular diseases, cancer, diabetes, renal disease and did not follow special diets.

Socio-demographic assessment

Age, smoking, first menarche age, literacy level, history of medication, supplementation and monthly family income were recorded from the pretested baseline questionnaire. Due to participants' religious and cultural taboos, questions regarding alcohol and opium were omitted (21). Details of anthropometric and physical activity assessments have been reported previously (22).

Dietary assessment

The dietary data were collected through a valid semi-quantitative food frequency questionnaire (FFQ) (23). All participants were asked about their usual intakes over the past year, although in the case group we considered a last year before PCOS diagnosis. Nutrient Data Laboratory of United States Department of Agriculture (USDA) database was used to calculate dietary TAC for selected foods by the oxygen radical absorbance capacity method. Results were expressed as mmol of Trolox Equivalents (mmol TE/day) (24). Using residual method, dietary TAC was adjusted for total energy intake.

Statistical analysis

Data analysis was done in statistical package for the social sciences software (SPSS Inc., Chicago, IL, USA, version 20) ($P<0.05$). Kolmogorov-Smirnov test, histogram and Q-Q plot were used for normality of continuous variables. Dietary intake variables were not normally distributed. Therefore, transformations such as logarithm, square root, reciprocal, cube root, and square were done using parametric tests. Analysis of covariance (ANCOVA) adjusting for total energy was used to examine significant differences in antioxidant intakes. OR and 95% confidence intervals (CIs) were calculated by binary conditional logistic regression models to investigate the association of odds PCOS in each tertile dietary antioxidant variables. A tertile cut-off point was used to categorize dietary TAC, Vitamin C, α-tocopherol, zinc, β-carotene, lycopene selenium and magnesium were divided into the tertiles. The first tertile was presented as a reference category. The following variables were adjusted; body mass index (BMI), sex, age, physical activity, familiar history of PCOS, smoking and total energy intake.

Results

Final analysis was conducted on 303 PCOS cases (97.7% participation rate) and 588 controls (97.7%). Twenty-one participants were initially excluded given the log scales of total energy intake, which were either >3 or <3 standard deviation (SD) from the mean.

The mean ± SD for age was 29.1 ± 5.9 and 28.8 ± 6.2 in cases and controls represents the age-matched design. In comparison to the controls, BMI (33.7 ± 6.1

vs. 24.2 ± 4.9) and waist circumference (WC) (97.2 ± 8.2 vs. 80.1 ± 6.5) of the case group were higher along with the incidence of PCOS in their families (P<0.05). Mean metabolic equivalent (MET) (47.3 ± 5.9 hour/day vs. 58.1 ± 7.1 hour/day) and dietary fiber intake (13 ± 3.9 vs. 16 ± 4.1) were significantly lower in cases. In addition, the mean energy intake (3009 ± 709 vs. 2139 ± 605) in the cases was significantly higher in comparison with controls (P<0.05).

Table 1 lists the mean intakes of dietary antioxidant variables, both for cases and controls. Cases had lower dietary TAC, α-tocopherol, vitamin C and magnesium (P<0.005). No differences were found between cases and the control group as regards lycopene, zinc, and selenium.

Table 1: Energy-adjusted antioxidant dietary variables among PCOS cases and controls

Dietary factors	PCOS women (n=303)	Control women (n=588)	P value*
Dietary TAC (mmol/day)	11.9 ± 3.9	14.1 ± 4.3	0.001
α-Tocopherol (mg/day)	5.5 ± 1.9	7.3 ± 1.5	0.035
Vitamin C (mg/day)	69.3 ± 32.3	84.1 ± 45.3	<0.001
β-Carotene (µg/day)	1691 ± 133	1727 ± 152	0.069
Lycopene (µg/day)	1885 ± 178	1902 ± 196	0.122
Zinc (mg/day)	11.9 ± 4.7	11.2 ± 3.9	0.234
Selenium (µg/day)	52.3 ± 20.6	49.5 ± 22.3	0.321
Magnesium (mg/day)	189.2 ± 44.9	227.8 ± 46.7	<0.001

Data are presented as mean ± SD. *; Based on ANCOVA, PCOS; Polycystic ovary syndrome, and TAC; Total antioxidant capacity.

Associations of dietary antioxidant variables with odds of PCOS are shown in Table 2. There was a significant relationship between the dietary TAC with an odds ratio (OR) of PCOS in the base model (OR of the highest tertile compared to lowest one: 0.77, 95% CI: 0.49-0.91). After adjusting for BMI, WC, energy intake, dietary intake of fiber, familial history of PCOS and physical activity, the highest tertile of the dietary TAC score was associated with a reduced odds ratio of PCOS (OR: 0.81, 95% CI: 0.59-0.96). There was a significant linear decrease among the dietary TAC tertiles for the odds ratio of PCOS (P trend=0.038). There was an inverse relationship between the dietary intake of α-tocopherol and the odds of PCOS in both base and fully adjusted models. The multivariable OR (95% CI) for PCOS was 0.73 (0.56-0.88) in the highest tertiles of α-tocopherol, compared to the lowest tertile. The adjusted ORs in the highest tertile of vitamin C, β-carotene and magnesium were 0.79 (95% CI: 0.83-0.97), 0.81 (95% CI: 0.67-0.98) and 0.91 (95% CI: 0.55-0.98) respectively, with a significant trend.

Table 2: ORs* of polycystic ovary syndrome by tertiles of the dietary total antioxidant capacity in Iranian population

Dietary antioxidant variables	Tertiles of energy-adjusted intake			P for trend
	1st	2nd	3rd	
Dietary TAC (mmol/day)				
No. cases/No. controls	131/196	93/196	79/196	
Base model†	1.00 (Ref.)	0.87 (0.53-1.07)	0.77 (0.49-0.91)	0.015
Full model‡	1.00 (Ref.)	0.89 (0.48-1.03)	0.81 (0.59-0.96)	0.038
α-Tocopherol (mg/day)				
No. cases/No. controls	138/196	89/196	76/196	
Base model†	1.00 (Ref.)	0.73 (0.53-1.03)	0.69 (0.50-0.83)	<0.001
Full model‡	1.00 (Ref.)	0.77 (1.23-1.02)	0.73 (0.56-0.88)	0.003
Vitamin C (mg/day)				
No. cases/No. controls	143/196	83/196	77/196	
Base model†	1.00 (Ref.)	0.75 (0.43-1.08)	0.74 (0.73-1.05)	0.069
Full model‡	1.00 (Ref.)	0.82 (0.83-1.03)	0.79 (0.83-0.97)	0.039
β-Carotene (µg/day)				
No. cases/No. controls	124/196	90/196	89/196	
Base model†	1.00 (Ref.)	0.80 (0.49-1.19)	0.77 (0.65-1.08)	0.105
Full model‡	1.00 (Ref.)	0.84 (0.60-1.18)	0.81 (0.67-0.98)	0.046
Lycopene (µg/day)				
No. cases/No. controls	122/196	93/196	88/196	
Base model†	1.00 (Ref.)	0.96 (0.69-1.22)	0.99 (0.73-1.18)	0.112
Full model‡	1.00 (Ref.)	0.94 (0.79-1.03)	0.93 (0.63-1.07)	0.059
Zinc (mg/day)				
No. cases/No. controls	100/196	88/196	115/196	
Base model†	1.00 (Ref.)	1.13 (0.71-1.29)	0.99 (0.72-1.39)	0.305
Full model‡	1.00 (Ref.)	1.05 (0.83-1.15)	1.02 (0.95-1.19)	0.114
Selenium (µg/day)				
No. cases/No. controls	130/196	98/196	75/196	
Base model†	1.00 (Ref.)	1.09 (0.78-1.24)	1.27 (0.62-1.33)	0.286
Full model‡	1.00 (Ref.)	1.11 (0.72-1.21)	1.25 (0.65-1.39)	0.273
Magnesium (mg/day)				
No. cases/No. controls	128/196	90/196	85/196	
Base model†	1.00 (Ref.)	0.91 (0.59-1.03)	0.89 (0.50-1.04)	0.062
Full model‡	1.00 (Ref.)	0.93 (0.53-0.98)	0.91 (0.55-0.98)	0.047

Adjusted odds ratio (OR) estimates and 95% confidence intervals (CIs) for polycystic ovary syndrome (PCOS), according to the tertile of each dietary antioxidant variables. *; A conditional logistic regression model, †; Adjusted for age (5-year categories), ‡; Adjusted for age (5-year categories), body mass index (BMI, Kg/m^2), waist circumference (WC, cm) intake (Kcal/d), dietary intake of fiber (g/d), familial history of PCOS (yes/no) and physical activity (MET/h/d).

Discussion

To be best of our knowledge, the association of dietary TAC with PCOS was assessed for the first time in a

developing country in the present study. Higher intake of dietary TAC, α-tocopherol, vitamin C, β-carotene and magnesium were inversely associated with lower odds of PCOS, even after adjusting for confounding factors. Intake of the α-tocopherol, vitamin C and β-carotene were significantly associated with reducing the odds of PCOS. Our data are consistent with Shahrokhi and Naeini (13) study that demonstrated relationship between the higher intakes of vitamin C, vitamin E, and β-carotene and the reduced odds of PCOS. Barrea et al. (25) reported a relationship between the Mediterranean diet (rich in antioxidants) and decreased odds of PCOS that consistent with our findings these data show a relationship between a diet high in the TAC and decreased odds of PCOS. Similarly with the current study, we previously found a significant negative association between adherence to the antioxidant nutrient pattern and odds of PCOS (15). Furthermore, clinical trials demonstrated the beneficial effects of dietary antioxidants on the PCOS management (26). It was interesting that, Shahrokhi and Naeini (13) and present study are contradictory about the relationship of the zinc and selenium intakes with PCOS. In the present study, we found no significant association between zinc and selenium with the odds of PCOS, while Shahrokhi and Naeini (13) showed dietary selenium and zinc were inversely associated with PCOS at a significant level.

As to the important role of diet in the chronic diseases, examine the general dietary antioxidant intake seems essential, particularly, the different antioxidants proportion of diet in combination with synergistic effects that prevent diseases via several mechanisms. Dietary TAC considers the synergistic interactions of antioxidant nutrients and can be used to assess the relationship between dietary intake and several chronic disorders. Verit et al. (27) found that TAC was the most important prediction element to PCOS. Kanafchian et al. (28) concluded TAC is decreased in the PCOS patients, which may be due to increased oxidative stress. Enechukwu et al. (29) showed that decreased TAC correlates with the cause of PCOS in these patients. However, Ghowsi et al. (30) and Shahrokhi and Naeini (13) found that there were not any significant differences in the serum TAC in the PCOS patients and the healthy control group.

An imbalance between the antioxidant and free radical production in the ovaries can cause negative effects on the oocyte quality, productivity, development, and growth of the placenta (31, 32). Lowered oxidative stress is related to better prognosis of PCOS (33). PCOS can lead to a decrease in the quality of life, especially if it is accompanied by chronic diseases like type 2 diabetes mellitus, obesity, dyslipidemia, hypertension, heart disease in which oxidative stress may intensify complications of these diseases (34). Antioxidants have a relationship with apoptosis in the ovarian tissue via different mechanisms. These are connected with proper growth and action of interstitial cells (35). Antioxidants, which impede or restrict, the harmful results of oxygen radicals have a vital role in the reproductive system functional and fertility process in the female (36). The dietary approaches to stop hypertension (DASH) diet is featured with using of the high amount of fruit, vegetables and legumes (37). A review study showed that the DASH diet and lower levels of oxidative stress biomarkers are related to each other (38). Obese women with the DASH eating plan are at the risk of the improvement of insulin resistance and inflammatory factors (37). Also, a dietary trigger like glucose can induce oxidative stress and cause an inflammatory response even without excess of adiposity in the PCOS patients. Based on a previous study, Mediterranean-inspired low glycemic load anti-inflammatory diet can improve hormones, body composition, and menstrual cyclicity, metabolic status, and inflammatory factors (39).

Our study has several limitations. Although, the validity and reproducibility of FFQ among Iranians has been well supported, our data may suffer from measurement error. However, we excluded participants that under/over reported their energy intakes. The nature of case-control design with its inherent biases would not allow establishing causation. Since cases were chosen out of a newly-diagnosed PCOS patients, there is less concern about recall bias. Since control group were recruited from the same hospital, similar socioeconomic status, a great element in the developing countries that influence nutritional status/intake can be greatly controlled. In order to control selection bias, we selected our control group of the same clinical center because their participation rate were higher. Residual confounding may not be completely ruled out because of imprecise measurements of covariates. However, there is a small risk that measurement errors in confounders were extreme since the crude and multivariable results were basically similar. Moreover, studies have reported that clinic PCOS patients tend to be more overweight than community PCOS patients, which can significantly affect the severity of PCOS (40). Similarly, the women in our study were more severely affected. We did not match cases and controls for BMI due to overmatching as it could narrow the exposure range.

One of the strengths present study was the high participation rate. When dietary information is obtained at onset of disease, it may not always reflect a typical eating pattern, thus cases may have altered their diet according to their disease symptoms. Therefore, to reduce the possibility of changing the habitual dietary intake, cases were included in the study 3 months prior to the interview. Controls were also free of any major risk factors for PCOS. Another strength was the use of FFQ along with a detailed assessment with adjustment for potential confounders. Compared to a single 24-h dietary recall, the FFQ allowed us to collect rarely consumed food items as well as seasonal variations.

Conclusion

Our results indicated that a high TAC diet plays a role in lowering the odds ratio of developing PCOS, although large prospective studies are needed to expand on these findings.

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Authors' Contributions

G.E., N.S.; Conceptualized and designed the study and wrote the manuscript. G.E., N.S., S.R., M.N.; Analyzed data, interpreted the data, provided professional comments. N.S., S.R., M.N., S.N.M.; Collected data. S.M., S.R., M.N., S.N.M.; Critically revised the manuscript for intellectual content and data accuracy. G.E.; Had responsibility for final content. All of the authors read and approved the final manuscript.

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Original Article

Can Laparoscopic Cystectomy Improve Pregnancy Outcomes in Endometrioma? A Prospective Clinical Trial Study

Sedigheh Hosseinimousa, M.D.[1*], Leili Safdarian, M.D.[1], Ashraf Aleyasin, M.D.[1], Marzieh Aghahosseini, M.D.[1], Marzieh Talebian, M.D.[2]

1. Department of Obstetrics and Gynecology and Reproductive, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran
2. Department of Obstetrics and Gynecology, Kashan University of Medical Sciences, Kashan, Iran

Abstract

Background: The purpose of this prospective study was to compare the ovarian response and pregnancy outcomes in the infertile women with endometrioma undergoing assisted reproductive technologies (ART) in two groups, who were underwent laparoscopic cystectomy and received gonadotropin releasing hormone-agonist (GnRH-agonist) and who only received GnRH-agonist without any surgery.

Materials and Methods: In this prospective clinical trial study, 79 infertile women with asymptomatic endometriomas cyst (2-6 cm) were enrolled and randomly assigned to two groups. First group underwent laparoscopic cystectomy and received GnRH-agonist. Second group only received GnRH-agonist without any surgery. Following ovulation induction, all patients underwent intracytoplasmic sperm injection (ICSI). Different parameters such as the number of retrieved oocytes and embryos; were made our outcomes that analyzed using SPSS.

Results: The pregnancy rate, chemical and clinical, and live birth rate were higher in the combined group, although these differences were not statistically significant (48.48% vs. 30.8%, P=0.12, 36.36% vs. 25.6%, P=0.32, 36.36% vs. 23.1%, P=0.29). The number of injections, antral follicles, retrieved oocytes, mature oocytes, total embryos, transferred embryos and duration of stimulation were similar in two groups.

Conclusion: Laparoscopic cystectomy followed by receiving GnRH-agonist improves pregnancy outcomes in endometrioma prior to treatment with ART (registration number: IRCT201106116689N2).

Keywords: Cystectomy, Endometrioma, Infertility, GnRH-Agonist

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Introduction

Endometriosis is defined as endometrial glands and stroma that occur outside of the uterine cavity. Evidence suggests that peritoneal microenvironment is altered by immune cells, extracellular matrix metalloproteinase and pro-inflammatory cytokines, creating the conditions for abnormal endometrial cell proliferation and survival (1, 2). Infertility is the most important complaints in patients with endometrioma (ovarian endometriotic cyst). Endometriomas reduces the number of follicles and oocytes quality (3-5). *In vitro* fertilization (IVF) is considered as an effective method for treatment of infertility in patients with endometrioma (6). In the presence of endometrial cyst lower embryo quality, lower implantation rate and higher pregnancy complications were seen compared to the absence of endometrial cyst during IVF cycles (7). There are different treatment options for women with ovarian endometriotic cysts before IVF, including surgical treatment, medical treatment, and a combination of surgical and medical treatments and expectant management among infertile women (8-12)

The gonadotropin releasing hormone-agonist (GnRH-agonist) is one of the medical options. Since the endometrioma is an estrogen-dependent disorder, GnRH agonist can produce a hypo-estrogenic environment by suppression of the hypothalamus and improve pregnancy rates at IVF (12, 13).

The purpose of the present prospective study was to investigate the ovarian response and pregnancy outcomes after laparoscopic cystectomy plus GnRH- agonist versus GnRH- agonist alone among infertile women suffering from endometrioma.

Materials and Methods

Obtaining the formal approvals from the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (90-02-30-14261-40996) this prospective

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***Corresponding Address: P.O.Box: 1411713135, Department of Obstetrics and Gynecology and Reproductive, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran**
Email: hoseinimosa@sina.tums.ac.ir

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clinical trial study was conducted in the Shariati Hospital of Tehran University of Medical Sciences, Tehran, Iran. Also, this study was restarted at the Iranian Registry of Clinical Trials (IRCT201106116689N2). All participants signed an informed consent before entering the study.

All women less than 40 years old with endometriosis and asymptomatic (without pain, torsion or rupture) endometriomal cyst (2 to 6 cm diameter) who were candidate to intracytoplasmic sperm injection (ICSI) were invited to this study. Who suffered of endometriomal cyst smaller than 2 cm or larger than 6 cm and who have history of surgery for endometrioma or other ovarian cysts were excluded. Also, women that their husbands suffered of azoospermia were omitted of this study.

In this prospective study, all infertile women who met the inclusion criteria were randomly assigned to two groups by a nurse from 2015 to 2019. This study was blinded for the nurse who randomized the participants and the statistician who analyzed the results. Randomization was done with an allocation sequence generated by block randomization by the trial statistician. All participants signed an informed consent before entering the study.

During this period, ninety-one of the women were assessed. Twelve of them did not have inclusion criteria. Six patients did not agree to be in a group and were excluded from the study. Finally, 73 participants were divided into 2 almost equal groups given a block size of 40.

Endometriosis was diagnosed in all patients previously by laparoscopy and sonography. Ultrasound diagnosis of endometrioma was present of round shaped homogeneous hypoechoic tissue. All transvaginal ultrasound examinations underwent by an expert physician gynecologist. The non-surgical diagnosis of endometrioma with transvaginal ultrasound is a valid method (14, 15). The combined group (laparoscopic cystectomy plus GnRH-agonist) underwent the laparoscopic cystectomy by a single experienced surgeon and the surgical technique consisted of resection of the endometriotic cyst wall. Then patients received 3 doses of Diphereline 3.75 mg (Beaufouipfen, France, IM) in 3 consecutive months. The GnRH-a alone group received three doses of Diphereline 3.75 mg (Beaufouipfen, France, IM) during three consecutive months.

Ovarian stimulation was conducted in two groups by Gonal F (Serono, Switzerland, 300-450 IU daily), ten days after the third injection of Diphereline. Seven days later, Gonal F was replaced by human menopausal gonadotropin (hMG, Ferring, Germany, 300-450 IU daily) until the observation of 18 mm follicles in the transvaginal ultrasound. With observation of at least two 18-mm follicles, human Chorionic Gonadotropin (hCG, Ferring Co, Germany, 10000 IU, IM) was injected and trans-vaginal oocyte retrieval was performed under general anesthesia after 36 hours. Fertilization was performed through ICSI. For blinding the study, counting the number of retrieved oocytes; determining the quality of oocytes and embryos and fertilization were performed by an embryologist who was not aware of the treatment groups. Transcervical embryo transfer was carried-out after three days. The chemical pregnancy was diagnosed based on the rising concentration of serum hCG levels. This measurement was performed in the Shariati hospital Laboratory service based on the standard serology method 14 days after embryo transfer. The clinical pregnancy was detected by observation of the pregnancy sac in transvaginal ultrasound, two weeks later of the hCG level rising.

Finally, the number of retrieved oocytes and embryos; quality of retrieved oocytes and embryos; fertilization rate, chemical and clinical pregnancy rates, abortion and live birth rates were compared between two groups by a statistician.

Statistical analysis

This study was blinded for the statistician who analyzed the results. All data were analyzed by SPSS software (Version 25, Armonk, New York, USA). The groups were initially compared on baseline variables with chi-square tests and independent samples t tests. Also, multiple logistic regression was used to examine the variables. Data were expressed as the mean ± standard deviation (mean ± SD) or percent (number). Statistical significance was defined as $P<0.05$.

Results

There were 79 patients who were divided into two groups randomly based on Bernoulli distribution and all of them underwent ovulation induction (Fig.1).

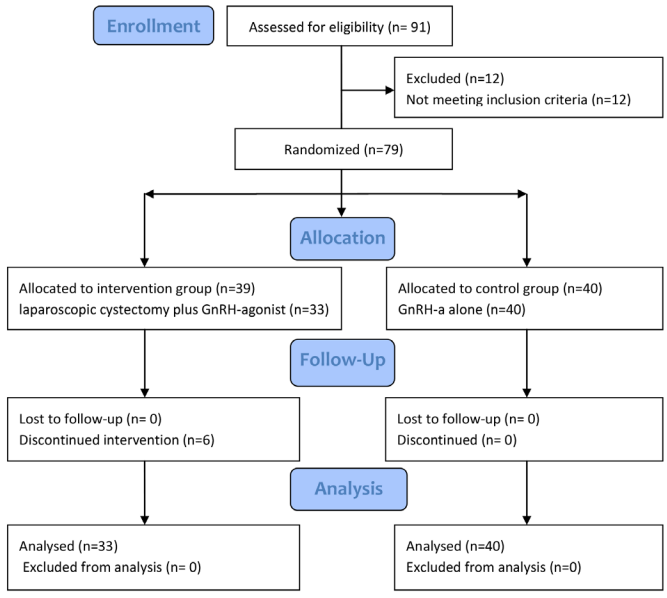

Fig.1: Study flowchart.

The demographic characteristics of two groups are shown in Table 1.

Table 1: Demographic characteristic of our study groups

Characteristic	Combined group (n=33)	GnRH-a alone group (n=40)	P value
Mean age (years ± SD)	30.3 ± 4.61	31.44 ± 4.49	0.125
BMI (kg/m^2)	23.91 ± 3.24	26.90 ± 3.78	0.004
Cyst diameter (mm)	49.18 ± 11.99	31.87 ± 10.63	<0.001
Duration of infertility (years ± SD)	6.21 ± 3.89	6.71 ± 4.30	0.309
Bilateral cyst (%)	18	8	0.108
Day 3 FSH (m IU/ml)	4.75 ± 2.22	6.58 ± 3.61	0.041
Day 3 LH (m IU/ml)	5.14 ± 3	5.30 ± 5.25	0.872
Day 3 estradiol (pg./ml)	56.24 ± 22.97	48.63 ± 37.34	0.435
Day 21 progesterone (pg./ml)	10.88 ± 9.20	8.13 ± 9.02	0.215
CA125 (m IU/ml)	51.9 ± 37.25	22.98 ± 15.37	0.004
AMH (ng/ml)	1.45 ± 0.95	1.70 ± 0.99	0.201

Crude and adjusted odds ratio for the association between the variables were reported as a confidence interval (CI) of 95%. Values are presented as mean ± SD. P<0.05 is defined for statistical significance. BMI; Body mass index, FSH; Follicle-stimulating hormone, LH; Luteinizing Hormone, GnRH; Gonadotropin-releasing hormone, and AMH; Anti mullerian hormone.

The IVF outcomes of two groups are shown in Table 2.

After laparoscopic cystectomy and receiving 3 doses of Diphereline, there were 16 chemical pregnancies in 33 patients. All of chemical pregnancies lead to live births (n=12, 3 twins /12); there were three abortions and one ectopic pregnancy (EP). Among 40 patients who received 3 doses of Diphereline before ART, there were 12 chemical pregnancies who lead to nine live births (3 twins); there were two abortions and one EP. Pregnancy outcomes of two groups are compared in Table 3.

Based on Table 1 Cyst size and CA125 level in combined group were higher; BMI and baseline FSH were lower than another group. Therefore, two groups were adjusted for BMI, Cyst diameter, FSH and CA125 levels in Table 4. There was no statistically significant difference between two groups regarding the live birth rate (Table 4).

Table 2: Comparison of IVF outcomes in our study groups

Characteristic	Combined group (n=33)	GnRH-a alone group (n=40)	P value
Duration of stimulation, day	10.07 ± 2.67	10.42 ± 1.55	0.529
Gonadotropin dose (IU)	3565.50 ± 1936.50	3578.25 ± 1593.75	0.797
No. of antral follicles	10.78 ± 5.97	11.92 ± 5.38	0.173
No. of retrieved oocytes	8.47 ± 6.52	9.55 ± 7.06	0.442
No. of mature oocytes	4.62 ± 3.62	5.30 ± 3.93	0.380
No. of total embryos	3.46 ± 2.77	3.62 ± 2.51	0.797
No. of grade 1 embryos	2.34 ± 1.82	2.15 ± 1.62	0.682
No. of transferred embryos	2.62 ± 1.59	2.38 ± 1.11	0.588
Cancellation rate, n (%)	2 (6.06)	1 (2.5)	0.398
Fertilization rate (%)	79	68	0.471

Crude and adjusted odds ratio for the association between the variables were reported as a confidence interval (CI) of 95%. Values are presented as mean ± SD. P<0.05 is defined for statistical significance. IVF; *In vitro* fertilization and GnRH; Gonadotropin-releasing hormone.

Table 3: Pregnancy outcomes in our study groups

Characteristic	Combined group (n=33)	GnRH-a alone group (n=40)	P value
Chemical pregnancy rate	16 (48.48)	12 (30.8)	0.124
Clinical pregnancy rate	12 (36.36)	10 (25.6)	0.325
Live birth rate	12 (36.36)	9 (23.1)	0.292
Abortion rate	3 (9.09)	2 (5)	0.740

Values are presented as n (%). P<0.05 is defined for statistical significance.

Table 4: Crude and adjusted OR for the association between type of intervention and live birth rate

Intervention	Crude OR (95% CI)	P value	Adjusted odds ratio* (95% CI)	P value
Combined/GnRH-a alone	1.91 (0.68-5.32)	0.220	1.08 (0.51-22.77)	0.961

BMI; Body mass index, FSH; Follicle-stimulating hormone, OR; Odds ratio, CI; Confidence interval, GnRH; Gonadotropin-releasing hormone, and *; Adjusted for: BMI, Cyst diameter, FSH, CA125.

No adverse effect or harm related to the drug and/or operation was observed in these groups.

Discussion

Endometriosis is a chronic inflammatory estrogen-dependent disease in which the endometrial glands and stroma grow outside the uterus. Infertility is the most important complaints in the patients with endometrioma (ovarian endometriotic cyst). IVF is considered as an effective method for treatment of infertility in patients with endometriomas (6).

Removal of endometriomas before IVF in infertile women is controversial. Since oocyte retrieval is difficult in the presence of endometrioma and because of the risk of follicular fluid contamination many clinicians consider cystectomy prior to the ART. Furthermore, bloody component of endometriomas is an excellent cultural environment and following the puncture of the cyst during the oocytes retrieving procedure, there are some risks for infection and pelvic abscess formation (16).

On the other hand, because of pseudo cyst nature of endometrioma without any capsule, it invades the ovarian cortex and its resection will be accompanied with the resection of natural ovarian tissues. It was shown that primordial follicles were found in more than 50% of resected endometrial cysts (17). Additionally, the ischemic injury resulted from the electro-coagulation and the related local inflammation will injure ovaries. Through the evaluation of the numbers of antral follicles and oocytes and anti-Mullerian hormones, many studies have reported ovarian reserve reduction after cystectomy (18-20).

Based on the European Society of Human Reproduction and Embryology (ESHRE) guideline (12), GnRH agonists for a period of 3-6 months prior to treatment with ART is one of the medical options used for improvement of clinical pregnancy rates in the infertile women with endometriosis. Sallam et al. (21) study showed that 3-6 months of treatment with GnRH agonists before the start of IVF associated with 4 times increase in the pregnancy rate of endometriosis patients. The theory for improvement of pregnancy rates at IVF with GnRH agonist pre-treatment is normalization of inflammation. Postoperative medical therapy with GnRH agonist can produce a hypo-estrogenic environment for treating microscopic foci which were not surgically removed (22).

In our study, although the rates of chemical and clinical pregnancy and live birth rate were higher in the combined group than GnRH-agonist alone group but these differences were not statistically significant.

Decleer et al. (23) divided patients with mild peritoneal endometriosis in the two groups. A group received GnRH agonist post-surgical for a 3-month period and the other group treated immediately after laparoscopy with stimulation for IVF. They detected no differences in the number of MII oocytes, embryos and pregnancy rate between their groups.

In the Dong et al. (24) study, 153 women underwent laparoscopic cystectomy then IVF and 68 women with an endometriomal cyst directly received IVF procedure. They found no significant difference regarding the numbers of retrieved oocytes, embryos; chemical and clinical pregnancy rates and live birth rate, that their results were consistent with our results.

Zhao et al. (13) concluded that prolonged GnRH-a protocol after ovarian endometrioma cystectomy may be an optimal choice in patients with Diminished ovarian reserve.

In Lee's study (25), patients with endometrioma were divided into three groups (surgery, aspiration of endometriomas and control) before ICSI, although the numbers of retrieved oocytes, were similar to our study. The clinical pregnancy rate was similar in all groups.

Alborzi et al. (26) enrolled patients with endometriosis in 3 groups: Letrozole group (2.5 mg/day, Femara, Novartis Pharma AG, Basel, Switzerland), GnRH-agonist group (Amp Diepherelin3.75 mg, Beaufour Ipsen Pharma Paris, France) and control group. The chemical pregnancy rate in stages III and IV of endometriosis in the all groups were similar to our GnRH-agonist alone group.

Yang et al. (27) reported that GnRH-a for six months after laparoscopic surgery can improve pregnancy rate in endometriosis. The pregnancy rate in this group was significantly higher than laparoscopic surgery group (30.77% vs. 9.23%, P=0.002).

In the Barra et al. (28) and Laganà et al. (29) study, IVF outcomes improved in patients with endometriosis who received Dienogest (progestin) for 3 months.

In the present study, the rates of chemical and clinical pregnancy and live birth were higher in the combined group than GnRH-agonist alone group but these differences were not statistically significant.

Our study had some limitations that should be mentioned. The sample size of this study was relatively small. Therefore, further randomized controlled trials are required to confirm the results of this study.

Conclusion

This study suggests that in the infertile women with endometriomal cyst Laparoscopic cystectomy followed by receiving GnRH-agonist prior to treatment with assisted reproductive technologies (ART) improve the rates of chemical and clinical pregnancy and live birth.

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Authors' Contributions

S.H., L.S.; Conceived and designed the experiments. A.A., M.A., S.H.; Performed the experiments. S.H., M.T.; Recorded the results. S.H.; Wrote the manuscript. All authors performed editing and approving the final version of this manuscript for submission.

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Original Article

Pharmacotherapy or Psychotherapy? Selective Treatment Depression in The Infertile Women with Recurrent Pregnancy Loss: A Triple-Arm Randomized Controlled Trial

Zahra Basirat, M.D.[1], Farzan Kheirkhah, M.D.[2], Mahbobeh Faramarzi, Ph.D.[1*], Seddigheh Esmaelzadeh, M.D.[1], Soraya Khafri, Ph.D.[2], Zahra Tajali, B.Sc.[1]

1. Department of General Courses, Infertility and Reproductive Health Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran

2. Social Determinants of Health Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran

Abstract

Background: Recurrent pregnancy loss (RPL) and infertility are associated with significant psychiatric complications. The study aimed to investigate the effectiveness of cognitive behavioral therapy (CBT) and sertraline in the treatment of in depression, anxiety, and infertility stress of depressed infertile women with RPL in comparison with usual care.

Materials and Methods: A triple-arm randomized controlled trial was carried out on the 60 depressed infertile women with RPL, a population of Infertility Center of Babol city, Iran, who were randomly assigned into three groups: pharmacotherapy with sertraline (n=20), psychotherapy with CBT (n=20), and a usual care as control group (n=20). The participants of psychotherapy received CBT sessions (90 minutes each) over 10 weeks. The participants in the pharmacotherapy group took 50 mg/day sertraline daily for 22 weeks. Outcomes were assessed using the Beck Depression Inventory (BDI-II), fertility problem inventory (FPI), and State-Trait Anxiety Inventory Form Y (STAI-Y) at the beginning of the trial, 10-weeks post-trial, and three months of follow-up. Using statistical package for the social sciences (SPSS) software, data were analyzed.

Results: CBT considerably reduced the depression symptoms more than sertraline with a moderate effect size at the post-trial (g=0.11, 95% CI: -0.03 to -0.50). Sertraline showed reduced the scores of state-anxiety more considerably in comparison with control group by a large effect size of post-trial (g=-1.04, 95% CI: -1.70 to -0.38). CBT reduced the total scores of FPI more considerably than sertraline, with a large, small size at follow up-trial [95% CI=-0.03(-0.65, -0.58)]. Both CBT and sertraline were superior to the control group in reducing depression and infertility stress.

Conclusion: Depression and infertility stress diminished under CBT and sertraline in depressed infertile women with RPL, with a significant advantage of CBT. Sertraline was superior to CBT in reduction of anxiety (registration number: IRCT201304045931N3).

Keywords: Anxiety, Cognitive Behavior Therapy, Depression, Infertility, Recurrent Early Pregnancy Loss

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Introduction

Recurrent pregnancy loss (RPL) is defined as two or more consecutive miscarriages before gestation week 22. It has been estimated to be prevalent in approximately 5% of clinically diagnosed pregnancies (1, 2). An overlap etiology and a number of collaborative pathologies were shown for infertility and RPL (3). Although, the risk of RPL increases in the women who are conceived with assisted-reproductive technology (ART) (4). Infertile females experience some psychological problems, including poor quality of life, depression, anxiety, sexual dysfunction function, and marital dissatisfaction (5-7). A recent meta-analysis reported 44.32% depression prevalence in the infertile women (8). Also, infertile women are at higher risk of psychological problems than infertile men (9). Women with RPL suffer from many psychiatric morbidities such as depression, anxiety, complicated grief, and suicide (10). Also, the psychiatric morbidity of infertility may be exacerbated by the RPL (11, 12).

Whilst the evidence for the effect of psychotherapy and pharmacotherapy in the mental health improvement of infertile women is robust, support for their use in infertile women with RPL is sparse. Most research has focused

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***Corresponding Address: P.O.Box: 4717647745, Department of General Courses, Infertility and Reproductive Health Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran**
Email: mfaramarzi@mubabol.ac.ir

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only on the infertile women who experiencing depression after *in vitro* fertilization (IVF) failure (13), while few studies have evaluated psychotherapy for depressed infertile women with RPL. To the best of our knowledge, no randomized controlled trial study has explored the effect of psychotherapy for women with RPL; it is unclear whether these findings can be generalized to depressed women with RPL. Nakano et al. (14) reported that cognitive behavioral therapy (CBT) reduced anxiety or depression scores in the 14 women with RPL. Patel et al. (15) reported that mindfulness psychotherapy improved the emotional adjustment in an infertile couple with RPL. Also, some research reported that CBT was useful for patients with a single perinatal loss (16, 17). Although, some research has proposed pharmacotherapy during pregnancy as a risk factor of pregnancy loss (18, 19). There are studies that recommended preconception counseling by psychologist/psychiatrists or antidepressants treatment for RPL women with severe depression (20, 21). To our knowledge, no study has been published to date that compare the effectiveness of psychotherapy and pharmacotherapy for depression treatment in the women with RPL.

We designed this study to investigate the effect of psychotherapy and pharmacotherapy in RPL women to compare the effect of the two methods of depression treatment in women with RPL. To the best of our knowledge, this is the first three arm randomized controlled trial that compares the effectiveness of CBT with sertraline for the treatment of depression in the infertile women with RPL. The hypotheses of the study were to examine: i. Whether CBT or sertraline is superior than usual care in reducing the score of depression in depressed infertile women with RPL, ii. Whether CBT or sertraline is superior than usual care in lowering the score of anxiety or infertility stress of depressed infertile women with RPL, iii. Which approach, pharmacotherapy or psychotherapy, is superior for mitigating the symptoms of depression, anxiety, and infertility stress among depressed infertile women with RPL.

Materials and Methods

Study type, setting, and duration

A triple-arm parallel-group randomized controlled trial design was conducted from November 2016 to December 2019 in the Fatemeh Zahra Infertility and Reproductive Health Research Center (Mazandaran, Iran), a single university-affiliated IVF center. The trial protocol was approved by the Ethics Committee of Babol University of Medical Sciences, Mazandaran, Iran and was registered in the Iranian Registry of Clinical Trials (IRCT201304045931N3). A written informed consent was obtained before the participants.

Study participants and procedure

All participants were recruited from Recurrent Abortion Clinic of the center Fatemeh Zahra Infertility and Reproductive Health Research Center. Eligibility criteria were included: i. Two or more consecutive miscarriages, ii. At least 5 years of education, iii. 18-40 years of age, iv. Meeting the criteria for probable diagnosis of depression with interview using the Structured Clinical Interview for DSM-5 Disorders (SCID-5-CV), v. Not undergoing fertility treatment until 6 months afterward. The participants were excluded if through clinical interviewing, the psychologist reported: i. Diagnosis of severe depression, bipolar disorders, schizophrenia, or suicide, ii. Having psychotherapy in the last three months, and current use of antidepressants. The excluded patients who suffered from severe mental disorders were referred to a psychiatrist to receive a suitable treatment.

A midwife assessed the inclusion criteria for the patients. If the patients met the inclusion criteria, they were invited to study and completed the demographic questionnaire. Women with initial eligibility in primary assessment were referred to our psychologist to receive a face to-face interview based on Structured Clinical Interview for DSM-5 Disorders (SCID-5-CV) (22). All participants completed three questionnaires, including the Beck Depression Inventory, second edition (BDI-II), Fertility Problem Inventory (FPI), and State-Trait Anxiety Inventory Form Y (STAI-Y) at baseline, 10-weeks post-trial, and three months of follow-up.

Sample size calculation

Available sampling was performed on the infertile women who referred to our center. As we could not find any research comparing the efficacy of CBT and sertraline on the infertile women with RPL, power calculation was performed based on published RCT of CBT and other pharmacotherapies in the infertile women (14, 23). Also, we conducted a pilot study to calculate the differences between the three groups of the study. To detect the smallest differences, 2.5 on the BDI-II, the minimum sample size for each group (α=0.05, power of 80%) was 16 participants. Thus, we recruited a minimum volunteer of 60 participants, with an attrition risk of 20%.

Randomization

Sixty depressed infertile women with RPL were divided randomly into three groups: pharmacotherapy with sertraline (n=20), psychotherapy with CBT (n=20), and a usual care as control group (n=20). Randomization was completed by an independent midwife according to 1:1:1 ratio using a computer random number generator. Also, allocation randomization was done using sequentially numbered sealed opaque envelopes and concealed from the researcher. The midwife assigned the participants manually and informed them via phone call. One of the study coordinators who was unaware of the trial allocation or the recruitment of the participants, evaluated the treatments.

Study interventions

Psychotherapy group

This experimental group received CBT enhanced with

Functional Analytic therapy (FACBT). Kohlenberg and Tsai (24) introduced FACBT to enhance the focus on the client-therapist relationship and to gain a broader insight into the cause of the problem and treatment. This model includes seven specific enhancement techniques the CBT therapist can use to address the needs of the patients. The seven techniques include expanded rationale, greater use of the patient-therapist relationship, employing case conceptualization, noticing and recognizing Clinically Relevant Behavior (CRB), asking questions to evoke CRBs, increasing self-awareness to detect CRBs, and applying modified thought records.

A female psychologist, who was expert in infertility branch, conducted the sessions. Psychotherapy was conducted in ten group sessions (90 minutes each) over 10 weeks. Each group consisted of 10 participants. The psychotherapy treatment was based on FACBT (24) as well as five domains of specific infertility stress (25). Table 1 summarized the contents of the sessions.

Pharmacotherapy group

The patients were visited at baseline as well as 2, 6, 10, 16, and 22 weeks post-trial for adjusting the medication and recording the symptoms plus adverse events. Also, there were optional supplementary visits or telephone contacts at any time.

Sertraline (Abidi Pharmaceutical Co., Tehran, Iran) treatment was begun at 50 mg/day. Dose changes were based on the response and side effects. If the symptom reduction was achieved, patients continued the initial dose of the sertraline. However, if the symptoms were not mitigated, the dose could gradually be raised to a maximum of 200 mg/day.

Usual care group

Participants of this group received usual care of the infertile without any psychological support.

Study outcomes

Primary outcomes

Beck Depression Inventory, second edition

This scale is a 21-item self-report inventory measuring the severity of depression. Each item is scored on a four-point Likert scale, ranging from 0 to 3. Total scores range 0-63 with higher scores indicate more severe depressive symptoms (26). We used Persian validated BDI-II (27). The Persian version of the BDI-II had high internal consistency (Cronbach's alpha=0.87 for) and acceptable reliability of test-retest (r=0.74).

Fertility problem inventory

This scale was developed by Newton to assess infertility stress (27). It consists of 46 questions. Each item is scored on a six-point Likert scale, ranging from 1 (strongly disagree) to 6 (strongly agree). Some items have reversed scores. The total score of FPI ranges from 46 to 276, where higher scores indicate higher levels of infertility stress. The FPI includes five subscales: social concern (worry about comments of family or friend about her infertility), sexual concern (reduction or difficulty of sexual arousal or enjoyment), relationship concern (worry of talking about infertility with relatives or friends), rejection of parenthood (negative view of life without child), and the need for parenthood (considering parenting as essential goal of life) (25). We used the Persian validated FPI. The validity of the Persian version of FPI was high for all domains (Cronbach's alpha coefficient 70%) (28).

State-Trait Anxiety Inventory Form Y

This scale that first developed by Spielberger et al. (29), is one of the most widely used instruments for capturing anxiety. The scale provides two different components of anxiety: state and trait. This study used the anxiety state component that includes 20 items answered on a 4-point Likert scale. The possible scores range from 20 to 80. We used Persian validated STAI-Y. The Cronbach's alpha for internal consistency of the Persian version of STAI-Y was 0.846 for state anxiety and 0.886 for trait anxiety. Also, the reliability and internal consistency were good (30).

Secondary outcomes

The secondary outcomes included treatment compliance and treatment satisfaction. Treatment compliance for psychotherapy group was defined as the mean number of attendance of the participants in the CBT sessions (from 10 sessions). Treatment adherence for pharmacotherapy was defined as the mean number of formal contact sessions with a psychiatric (from 5 sessions through phone or visit in our infertility clinic). For treatment satisfaction, the participants answered to a question and rated their feeling about the program from 1 (very low satisfaction) to 5 (very high satisfaction).

Statistical analysis

To examine participants' demographic characteristics, cross-tabulations stratified by three groups were used. ANOVA tests were applied to examine group differences in clinical characteristics at baseline. Also, t test and Chi Square test were applied to examine differences in adherence or satisfaction between the CBT and sertraline groups.

We used intention-to-treat analysis to manage the missing outcomes via multiple imputation chained technique (MICE). For the participants, linear mixed models with random intercepts, time, treatment group, and time-group interaction as fix factors were used to estimate each outcome measure in the our groups. Pairwise contrasts were used to compare group differences in the pre-to-post and pre-to-follow-up outcome scores. Also, pooled standard deviation adjusted for sample size (Hedges' g) was employed to examine the effect sizes. The effect sizes were defined as small (g=0.20), medium

(g=0.50), and large (g=0.80) (31). The data were analyzed using statistical package for the social sciences (SPSS) software version 18.0 (SPSS Inc., Chicago, IL., USA). We considered P<0.05 as significant.

Results

Baseline demographic and clinical characteristics

Of 60 women who entered the trial, 50 completed the trial from baseline to post-trial and follow-up (CBT group: n=19, sertraline group: n=13, control group: n=18). Figure 1 reveals the recruitment of the participants from the beginning of the study to post-trial and follow-up.

Table 2 describes the demographic characteristics of the participants in three groups of the trial. The women, aged 31.7 years (± SD=5.9). The majority of them had high school or university level of education. There were no significant differences with respect to age, education, infertility duration, and the number of miscarriages among these three groups.

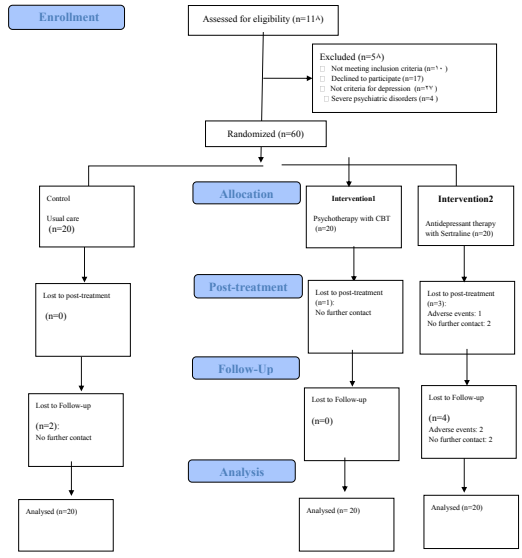

Fig.1: Flowchart of participants over the trial.

Table 1: Sessions outline of (FACBT)*

Sessions	Contents
1. Introduction of targets	Introduce program benefits, building empathy, Focused on setting strategies, problems related to infertility. Building a therapeutic alliance and obtaining information from the patient, identifying automatic thoughts about infertility, Home assignment: Coping diary. 30 minutes of daily attention to the five aspects of infertility stress.
2. Infertility stress	Reflection on, the last week and repetition. Emphasis on the therapeutic alliance. Training of A-B-C model of FCBT. Helping the patient to recognize that stressful thoughts about infertility teaching the use of the thought record. Home Assignment: Thought record (distinguish between rational and irrational thoughts). Attention to one stressful infertility experience.
3. Infertility stress related to the need for parenthood	Reflection on, the last week and repetition. Evoke Clinically Relevant Behaviors (CRB1s). Understanding thinking Errors. Dealing with automatic infertile thoughts in life and social concerns during a / the (please choose one of them) session. Home assignment: Seeking to fictional generalization and CRBs of infertility stress, especially the need for parenthood.
4. Infertility stress related to a child-free lifestyle	Reflection on, the last week and repetition. Self as control. Control as the infertility problem. Reinforcing CRB2s related to a child-free lifestyle. Attention to the acceptance of unchangeable events. Home assignment: Seeking to fictional generalization and CRBs** of infertility stress, especially the need for parenthood. Attention to the acceptance of a child-free lifestyle.
5. Infertility stress related to social concerns	Reflection on, the last week and repetition. Values clarification and commitment. Awareness in daily. Advancing CRB3s related to a child-free lifestyle. Dealing with automatic infertile thoughts in life related to social concerns. Home assignment: Seeking to fictional generalization and CRBs of infertility stress, especially social concerns.
6. Infertility stress related to the failure of ART	Reflection on, the last week and repetition. Dealing with difficult emotions about the failure of ART. Home assignment: Seeking to fictional generalization and CRBs of ART.
7. Infertility stress related to communication	Reflection on the last week and repetition. Dealing with difficult relationships. Home assignment: Careful attention to their relationship with their husband and others. Seeking to fictional generalization and CRBs of communication.
8. Self-compassion	Reflection on, the last week and repetition. Helping patients to love themselves. Home assignment: Attention to loving yourself with. Attention to detaching themselves from the infertility stress, the need for parenthood, rejection of a child-free lifestyle, social concerns, marital relationship problems, and marital problems. In addition, compassion for herself.
9. Calming down stressful thoughts related to infertility	Reflection on, the last week and repetition. Perspective Taking. Home assignment: Attention to the realization and nonjudgmental comprehension of the momentum of thoughts, especially about the ART failure, social concerns, marital concerns, and relationship concerns arising from unwanted thoughts about the five domains of infertility stress.
10. Relapse prevention	Helping the patient to develop a practice of her own, review of progress, insights, techniques, and the individual evaluation of the sessions. Reflection of the learned skills and final discussion.

***; The cognitive behavior therapy, enhancing with functional analytic therapy for women with infertility, **; The therapist used many techniques every session like, evoke CRBs, emotional validation, Increase the current effectiveness of certain stimuli infertility stimuli, or events as reinforcement, and positive reinforcement, and ART; Assisted reproductive technologies.**

Table 2: Demographic characteristics of the population study

Variables	CBT	Sertraline	Control	All patients
Age (Y)	32.7 ± 6.8	30.2 ± 4.9	32.2 ± 5.8	31.7 ± 5.9
Education (Y)				
<12	10 (50)	10 (50)	6.0 (30)	26 (43.3)
≥12	10 (50)	10 (50)	14 (70)	34 (46.7)
Job				
Employee	1.0 (5)	2.0 (10)	2.0 (10)	5.0 (8.3)
Unemployed	19 (95)	18 (90)	18 (90)	55 (81.7)
Number of abortion				
2	9.0 (45)	10 (50)	11 (55)	20 (33.3)
3	5.0 (25)	5.0 (25)	4.0 (20)	14 (23.3)
≥4	6.0 (30)	5.0 (25)	5.0 (25)	26 (43.4)
Duration of infertility (month)	69.6 ± 54.3	34.9 ± 8.2	60.6 ± 56.1	64.4 ± 49.0

Data are presented as mean ± SD or n (%). CBT; Cognitive behavior therapy.

Table 3: Within group effect sizes of the interventions from pre-treatment to post-treatment and follow-up in three groups of the trials

Outcomes	Description			Within group effect size pre-treat with post-treat	Within group effect size pre-treat with follow-treat
	Pre-treat	Post-treat	Fallow-up		
Depression					
CBT	23.1 ± 9.89	13.4 ± 12.2	16.1 ± 11.4	0.84 (0.30, 1.37)	0.54 (-0.01, 1.11)
Sertraline	23.4 ± 9.9	14.7 ± 10.2	22.9 ± 9.2	1.03 (0.53, 1.53)	0.08 (-0.21, 0.37)
Control	24.4 ± 8.1	24.2 ± 9.5	24.1 ± 9.5	0.02 (-0.30, 0.36)	0.04 (-0.29, 0.38)
Anxiety					
CBT	29.4 ± 7.3	45.4 ± 7.3	47.0 ± 6.7	0.42 (-0.15, 1.00)	0.25 (-0.35, 0.85)
Sertraline	51.5 ± 6.0	43.6 ± 5.6	46.9 ± 5.1	1.21 (0.58, 1.84)	0.83 (0.31, 1.35)
Control	47.5 ± 9.0	51.0 ± 8.5	50.2 ± 6.5	-0.38 (-0.86, 0.10)	-0.25 (-0.87, 0.36)
Infertility stress social concern					
CBT	34.9 ± 9.7	32.4 ± 9.8	30.6 ± 9.7	0.17 (-0.50, 0.85)	0.31 (-0.35, 0.97)
Sertraline	29.5 ± 8.3	28.2 ± 8.4	30.0 ± 6.9	0.16 (-0.23, 0.56)	-0.09 (-0.62, 0.43)
Control	30.0 ± 9.7	33.1 ± 11.5	35.8 ± 12.1	-0.23 (-0.73, 0.26)	-0.46 (-0.96, 0.04)
Sexual concern					
CBT	27.8 ± 7.6	24.4 ± 9.7	23.8 ± 9.6	0.28 (-0.32, 0.90)	0.28 (-0.42, 1.00)
Sertraline	24.2 ± 9.0	21.3 ± 8.8	23.7 ± 6.5	0.32 (-0.14, 0.79)	0.06 (-0.39, 0.53)
Control	22.9 ± 7.3	26.1 ± 8.9	26.0 ± 10.6	-0.40 (-0.84, 0.03)	-0.28(-0.82, 0.25)
Relationship concern					
CBT	35.9 ± 8.9	29.9 ± 9.3	30.5 ± 8.4	0.42 (-0.28, 1.13)	0.41 (-0.27, 1.10)
Sertraline	32.4 ± 10.0	29.4 ± 8.6	28.3 ± 6.8	0.36 (-0.05, 0.77)	0.52 (0.08, 0.95)
Control	30.2 ± 8.8	34.5 ± 8.7	34.5 ± 10.2	-0.50 (-0.96, -0.03)	-0.40 (-0.92, 0.12)
Reject of life without parenthood					
CBT	30.9 ± 9.8	26.9 ± 9.8	27.5 ± 9.0	0.35 (-0.16, 0.87)	0.30 (-0.23, 0.84)
Sertraline	23.3 ± 5.9	23.6 ± 4.8	28.6 ± 6.2	-0.06 (-0.43, 0.31)	-0.75 (-1.32, 0.18)
Control	31.1 ± 7.8	33.9 ± 7.3	32.2 ± 6.7	-0.63 (-0.96, -0.30)	0.94 (-1.34, -0.54)
Need for parenthood					
CBT	35.9 ± 12.4	29.5 ± 10.4	26.6 ± +9.7	0.58 (0.11, 1.05)	-0.03 (-0.69, 0.62)
Sertraline	38.7 ± 9.8	35.6 ± 10.1	39.2 ± 9.7	0.36 (-0.03, 0.77)	-0.05 (-0.40, 0.28)
Control	39.5 ± 9.5	39.3 ± 11.3	44.0 ± 10.3	0.02 (-0.31, 0.37)	-0.65 (-1.02, -0.28)
Total score					
CBT	165.6 ± 36.4	143.1 ± 34.3	149.1 ± 31.9	0.42 (-0.24, -1.10)	0.30 (-0.39, -1.01)
Sertraline	148.3 ± 34.6	138.2 ± 33.5	150.12 ± 9.0	0.34 (-0.05, 0.74)	-0.06 (-0.47, 0.35)
Control	154.1 ± 23.2	163.3 ± 42.7	175.6 ± 40.0	-0.25 (-0.70, 0.19)	-0.61 (-1.10,- 0.11)

Data are presented as mean ± SD or g (95% CI). Rang scores: Depression, 0-63; State anxiety, 20-80; Trait anxiety, 20-80; Social concern (1-60), sexual concern (1-48), relationship concern (1-60), rejection of life without child (1-48), need for parenthood (1-60), total scores of infertility stress (46-276). CBT; Cognitive behavioral therapy, *; Linear mixed models with random intercept time, treatment group, and time-group interaction as fix factors were used to estimate each outcome measure in three groups of the trial, P<0.05. The effect sizes (Hedges' g) were defined as small (g=0.20), medium (g=0.50), and large (g=0.80).

Treatment outcomes

Depression

In the CBT group, the score of depression, decreased more significantly in the post-trial than pre-trial with a large effect size [g (95% CI)=0.84 (0.30, 1.37)] and at follow-up over pre-trial with a moderate effect size [g (95% CI)= 0.54 (0.14, 1.11)]. In the sertraline group, the depression symptoms diminished more significantly at post-trial than at pre-trial with a large effect size [g (95% CI)=1.03 (0.53, 1.53)]. In the control group, the depression symptoms did not change significantly from pre-trial to post-trial and also, in the follow-up (Table 3).

There were significant group- time interactions for the severity of depression symptoms according to the BDI-II [F (4, 196.08)=4.96, P=0 .001)]. CBT decreased the depression symptoms more significantly, while the sertraline group showed a moderate effect size at the post-trial [g (95% CI)=0.11(-0.03, -0.50)] and large effect size at follow-up [g (95% CI)=-1.60 (-1.31, -0.03)]. The depression, diminished more significantly in the CBT group than in the control group with a large effect size at the post-trial [g (95% CI)=-1.00(-1.66, -0.27)] and follow-up [g (95% CI)=0.78 (-1.42, -0.11)]. Also, in the sertraline group, we observed a significant decrease in the depression symptoms in comparison with the control group, with a large effect size at the post-trial [g (95% CI)= -0.97 (-1.63, -0.32)], but not at follow-up (Table 4).

In the CBT and control groups, anxiety scores did not change significantly at post-trial than pre-trial and also, at follow-up in comparison with pre-trial. In the sertraline group, the score of anxiety dropped more significantly at post-trial than at pre-trial with a large effect size [g (95% CI)=1.21 (0.48, 1.84)] and at follow-up in comparison with pre-trial with a large effect size [g (95% CI)=0.83 (0.31, 1.35)].

There were significant group-time interactions among three groups for the severity of anxiety symptoms, according to state-anxiety [F (4, 174.33)=5.20, P=0 .001)]. There were no significant differences between the CBT group and the sertraline group in reducing the anxiety at post-trial and at follow-up. The scores of state-anxiety did not change significantly in the CBT group over the control group at post-trial and follow-up. Sertraline group lowered the scores of state-anxiety more significantly than the control group did with a large effect size of the post-trial [g (95% CI)=-1.04 (-1.70, -0.38)], but not follow-up.

Infertility stress

In the CBT group, the total score of infertility stress diminished more considerably at post-trial with a moderate effect size [g (95% CI)=0.42 (-0.24, -1.10)] and at follow-up with a moderate effect size [g (95% CI)= 0.32 (-0.39, -1.01)]. Of five subscales of FPI, CBT group showed the 'need to parenthood concerns' scale more considerably at post-trial with a moderate effect size [g (95% CI)=0.58 (0.11, 1.05)] and at follow-up in comparison with pre-trial with a moderate effect size [g (95% CI)=0.30 (-0.39, -1.01)]. The CBT group showed a more significant decrease in the total scores of infertility stress in comparison with the sertraline group, with a large, small size at follow-up [g (95% CI)=-0.03 (-0.65, -0.58)].

In the sertraline group, the total score of infertility stress did not change significantly at post-trial and follow-up. Of five subscales of FPI, only the sertraline group showed a decrease in scores of 'marital relationship concerns' more considerably in the post-trial with a moderate effect size [g (95% CI)=0.52 (0.08, 0.95)].

In the control group, the total score of infertility stress and the social concerns did not change considerably in the post-trial, but those scores increased significantly more at follow-up with a large effect size [g (95% CI)=0.61 (-1.10, -0.11). Also, the concerns about 'rejection of parenthood increased significantly in post-trial with a large effect size [g (95% CI)=-0.63(-0.96, -0.03)] and follow-up [g (95% CI)=-0.40 (-0.92, -0.12)]. Also, the score of 'concerns about the need of parenthood' increased significantly in the follow-up period than at pre-trial with a large effect size [g (95% CI)=-0.65 (-1.34, -0.54)].

Table 4: Between effect sizes of the interventions from pre-treatment to post-treatment and follow-up in three groups of the trials

Outcomes	CBT and sertraline*		CBT and control**		Sertraline and control*	
Depression	**Post**	**Follow up**	**Post**	**Follow up**	**Post**	**Follow up**
Anxiety	-0.11 (-0.73, -0.50)*	-0.67 (-1.31, -0.03)*	-1.00 (-1.66, -0.27)*	-0.78 (-1.42, -0.11)*	-0.97 (-1.63, -0.32)*	-0.13 (-0.75, 0.48)
Infertility stress	0.27 (-0.34, 0.90)*	0.02 (-0.59, 0.64)	-0.72 (-1.36, 0.11)*	-0.50 (-1.13, 0.18)*	-1.04 (-1.70, -0.38)*	-0.59 (-1.22, 0.04)*
Social concern	0.46 (-0.16, 1.09)	0.03 (-0.58, 0.65)	-0.06 (-0.68, 0.53)	-0.48 (-1.11, -0.10)	-0.49 (-1.12, 0.13)	-0.57 (-1.20, 0.05)
Sexual concern	0.34 (-0.27, 0.96)	0.01 (-0.60, 0.63)	-0.18 (-0.81, 0.65)	-0.22 (-0.84, 0.11)	-0.55 (-1.18, 0.07)	-0.27 (-0.89, 0.35)
Relationship	0.05 (-0.56, 0.67)	0.29 (-0.32, 0.91)	-0.52 (-1.15, -0.08)	-0.44 (-1.07, 0.12)	-0.59 (-1.22, 0.03)	-0.73 (-1.38, -0.09)*
Reject of parenthood	0.44 (-0.18, 1.06)	-0.13 (-0.75, 0.48)	-0.82 (-1.47, 0.09)	-0.99 (-1.64, -0.10)	-1.70 (-2.42, -0.98)*	-1.05 (-1.71, -0.39)
Need to parenthood	-0.61 (-1.24, 0.02)	-0.27 (-0.89, 0.34)	-0.92 (-1.58, -0.34)	-0.75 (-1.39, -0.14)	-0.35 (-0.98, 0.26)	-0.49 (-1.11, 0.13)*
Total scores	0.14 (-0.47, 0.77)	-0.03 (-0.65, -0.58)*	-0.53 (-1.16, -0.18)	-0.74 (-1.38, -0.33)	-0.67 (-1.30, -0.03)*	-0.74 (-1.38, -0.10)*

Data are presented as g (95% CI). CI; Confidence interval, CBT; Cognitive behavioral therapy, **; Linear mixed models with random intercept time, treatment group, and time-group interaction as fix factors were used to estimate each outcome measure in three groups of the trial, *; P<0.05. The effect sizes (Hedges' g) were defined as small (g=0.20), medium (g=0.50), and large (g=0.80).

There were significant group-time interactions between three groups for the total score of infertility stress, according to FPI [F (4, 2097.24)=2.97, P=0.022)]. Also, there were significant group-time interactions for two subscales of FPI, including marital concern [F (4, 189.6)=3.05, P=0 .008)] and rejection of parenthood [F (4, 127.23)=4.15, P=0 .004)]. The CBT group had a reduction at total scores of infertility stress more considerable than the sertraline group, with a large, small size at follow up [g (95% CI)=-0.03 (-0.65, -0.58)]. The CBT group had a reduction in the infertility stress scores more than the control group, with a large effect size in the post-trial [g (95% CI)=-0.53 (-1.16, -0.18)] and follow-up [g (95% CI)=-0.74 (-1.38, -0.33)]. Also, sertraline group showed less infertility stress than the control group, with a large effect size at the post-trial [g (95% CI)=-0.67 (-1.30, -0.03)] and follow-up [g (95% CI)=-0.74 (-1.38, -0.10)]. The CBT group had a decrease at scores of "the need to parenthood of infertility stress" more than the control group, with a large effect size at the post-trial [g (95% CI)=-0.92 (-1.58, -0.34)] and follow-up [g (95% CI)=-0.75 (-1.39, -0.14)]. Also, CBT group had a reduction in scores of 'rejection of parenthood' more than the control group did at follow-up with a large effect size [g (95% CI)=-0.99 (-1.64, -0.10)]. The sertraline group also had a reduction at scores of the following subscales of infertility of stress more than control did; social concerns at post-trial with a moderate effect size [g (95% CI)=-0.49 (-1.12, -0.13)], marital relationship concerns at follow-up with a large effect size [g (95% CI)=-0.73 (-1.38, -0.09)], and rejection of parenthood with a moderately small size of post-trial [g (95% CI)=-1.70 (-2.42, -0.98)] and follow-up [g (95% CI)=-0.49 (-1.11, -0.13)].

Secondary outcomes

Treatment compliance

Dropout rates were 5% (19/20) in the CBT group, 35% (13/20) in the sertraline arm, and 10% (18/20) in the usual care group. Out of 20 women of the CBT group, 19 persons provided post-trial (95%) and follow-up data (95%). 17 women of the sertraline group, (17/20, 85%) provided post-trial and 13 persons (65%) provided follow-up data. Women in the CBT group were more likely than those in the sertraline group to complete trial at the follow-up assessments [$\chi 2(1)=5.625$, P=0.02; OR (95% CI)=1.46 (1.04, 2.04)]. The CBT group (n=15 /20) attended 8.10 ± 1.83 sessions (mean ± SD) from 10 sessions (with the psychologist), (75% compliance). The mean number of sertraline sessions contacted with the psychiatrists was 2.60 (SD 1.23) from 5 sessions of formal contact with psychiatric. Also, 11 women of the sertraline group contacted with psychiatrist 3 to 5 sessions of formal contract for the treatment (55% compliance).

Treatment satisfaction

The mean score treatment satisfaction of the participations in the CBT group was very significantly higher (4.26 ± 0.99, rated; 1-5) than the scores of those treatment satisfaction with sertraline group (2.12 ± 1.08, t=6.081, P<0.001).

Discussion

Here, we compared the efficacy of psychotherapy with pharmacotherapy in improving depression, anxiety, and pregnancy stress of depressed infertile women with RPL. We found that both CBT and sertraline led to moderate to large improvements in the scores of depression and infertility stress in these women. Regarding depression amelioration, both CBT and sertraline were superior to the control group, and CBT was superior to sertraline, with a moderate to large effect size of post-trial and follow-up.

This study has been the first RCT to compare the effect of CBT with sertraline in depressed infertile women with RLP history, therefore, we could not find any research to use sertraline in the treatment of depression in the RPL women. Although, there was an RCT that had compared the effect of CBT vs. sertraline in the diabetic patients who suffered from major depression. They reported that both CBT and sertraline improved the depression in their patients, with a superiority for sertraline (32).

In line with our results, Nakano et al. (14) investigated the effect of individual CBT on the 14 patients with RLP and depression/anxiety. They observed that CBT was useful in the improving the scores of depressions based on the BDI-II measurement. Although in both studies, CBT reduced the depression of women, there have been differences between our study and Nakano' study. Respectively, these differences include: population (the infertile women of vs. non infertile women), the number of groups in the study (three groups, including CBT, sertraline, and control vs. one group, only CBT), and the design of the study (RCT vs. interventional study with pretest-posttest design).

The important question of these results is that how the efficacy of CBT in the treatment of depression, persisted until 3-month follows-up against sertraline. There are some assumptions. First, in psychotherapies such as CBT, the thoughts can be altered, which may be persisted for a long time or even forever. Secondly, the CBT group had more treatment adherence in comparison with the sertraline group. The attendance and cooperation in the treatment of CBT group were greater than the sertraline group. Also, participants in the CBT group also, received group psychotherapy with more benefits than individual therapy such as giving valuable support from the group, sharing feelings and experiences in the group, and receiving corrected feedback. Finally, patients who received sertraline had worries regarding the effect of sertraline on their fertility or their future children.

In the present study, sertraline, showed a reduce score of anxiety more significantly than the control group, with a large effect size at post-trial, but not at follow-up. There were no significant differences between CBT

and sertraline in reducing the anxiety at post-trial and at follow-up. Inconsistent with our results, a study reported that CBT reduced the anxiety of depressed women with RPL (14). Also, results of a systematic review reported that psychological support and interventions may reduce levels of stress, anxiety or depression on subsequent pregnancy of women with a miscarriage history (33). Our previous RCTs also revealed that CBT was an efficient approach in reducing the anxiety in infertile women (18, 34).

It is important to explain why CBT did not improve the anxiety of infertile women. It may be related to the treatment approach. First, we used a model of CBT enhanced with FACBT which emphasized infertility-specific stress, rather than general anxiety. Secondly, the focuses of therapy were treatment of depression, not anxiety symptoms. Finally, the practices for anxiety improvement were minor. Wenzel (35) suggested that interventional strategies such as cognitive restructuring, behavioral activation and mindfulness are essential in the patients with RPL. Focus on improving anxiety along with the depression is recommended for future psychotherapies research.

In the present study, CBT reduced the total scores of infertility stress more considerably than sertraline, with a large, small size at follow-up, but not at post-trial. Both CBT and sertraline were superior to the control group in mitigating infertility stress. We propose two assumptions. First, considering the mean value of infertility stress of depressed the infertile women at the baseline, we found that the mean level of infertility stress in the CBT group was higher than the sertraline group (165 ± 36.4 vs. 148.3 ± 34.3, respectively) in pre-treatment. Although, both CBT and sertraline reduced the total score of infertility stress at the post-treatment (138 ± 33.5 vs. 149.1 ± 34.3, respectively), this reduction was not considerable at post-trial. When the infertility stress mitigation in the CBT group continued to the follow-up, the different effect of CBT and sertraline would be significant. Secondly, as the effectiveness of CBT was required for practice of skills, reducing symptoms of the infertility stress in CBT lasted longer compared with sertraline.

Note that in this study, both CBT and sertraline changed only in the some subscales of infertility stress. For explaining these effects, we propose three reasons. First, considering the mean scores and range of scores at baseline, it is found that the mean of these two subscales was higher than that of the three others infertility subscales from the beginning. Secondly, the main effect of CBT and sertraline in mitigating infertility stress, especially the total score of the FPI, was on reducing "rejection of life without parenthood" and "need to parenthood". Third, these subscales are very important in the infertile women with RPL history in comparison with the control group. And, two subscales of FPI "rejection of life without parenthood" and "need to parenthood" increased in post-trial and follow-up.

These findings have particularly important clinical implications for gynecologists, psychiatrists, and psychologist. This study suggests that both CBT and sertraline are sufficient in the reducing depression and infertility stress of infertile women with RPL, history with a significant advantage favoring CBT. On the other hand, sertraline was superior to CBT in mitigating the anxiety score. The CBT group showed greeter adherence and satisfaction with the treatment than sertraline. Further study is required to investigate how to increase the adherence and satisfaction with pharmacotherapy in the infertile women with RPL.

While these findings are promising, there are some limitations to be noted. First, the disproportional number of dropouts from the CBT group and sertraline group was not addressed well. The dropouts of the pharmacotherapy were high. Of the six participants who discontinued taking sertraline, three patients explained that they experienced side effects such as agitation, nausea, and vomiting. Also, three other patients did not respond to our contacts with phone or social networks such as WhatsApp. Further research is required to assess the obstacles against infertile women with RPL history that taking anti-depressants medicine. Moreover, our results were provided from one infertility clinic of a small city. A multicenter study is a better choice for further studies. In addition, further research is needed to evaluate other psychotherapy interventions and other antidepressant effect on the anxiety and depression in this patients. Confirming our findings, require to test potential moderators influencing psychotherapy or sertraline response, and address an acceptance of therapy model.

Conclusion

This study provided preliminary support for the efficacy of CBT and sertraline therapy for infertile women with RPL history and offered a range of further research opportunities in this field. Future research is also necessary to demonstrate whether routine CBT/pharmacotherapy adjoined with treatments of ART would prevent the negative psychological consequences in these patients. Assessing whether adding CBT or sertraline to therapies is cost-effective for the treatment of depressed infertile women with RPL is also another research area.

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Authors' Contributions

M.F.; Participated in study design, drafted the manuscript. F.Kh.; Was responsible for overall supervision, revised the drafted manuscript. Z.B., S.E.; Conducted project managing. S.Kh.; Contributed extensively in interpretation

of the data and the conclusion. Z.T.; Contributed to data gathering. All authors contributed to the drafting of this manuscript and approved the final.

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Original Article

The Effective Factors on The Sexual Function of Polycystic Ovary Syndrome Women: A Cross-Sectional Study

Bita Fereidooni, Ph.D.[1], Ensiyeh Jenabi, Ph.D.[2*], Salman Khazaei, Ph.D.[3], Sara Abdoli, MSc.[4]

1. Midwife in Social Security Organization, Hamadan Branch, Hamadan, Iran
2. Autism Spectrum Disorders Research Center, Hamadan University of Medical Sciences, Hamadan, Iran
3. Department of Epidemiology, Hamadan University of Medical Sciences, Hamadan, Iran
4. Department of Nursing and Midwifery, Hamadan University of Medical Sciences, Hamadan, Iran

Abstract

Background: One of the most common endocrine disorders in women during reproductive age is polycystic ovary syndrome (PCOS). This study aimed to evaluate sexual functioning among women with PCOS in a sample of a region's population in the west of Iran.

Materials and Methods: This study was a cross-sectional study that was conducted on 130 women with PCOS who referred to three clinics of gynecology, infertility, and dermatology affiliated with Hamadan University of Medical Sciences, Iran, from September to November 2020. The measurement tools included demographic characteristics, hirsutism score, and sexual function was assessed using the female sexual function index (FSFI) questionnaire.

Results: In total, 60% of patients had reported sexual dysfunction related to lubrication, satisfaction, and pain as domains of sexual dysfunction. The number 109 (83.85%) of them had hirsutism and these patients had a lower score for lubrication (3.7 ± 1.47 vs. 4.47 ± 1.71, P=0.03), orgasm (3.2 ± 1.34 vs. 3.95 ± 1.37, P=0.02), satisfaction (3.4 ± 1.29 vs. 3.71 ± 1.33, P=0.03), and FSFI score (22.56 ± 5.78 vs. 25.42 ± 5.51, P=0.04). Women with higher education had reported higher scores of FSFI and its domains. Rural participants had a higher arousal score (3.93 ± 1.4 vs. 3.37 ± 1.28, P=0.04). Moreover, housekeeper women had higher scores regarding desire, pain, and total FSFI score.

Conclusion: Our results showed that there was a significant association between hirsutism and FSFI scores, different domains, including lubrication, orgasm, and satisfaction.

Keywords: Hirsutism, Iran, Polycystic Ovary syndrome

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Introduction

One of the most common endocrine disorders in women during reproductive age is polycystic ovary syndrome (PCOS) with a prevalence of between 5-24% in different countries (1). The PCOS phenotypic characteristics included enlarged ovaries, menstrual irregularities, and clinical and biochemical hyper androgens (2).

Different situations, as well as obesity, insulin resistance, lipid disorders, ovulatory infertility, and endometrial cancer, are associated with PCOS (3). Hirsutism, acne, alopecia, and infertility can lead and psychological problems (4).

Criteria for PCOS diagnosis are oligo/amenorrhea, clinical and/or biochemical hyperandrogenism, and polycystic ovaries (5). Patients with PCOS have various complications such as hirsutism, which can potentially affect their life quality and also, influence their self-body image, which can be considered challenging aspects of this disease.

Sexual dysfunction is defined as a problem during any phase of the sexual response cycle that prevents a person or couple from being satisfied with sexual activity and can be caused by physical, social, and psychological factors (6). Some studies reported that changes in the physical appearance associated with PCOS may be led to decreased sexual satisfaction (7, 8). Dashti et al. (8) in Malaysia reported that Sexual dysfunction occurred in 62.5% of women with PCOS and was associated with arousal and lubrication with 93.8 and 87.5%, respectively. Eftekhar et al. (9) in Tehran, Iran reported the frequency of sexual dysfunction was 57.7% in PCOS women. The domains of desire and arousal were reported 99.2 and 98.5%, respectively.

According to the fact that the study hasn't been conducted in the west of Iran, therefore, in this study, we evaluated the sexual functioning among a population of these patients of the west of Iran.

Materials and Methods

This study was a cross-sectional study among women with PCOS who referred to three clinics of gynecology, infertility, and dermatology affiliated with Hamadan

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***Corresponding Address: P.O.Box: 6517838696, Autism Spectrum Disorders Research Center, Hamadan University of Medical Sciences, Hamadan, Iran**
Email: en.jenabi@yahoo.com

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University of Medical Sciences, Iran, from September to November 2020. The Ethical Committee of Hamadan University of Medical Sciences, Hamedan, Iran, confirmed this protocol (IR.UMSHA.REC.1399.614). After explaining the aims of the study, written informed consent was obtained from the volunteer participants.

Women with the diagnosis of PCOS according to the Rotterdam diagnostic criteria were invited to contribute to the present study. These criteria included, Oligo/amenorrhea, clinical and/or biochemical hyperandrogenism, and polycystic ovaries (having 12 follicles or more in one or both ovaries and/or increased ovarian volume i.e. > 10 ml) (5).

The inclusion criteria for women were: age 18-45 years and married, not having non-classic adrenal hyperplasia, thyroid or metabolic disease, hyperprolactinemia, and not having psychiatric disorders. Pregnant women, breastfeeding mothers, and patients who received oral contraceptive pills (OCPS) or other hormonal medications that affect the hypothalamic-pituitary-gonadal (HPG) axis 3 months ago were excluded.

For sample size calculation, we considered the parameter in Eftekhar et al. (9) study that reported the 57.7% prevalence of sexual dysfunction in women with PCOS. We reached a sample size of 130, although considering the margin of error (d)=15%, non-response=5%, and alpha level=5%.

The measurement tools included some questionnaires and clinical evaluations. a. Background demographic questionnaire included age, location (urban and rural), job, education, height, weight, and infertility characteristics. This questionnaire was designed for this purpose. The face and content validity were performed. The tool validity was checked by 10 reproductive health experts. Also, the reliability of the questionnaire was approved by 15 women with PCOS. b. Hirsutism was evaluated based on the Ferriman-Gallwey scoring method by a trained interviewer under the supervision of a physician. The range of Ferriman-Gallwey scoring is from 0 to 36 and scores of 8 or higher are considered of having hirsutism (10). c. Using female sexual function index (FSFI) questionnaire, sexual function was assessed. FSFI self-report scale is a 19-item questionnaire for the dimensions of sexual functioning in women during the last month. This questionnaire contains six domains, including the desire (2 questions), subjective arousal (4 questions), lubrication (4 questions), orgasm (3 questions), satisfaction (3 questions), and pain (3 questions). The minimum total score is 2, and the maximum total score is 36. The higher scores show better function, while lower scores show no sexual activity during the past month (11, 12). This scale has been validated in Iran by Mohammadi et al. (13) that sexual dysfunction was defined as a total FSFI score of less than 28.

Table 1: The association between demographic characteristics with FSFI score and its domains

Demographic characteristics		n (%)	Desire	Arousal	Lubrication	Orgasm	Satisfaction	Pain	FSFI score
Location	Urban	101 (77.69)	3.69 ± 1.16	3.37 ± 1.28	3.75 ± 1.49	3.25 ± 1.37	3.77 ± 1.37	4.76 ± 1.04	22.59 ± 5.78
	Rural	29 (22.31)	3.72 ± 0.86	3.93 ± 1.4	4.07 ± 1.66	3.41 ± 1.35	4 ± 1.28	5.06 ± 1.1	24.19 ± 5.67
	P value		0.89	**0.04**	0.33	0.59	0.42	0.18	0.19
Age group (Y)	20-29	69 (53.08)	0.82 ± 1.17	3.7 ± 1.36	3.94 ± 1.58	3.23 ± 1.32	3.98 ± 1.37	4.79 ± 1.05	23.45 ± 5.83
	30-45	61 (46.92)	3.54 ± 0.99	3.26 ± 1.24	3.69 ± 1.48	3.37 ± 1.42	3.65 ± 1.31	4.87 ± 1.08	22.38 ± 5.7
	P value		0.15	0.06	0.34	0.55	0.18	0.68	0.29
Job	Housekeeper	105 (80.77)	3.8 ± 1.11	3.6 ± 1.38	3.93 ± 1.57	3.41 ± 1.41	3.9 ± 1.39	4.98 ± 1.02	23.91 ± 5.84
	Worker	25 (19.23)	3.24 ± 0.62	3.04 ± 0.93	3.8 ± 1.33	2.8 ± 1.02	3.5 ± 1.12	4.18 ± 1.06	20.14 ± 4.62
	P value		**0.02**	0.055	0.11	0.047	0.19	**<0.001**	**0.006**
Education	Primary	25 (19.23)	3.58 ± 1.28	3.46 ± 1.35	3.37 ± 1.63	3.04 ± 1.61	3.14 ± 1.02	4.94 ± 0.9	21.52 ± 5.66
	Guidance	45 (34.62)	3.52 ± 1.03	3.35 ± 1.3	3.48 ± 1.37	3.33 ± 1.45	3.88 ± 1.29	4.64 ± 1.12	22.2 ± 5.39
	High school	38 (29.23)	3.5 ± 1.06	3.13 ± 1.33	3.61 ± 1.4	2.8 ± 0.95	3.42 ± 1.31	4.51 ± 1.11	20.98 ± 5.39
	Diploma	13 (10.0)	4.68 ± 0.82	4.45 ± 0.74	5.63 ± 0.7	4.1 ± 0.73	5 ± 0.89	5.68 ± 0.5	29.55 ± 1.27
	Academic	9 (6.92)	4.24 ± 0.37	4.43 ± 1.09	5.07 ± 1.14	4.7 ± 0.99	5.41 ± 0.63	5.52 ± 0.49	29.38 ± 0.55
	P value		**0.003**	**0.004**	**<0.001**	**0.004**	**<0.001**	**0.001**	**<0.001**
BMI	Normal	31 (23.85)	3.72 ± 1.05	3.42 ± 1.07	4.1 ± 1.55	3.18 ± 1.48	4.05 ± 1.19	4.96 ± 0.87	23.46 ± 5.62
	Overweight	53 (40.77)	3.71 ± 1.13	3.61 ± 1.4	4.02 ± 1.64	3.32 ± 1.35	3.86 ± 1.53	4.77 ± 1.16	23.31 ± 6.07
	Obese	46 (35.38)	3.64 ± 1.1	3.4 ± 1.4	3.39 ± 1.32	3.33 ± 1.32	3.61 ± 1.19	4.79 ± 1.07	22.19 ± 5.67
	P value		0.94	0.69	0.06	0.87	0.35	0.22	0.54
Infertility	Yes	58 (44.62)	3.75 ± 1.17	3.34 ± 1.37	3.83 ± 1.56	0.38 ± 1.49	3.70 ± 1.43	4.92 ± 0.97	22.93 ± 5.59
	No	72 (55.38)	3.64 ± 1.03	3.61 ± 1.28	3.81 ± 1.52	3.22 ± 1.26	3.92 ± 1.27	4.75 ± 1.26	22.97 ± 5.72
	P value		0.57	0.25	0.94	0.50	0.34	0.37	0.97

Data are presented as mean ± SD or n (%). BMI; Body mass index and FSFI; Female sexual function index. Bold items are significant (P<0.05).

Statistical analysis

We used the independent t test and one-way ANOVA for comparison of background demographic characteristics with FSFI score (domains), and hirsutism. For data analysis, the Stata version 14 (StataCorp, College Station, TX) was used and P≤0.05 was considered significant.

Results

All 130 women with PCOS diagnosis answered the questionnaires. Of them, 101 (77.69%) were urban residents and 105 (80.77%) were housekeepers. Their mean age of them was 29.74 ± 5.3 (range: 20.44 years). Only 16.92% of them had a diploma or academic education and more than 75% had overweight or obese. The association between categorical variables with the FSFI score and its domains is shown in Table 1. Women with higher education had reported higher scores of FSFI and its domains (P<0.05). Rural residents had a higher arousal score in comparison with urban residents (3.93 ± 1.4 vs. 3.37 ± 1.28, P=0.04). Moreover, housekeeper women had higher scores regarding desire, pain, and total FSFI score (P<0.05). There was no significant association among body mass index (BMI) and age of our participants with FSFI score and its domains.

The mean (± SD) of domains of sexual dysfunction and frequency of sexual dysfunction is presented in Table 2. In total, 60% of patients had reported sexual dysfunction related to lubrication, satisfaction, and pain as domains of sexual dysfunction.

Table 2: The mean (SD) and frequency (%) of domains of sexual dysfunction among women with PCOS

Domain	Mean ± SD	Minimum	Maximum	Frequency (%)
Desire	3.69 ± 1.09	1.2	6	48 (36.92)
Arousal	3.49 ± 1.32	0.9	6	103 (79.23)
Lubrication	3.82 ± 1.53	1.2	6	130 (100)
Orgasm	2.93 ± 1.36	0.9	6	112 (86.15)
Satisfaction	3.82 ± 1.35	1.2	6	130 (100)
Pain	4.83 ± 1.06	1.6	6	130 (100)
FSFI score	22.95 ± 5.77	12.3	31.9	78 (60)

FSFI; Female sexual function index, PCOS; Polycystic Ovary Syndrome, and SD: Standard Deviation.

The relation between women's hirsutism based on Ferriman-Gallwey scoring with FSFI and its domains has reorted in the Table 3.

Discussion

Khademi et al. (14) in Iran reported that only 7 women of 100 infertile women reported normal sexual functioning. The most prevalent sexual problem among women was arousal with 80%. Another study in India showed that female sexual dysfunction among women in the domains of desire, arousal, lubrication, orgasm, satisfaction, and the pain was 44.0, 49.0, 37.0, 32.0, 37.0, and 34.6%, respectively (15). In our study, the frequency of sexual dysfunction was 60.0% in the PCOS affected. Therefore, the frequency of sexual dysfunction in our study is approximately similar to Eftekhar's (57.7%) and Dashti's (62.5%) studies. The difference in the frequency of sexual dysfunction might be due to the different evaluation tools and demographic characteristics of the women such as age and BMI.

Eftekhar et al. in Iran reported that BMI levels higher than normal had decreased desire and satisfaction among women with PCOS in their study (9). Kogure et al. (16) reported that desired and BMI were risk factors for sexual dysfunction, and overweight and obesity were risk factors for the degree of dissatisfaction. Ferraresi et al. (17) reported that the obese women had a higher risk for sexual dysfunction and lower FSFI scores. However, our findings showed that there was not a significant association between BMI and FSFI scores. The probable reason for this difference could be due to the self-report of weight and height by the women in the present study.
Also, Eftekhar et al. (9) showed that the effect of hirsutism was significant in all domains of the FSFI except for dyspareunia. Dashti et al. (8) in Malaysia showed that there was no significant association with any of the FSFI score domains between women with and without hirsutism, while in the present study, we observed a significant association between hirsutism and some of FSFI scores, including the domains of lubrication, orgasm, and satisfaction. In our study, the effect of hirsutism was not significant on dyspareunia, which is in line with Eftekhar's results. In addition, a systematic review has been performed in 2019 with 19 studies (18). There was no sexual dysfunction between women with PCOS and control subjects (18), which was the same as our results.
Limitations: We did not determine stress, depression, and psychological aspects in these women. Also, our study was a cross-sectional study that clinical/biochemical parameters were not evaluated. In addition, we had not defined a control group. Also, we have some suggestions such as an examination of the psychological aspects of women with PCOS. In addition, prospective cohort studies with a large sample are recommended to evaluate therapies performed to restore normal sexual function in women with PCOS history.

Table 3: The relation between women's hirsutism based on Ferriman-Gallwey scoring with FSFI and its domains

Hirsutism based on Ferriman-Gallwey scoring	n (%)	Desire	Arousal	Lubrication	Orgasm	Satisfaction	Pain	FSFI score
No	21 (16.15)	3.67 ± 0.82	3.57 ± 1.36	4.47 ± 1.71	3.95 ± 1.37	3.4 ± 1.29	5.02 ± 1.04	25.42 ± 5.51
Yes	109 (83.85)	3.7 ± 1.14	3.48 ± 1.32	3.7 ± 1.47	3.2 ± 1.34	3.71 ± 1.33	4.79 ± 1.06	22.56 ± 5.78
P value		0.91	0.76	**0.03**	**0.02**	**0.03**	0.36	**0.04**

FSFI; Female sexual function index. Bold items are significant (P<0.05). Data are presented as mean ± SD or n (%).

Conclusion

Our results showed that there was a significant association between hirsutism and FSFI scores, different domains, including lubrication, orgasm, and satisfaction. Therefore, proper intervention for women with PCO is essential.

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Authors' Contributions

E.J., B.F.; Study design, data analysis, interpretation, and manuscript writing. S.K., S.A.; Participated in the study implementation and content analysis, and revised drafts. All authors read and approved the final manuscript.

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Original Article

Primary Dysmenorrhea Associated with Psychological Distress in Medical Sciences Students in The North of Iran: A Cross-Sectional Study

Hajar Adib-Rad, Ph.D.[1], Farzan Kheirkha, M.D.[2], Mahbobeh Faramarzi, Ph.D.[2*], Shabnam Omidvar, Ph.D.[1], Zahra Basirat, M.D.[1], Mahmoud Haji Ahmadi, Ph.D.[3]

1. Infertility and Health Reproductive Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran
2. Social Determinants of Health Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran
3. Non-Communicable Pediatric Disease Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran

Abstract

Background: Primary dysmenorrhea is the usual medical status in medical students that are defined as pain during the menstrual period. This study was done to evaluate the psychological problems associated with dysmenorrhea.

Materials and Methods: Three hundred forty students aged 18 to 20 years participated in this cross-sectional study (194 with dysmenorrhea and 150 without dysmenorrhea). In this cross-sectional study, data were collected through the sociodemographic checklist, the verbal multidimensional scoring system (VMS), and the revised version of the Symptom Checklist-90 (SCL-90-R) questionnaire using the convenience sampling method. This questionnaire includes 9 Subscale and a GSI index. We considered psychological distress to be equivalent to the Global Severity İndex (GSI), which is obtained by dividing 90 questions by 90. The significance level of the tests was considered 0.05.

Results: The GSI of the SCL-90 score in the 194 students with dysmenorrhea and 150 students without dysmenorrhea was 1.02 ± 0.42 and 0.34 ± 0.15 respectively (P<0.001). In the group with dysmenorrhea, the severity of dysmenorrhea was significantly associated with a family history of dysmenorrhea and mother's education (P=0.012 and P=0.037, respectively). The strongest predictors of GSI>1 were a family history of dysmenorrhea and mother's education [odds ratio (OR)=2.33, 95% confidence interval (CI), 1.43-4.15 and OR=0.45, 95% CI, 0.24-0.87, respectively].

Conclusion: According to the result, dysmenorrhea is associated with psychological distress. Psychological interventions and counseling in addition to drug treatment are suggested for treatment of primary dysmenorrhea. Therefore, it is necessary to formulate strategies and health policies to recover psychological issues of menstrual health.

Keywords: Anxiety, Depression, Primary Dysmenorrhea, Psychological Distress

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Introduction

Primary dysmenorrhea is one of the most usual medical statuses during their childbearing age that is defined as pain during the menstrual period (1). In different studies, its prevalence has been reported to be 50 to 70.8% (2, 3). It is estimated that 3-40% of women have experienced moderate to severe dysmenorrhea (4). A numerous variety of symptoms during menstruation have been noted, including cramps, lower abdominal pain, headache, backache and leg pains, nausea, vomiting, diarrhea (5, 6), as well as exhaustion, dizziness, depressed mood and irritability (7).

Risk factors for menstrual pain include a family history of dysmenorrhea, high menstruation flow, earlier age at menarche, and length of menstrual periods (8). Pain and its subsequences oblige women to use pharmacological and non-pharmacological treatments such as yoga, aromatherapy, and herbs (9). Menstruation is a physiological appearance that has various biopsychosocial points. It has reflectance for women of all cultures and socioeconomic levels. In the late luteal phase, a majority of women experience at least some degree of incoordination of mind and body.

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***Corresponding Address: Social Determinants of Health Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran**
Email: mahbob330@yahoo.com

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Predictors of painful menstruation include eating habits, smoking, physical activity, or lifestyle (10). Stress, depressed mood, anxiety, and irritability may be caused by severe pain (11).

There is growing evidence of a psychological cause for primary dysmenorrhea. One study reported that person with severe menstrual pain not only report a various psychograph from people of their own age in conditions of their mental well-being but are also more discontented with their body figure (10). In addition, a study demonstrated that there is a relation between neuroticism and menstrual pain. Also, social support in person with menstrual pain was less than in person without menstrual pain (7). Primary dysmenorrhea is strongly related to depression and anxiety. Therefore, attention should be given to mental health screening in these people and psychological support may be necessary during their management (12). Studies are contradictory about the impact of dysmenorrhea on mood. Some studies reported that dysmenorrhea affects mood, and some reported that it is not related. Also, no study has been conducted in Iran that has measured the psychological problems of dysmenorrhea by SCL-90 questionnaire. Therefore, it's important to pay attention to the effect of dysmenorrhea on mood. This study was done to evaluate the psychological problems associated with dysmenorrhea.

Materials and Methods

Participants

In this cross-sectional study, three hundred forty four students of Babol University of Medical Sciences (194 with dysmenorrhea and 150 without dysmenorrhea) evaluated between 2017 and 2019 in the north of Iran. In this study, convenience sampling method was used. Inclusion criteria included students 18 to 20 years of age, mild to severe primary dysmenorrhea, no record of pelvic or abdominal operation, primary dysmenorrhea that has initiated after two years of menarche and consent to entering the research. The exclusion criteria included secondary dysmenorrhea, failure to record pain intensity, heavy coffee intake, smoking or other drugs (except drugs used for dysmenorrhea), having severe stress for 6 months before the study and people with psychiatric problems who were taking medication. Dysmenorrhea criteria included beginning of pain before or during the menses, menses pain lasting for 1-3 days during each menstrual cycle, low back pain and lower abdominal pain during menstruation (13).

The sample size was estimated using the G * Power Software, considering a significance level of 0.05 and power of 0.80, to gain an effect size of 0.18 between the variables: dysmenorrhea and psychological distress. It was estimated about 340 based on these criteria. We used the available samples during the sampling period and 416 people were invited to this study. The researcher gave a description about the aim of the study. Three hundred sixty nine members completed demographic/menstrual characteristics checklist and two questionnaires. We excluded four members with secondary dysmenorrhea and 21 members who answered incompletely to the questionnaires. Therefore, we enrolled 344 subjects in the final analysis including 194 members with menstrual pain and 150 students without menstrual pain. Therefore, sampling was performed on 344 students.

Measurements

In our study, data were collected through the demographic and menstrual characteristics, verbal multidimensional scoring system (VMS) and SCL-90-R questionnaires.

Demographic and menstrual characteristics checklist

The demographic and menstrual characteristics included body mass index (BMI), age, menarche age, interval and duration of menses, residence, the education level of mother and father, satisfaction with family income, and family history of dysmenorrhea.

The verbal multidimensional scoring system

This questionnaire consists of four questions to grading dysmenorrhea. VMS used to evaluate the score of dysmenorrhea degree of pain, restriction, and activities on the four degrees: without pain menses (nil), menses with pain but unusual use of analgesic or restriction of tasks (grade I), menses with medium pain with effect on daily activity and use of analgesic with relief (grade II), and menses with intense pain with the serious restriction on daily activity, useless use of analgesic, and symptoms such as nausea, affection, vomiting, headache, and diarrhea (grade III) (14). We selected students without dysmenorrhea based on the VMS scale, and students with painless menses (nil) on this scale were included in the dysmenorrhea group. In an Iranian study, the reliability and validity of VMS were calculated 0.81 and 0.78, respectively (15).

Symptom Checklist-90 R (SCL-90-R)

The SCL-90-R is a questionnaire in the field of psychiatry. This checklist contains 90 questions, 9 Subscale and a Global Severity İndex (GSI). Self-reported questionnaire evaluates the following 9 symptoms: phobic anxiety, obsessive-compulsive disorder (OCD), depression, aggression, anxiety, paranoia, sensitivity, somatization, and psychotic tendency. The total score is assessed as the GSI and GS>1 indicates psychological distress. We considered psychological distress to be equivalent to the GSI, which is obtained by dividing 90 questions by 90. Questions this checklist is with five answer items (0=never, 1=a little bit, 2=moderately, 3=quite a bit, 4=very). The average of each subcomponent is between 0-4. An average above 1 in GSI, as well as in each subcomponent is morbidity (16) . Its reliability in the study of Anisi et al. (17) Using internal consistency coefficients and test- re test with Cronbach's alpha method and Pearson correlation were obtained 0.98 and 0.82, respectively.

Ethical considerations

The Ethics Committee of Babol University of Medical Sciences approved the study (IR. MUBABOL. REC.2014.4232). Informed consent form was signed by students before the study on the declaration of Helsinki.

Data analysis

Data analysis was done by the statistical package for the social sciences (SP, version 22.0, SPSS Inc, Chicago, Illinois, USA) software package. Therefore, a t test was used for differences between the two groups, which were significant. Also, the t test and chi-square test were used for differences demographic/menstrual characteristics and psychological distress in members. Pearson Chi-Square and ANOVA tests were used to investigate the relationship between variables and dysmenorrhea severity in the group with dysmenorrhea. Also, the predictive factors of dysmenorrhea were examined using multiple logistic. The significance level of the tests was considered 0.05.

Results

The demographic/menstrual characteristics of the students are presented in Table 1.

Table 1: Demographic and menstrual characteristics in population study

Characteristic	With dysmenorrhea (n=194)	Without dysmenorrhea (n=150)	P
Age (Y)	19.35 ± 0.78	19.23 ± 0.75	0.254
BMI (kg/m^2)	21.83 ± 3.17	21.98 ± 3.35	0.913
Menstruation			
Menarche age (Y)	13.05 ± 1.46	13.00 ± 1.23	0.089
Menstrual cycle interval (days)	28.79 ± 4.26	28.8 ± 4.30	0.161
Menstrual cycle duration (days)	6.52 ± 1.08	6.52 ± 1.33	0.150
Residence			
Urban	97 (50.0)	77 (51.3)	
Rural	32 (16.5)	23 (15.4)	0.984
Dormitory	65 (33.5)	50 (33.3)	
Father's education			
Under the diploma	53 (27.3)	53 (35.3)	
Diploma	77 (39.7)	51 (34.0)	0.267
College	64 (33.0)	46 (30.7)	
Mother's education			
Under the diploma	97 (50.0)	162 (47.1)	
Diploma	79 (40.70)	135 (39.2)	0.026
College	18 (9.3)	47 (13.7)	
Family history of dysmenorrhea			
No	30 (15.5)	105 (70.0)	0.001
Yes	164 (84.5)	45 (30.0)	
Satisfaction with family income			
High	49 (25.3)	46 (30.7)	
Middle	141(72.7)	101 (67.3)	0.538
Low	4 (2.1)	3 (2.0)	

In the group with dysmenorrhea on the VMS, there was mild dysmenorrhea in 62 students (32.0%), moderate dysmenorrhea in 101 students (52.1%), and severe dysmenorrhea in 31 students (16.0%), which was a significant difference (P<0.001). Also, 58.8% of students with dysmenorrhea used medication to relieve pain. In the group with dysmenorrhea relationship between dysmenorrhea severity and demographic characteristics, presented in the Table 2. Results showed that severity dysmenorrhea was significantly associated with family history of dysmenorrhea and mother's education (P=0.012 and P=0.037, respectively).

Table 2: Dysmenorrhea severity and demographic characteristics in the group with dysmenorrhea

Characteristic	Mild (n=62)	Moderate (n=101)	Severe (n=31)	P value†
BMI (kg/m^2)	21.56 ± 2.88	22.21 ± 3.42	21.11 ± 2.73	0.174
Residence				
Urban	32 (33)	47 (48.5)	18 (18.6)	
Rural	9 (29)	20 (64.5)	2 (6.5)	0.483
Dormitory	21 (31)	34 (51.5)	11 (16.7)	
Father's education				
Under the diploma	15 (28.3)	34 (64.2)	4 (7.5)	
Diploma	30 (39)	33 (42.9)	14 (18.2)	0.089
College	17 (26.6)	34 (53.1)	13 (20.3)	
Mother's education				
Under the diploma	31 (32)	55 (56.7)	11 (11.3)	
Diploma	23 (29.1)	41 (51.9)	15 (19)	0.037
College	8 (44.4)	5 (27.8)	5 (27.8)	
Family history of dysmenorrhea				
Yes	45 (30.8)	77 (52.7)	24 (16.5)	0.012
No	17 (35.4)			
Satisfaction with income		24 (50.0)	7 (14.6)	
High	14 (28.6)	27 (55.1)	8 (16.3)	0.837
Middle	25(31.2)	40 (50.0)	15 (18.7)	
Low	23 (35.4)	34 (52.3)	8 (12.3)	

Data are presented as mean ± SD or n (%). †; The data were evaluated using Chi-square and ANOVA tests and BMI; Body mass index.

Table 3 shows psychological distress in the population study. The mean GSI of the SCL-90 score in the student with and without dysmenorrhea was 1.02 ± 0.42 and 0.34 ± 0.15 respectively (P<0.001). Also, in the dysmenorrhea group with psychological distress (GSI>1), somatization associated with pain intensity (P=0.033) and pain medication (P=0.039), OCD with pain medication (P=0.015), aggression with family income satisfaction (P=0.008), phobic anxiety with pain intensity (P=0.006), and psychoticism associated with menarche (P=0.002) and menstrual regulation (P=0.005). The results of logistic regression for GSI>1 in the group with dysmenorrhea are shown in Table 4. Six factors including BMI, pain intensity, residence, mother's education, father's education and a family history of dysmenorrhea were included in the analysis. According to this analysis, the strongest

predictors of GSI>1 were a family history of dysmenorrhea and mother's education (OR=2.33, 95% CI: 1.43-4.15 and OR=0.45, 95% CI: 0.24-0.87, respectively).

Table 3: Psychological distress in population study

Psychological score SCL-90-R	With dysmenorrhea (n=194)	Without dysmenorrhea (n=150)	P value†
Somatisation	0.91 ± 0. 51	0.35 ± 0.24	0.001
OCD	1.23 ± 0.51	0.43 ± 0.23	0.001
Interpersonal sensitivity	1.11 ± 0.56	0.44 ± 0.26	0.001
Depression	1.12 ± 0.61	0.33 ± 0.19	0.001
Anxiety	0.95 ± 0.58	0.32 ± 0.21	0.001
Hostility	1.01 ± 0.56	0.31 ± 0.22	0.001
Phobic anxiety	0.71 ± 0.45	0.27 ± 0.25	0.001
Paranoid ideation	1.31 ± 0.64	0.39 ± 0.26	0.001
Psychoticism	0.85 ± 0.46	0.22 ± 0.17	0.001
GSI	1.02 ± 0.42	0.34 ± 0.15	0.001

Data are presented as mean ± SD. SCL-90-R; Symptom Checklist-90-Revised, OCD; Obsessive-compulsive disorder, GSI; Global severity index, and †; The data were evaluated using t tests.

Table 4: Predictive factors of GSI>1 in multiple logistic regression analysis

Variable	OR	95% CI	P value†
BMI (kg/m^2)	1.11	0.66-1.87	0.690
Pain intensity	1.41	0.82-2.42	0.207
Residence	0.92	0.65-1.29	0.642
Mother's education	0.45	0.24-0.87	0.024
Father's education	1.32	0.57-2.33	0.521
Family history of dysmenorrhea	2.33	1.43-4.15	0.001

GSI; Global severity index, OR; Odds ratio, CI; Confidence interval, BMI; Body mass index, and †; The data were evaluated using multiple logistic regressions.

Discussion

Our study revealed that the mean global GSI in the student with and without dysmenorrhea was 1.02 ± 0.42 and 0.34 ± 0.15, respectively. In line with our study and based on some researches, students with dysmenorrhea reported mental distress. The intensity of dysmenorrhea and PMS were related to psychological distress (7, 18). Also, Fukushima et al. (19) reported that 15% of medical students had dysmenorrhea. In another study, researchers reported 70.8% of dysmenorrhea (3). One study reported that students with dysmenorrhea may not have adequate social support, and inadequate social support in students was attributed to high levels of alexithymia in this population. The researchers stated that many patients with alexithymia have relationship disorders (7). We can justify that dysmenorrhea cause psychological distress. The pain and discomfort caused by dysmenorrhea cause the student to become tired and affect her mood. Also, the student force and potency is reduced because of interaction with family and friends changes. This communication problem can cause psychological distress. Students must be under strong family support during menstruation. If this support is not provided, they will suffer, which can lead to a vicious cycle and increase their sense of pain.

Our finding showed that severity dysmenorrhea was significantly associated with mother's education not father. In students with a mother college education, mild dysmenorrhea was more common than moderate and severe dysmenorrhea. Also, moderate dysmenorrhea was more in people who had a positive a family history of dysmenorrhea in the core family members than students who did not have a family history of dysmenorrhea. This states that heredity play a role in dysmenorrhea. Bhusal et al. (20) reported that adolescents' level of knowledge about menstrual health was significantly related to living in rural areas, studying in private schools, mother's education to the extent of reading and writing, father's education at the level of 10, and living alone with mother. In other studies, primary dysmenorrhea was not related to maternal and paternal education but was related to BMI (21, 22). On the other, some studies reported that it was associated with a family history of dysmenorrhea (23, 24). Socio-cultural issues in any society can affect student dysmenorrhea in different ways. We should note that mother's education can be effective in supporting girls during dysmenorrhea. Indeed, this indicates that the mother's higher education can be effective in the care and health of their girls. The lack of signification between the father's education and the severity of dysmenorrhea may be due to the less relationship of fathers with their daughters' menstrual problems in Iran, which indicates a cultural issue.

The present study showed that some SCL-90 subscales were associated with pain intensity, pain medication, family income satisfaction, menarche and menstrual regulation. In line with our research and based on several researches, there are relationships between primary dysmenorrhea with pain medication, menarche and social class (3, 12, 25). Medication reduces the symptoms of dysmenorrhea as well as non-drug treatment such as relaxation. Pain management can alleviate the pain and suffering caused by primary dysmenorrhea and lead to peace of mind (26-28).

According to our study, the logistic regression showed that the strongest predictors of GSI>1 were a family history of dysmenorrhea and mother's education. In line with our study, the multiple logistic regression in one study showed that social support, alexithymia, neurotic personality, and a family history of dysmenorrhea were significantly associated with dysmenorrhea that were as its main risk factors. Low social support was strongest predictor of primary dysmenorrhea in this study. Also, alexithymia was second strongest predictor of primary dysmenorrhea (7). Alexithymia has negative effect and is associated with chronic pain (29). Many factors affect menstruation, including social demographic issues, sever physical exercise, sleep disorders, and psychological stress (30, 31). Stress is a key problem in many menstrual irregularities (32). In one study, sleep hours, alcohol intake, perceived stress, BMI and a history of diagnosed anemia were risk factors associated with menstrual

irregularity (33). Also, in one study which was done in Pakistan, 34.1% of students had menstrual changes (34). Moreover, a study in China determined that severe stress were related to menstrual disorder (31). Conversely, One study in India reported that there is no relation between severe stress and menstrual disorder (35). It should be stated that studies reported that self-medication without consulting health professionals in young women with period disorders is very usual (36).

Certain limitations should be said. We have used a self-administered questionnaire, and data were reported on the memory. They may have not provided the correct information. This study was done only on the student aged 18 to 20 years which the results can't be generalized to older ages. Another limitation of the study is that the study was carried out in students from a single Iranian medical university. If a larger study had been performed, more risk factors would probably have been identified. The cross-sectional quiddity of this research avoids any consequence about causal property. Interventional and prospective cohort researches are a more valid track of evaluating correlation between different risk factors and menstrual pain. Thus, we suggest that future studies would be done in this field.

Conclusion

Finally, our results showed that dysmenorrhea is associated with psychological distress. We need to promote the awareness of students, health professionals, and physicians about the psychological issues of menstruation. Psychological interventions and counseling in addition to drug treatment are suggested to treatment of primary dysmenorrhea. Therefore, it is necessary to use strategies and health policies to decrease psychological distress of women with dysmenorrhea.

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Authors' Contributions

H.A.-R.; Study conception and design, Literature Search, data Collection, manuscript preparation, analysis and interpretation of data and drafting of manuscript. F.Kh.; Study conception and design, manuscript preparation. M.F.; Study conception and design, manuscript preparation, analysis and interpretation of data and drafting of manuscript. Sh.O.; Literature Search, data collection, manuscript preparation. Z.B.; Study conception and design, manuscript preparation. M.H.A.; Analysis and interpretation of data. All authors read and approved the final manuscript.

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Original Article

Altruistic Donation of Surplus Embryos to Known and Unknown Recipients, The Dutch Approach

J.J.P.M. Pieters, M.D., Ph.D.*, M.H.A.M. van Miltenburg, Ph.D.

TFP-Medisch Centrum Kinderwens Leiderdorp, Leiderdorp, The Netherlands

Abstract

Background: Previous studies have shown that embryo donation can be a successful treatment for infertile couples, however the willingness of Dutch couples to donate or accept embryos was unknown. The aim of this article is to describe the protocol and results for altruistic embryo donation of the only embryo bank in the Netherlands.

Materials and Methods: This is a descriptive study. Since 2011, donated cryo-embryos from couples that have undergone *in vitro*-fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatments, are being stored in our embryo bank. The majority of the donated embryos were frozen on day 3 or 4 by slow freezing techniques. We perform a thorough medical and psychological screening of donor couples and recipients, according to the protocol drawn up in close collaboration with the Dutch Ministry of Health.

Results: Up to June 2021, 54 women have received embryos from our embryo bank, all single embryo transfers. While the clinical pregnancy rate in 'unknown' embryo donations was relatively high (25.3%), the live birth rate shows limited success (12.6%), partly due to high pregnancy loss through miscarriage. In known donation procedures, the recipients tend to undergo more procedures, depending on the number of donated cryo-embryos. Twenty-eight women received embryos from known donors, with a clinical pregnancy rate per embryo transfer of 24%, and live birth rate of 14%. In total, 82 recipients were granted donated cryo-embryos, twenty had an ongoing pregnancy (24.4%), nineteen of whom have given birth to a healthy child (23%).

Conclusion: Altruistic embryo donation of embryos appears to be satisfying for the donors, as they are not obliged to destroy their embryos, but instead help others build a family. Although success rates are still limited, partly due to the relatively high miscarriage rates and inferior freezing techniques, to this date nineteen out of 82 recipients have given birth to a healthy child.

Keywords: Altruism, Embryo Donation, Protocol, Screening

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Introduction

Many women and couples have to face the fate of unsuccessful infertility treatments and childlessness. They are left with the option of remaining without a child or decide to build a family through adoption. Embryo donation may create an ultimate possibility to have a child, in women who have no more options for treatment. Hundreds of thousands, perhaps millions of surplus embryos are stored at fertility laboratories all over the world. They may be forgotten after successful treatments, or parents are not able to decide what to do with them after their family is complete. The feeling of preciousness of these embryos could make the option to destroy very difficult (1). Couples may have endured a long road of multiple fertility treatments, before becoming parents themselves. Donating their surplus cryo-embryos provides a possibility of life for these embryos and therewith a feeling of satisfaction in the people who have decided that their family is complete (2, 3). While many countries have implemented embryo donation as a standard care for infertile couples, embryo donation was not an option in the Netherlands until 2011 when we set up the first embryo bank in our country. This innovation of care for the Dutch health system now enables embryo donation procedures for childless couples, and as yet we remain the only Dutch fertility center facilitating embryo donation (2).

The option to receive a donated surplus embryo provides a unique possibility of parenthood in patients that have no more possibilities and remain childless or perhaps decide to adopt. Accepting a donated embryo is not comparable to adoption. By Dutch law, the woman who is pregnant and gives birth to the child, is biologically and juridically the mother of this child, hence there is no need to adopt. Nor does her partner (when wedded or legally bound) need to adopt the child, but this may be different in other countries. The description "embryo-adoption" has been widely used and can be found in literature, but as such is not a correct term. The child will indeed not be genetically one's own, but will be "biologically, juridically and psychologically

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***Corresponding Address: TFP-Medisch Centrum Kinderwens Leiderdorp, Leiderdrop, the Netherlands**
Email: jacqueline.pieters@tfp-fertility.com

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one's own" from the very beginning of the pregnancy and has no juridical relation to the donating couple.

Many articles have been written on the procedures of embryo donation and the medical pregnancy risks for mother and child (4-7), on the possible psychological and social risks for donors and recipients (8, 9) and on the psychological development of the children and their bonding mechanism (10, 11). Different countries have different points of view on disclosure or non-disclosure, matching or not matching, and commercializing or non-commercializing (12-15).

In the Netherlands, gamete donation is only possible in a non-commercial and disclosed way (ie non-anonymous and registered). Dutch law was changed in 2004 in order to prevent anonymity, now stating that all donors must be registered and agree to share person identifying details. Thus, children of 16 years of age and older may request identifying information about their donor. In numerous countries gamete donation is commercial and anonymous. Even within Europe, there is a big difference in laws and insight on this matter between the different countries. Recently, the Council of Europe has stated that anonymous donation should not be permitted as non-anonymous donation is in the best interest of the child. The aim of this article is to describe the protocol and results for altruistic embryo donation of the only embryo bank in the Netherlands.

Non-commercial, non-anonymous embryo donation, a prospective and ongoing study

Fertility Clinic TFP Medisch Centrum Kinderwens in Leiderdorp, is the first and only government approved embryo bank in the Netherlands. We opened for procedures of embryo donation in 2011, to meet repeated requests from parents who had completed their family after IVF procedure and were facing a decision on their stored cryo-embryos. Some of these parents decided they want to give these surplus cryo-embryos "a chance at life" and help others build a family. We set-up a protocol for embryo donation procedures, in close collaboration with and monitored by the Dutch Ministry of Health. We followed the existing guidelines by ESHRE (16) and ASRM (17-20). We established a protocol that has been thoroughly checked and finally approved by the Dutch Ministry of Health in 2011.

In this article, we describe our embryo donation procedures, from counseling and screening in donation to accepting surplus cryo-embryos through our embryo bank, and share the results of these procedures.

Materials and Methods

Information brochures

Brochures for potential donors and acceptors can be found on our website: https://tfp-fertility.com/en-nl/tfp-mc-kinderwens-leiderdorp.

The Dutch approach

Since 2011, we have performed embryo donation procedures in a standardized protocolled manner, approved by the Medical Ethical Committee of TFP Medisch Centrum Kinderwens.

Intake of donating couples

Embryo donors are heterosexual couples, who have completed family building and wish to donate their surplus cryo-embryos to unknown or known others. We do not accept surplus cryo-embryos that were established using donor gametes, for two important reasons: i. future difficulties in explaining its genetic origin to the child and just as importantly: ii. So as not to surpass the limit of the number of offspring of a donor (in the Netherlands, a maximum of 25 children per donor is decreed in the appropriate guidelines, recently we have set the limit to a maximum of 12 families per donor). Cryo-embryos will only be accepted for donation in the embryo bank after the youngest child has reached the age of one year, this is to ensure that parents have enough time to rethink their donation and are completely sure of not wanting another child (21).

During the first consultation with the gynaecologist (J.P.), medical files are reviewed, and a medical and genetic anamnesis is performed to acquire information for possible genetic or health risks for the future child. In the majority of donating couples no clear cause of infertility was found, hence the cause was documented as idiopathic (Fig.S1, See Supplementary Online Information at www.ijfs.ir). Other couples were subjected to *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) due to reduced sperm quality or tubal pathology (Figs.S1, S2, See Supplementary Online Information at www.ijfs.ir). Donated embryos were frozen years ago, when slow freezing of embryos on day 3 or 4 was the standard in most Dutch laboratories (Fig.S3, See Supplementary Online Information at www.ijfs.ir). We assess donors' health, medication, smoking habits and the health of their children, as well as the possible medical problems of their own parents and brothers and sisters (genetic family tree). If a serious physical and/or psychological disorder is present in the donors -or their family, we cannot accept the embryos for our bank (Supplementary 1, See Supplementary Online Information at www.ijfs.ir). Next, the couple has a consultation with a social worker who discusses all consequences of embryo donation, checking for risks of not being able to let go. Relevant articles of Dutch law are discussed and explained, which entails that a child will have the right to know his or her genetic parents and may even try to get in touch with them and their children, who are 100% genetic siblings to these children. After the consultation with a social worker, the couple is screened for infectious diseases as has been done at the time of their IVF-treatment. This is stated by European law on donation of cells and tissues (12, 22): HBV, HBC, HIV and lues. This screening accounts for unknown as well as for known donating procedures.

Donating couples complete our anamnestic file by signing a contract to "give up their ownership" of their surplus

embryos (Supplementary 2, See Supplementary Online Information at www.ijfs.ir). Donating couples are informed that they will not receive any identifying information on a child that may be born as a result of their donation. If they like, they may write a letter to the future child, explaining their donation and express their best wishes for the child. These letters are evaluated to assure that the content will never negatively affect the child. We only accept surplus cryo-embryos for donation, if we find no risk for medical or genetic diseases and we ask the donating couples to keep us informed of any medical problems that may occur in the future. Of course, in fully disclosed donating procedures, donors and recipients will be able to meet with each other from time to time. We inform them on the importance of contractual agreements and we counsel on the change in mutual relationship.

Medical files of the donating couples and embryos are given a unique code to ensure the privacy of the donating couple. If the embryos are stored at a different clinic, we will assist to transport the embryos to our embryo-bank (Fig.1).

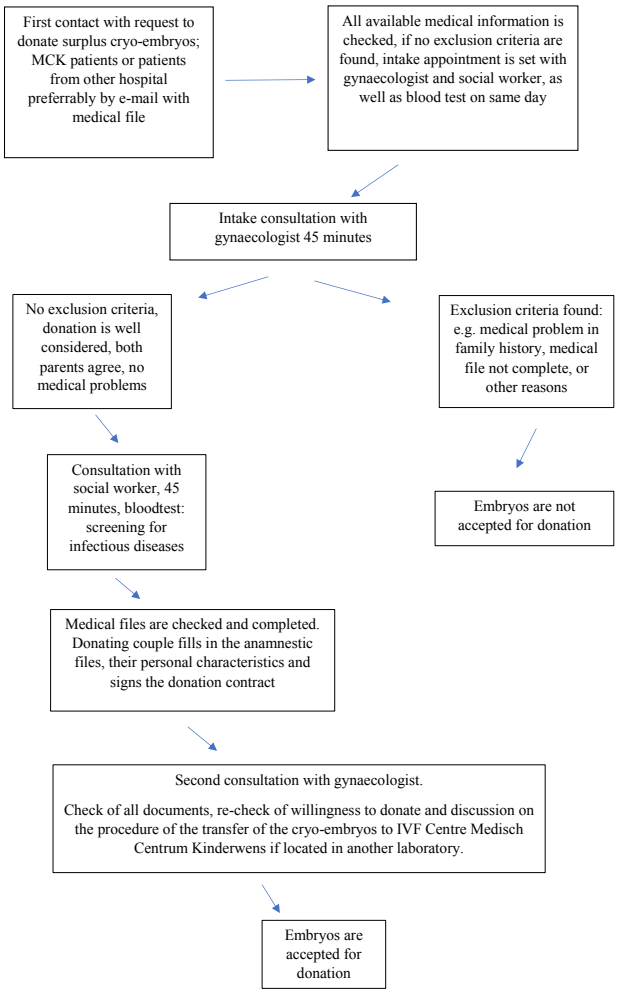

Fig.1: Donating couples.

Intake of recipient(s)

The accepting party, being heterosexual, lesbian or single, is counseled in a similar manner to donating couples: two or more consultations with the gynaecologist (J.P.), as well as at least one consultation with our social worker. During these consultations, an assessment is made to ensure that no other option for parenthood is left, which is one of the strict indications for an embryo donation procedure. We perform a medical and psychological screening and assemble all information on former treatments in the recipients' medical file. This screening accounts for unknown as well as for known donating procedures. Most recipients were diagnosed with idiopathic or male subfertility (Fig.S1, See Supplementary Online Information at www.ijfs.ir). All recipients were selected to have a healthy BMI, they had no concurrent diseases, they did not smoke and were advised to take folic acid on a daily basis (since 2018 daily vitamin D is also advised).

In these women, special attention is focused on their past fertility treatment and their ability to accept their infertility. At the moment of discussing the option of embryo donation, they have reached the point where they will remain childless or choose to adopt. Medical, social and psychological status is evaluated, as well as their willingness to be open to the child on disclosure of its genetic background (23, 24).

Pregnancy as a result of embryo donation has specific medical risks, comparable to egg donation pregnancies, such as miscarriage, bleeding, placental problems, hypertensic disorders, intra-uterine growth retardation and premature birth (4, 6, 7). These higher risks for complications in pregnancy urge us to evaluate the physical condition of the recipient and counsel her on these specific risks (Fig.2).

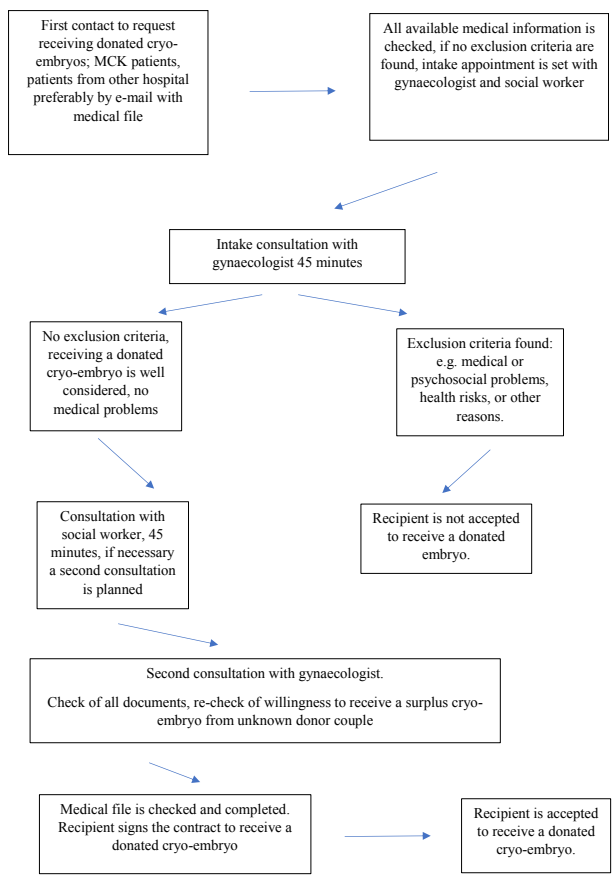

Fig.2: Recipients.

Embryobanking for donation of surplus cryo-embryos to an unknown other or to a relative or friend

In performing procedures of embryo donation in our fertility clinic, we thoroughly check and re-check the donor's motivations for donating their surplus cryo-embryo(s) to unknown recipients in multiple counseling sessions with a gynecologist (J.P.) and our social worker. We discuss the screening protocol of the (known or unknown) recipients and the in- and exclusion criteria (MCK brochures in English, Supplementary 3, See Supplementary Online Information at www.ijfs.ir). After having completed the counseling procedure of the donors and finishing all the paperwork, an extra telephone appointment with the donors is planned, in order to tell them the negative result of their infection screening tests and moreover, to check again their wish to donate. If there are no missing data, all questions have been answered and all test results have checked to be good, they are asked to complete and sign the the donation contract and, if applicable, the cryo-embryos are transported to our laboratory (supervisor M.v.M.). Most donated embryos were frozen on day 3 and 4 using a slow freeze protocol (Fig.S3, See Supplementary Online Information at www.ijfs.ir), their morphological grades ranging from 6-cell embryos to the morula stage. Only a small proportion of embryo donation transfers were blastocyst transfers (Fig.S3, See Supplementary Online Information at www.ijfs.ir), with morphological grades ranging from early blastocysts to hatching blastocysts. Morphological classification of embryos/blastocysts was performed using the scoring method described in the Istanbul consensus on embryo assessment (25). Donated embryos are stored in our embryo bank with a personal, anonymous code that is linked to their medical file.

Follow-up research among donors and recipients

We inform all donating couples that we would like to re-contact with them in a few years to assess how they feel at that moment in time about having donated their cryo-embryo(s). Also, we ask them to keep us informed on any changes in medical or psychiatric circumstances. This accounts for themselves as well as their currently healthy and well-developing children.

We will re-connect with the recipients who had a successful embryo donation and will try to contact them yearly in order to follow up with the parents and children. We intend to publish the results of our follow-up research in the coming years.

Results

Procedures and results

Since our start in 2011, 54 unknown and 28 known recipients have received embryos from our embryo bank, all single embryo transfers. We describe the diagnoses and fertility treatments of donors and recipients in Figure S1 (See Supplementary Online Information at www.ijfs.ir). Considering that some of the donated embryos were frozen over a decade ago, it is evident that the quality of embryos donated was variable. Freezing methods at that time were inferior to current freezing methods such as vitrification. Therefore, on average, more than one embryo was thawed in order to transfer one qualitatively good embryo (Tables 1, 2). Given the limited availability of embryos in our bank, women were granted a maximum of two embryo transfers. In donating procedures with a known recipient, all donated surplus cryo-embryos were specifically linked to the file, giving these recipients sometimes more than two options for pregnancy.

We performed 95 embryo transfers resulting in 24 clinical pregnancies (25%) and 12 live-births (12.6%) in donating procedures with an unknown recipient. The average age of embryo acceptors was 39 years, the average age of the donating women was 34.9 years.

We performed 50 donating procedures with a known recipient, resulting in 12 clinical pregnancies (24%) and 7 live births (14%). In known donation procedures, the average age of the acceptor was 37.8 years and the average age of the donating women was 33.9 years.

Collectively, 82 recipients (both known and unknown donations) were granted donated cryo-embryos, with an average of 1.76 embryo transfers per recipient. Of these embryo transfers, twenty had an ongoing pregnancy (24.4%), nineteen of them have given birth to a healthy child (23%).

While donation of gametes, such as oocytes, may often be linked to undesired side-effects such as hypertension, intra-uterine growth retardation and premature birth (5, 6, 26); we observed an expected percentage of medical problems in our embryo donation procedures (Table 3). In addition, all children were healthy at delivery and showed normal growth during follow-up.

Table 1: Characteristics of embryo donation procedures with unknown recipients

Variables	n or n (%)
Recipients	54
Embryos thawed	187
Number of embryos thawed per transfer	2.0
Number of embryo transfers	95
Number of embryo transfers per recipient	1.8
Cancelled embryo transfers	0 (0)
Clinical pregnancy per transfer	24 (25.3)
Ongoing pregnancy per transfer	13 (13.7)*
Live births per transfer	12 (12.6)
Average age of embryo donor	34.9 (27-41)
Average age embryo acceptor	39 (29-45)

All transfers were single embryo transfers. *; One pregnancy was terminated, because of multiple malformations.

Table 2: Characteristics of embryo donation procedures with known recipients

Variables	n or n (%)
Recipients	28
Embryos thawed	85
Number of embryos thawed per transfer	1.7
Number of embryo transfers	50
Number of embryo transfers per recipient	1.8
Cancelled embryo transfers	2 (3.8)
Clinical pregnancy per transfer	12 (24)
Ongoing pregnancy per transfer	7 (14)
Live births per transfer	7 (14)
Average age of embryo donor	33.9 (25-41)
Average age of embryo acceptor	37.8 (29-47)

All transfers were single embryo transfers.

Table 3: Follow up: pregnancy, delivery, and complications

Unknown donation	n	Known donation	n
Live birth	12	Live birth	7
Hypertension, pre-eclampsia	3	Hypertension, pre-eclampsia	None
Placental problems, bleeding	None	Placental problems, bleeding	1
Premature birth (<36 weeks), induction	None	Premature birth (<36 wk), induction	1
Premature birth (<36 weeks), caesarian	1	Premature birth (<36 wk), caesarian	None
Fetal distress, vacuum/ forceps delivery	None	Fetal distress, vacuum/ forceps delivery	None
Fetal distress, caesarian	None	Fetal distress, caesarian	None
Small for gestational age	None	Small for gestational age	None
Healthy child	12	Healthy child	7

Discussion

Embryo donation may create the ultimate possibility to have a child in women who have no more options for treatment. Donating surplus cryo-embryos provides a feeling of satisfaction in the people who have decided that their family is complete (27, 28).

Intensive screening procedures for donating couples as well as the recipients are important, in order to assess all possible medical, social or psychological problems for the future child, its parents and for the donating couples as well. We counsel complete and early openness to the child, as the child will be able to potentially meet its genetic parents and siblings at the age of 16. Dutch law ensures that all children conceived of donor gamete conception, will be able to find and ultimately meet with their donors. The important difference with other children that grow up as a single child, is that the children born through embryo donation procedures have full genetic siblings and may get in contact with their siblings later in life.

The results of embryo donation procedures from 2011 until today from the first government approved embryo bank of the Netherlands, show that the procedures have a limited success percentage (ongoing pregnancy) per embryo transfer to an unknown recipient of 13.7%. When observing the procedures for known recipients, we find a comparable success percentage of 14%. In most of these donations, the cryopreservation procedures as well as the thawing procedures were done in our own laboratory. It is generally known that blastocyst transfers demonstrate a higher live birth rate (29). As we have performed mostly day 3 embryo-transfers in the beginning years of our embryo bank procedures, this may explain our low ongoing pregnancy rate. Given the recent shift towards donation of day 5 blastocyst vitrified embryos, we anticipate higher pregnancy outcomes in the foreseeable future. Also, the advanced age of the receiving and the donating woman, is an important factor, as is known in oocyte procedures (30, 31). The advanced average age of the recipients in our program with unknown embryo donation may explain the high pregnancy loss through miscarriage (32, 33).

In earlier reports on embryo donation, a success percentage of Live Birth Rate was given as 14-40% (34, 35); most publications on embryo donation do not provide any success percentages but focus on ethical, social and moral issues. ASRM Ethics Committee published a statement in which it is clarified that scientific research indicates that even in natural conception, an estimated of 70% of all human embryos fail to result in live birth (36, 37). The Committee states that if the donated embryos are provided by people with impaired fertility, the percentage that will result to live birth may be lower than in natural conceiving population where no need for fertility treatment exists (18).

Dutch guidelines are very strict on medical intervention and fertility treatments, IVF/ICSI is only permitted if there is a clear medical indication. Our limited success percentage of LBR after embryo donation may partly be explained by the fact that the population reaching the stage of IVF/ICSI has a worse prognosis than in countries where these fertility treatments are offered with less restrictions and at a younger age.

In this report of our embryo donation pregnancies, we have observed a high pregnancy loss due to miscarriage; overall, the total pregnancy rate per embryo transfer was 25.5%. We presume that allograft immune reaction might be present in embryo donation, comparable to pregnancies in oocyte donation procedures, which may lead to miscarriage (38, 39).

We registered all pregnancy follow ups and found an expected percentage of pregnancy complications (33% in unknown embryo donation pregnancies and 28.5% in known procedures). The recipients' advanced age may have contributed to the proportion of pregnancy complications. All children that were born, were in good health and they have continued to develop well until now. One pregnancy had to be terminated because of severe fetal malformations.

We accept surplus cryo-embryos in our embryo

bank, in order to donate them to recipients that are unknown to the donors- but well-known to us after the screening consultations. This creates a situation of great responsibility, towards the donors as well as towards the recipients. When donors and recipient are well-known to each other, the existing relationships may change considerably, with the birth of a child but also when the embryo donation procedure is not successful. We feel responsible towards the donor couples, who handed over to us their cryo-embryos in full confidence that we will perform an adequate screening of the (known or unknown) recipients. The donors ask us to take good care of the embryos, they trust us to screen the recipient for her medical, social and psychological health and hope for the future child to have a happy life and a good future. The screening procedure of the unknown recipients is of course a snapshot of the current situation. This point is elaborately discussed with the donor couples, but we always keep the donors' concern in mind.

Our responsibility to the recipients lies in the thorough screening of all medical files of the donors, the search for possible genetic, medical, psychological or other risk for their future child after a successful donation procedure. Also here, we counsel the recipients that we can only screen the situation as is at the moment of screening, but that we ask donors to contact us if any medical or other problems may become clear in later years.

Thus, our embryo bank functions as a mediator, as we are the only professionals that get to know and counsel the donor couples as well as the recipients. The significant responsibility for the happiness of donors, recipients and the future children is always our priority.

Only in many years from now, will we know if the children originating from an embryo donation procedure of our embryo bank to an unknown recipient, will try to get in contact with their genetic family. Only then we can find out how the child, the biological mother and the donor couples have experienced the donation procedure. As has been stated in earlier reports, more data on these topics are warranted (40).

Conclusion

Donating one's surplus cryo-embryo to an unknown recipient shows to be an act of ultimate altruism for all donor couples we have seen in our clinic. In their own words: "we are happy to help someone else with our embryo to give them a chance for creating a family and raising their own child". The donation of these surplus embryos is not considered as giving away children, but the donated embryos are indicated as "a chance at happiness". Donating surplus cryo-embryos to known recipients may have an impact on existing relationships and thorough counseling and screening for medical or psychological risks is mandatory.

The recipients understandably are very happy to have been given this ultimate possibility of having a "child of their own". If the procedure is successful, they may experience pregnancy and childbirth. Even if the embryo donation procedure is unsuccessful, they are thankful to the donors to have offered them this last opportunity to possibly fulfill their wish for a child.

As a result of donating embryos from our embryo bank, until today 82 women have been offered an additional opportunity for motherhood. Nineteen of these recipients have given birth to a healthy child. We are confident that embryo donation procedures provide a safe addition to our pallet of treatment options when dealing with unsuccessful fertility treatments.

We will certainly aim at collecting data in the coming years, in order to learn more about these precious and vulnerable procedures.

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Authors' Contributions

J.P.; Designed the initial protocol in 2011 for the first and only embryo bank in the Netherlands. Since then, she screens personally all donors and recipients in close collaboration with the medical social workers of the clinic. M.v.M.; Shares responsibility for the embryo bank and provided the data and data analysis for this manuscript. J.P., M.v.M.; Wrote and edited the manuscript. All authors read and approved the final manuscript.

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Original Article

Comparison of Side Effects of COVID-19 Vaccines: Sinopharm, AstraZeneca, Sputnik V, and Covaxin in Women in Terms of Menstruation Disturbances, Hirsutism, and Metrorrhagia: A Descriptive-Analytical Cross-Sectional Study

Amirsaleh Abdollahi, M.D.[1], Iman Naseh, M.D., Ph.D.[2*], Fatemeh Kalroozi, Ph.D.[3], Mohammad Hassan Kazemi-Galougahi, Ph.D.[4], Maryam Nezamzadeh, M.Sc.[5], Shayan Sabeti Billandi, M.D.[1], Mojtaba Yousefi Zoshk, Ph.D.[6]

1. Mazandaran University of Medical Sciences, Sari, Iran
2. Department of Infectious Diseases, Faculty of Medicin, Aja University of Medical Sciences, Tehran, Iran
3. Department of Pediatric Nursing, Faculty of Nursing, Aja University of Medical Sciences, Tehran, Iran
4. Department of Social Medicine, Faculty of Medicine, Aja University of Medical Sciences, Tehran, Iran
5. Department of critical Nursing, Faculty of Nursing, Aja University of Medical Sciences, Tehran, Iran
6. Department of Trauma, Faculty of Medicin, Aja University of Medical Sciences, Tehran, Iran

Abstract

Background: Present study assessed whether Sinopharm, AstraZeneca, Sputnik V, and Covaxin's vaccinated women reveal a distinct incidence of menstruation disturbances, hirsutism, and metrorrhagia.

Materials and Methods: Data collection was performed from June to August 2021, and 427 women working in seven selected hospitals in Tehran were studied in this descriptive-analytical cross-sectional study. All of these women had received one or both doses of the vaccines with one of the assessed vaccines. Required data was collected via questionnaire and imported to SPSS 16 for further assessment and analysis. Fisher's Exact Test and Chi-Squared test were main statistical tests used to understand whether any significant relation exists or not.

Results: The participant's mean age and body mass index (BMI) were 29.78 ± 10.55 and 23.27 ± 3.82, respectively. Three hundred ninety-five cases (92.4%) had received both doses of the vaccines. Also, 154 cases (36.1%) had a history of COVID-19. A total of 38 cases (8.8%) of menstruation disturbances, 20 cases (4.6%) of metrorrhagia, and 7 cases (1.6%) of hirsutism were reported after receiving the vaccines. There was a significant difference among the vaccinated groups with the vaccines as mentioned earlier in terms of menstruation disturbances (hypermenorrhea, dysmenorrhea, Amenorrhea) (P=0.01). The highest and the lowest incidence of menstruation disturbances were recorded in the group vaccinated with Covaxin (17.6%) and Sputnik V (5%), respectively. There was also no significant difference amongst the vaccinated groups with the four vaccines regarding the incidence of metrorrhagia and hirsutism (P=0.10 and P=0.12, respectively). There was no significant relationship between all three complications incidence with the previous infection concerning all vaccines (coefficient=0.46, 1.27, -0.15 respectively for menstruation disturbances, metrorrhagia, and, hirsutism).

Conclusion: Seemingly, Covaxin revealed the most side effects in terms of menstruation disturbances. As a result, professionals must carry out several studies with reasonable samples to recommend the vaccine to those women confidently.

Keywords: Side Effects, Menstrual Cycle, Hirsutism, Metrorrhagia, COVID-19 Vaccines

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Introduction

COVID-19 is regarded as the most challenging pandemic disease, engaging global health and the economy. Since its outbreak in Wuhan, China, various initiatives, measures, and tools have been devised to control the pandemic. For instance, mask shields (1, 2), traffic and lockdown restrictions (3, 4), and social distancing (5, 6) are clear examples of disease control measures.

Interestingly, with the development of the vaccines in 2020, general vaccination became the most important method of controlling COVID-19, which, in turn, could reduce the pandemic prevalence and mortality rate (7, 8). Of course, general vaccination would be coupled

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***Corresponding Address: P.O.Box: 1411718541, Department of Infectious Diseases, Faculty of Medicin, Aja University of Medical Sciences, Tehran, Iran**
Email: imna3030@gmail.com

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with further studies in this regard (9). The most common complications of vaccination include fever, fatigue, headache (10), restlessness, injection site pain, and joint pain (11). On the other hand, researchers have always sought to scrutinize these vaccines' effects on particular groups, including women, such as their hormones, menstrual status, metrorrhagia, and hirsutism (14-12).

Menstruation is overshadowed with the following factors (15) as: sleep (16), stress (17, 18), nutrition (19, 20), occupation and its hazards (21), age (22), race and environment (26-23). Notwithstanding the relatively limited studies, this work offers valuable insights and compares the side effects of four types of COVID-19 vaccines (i.e., Sinopharm, AstraZeneca, Sputnik V, and Covaxin) in women in terms of menstruation disturbances, hirsutism, and metrorrhagia.

Materials and Methods

This study is part of a large-scale undertaken to investigate the side effects resulting from Sinopharm, AstraZeneca, SputnikV, and Covaxin vaccines among the female participants regarding menstruation disturbances, hirsutism, and metrorrhagia. A descriptive-analytical cross-sectional study was performed in 427 female participants working as part of a medical care team in seven selected hospitals in Tehran, Iran.

All subjects were enrolled in the study between June and August 2021. Of course, they had already received one of the four vaccines, Sinopharm, AstraZeneca, SputnikV, and Covaxin (92.4% two doses, 7.6% one dose), and at least more than twenty days had passed since they received the first dose. All participants have chosen their vaccine deliberately from available vaccine types named previously. Also, they had no history of menstrual irregularities (such as hypermenorrhea-dysmenorrhea), hirsutism, and metrorrhagia before vaccination. It should be noted that all the above-mentioned staff were regarded as our study sample (N=n). Data were collected through a modified questionnaire.

The design of the questionnaires was based on the valid documents of the Center for Disease Control and Prevention (CDC) and World Health Organization (WHO) which was thoroughly reviewed by five gynecologists, and the necessary corrections were made. To confirm the research tool's validity, the questions were checked with the content validity index (CVI), and then questions with CVI below 0.7 were removed, while the questions with CVI between 0.7 and 0.79 were reviewed. Finally, the CVI of the tool was reviewed and calculated again. To ensure the reliability of the research tool, Cronbach's alpha coefficient (27) was also evaluated, and the questionnaire's reliability was substantiated (α=0.86).

The designed questionnaire encompassed four open-ended questions, seven two-choice questions, and two multiple-choice questions. Those questions covered demographic information such as age, sex, height, weight, marital status, type of vaccine received, number of doses of vaccine received, and questions about the underlying disease (such as hypertension, hyperthyroidism or hypothyroidism, kidney, heart, lung, skin disease, and diabetes), and history of COVID-19 disease before receiving the vaccine. Also Three questions pertained to possible vaccine side effects in women. these questions included questions about menstruation disturbances (hypermenorrhea-dysmenorrhea-amenorrhea), metrorrhagia, and hirsutism after vaccination (IR.AJAUMS.REC.1400.163).

In receiving written informed consent from the participants, researchers referred to the selected medical centers and administered the questionnaire among the women on the care-treatment team of the seven mentioned centers. Inclusion criteria consist of these conditions: i. Being employed in 7 chosen centers, ii. Receiving one or both doses of vaccine with one of the four selected types of vaccine, iii. Being vaccinated at least twenty days earlier, and iv. Having no history of menstruation disturbances, hirsutism, and metrorrhagia. Besides, our exclusion criterion was an incomplete questionnaire. All participants were asked on the study's objectives and assured that all information would remain confidential. The research tool was distributed among participants. Having collected all the questionnaires, the data was analyzed using SPSS 16, International Business Machines Corporation (IBM), NY, USA.

Having matched the questionnaires and the software data, possible significant relationships between the study variables were determined using the Chi-Squared and Fisher's Exact Test with a significance level of 0.05. A multivariate analysis was done by logistic regression to determine the effect of demographic variables on menstruation disturbances, metrorrhagia, and hirsutism. Also, the crosstab method was applied to assess the effect of COVID-19 history on the aforementioned side effects. Central statistical and dispersion indices such as mean and standard deviation were also analyzed.

Results

According to the results, 427 women working in seven selected hospitals would complete the questionnaires (mean age: 29.78 ± 10.55). As for body mass index (BMI) index, 274 (64.1%) were normal and 102 (23.8%) were overweight. Also, 363 (86.9%) did not report any underlying disease; hypothyroidism or hyperthyroidism, hypertension, and a history of allergies were the most commonly reported diseases, respectively. Additionally, 157 (36.1%) reported a history of COVID-19 with a positive PCR test. Also, 203 (46.6%) had received Sinopharm vaccine, 116 (26.6%) AstraZeneca vaccine, 80 (18.8%) SputnikV vaccine and 34 (8%) Covaxin vaccine (Table 1).

Table 1: Demographic characteristics of participants

Vaccines Variables	Sputnik n (%)	Covaxin n (%)	AstraZeneca n (%)	Sinopharm n (%)
Marital status				
Single	20 (25)	15 (40)	34 (31.1)	147 (73.4)
Married	60 (75)	19 (60)	78 (68.9)	54 (26.6)
Number of received dose				
One	6 (7.3)	1 (2.9)	22 (19.8)	3 (1.5)
Two	74 (92.7)	33 (97.1)	90 (80.2)	198 (98.5)
History of underlying disease				
No disease	65 (82)	26 (79.8)	95 (84.7)	175 (87.5)
Hypertension	1 (1.2)	1 (2.9)	2 (1.8)	3 (1.5)
Hypo-or hyperthyroidism	2 (2.4)	1 (2.9)	3 (2.7)	3 (1.5)
Allergy	2 (2.4)	1 (2.9)	2 (1.8)	2 (1)
Neurological disease	1 (1.2)	0 (0)	0 (0)	1 (0.5)
Kidney disease	1 (1.2)	0 (0)	0 (0)	2 (1)
Lung disease	0 (0)	0 (0)	1 (0.9)	0 (0)
Diabetes	1 (1.2)	0 (0)	2 (1.8)	3 (1.5)
Skin disease	1 (1.2)	1 (2.9)	1 (0.9)	4 (2)
Heart disease	1 (1.2)	0 (0)	2 (1.8)	1 (0.5)
Liver disease	0 (0)	0 (0)	1 (0.9)	2 (1)
Addiction	2 (2.4)	1 (2.9)	1 (0.9)	3 (1.5)
Others	3 (3.6)	1 (2.9)	2 (1.8)	2 (1)
History of allergy to influenza vaccine	12 (5.6)	3 (9.4)	6 (1.9)	7 (0.8)
Previous COVID-19 Infection	24 (29.3)	8 (23.5)	38 (34.5)	84 (42)
Age (Y)				
<20	0 (0)	0 (0)	1 (0.5)	24 (12)
20-29	23 (29.4)	14 (40)	25 (22.9)	131 (65.8)
30-39	27 (32.4)	9 (26.7)	33 (29.7)	22 (11.4)
40-49	23 (29.4)	2 (6.7)	42 (37.5)	15 (7.6)
>=50	7 (8.8)	9 (26.7)	11 (9.4)	5 (3.3)
BMI (kg/m^2)				
Underweight (<18.5)	5 (6.3)	3 (6.5)	2 (1.6)	25 (12.5)
Normal (18.5-25)	42 (53.1)	21 (70)	68 (60.7)	123 (61.4)
Overweight (25-29.9)	25 (31.3)	8 (29)	33 (29.5)	40 (20.1)
Obese (>30)	8 (9.4)	2 (4.5)	10 (8.2)	13 (6)

BMI; Body mass index.

The study showed that 8% of women's Sinopharm vaccine, 10.7% for AstraZeneca vaccine, 5% receiving SputnikV vaccine, and 17.6% of women receiving Covaxin reported menstrual irregularities (hypermenorrhea-dysmenorrhea-menorrhea). The difference between the effects of these four types of vaccines on menstruation via the Chi-Squared test showed a significant difference among all of them (P=0.01). For this reason, we compared the four types of vaccines in terms of menstruation disturbances in pairs (6 cases) and, interestingly, only a significant difference was observed between Covaxin and SputnikV vaccines in terms of menstruation disturbances (P=0.02).

Based on the responses and the type of vaccine analyses, it was shown that the group vaccinated with Bharat Biotech vaccine (n=4, 11.8%) and SputnikV vaccine (2.5%, n=2) experienced the highest and lowest metrorrhagia incidence, respectively. The two groups of women vaccinated with the AstraZeneca vaccine (6.3%, n=7) and Sinopharm (3.5%, n=7) were placed in the second and third ranks. It should be noted that Fisher's Exact Test did not indicate a significant difference in the incidence of metrorrhagia (P=0.12).

The participants developed hirsutism were assigned in the following groups in a descending order: (0.5%, n=1) Sinopharm vaccination group (1.3%, n=1) SputnikV vaccination group (2.9%, n=1) Covaxin vaccination

group, and AstraZeneca vaccine (3.6%, n=4) (Fig.1). However, Fisher's exact test did not show any significant difference in this connection (P=0.10, Table 2).

Furthermore; a multivariate analysis by logistic regression has been done to assess possible effect of demographic variables on developed side effects (i.e., menstruation disturbances, metrorrhagia, and hirsutism). There was no significant relationship between the incidence of mentioned side effects and demographic variables (Table 3).

Table 2: Frequency of three complications of menstruation disturbances, metrorrhagia, hirsutism among participants receiving the four vaccines

Disorder type		AstraZenec	Sputnik-V	Covaxin	Sinopharm	P value*
Menstruation disturbances	Unreported	100 (89.3)	76 (95)	28 (82.4)	185 (92)	0.01
	Reported	12 (10.7)	4 (5)	6 (17.6)	16 (8)	
Metrorrhagia	Unreported	105 (93.7)	78 (97.5)	30 (88.2)	194 (96.5)	0.12
	Reported	7 (6.3)	2 (2.5)	4 (11.8)	7 (3.5)	
Hirsutism	Unreported	108 (96.4)	79 (98.8)	33 (97.1)	200 (99.5)	0.10
	Reported	4 (3.6)	1 (1.3)	1 (2.9)	1 (0.5)	

Data are presented as n (%). P values were obtained from Fisher's exact test.

Table 3: Multivariate analysis of menstruation disorder, hirsutism, and metrorrhagia

Disorder	Variables	Groups	Coefficient	SE	OR	95% CI for OR		P value
						Lower	Upper	
Menstruation disturbances	Age (Y)		0.014	.027	1.014	0.962	1.068	0.603
	BMI (kg/m^2) (reference: normal)	Thin	-0.614	1.079	.541	0.065	4.486	0.569
		Overweight	-0.021	0.527	.979	0.349	2.751	0.968
		Obese	0.543	0.757	1.721	0.390	7.586	0.473
	Covid-19 morbidity history	Yes	0.468	0.425	1.597	0.694	3.674	0.270
	Marital status	Married	-0.008	0.526	0.992	0.354	2.782	0.989
	Vaccine type (reference: Sinopharm)	AstraZeneca	-0.419	0.826	0.658	0.130	3.318	0.612
		Covaxin	1.083	0.772	2.954	0.651	13.412	0.161
		Sputnik-V	0.048	0.580	1.049	0.336	3.271	0.935
	Constant		-2.978	0.738	0.051			0.000
Metrorrhagia	Age (Y)		0.027	0.037	1.027	0.955	1.105	0.469
	BMI (kg/m^2) (reference: normal)	Thin	-17.614	7903.849	0.000	0.000		0.998
		Overweight	-0.548	0.843	0.578	0.111	3.015	0.522
		Obese	0.624	0.975	1.867	0.276	12.625	0.522
	Covid-19 morbidity history	Yes	1.274	0.661	3.574	0.979	13.044	0.054
	Marital Status	Married	0.266	0.718	1.304	0.319	5.331	0.711
	Vaccine type (reference: Sinopharm)	AstraZeneca	-18.253	6839.732	0.000	0.000		0.998
		Covaxin	1.395	0.991	4.034	0.578	28.147	0.159
		Sputnik-V	-0.047	0.806	0.954	0.197	4.629	0.954
	Constant		-4.654	1.154	0.010			0.000
Hirsutism	Age (Y)		0.024	0.049	1.024	0.930	1.127	0.630
	BMI (kg/m^2) (reference: normal)	Thin	-16.042	8076.7	0.000	0.000		0.998
		Overweight	-0.462	1.179	0.630	0.063	6.348	0.695
		Obese	0.972	1.299	2.642	0.207	33.729	0.455
	Covid-19 morbidity history	Yes	-0.152	0.918	0.859	0.142	5.188	0.868
	Marital status	Married	-0.753	0.923	0.471	0.077	2.878	0.415
	Vaccine type (reference: Sinopharm)	AstraZeneca	-15.9	7000.3	0.000	0.000		0.998
		Covaxin	2.575	1.549	13.127	0.630	273.412	0.097
		Sputnik-V	2.455	1.263	11.646	0.980	138.429	0.052
	Constant		-5.447	1.649	0.004			0.001

SE; Standard error, OR; Odds ratio, CI; Confidence intervals, and BMI; BMI; Body mass index. P values obtained by logistic regression analysis.

Table 4: Frequency of three complications: menstruation disturbances, hirsutism, and metrorrhagia in two groups with and without a history of COVID-19

Vaccine type		Sputnik-V		Covaxin		AstraZeneca		Sinopjarm	
Side effect		Positive COVID-19 history	Negative COVID-19 history	Positive COVID-19 history	Negative COVID-19 history	Positive COVID-19 history	Negative COVID-19 history	Positive COVID-19 history	Negative COVID-19 history
Menstruation disturbance	Yes	3 (13)	1 (1.8)	3 (37.5)	3 (11.5)	4 (10.3)	8 (11)	8 (9.3)	8 (7)
	No	20 (87)	56 (98.2)	5 (88.5)	23 (88.5)	35 (89.7)	65 (89)	78 (90.7)	107 (93)
	Total	23 (100)	57 (100)	8 (100)	26 (100)	39 (100)	73 (100)	86 (100)	115 (100)
	P value*	P=0.06		P=0.12		P=0.59		P=0.60	
Metrorrhagia	Yes	1 (4.3)	1 (1.8)	2 (25)	2 (7.7)	3 (7.7)	4 (5.5)	5 (5.8)	2 (1.7)
	No	22 (95.7)	56 (98.2)	6 (75)	24 (92.3)	36 (92.3)	69 (94.5)	81 (94.2)	113 (98.3)
	Total	23 (100)	57 (100)	8 (100)	26 (100)	39 (100)	73 (100)	86 (100)	115 (100)
	P value*	P=0.49		P=0.22		P=0.69		P=0.14	
Hirsutism	Yes	1 (4.3)	0 (0)	0 (0)	1 (3.8)	2 (5.1)	2 (2.7)	0 (0)	1 (0.9)
	No	22 (95.7)	57 (100)	8 (100)	25 (96.2)	37 (94.9)	71 (97.3)	86 (100)	114 (99.1)
	Total	23 (100)	57 (100)	8 (100)	26 (100)	39 (100)	73 (100)	86 (100)	115 (100)
	P value*	P=0.28		P=0.76		P=0.60		P=0.57	

Data are presented as N (%). P values were obtained from Fisher's exact test.

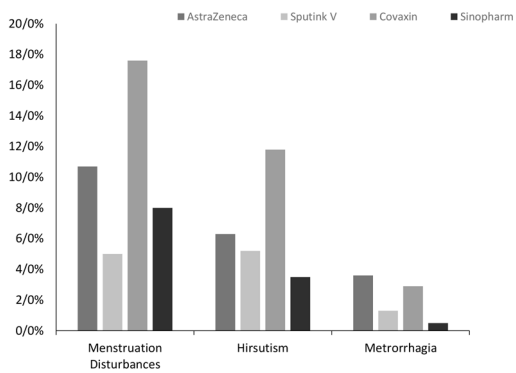

Fig.1: Comparison of 4 types of vaccines in terms of women's menstruation disturbances, metrorrhagia and hirsutism.

Overally, regardless of the vaccine, 38 (8.8%) reported menstruation disturbances, 20 (4.6%) metrorrhagia, and 7 (1.6%) hirsutism. Also, 43 (10%) had experienced at least one of the three afor-mentioned complications. Besides, 19 women reported simultaneous onset of two or three complications as follows: three reported onsets of all three complications (2 in the Covaxin group and 1 in AstraZeneca, respectively), 16 reported the onset of menstrual irregularities and metrorrhagia (5 AstraZeneca, 2 SputnikV, 3 Covaxin and 6 Sinopharm). However; 24 others reported only one of three complications, and 19 (5 AstraZeneca, 2 SputnikV, 2 Covaxin, and 10 Sinopharm) referred to menstruation disturbances. Additionally, four participants (2 AstraZeneca, 1 SputnikV We, 1 Covaxin) reported hirsutism, and 1 (1 Sinopharm) reported metrorrhagia.

Furthermore, the present study considered the relationship between pre-vaccination with COVID-19 infection and the onset of three side effects - i.e., Changes in menstruation - the incidence of metrorrhagia, and hirsutism. We compared those affected with COVID-19 and those who reported no history in this regard before vaccination. However; there was no significant difference between people with and without a history of COVID-19 in any of the three complications incidence of menstruation disturbances, hirsutism, and metrorrhagia concerning all 4 vaccines (Table 4).

Discussion

The primary purpose of the present study was to compare the side effects of 4 types of covid -19 vaccines i.e., Sinopharm, AstraZeneca, SputnikV, and Covaxin in women in terms of change in menstruation, hirsutism, and metrorrhagia. The result of the studies showed that the amount of change in menstruation in vaccinated groups with four AstraZeneca vaccines, SputnikV, Covaxin, and Sinopharm, was significantly different. The most commonly vaccine-induced change in menstruation was reported for Covaxin (17.6%), while its lowest change was recorded in SputnikV (5%). Also, accordingly, AstraZeneca (10.7%) and Sinopharm (8%) vaccines ranked second and third.

Two complications of hirsutism and metrorrhagia were also examined. The results indicated that all types of vaccines in this study did not differ significantly regarding both complications. The most common complication (in terms of number) was menstruation disturbances (38 cases (8.8%), followed by metrorrhagia, 20 cases (4.6%), and finally, 7 cases (1.6%) of hirsutism.

Only a limited number of similar studies examined the effect of COVID-19 vaccines on gynecological disorders such as menstruation, hirsutism, and metrorrhagia, and, therefore, more research is needed. As a result, we believe our study is novel. Literature has been mostly restricted to limited comparisons of the incidence of blood clots following the injection of Pfizer-Moderna

(26) and AstraZeneca vaccines. Previous research has suggested that thrombocytopenia may be a factor in heavy menstrual bleeding (27) or metrorrhagia (28). Studies have also shown that produced antibodies may invade platelets and be a precursor to idiopathic purpura (ITP) thrombocytopenia (29).

According to the Mathioudakis study, pre-vaccination infection with COVID-19 was associated with an increased incidence of side effects following receiving the vaccine by 1.08 (30). However, according to the present study, COVID-19 and the occurrence of three side effects of menstruation disturbances-hirsutism and metrorrhagia were evaluated, and the results did not indicate a significant difference in the incidence of these three complications between the two groups with history and without a history of COVID-19.

Late studies have indicated that coagulopathy disorders commonly emerge as complications of SARS-Cov-2 disease and its long-term effects (31). The Aforementioned side effect may appear in the form of Venous Thromboembolism (VTE), Disseminated Intravascular Coagulation (DIC) (32). In addition, coagulopathy disorders may cause metrorrhagia (33). However; our study indicated no significant relation between past COVID-19 history and metrorrhagia.

In this study, a total of 427 Iranian women were studied. As a result, the racial difference factor was ineffective in this experiment (21, 23). The participants were in the same age group and had the same place of employment and job proximity (19) (all engaged in health services), knowing that workplace stress is one of the factors affecting the menstrual cycle (15, 16). Their body mass index was also approximately the same. Needless to say that fitness and weight are factors that also affect the menstrual cycle (17, 18).

Using a questionnaire is a limitation of this study. Further studies are crucial, and more specific and sensitive methods should be used. Due to the funding limitation of this study, a direct diagnosis of menstruation disturbances was not possible. Because of the cross-sectional methodology, it is not possible to conclude any causal inference, and only the prevalence of menstruation disturbances can be assessed directly (34). Also, it is not possible to evaluate the long-term relationship between vaccines and menstruation disturbances. Also Sampling strategy can be disputable. Choosing all women among health workers can be a selection bias that does not represent all women in the population. We would attempt to match the sample group as similar as possible to minimize the effect of other compelling factors. Other strengths of this study include the high statistical population and its minimal missing data. This study was conducted in a hospital setting amongst medical staff members. Most of the participants showcased mastery over general health knowledge who were even working professionally in this field. Therefore, they presented more accurate and complete information about their health status (35, 36). However, the collected data through a questionnaire potentially represent errors or biases like nonresponse bias or recall bias common in cross-section studies (34). It was also possible that some respondents did not answer the questions honestly (the complications mentioned above are deemed taboo in some groups and cultures (37). However; efforts were made to minimize these errors by choosing the right time between the vaccine injection and filling out the questionnaire and selecting a knowledgeable group.

Nonetheless, due to the vaccines' novelty and the ongoing licensing process in various countries, more research is required to determine their effect on the menstrual cycle (38). One of the most up-to-date studies discusses the effect of COVID-19 on women's pregnancy (39) which underpins the significance of our study.

Some vaccines-based experimental groups were not significant due to the limited number of injectable doses and hence there is a potential for being bias due to the small sample size. Also, due to the urgent necessity to inject these vaccines to prevent the further spread of COVID-19 and reduce its risk, it is not possible to test these vaccines in a clinical trial, and such studies are minimal. Consequently, the effects of these vaccines can be studied in more precise ways in the future. Due to the ever-growing concerns in women about the incidence of the complications mentioned above and also the strong possibility of injecting, a dose or booster doses of the vaccines in the future (40), is recommended that more research be implemented on this issue.

It is also recommended that group studies and clinical trials be put on the agenda to understand the effects of these vaccines better. Additionally, injection of these vaccines in pregnant women and those prone to sex hormone disorders, as well as those women undergoing infertility treatment, should be performed with extra caution.

Conclusion

This study indicated no association between COVID-19 history and assessed feminine side effects (i.e.menstruation disturbances, metrorrhagia, and hirsutism). Also, a significant difference between vaccines regarding menstruation disturbances was found. This side effect was more frequent in Covaxin recipients than in other groups. It should be noted that further study is needed to evaluate the hypothesis.

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Authors' Contributions

I.N., A.A., M.Y.Z.; Designed the study. A.A., F.K., M.N.; Contributed to data collection and creation of data resources. F.K., A.A.; Checked and verified the dataset and prepared it for analysis. M.H.K.-G.; Did the statistical analysis. A.A., S.S.B., M.H.K.-G., M.N., F.K.; Wrote the manuscript. F.K.,

M.N., M.H.K.-G., I.N.; Reviewed and edited the manuscript. I.N., F.K., M.Y.Z.; Supervised the work. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Short Communication

Medical Arrangement Strategies for Infertility Female Patients during COVID-19 Mini-Outbreak

Hong-Xing Li, M.Sc.[1, 2], Yan Pang, M.Sc.[3], Di Cao, M.M.[1], Xiao-Ling Ma, Ph.D.[1, 2*]

1. Reproductive Medical Center, The First Hospital of Lanzhou University, Lanzhou, China
2. Key Laboratory for Reproductive Medicine and Embryo of Gansu Province, Lanzhou, China
3. PET-CT Center of Gansu Provincial Hospital, Lanzhou, China

Abstract

Over the past two years, COVID-19 pandemic is an unprecedented health emergency. All countries have taken their own measures to mitigate the spread of the virus in the first and subsequent mini-outbreaks of infection. In view of the current situation of small outbreaks of COVID-19, guidelines on epidemic prevention should be developed specifically for reproductive medical centers. It is necessary to establish a dynamic patient assessment and management system to identify patients who need priority fertility treatment during epidemic control. Female Patients were assigned as grade A and required hospitalization in the inpatient ward after egg retrieval. Patients who underwent controlled ovarian stimulation were classified as grade B, and they can choose to be hospitalizedat home according to their own convenience. Patients undergoing frozen embryo transfer (FET) cycle or planned downregulation with gonadotropin-releasing hormone agonists were defined as grade C, who could continue the assisted reproductive technology (ART) treatment cycle with negative COVID-19 nucleic acid test and there was no fever or respiratory symptoms. This brief comment summarizes the working procedure of the reproductive medical center in the first hospital of Lanzhou University in China to minimize the probability of hospital infection and ensure the safe conduct of assisted reproductive technology therapy.

Keywords: Assisted Reproductive Technology, COVID-19, Ovarian Hyperstimulation

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The aim of the study is to design medical arrangement strategies for infertility patients during COVID-19 mini-outbreak according to the working procedure of our reproductive medical center. Over the past two years, COVID-19 pandemic and mini-outbreak is an unprecedented health emergency in most cities (1). Currently the dominant global variant of COVID-19 is the delta (B.1.617.2) and omicron (B.1.1.529) strain. The emerging Omicron variant may become the new dominant strain (2-4). During the COVID-19 mini-outbreak period, routine *in vitro* fertilization (IVF) work routine has been changed fundamentally according to the epidemic situation and government policies. It is a grave problem of iatrogenic transmission and hospital infection because COVID-19 is transmitted mainly through droplets. Many doctors and nurses have also been infected by COVID-19, for example, the percentage of healthcare workers who were anti-SARS-CoV-2 IgG-positive at Kaunas hospitals was 1.16% (5). Clinical studies have shown that the proportion of hospital infection patients was once as high as 41.3% (6). Furthermore, cryopreservation of reproductive cells and embryos represents a very important aspect of assisted reproductive technology (ART) (7). Although there is no obvious clinical evidence until now, liquid nitrogen has been contaminated by COVID-19 during production process, it may be a source of infection of embryos (8, 9). Therefore, the cryopreservation of sperm and embryos must be virus-free. We have designed COVID-19 prevention measures for reproductive medical center by referring to the Health industry standard WS/T311 2009 Technical Specifications for Hospital Isolation in China in order to reduce exposure opportunities of patients in hospitals. In our hospital, all patients and partners were required two consecutive nucleic acid negative proofs of COVID-19 tests within 72 hours before inpatient hospitalization. In this article, the discussion was only about female infertility patients, the male partner had negative COVID-19 nucleic acid test and no fever or respiratory symptoms.

This study was approved by the Ethics Committee of the First Hospital of Lanzhou University (LDYYLL2019-42).

First-visit patients in infertility clinic should be suspended if possible. The other outpatients are

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***Corresponding Address: Reproductive Medical Center, The First Hospital of Lanzhou University, Lanzhou, China**
Email: ldyyszzx@163.com

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diagnosed and treated at different time interval. Seats in the waiting area are scattered. Strictly abide by the principle of "one doctor, one care, and one patient". Patients need to be accompanied only in special circumstances such as inconvenient walking. And patients should wear masks, undergo temperature detection and fill in an epidemiological questionnaire before entering the reproductive center.

On the premise of virus protection, patients who have already started the treatment of downregulation or controlled ovarian hyperstimulation are suggested to refer to the original treatment plan if possible (Fig.1). Simply speaking, patients undergoing the treatment cycle should first go to the fever clinic if their body temperature were higher than 37.3°C or go to the respiratory department if they have cough and other respiratory symptoms.

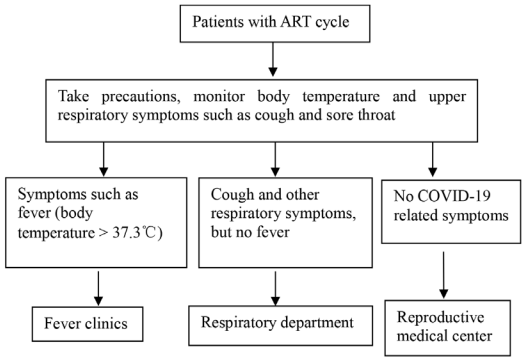

Fig.1: Treatment process of patients undergoing assisted reproductive technology (ART) cycle.

In view of the present situation, it is necessary to establish stratified dynamic assessment and treatment arrangement strategy for infertile patients during COVID-19 mini-outbreak (Table 1). Briefly, patients may suffer from ovarian hyperstimulation syndrome after egg retrieval who were assigned as grade A. So they were required hospitalization in the inpatient ward. Every patient and the partner lived in a single ward room which was equivalent to selfisolation. It is much safer for patients to be hospitalized than to come to the hospital frequently in our area. Patients were classified as grade B underwent controlled ovarian stimulation who need inject follicle-stimulating hormone every day. So they can choose to be hospitalized or be at home according to their own convenience. Patients undergoing frozen embryo transfer (FET) cycle or planned downregulation with gonadotropin-releasing hormone agonists were defined as grade C who could continue the ART treatment cycle with negative COVID-19 nucleic acid test and no fever or respiratory symptoms. If it is permitted, the patients and partners should be suggested to self-isolate them from the beginning of controlled ovarian stimulation to the ART procedure when it is finished. If a patient develops symptoms or screens positive during treatment, reverse transcription-polymerase chain reaction (RT-PCR) should be tested and treatment would not proceed until the patient screens negative according to the national guidelines. Patients and partners who were symptomatic after oocyte retrieval should be advised to freeze all their embryos (10).

Table 1: Stratified dynamic assessment and treatment arrangement strategy for infertile female patients during COVID-19

Hierarchical evaluation	Classification of patients	Medical arrangement
Grade A	Patients after egg retrieval	Hospitalization in the inpatient ward
Grade B	Controlled ovarian stimulation patients from other cities	Hospitalization in the inpatient ward (escorts are only allowed under special circumstances)
	Controlled ovarian stimulation patients in this region	Choose home or inpatient ward according to the patient's convenience
Grade C	Patients with planned downregulation or Patients undergoing frozen embryo transfer (FET) cycle	Patients with negative COVID-19 nucleic acid test and no fever or respiratory symptoms can start treatment cycle
Grade D	First-visit patients in infertility clinic	Postpone treatment as much as possible

Patients who need pregnancy confirmation and ultrasound follow-up were suggested to visit the nearest hospital. The relevant medical examination data of local medical treatment process can be retained. To reduce unnecessary doctor-patient contact during COVID-19 mini-outbreak, we use telemedicine to minimize the frequency of repeated patients' visits at the center. If there is any problem during the fetal inspection after ultrasound examination in local hospital, the patient could consult doctor in charge through online platform or telephone. Preparations must be done before calling, medical card number or identity document (ID) number, recent medication records, and paper and pen if necessary.

In short, during the COVID-19 mini-outbreak period, the work of assisted reproductive medicine should focus on the public perspective, prepare sufficient response plans and advocate online diagnosis, and continue to work with patients to ensure efficient prevention, coordination and response measures to curb outbreaks. Besides, it is also necessary to refine the hospital process, relieve the physical and mental pressure and economic pressure of patients, and minimize social panic to the greatest extent.

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Authors´ Contributions

H.-X.L., Y.P., X.-L.M.; Contributed to conception and design. Y.P., D.C.; Were responsible for overall supervision. H.-X.L.; Drafted the manuscript, which was revised by D.C., X.-L.M. All authors read and approved the final manuscript.

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Short Communication

Association of Inherited Thrombophilia with Recurrent Pregnancy Loss in A Population of Lebanese Women: A Case Control Study

Sara Khalife, Ph.D.[1*], Regina Geitani, Ph.D.[2]

1. Department of Medical Laboratory Technology, Faculty of Health Sciences, Beirut Arab University, Tripoli, Lebanon
2. Department of Medical Laboratory Sciences, Faculty of Health, Saint Holy Family University, Batroun, Lebanon

Abstract

Recurrent pregnancy loss (RPL) complication is a challenge of reproductive medicine due to its often unknown etiology. A case-control study was carried out between June 2019 and April 2020 to examine the correlation between RPL and inherited thrombophilia (IT), namely mutations in factor V Leiden (*FVL G1691A*), prothrombin (*FII G20210A*), and methylenetetrahydrofolate reductase (*MTHFR C677T*). A total of 120 Lebanese women with RPL was studied and compared, for the frequency of these mutations, to 100 healthy reproductive Lebanese women. The association between the zygosity status of the three tested mutations, the existence of more than one prothrombotic single nucleotide polymorphisms (SNPs), and the increased risk of RPL were examined using Chi-square or two-tailed fisher exact test, and the student t test. The predictive factors of RPL were analyzed using a multiple logistic regression model. $P<0.05$ was considered to be statistically significant. Our results showed statistically significant higher frequencies of *FVL G1691A* and *FII G20210A* mutations among the cases with RPL compared to the control group. Thus, RPL is associated with *FVL G1691A* and *FII G20210A* mutations. These mutations seem to increase the risk of RPL in the Lebanese women. Therefore, we suggest thrombophilia screening and adequate genetic counseling for women with RPL and at high-risk to plan for primary prevention, avoiding thromboembolic or obstetric accidents, and reducing the associated morbidity and mortality among Lebanese women.

Keywords: Abortion, Factor V Leiden (G1691A), Lebanon, MTHFR (C677T), Prothrombin (G20210A)

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Recurrent pregnancy loss (RPL) is defined as the occurrence of two or more spontaneous pregnancy losses prior to 20 weeks of gestation. Approximately, 2 to 5% of women are affected by this clinical condition, which poses a challenging therapeutic dilemma in reproductive medicine (1). Even though the etiology of RPL is not clearly stated, anatomical abnormalities, autoimmune diseases, infections, genetic disorders, endocrine factors, and thrombophilia have been postulated as a possible root cause for RPL (2). Yet, more than 50% of the cases are classified as idiopathic RPL (3).

It was recently estimated that up to 50% of cases with RPL are due to thrombophilia (4). However, its implication in RPL varied between studies because of differences in the inclusion criteria and the ethnic origin of the subjects (5). Several studies have addressed the role of inherited thrombophilia (IT) as a risk factor for RPL (5, 6). The most common causes of IT include, factor V Leiden mutation (*FVL G1691A*), prothrombin gene mutation (*FII G20210A*), and homozygosity for the methylenetetrahydrofolate reductase deficiency (*MTHFR C677T*) (7).

In obstetrics, IT was shown to be a risk factor for maternal venous thromboembolism (TE) (8). Despite of the increasing number of studies that showed an association between RPL and IT, conflicting results exist (9). In addition, much uncertainty exists regarding the utility of thrombophilia testing in the routine investigation of RPL (3).

This study aimed to determine the frequency of *FVL G1691A*, *FII G20210A*, and *MTHFR C677T* mutations in a population of Lebanese women with RPL history and also, survey its correlation with RPL. Between June 2019 and April 2020, this case-control study was carried out in several Obstetrics and Gynecology clinics located in the nine governorates of Lebanon. The women with RPL; who experienced two or more pregnancy losses prior to 20 weeks of gestation participated in our case group (n=120). And a group of 100 healthy Lebanese women with no history of pregnancy loss and with at least 2 successful pregnancies made our control group in this study. Both cases and control subjects were Lebanese women. Women with anatomical abnormalities, vaginal infections, and systemic diseases were excluded from the case group, whereas women with

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***Corresponding Address: Department of Medical Laboratory Technology, Faculty of Health Sciences, Beirut Arab University, Tripoli, Lebanon**
Email: sara.khalifeh@bau.edu.lb

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a history of pregnancy complications or miscarriage were excluded from the control group.

A standardized questionnaire was used to collect general data and to assess the medical history of all participants.

Ethical approval was obtained from the Ethics Committee of Beirut Arab University, Lebanon (IRB number: 2019H-0099-HS -R-0368). The procedures used in this study were in accordance with the ethical standards of Beirut Arab University institutional research committee. Written informed consent was obtained from all individual participants included in the study.

Three ml of venous blood was collected from each participant in Ethylene diamine tetra acetic acid tubes for DNA extraction. Genomic DNA was extracted using the Macherey-Nagel Nucleospin Blood kit (NucleoSpin blood; Macherey-Nagel GmbH & Co KG, (740951.50, Germany). Amplification reactions were performed using the MJ MiniTM Bio-Rad thermal cycler, according to the protocol described in the ThromboStrip- Opegen kit (3.117.016.53.000, Operon, Zaragoza, Spain). The following coagulation genes: *FVL G1691A, FII G20210A,* and *MTHFR C677T* were simultaneously amplified by polymerase chain reaction (PCR).

The detection of mutations was performed using the ThromboStrip- Opegen kit according to the manufacturer's instructions. Briefly, Hybridization of PCR products was performed at 42°C in a thermo-shaker adjusted to a speed of 450 rpm with a strip membrane bearing covalently-linked DNA probes that recognize each gene amplified by PCR. Following hybridization, several washes were done to eliminate nonspecific binding. The hybridization was then detected by incubating the membrane strip with a streptavidin-peroxidase conjugate, followed by the addition of peroxidase substrate (3,3′,5,5′-Tetramethylbenzidine or TMB). The probes for each gene, one for the normal sequence, one for the mutated sequence, and control probe lines of strip positioning, showed the pattern of each variant. Three possible results could be expected: no mutation, homozygous or heterozygous mutant.

Data were analyzed with a general linear model procedure of Statistical Package Software for Social Science (IBM SPSS, version 22.00, IBM Corp, Armonk, N.Y, USA). The Chi-square or 2-tailed Fisher exact test, and the student t-test were used to compare maternal characteristics and genotype frequencies between cases and controls. The predictive factors of RPL were analyzed using a multiple logistic regression model. $P<0.05$ was considered to be statistically significant.

A total of 220 study participants was assigned to two groups: cases (n=120) and controls (n=100). The mean age in both groups was 28.7 and 30.2 years, respectively. There were no significant differences by mean age, body mass index (BMI), smoking habits, parity, consanguinity, and family history of TE between these two groups ($P>0.05$, Table 1).

Hypertensive disorders and family history of TE were reported in both groups. However, individuals in the case group were more hypertensive in comparison with the control group (P=0.04, Table 1). Current medications undertaken by cases and controls have not been reported.

Table 1: General characteristics of our study participants

Characteristics	Cases (n=120)	Control (n=100)	P value
Age (Y)	28.7 ± 3.1	30.2 ± 2.7	0.97
BMI (kg/m^2)	30.3 ± 2.1	28.9 ± 3.3	0.99
Smoking habits	48 (40)	33 (33)	0.28
Hypertension	19 (15.8)	7 (7)	0.04†
Parity	1.89 ± 1.7	3.1 ± 1.2	0.74
Consanguinity	13 (10.83)	7 (7)	0.32
Family history of TE	27 (22.5)	19 (19)	0.52

Data are presented as mean ± SD or n (%). Chi-square or two-tailed fisher's exact test, and the student t test were used. †; Statistically significant, BMI; Body mass index, SD; Standard deviation, and TE; Thromboembolism.

Higher frequencies of *FVL G1691A* and *FII G20210A* mutations were observed in the study cases in comparison with the control group (Table 2). In contrast, no significant difference was shown in the frequency of *MTHFR C677T* mutation between the two groups, respectively (66.66 vs. 62%, P=0.57).

Table 2: Prevalence of *FVL G1691A, FII G20210A,* and *MTHFR C677T* variants in cases with RPL and the control group

Variable	Cases (n=120)	Control (n=100)	P value
FVL G1691A mutation	25 (20.83)	9 (9)	0.01†
FII G20210A mutation	10 (8.33)	2 (2)	0.03†
MTHFR C677T mutation	80 (66.66)	63 (62)	0.57
>1 mutation	28 (23.33)	10 (10)	0.009†

Data are presented as n (%). Chi-square test was used. RPL; Recurrent pregnancy loss and †; Statistically significant.

The frequency of occurrence of more than one mutation in the same subject was significantly higher in the cases with RPL history compared to the control group, respectively (23.33% vs. 10%, P=0.009).

In addition, the frequency in heterozygous women for the FII (AG) mutation was significantly higher in the case group than the control group (6.66% vs. 1%, respectively), (P=0.03). In contrast, no statistical difference was observed between our groups in the *FVL* (AG) (14.16% vs. 8%, P=0.15) and *MTHFR* (CT) (56.66% vs. 57%, P=0.96) heterozygosity frequency, respectively (Table 3).

Moreover, the frequency of homozygotes (AA) was significantly higher in the cases with RPL than the control group (6.66% vs. 1%, P=0.03). However, no statistical difference was observed in the frequencies of homozygotes for the FII (AA) mutation (1.66% vs. 1%, P=0.67) and *MTHFR* (TT) mutation (10% vs. 5%, P=0.16) in the case and the control groups, respectively.

Multiple logistic regression was used to calculate the odds ratios (ORs) and to measure the predictive factors

of RPL. *FVL G1691A* and *FII G20210A* mutations seem to increase the risk of RPL by almost 3-fold and > 4-fold (OR: 2.70, 95% CI: 1.17 to 6.00; OR: 4.45, 95% CI: 0.95 to 20.82, respectively). The *MTHFR C677T* mutation was not associated with an increased risk for RPL (OR: 1.17, 95% CI: 0.67 to 2.04). Data are summarized in Table 4.

Table 3: Genotype distribution of *FVL G1691A, FII G20210A* and *MTHFR C677T* in women with RPL and the control group

Variable	Genotype	Cases (n=120)	Controls (n=100)	P value
FVL G1691A	GG	95 (79.16)	91 (91)	0.01†
	AA	8 (6.66)	1 (1)	0.03†
	AG	17 (14.16)	8 (8)	0.15
	Total mutation	25 (20.83)	9 (9)	0.01†
FII G20210A	GG	110 (91.66)	98 (98)	0.03†
	AA	2 (1.66)	1 (1)	0.67
	AG	8 (6.66)	1 (1)	0.03†
	Total mutation	10 (8.33)	2 (2)	0.03†
MTHFR C677T	CC	40 (33.33)	38 (38)	0.47
	TT	12 (10)	5 (5)	0.16
	CT	68 (56.66)	57 (57)	0.96
	Total mutation	80 (66.66)	62 (62)	0.47

RPL; Recurrent pregnancy loss and †; Statistically significant.

Table 4: Predictive factors of RPL in the multiple logistic regression analysis

Variable	Cases (n=120)	Control (n=100)	OR	95% CI
FVL G1691A mutation	25	9	2.70†	1.17-6.00†
FII G20210A mutation	10	2	4.45†	0.95-20.82†
MTHFR C677T mutation	80	63	1.17	0.67-2.04
> 1 mutation	28	10	2.73†	1.25-5.96†
Hypertension	19	7	2.49†	1.00-6.21†

RPL; Recurrent pregnancy loss, OR; Odds ratio, CI; Confidence interval, and †; Statistically significant.

In this study, a relatively high prevalence of *FVL G1691A, FII G20210A*, and *MTHFR C677T* variants has been observed in our groups, case and control, (20.83% vs. 9%, 8.33% vs. 2%, and 66.66% vs. 62%, respectively) which was in line with previous reports on the Lebanese population (10, 11). Similar results were seen in related ethnic populations such as Palestinian, Jordanian, Turkish, Syrian, Greek, and Greek-Cypriot, suggesting that eastern Mediterranean populations have a relatively high prevalence of these mutations (12-15).

Consistent with our results, the *FII G20210A* mutation was reported and identified as a risk factor for early RPL (16), and the *FVL G1691A* mutation as a common risk factor associated with early and late RPL (17, 18).

In addition, our results are supported by a meta-analysis whose findings show an increased risk of venous TE in pregnancy with *FVL G1691A* and *FII G20210A* carrier state (19).

Similarly, in Saudi Arabia, *FVL G1691A* and *FII G20210A* mutations were found to increase significantly the risk of RPL (20), which is in agreement with other findings in Iran and Turkey (17, 21). However, contradicting findings were reported in these same countries showing no correlation between the occurrence of RPL and mutations in *FVL* and *FII* (22, 23).

Moreover, regional and ethnic variations have been shown to affect the risk of RPL associated with *FVL G1691A* mutation. Indeed, a significant correlation has been found between *FVL G1691A* mutation and RPL in studies conducted in Asia, Africa, Europe, and the Middle-east, rather than Latin and North America (24). Our study supports this finding and identifies the *FVL G1691A* mutation as a risk factor for RPL in the Lebanese population.

Our case group showed a significant higher prevalence of heterozygous *FII* (AG) mutation in comparison with the control group, supporting Foka et al. (25) study that an increased frequency of *FII G20210A* was reported in women with RPL. Homozygous *FII* (AA) mutation was observed in our study cases with RPL and the control group at a prevalence of 1.66% and 1%, respectively. However, the difference was not statistically significant (P=0.67). Although, it is well established that the heterozygous and homozygous types of *FII G20210A* mutation predispose to a 3 to 8, and 18 to 80 times higher risk of thrombotic events, respectively (26), in our study homozygous *FII* (AA) mutation was not found to be a risk factor for RPL. This could be explained by the fact that RPL is a multifactorial condition, and one risk factor could not be enough for its occurrence.

Interestingly, when analyzing the frequency of women heterozygous for the *FVL* G1691A mutation in both groups, heterozygous *FVL* (AG) mutation alone, was not found to be a risk factor for RPL (P=0.1512). However, in contrast to our findings, a recent study conducted in Turkey, as well as other reports and meta-analyses, confirm that an increased risk of RPL was reported in women carriers of the *FVL G1691A* mutation (17, 18).

Our results suggest that homozygous *FVL* (AA) mutation could increase the risk of RPL, supporting previous study that showed an increased risk of developing venous TE during pregnancy with the *FVL G1691A* mutation, and largely when women bear the homozygous type of the mutation (8).

Assessing the prevalence of *MTHFR C677T* variant, it was not found to be a significant risk factor for RPL even in homozygosity pattern (P=0.57), that was in contrast to a study that showed homozygous women for the *MTHFR* (TT) mutation had a 2-3 fold-increased risk of early fetal loss in comparison to CC genotype women (27).

In addition, our study has shown an increased risk for RPL in women presenting more than one mutation, which was in agreement with previous findings that showed women with concurrent polymorphism for the three tested

mutations are at a greater risk for RPL in comparison with women with a single mutation (28).

In the present study, *FVL G1691A* and *FII G20210A* mutations were found to be associated with almost 3-fold and > 4-fold increased risk of RPL, respectively. However, the *MTHFR C677T* mutation was not associated with an increased risk for RPL. These data were in accordance with a previous report in which women with *FVL G1691A* or *FII G20210A* mutations, but not *MTHFR C677T* mutation had higher risks of developing RPL (24).

Surprisingly, our results are in contrast with a previous report on the Lebanese population, where no association has been found between adverse pregnancy outcomes and *FVL G1691A, FII G20210A*, and *MTHFR C677T* mutations (11). The described inconsistency could be due to differences in the type of obstetric complications, control selection, and inclusion and exclusion criteria. In addition, thrombophilia is a multifactorial disorder involving both genetic and environmental risk factors.

This gene-environment interplay could affect the pathogenesis of thrombophilia and could result in biased estimates even though confounding factors were controlled in our study. The influence of unknown confounders cannot be ruled out. This could be the most important limitation of our study, in addition to limited data collected from the study participants due to timing and convenience.

In our study, a statistically significant association has been found between RPL and mutations in *FVL* and *FII* in Lebanese women. However, even though an increasing number of studies have found such a correlation, yet, there is no consensus for genetic testing and counseling in the RPL cases. Altogether, our results could offer a strong argument in support of a change in current practices. Therefore, thrombophilia screening could be advocated for women at high risk of thrombotic episodes, allowing a better prognosis. Finally, early diagnosis of thrombophilia, genetic counseling, and gynecological monitoring could be of high benefit to prevent pregnancy complications in women with RPL and/or at high risk by proposing adequate therapeutic management and prophylaxis.

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Authors' Contributions

S.K., R.G.; Contributed to conception, design, and Drafted the manuscript. S.K.; Contributed to all experimental work, data, statistical analysis, and interpretation of data.

All authors read and approved the final manuscript.

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

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

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

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International Journal of Fertility and Sterility (Int J Fertil Steril)
P.O. Box: 16635-148, Iran
Tel/Fax: + 98-21-22510895
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