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Functional Roles of lncRNAs in Recurrent Pregnancy Loss: A Review Study

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Abstract

Recurrent pregnancy loss (RPL) or recurrent miscarriage is the failure of pregnancy before 20-24 weeks that influences around 2-5% of couples. Several genetic, immunological, environmental and physical factors may influence RPL. Although various traditional methods have been used to treat post-implantation failures, identifying the mechanisms underlying RPL may improve an effective treatment. Recent evidence suggested that gene expression alterations presented essential roles in the occurrence of RPL. It has been found that long non-coding RNAs (lncRNAs) play functional roles in pregnancy pathologies, such as recurrent miscarriage. lncRNAs can function as dynamic scaffolds, modulate chromatin function, guide and bind to microRNAs (miRNAs) or transcription factors. lncRNAs, by targeting various miRNAs and mRNAs, play essential roles in the progression or suppression of RPL. Therefore, targeting lncRNAs and their downstream targets might be a suitable strategy for diagnosis and treatment of RPL. In this review, we summarized emerging roles of several lncRNAs in stimulation or suppression of RPL.

Keywords: Diagnosis, Implantation, lncRNAs, miRNAs, Recurrent Miscarriage

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Introduction

Recurrent pregnancy loss (RPL) is an important issue in reproductive health (1, 2). RPL is generally termed as three or more consecutive and spontaneous death of the fetal before the 20th week of pregnancy or before the fetal weighs 500 g (3). This disorder affects about 5% of couples who are interested in having a child (4). About 50% of women who suffer from this disorder have no significant symptoms on routine checkups (5). Etiology of this disease in 50% of cases remains unclear (6). Several factors such as chromosomes and genetic abnormalities (7, 8), infections (9), endocrine disorders (10), progesterone deficiency (11), uterine defects (12), anatomical disorders, placental abnormalities (13), alcohol and smoking (14), exposure to factors such as lead, mercury, ethylene oxide and ionizing radiation, and stressful conditions (15) are common established causes of RPL. Recent evidence showed that gene alternations play an important role in the occurrence of RPL (16). Long non-coding RNAs (lncRNAs), by targeting DNA, RNA, and proteins, can affect their transcription and translation (17). Recently, role of lncRNAs in regulating pregnancy and RPL has been considered (18, 19). lncRNAs have different expressions in the chorionic villi of patients with RPL (20). lncRNAs can impact embryonic implantation by targeting trophoblast cell proliferation, migration, invasion and apoptosis (21, 22). Therefore, lncRNAs could be useful biomarkers for the diagnosis and treatment of patients with RPL (23). In this review, we summarized potential roles of lncRNAs in RPL.

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Characteristics of long non-coding RNAs

So far, 61533 total human genes are characterized, 19982 of which are related to the protein-coding genes, 18811 are specific to the lncRNA genes, and 7567 was found to be related to the small ncRNA genes (gencodegenes.org/human/stats.html). ncRNAs are categorized into structural (rRNAs and tRNAs) and regulatory (small, medium, and lncRNAs) ncRNAs (24). lncRNAs (>200 nt) with a similar structure to mRNAs are transcribed by RNA polymerase II. lncRNAs have transcriptional termination codons or stop codons with the potential to signal ending translation, therefore lncRNAs cannot construct a full-length protein (17, 25, 26). They can be categorized into long intergenic ncRNAs, sense lncRNAs, antisense lncRNAs, intronic lncRNAs and bidirectional lncRNAs (27-29). lncRNAs, as decoys, can bind to microRNAs (miRNAs) or transcription factors to block their functions (30, 31). lncRNAs, as the guide molecules, bind to the regulatory proteins and guide them to the specific target area (32). Besides, lncRNAs as the scaffold molecules provide a central platform for different types of macromolecular complexes (33) (Fig.1). Studies showed that lncRNAs may play role in pregnancy pathologies, such as miscarriage (34, 35).

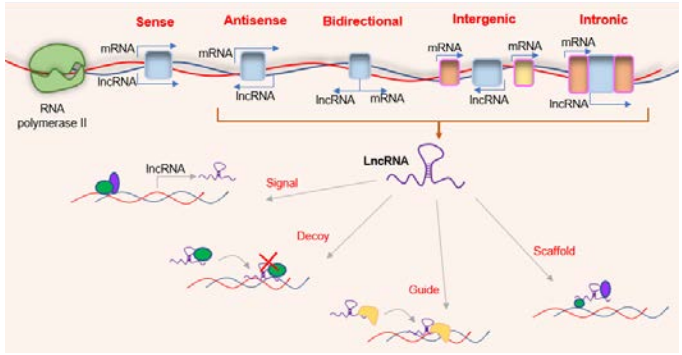

Fig.1: Biogenesis and functions of long non-coding RNAs (lncRNAs). lncRNAs could be classified into intergenic, sense, antisense, intronic, and bidirectional lncRNAs. They can act as molecular signals to regulate transcription, bind to microRNAs, chromatin modifiers or transcription factors to affect transcription and translation, and as the scaffold molecules providing a central platform.

Functional roles of lncRNAs in recurrent pregnancy loss

It has been reported that lncRNAs present essential roles in the stimulation or suppression of placental trophoblast invasion into endometrial stromal cells, by targeting various miRNAs and transcription factors (36) (Table 1). Here, we summarized potential roles of several lncRNAs in RPL (Fig.2).

Table 1: Functional roles of lncRNAs in recurrent pregnancy loss

lncRNA	Expression in RPL	Target		Result	Ref
		Suppression	Stimulation		
TCL6		…	EGFR	Suppress the proliferative ability of trophoblasts	(37)
SNHG7-1	Down	miR-34a	Wnt/β-catenin	Suppress the proliferative and invasive ability of trophoblasts to induce RPL progression	(38)
Lnc-SLC4A1-1	Up	TNF-α/IL-1β	NF-κB/CXCL8	Stimulate apoptosis and immune responses in trophoblast cells	(18)
H19	Down	miR-106a-5p	VEGFA	High invasion of extravillous trophoblast cells, regulate angiogenesis, induce apoptosis	(39)
		miRNA let-7	ITGB3	Suppress trophoblast cell adhesion and invasion	(40)
		miR-675	NOMO1	Suppress trophoblast cell proliferation	(41)
EPB41L4A-AS1		HIF-1α	VDAC1	Inhibition of the aerobic glycolysis and cell growth, and induction of apoptosis	(42)
TINCR		…	…	Progression of miscarriage	(43)
MALAT-1	Down	…	…	Reduce cell migration and proliferation, regulate angiogenesis, and induce apoptosis	(44)
ANRIL		…	…	Progression of miscarriage	(45)
HULC		…	…	Progression of miscarriage	(46)
Lnc-49a	Down	CD49a	IFN-γ	Reduce the adhesion, migration, and cytotoxic activity of dNK cells, stimulate the progression of miscarriage	(47)
MEG3	Down	RASA1	Ras-MAPK	Reduce implantation, proliferation, invasion, and induce apoptosis of primary trophoblasts	(48)
ZEB2-AS1		CST3	…	Accelerate proliferation, migration, and invasion of chorionic trophoblasts	(49)
SOX2OT		…	…	Progression of miscarriage	(50)
NEAT1	Up	miR-373	FLT1	Reduce proliferation, migration, invasion, and colony formation of trophoblast cells, and accelerate apoptosis	(51)
CCAT2		…	…	Reduce risk of miscarriage	(52)
HOTAIR	Down	PIK3-AKT	TTP	Suppress trophoblast cell proliferation, migration, and invasion	(53)
Lnc-HZ08	Up	PI3K/p-AKT/ P21/CDK2	…	Suppress trophoblast cell proliferation, migration, and invasion	(54)
Lnc-HZ01	Up	MXD1	METTL14	Suppress trophoblast cell proliferation and induce risk of miscarriage	(55)

RPL; Recurrent pregnancy loss, TNF-α; Tumour necrosis factor α, IL; Interleukin, HIF-1α; Hypoxia-inducible factor 1α, VDAC1; Voltage-dependent anion channel 1, VEGFA; Vascular endothelial growth factor A, RASA1; Ras p21 protein activator 1, CST3; Cystatin C, TTP; Tristetraprolin, EGFR; Epidermal growth factor receptor, IFN-γ; Interferon-γ granzyme B, ITGB3; Integrin β3, NOMO1; Nodal modulator 1, FLT-1; Fms-like tyrosine kinase-1, and METTL14; Methyltransferase 14, N6-Adenosine-Methyltransferase Subunit.

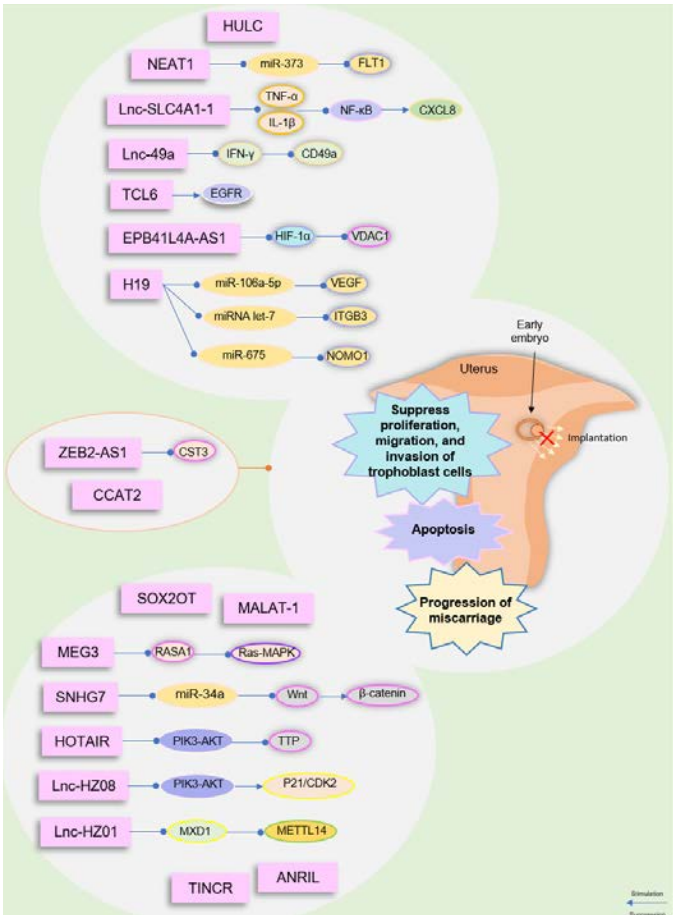

Fig.2: lncRNAs by targeting several miRNAs/mRNAs play important roles in the induction or suppression of recurrent pregnancy loss (RPL). Some lncRNAs, including TCL6, SNHG7, H19, lnc-49a, lnc-SLC4A1-1, EPB41L4A-AS1, TINCR, MALAT-1, ANRIL, HULC, MEG3, HOTAIR, and SOX2OT present critical roles in RPL development. These lncRNAs can inhibit trophoblastic cell proliferation, migration, invasion, and induce apoptosis. In contrast, two lncRNAs such as CCAT2 and ZEB2-AS1 target trophoblastic cell proliferation, migration, and invasion for reduction of the risk of miscarriage.

TCL6

lncRNA TCL6 stands for lncRNA T-cell leukemia/lymphoma 6 which is located in the breakpoint cluster region on chromosome 14q32. It has been reported to be involved in the preeclampsia pathogenesis by suppressing proliferative ability of trophoblasts (56, 57). Recently, the real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis reported that overexpressed lncRNA-TCL6 of placental villus was correlated with threatened abortion compared to normal pregnancy. Regarding its aberrant expression, biological investigation of lncRNA TCL6 using si-lncRNA TCL6-1 revealed that knock-down of lncRNA TCL6 could significantly induce proliferation and invasion of trophoblasts. Epidermal growth factor receptor (EGFR) signaling plays an essential role in early human embryo implantation and pregnancy disorders. There is a negative correlation between lncRNA TCL6 and the EGFR pathway. In this regard, inhibitory role of the lncRNA TCL6 on proliferation activity of trophoblast cells could be inversed by EGFR knock-down. Taken together, lncRNA TCL6 could induce early abortion and inhibit placental implantation by targeting the EGFR signaling (37).

SNHG7

Despite the regulatory role of lncRNAs in trophoblast proliferation and apoptosis, function of SNHG7 in RPL remains challenging (58). Small nucleolar host gene 7 (SNHG7) is a 2176 base pairs (bp) lncRNA on chromosome 9q34.3 (59). Gene expression analysis of SNHG7, using qRT-PCR, reported its down-regulated expression in recurrent spontaneous abortion (RSA) villi compared to normal pregnancy villi. SNHG7 silencing was also shown to prohibit trophoblast proliferation by MTT assay, along with increase apoptosis using flow cytometry, thereby confirming SNHG7 function in RPL development (38). Since the discovery of lncRNAs as indirect regulators of mRNA expression levels using sequestering miRNA, several studies identified SNHG7 as a molecular target of miR-34a (60, 61). In this regard, inhibition of miR-34a reversed suppressed proliferation of trophoblasts which was induced by SNHG7 silencing (38). It has been shown that WNT1 through the Wnt/β-catenin pathway, acted as a miR-34a down-stream target. Dysregulated expression of the Wnt/β-catenin pathway might be correlated to RPL pathogenesis (62). SNHG7 silencing was also reported to modulate proliferation and invasion of trophoblasts with the Wnt/β-catenin pathway inactivation. Due to the involvement of SNHG7/miR-34a/Wnt/β-catenin axis in RPL progression, the latter could be underlying potential molecular targets for RPL treatment (38).

lnc-SLC4A1-1

lnc-SLC4A1-1 was found to target NF-κB and CXCL8, inhibiting the inflammatory response by TNF-α and IL-1β. Therefore, high expression of this lncRNA can trigger apoptosis and stimulate immune responses in trophoblast cells (18).

H19

H19 is a lncRNA with 2.3 kbp length in the cytoplasm. High expression of H19 was reported during embryonic development and its low expression after birth (63). H19 can modulate adhesion and invasion of trophoblasts within early pregnancy (40, 64). Reduced expression of H19 resulted in low invasion of extravillous trophoblast cells, associated with the pathogenesis of both intrauterine growth restriction and preeclampsia (65). Significant expression of H19 in trophoblast of early pregnant women modulated the angiogenic ability of extravillous trophoblast cells by targeting the miR-106a-5p/VEGFA axis (66). Decreased expression of H19 in placental villi tissue of RPL patients showed the same expression trend in anti-apoptotic Bcl-2 level along with a negative impact on pro-apoptotic Bax expression. Therefore, H19 regulates RPL pathogenesis through inducing apoptosis and can be presented as a novel therapeutic target for RPL patients (39). In

another study, H19 was reported to block miRNA let-7 and stimulated expression of integrin β3 (ITGB3), as the target gene of let-7. It was also shown that the H19/let-7/ITGB3 axis could accelerate adhesion and invasion ability of trophoblast cells. Therefore, low expression of H19 triggered RPL progression (40). Besides, H19 has been found to target miR-675 and upregulate nodal modulator 1 (NOMO1) protein expression to induce cell proliferation and phosphorylation of Smad 2. Therefore, low expression of this lncRNA was participated in the proliferation of trophoblast cells (41).

EPB41L4A-AS1

lncRNA EPB41L4A-AS1 is located on the 5q22.2 chromosome, while it is down-regulated in tumors. Its down-regulation in malignancies started aerobic glycolysis, called the Warburg effect, inducing fast tumor growth (42). Trophoblasts as rapid-growing cells require aerobic glycolysis to support their fast growth. Although the biological function of EPB41L4A-AS1 in cancers is evident, its functional effect on early pregnancy placental tissue and RPL needs to be elucidated. EPB41L4A-AS1 expression was found to be upregulated in placental tissue in early RPL compared to the normal pregnancy. Recent mRNA microarray analysis reported a significant increase in glycolysis-related genes inconsistent with the expression of fatty acid oxidative phosphorylation genes (67). Both glycolysis along with fatty acid oxidative phosphorylation process were involved in extravillous trophoblasts metabolism that was impaired in abortion (68). Increased expression of EPB41L4A-AS1 prohibited HIF-1α as a cell cycle transcription factor and induced VDAC1 expression as a key apoptosis regulatory protein (42, 69, 70). Altogether, overexpression of EPB41L4A-AS1 was responsible for the disrupted metabolic program in human villous trophoblast through inhibition of the aerobic glycolysis and cell growth, and promotion of apoptosis that might be key reasons of RPL (42).

TINCR

Terminal differentiation-induced non-coding RNA (TINCR) is a 3.7 kb lncRNA located on human chromosome 19. TINCR is involved in epidermal and somatic tissue differentiation and progression of miscarriage. Although the association of genetic susceptibility of some lncRNAs in RPL has been found (71), there was no significant correlation between the TINCR gene rs2288947 A>G polymorphism and RPL (43).

MALAT-1

Metastasis-associated lung adenocarcinoma transcript-1 (MALAT-1) is the lncRNA with more than 8000 bp length. It is up-regulated in invasive placentation (72). Expression of MALAT-1 was shown to be decreased in women with RPL as well as reducing cell migration and proliferation, while it induced apoptosis (44). The rs619586 G variant of MALAT-1 decreased susceptibility to RPL (73).

ANRIL

Recently, ANRIL has been introduced as a new large antisense lncRNA with 403 kb length locating on the chromosome 9p21 locus. ANRIL polymorphism is correlated with susceptibility to miscarriage (74, 75). The Rs2151280 GG allele of ANRIL was correlated with an increased risk of RPL and it might be associated with initiation of abortion (45).

HULC

Highly upregulated in liver cancer (HULC) is a lncRNA with 500 nucleotides located on chromosome 6p24.3. It is highly expressed in human hepatocellular carcinoma (76). This lncRNA regulates cell invasion, proliferation and migration (77). SNP genotyping of lncRNA HULC showed that three variant genotypes of the HULC, including rs1041279 C>G, rs7770772 G>C and rs17144343 G>A, reduced RPL. Such protective impact was very noticeable in women aged under 35 as well as those with at least four abortions. Therefore, lncRNA HULC might be a potent biomarker for diagnosis of RPL (46).

lnc-49a

lnc-49a is a novel lncRNA, positively regulated CD49a expression of human decidual natural killer (dNK) cells. This lncRNA was reported to reduce adhesion, migration, and cytotoxic activity of dNK cells, while accelerated interferon-γ granzyme B (IFN-γ) expression (47). Therefore, lnc-49a could stimulate progression of RPL, by suppressing cellular migration and invasion.

MEG3

lncRNA maternally expressed gene 3 (MEG3) is located on chromosome 14q32.3 and modulated proliferation and invasion of trophoblasts (78). A recent analysis in aborted villous tissue showed a dramatic decrease level of MEG3 expression, compared to the normal pregnant women. This was negatively associated with the unexplained RPL. Analysis of possible down-stream targets of MEG3 identified the Ras p21 protein activator 1 (RASA1) gene that was overexpressed in villous tissue of aborted women in comparison to normal villous tissue (48). RASA1 obstructed the Ras signaling by binding to the activated form of Ras (Ras-GTP), thereby repressed this pathway (79, 80). Indeed, the Ras-MAPK signaling modulated cell proliferation and apoptosis. MEG3 was shown to stimulate the Ras-MAPK signaling via suppressing RASA1 in embryonic villi of women with unexplained RSA. It can be suggested that an under-expressed level of MEG3 can increase implantation, proliferation, invasion and suppress apoptosis in aborted primary trophoblasts (48).

ZEB2-AS1

lncRNA zinc finger E-box binding homeobox 2 antisense RNA1 (ZEB2-AS1) is involved in the prediction

and progression of cancers (81). It has been reported that ZEB2-AS1 targeted cystatin C (CST3) which was associated with trophoblast inactivity along with the susceptibility to developing preeclampsia (82, 83). Both FISH assay and qRT-PCR analysis revealed low expression of ZEB2-AS1 level in recurrent aborted mice. In addition, CST3 expression was dramatically upregulated in the placental tissue of aborted mice. Furthermore, CST3 overexpression inhibited proliferative activity, migration capacity, and invasion of mouse chorionic trophoblasts. In contrast, high expression of ZEB2-AS1 prohibited CST3 expression, thereby promoting the biological function of mouse chorionic trophoblast cells. Therefore, ZEB2-AS1 can be introduced as a protective lncRNA against RPL via promoting trophoblast activity through suppression of CST3 expression (49).

SOX2OT

lncRNA SOX2 overlapping transcript (SOX2OT) mapping on the *SOX2* gene was found to represent an oncogenic activity in the pathogenesis of breast cancer (84, 85). Since the association of abortion as one type of reproductive risk factor for breast cancer (86), it is hypothesized that lncRNA SOX2OT has a possible correlation with RPL (87). It has been suggested that the SOX2OT polymorphism functions as an increased risk factor for RPL with no dramatic relationship to the number of abortion in different age (50).

NEAT1

Nuclear-enriched abundant transcript-1 (NEAT1) lncRNA is located on chromosome 11q13 and binds with various paraspeckle bodies (88). Expression of NEAT1 was demonstrated to be increased in placental samples of preeclampsia than the control group. During preeclampsia, NEAT1, as a competing endogenous RNAs (ceRNAs), can bind with miR-373 and enhance expression of Fms-like tyrosine kinase-1 (FLT-1). Therefore, the NEAT1/miR-373/FLT-1 may provide a new approach for understanding the pathogenesis of RPL by targeting trophoblast cell proliferation and apoptosis (51).

CCAT2

Colon cancer-associated transcript 2 (CCAT2) is a lncRNA with 1752 bp located on chromosome 8q24.21 which represents an oncogenic role in colorectal cancer. Effect of CCAT2 polymorphism on RPL indicated a reduced risk of RPL of the CCAT2 rs6983267 allele, and provided new lncRNA to protect RPL especially in women younger than 35 years old (52).

HOTAIR

lncRNA hox antisense intergenic RNA (HOTAIR) has been demonstrated to participate in normal cell development and its dysregulation involved in cancer progression (89). Low expression of HOTAIR is reported in trophoblasts from RPL women compared to those with normal pregnancy. HOTAIR modulated invasion and migration capacity of trophoblast, thereby enhanced the RPL pathology (71). Tristetraprolin (TTP) stands as a tandem zinc-finger mRNA binding protein and modulated stability of various targets by binding to specific mRNAs. Upregulated TTP disrupted trophoblast invasion in patients with RPL, through HOTAIR destabilization. Therefore, HOTAIR/TTP can be a possible target for management of trophoblast cell invasion (53).

lnc-HZ01

Lnc-HZ01 is a novel lncRNA that stimulates occurrence of miscarriage by enhancing the protein stability of MAX dimerization protein 1 (MXD1). c-JUN is a transcription factor of MXD1 that enhances MXD1 stability in the nucleus of trophoblast cells with the USP36 enzyme. METTL14 is a pivotal methyltransferase that can promote lnc-HZ01 RNA stability in villous tissues through m6A methylation on lnc-HZ01. Besides, lnc-HZ01 has been demonstrated to regulate trophoblast cells proliferation by up-regulating mRNA and protein levels of eukaryotic translation initiation factor (4E EIF4E). In human villous tissues, MXD1 could stimulate 4E EIF4E transcription and lnc-HZ01 in a positive self-feedback loop. Therefore, lnc-HZ01 repressed trophoblast cell proliferation and stimulated RPL through the MXD1/EIF4E pathway (55).

lnc-HZ08

lnc-HZ08 is a highly expressed lncRNA in human trophoblast cells. High expression of lnc-HZ08 could reduce PI3K level in trophoblast cells by targeting the PI3K/p-AKT/p-P21/CDK2 pathway. Therefore, this lncRNA was displayed to induce miscarriage via suppressing trophoblast cells proliferation, migration, and invasion (54).

Conclusion

RPL is one of the most serious clinical problems in reproductive health that affects family well-being dramatically. Consequently, clinical research should focus on how to improve the pregnancy success rate for women with recurrent miscarriage. In recent years, lncRNAs have become a focus of research because they regulate the occurrence and development of a wide variety of diseases. In this study, we literature the most recent advances in lncRNAs research and potential molecular pathways involved in RPL progression. These studies provided only a little information, while many of the functions of lncRNAs remain unknown. Despite the advancements of ncRNAs research in recent years, ncRNAs have the following limitations for clinical use: i. In the circulatory system, ncRNAs are often dynamic and samples from patients over time only provide an indication of the current expression status, ii. function of the most ncRNAs are explored at the cellular level, and animal experiments to investigate the functions and mechanisms of ncRNAs are urgently needed, and iii. It is difficult to know whether ncRNAs will affect expression of the other genes when

used as a targeted therapy, while it requires more clinical experimental evidence.

It can be concluded that silencing lncRNAs, including TCL6, SNHG7, H19, EPB41L4A-AS1, TINCR, MALAT-1, ANRIL, lnc-49a, lnc-SLC4A1-1, HULC, PVT1, HOTAIR, MEG3, and SOX2OT, as well as silencing transcription and inflammatory factors such as NF-κB, IFN-γ, EGFR, VDAC1, VEGF, and β-catenin may prevent RPL development. Besides, functional roles of some lncRNAs (ZEB2-AS1 and CCAT2), miRNAs (miR-34a, miRNA let-7, miR-675, and miR-106a-5p), and transcription and inflammatory factors (RASA1, HIF-1α, CST3, TNF-α, IL-1β, and TTP) in the stimulation of trophoblastic cell proliferation, migration, and invasion should be considered as novel biomarkers or potential inducers for reduction of the risk of miscarriage.

Taken together, we recommend that researchers conduct larger population samples to demonstrate clinical potential of lncRNAs in diagnosing and treating RPL. In addition, to use RNA for disease treatment, more efficient and cost-effective methods for synthesizing and purifying RNA are needed.

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Authors' Contributions

Sh.A., M.A.G.D., F.Gh., Z.R., R.M.J., M.K., M.N., M.F.; Have made contributions to the writing of the manuscript. All authors have approved the submitted version of the article and agreed to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work. All authors read and approved the final manuscript.

References

1. Azin SA, Golbabaei F, Warmelink JC, Eghtedari S, Haghani S, Ranjbar F. Association of depression with sexual function in women with history of recurrent pregnancy loss: descriptive-correlational study in Tehran, Iran. Fertil Res Pract. 2020; 6(1): 1-9.
2. El Hachem H, Crepaux V, May-Panloup P, Descamps P, Legendre G, Bouet PE. Recurrent pregnancy loss: current perspectives. Int J Womens Health. 2017; 9: 331-345.
3. Dimitriadis E, Menkhorst E, Saito S, Kutteh WH, Brosens JJ. Recurrent pregnancy loss. Nat Rev Dis Primers. 2020; 6(1): 98.
4. Habets DHJ, Pelzner K, Wieten L, Spaanderman MEA, Villamor E, Al-Nasiry S. Intravenous immunoglobulins improve live birth rate among women with underlying immune conditions and recurrent pregnancy loss: a systematic review and meta-analysis. Allergy Asthma Clin Immunol. 2022; 18(1): 23.
5. Otani-Matsuura A, Sugiura-Ogasawara M, Ebara T, Matsuki T, Tamada H, Yamada Y, et al. Depression symptoms during pregnancy and postpartum in patients with recurrent pregnancy loss and infertility: the Japan environment and children's study. J Reprod Immunol. 2022; 152: 103659.
6. Mekinian A, Cohen J, Alijotas-Reig J, Carbillon L, Nicaise-Roland P, Kayem G, et al. Unexplained recurrent miscarriage and recurrent implantation failure: is there a place for immunomodulation? Am J Reprod Immunol. 2016; 76(1): 8-28.
7. Gaboon NE, Mohamed AR, Elsayed SM, Zaki OK, Elsayed MA. Structural chromosomal abnormalities in couples with recurrent abortion in Egypt. Turk J Med Sci. 2015; 45(1): 208-213.
8. Houmaid H, El Bekkay C, Nassereddine S, Talbi H, Amehdare L, Hilali A. Chromosomal abnormalities in 238 couples with recurrent miscarriages in Morocco. Open J Genet. 2018; 8(02): 15-22.
9. Abdoli A, Dalimi A, Soltanghoraee H, Ghaffarifar F. Molecular detection and genotypic characterization of toxoplasma gondii in paraffin-embedded fetoplacental tissues of women with recurrent spontaneous abortion. Int J Fertil Steril. 2017; 10(4): 327-336.
10. Amrane S, McConnell R. Endocrine causes of recurrent pregnancy loss. Semin Perinatol. 2019; 43(2): 80-83.
11. Devall AJ, Coomarasamy A. Sporadic pregnancy loss and recurrent miscarriage. Best Pract Res Clin Obstet Gynaecol. 2020; 69: 30-39.
12. Kuroda K. Impaired endometrial function and unexplained recurrent pregnancy loss. Hypertens Res Pregnancy. 2019: 7(1): 16-21.
13. Quenby S, Gallos ID, Dhillon-Smith RK, Podesek M, Stephenson MD, Fisher J, et al. Miscarriage matters: the epidemiological, physical, psychological, and economic costs of early pregnancy loss. Lancet. 2021; 397(10285): 1658-1667.
14. Alves C, Rapp A. Spontaneous abortion. Available from: https://www.ncbi.nlm.nih.gov/books/NBK560521/ (26 Jul 2022).
15. Kolte A, Olsen L, Mikkelsen E, Christiansen O, Nielsen H. Depression and emotional stress is highly prevalent among women with recurrent pregnancy loss. Hum Reprod. 2015; 30(4): 777-782.
16. Arias-Sosa LA, Acosta ID, Lucena-Quevedo E, Moreno-Ortiz H, Esteban-Pérez C, Forero-Castro M. Genetic and epigenetic variations associated with idiopathic recurrent pregnancy loss. J Assist Reprod Genet. 2018; 35(3): 355-366.
17. Xu WW, Jin J, Wu XY, Ren QL, Farzaneh M. MALAT1-related signaling pathways in colorectal cancer. Cancer Cell Int. 2022; 22(1): 126.
18. Huang Z, Du G, Huang X, Han L, Han X, Xu B, et al. The enhancer RNA lnc-SLC4A1-1 epigenetically regulates unexplained recurrent pregnancy loss (URPL) by activating CXCL8 and NF-kB pathway. EBioMedicine. 2018; 38: 162-170.
19. Chen X, Guo DY, Yin TL, Yang J. Non-coding RNAs regulate placental trophoblast function and participate in recurrent abortion. Front Pharmacol. 2021; 12: 646521.
20. Wang L, Tang H, Xiong Y, Tang L. Differential expression profile of long noncoding RNAs in human chorionic villi of early recurrent miscarriage. Clin Chim Acta. 2017; 464: 17-23.
21. Yang D, Ding J, Wang Y, Yuan M, Xian S, Zhang L, et al. YY1-PVT1 affects trophoblast invasion and adhesion by regulating mTOR pathway-mediated autophagy. J Cell Physiol. 2020; 235(10): 6637-6646.
22. Farzaneh M, Najafi S, Dari MAG, Sheykhi-Sabzehpoush M, Dayer D, Cheraghzadeh M, et al. Functional roles of long noncoding RNA MALAT1 in gynecologic cancers. Clin Transl Oncol. 2023; 25(1): 48-65.
23. Tong YS, Wang XW, Zhou XL, Liu ZH, Yang TX, Shi WH, et al. Identification of the long non-coding RNA POU3F3 in plasma as a novel biomarker for diagnosis of esophageal squamous cell carcinoma. Mol Cancer. 2015; 14: 3.
24. Patty BJ, Hainer SJ. Non-coding RNAs and nucleosome remodeling complexes: an intricate regulatory relationship. Biology (Basel). 2020; 9(8): 213.
25. Anbiyaiee A, Ramazii M, Bajestani SS, Meybodi SM, Keivan M, Khoshnam SE, et al. The function of LncRNA-ATB in cancer. Clin Transl Oncol. 2022 (ahead of print).
26. Farzaneh M, Ghasemian M, Ghaedrahmati F, Poodineh J, Najafi S, Masoodi T, et al. Functional roles of lncRNA-TUG1 in hepatocellular carcinoma. Life Sci. 2022; 308: 120974.
27. Uesaka M, Nishimura O, Go Y, Nakashima K, Agata K, Imamura T. Bidirectional promoters are the major source of gene activation-associated non-coding RNAs in mammals. BMC Genomics. 2014; 15: 35.
28. Zhou M, Guo X, Wang M, Qin R. The patterns of antisense long non-coding RNAs regulating corresponding sense genes in human cancers. J Cancer. 2021; 12(5): 1499-1506.
29. Ousati Ashtiani Z, Abbasi S, Pourmand G, Ghafouri-Fard S. Overexpression of long intergenic noncoding RNAs in bladder cancer: a new insight to cancer diagnosis. Pathol Res Pract. 2022; 235: 153961.
30. Chen LL. Linking long noncoding RNA localization and function. Trends Biochem Sci. 2016; 41(9): 761-772.
31. Statello L, Guo CJ, Chen LL, Huarte M. Gene regulation by long non-coding RNAs and its biological functions. Nat Rev Mol Cell Biol. 2021; 22(2): 96-118.
32. Ma L, Bajic VB, Zhang Z. On the classification of long non-coding RNAs. RNA Biol. 2013; 10(6): 925-933.

33. Wu R, Su Y, Wu H, Dai Y, Zhao M, Lu Q. Characters, functions and clinical perspectives of long non-coding RNAs. Mol Genet Genomics. 2016; 291(3): 1013-1033.
34. Che D, Fang Z, Mai H, Xu Y, Fu L, Zhou H, et al. The lncRNA ANRIL gene rs2151280 GG genotype is associated with increased susceptibility to recurrent miscarriage in a southern chinese population. J Inflamm Res. 2021; 14: 2865-2872.
35. Zhang Y, Wang S. The possible role of long non-coding RNAs in recurrent miscarriage. Mol Biol Rep. 2022; 49(10): 9687-9697.
36. Chen X, Guo DY, Yin TL, Yang J. Non-coding RNAs regulate placental trophoblast function and participate in recurrent abortion. Front Pharmacol. 2021; 12: 646521.
37. Liu LP, Gong YB. LncRNA-TCL6 promotes early abortion and inhibits placenta implantation via the EGFR pathway. Eur Rev Med Pharmacol Sci. 2018; 22(21): 7105-7112.
38. Xiang H, Yan H, Sun B, Feng F, Chen P. Decreased expression of long non-coding RNA SNHG7 cause recurrent spontaneous abortion through suppression proliferation and invasion of trophoblast cells via miR-34a. Am J Transl Res. 2019; 11(1): 463-472.
39. Bai RX, Tang ZY. Long non-coding RNA H19 regulates Bcl-2, Bax and phospholipid hydroperoxide glutathione peroxidase expression in spontaneous abortion. Exp Ther Med. 2021; 21(1): 41.
40. He D, Zeng H, Chen J, Xiao L, Zhao Y, Liu N. H19 regulates trophoblastic spheroid adhesion by competitively binding to let-7. Reproduction. 2019; 157(5): 423-430.
41. Gao WL, Liu M, Yang Y, Yang H, Liao Q, Bai Y, et al. The imprinted H19 gene regulates human placental trophoblast cell proliferation via encoding miR-675 that targets Nodal Modulator 1 (NOMO1). RNA Biol. 2012; 9(7): 1002-1010.
42. Liao M, Liao W, Xu N, Li B, Liu F, Zhang S, et al. LncRNA EPB41L4A-AS1 regulates glycolysis and glutaminolysis by mediating nucleolar translocation of HDAC2. EBioMedicine. 2019; 41: 200-213.
43. Huang W, Zhou H, Pi L, Xu Y, Fu L, Yang Y, et al. Association between the rs2288947 polymorphism of the lncRNA TINCR gene and the risk of recurrent miscarriage in a Southern Chinese population. J Clin Lab Anal. 2019; 33(6): e22919.
44. Wang Y, Liu HZ, Liu Y, Wang HJ, Pang WW, Zhang JJ. Downregulated MALAT1 relates to recurrent pregnancy loss via sponging miRNAs. Kaohsiung J Med Sci. 2018; 34(9): 503-510.
45. Che D, Yang Y, Xu Y, Fang Z, Pi L, Fu L, et al. The lncRNA MALAT1 rs619586 G variant confers decreased susceptibility to recurrent miscarriage. Front Physiol. 2019; 10: 385.
46. Fang Z, Che D, Qing S, Li Q, Men H, Yuan L, et al. The lncRNA SOX2OT rs9839776 C>T Polymorphism indicates recurrent miscarriage susceptibility in a southern chinese population. Mediators Inflamm. 2019; 2019: 9684703.
47. Li H, Hou Y, Zhang S, Zhou Y, Wang D, Tao S, et al. CD49a regulates the function of human decidual natural killer cells. Am J Reprod Immunol. 2019; 81(4): e13101.
48. Zhang J, Liu X, Gao Y. The long noncoding RNA MEG3 regulates Ras-MAPK pathway through RASA1 in trophoblast and is associated with unexplained recurrent spontaneous abortion. Mol Med. 2021; 27(1): 70.
49. Yu N, Chen X, Du M, Li H, Wang Y, Jiang F, et al. Long non-coding RNA ZEB2-AS1 augments activity of trophoblast cells and prevents the development of recurrent spontaneous abortion in mice through EZH2-Mediated CST3 inhibition. Reprod Sci. 2022; 29(3): 963-974.
50. Fang Z, Che D, Qing S, Li Q, Men H, Yuan L, et al. The lncRNA SOX2OT rs9839776 C>T polymorphism indicates recurrent miscarriage susceptibility in a southern Chinese population. Mediators Inflamm. 2019; 2019: 9684703.
51. Teng L, Liu P, Song X, Wang H, Sun J, Yin Z. Long non-coding RNA nuclear-enriched abundant transcript 1 (NEAT1) represses proliferation of trophoblast cells in rats with preeclampsia via the MicroRNA-373/FLT1 Axis. Med Sci Monit. 2020; 26: e927305.
52. Che D, Huang W, Fang Z, Li L, Wu H, Pi L, et al. The lncRNA CCAT2 rs6983267 G allele is associated with decreased susceptibility to recurrent miscarriage. J Cell Physiol. 2019; 234(11): 20577-20583.
53. Tian FJ, He XY, Wang J, Li X, Ma XL, Wu F, et al. Elevated tristetraprolin impairs trophoblast invasion in women with recurrent miscarriage by destabilization of HOTAIR. Mol Ther Nucleic Acids. 2018; 12: 600-609.
54. Xie J, Liang T, Zhao J, Xu Z, Tian P, Wang R, et al. Lnc-HZ08 regulates BPDE-induced trophoblast cell dysfunctions by promoting PI3K ubiquitin degradation and is associated with miscarriage. Cell Biol Toxicol. 2022; 38(2): 291-310.
55. Xu Z, Tian P, Guo J, Mi C, Liang T, Xie J, et al. Lnc-HZ01 with m6A RNA methylation inhibits human trophoblast cell proliferation and induces miscarriage by up-regulating BPDE-activated lnc-HZ01/MXD1 positive feedback loop. Sci Total Environ. 2021; 776: 145950.
56. Wu JL, Wang YG, Gao GM, Feng L, Guo N, Zhang CX. Overexpression of lncRNA TCL6 promotes preeclampsia progression by regulating PTEN. Eur Rev Med Pharmacol Sci. 2019; 23(10): 4066-4072.
57. Wang H, Shen G, Liu M, Mao L, Mao H. Expression and clinical significance of lncRNA TCL6 in serum of patients with preeclampsia. Exp Ther Med. 2022; 23(1): 41.
58. Chen X, Guo DY, Yin TL, Yang J. Non-coding RNAs regulate placental trophoblast function and participate in recurrent abortion. Front Pharmacol. 2021; 12: 646521.
59. Bian Z, Ji W, Xu B, Huang W, Jiao J, Shao J, et al. The role of long noncoding RNA SNHG7 in human cancers (Review). Mol Clin Oncol. 2020; 13(5): 45.
60. Pei LJ, Sun PJ, Ma K, Guo YY, Wang LY, Liu FD. LncRNA-SNHG7 interferes with miR-34a to de-sensitize gastric cancer cells to cisplatin. Cancer Biomark. 2021; 30(1): 127-137.
61. Olgun G, Sahin O, Tastan O. Discovering lncRNA mediated sponge interactions in breast cancer molecular subtypes. BMC Genomics. 2018; 19(1): 650.
62. Luo D, Zou W, He Y, Xian H, Wang L, Shen J, et al. Modified dynamic hip screw loaded with autologous bone graft for treating Pauwels type-3 vertical femoral neck fractures. Injury. 2017; 48(7): 1579-1583.
63. Zeng H, Fan X, Liu N. Expression of H19 imprinted gene in patients with repeated implantation failure during the window of implantation. Arch Gynecol Obstet. 2017; 296(4): 835-839.
64. Zuckerwise L, Li J, Lu L, Men Y, Geng T, Buhimschi CS, et al. H19 long noncoding RNA alters trophoblast cell migration and invasion by regulating TβR3 in placentae with fetal growth restriction. Oncotarget. 2016; 7(25): 38398-38407.
65. Xu J, Xia Y, Zhang H, Guo H, Feng K, Zhang C. Overexpression of long non-coding RNA H19 promotes invasion and autophagy via the PI3K/AKT/mTOR pathways in trophoblast cells. Biomed Pharmacother. 2018; 101: 691-697.
66. Zeng H, He D, Xie H, Zhao Y, Peng Z, Deng H, et al. H19 regulates angiogenic capacity of extravillous trophoblasts by H19/miR-106a-5p/VEGFA axis. Arch Gynecol Obstet. 2020; 301(3): 671-679.
67. Zhu Y, Liu Q, Liao M, Diao L, Wu T, Liao W, et al. Overexpression of lncRNA EPB41L4A-AS1 induces metabolic reprogramming in trophoblast cells and placenta tissue of miscarriage. Mol Ther Nucleic Acids. 2019; 18: 518-532.
68. Yang B, Zhang L, Cao Y, Chen S, Cao J, Wu D, et al. Overexpression of lncRNA IGFBP4-1 reprograms energy metabolism to promote lung cancer progression. Mol Cancer. 2017; 16(1): 154.
69. Goda N, Ryan HE, Khadivi B, McNulty W, Rickert RC, Johnson RS. Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. Mol Cell Biol. 2003; 23(1): 359-369.
70. Camara AKS, Zhou Y, Wen PC, Tajkhorshid E, Kwok WM. Mitochondrial VDAC1: a key gatekeeper as potential therapeutic target. Front Physiol. 2017; 8: 460.
71. Zhang Y, Jin F, Li XC, Shen FJ, Ma XL, Wu F, et al. The YY1-HOTAIR-MMP2 signaling axis controls trophoblast invasion at the maternal-fetal interface. Mol Ther. 2017; 25(10): 2394-2403.
72. Tseng JJ, Hsieh YT, Hsu SL, Chou MM. Metastasis associated lung adenocarcinoma transcript 1 is up-regulated in placenta previa increta/percreta and strongly associated with trophoblast-like cell invasion in vitro. Mol Hum Reprod. 2009; 15(11): 725-731.
73. Che D, Fang Z, Mai H, Xu Y, Fu L, Zhou H, et al. The lncRNA ANRIL gene rs2151280 GG genotype is associated with increased susceptibility to recurrent miscarriage in a southern chinese population. J Inflamm Res. 2021; 14: 2865-2872.
74. Holdt LM, Teupser D. Long noncoding RNA ANRIL: lnc-ing genetic variation at the chromosome 9p21 locus to molecular mechanisms of atherosclerosis. Front Cardiovasc Med. 2018; 5: 145.
75. Pasmant E, Laurendeau I, Héron D, Vidaud M, Vidaud D, Bièche I. Characterization of a germ-line deletion, including the entire INK4/ARF locus, in a melanoma-neural system tumor family: identification of ANRIL, an antisense noncoding RNA whose expression coclusters with ARF. Cancer Res. 2007; 67(8): 3963-3969.
76. Yu X, Zheng H, Chan MT, Wu WK. HULC: an oncogenic long noncoding RNA in human cancer. J Cell Mol Med. 2017; 21(2): 410-417.
77. Kong D, Wang Y. Knockdown of lncRNA HULC inhibits proliferation, migration, invasion, and promotes apoptosis by sponging miR-122

in osteosarcoma. J Cell Biochem. 2018; 119(1): 1050-1061.
78. Ghafouri-Fard S, Taheri M. Maternally expressed gene 3 (MEG3): a tumor suppressor long non coding RNA. Biomed Pharmacother. 2019; 118: 109129.
79. Zeng X, Hunt A, Jin SC, Duran D, Gaillard J, Kahle KT. EphrinB2-EphB4-RASA1 Signaling in Human Cerebrovascular Development and Disease. Trends Mol Med. 2019; 25(4): 265-286.
80. Chen D, Teng JM, North PE, Lapinski PE, King PD. RASA1-dependent cellular export of collagen IV controls blood and lymphatic vascular development. J Clin Invest. 2019; 129(9): 3545-3561.
81. Xu C, Cui H, Li H, Wu Y, An H, Guo C. Long non-coding RNA ZEB2-AS1 expression is associated with disease progression and predicts outcome in gastric cancer patients. J BUON. 2019; 24: 663-671.
82. Afonso S, Romagnano L, Babiarz B. The expression and function of cystatin C and cathepsin B and cathepsin L during mouse embryo implantation and placentation. Development. 1997; 124(17): 3415-3425.
83. Thilaganathan B, Ralph E, Papageorghiou AT, Melchiorre K, Sheldon J. Raised maternal serum cystatin C: an early pregnancy marker for preeclampsia. Reprod Sci. 2009; 16(8): 788-793.
84. Tang X, Gao Y, Yu L, Lu Y, Zhou G, Cheng L, et al. Correlations between lncRNA-SOX2OT polymorphism and susceptibility to breast cancer in a Chinese population. Biomark Med. 2017; 11(3): 277-284.
85. Askarian-Amiri ME, Seyfoddin V, Smart CE, Wang J, Kim JE, Hansji H, et al. Emerging role of long non-coding RNA SOX2OT in SOX2 regulation in breast cancer. PLoS One. 2014; 9(7): e102140.
86. Brind J, Condly SJ, Lanfranchi A, Rooney B. Induced abortion as an independent risk factor for breast cancer: a systematic review and meta-analysis of studies on south asian women. Issues Law Med. 2018; 33(1): 32-54.
87. Erlandsson G, Montgomery SM, Cnattingius S, Ekbom A. Abortions and breast cancer: record-based case-control study. Int J Cancer. 2003; 103(5): 676-679.
88. Zhang M, Guo J, Liu L, Huang M, Li Y, Bennett S, et al. The role of long non-coding RNA, nuclear enriched abundant transcript 1 (NEAT1) in cancer and other pathologies. Biochem Genet. 2022; 60(3): 843-867.
89. Mozdarani H, Ezzatizadeh V, Rahbar Parvaneh R. The emerging role of the long non-coding RNA HOTAIR in breast cancer development and treatment. J Transl Med. 2020; 18(1): 152.

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Interleukin-6 as A Useful Predictor of Endometriosis-Associated Infertility: A Systematic Review

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Abstract

Endometriosis is a chronic inflammatory disease defined by the presence of endometrial-like tissue outside the uterine cavity. Several authors have reported on the association between changes in inflammatory marker levels and the maintenance or progression of endometriosis and associated infertility. Interleukin-6 (IL-6) is the most studied cytokine in endometriosis and has important functions in reproductive physiology. The aim of this study is to review systematically available evidence about altered IL-6 concentrations in endometriosis-related infertility. This is a systematic review including all studies until December 2022 in which IL-6 in serum, peritoneal fluid, follicular fluid, or endometrial biopsy specimens was measured and that correlated their findings with endometriosis-associated infertility. Fifteen studies were included in the systematic review. There seems to be a correlation between elevated serum and peritoneal fluid IL-6 concentrations and the occurrence of endometriosis-associated infertility. IL-6 may be a potential diagnostic or biomarker tool for the prediction of endometriosis-related infertility. However, the numerous biases affecting the available studies, and challenges in endometriosis research reproducibility must be considered. Future investigations should pay attention to factors that may affect the results, such as the choice of suitable control groups, and carefully consider other pathological conditions affecting the patients, endometriosis stage, and type of lesion.

Keywords: Endometriosis, Infertility, Interleukin-6

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Introduction

Endometriosis is a chronic inflammatory disease defined by the presence of endometrial-like tissue outside the uterine cavity (1, 2). It affects 5 to 10% of reproductive-age women (1, 2), causing low quality of life, depression, and various pain symptoms, including dysmenorrhea, dyspareunia, chronic pelvic pain, dyschezia, and dysuria (3, 4). Many authors have studied the association between this condition and infertility (3, 5). Several theories have been proposed to try to clarify the pathogenetic mechanisms underlying endometriosis. The most common involves pelvic and systemic inflammation stimulating the activation of immune cells and the secretion of cytokines and chemokines. Alterations in concentrations of these inflammatory mediators have been extensively described in women affected by this disease (6). Interleukin-6 (IL-6) is the most studied interleukin in endometriosis (4) and, considering its important functions in reproductive physiology, altered concentrations in these patients may lead to fertility problems (7).

Given this, this study aims to review systematically the available papers documenting IL-6 levels in serum (S), peritoneal fluid (PF), follicular fluid (FF), or endometrial biopsy specimens (ES) in patients with endometriosis-associated infertility.

Material and Methods

A bibliographic search using Medline, Embase, Cochrane database of Systematic Reviews, and ClinicalTrials.gov to December 2022 was performed querying for randomized controlled trials and prospective studies evaluating IL-6 in patients with endometriosis-associated infertility.

We used the medical subject heading (MeSH) term interleukin-6 (MeSH Unique ID: D015850) in combination

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with: Endometriosis (MeSH Unique ID: D004715); and Infertility (MeSH Unique ID: D007246).

To be included, each study had to measure IL-6 levels in S, PF, FF, or ES samples from endometriosis patients and compare the data with the presence of endometriosis-related infertility. Only papers written in English were included. Commentaries, letters to editors, editorials, and conference abstracts were excluded.

A systematic review was performed to find any statistically significant difference between groups evidenced by the results of chi-squared tests, Student's t tests, Mann-Whitney U-tests, Kruskal-Wallis tests, and Wilcoxon signed rank tests reported in the selected papers. Differences were expressed as means/median ± standard deviation (SD) and a $P<0.05$ was considered statistically significant. The systematic review was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (8) (Fig.1).

One author (G.G.I.) independently screened titles and abstracts of each citation and included those for full-text review. Each retrieved full-text article was independently evaluated for inclusion by another author (F.D.G.). Any potential disagreement was solved by discussion by a third author (F.A.G.).

Results

Our systematic bibliographic research strategy identified 656 articles. After screening of abstracts and titles and removal of 73 duplicates, 107 full-text records were assessed for eligibility. Finally, 15 studies were included in the systematic review (Fig.1).

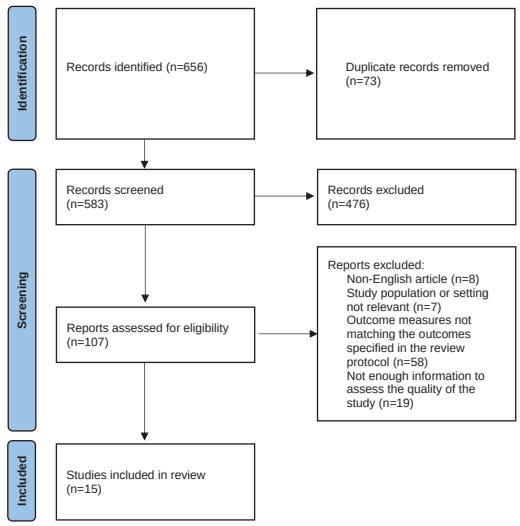

Fig.1: PRISMA flowchart.

IL-6 distribution among endometriosis-associated infertility and infertility without endometriosis, endometriosis without infertility, and non-endometriosis without infertility, respectively are displayed in Tables 1 (9-20), 2 (21, 22) and 3 (15, 23).

Table 1: Interleukin-6 distribution among women with endometriosis-related infertility and without endometriosis

Author, Year	E (n)	C (n)	C type	Type of sample	E IL-6	C IL-6	P value	Additional information
Buyalos et al. (9), 1992	10	10	Tubal infertility	FF		ns	αNA	
	10	10	Antisperm antibody	FF		ns	αNA	
Harada et al. (10), 1997	19	19	Diagnostic laparoscopy for infertility	PF	>	<	<0.05	
Iwabe et al. (11), 2002	53	40	Diagnostic laparoscopy for infertility	PF	>	<		
Jørgensen et al. (12), 2022	42	32	Diagnostic laparoscopy for infertility	ES		ns	0.99	
Kalu et al. (13), 2007	18	22	Diagnostic laparoscopy for infertility	PF	>	<	0.015	
	15	20	Diagnostic laparoscopy for infertility	S		ns	0.946	
Liu et al. (14), 2000	14	11	Diagnostic laparoscopy for infertility	PF	>	<	<0.01	
Moberg et al. (15), 2015	37	23	Diagnostic laparoscopy for infertility	PF	>	<	<0.0001	
Pellicer et al. (16), 1998	8	7	Diagnostic laparoscopy for infertility	S	<	>	<0.05	
	12	11	Assisted reproduction without endometriosis	FF	>	<	<0.05	
	8	7	Diagnostic laparoscopy for infertility	S	>	<	<0.05	Natural cycle
	12	11	Assisted reproduction without endometriosis	S	>	<	<0.05	Before HCG
	12	11	Assisted reproduction without endometriosis	S		ns	αNA	After HCG
Singh et al. (17), 2016	200	140	Tubal infertility	FF	>	<	<0.05	
	200	140	Tubal infertility	S	>	<	<0.05	
Skrzypczak et al. (18), 2005	28	20	Infertile without endometriosis	PF		ns	αNA	
	20	10	Fertile	PF		ns	αNA	
Wu et al. (19), 2017	17	17	Tubal, male, myoma or idiopathic infertility	FF	>	<	0.01	
Wunder et al. (20), 2006	47	279	Assisted reproduction without endometriosis	FF		ns	0.34	

Interluekin-6 (IL-6) levels for exposure groups were compared to those for controls reporting the higher or lower values between the two groups in case of statistical significance. αNA; α error not available, C; Control, E; Endometriosis, ES; Endometrial biopsy specimens, FF; Follicular fluid, ns; Non-significant, PF; Peritoneal fluid, S; Serum, and HCG; Human chorionic gonadotropin.

Table 2: Interleukin-6 distribution among women with endometriosis-related infertility and endometriosis without infertility

Author (References), Year	I (n)	C (n)	C type	Type of fluid	I IL-6	C IL-6	P value
Gica et al. (21), 2020	22	14	Diagnostic laparoscopy for endometriosis	S	>	<	0.00
Moberg et al. (15), 2015	37	28	Diagnostic laparoscopy for endometriosis	PF	>	<	<0.0001

Interluekin-6 (IL-6) levels for exposure groups were compared to those for controls reporting the higher or lower values between the two groups in case of statistical significance. C; Control, I; Infertility, n; Number, PF; Peritoneal fluid, and S; Serum.

Table 3: Interleukin-6 distribution among women with endometriosis-related infertility and non-endometriosis without infertility

Author (References), Year	E (n)	C (n)	C type	Type of fluid	E IL-6	C IL-6	P value
Ueki et al. (22), 1994	7	9	Fertile with myoma or ovarian cyst	PF	>	<	αNA
Wang et al. (23), 2018	55	30	Fertile without endometriosis	PF	>	<	<0.05

Interluekin-6 (IL-6) levels for exposure groups were compared to those for controls reporting the higher or lower values between the two groups in case of statistical significance. αNA; α error not available, C; Control, E; Endometriosis, n; Number, and PF; Peritoneal fluid.

One study reported higher values of S IL-6 in patients with endometriosis-related infertility than controls (17). Other authors compared women with endometriosis and controls, studying some patients with endometriosis in natural cycles to others in stimulated cycles. They concluded S IL-6 was increased in the natural cycles of patients with endometriosis and modulated by ovarian stimulation, showing a significant decrease in stimulated cycles and a significant increase just after human chorionic hormone administration (16). One study found no difference in S IL-6 levels between endometriosis-associated infertility patients and the control group (13).

All of the 6 included studies evaluating IL-6 concentration in PF found higher levels in the endometriosis-associated infertility group than in controls (10, 11, 13-15, 18). Interestingly, Harada et al. (10) found a statistically significant correlation between the American Fertility Society scoring system and the log-transformed IL-6 levels in PF from patients with endometriosis ($r=0.723$, $P<0.01$).

Higher FF IL-6 concentrations were found in three papers in which infertile women with endometriosis were compared to patients without endometriosis (11, 16, 17). Two studies found no difference in FF IL-6 concentrations between endometriosis-associated infertility patients and control groups (9, 20).

The only study evaluating IL-6 levels in ES between infertile patients with endometriosis and a control group failed to show any difference (12). Moreover, higher values of S (21) and PF (15) IL-6 were reported among patients with endometriosis-associated infertility compared with those who underwent diagnostic laparoscopy for endometriosis. Finally, higher values of PF IL-6 were shown among women with endometriosis-associated infertility compared to women without endometriosis or infertility (22, 23).

Discussion

The present systematic review reinforces the hypothesis that there is a correlation between elevated S and PF IL-6 concentrations and endometriosis-related infertility. However, there was no clear evidence of a correlation between FF and ES IL-6 concentrations and endometriosis-associated infertility.

Nowadays 30 and 50% of patients with endometriosis develop infertility, and about 25 to 50% of infertile women are diagnosed with endometriosis (3, 21).

Several theories have been proposed to explain this pathogenesis and, currently, the chronic inflammation theory seems to be the most plausible (6). Therefore, research into inflammatory factors as a cause of endometriosis is growing. Many authors have stated that inflammatory markers are highly influential in endometriosis (21), and their evaluations represent non-invasive tests for this disease (24).

IL-6 is the most investigated interleukin concerning endometriosis and is an important pleiotropic cytokine for assisting with the diagnosis of this disease. It is secreted in response to injury by various immune cells (10), and participates in several immunological mechanisms (4, 25). Many studies have demonstrated higher S (26), PF (27) and FF (28) IL-6 concentrations in women with endometriosis. IL6 also plays an important role in reproductive mechanisms, such as the production of steroid hormones by the ovaries (10), folliculogenesis, and oocyte maturation by ovarian angiogenesis and enhancement of vascular permeability (7). IL-6 is an activator of macrophages (29), which can amplify angiogenesis and regulate the immune environment of the endometrium (5, 30). In this regard, IL-6 is felt to contribute to infertility often associated with endometriosis (3). IL-6 family proteins, especially leukemia inhibitory factor (LIF), also play an important role in the early stages of embryonic implantation (15, 31) and therefore one hypothesis is that their reduced expression may cause endometriosis-related infertility (15, 32). LIF, its receptor LIFR, and the IL-6 family signaling molecule glycoprotein (gp)130 are predominantly expressed in the mid-secretory endometrium, where embryo implantation occurs (15, 33, 34). The binding of LIF to the high-affinity receptor complex formed by LIFR and gp130 activates the intracellular signaling transduced mainly through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, which can be inhibited

by suppression of cytokine signaling 1 (SOCS1) (15, 35). The latter has been detected in the endometrium in both the proliferative and secretory phases (15, 34). LIF also has other functions in the endometrium, such as regulating the immune environment during implantation, controlling interactions between decidual leukocytes and the embryo, and altering the expression of glycans on the cell surface (36, 37). In mice, the intrauterine administration of anti-LIF has been shown to cause pregnancy blockage (38). In accordance with this, some authors found a lower expression of LIF in the mid-secretory endometrium of infertile women with endometriosis than in fertile women (32, 37), suggesting that altered endometrial expression of LIF, LIFR and gp130 may explain the higher rate of infertility in women suffering from endometriosis (14, 15).

Prima et al. (39) also showed a negative correlation between PF IL-6 levels and the Endometriosis Fertility Index (EFI) score, an indicator to predict pregnancy in patients undergoing surgery that combines patient history factors (age, duration of infertility and previous pregnancy history) and intraoperative findings (surgical factors). Contrary to the discordant studies included in this review relative to differences in FF IL-6 concentrations, Altun et al. (40) reported an increased likelihood of clinical pregnancies n women without endometriosis and with low FF IL-6 concentrations Therefore, evaluating local and systemic IL-6 levels may be clinically useful to predict endometriosis-related infertility, especially in S and PF.

Factors that may limit the conclusions of this review are that the control groups differed among the included studies and that the results could have been influenced by the sample size, the presence of other inflammatory pathological conditions, and the use of hormones and therapies. The technical sensitivity of the assays used can be an additional confounding factor and it is possible that the IL-6 detected by some researchers may be non-functional or antagonized by anti-inflammatory cytokines or cytokine inhibitors. Another point to note is that IL-6 levels depend on the menstrual cycle phase, the endometriosis stage, and the type of lesion.

Conclusion

Local and systemic IL-6 levels may prove useful in the future as diagnostic or biomarker tools to predict endometriosis-related infertility. However, the numerous biases affecting the available studies, and challenges in endometriosis research reproducibility must be considered. Future investigations should pay attention to the choice of suitable control groups and carefully consider other pathological conditions affecting the patients, the endometriosis stage, and the type of lesion as these may affect the results.

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Authors' Contributions

G.G.I.; Conceptualization, Investigation, Methodology, Writing draft, Project administration, and Validation. F.D.G., F.A.G., F.G., D.B., C.L.; Investigation, Writing review, and Editing. M.P.; Supervision and Writing review. All authors read and approved the manuscript.

References

1. Bulun SE, Yilmaz BD, Sison C, Miyazaki K, Bernardi L, Liu S, et al. Endometriosis. Endocr Rev. 2019; 40(4): 1048-1079.
2 Newmark AL, Luciano DE, Ulrich A, Luciano AA. Medical management of endometriosis. Minerva Obstet Gynecol. 2021; 73(5): 572-587.
3. Agarwal SK, Chapron C, Giudice LC, Laufer MR, Leyland N, Missmer SA, et al. Clinical diagnosis of endometriosis: a call to action. Am J Obstet Gynecol. 2019; 220(4): 354.e1-354.e12.
4. Malvezzi H, Hernandes C, Piccinato CA, Podgaec S. Interleukin in endometriosis-associated infertility-pelvic pain: systematic review and meta-analysis. Reproduction. 2019; 158(1): 1-12.
5. Al-Jefout M. Laparoscopy for diagnosis and treatment of endometriosis. In: Darwish A, editor. Advanced gynecologic endoscopy. IntechOpen, 2011.
6. Jiang L, Yan Y, Liu Z, Wang Y. Inflammation and endometriosis. Front Biosci (Landmark Ed). 2016; 21(5): 941-948.
7. Li C, Zhao HL, Li YJ, Zhang YY, Liu HY, Feng FZ, et al. The expression and significance of leukemia inhibitory factor, interleukin-6 and vascular endothelial growth factor in Chinese patients with endometriosis. Arch Gynecol Obstet. 2021; 304(1): 163-170.
8. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ. 2021; 372: n71.
9. Buyalos RP, Watson JM, Martinez-Maza O. Detection of interleukin-6 in human follicular fluid. Fertil Steril. 1992; 57(6): 1230-1234.
10. Harada T, Yoshioka H, Yoshida S, Iwabe T, Onohara Y, Tanikawa M, et al. Increased interleukin-6 levels in peritoneal fluid of infertile patients with active endometriosis. Am J Obstet Gynecol. 1997; 176(3): 593-597.
11. Iwabe T, Harada T, Terakawa N. Role of cytokines in endometriosis-associated infertility. Gynecol Obstet Invest. 2002; 53 Suppl 1: 19-25.
12. Jørgensen H, Fedorcsak P, Isaacson K, Tevonian E, Xiao A, Beste M, et al. Endometrial cytokines in patients with and without endometriosis evaluated for infertility. Fertil Steril. 2022; 117(3): 629-640.
13. Kalu E, Sumar N, Giannopoulos T, Patel P, Croucher C, Sherriff E, et al. Cytokine profiles in serum and peritoneal fluid from infertile women with and without endometriosis. J Obstet Gynaecol Res. 2007; 33(4): 490-495.
14. Liu Y, Luo L, Zhao H. Changes of cytokines levels in peritoneal fluids of patients with endometriosis and its effect on reproductive activity. J Tongji Med Univ. 2000; 20(2): 163-165.
15. Moberg C, Bourlev V, Ilyasova N, Olovsson M. Endometrial expression of LIF and its receptor and peritoneal fluid levels of IL-1α and IL-6 in women with endometriosis are associated with the probability of pregnancy. Arch Gynecol Obstet. 2015; 292(2): 429-437.
16. Pellicer A, Albert C, Mercader A, Bonilla-Musoles F, Remohí J, Simón C. The follicular and endocrine environment in women with endometriosis: local and systemic cytokine production. Fertil Steril. 1998; 70(3): 425-431.
17. Singh AK, Dutta M, Chattopadhyay R, Chakravarty B, Chaudhury K. Intrafollicular interleukin-8, interleukin-12, and adrenomedullin are the promising prognostic markers of oocyte and embryo quality in women with endometriosis. J Assist Reprod Genet. 2016; 33(10): 1363-1372.
18. Skrzypczak J, Szczepańska M, Puk E, Kamieniczna M, Kurpisz M. Peritoneal fluid cytokines and sICAM-1 in minimal endometriosis: search for discriminating factors between infertility and/or endometriosis. Eur J Obstet Gynecol Reprod Biol. 2005; 122(1): 95-103.
19. Wu G, Bersinger NA, Mueller MD, von Wolff M. Intrafollicular inflammatory cytokines but not steroid hormone concentrations are increased in naturally matured follicles of women with proven endometriosis. J Assist Reprod Genet. 2017; 34(3): 357-364.
20. Wunder DM, Mueller MD, Birkhäuser MH, Bersinger NA. Increased ENA-78 in the follicular fluid of patients with endometriosis. Acta Obstet Gynecol Scand. 2006; 85(3): 336-342.
21. Gica N, Panaitescu AM, Iancu G, Botezatu R, Peltecu G, Gica C. The role of biological markers in predicting infertility associated with non-obstructive endometriosis. Ginekol Pol. 2020; 91(4): 189-192.

22. Ueki M, Tsurunaga T, Ushiroyama T, Ueda M. Macrophage activation factors and cytokines in peritoneal fluid from patients with endometriosis. Asia Oceania J Obstet Gynaecol. 1994; 20(4): 427-431.
23. Wang XM, Ma ZY, Song N. Inflammatory cytokines IL-6, IL-10, IL-13, TNF-α and peritoneal fluid flora were associated with infertility in patients with endometriosis. Eur Rev Med Pharmacol Sci. 2018; 22(9): 2513-2518.
24. Socolov R, Socolov D, Sindilar A, Pavaleanu I. An update on the biological markers of endometriosis. Minerva Ginecol. 2017; 69(5): 462-467.
25. Kaur S, Bansal Y, Kumar R, Bansal G. A panoramic review of IL-6: Structure, pathophysiological roles and inhibitors. Bioorg Med Chem. 2020; 28(5): 115327.
26. Wang F, Wang H, Jin D, Zhang Y. Serum miR-17, IL-4, and IL-6 levels for diagnosis of endometriosis. Medicine (Baltimore). 2018; 97(24): e10853.
27. Jiang J, Jiang Z, Xue M. Serum and peritoneal fluid levels of interleukin-6 and interleukin-37 as biomarkers for endometriosis. Gynecol Endocrinol. 2019; 35(7): 571-575.
28. Prins JR, Marissen LM, Scherjon SA, Hoek A, Cantineau AEP. Is there an immune modulating role for follicular fluid in endometriosis? A narrative review. Reproduction. 2020; 159(1): R45-R54.
29. Ayaub EA, Tandon K, Padwal M, Imani J, Patel H, Dubey A, et al. IL-6 mediates ER expansion during hyperpolarization of alternatively activated macrophages. Immunol Cell Biol. 2019; 97(2): 203-217.
30. Song Y, Su RW, Joshi NR, Kim TH, Lessey BA, Jeong JW, et al. Interleukin-6 (IL-6) activates the NOTCH1 signaling pathway through E-proteins in endometriotic lesions. J Clin Endocrinol Metab. 2020; 105(5): 1316-1326.
31. Cheng X, Liu J, Shan H, Sun L, Huang C, Yan Q, et al. Activating transcription factor 3 promotes embryo attachment via up-regulation of leukemia inhibitory factor in vitro. Reprod Biol Endocrinol. 2017; 15(1): 42.
32. Schmitz CR, Oehninger S, Genro VK, Chandra N, Lattanzio F, Yu L, et al. Alterations in expression of endometrial milk fat globule-EGF factor 8 (MFG-E8) and leukemia inhibitory factor (LIF) in patients with infertility and endometriosis. JBRA Assist Reprod. 2017; 21(4): 313-320.
33. Yoo I, Chae S, Han J, Lee S, Kim HJ, Ka H. Leukemia inhibitory factor and its receptor: expression and regulation in the porcine endometrium throughout the estrous cycle and pregnancy. Asian-Australas J Anim Sci. 2019; 32(2): 192-200.
34. Yue X, Wu L, Hu W. The regulation of leukemia inhibitory factor. Cancer Cell Microenviron. 2015; 2(3): e877.
35. Zhao Y, He D, Zeng H, Luo J, Yang S, Chen J, et al. Expression and significance of miR-30d-5p and SOCS1 in patients with recurrent implantation failure during implantation window. Reprod Biol Endocrinol. 2021; 19(1): 138.
36. Massimiani M, Lacconi V, La Civita F, Ticconi C, Rago R, Campagnolo L. Molecular signaling regulating endometrium-blastocyst crosstalk. Int J Mol Sci. 2019; 21(1): 23.
37. Carvalho LFP, Hui CYY, Agarwal A. Endometriosis and infertility: biomarkers affecting implantation rate. Expert Rev Obstet Gynecol. 2013; 8(5): 467-473.
38. Mehri N, Jamshidizad A, Ghanei Z, Karkhane AA, Shamsara M. Optimizing the expression and solubilization of an E. coli-produced leukemia inhibitory factor for anti-LIF antibody production and use thereof for contraception in mice. Mol Biotechnol. 2021; 63(12): 1169-1182.
39. Prima AT, Hendry D, Bachtiar H. Correlation between levels of interleukin-6 in peritoneal fluid with degree of pain, adhesion, and endometriosis fertility index score in endometriosis. AOJ. 2021; 5(2): 193-205.
40. Altun T, Jindal S, Greenseid K, Shu J, Pal L. Low follicular fluid IL-6 levels in IVF patients are associated with increased likelihood of clinical pregnancy. J Assist Reprod Genet. 2011; 28(3): 245-251.

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Association between Serum HLA-G Levels in The First Trimester of Pregnancy and The Onset of Preeclampsia: A Systematic Review and Meta-analysis Study

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Abstract

Human leukocyte antigen G (HLA-G) levels are among the biomarkers suggested for pre-eclampsia (PE). This study is aimed at determining the possible relationship between low soluble HLA-G (sHLA-G) levels in maternal blood at the beginning of pregnancy and subsequent PE. We searched the international scientific databases of Web of Science, Embase, PubMed, Cochrane, and Scopus. We extracted the studies investigating the relationship between the serum levels of HLA-G in the first trimester of pregnancy and the onset of PE using the appropriate keywords. The collected data were analyzed using the random-effects meta-analysis model and STATA (version 14). A total of 5 studies met the eligibility criteria, and the total sample size was 668 subjects. The mean and SD age of case subjects was 31.41 ± 4.16 years, while it was 30.56 ± 3.5 for control subjects. According to the findings, there was an inverse relationship between HLA-G serum level in the first trimester of pregnancy and the subsequent onset of PE, standard mean difference (SMD)=-1.51 [95% confidence interval (CI): -2.26, -0.75, I2=90.8%, P=0.000]. Based on these results, low sHLA-G level in early pregnancy has a positive correlation with subsequent PE, and the significant role of sHLA-G in the early stages of placentation can be proven.

Keywords: First Trimester, Preeclampsia, Pregnancy

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Introduction

Pre-eclampsia (PE) is regarded as one of the most common disorders of pregnancy, which is usually manifested by the onset of hypertension along with proteinuria after the 20th week of pregnancy (1). Previous epidemiological data showed that about 3-5% of pregnancies worldwide are pregnancies are affected by PE (2). This condition not only leads to morbidity and mortality in some pregnant women but also causes fetus health issues such as growth restriction and preterm birth (3, 4).

The main feature of PE is defected placentation. The reduced ability of trophoblasts to invade the placental spiral arteries, as well as incomplete endothelial remolding of these arteries are considered the main mechanisms leading to the onset of PE (5). Despite the above-mentioned, clear-cut information cannot be provided on the exact pathogenesis of PE since various factors play a role in this regard (6). Risk factors including a genetic background of hypertension, maternal age, diabetes, cardiovascular conditions, and obesity have been listed to be associated with PE (7-9). In the case of the immune system, inflammatory conditions have been demonstrated to play a critical role in PE pathogenesis (10, 11). In this regard, through a review of the conducted studies, increased levels of inflammatory cytokines and declined levels of regulatory and anti-inflammatory cytokines have been found to play a role (12-15). Moreover, decreased frequency of immune cells with regulatory properties and

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increased number of immune cells with inflammatory properties during all stages of pregnancy have been focused on as the main features of PE (16). Uncontrolled immune activation and pro-inflammatory condition during the early weeks of gestation and in the first trimester of pregnancy are associated with the onset of PE after the 20th week of pregnancy (17). Several efforts have been done to find inflammatory biomarkers in early gestation to predict PE (18).

Many factors are involved in the immune system regulation at the feto-maternal interface, among which human leukocyte antigen G (HLA-G) is believed to be a critical factor that prevents fetus immune rejection and provide fetal growth and appropriate placentation. During a normal pregnancy, extravillous trophoblasts express an abundant amount of HLA-G (19). Interestingly, upregulated expression of HLA-G in trophoblasts is simultaneous with the invasive ability (20). HLA-G has several isoforms, which are different in subunits and the existance of soluble forms. HLA-G1 and HLA-G5 are the most frequent soluble ones and consist of four subunits (21). During pregnancy, HLA-G1 regulates the proliferation and activation of T cells and NK cells to support feto-maternal immune tolerance (22). HLA-G5 has been shown to regulate and control the production of cytokines by alloantigen-activated T cells (23). In the case of patients with PE, studies have shown that placental trophoblasts are defective in upregulating HLA-G expression and the serum level of soluble HLA-G is significantly lower than in patients without PE (24, 25). However, most studies in this regard have been done in cases with PE in their second or third trimester of pregnancy (26, 27). To determine the possible role of HLA-G in the onset of PE it is necessary to investigate the expression of this molecule during the first few weeks of pregnancy and the placentation process.

The role of HLA-G and its potential use as a biomarker to predict the onset of PE is currently unclear. Therefore, the current study aimed to review and analyze the published data available on the association of serum HLA-G during the first trimester of pregnancy and the onset of PE.

Materials and Methods

We conducted this systematic evidence review according to best practices and report our findings according to the preferred reporting items for systematic reviews and meta-analyses (PRISMA) checklist (28).

Search strategies

A comprehensive search was performed to extract the published studies on the relationship between the sHLA-G serum level in the first trimester of pregnancy and the onset of PE. The keywords used included “pregnancy”, “human leukocyte antigen G”, “HLA-G”, “preeclampsia”, “first trimester”, “onset of preeclampsia”, “PE", “frequency, and “genotype. Different combinations of these keywords and Boolean operators (“OR” and “AND”) were used for searching international databases, including ISI, PubMed, Embase, and Scopus. Google Scholar was also searched to find the studies not included in the mentioned databases (Table 1). Also, the references of the extracted studies were checked to find potentially relevant studies. All records were then imported into the endnote and duplicated records were deleted.

Table 1: Search strategy used in each database in this review

Electronic database	Search terms (including truncations)
ISI	“Pregnancy" OR “First Trimester”
	AND "Genotype"
	AND “Human leukocyte antigen G” OR “HLA-G”
	AND “Onset of Preeclampsia” OR “PE"
PubMed	“Preeclampsia”OR “PE"
	AND
	" Pregnancy" OR“First Trimester”
	AND “Human leukocyte antigen G” OR “HLA-G”
Scopus	“Human leukocyte antigen G” OR “HLA-G”
	AND "Genotype"
	AND “Preeclampsia”OR “PE
	AND “First Trimester of pregnancy”
Embase	“First Trimester of pregnancy”
	AND
	“Human leukocyte antigen G” OR “HLA-G”
	AND
	“Onset of Preeclampsia” OR “Preeclampsia "

Study selection

After deleting the duplicated studies, the titles and abstracts of the remaining were checked to find the eligible studies based on the following inclusion and exclusion criteria:

Inclusion criteria were the original case-control studies on the relationship between the sHLA-G serum level in the first trimester of pregnancy and the onset of PE with extractable intended data and available full texts. Exclusion criteria included review articles, meta-analyses, congress abstracts, studies in languages other than English, and retracted papers.

Using studies eligible based on the above criteria were selected by two of the authors independently while they were rechecked and confirmed by all authors.

Data extraction

Data from the selected studies were extracted by two different authors. Data such as the related data, including authors’ names, location, publication date, age and the number of case and control participants, gestational age, and sHLA-G serum level (ng/ml) were extracted by two of the authors. Data were reviewed for potential mistakes by other authors and then confirmed by all authors.

Risk of bias in individual studies (Quality assessment)

The Newcastle-Ottawa scale (NOS) for cross-sectional and case-control studies was used to assess the risk of bias in individual studies (18) with 9 points for case-control studies and 8 points for cross-sectional studies indicating high quality and low risk of bias: 1-3, 4-6, and 7-9, were categorized as of low, intermediate, and high quality, respectively for case-control studies and 1-3, 4-5, and 6-8 were categorized as low, intermediate, and high quality, respectively for cross-sectional studies (Table 2).

Risk of bias across studies

Publication bias was evaluated by Begg's Funnel plots and Egger test. P<0.05 were considered valid for heterogeneity.

Data synthesis and analysis

Variables such as the sample size, mean, and standard deviation of expected data were grouped. Each study's weight was assigned based on its inverse variance. The Q test and I^2 index were done at an α-level error of lower than 10% significance to assess test heterogeneity within the included studies. To analyze our collected heterogeneous data, the random effect model was utilized. Moreover, Stata Version 14.2 was used to analyze all of the data statistically.

Results

The present meta-analysis included five original studies published on the relationship between sHLA-G serum level in the first trimester of pregnancy and the onset of PE (Table 3). The study selection process is shown in Figure 1. The total sample size was 668 (155 case subjects and 513 control subjects), with mean ages of 31.41 ± 4.16 and 30.56 ± 3.5 years for the case and control subjects, respectively. The overall estimate of the standard mean difference between groups using the random-effects model was -1.51 [95% confidence interval (CI): -2.26, -0.75, I2=90.8%, P=0.000] (Fig.2). Accordingly, it can be concluded that a significant relationship was found between HLA-G serum level in the first trimester of pregnancy and the onset of PE in pregnant women, and the risk of developing PE later in pregnancy was increased by low sHLA-G serum levels. Based on Figure 3, no evidence of publication bias was found employing Begg's funnel plots and Egger test.

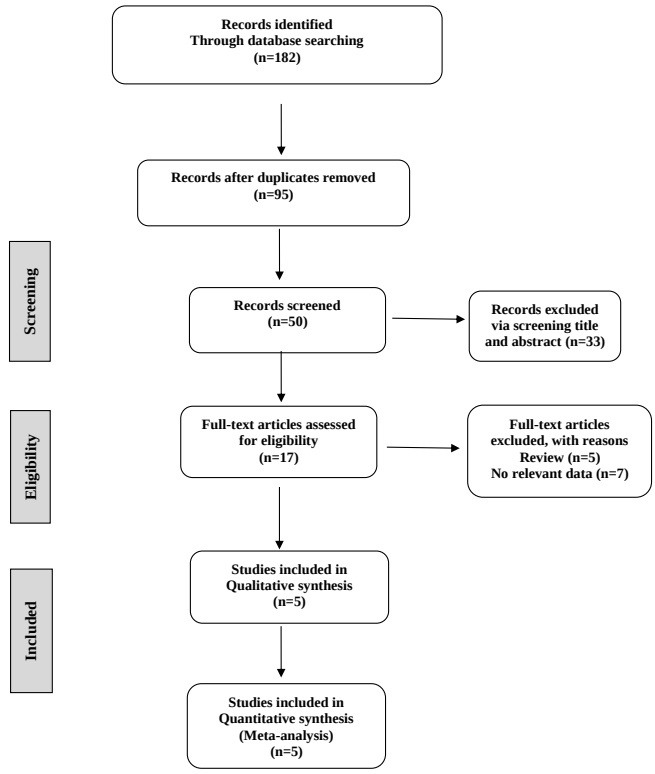

Fig.1: Prisma flow diagram illustrating the selection of articles.

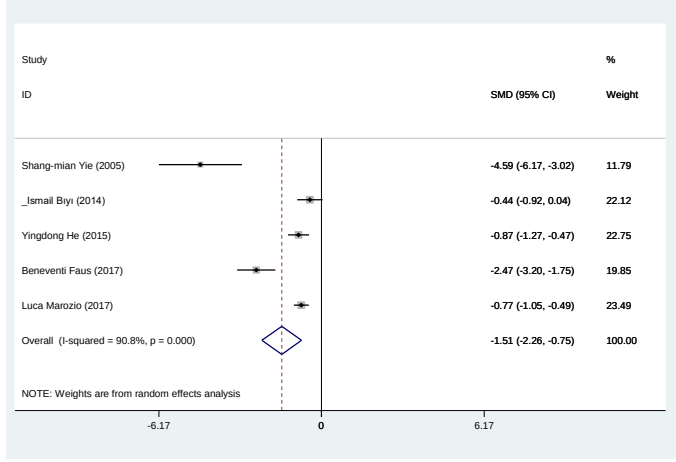

Fig.2: Forest plots for case-control studies of the relationship between the variables of HLA-G serum level in the first trimester of pregnancy and onset of PE in the investigated studies with 95% confidence interval (based on random model). PE; Preeclampsia, HLA-G; Human leukocyte antigen G, SMD; Standard mean difference, and CI; Confidence interval.

Table 2: Risk of bias in individual studies

First author's name	Type of study	Selection				Comparability	Outcome			Total
		1	2	3	4	1	1	2	3	
Yie et al. (29)	Case-control	*	*			*	*	*		5
Biyik et al. (30)	Case-control	*			**	*	**			6
Beneventi et al. (31)	Case-control	*	*	*	*	*	*	*	*	8
Marozio et al. (32)	Case-control	*	*			*	**			5
He et al. (33)	Case-control	*	*	*		*	**			6

Table 3: Characterizations of articles reviewed in the present study

First Author (reference)	Publication year	Sample size		Mean age ± SD		Gestational age ± SD		sHLA-G serum level (ng/ml) ± SD	
		Case	Control	Case	Control	Case	Control	Case	Control
Yie et al. (29)	2005	12	12	28 ± 5	29.8 ± 2.2	8.2 ± 6.6	7.8 ± 0.5	125 ± 16.87	195 ± 13.4
Biyik et al. (30)	2014	19	154	28.7 ± 6.64	27.24 ± 6.21	12.48 ± 0.7	12.3 ± 0.8	23.89 ± 12.82	36.91 ± 30.72
Beneventi et al. (31)	2017	17	42	34.26 ± 1.76	36.2 ± 1.3			22.32 ± 5.2	47 ± 11.3
Marozio et al. (32)	2017	65	234	31.7 ± 4.8	31.7 ± 4.9			96.86 ± 14.42	105.4 ± 10.03
He et al. (33)	2015	42	71	32.03 ± 2 .63	30.8 ± 2.9			2.52 ± 2.38	5.26 ± 3.53

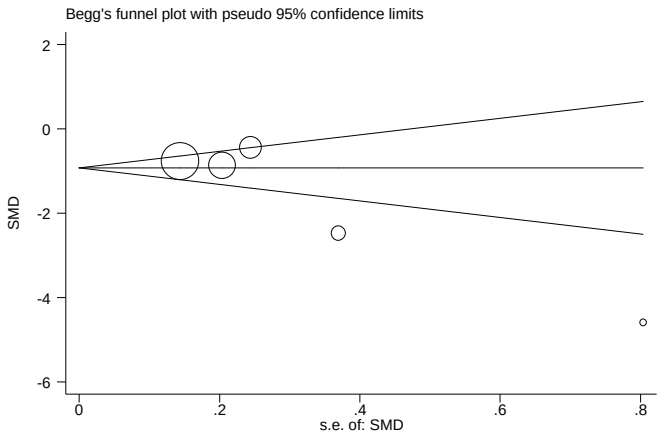

Fig.3: Begg's funnel plot for publication bias diagram in the investigated studies, the circles show the weight of the studies. SMD; Standard mean difference.

Discussion

Based on our findings, it seems that a clinical index determination for severe PE prediction especially in the first trimester is crucial, since its development has a negative effect on the prognosis of the mother and her baby. Therefore, the present meta-analysis was performed to investigate the controversial results on the potential relationship between serum levels of sHLA-G in the first trimester of pregnancy, and the onset of PE in pregnant women. To our knowledge, this is the first meta-analysis investigating this potential relationship. Our results suggest a significant relationship between sHLA-G serum level in the first trimester of pregnancy and the onset of PE.

Through our literature review, some studies were found to address the relationship between HLA-G and gestational time, including those in the current meta-analysis. For instance, Alegre et al. (34) and Yie et al. (29), have conducted studies in which a comparison is made between pregnant and non-pregnant women in terms of plasma sHLA-G levels. Based on their results, in the case of pregnant women, sHLA-G levels were detectable, while it was not the case for non-pregnant women. Another study, it was found by Klitkou et al. (35) that sHLA-G levels they were lower in fetal blood in comparison to the maternal blood, which shows the role of the placenta as a barrier. Besides, they observed that throughout the 20 weeks of gestation, sHLA-G levels were lower in maternal blood compared to fetal blood. Interestingly, a recently conducted study showed the possible association of sHLA-G with PE (36), while another recent study reported that sHLA-G levels did not predict complicated pregnancies (including PE). However, it should be noted that the latter study addressed various types of complicated pregnancies, and the selected sample size was found to be relatively small (30).

Through the study conducted by Yie et al. (29), the following results were obtained: a decrease occurred in the serum and placental HLA-G levels during PE in comparison to normal pregnancy, and a significant association was found between serum and placental levels of HLA-G. The results indicate that in maternal plasma, the trophoblast is the main source of sHLA-G. In a report by Steinborn et al. (37), it was revealed that lower sHLA-G levels are shown in women with placental abruption in comparison to the ones in normal pregnancy at the same gestational age.

It has also been found that below 43.5 IU/mL sHLA-G levels at the end of the first trimester lead to an increase in developing placenta-mediated complications, particularly severe PE. This usually happens through the later course of pregnancy which is reported to be related to abnormal placentation and placental abruption (32). Recent studies regarding the HLA-G and its association with PE have also highlighted the role of HLA-G in PE. A study published in 2020 suggested that placental down-regulation of HLA-G and its receptors contribute to the onset of PE through disturbance in interferon immune activation (38). Another recent study in 2021 has shown that not only decreased placental expression of HLA-G may be associated with PE, but changes in HLA-G isoform may also be a critical factor in PE (39).

The small sample size and address only sHLA-G levels constitute the limitations of this study. Accordingly, we cannot totally rule out the possibility that it could be a source of bias. However, our results showed that sHLA-G1 levels were independent of other factors such as body mass index (BMI).

Conclusion

To summarise, in comparison to the normal controls, plasma sHLA-G1 levels were found to be significantly lower in women with late-onset severe PE. One could conclude that although the low sHLA-G1 expression is shown to be significantly related to the occurrence of PE,

high sHLA-G1 levels may be considered as one of the factors to maintain a normal pregnancy. Although first-trimester sHLA-G1 seems to have predictive values for PE, large-scale cohort studies are needed to justify this.

Acknowledgements

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Authors Contributions

S.M., F.M.; Designed the conception of the study. M.Gh.H., V.T., E.V.; Focus on the statistical analysis. S.Sh., R.H., S.M.; Technical support and conceptual advice. All authors contributed to the draft of the manuscript, revised it critically, and approved the final version.

References

1. Dekker GA, Robillard PY. Preeclampsia: a couple's disease with maternal and fetal manifestations. Curr Pharm Des. 2005; 11(6): 699-710.
2. Ananth CV, Keyes KM, Wapner RJ. Pre-eclampsia rates in the United States, 1980-2010: age-period-cohort analysis. BMJ. 2013; 347: f6564.
3. Omani-Samani R, Ranjbaran M, Amini P, Esmailzadeh A, Sepidarkish M, Almasi-Hashiani A. Adverse maternal and neonatal outcomes in women with preeclampsia in Iran. J Matern Fetal Neonatal Med. 2019; 32(2): 212-216.
4. Souza JP, Gülmezoglu AM, Vogel J, Carroli G, Lumbiganon P, Qureshi Z, et al. Moving beyond essential interventions for reduction of maternal mortality (the WHO Multicountry Survey on Maternal and Newborn Health): a cross-sectional study. Lancet. 2013; 381(9879): 1747-1755.
5. Kaufmann P, Black S, Huppertz B. Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia. Biol Reprod. 2003; 69(1): 1-7.
6. Sircar M, Thadhani R, Karumanchi SA. Pathogenesis of preeclampsia. Curr Opin Nephrol Hypertens. 2015; 24(2): 131-138.
7. Das UN. Cytokines, angiogenic, and antiangiogenic factors and bioactive lipids in preeclampsia. Nutrition. 2015; 31(9): 1083-1095.
8. Hod T, Cerdeira AS, Karumanchi SA. Molecular mechanisms of preeclampsia. Cold Spring Harb Perspect Med. 2015; 5(10): a023473.
9. Mol BWJ, Roberts CT, Thangaratinam S, Magee LA, de Groot CJM, Hofmeyr GJ. Pre-eclampsia. Lancet. 2016; 387(10022): 999-1011.
10. Marusic J, Prusac IK, Tomas SZ, Karara JR, Roje D. Expression of inflammatory cytokines in placentas from pregnancies complicated with preeclampsia and HELLP syndrome. J Matern Fetal Neonatal Med. 2013; 26(7): 680-685.
11. Redman CW, Sargent IL. Pre-eclampsia, the placenta and the maternal systemic inflammatory response--a review. Placenta. 2003; 24 Suppl A: S21-S27.
12. Pinheiro MB, Martins-Filho OA, Mota AP, Alpoim PN, Godoi LC, Silveira AC, et al. Severe preeclampsia goes along with a cytokine network disturbance towards a systemic inflammatory state. Cytokine. 2013; 62(1): 165-173.
13. Tosun M, Celik H, Avci B, Yavuz E, Alper T, Malatyalioğlu E. Maternal and umbilical serum levels of interleukin-6, interleukin-8, and tumor necrosis factor-alpha in normal pregnancies and in pregnancies complicated by preeclampsia. J Matern Fetal Neonatal Med. 2010; 23(8): 880-886.
14. Gharesi-Fard B, Mobasher-Nejad F, Nasri F. The expression of T-helper associated transcription factors and cytokine genes in preeclampsia. Iran J Immunol. 2016; 13(4): 296-308.
15. Tok A, Seyithanoğlu M, Ozer A, Erkayıran U, Karaküçük S, Çelebi A. The serum level of soluble CXCL16 is increased in preeclampsia and associated with hepatic/renal damage. J Matern Fetal Neonatal Med. 2021; 34(9): 1435-1440.
16. Ahn H, Park J, Gilman-Sachs A, Kwak-Kim J. Immunologic characteristics of preeclampsia, a comprehensive review. Am J Reprod Immunol. 2011; 65(4): 377-394.
17. Founds SA, Terhorst LA, Conrad KP, Hogge WA, Jeyabalan A, Conley YP. Gene expression in first trimester preeclampsia placenta. Biol Res Nurs. 2011; 13(2): 134-139.
18. Tangerås LH, Austdal M, Skråstad RB, Salvesen KÅ, Austgulen R, Bathen TF, et al. Distinct first trimester cytokine profiles for gestational hypertension and preeclampsia. Arterioscler Thromb Vasc Biol. 2015; 35(11): 2478-2485.
19. Rizzo R, Vercammen M, van de Velde H, Horn PA, Rebmann V. The importance of HLA-G expression in embryos, trophoblast cells, and embryonic stem cells. Cell Mol Life Sci. 2011; 68(3): 341-352.
20. Guillard C, Zidi I, Marcou C, Menier C, Carosella ED, Moreau P. Role of HLA-G in innate immunity through direct activation of NF-kappaB in natural killer cells. Mol Immunol. 2008; 45(2): 419-427.
21. Carosella ED, Rouas-Freiss N, Paul P, Dausset J. HLA-G: a tolerance molecule from the major histocompatibility complex. Immunol Today. 1999; 20(2): 60-62.
22. Jiang F, Zhao H, Wang L, Guo X, Wang X, Yin G, et al. Role of HLA-G1 in trophoblast cell proliferation, adhesion and invasion. Biochem Biophys Res Commun. 2015; 458(1): 154-160.
23. Lombardelli L, Aguerre-Girr M, Logiodice F, Kullolli O, Casart Y, Polgar B, et al. HLA-G5 induces IL-4 secretion critical for successful pregnancy through differential expression of ILT2 receptor on decidual CD4- T cells and macrophages. J Immunol. 2013; 191(7): 3651-3662.
24. Hackmon R, Koifman A, Hyodo H, Glickman H, Sheiner E, Geraghty DE. Reduced third-trimester levels of soluble human leukocyte antigen G protein in severe preeclampsia. Am J Obstet Gynecol. 2007; 197(3): 255. e1-5.
25. Steinborn A, Varkonyi T, Scharf A, Bahlmann F, Klee A, Sohn C. Early detection of decreased soluble HLA-G levels in the maternal circulation predicts the occurrence of preeclampsia and intrauterine growth retardation during further course of pregnancy. Am J Reprod Immunol. 2007; 57(4): 277-286.
26. Zhu X, Han T, Yin G, Wang X, Yao Y. Expression of human leukocyte antigen-G during normal placentation and in preeclamptic pregnancies. Hypertens Pregnancy. 2012; 31(2): 252-260.
27. Darmochwal-Kolarz D, Kolarz B, Rolinski J, Leszczynska-Gorzelak B, Oleszczuk J. The concentrations of soluble HLA-G protein are elevated during mid-gestation and decreased in pre-eclampsia. Folia Histochem Cytobiol. 2012; 50(2): 286-291.
28. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. J Clin Epidemiol. 2009; 62(10): e1-34.
29. Yie SM, Li LH, Li YM, Librach C. HLA-G protein concentrations in maternal serum and placental tissue are decreased in preeclampsia. Am J Obstet Gynecol. 2004; 191(2): 525-529.
30. Bıyık I. Maternal serum soluble HLA-G in complicated pregnancies. J Matern Fetal Neonatal Med. 2014; 27(4): 381-384.
31. Beneventi F, Locatelli E, De Amici M, Martinetti M, Spinillo A. Soluble HLA-G concentrations in obese women during pregnancy and in cord blood. J Reprod Immunol. 2017; 119: 31-37.
32. Marozio L, Garofalo A, Berchialla P, Tavella AM, Salton L, Cavallo F, et al. Low expression of soluble human leukocyte antigen G in early gestation and subsequent placenta-mediated complications of pregnancy. J Obstet Gynaecol Res. 2017; 43(9): 1391-1396.
33. He Y, Chen S, Huang H, Chen Q. Association between decreased plasma levels of soluble human leukocyte antigen-G and severe pre-eclampsia. J Perinat Med. 2016; 44(3): 283-290.
34. Alegre E, Díaz-Lagares A, Lemaoult J, López-Moratalla N, Carosella ED, González A. Maternal antigen presenting cells are a source of plasmatic HLA-G during pregnancy: longitudinal study during pregnancy. Hum Immunol. 2007; 68(8): 661-667.
35. Klitkou L, Dahl M, Hviid TV, Djurisic S, Piosik ZM, Skovbo P, et al. Human leukocyte antigen (HLA)-G during pregnancy part I: correlations between maternal soluble HLA-G at midterm, at term, and umbilical cord blood soluble HLA-G at term. Hum Immunol. 2015; 76(4): 254-259.
36. Djurisic S, Hviid TV. HLA class Ib molecules and immune cells in pregnancy and preeclampsia. Front Immunol. 2014; 5: 652.
37. Steinborn A, Rebmann V, Scharf A, Sohn C, Grosse-Wilde H. Placental abruption is associated with decreased maternal plasma levels of soluble HLA-G. J Clin Immunol. 2003; 23(4): 307-314.
38. Wedenoja S, Yoshihara M, Teder H, Sariola H, Gissler M, Katayama S, et al. Fetal HLA-G mediated immune tolerance and interferon response in preeclampsia. EBioMedicine. 2020; 59: 102872.
39. Persson G, Stæhr CS, Klok FS, Lebech M, Hviid TVF. Evidence for a shift in placental HLA-G allelic dominance and the HLA-G isoform profile during a healthy pregnancy and preeclampsia. Biol Reprod. 2021; 105(4): 846-858.

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Gene Expression Levels of *CSF-1* and *CSF-1R* Endometrial under The Influence of Prolactin Level in Unexplained Miscarriage: A Case-Control Study

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Abstract

Background: Hormones such as prolactin, by influencing expression of the endometrial genes, play a pivotal role in embryo implantation and development. The present study aimed to evaluate serum level of prolactin and its effect on altering expression level of colony-stimulating factor-1 (*CSF-1*) and colony-stimulating factor-1 receptor (*CSF-1R*) genes in endometrial tissue during *in vitro* fertilization (IVF) pregnancy in the infertile women and recurrent pregnancy loss (RPL), compared to fertile women, who lost their pregnancies at gestational age <20 weeks.

Materials and Methods: In this case-control study, 40 infertile women, 40 IVF pregnant women with RPL and 40 fertile women who lost their pregnancies at <20 weeks of gestation for unknown reasons were selected. Prolactin serum level was assessed using ELISA technique and expression of *CSF-1* and *CSF-1R* genes was determined in endometrial tissue, using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Results: Mean prolactin level of the infertile group was 24.38 ± 1.43 ng/mL and it had statistically significant relationship with the fertile group (P<0.001). Expression level of the *CSF-1* and *CSF-1R* genes were higher in the fertile than infertile groups by 2.88 times (P<0.0001) and 2.64 times (P<0.0001), while it was respectively 2.28 (P<0.0001) and 1.69 (P<0.0001) times higher compared to the RPL group. Risk factors for pregnancy loss, such as aging, increased body mass index (BMI), smoking and diabetes caused decreasing changes in gene expression (*CSF-1* and *CSF-1R*) and the differences were statistically significant, except in the infertile group.

Conclusion: The present study showed a significant relationship of *CSF-1* and *CSF-1R* expression levels with pregnancy loss. Risk factors such as aging, obesity, smoking and diabetes decreased both genes expression levels.

Keywords: Genes, Infertility, Miscarriage, Prolactin, Recurrent Pregnancy Loss

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Introduction

Recurrent miscarriage occurs in 1-2% of women with reproductive age. Different terms and guideline is proposed to describe recurrent pregnancy loss. For example, it is termed as “recurrent pregnancy loss" and “recurrent miscarriage” by respectively the European society of human reproduction and embryology (ESHRE) and royal college of obstetricians and gynaecologists (RCOG) of UK. The new definition of ESHRE and the American society for reproductive medicine (ASRM) for recurrent miscarriage is the loss of two or more consecutive pregnancies, whereas the earlier description was defined as three or more consecutive pregnancy losses (1).

Recurrent pregnancy loss (RPL) is the most common complication of pregnancy. Almost 70% of human conceptions do not survive a live birth. Approximately 50% of all pregnancies end in miscarriage before clinical diagnosis even with fetal heart activity (2). RPL is characterized by two or three consecutive miscarriages prior to the 20th week of gestation (3). Pathogenic factors which have been known in only 50% of the cases include immune, endocrine, genetic and infectious factors, as well as metabolic disorders, anatomical abnormalities and other unknown causes (2, 3).

As a hormone secreted by the anterior pituitary gland, central nervous system, immune system, uterus, the tissue involved in pregnancy, and even the mammary glands, prolactin has several chemical forms after translation. It emerges a range of chemical changes, such as phosphorylation or glycosylation (4). It relies on the estrogen, progesterone, glucocorticoids, insulin,

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thyroid hormone and parathyroid hormone. Prolactin also enhances uptake of some amino acids and glucose as well as the production of milk sugar and milk fats (5).

Prolactin is a member of the growth hormone-placental lactogen family, arisen from a common ancestral gene, about 500 million years ago. More than 300 different functions (6), including growth, development, reproduction, metabolism, water and electrolyte balance, brain and behavior, and immune system regulation, are reported for prolactin (7), most of which are related to lactation and reproduction (8). Thus, its secretion is increased during pregnancy (9). Prolactin receptors are located on endometrial cells and, by binding to the hormone, help the endometrium capability to accept egg and create a suitable environment for blastocyst implantation (10). The prolactin effects are exerted through the CSF-1R receptor. This receptor is a member of the prolactin family, belonging to the tyrosine kinase receptors which are also expressed in macrophages and dendritic cell ancestors. This receptor controls proliferation, differentiation and survival of macrophages (11). In addition, CSF-1R is more expressed in the cell columns of extravillous trophoblasts, anchoring placenta to uterus (12).

Colony-stimulating factors (i.e. M-CSF, CSF1, GM-CSF, CSF2, G-CSF and CSF3) are a family of cytokines, among which M-CS and GM-CSF are expressed during pregnancy in the oviduct and uterus (13). CSF-1 (M-CS) is a factor that promotes growth of immune cells, especially monocytes. It is mainly produced by fibroblast cells, but there are various reports of its presence in the other tissue (14). This glycosylated homodimer with a disulfide bond is also expressed in the endometrium, decidua and placenta. In the endometrial glands, high levels of CSF-1 are seen during the secretory phase compared to the proliferative phase. Decidua also shows high levels of mRNA and CSF-1 protein in the secretory phase compared to the proliferative phase endometrium. In addition, it seems that endometrial cells near the anchoring villi of trophoblast are the main sources of CSF-1 for the placental-uterine interface (12).

Recurrent miscarriage and RPL mostly occur in the first trimester of pregnancy, prior to the week 20 of gestation; in addition, abortion (28th week of gestation) or premature birth (after 28th week of gestation) events occur in 10-20% of fertile couples (15). The current study aimed to evaluate prolactin hormone change and its relationship on the expression of *CSF-1* and *CSF-1R* genes during miscarriage prior to the week 20th of gestation in three groups of women with infertility, RPL and healthy fertile.

Materials and Methods

In this case-control study, three groups of women with infertility, RPL and healthy fertile were selected from patients referred to Yazd Infertility Center (Yazd, Iran), as well as Yas and Mirza Kuchak Khan Hospitals in Tehran, Iran. Women with children who have had at least two times normal pregnancy were assigned to the fertile group. Women with unknown causes of infertility and normal menstrual cycles with passing at least five years from their marriages were enrolled in the infertile group. Additionally, women who passed at least five years from their marriages and experienced a miscarriage at least twice without any children were assigned to the RPL group. Each group included 40 subjects (the sample size was estimated based on the following assumption: type1 and 2 errors: 0.05 and 0.20, respectively; expected implantation rate in the control group: 65%; expected frequency of abortion: 35%). Women in both infertility and RPL groups attempted to conceive via IVF, but subjects in the fertile group had normal pregnancies. The inclusion criteria were as follow: having a normal ovarian function, regular menstrual cycles, normal fallopian tubes, lack of uterine abnormalities, lack of endometriosis signs in ultrasound or laparoscopic examinations, and miscarriage of unknown causes with a normal embryonic karyotype prior to the week 20th of gestation. In addition, their spouses should have a normal volume and analysis of semen, based on the World health organization (WHO) reference values.

The selected individuals were within the age range of 25 and 35 years. Serum samples were taken from all subjects before undergoing curettage and stored at -20°C. Endometrial specimens were also collected using the Novak curette/Pipelle catheter and stored at -20°C after transferring to vials containing RNA-later. Other information about their age, height, weight and blood pressure was extracted from their files.

Determining serum concentration of prolactin, using ELISA

Serum prolactin concentrations were assessed by the commercially available kits (REF: DKO011, LOT No.: 4808A, DiaMetra, Italy) based on ELISA.

RNA extraction and cDNA synthesis

Firstly, endometrial tissue samples (approximately 100-150 mg) were rinsed with saline to remove RNA-later. Then, whole RNA was extracted from the tissue using a commercially available kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Then, the extracted total RNA was evaluated using spectrophotometry and gel electrophoresis. To synthesize cDNA, 1 mg of the total RNA of each sample was mixed with random hexamer primers, RT (Reverse Transcriptase) enzymes and enzyme buffer, according to the kit instructions (Gene All Inc., South Korea) and placed in a thermocycler.

Expression level analysis of *CSF-1* and *CSF-1R* by quantitative reverse transcription polymerase chain reaction

Beta-actin housekeeping gene was used to evaluate expression levels of *CSF-1* and *CSF-1R* genes. Sequences of the designed primers and length of the proliferated segments are shown in Table 1.

Table 1: Primer sequence and polymerase chain reaction (PCR) product of *CSF-1* and *CSF-1R* genes

Gene	Primer sequence (5'-3')	PCR product size (bp)
CSF-1	F: AAGTTTGCCTGGGTCCTCTC R: TCCACTCCCAATCATGTGGC	290
CSF-1R	F: TGAGCTCACCCTTCGATACC R: CCTCAGGGTATGGGTCATCC	188
B-actin	F: TGGGCATCCACGAAACTAC R: GATCTCCTTCTGCATCCTGT	135

SYBR Green PCR Master Mix kit was used to perform qRT-PCR. The reaction was carried out with a mixture containing 10 µl master mix SYBR Green (Qiagen, Germany), 1 µl of each primer (10 pmol/µl), 1 µl of cDNA (50 ng) and 7 µl of dH_2O based on the following program: initial denaturation at 95˚C for 10 minutes, then denaturation at 95˚C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72˚C for 20 seconds in a total of 35 cycles. The melting curve analysis was performed at the translation rate of 1°C/second from 95˚C to 60˚C for 60 seconds. To analyze the samples, the PCR cycle threshold curve was plotted based on the exponential phase, and the $2^{-\Delta\Delta Ct}$ method was used to analyze the data.

Statistical analysis

SPSS version 25 software (IBM, USA) was used for data analysis. The inter- and intra-group comparisons in terms of the mean age, BMI and prolactin levels were performed by one-way ANOVA and the post hoc Tukey test. The results were expressed as mean ± SD. Due to the non-normality of the data, nonparametric Kruskal-Wallis test was used to compare gene expression among the three groups. Pearson's correlation coefficient was used to evaluate the relationship between the prolactin level and the expression level of *CSF-1* and *CSF-1R* genes. Significant level was considered P<0.05.

Ethical considerations

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the Tehran Islamic Azad University of Medical Science (IR.IAU.TMU.REC.1397.007, Tehran, Iran). Informed consent was obtained from all participants and their information was received through a questionnaire.

Results

Demographic characteristics of the subjects in the fertile, RPL and infertile groups, including age, body mass index (BMI), smoking habit, diabetes status and prolactin levels, are shown in Table 2.

Assessment of prolactin levels in the study groups

Mean prolactin level had a significant difference between the fertile and infertile groups (41.84 ± 4.32 vs. 24.38 ± 1.43 ng/mL, P<0.001). The same result was obtained between the fertile and RPL (32.45 ± 4.16 ng/mL, P=0.121). The results are shown in Figure 1.

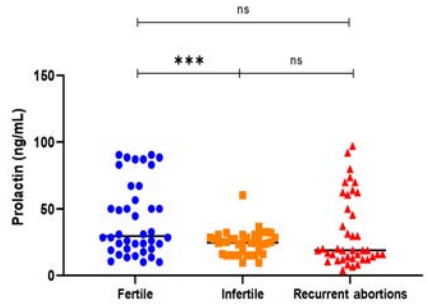

Fig.1: Comparison of the prolactin levels among fertile, infertile and miscarriage. Comparison of the three study groups showed that the highest and lowest levels of prolactin belonged to the fertile and infertile groups respectively, and a significant difference was observed among these groups. ***; P<0.0001 and ns; P>0.01.

Assessment of the expression level of *CSF-1* and *CSF-1R* genes in the study groups

Expression level of the *CSF-1* gene in the endometrial tissue was higher in the fertile than the infertile group by 2.88 times (P<0.0001). Its expression level was 2.28 times higher than the RPL ones (P<0.0001). Its expression level between the RPL was 1.26 times higher than the infertile group (P=0.011).

Expression level of the *CSF-1R* gene was 2.64 times higher in the fertile group than infertile ones (P<0.0001), and 1.69 times higher than the RPL group. The difference was statistically significant (P<0.0001). Expression level of the *CSF-1R* gene in the RPL group was 1.56 times higher than the infertile group, and the difference was statistically significant (P=0.002). Data are shown in Figure 2.

Table 2: Characteristics of the fertile, infertile and recurrent abortion women

Variable	Fertile	Infertile	Recurrent abortion	P value between the groups
Age (Y)	32.93 ± 1.25	33.20 ± 1.30	32.03 ± 1.02	0.769
BMI (kg/m^2)	23.76 ± 0.56	27.43 ± 0.48	26.23 ± 0.58	<0.001
Prolactin (ng/mL)	41.84 ± 4.32	24.38 ± 1.43	32.45 ± 4.16	0.003
Smoker				0.303
Positive	8 (20)	4 (10)	9 (22.5)	
Negative	32 (80)	36 (90)	31 (77.5)	
Diabetes				0.277
Positive	3 (7.5)	6 (15)	8 (20)	
Negative	37 (92.5)	34 (85)	32 (80)	

Data are presented as mean ± SD or n (%). BMI; Body mass index.

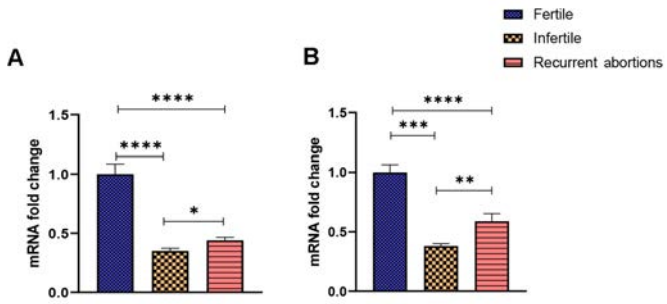

Fig.2: Comparison of *CSF-1* and *CSF-1R* gene expressions between the studied groups. Expression changes of the **A.** *CSF-1* and **B.** *CSF-1R* genes among the three study groups are shown based on the fold change. The lowest and highest expression levels of the *CSF-1* and *CSF-1R* gene were observed in the infertile and fertile groups, respectively. *; P<0.01, **; P<0.001, ***; P<0.0001, ****; P<0.00001, and ns; P>0.01.

Relationship of prolactin on the expression level of *CSF-1* and *CSF-1R* genes in the study groups

Effect of ≤40 and >40 ng/mL prolactin concentrations was evaluated on expression level of *CSF-1* and *CSF-1R* genes in the fertile, infertile and RPL groups. Expression level of *CSF-1* gene in subjects with prolactin serum concentration >40 ng/mL was increased 1.63, 1.01 and 1.36 times in the fertile, infertile and RPL groups, respectively, compared to the ones with prolactin serum levels ≤40 ng/mL. Differences for the infertile group were insignificant.

Expression level of *CSF-1R* gene in the individuals with prolactin serum concentration >40 ng/mL was respectively increased 2.86, 1.07 and 1.54 times in the fertile, infertile and RPL groups, respectively than those with prolactin serum levels ≤40 ng/mL; the differences were statistically significant unless infertile group. Data are shown in Figure 3.

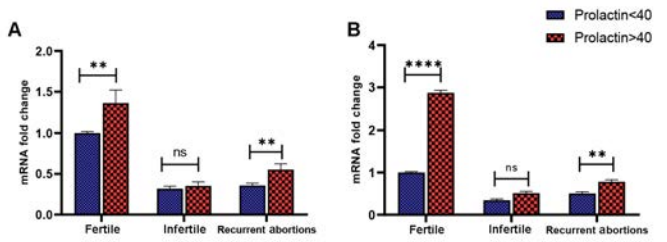

Fig.3: Changes in the expression levels of *CSF-1* and *CSF-1R* genes under the influence of prolactin hormone. Expression changes of the **A.** *CSF-1* and **B.** *CSF-1R* genes among the three study groups are shown based on the fold change. By increasing serum concentration of prolactin to >40 ng/mL, expression level of the *CSF-1R* gene was increased in the three groups compared to those with prolactin serum levels ≤40 ng/mL; although the differences were insignificant for infertile group. **; P<0.001, ****; P<0.00001, and ns; P>0.01.

Effect of different variables on *CSF-1* and *CSF-1R* expression levels in the study groups

Comparison of the subjects in the two age groups of ≤30 and >30 years showed significant differences in the *CSF-1* and *CSF-1R* gene expression levels of the fertile and RPL group. In comparison, there was no significant difference in the expression level of genes between the infertile subgroups. However in the age ≤30 of the fertile, infertile and RPL groups, expression level of the *CSF-1* gene in endometrial tissue was 1.34 (P=0.0096), 1.02 (P=0.736) and 1.23 (P=0.031) times higher than the age group >30 years. Expression level of the *CSF-1R* gene was higher 1.42 (P=0.0015), 1.33 (P=0.620) and 1.34 (P=0.031) times in the fertile, infertile and RPL groups, respectively.

Evaluation of the subjects in the BMI groups ≥25 and 25> kg/m^2 showed intragroup differences for the expression of the *CSF-1* and *CSF-1R* genes. In other words, expression level of the *CSF-1* and *CSF-1R* genes was decreased with increasing BMI. Comparison of the subjects with a BMI ≥25 kg/m^2 showed that *CSF-1* gene expression level was 1.58 (P=0.006), 1.25 (P=0.032) and 1.48 (P=0.049) times higher than the fertile, infertile and RPL groups, respectively. Additionally, comparison of the individuals with BMI ≥25 kg/m^2 showed that expression level of *CSF-1R* was 2 (P<0.0001), 1.20 (P=0.048) and 1.33 (P=0.034) times higher than the fertile, infertile and RPL groups, respectively.

Intragroup comparisons showed significant differences between diabetic and non-diabetic subjects in each group by evaluating the *CSF-1* and *CSF-1R* gene expression levels. Comparison of the diabetic subjects with non-diabetic individuals showed that *CSF-1* expression levels were decreased 2.22 (P=0.0004), 1.58 (P=0.0073) and 2.13 (P=0.0002) times in the fertile, infertile, and RPL groups respectively. Comparison for the *CSF-1R* expression levels were decreased 1.42 (P=0.0028), 1.33 (P=0.029) and 1.34 (P=0.019) times in the fertile, infertile and RPL groups, respectively.

Intragroup comparisons between smokers and non-smokers showed significant differences. Expression levels of *CSF-1* genes in smoker subjects were decreased 1.58 (P=0.017), 1.25 (P=0.045) and 1.49 (P=0.026) times in the fertile, infertile and RPL groups, respectively, compared to their counterparts with non-smokers. Expression levels of *CSF-R1* genes in the smoker subjects were 2 (P=0.039), 1.20 (P=0.025) and 1.33 (P=0.030) times lower than the non-smokers who counterparts in the fertility, infertility and RPL groups respectively.

Intragroup comparisons between gestational age ≤10 and >10 weeks showed no significant difference in the gene expression levels of the infertile and PRL groups. The fertile group with a history of miscarriage, less than ten weeks, was compared to those with a history of miscarriage more than ten weeks. CSF-1 and CSF-1R gene expression levels were increased 1.2 (P=0.002), and 1.16 (P=0.012) times, respectively. All data are shown in Figure 4.

Correlation coefficient of prolactin level with expression level of *CSF-1* and *CSF-1R* genes

Pearson correlation test for correlation between prolactin and *CSF-1* gene expression showed that the fertile group had a positive correlation (r=0.64, P=0.0017), the infertile group had no significant correlation (r=0.052, P=0.575) and the RPL group had statistically significant positive correlation (r=0.415, P=0.008). The same study, performed for the *CSF-1R* gene, showed that this correlation was only seen in the fertile group (r=0.452, P=0.015), but there was not any association between the infertile (r=0.098, P=0.285) and RPL groups (r=0.167, P=0.067).

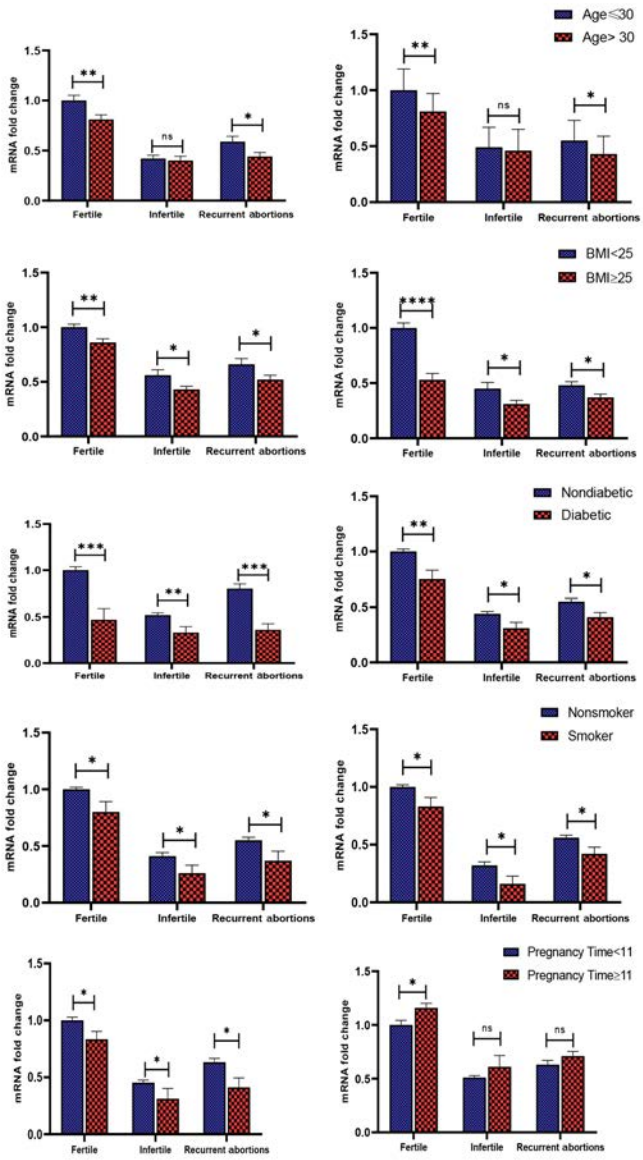

Fig.4: Changes in the expression levels of *CSF-1* and *CSF-1R* under the influence of different parameters. Effect of age, BMI, diabetes, smoking, gestational age and prolactin level on expression level of the studied genes was observed in the current study. BMI; Body mass index, *; P<0.01, **; P<0.001, ***; P<0.0001 and ns; P>0.01.

Discussion

Many factors can contribute to the success of IVF-ET in fertilization and embryo transfer. The main independent variables are age of the women, serum concentration of the anti-Mullerian hormone, number of the transferred embryos and their qualities. Some researchers showed that growth factors, hormones and cytokines, produced by macrophage cells, were involved in the implantation process (16).

During pregnancy, prolactin rises above the normal level of 10-25 ng/mL and reaches to a peak of 200-400 ng/mL within eight weeks of gestation (17). In the current study, mean prolactin serum concentration was determined in pregnancy losses at eight weeks of gestation as 41.84, 24.38 and 32.45 ng/mL for respectively the fertile, infertile and RPL groups; but in pregnancies losses <20 weeks of gestation, expression levels of *CSF-1* gene in subjects with prolactin serum concentration >40 ng/mL was increased 1.63, 1.01 and 1.36 times; additionally, expression levels of *CSF-1R* gene were 2.86, 1.07 and 1.54 times in the fertile, infertile and RPL groups respectively, compared to the individuals with prolactin serum levels ≤40 ng/mL indicating that prolactin serum concentration was increased by gestational age and it affects the embryo survival. Findings showed that increasing concentration of serum prolactin in this group was not sufficient for the survival of embryo. It also contributes to pregnancy loss. Moreover, investigating the role of prolactin biomarker confirmed its effect on fertility.

It is expected that maternal prolactin serum concentration is significantly elevated from 10 to 20 weeks of gestation (17). Although the highest and lowest average serum prolactin levels were significantly detected between the fertile and infertile groups, no significant elevation was determined between the fertile and PRL groups.

In pregnant women, elevated levels of *CSF-1* and *CSF-1R* expression were observed in the endometrial epithelium and fetal trophoblast, respectively. Studies showed that activation of trophoblast *CSF-1R* and increased level of local *CSF-1* expression were essential for the implantation of a normal fetus and placental development (18). Prolactin also affected endometrial tissue through the *CSF-1R* (10). In the studied subjects, expression level of *CSF-1* and *CSF-1R* genes in endometrial tissue, as well as the gene expression changes under the influence of prolactin concentration, were compared among the fertile, infertile and RPL groups. Expression level of the *CSF-1* and *CSF-1R* genes was higher in the endometrial tissue of the fertile group rather than the infertile ones. With increasing prolactin serum levels, expressions of *CSF-1* and *CSF-1R* were increased in the three groups. In the endometrial tissue of infertile group, expression levels of *CSF-1* and *CSF-1R* were lower than those of the fertile and RPL groups. Increased prolactin serum levels did not change expression of the *CSF-1* and *CSF-1R* genes in the infertile group. Increasing prolactin serum concentration caused increasing *CSF-1* and *CSF-1R* expression levels in the fertile women.

A recent study showed that CSF-1 expression level increased up to 1000 times during gestation in the endometrium of pregnant mice, due to its synthesis in the uterine lumen and glandular secretory epithelium controlled by the maternal endocrine hormones. Increasing local CSF-1 synthesis in the uterus was associated with proliferation and differentiation of cells. Additionally, association of CSF-1 receptor was determined between the uterus and placenta for implantation. CSF-1 also played role in regulating processes which are essential for implantation and preimplantation (19). A study, performed by Cai et al. (13), indicated that increasing M-CSF affected fetal development and increased trophectoderm (TE) cell count in mice.

Pregnancy loss risk factors are age, weight and general

health status of mother. Risk of spontaneous miscarriage was increased by increasing maternal age (20). In the present study, in addition to prolactin serum levels, other parameters, such as age, BMI, diabetes, smoking habits, and gestational age, CSF-1 and CSF-1R expression were evaluated in the all three groups. Intragroup comparisons showed significant difference between the subjects, aged > 30 and ≤30, in terms of gene expression expected the infertile group. But, expression level of the both genes was decreased by increasing age in the fertile women and those with RPL. Intragroup comparisons showed a significant difference between the subjects with BMI ≥25 and <25. Additionally, *CSF-1* and *CSF-1R* gene expressions were decreased in three groups by increasing BMI.

Intragroup comparisons showed a significant difference in the gene expression pattern between diabetic and non-diabetic subjects. However, expression level of the both genes was reduced in diabetic individuals. In addition, the difference was statistically significant. Intergroup comparisons showed significant differences between the smokers and non-smokers in gene expression levels. In terms of gestational age, expression of the both genes had a significant increase in the fertile women who lost their pregnancies at <20 weeks of gestation, but the infertile and PRL groups had no significant changes.

Conclusion

Overall, hormones, such as prolactin, are among the development factors of fetus, which can help fetal growth and successful continuation of pregnancy, through affecting the genes involved in conceptus–endometrial interactions. Decreased expression of *CSF-1* and *CSF-1R* can be considered as disruptive factors of implantation and fetal growth, leading to miscarriage in the studied groups. Factors, such as aging, increased BMI, smoking and diabetes, also caused decreasing changes in gene expression (*CSF-1* and *CSF-1R*). It seems that decreasing expression level of these genes disrupted conceptus-endometrium interaction, resulting in miscarriage. For future investigations, it is suggested to examine expression of the other genes involved in embryo implantation and their functional role in recurrent pregnancy loss. Additionally, discovery of the other molecular factors, involved in implantation, lead to increase in fertility success.

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Authors' Contributions

Z.T.F.; Designed and directed the project, planned the qRT-PCR method, data and statistical analysis, in addition to interpretation of data. Z.S.J.; Contributed to sample preparation, performed the experiments, and wrote the paper. All authors read and approved the final manuscript.

References

1. Hennessy M, Dennehy R, Meaney S, Linehan L, Devane D, Rice R, et al. Clinical practice guidelines for recurrent miscarriage in high-income countries: a systematic review. Reprod Biomed Online. 2021; 42(6): 1146-1171.
2. Hyde KJ, Schust DJ. Genetic considerations in recurrent pregnancy loss. Cold Spring Harb Perspect Med. 2015; 5(3): a023119.
3. Pei CZ, Kim YJ, Baek KH. Pathogenetic factors involved in recurrent pregnancy loss from multiple aspects. Obstet Gynecol Sci. 2019; 62(4): 212-223.
4. Freeman ME, Kanyicska B, Lerant A, Nagy G. Prolactin: structure, function, and regulation of secretion. Physiol Rev. 2000; 80(4): 1523-1631.
5. Kim YJ. Pivotal roles of prolactin and other hormones in lactogenesis and the nutritional composition of human milk. Clin Exp Pediatr. 2020; 63(8): 312-313.
6. Harris J, Stanford PM, Oakes SR, Ormandy CJ. Prolactin and the prolactin receptor: new targets of an old hormone. Ann Med. 2004; 36(6): 414-25.
7. Binart N, Bachelot A, Bouilly J. Impact of prolactin receptor isoforms on reproduction. Trends Endocrinol Metab. 2010; 21(6): 362-368.
8. Dobolyi A, Oláh S, Keller D, Kumari R, Fazekas EA, Csikós V, et al. Secretion and function of pituitary prolactin in evolutionary perspective. Front Neurosci. 2020; 14: 621.
9. Majumdar A, Mangal NS. Hyperprolactinemia. J Hum Reprod Sci. 2013; 6(3): 168-175.
10. Kamel A, Halim AA, Shehata M, AlFarra S, El-Faissal Y, Ramadan W, et al. Changes in serum prolactin level during intracytoplasmic sperm injection, and effect on clinical pregnancy rate: a prospective observational study. BMC Pregnancy Childbirth. 2018; 18(1): 141.
11. Thiriet M. Signaling at the cell surface in the circulatory and ventilatory systems. New York: Springer; 2012; 101-172.
12. Yen & Jaffe's Reproductive endocrinology: physiology, pathophysiology, and clinical management. Strauss JF, Barbieri R, editors. 6th ed. Elsevier Health Sciences: 2009; 220.
13. Cai L, Jeong YW, Jin YX, Lee JY, Jeong YI, Hwang KC, et al. Effects of human recombinant granulocyte-colony stimulating factor treatment during in vitro culture on porcine pre-implantation embryos. PLoS One. 2020; 15(3): e0230247.
14. Hamilton JA. GM-CSF in inflammation. J Exp Med. 2020; 217(1): e20190945.
15. Mierla D, Szmal C, Neagos D, Cretu R, Stoian V, Jardan D. Association of prothrombin (A20210G) and Factor V Leiden (A506G) with recurrent pregnancy loss. Maedica (Bucur). 2012; 7(3): 222-226.
16. Kunicki M, Łukaszuk K, Woclawek-Potocka I, Liss J, Kulwikowska P, Szczyptańska J. Evaluation of granulocyte colony-stimulating factor effects on treatment-resistant thin endometrium in women undergoing in vitro fertilization. Biomed Res Int. 2014; 2014: 913235.
17. Speroff L, Fritz MA. Clinical gynecologic endocrinology and infertility. 7th ed. Philadelphia: Lippincott Williams & Wilkins; 2012: 301.
18. Sapi E. The role of CSF-1 in normal physiology of mammary gland and breast cancer: an update. Exp Biol Med (Maywood). 2004; 229(1): 1-11.
19. Furth Rv. Mononuclear phagocytes: biology of monocytes and macrophages. Berlin: Springer Science & Business Media; 2013; 13-17.
20. Iqbal Z, Jilanee SDA, Uppada LP, Imtiaz S, Khan H, Shah SMH, et al. Evaluating the clinical risk factors associated with miscarriages in women in Karachi, Pakistan. Cureus. 2021; 13(10): e19057.

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The Effect of Altered *Mucin1*, *FGF2*, and *HBEGF* Gene Expression at The Ectopic Implantation Site and Endometrial Tissues in The Tubal Pregnancy Pathogenesis: A Case-Control Study

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Abstract

Background: Ectopic pregnancy (EP) is defined as implantation and development of an embryo outside of the uterine tissue. Women undergoing assisted reproductive technologies (ART), particularly frozen embryo transfer (FET), are in high-risk populations for EP. *Mucin1* (*MUC1*), fibroblast growth factor-2 (*FGF2*), and Heparin-binding epidermal growth factor (*HBEGF*) genes are involved in the endometrial receptivity pathway, leading to normal eutopic implantation; Although, their relevance in the tubal pregnancy after FET is unknown. We aimed evaluation of *Mucin1*, *FGF2*, and *HBEGF* expression fold as endometrial receptive markers in the EP patients following the FET cycle.

Materials and Methods: A case-control study was conducted on ten patients (five EP patients and five women in the pseudo-pregnancy group, as the control samples). Pseudo-pregnancy group was established in women who were candidates for hysterectomy for benign diseases. Fallopian tube biopsies and corresponding endometrial tissues from these patients were taken during the hysterectomy. However, the fallopian tube and endometrial tissues of EP patients were obtained during salpingectomy. The mRNA expressions of *MUC1*, *FGF2*, and *HBEGF* genes in the fallopian tube and endometrial tissues were measured by real-time polymerase chain reaction (PCR) assay.

Results: *MUC1* mRNA expression level in the endometrium of the case group was higher than in the control group (P=0.04); however, its mRNA expression in the fallopian samples of the case group in comparison with the control group was significantly decreased (P=0.001). The *HBEGF* mRNA expression level was not significantly different between the case and control endometrium, whereas its expression was significantly increased in the case fallopian samples compared with the control ones (P=0.001). The same pattern was observed for *FGF2* mRNA expression level in the fallopian samples of the case group but was significantly reduced in the endometrial samples in comparison with the control samples (P=0.03).

Conclusion: *MUC1*, *FGF2*, and *HBEGF* gene mRNA expression changes may explain the embryo rejection from the uterus and the establishment of a receptive phenotype in fallopian cells.

Keywords: Ectopic Pregnancy, *FGF2*, Frozen Embryo Transfer, *HBEGF*, *Mucin1*

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Introduction

An embryo moving in the fallopian tube to the uterus implantation site and also a suitable milieu for the embryo during this short lifetime of development in the tubal environment, is necessary to achieve a successful embryo implantation (1). The interactions and paracrine signaling network among the tubal epithelium, smooth muscle, and immune cells coordinate pivotal functions for successful later embryo implantation (1, 2). Any alterations in these interactions create a different environment that may influence an embryo tubular implantation (3).

A tubal pregnancy is defined as an embryo implantation within the fallopian tube, which is more prevalent, more than 90 %, type of the ectopic pregnancy (EP) (4). On the other hand, women undergoing assisted reproductive technologies (ART), particularly frozen embryo transfer (FET) procedures, are in high-risk populations for EP, in such a way that in an ART pregnancy, the increasing

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rate of an EP incidence, more than 8.6%, was reported in comparison with the actual non-ART pregnancy (natural conception) (5).

To prepare for embryo implantation, a considerable changes occur in the composition of the apical plasma membrane of the uterine epithelium. Among these changes, the dynamic expression of the genes involved in the endometrial receptivity pathway such as Mucin 1 (*MUC1*), fibroblast growth factor 2 (*FGF2*), and Heparin-binding epidermal growth factor (*HBEGF*) are previously proved (6). While the MUC1 gene plays an anti-binding role during implantation *FGF2* and *HBEGF* genes mediate blastocyst adhesion to the endometrial cells (7, 8).

Furthermore, substantial studies have demonstrated that a molecular basis that includes abnormal gene and protein expression, as well as the aberrant function and structure of the endometrial and fallopian tissues, are among the primary causes of EP (9, 10). Although, the functions of the *MUC1*, *HBEGF* and *FGF2* genes are well-established in the normal eutopic pregnancy, their relevance in the tubal pregnancy after FET is unknown. In the case of a tubal pregnancy, it has been suggested that signals emerging from the fallopian cells can compete with the uterus signals and attract the embryo toward misplaced implantation (11). A greater understanding of the functions and structure of fallopian tube epithelial cells, can lead to knowledge about protective mechanisms against the ectopic implantation that made our aim of the present study.

Materials and Methods

This case-control study was approved by the Research Ethics Committee of the Royan Institute, Tehran, Iran (IR.ACECR.ROYAN.REC.1399.091) and the Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.RETECH.REC.1398.490) and informed written consent was obtained prior to the collection of tissue samples from each patient.

Study design and participants

Ten patients who had referred to the Royan Infertility Clinic, Tehran, Iran, from April 2021 to February 2022 were invited to participate in the present study. Volunteers who included in the study met the following criteria:

Inclusion criteria for all participants were aged between 30-40 years, normal body mass index (BMI) according to World Health Organization (WHO) categories ($18.5 \leq BMI < 25$ kg/m^2) (12, 13), and having the history of the regular menstrual cycle. Exclusion criteria include a history of pelvic inflammatory disease (PID) such as Gonorrhea, Chlamydia and/or salpingitis, fallopian tube problems such as adhesion or hydrosalpinx, history of tubal ligation, heterotopic pregnancy, receiving methotrexate, the record of using intrauterine devices such as intrauterine device (IUD), a history of the EP, a history of endometriosis, uterine abnormalities, myoma, hyperplasia, polycystic ovary syndrome (PCOS) and thyroid disease.

We categorized our participants in two equal case and control groups (n=5).

Ectopic pregnancy group

Five fallopian tube tissues were collected from women in the case group diagnosed with an ectopic embryo implantation in the ampulla of the fallopian tube during salpingectomy. All of them were in the 6 to 8 weeks of their gestational age. All participants in this group conceived after embryo transfer after freeze embryo transfer cycle and were taking exogenous progesterone (P4, Progestin®, Aburaihan Pharmaceutical Co, Tehran, Iran) and estradiol (E2) (Aburaihan Pharmaceutical Co, Tehran, Iran) for endometrial preparation and continued both hormones as luteal phase support (14). Each fallopian tube sample containing an embryo and a gestational sac that is defined as a tubal pregnancy was removed from the ampoule, about 10 mm distance from the gestational sac was dissected from the specimen during surgery.

Pseudo-pregnancy group (as the control group)

Ethical constraints prohibited the use of the fallopian tissue from women bearing a viable embryo. Therefore, patients who were candidates for hysterectomy for benign diseases were requested to receive human chorionic gonadotrophin (hCG, 5000 IU, Pregnyl; Organon, Norway), in the mid-luteal phase. This method makes hormonal conditions that are identical to those found in normal pregnancies. This protocol known as a pseudo-pregnancy that has previously been used by other research groups (7, 15). Fallopian tube biopsies from the ampulla and corresponding endometrial tissues of these patients were taken during hysterectomy.

Tissue samples processing, RNA extraction and cDNA synthesis

Biopsy specimens of the ampulla and endometrium tissues were directly placed in the RNAlater solution (AM7020, Ambion, Austin, TX, US), then were freezed by the snap freeze method (samples were rapidly frozen by placing tissues in liquid nitrogen for ten seconds) and finally, stored at -80°C until RNA extraction.

The extraction of RNA was carried out according to the manufacturer's protocol. (Trizol, Cat No: 15596026, Invitrogen, USA). Using DNase I (Fermentas, Cat No: E00381, Sanktleon-rot, Germany), probable genomic DNA contamination, was eliminated of the extracted RNA. The complementary DNA (cDNA) was produced using the first strand cDNA synthesis kit (Cat No: k1632, Thermo Scientific™ RevertAid™, Lithuania) as directed by manufacturer.

Relative expression levels of each target gene were normalized by the beta-actin gene (*β-actin*, as the housekeeping or internal control gene) (Table 1).

Table 1: Primer sequences used for real-time polymerase analysis

Gene	Forward primer (5´-3´)	Annealing temperature (°C)	Product size (bp)
MUC1	F: CAGCCTCTCTTACACAAACCCA R: AGAACCTGAGTGGAGTGGAATG	60	122
HBEGF	F: CATCCCCACAATCTGGCTTAGT R: ACCCCTACATCCTGACCATACA	60	157
FGF2	F: CTGTACATTTTTGGGGTCAGCTG R: CCAGCATTTCGGTGTTGAAGAA	60	167
β-actin	F: CAAGATCATTGCTCCTCCTG R: ATCCACATCTGCTGGAAGG	60	90

In a final reaction volume of 20 µL, each Real time polymerase chain reaction (PCR) reaction sample contained 5 µL of SYBR Green PCR Master Mix (Cat No: RR820L, Takara, China,), 11 µL of dH_2O (Cat No: W4502, Sigma-aldrich®,. Life Technologies TM), 1 µL of each forward and reverse primer (Metabion, Martinsried, Germany), and 2 µL of single-strand cDNA. Real time PCR conditions are described as following: initial denaturation (one cycle at 95°C for 10 minutes), followed by 40 cycles of denaturation (95°C for 10 seconds), annealing (60°C for 60 seconds, depends on the primer) and extension (72°C for 30 seconds), and a final extension (one cycle at 72°C for 10 minutes).

The relative standard curve and $2^{-\Delta\Delta Ct}$ techniques were used to determine the expression level of each target gene (16).

Statistical analysis

The IBM SPSS statistics 21 program was used to perform the statistical computations (IBM Corp., Armonk, NY). The normal distribution of the values was analyzed by the Student's t test with a two-tailed distribution. The non-normal distributions were examined using the Mann-Whitney non-parametric test ($P \leq 0.05$). The Levene's test for equality of variances were performed. The level of significance was set at $P \leq 0.05$.

Results

Demographic information

There were no statistically significant differences between the patients with the EP group and the control group in terms of patients' age (37.00 ± 2.34 vs. 38.80 ± 0.83) and BMI (23.90 ± 0.95 vs. 24.32 ± 0.44), respectively. The other demographic information is summarized in Table 2.

Table 2: Demographic information of patients in the control and ectopic pregnancy groups

Variable	Control group	EP group	P value
Age (Y)	37.00 ± 2.34	38.80 ± 0.83	0.14
BMI (kg/m^2)	23.90 ± 0.95	24.32 ± 0.44	0.39
hCG dose (IU)	3.8 ± 0.83	-	NA
Gestational age (Y)	-	6.2 ± 0.83	NA
Number of transferred embryos	-	2.0 ± 0.7	NA

Values are reported as means ± standard deviations (SD). BMI; Body mass index, hCG; Human chorionic gonadotrophin, EP; Ectopic pregnancy, and NA; Comparison not applicable.

Genes expression at the level of mRNA in endometrium and fallopian samples of patients with ectopic pregnancy and control groups

The RNA expression level of *MUC1* in the endometrium of the case group (0.33 ± 0.06) was significantly higher than the control group (0.05 ± 0.002, P=0.04); in contrast, its expression in fallopian samples was significantly decreased (0.068 ± 0.01 vs. 3.04 ± 0.8, for case and control group, respectively) (P=0.001, Fig.1).

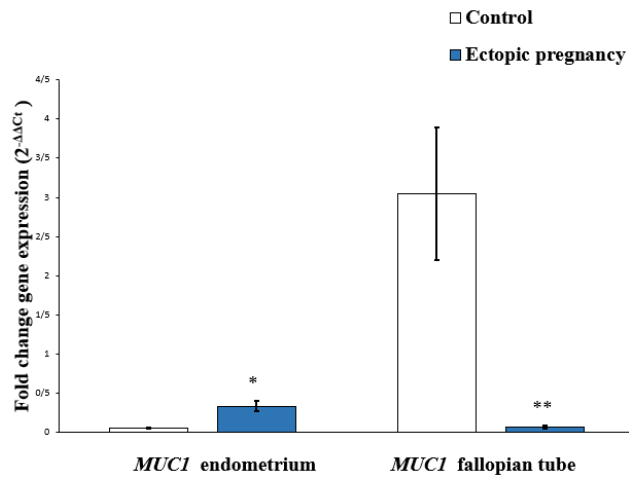

Fig.1: *MUC1* mRNA expression in endometrium and fallopian samples. *; P<0.05 and **; P<0.01.

As shown in Figure 2, the level of mRNA expression of *HBEGF* in the endometrium of our case group was not significantly different from the control group (0.33 ± 0.03 vs. 0.30 ± 0.03, respectively) (P=0.6), whereas its expression was significantly increased in the fallopian samples of the case group in comparison with the control group (1.46 ± 0.28 vs. 0. 61 ± 0.06, respectively) (P=0.001). The same pattern was observed for *FGF2* mRNA expression level in the fallopian samples of case and control groups, (3.68 ± 0.77 vs. 2.01 ± 0.75 respectively) (P=0.04, Fig.3); however, its expression was significantly decreased in the EP endometrial samples of the case group (1.24 ± 0.05) in comparison with the control group (1.87 ± 0.04, P=0.03).

Protein-protein interaction analysis using the STRING database showed that *MUC1*, *FGF2*, and *HBEGF* have a related signaling pathway (Fig.4).

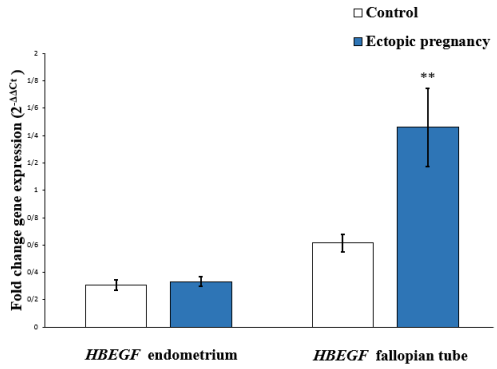

Fig.2: *HBEGF* mRNA expression in endometrium and fallopian. **; P<0.01.

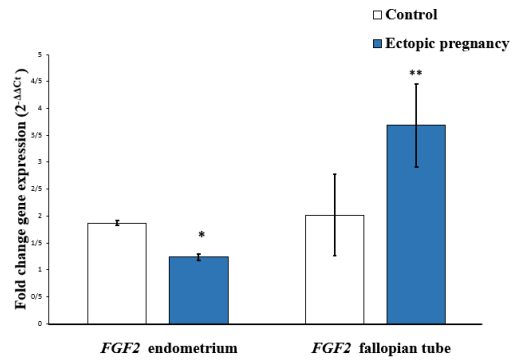

Fig.3: *FGF2* mRNA expression in endometrium and fallopian samples. *; P<0.05 and **; P<0.01.

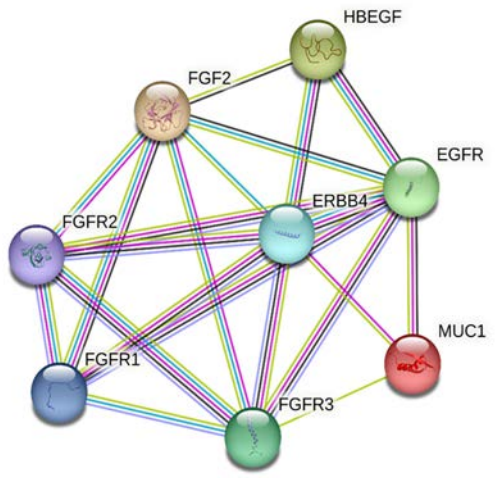

Fig. 4: Molecular interactions of three analyzed factors: FGF2, HBEGF, and MUC1. STRING database protein-protein interaction analysis.

Discussion

Different mRNA expression levels of *MUC1*, *HBEGF* and *FGF2* genes were observed in the fallopian and endometrium samples of our case group in comparison with our control group.

There are different molecular factors that lead to a tubal pregnancy, although it may directly occur due to an impaired embryo transfer in the uterine environment (17). The risk factors of an ectopic pregnancy have remained uncertain, and the etiology and molecular mechanisms behind its higher incidence in fresh/FET cycles following assisted reproductive technology are unspecified as well.

An increase mRNA expression level of *MUC1* in the fallopian tubes in comparison with the endometrium provides a gradient signal to prevent implantation in an ectopic site in normal pregnancy (18), it may be because of its anti-adhesive action. Therefore, the down-regulation of *MUC1* mRNA expression in the endometrial luminal epithelium and during the window of implantation has a role in establishing normal eutopic implantation. The same mechanism occurs in the epithelium of the fallopian tube, in which the reduction in the *MUC1* mRNA expression level facilitates an embryo attachment. In the current study, *MUC1* mRNA expression level is downregulated in the fallopian tissues, consistent with other research (7, 18); however, there was an increased mRNA expression level in the endometrial samples. It seems this alteration in the endometrial *MUC1* mRNA expression level contributes to the suboptimal embryo-endometrial dialogue and also, leads to the rejection of the embryo from the uterus. Interestingly, *in vitro* study suggested that the human embryo promotes the reduction in the protein expression of *MUC1* to facilitate endometrial cell attachment (19). It is possible that an alter capability of some embryos after a freeze-thawing procedure leads to the decrease of *MUC1* mRNA expression in the endometrium and guides the embryo to the other direction (e.g., fallopian tube).

On the other hand, both *FGF2* and *HBEGF* genes are important factors for the endometrial remodeling and trophoblast adhesion improvement (20). The *HBEGF gene*, that is expressed in the glandular and luminal epithelium of the endometrium and fallopian tube, plays a role in the decidualization process, consequently during the embryo implantation process (21). Interestingly, FGF2 and HBEGF protein expressed at the surface of endometrial epithelial cells mediates the process of blastocyst binding to the endometrium (22).

The FGF2 protein plays an important role in the regulation of cell survival, cell division, angiogenesis, and cell differentiation (23). The FGF2 protein is produced by human endometrial epithelial cells, while its in vitro protein expression level is regulated by the recombinant hCG hormone supplementation, also provides the endometrial receptivity enhancement (24).

The human blastocysts with high implantation potential regulate the expression level of genes that are involved in the endometrial receptivity through the secretion of regulatory molecules (22).

Endometrial selectivity and receptivity are two words that describe the endometrium's function as a biosensor of embryo quality. Selectivity is an endometrium-programmed feature that recognizes and rejects embryos with inadequate developmental ability. On the other hand, the receptive phenotype allows the endometrium to provide an ideal milieu for the embryo growth (25). A poor-quality embryo may be rejected by the endometrium, however, the fallopian tube's epithelium is unable to accomplish the same that results in an ectopic implantation. Changes in the expression patterns of genes

involved in the endometrial reception or in the biology of the fallopian tube have a potential role in underlying causes of EP (26, 27).

Some elements, including tubal damages, the type of embryo transfer technique, multiple embryos in an ET cycle, and a high volume of the transfer medium, could have an adverse effect on an IVF-ET procedure success (28). Although, these risk factors do not explain how and why the embryo passes through the uterus and enters and implants in the fallopian tube. Several studies on the ectopic implantation of the embryo have found that leukemia inhibitory factor (LIF), *HOXA10*, and *MUC1* gene expression were significantly changed at the site of EP (29). Our investigation found changes in both the endometrium and the fallopian tissues, which is consistent with previous research (20, 29). However, our investigations are unable to distinguish between cause and consequence.

Surprisingly, *MUC1*, *FGF2*, and *HBEGF* genes are targets of the hormones, P4 and E2, and also, their expressions are affected by these hormones (8, 19, 30-34). Both P4 and E2 are master regulators of uterine receptivity. During the frozen embryo transfer cycle, that endometrial preparation with exogenous steroids, P4 and E2, and the embryo is developed and the luteal phase is supported. The optimal doses for luteal phase support are essential to provide a suitable physiological hormone level. It is shown that elevated E2 and P4 levels are risk factors for EP following FET cycles. Because of its impact on uterine contractions and tubal movements, the extra-oral administration of E2 between two phases, ovarian puncture and embryo transfer, may increase the EP occurrence rate (35). On the other side, the P4 hormone may lead to an EP occurrence which may cause a malfunction in the fallopian tube ciliary (36, 37). Therefore, defective responses to hormonal therapy may be an EP index model that suggests further studies.

There were some drawbacks in our study. The gestational age in patients with EP lasted longer than the time we were able to create a condition of pseudo-pregnancy with repeated hCG injections. We considered that prolonging the pseudo-pregnant condition and, as a result, delaying elective surgery for a longer period would be unethical. Because truly ethical disqualification decisions that prohibit the fallopian tissue biopsy from those women having viable embryos, we used a pseudo-pregnancy model which could potentially influence our results. There are only limited reports of this model (7, 15, 38), we consider future research also to be needed to improve our understanding.

Conclusion

It seems, an altered expression of *MUC1*, *FGF2*, and *HBEGF* genes may underpin an embryo rejection from the uterus and induce a receptive phenotype in the fallopian epithelial cells. Understanding the molecular mechanisms of an embryo implantation site, ectopic and eutopic is essential. Further study of high doses of P4 and E2 for luteal phase support in FET cycles, to develop effective prevention strategies against ectopic implantation.

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Authors' Contributions

N.N.; Writing manuscript. F.Gh., F.K., R.A; Provided concept, Method of study, and Supervision. F.Gh., Z.Ch.; Patient recruitment. F.Gh.; Performed laparoscopic biopsies. N.N., S.A., Z.Ch.; Performed laboratory examinations. N.N., A.R., S.A., A.N., Z.Ch.; Formal analysis and Investigation. F.Gh., F.K., Z.Ch.; Writing-Reviewing and Editing manuscript. All authors read and approved the final manuscript.

References

1. Shaw JL, Horne AW. The paracrinology of tubal ectopic pregnancy. Mol Cell Endocrinol. 2012; 358(2): 216-222.
2. Aghajanpour S, Hosseini E, Amirchaghmaghi E, Zandieh Z, Amjadi F, Yahyaei A, et al. Differential expression of innate/adaptive immunity genes induced by endometrial scratching as a hopeful approach for implantation boosting in unexplained, repeated implantation failure: An RCT. J Reprod Immunol. 2021; 148: 103426.
3. Tong S, Skubisz MM, Horne AW. Molecular diagnostics and therapeutics for ectopic pregnancy. Mol Hum Reprod. 2015; 21(2): 126-135.
4. Nama V, Manyonda I. Tubal ectopic pregnancy: diagnosis and management. Arch Gynecol Obstet. 2009; 279(4): 443-453.
5. Clayton HB, Schieve LA, Peterson HB, Jamieson DJ, Reynolds MA, Wright VC. Ectopic pregnancy risk with assisted reproductive technology procedures. Obstet Gynecol. 2006; 107(3): 595-604.
6. Berger C, Boggavarapu NR, Menezes J, Lalitkumar PG, Gemzell-Danielsson K. Effects of ulipristal acetate on human embryo attachment and endometrial cell gene expression in an in vitro co-culture system. Hum Reprod. 2015; 30(4): 800-811.
7. Al-Azemi M, Refaat B, Aplin J, Ledger W. The expression of MUC1 in human fallopian tube during the menstrual cycle and in ectopic pregnancy. Hum Reprod. 2009; 24(10): 2582-2587.
8. Wang H, Shi G, Li M, Fan H, Ma H, Sheng L. Correlation of IL-1 and HB-EGF with endometrial receptivity. Exp Ther Med. 2018; 16(6): 5130-5136.
9. Cai W, Yang L, Zhang R, Yang Y, Li S, Zhang J. Abnormally increased DNA methylation in chorionic tissue might play an important role in development of ectopic pregnancy. Reprod Biol Endocrinol. 2021; 19(1): 101.
10. Duncan WC, Shaw JL, Burgess S, McDonald SE, Critchley HO, Horne AW. Ectopic pregnancy as a model to identify endometrial genes and signaling pathways important in decidualization and regulated by local trophoblast. PLoS One. 2011; 6(8): e23595.
11. Jia-Rong Z, Shuang-Di L, Xiao-Ping W. Eutopic or ectopic pregnancy: a competition between signals derived from the endometrium and the fallopian tube for blastocyst implantation. Placenta. 2009; 30(10): 835-839.
12. WHO Consultation on Obesity and World Health Organization. Obesity: preventing and managing the global epidemic: report of a WHO consultation. World Health Organization. 2000; 894: i-xii, 1-253.
13. WHO Expert Consultation. Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. Lancet. 2004; 363(9403): 157-163.
14. Ghaffari F, Chekini Z, Vesali S. Duration of estradiol supplementa-

tion in luteal phase support for frozen embryo transfer in hormone replacement treatment cycles: a randomized, controlled phase III trial. Arch Gynecol Obstet. 2022; 305(3): 767-775.
15. Refaat B, Amer S, Ola B, Chapman N, Ledger W. The expression of activin-betaA- and -betaB-subunits, follistatin, and activin type II receptors in fallopian tubes bearing an ectopic pregnancy. J Clin Endocrinol Metab. 2008; 93(1): 293-299.
16. Nikmard F, Hosseini E, Bakhtiyari M, Ashrafi M, Amidi F, Aflatoonian R. The boosting effects of melatonin on the expression of related genes to oocyte maturation and antioxidant pathways: a polycystic ovary syndrome- mouse model. J Ovarian Res. 2022; 15(1): 11.
17. Jiang H, Yang XY, Zhu WJ. Networks of E-cadherin, β1 integrin, and focal adhesion kinase in the pathogenesis of tubal pregnancy. Gynecol Endocrinol. 2019; 35(4): 346-350.
18. Brito LR, Guedes Neto Ede P, Furich DG, Savaris RF. MUC1 (VPM654 and EPR1023) expression in mucosa of fallopian tubes with ectopic pregnancy is altered. Appl Immunohistochem Mol Morphol. 2016; 24(8): 569-574.
19. Kubota K, Miwa M, Hayashi KG, Hosoe M, Sakatani M. Steroidal but not embryonic regulation of mucin 1 expression in bovine endometrium. J Reprod Dev. 2021; 67(6): 386-391.
20. de Ruijter-Villani M, van Boxtel PR, Stout TA. Fibroblast growth factor-2 expression in the preimplantation equine conceptus and endometrium of pregnant and cyclic mares. Theriogenology. 2013; 80(9): 979-989.
21. Chobotova K, Karpovich N, Carver J, Manek S, Gullick WJ, Barlow DH, et al. Heparin-binding epidermal growth factor and its receptors mediate decidualization and potentiate survival of human endometrial stromal cells. J Clin Endocrinol Metab. 2005; 90(2): 913-919.
22. Eivazi S, Tanhaye Kalate Sabz F, Amiri S, Zandieh Z, Bakhtiyari M, Rashidi M, et al. MiRNAs secreted by human blastocysts could be potential gene expression regulators during implantation. Mol Biol Rep. 2023; 50(2): 1375-1383.
23. Akl MR, Nagpal P, Ayoub NM, Tai B, Prabhu SA, Capac CM, et al. Molecular and clinical significance of fibroblast growth factor 2 (FGF2 /bFGF) in malignancies of solid and hematological cancers for personalized therapies. Oncotarget. 2016; 7(28): 44735-44762.
24. Paiva P, Hannan NJ, Hincks C, Meehan KL, Pruysers E, Dimitriadis E, et al. Human chorionic gonadotrophin regulates FGF2 and other cytokines produced by human endometrial epithelial cells, providing a mechanism for enhancing endometrial receptivity. Hum Reprod. 2011; 26(5): 1153-1162.
25. Macklon NS, Brosens JJ. The human endometrium as a sensor of embryo quality. Biol Reprod. 2014; 91(4): 98.
26. Feng Y, Zou S, Weijdegård B, Chen J, Cong Q, Fernandez-Rodriguez J, et al. The onset of human ectopic pregnancy demonstrates a differential expression of miRNAs and their cognate targets in the Fallopian tube. Int J Clin Exp Pathol. 2013; 7(1): 64-79.
27. Wang L, Feng Y, Zou S, Brännström M, He L, Billig H, et al. Linking DNA methylation to the onset of human tubal ectopic pregnancy. Am J Transl Res. 2013; 5(2): 116-125.
28. Rombauts L, McMaster R, Motteram C, Fernando S. Risk of ectopic pregnancy is linked to endometrial thickness in a retrospective cohort study of 8120 assisted reproduction technology cycles. Hum Reprod. 2015; 30(12): 2846-2852.
29. Bianchi E, Sun Y, Almansa-Ordonez A, Woods M, Goulding D, Martinez-Martin N, et al. Control of oviductal fluid flow by the G-protein coupled receptor Adgrd1 is essential for murine embryo transit. Nat Commun. 2021; 12(1): 1251.
30. Horne AW, Lalani EN, Margara RA, White JO. The effects of sex steroid hormones and interleukin-1-beta on MUC1 expression in endometrial epithelial cell lines. Reproduction. 2006; 131(4): 733-742.
31. Leach RE, Khalifa R, Armant DR, Brudney A, Das SK, Dey SK, et al. Heparin-binding EGF-like growth factor modulation by antiprogestin and CG in the baboon (Papio anubis). J Clin Endocrinol Metab. 2001; 86(9): 4520-4528.
32. Xie H, Wang H, Tranguch S, Iwamoto R, Mekada E, Demayo FJ, et al. Maternal heparin-binding-EGF deficiency limits pregnancy success in mice. Proc Natl Acad Sci USA. 2007; 104(46): 18315-18320.
33. Michael DD, Alvarez IM, Ocón OM, Powell AM, Talbot NC, Johnson SE, et al. Fibroblast growth factor-2 is expressed by the bovine uterus and stimulates interferon-tau production in bovine trophectoderm. Endocrinology. 2006; 147(7): 3571-3579.
34. Chung D, Gao F, Jegga AG, Das SK. Estrogen mediated epithelial proliferation in the uterus is directed by stromal Fgf10 and Bmp8a. Mol Cell Endocrinol. 2015; 400: 48-60.
35. Weiss A, Beck-Fruchter R, Golan J, Lavee M, Geslevich Y, Shalev E. Ectopic pregnancy risk factors for ART patients undergoing the GnRH antagonist protocol: a retrospective study. Reprod Biol Endocrinol. 2016; 14: 12.
36. Paltieli Y, Eibschitz I, Ziskind G, Ohel G, Silbermann M, Weichselbaum A. High progesterone levels and ciliary dysfunction--a possible cause of ectopic pregnancy. J Assist Reprod Genet. 2000; 17(2): 103-106.
37. Bylander A, Nutu M, Wellander R, Goksör M, Billig H, Larsson DG. Rapid effects of progesterone on ciliary beat frequency in the mouse fallopian tube. Reprod Biol Endocrinol. 2010; 8: 48.
38. Refaat B, Ledger W. The expression of activins, their type II receptors and follistatin in human Fallopian tube during the menstrual cycle and in pseudo-pregnancy. Hum Reprod. 2011; 26(12): 3346-3354.

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Evaluation of Microbial Profile in Patients with Polycystic Ovary Syndrome and Periodontal Disease: A Case-Control Study

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Abstract

Background: Polycystic ovary syndrome (PCOS) and oral health are found to share a reciprocal link. Previous substantiating evidences suggest that PCOS may have a confounding effect on periodontal health and may quantitatively modify the composition of the oral microbiome. To analyze the role of PCOS as a risk factor in causing periodontal disease, we compared and evaluated the levels of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in patients with polycystic ovary syndrome, polycystic ovary syndrome, chronic periodontitis, polycystic ovary syndrome, and gingivitis, and healthy controls.

Materials and Methods: In this case-control study, 40 female participants are enrolled and grouped into four groups which included healthy female individuals, patients with PCOS, patients with PCOS and gingivitis, and patients with PCOS and periodontitis. Periodontal examination is assessed primarily on all the participants using a UNC-15 probe. Dental plaque is then collected using a sterile curette in one stroke and transferred into an Eppendorf tube containing TE Buffer (Tris-EDTA buffer) solution. The level of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* was estimated by real-time polymerase chain reaction (PCR).

Results: The levels of *Fusobacterium nucleatum* were observed to be significantly higher in group with patients with PCOS and periodontitis.

Conclusion: PCOS may have an impact on the composition of oral microflora causing repercussions in periodontal health.

Keywords: Dental Plaque, Periodontitis, Polycystic Ovary Syndrome

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Introduction

Women of reproductive age may be affected by the complex, heterogeneous endocrine disorder known as polycystic ovary syndrome (PCOS) (1). The primary features of this condition encompass chronic anovulation, menstrual problems, and clinical or biochemical signs of hyperandrogenism (2). The Rotterdam criterion defines PCOS patients as those female patients with the presence of at least two of the following: polycystic ovaries, oligomenorrhea, and/or anovulation, hyperandrogenism (clinical and/or biochemical). According to the PCOS Consensus Workshop Group, the prevalence rate of PCOS has dispersed globally, ranging from 9.13 to 36% in India (3).

Periodontitis on the other hand is considered as a condition distinguished by inflammation of the supporting tissues of the teeth. The strong interplay between putative periodontal pathogens and host inflammatory response is attributed to the subsequent destruction of supporting tissue and tooth loss. The endocrine system has a significant impact on the homeostasis of the periodontium which is maintained through complex multifactorial relationships. The estrogen receptor (ER) and a progesterone receptor (PgR), present in the periodontium indicate that the periodontium is one of the target tissues for these hormones (4). The direct effects of estrogens were mediated through two distinct subtypes of receptors, ERα and Erβ (5). The ERα receptor is predominantly present in the different

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tissues such as the endometrium, ovaries, and mammary glands, whereas the ERβ receptor is present in the non-reproductive tissues, including periodontal ligament (6), gingival epithelium (7), and salivary glands (8). This explains the reason that changes in the amounts of circulating steroid hormones can affect periodontal tissues. Changes that take place in the level of sex hormones during different stages of females are observed to directly or indirectly affect oral health, subsequently influencing a person's susceptibility to periodontal disease. PCOS is defined by alteration in the level of sex hormone, where the progesterone levels remain low and the levels of estrogen and male androgenic hormones go high. The association of PCOS with an increase in the levels of inflammatory markers like C reactive protein, pro-inflammatory cytokines like interleukin-6, tumor necrosis factor-α (TNF-α), and chemokines, demonstrate this low-grade systemic inflammation (9).

On the other hand, periodontal diseases, like gingivitis and periodontitis, are common prevalent chronic infectious diseases. Such diseases are predominantly brought in by a bacterial plaque that grows in the subgingival area and are also found to increase systemic and local inflammatory markers such as TNF-α, C reactive protein, and interluekin-6 (IL-6) which is attributed to the destruction of periodontal tissue (10). Insulin resistance which is a prominent characteristic of PCOS is linked to low-grade inflammation through inflammatory cytokines like TNF-α, IL-1, and IL-6 and their signaling pathways (11). On the contrary, hormonal level alterations in PCOS are found to affect the salivary levels of potential periodontal pathogens and/or their systemic immune responses in the presence of gingival inflammation. This might be accounted to the deposition of active progesterone and oestrogen hormones, in the periodontal tissues' which supply the vital nutrients needed for the growth of the bacteria. Since insulin resistance is one of the main symptoms of the PCOS, periodontal organisms in subgingival plaque produce lipopolysaccharides that can cause considerable production of IL-1 and TNF-α. The insulin resistance can get exacerbated upon persistent elevation of cytokines which can ultimately in turn increase the condition of PCOS (12). Meanwhile, few studies showed an elevated oxidative stress level and alterations in the oral microbial flora of PCOS patients (12, 13). Evaluating the link between periodontal disease and PCOS, poor periodontal parameters were seen in patients with PCOS. While studies reported higher severity of periodontal disease in PCOS patients, hence it is mandatory to undergo periodontal examination and therapy periodically (14).

Hormonal disorders such as PCOS and oral health was shown to share a reciprocal link with each other. PCOS was found to bring about a repercussion in periodontal health by influencing the composition of the oral microflora. But there exists a paucity of literature evaluating change brought in by hormonal disorders, such as PCOS on the diversity of oral microbiota. The correlation between oral microbiota in dental plaque with the gingival inflammation in presence of PCOS is not been established to date. Therefore, this study aims to analyse the role of PCOS as a risk factor in causing periodontal disease.

Materials and Methods

Ethical guidelines

The present case-control study protocol was conducted in full accordance and approval of the Institutional Ethics Committee, SRIHER with the protocol number CSP/20/JAN/83/51. The nature of the study was discussed with patients orally and written informed consent was acquired from each patient prior to the initiation of the study.

Study population and study design

This case-control study was performed between December 2019 to March 2020 and included patients attending the Department of Gynaecology and Obstetrics and Department of Periodontology, Sri Ramachandra Institute of higher education and research. Forty female patients between the age of 18 to 40 years were recruited for the study. During screening, patients who fulfilled the inclusion criteria were provided with an informed consent form and were recruited into the study. The inclusion criteria for the PCOS group was based on the criteria by Rotterdam (2). The control group was healthy female individuals without PCOS and periodontitis. Patients in the periodontitis group were classified according to the definition of Eke et al. (15). Patients in the gingivitis group were classified according to the American Academy of Periodontology 1998. Patients with other medical conditions and those who had been under medications that could affect their periodontal status for the last 6 months were excluded from the study.

Using a one-way ANOVA test with a power of 0.95, alpha of 0.05 with an effect size of 19.25, a sample of 40 patients including the dropouts (10 members in each group) was calculated. Four groups were included in the study which include healthy female individuals, patients with PCOS, patients with PCOS and gingivitis and patients with PCOS and periodontitis.

Each patient's clinical periodontal evaluation, such as probing depth (PD), plaque index (PI), and bleeding on probing, was recorded after enrolment. Apart from third molars, with the help of Williams periodontal probe (Hu-Friedy, Chicago, IL) six sites of each tooth (mesiobuccal, mid-buccal, distobuccal, mesio-lingual, mid-lingual, and disto-lingual locations) were measured clinically. If bleeding on probing (BOP) happened within 15 seconds of applying the probe, it was considered positive. A single calibrated examiner conducted after the clinical periodontal measures. Subsequently, the periodontal inflammatory surface area for each patient was calculated.

Subgingival dental plaque sample collection

The tooth with the deepest PD was dried and isolated using cotton rolls. The subgingival plaque was collected by

inserting a sterile Gracey 5/6 curette 2 mm (Hue Friedy, Switzerland) into the gingival pocket followed by a pull stroke three subsequent times without evoking bleeding. The strokes had to sufficient force to remove plaque while no attempt was made to remove cementum from the root surface.

The Gracey 5/6 curette (Hue Friedy, Switzerland) was chosen to standardize as it permits the most effective method of placing the curette inside the transport tube. Immediately upon collection of the samples, the curettes were shaken into a transport tube with 200 µl of transport solution (Tris-Ethylenediaminetetraacetic acid buffer solution) (Himedia, Mumbai, India). The samples were then sealed and sent immediately to the testing facility for quantification of the periodontal pathogen *P. gingivalis* and *F. nucleatum*.

Sample processing

Following the transfer of the sample to the tube consisting of Tris-Ethylenediaminetetraacetic acid buffer (T.E.buffer) containing tris base powder and Ethylenediaminetetraacetic acid (Himedia, Mumbai, India). It was centrifuged (Model SLM-MCF-10K, Biobee tech, Bengaluru, India) at 5,000 rpm for 5 minutes at room temperature. After discarding the supernatant, 500 µl of fresh T.E. buffer was added again and 3 minutes of centrifugation at 5000 rpm was done. The same procedure was subsequently repeated 3 times with new T.E. buffer.

DNA extraction procedure

The sample processing was followed by DNA extraction by using modified proteinase-k method (16). After discarding the supernatant, 50 µl of lysis buffer I containing 1% Triton X-100, Tris-HCL pH=8.0, 10 mM, and EDTA 1 mM (Himedia, Mumbai, India) was added, vortexed, and allowed to stand for 5 minutes. Then, 10 µl of proteinase-K (100 µg/ml, Genei laboratories pvt. ltd, Bengaluru, India) and 50 microliters of Lysis buffer II contain Tris-HCl, pH=8.0, 50 mM, KCl, 50 mM, $MgCl_2$, 2.5 mM, Tween-20, 0.45%, and Nonidet P-40, 0.45% (Himedia, Mumbai, India) were added, and the mixture was vigorously vortexed. The samples are then held in a 60°C for two hours and then transferred to a boiling water bath for 10 minutes. Following this, the samples were centrifuged at 10,000 rpm for 3 minutes, and the DNA-containing supernatant is then collected in a new tube. The DNA was purified by using 3 M sodium acetate and absolute ethanol and reconstituted in 100 µl water. For PCR analysis, the DNA was kept at -200°C.

Bacterial quantification by quantitative real-time polymerase chain reaction

The qPCR reactions were used to determine the presence and levels of two different oral taxa in the subgingival plaque samples. Using an Eppendorf Real plex master cycler, real-time qPCR amplification and detection reactions were carried out in a 96-well format. Also, an 8 pm/µl of each P. gingivalis and F. nucleatum-specific primers were used in a total volume of 25 µl for the PCR reactions, along with 2 µl of template DNA and 12.5 µl of TB Green Premix Ex Taq (Tli RNaseH Plus) (Takara Bio inc., Kusatsu, Japan). All species-specific primers used in the qPCR reaction are given in Table 1 (17, 18). The reaction mixture was prepared in 0.2 ml qPCR tubes and kept in a thermal cycler (Realplex Master cycler, Eppendorf, Hamburg, Germany). qPCR reaction conditions were 95°C for 3 minutes, and 35 cycles of 95°C for 20 seconds and 60°C for 30 seconds, and 72°C for 30 seconds. Plotting the standard graph required running serial dilutions of DNA isolated (same procedure as described above) from the standard strains of P. gingivalis ATCC No. 33277 (Known quantity, (10^8 to 10^3 CFU/ml) and *F. nucleatum* ATCC No. 25886 (Known quantity, 10^8 to 10^3 CFU/ml). Deionised water served as a negative control. The amplification curves were obtained with fluorescent probes. Cycle threshold values (CT value) were obtained for DNA samples of known quantity (standards) and standard curve was plotted. Ct values of unknown samples were obtained from real time PCR runs. Then these CT values were plotted on the standard curve to get the corresponding quantity for unknown samples.

Statistical analysis

Statistical analysis was done using SPSS version 3.1.9.2 statistical software (International Business Machines Cooperation, Chicago). The data distribution was assessed by the Shapiro-Wilk normality test. After dividing the entire data below and above the median, Kruskal-Walli's test was used to compare all groups for non-normally distributed data (bacterial counts). For normally distributed variables (Periodontal Epithelial Surface Area & Periodontal Inflammatory Surface Area), the ordinary one-way ANOVA test was used. Statistical significance was set at $P \leq 0.05$ with a 95% level of confidence in the statistical analyses, which were carried out using the statistical program (SPSS version 3.1.9.2; International Business Machines Cooperation, Chicago).

Table 1: Species-specific primers

Organisms	Primer sequence (5´-3´)	Author (References)
Porphyromonas. gingivalis	F: AGGCAGCTTGCCATACTG CG R: ACTGTTAGCAACTACCGATGT	Kugaji et al. (17)
Fusobacterium nucleatum	F: GAAGAAACAAATGACGGT AACAAC R: GTCATCCCCACCTTCCTCCT	Yamaura et al .2005 (18)

Results

Demographic data

Full-mouth clinical assessments and demographic data were documented (Table 2). For all four groups, bleeding in probing (BOP), periodontal pocket depth (PPD), and clinical attachment loss (CAL) were determined. It was observed that the BOP, PPD, and CAL levels were significantly higher in the PCOS with periodontitis group.

Primary analysis results

Bacterial count analysis in plaque samples

The microbiological diversity of the dental plaque was assessed using qPCR. *Porphyromonas gingivalis* and *Fusobacterium nucleatum* bacteria were quantified. Although there was a difference in the level of *Porphyromonas gingivalis* among the four groups, the results were not statistically significant. Quantification of the level of *Fusobacterium nucleatum* showed an ascending growth pattern of the healthy group to group with PCOS and periodontitis (Table 2). On intergroup comparison in the levels of Fusobacterium nucleatum, a statistically significant difference was obtained between the healthy female individuals and patients with PCOS and periodontitis (P=0.04, Fig.1).

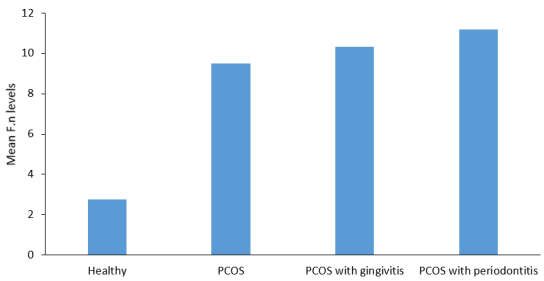

Fig.1: Subgingival *Fusobacterium nucleatum* levels. PCOS; Polycystic ovary syndrome.

Secondary results analysis

The periodontal epithelial surface area (PESA) accurately measures the surface area of the pocket epithelium, whereas periodontal inflammatory surface area (PISA) measures the amount of the inflamed periodontal tissue and as such assess the systemic inflammatory burden. A Microsoft Excel spreadsheet was developed to help with the estimation of Periodontal Inflammatory Surface Area and Periodontal Epithelial Surface Area using the formulas published by Hujoel et al. (20). PESA and PISA values were found to be statistically significant among the four groups (P=0.00). Among the intergroup comparison of periodontal inflammatory surface area scores, a significant difference was noted between healthy female individuals and patients with PCOS patients with gingivitis and periodontitis (P=0.00, Fig.2, Table 3).

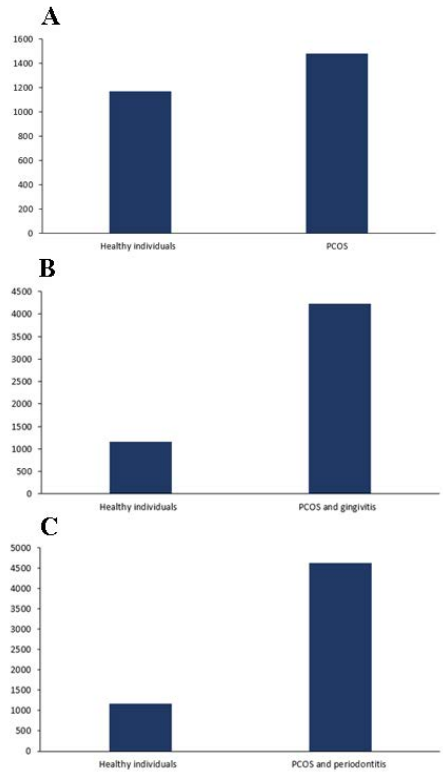

Fig.2: Intergroup PISA score comparison. **A.** PISA scores between healthy individuals and PCOS patients, **B.** PISA scores between healthy individuals and PCOS and gingivitis patients, and PISA scores between healthy individuals and PCOS and periodontitis patients. PISA; Periodontal inflammatory surface area and PCOS; Polycystic ovary syndrome.

Table 2: Demographic data and periodontal parameters

Demographic data	Healthy	PCOS	PCOS with gingivitis	PCOS with periodontitis
No of participants	10	10	10	10
Age (Y)	22.3 ± 3.3	22.9 ± 2.1	22.4 ± 3.1	27.6 ±3.4
Mean BOP	0.953 ± 0.33	1.055 ± 0.46	2.338 ± 1.65	2.647 ± 1.88
PPD (site specific)	0.59 ± 0.37	0.341 ± 0.53	3.861 ± 0.90	5.812 ± 0.91
CAL (site-specific)	1.211 ± 0.57	1.082 ± 0.57	3.431 ± 1.65	3.92 ± 1.75
MEAN PISA	116.590 ± 56.3	147.740 ± 60.49	423.320 ± 79.49	462.940 ± 84.5
MEAN PESA	818.010 ± 59.6	827.010 ± 80.1	941.180 ± 180.3	1090.840 ± 205.96

All data are based on mean ± SD. PCOS; Polycystic ovary syndrome, BOP; Bleeding on probing, PPD; Periodontal probing depth, CAL; Clinical attachment loss, PISA; Periodontal inflammatory surface area, and PESA; Periodontal Epithelial surface area calculation.

Table 3: Intergroup PISA score comparison

PESA and PISA scores	Sum of squares	df	Mean square	Sig.
PESA score (mm^2)				0.000
Between groups	485162.621	3	61720.874	
Within groups	132595.910	36	3683.220	
Total	617758.531	39		
PISA score (mm^2)				0.000
Between groups	979692.647	3	326564.216	
Within groups	17882.933	36	496.748	
Total	997575.580	39		

PISA; Periodontal inflammatory surface area and PESA; Periodontal epithelial surface area.

Discussion

The PCOS is an endocrine condition caused by a disruption in the hypothalamus pituitary axis, which results in abnormal gonadotropin secretion by the hypothalamus. This abnormal secretion of the gonadotropin hormone leads to an increased luteinizing hormone (LH) levels and normal or low follicle-stimulating hormone (FSH) levels (20). Bacterial plaque that causes periodontal diseases results in local and systemic elevations in levels of TNF-α, C reactive protein, and IL-6. These factors subsequently result in the destruction of periodontal tissue. Both, PCOS and periodontal disease, share chronic low-grade inflammation as a common risk factor. The existing studies reported that in comparison to healthy controls, patients with PCOS suffered from greater rates of BOP, periodontal probing depth (PPD), and CAL (21, 22).

Studies have also shown that periodontal disease may cause systemic inflammation and oxidative stress, both of which can aggravate insulin resistance. This suggests that there may be a bidirectional association between PCOS and PD (23). On the other hand, female sex hormones are found to affect the composition of oral microbiota in conditions like puberty, mensuration, and pregnancy. Also, studies confirm the increase in the levels of *Prevotella intermedia* in the subgingival plaque due to an increased plasma female sex hormones and alteration of the immune response (24, 25). There are limited data on PCOS effects on putative periodontal pathogens, which formed the principle objective of the current study.

Our findings suggest that PCOS has an amplifying effect on the levels of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* and their connection with gingival inflammation. Thus, the change in the microbiota in patients with PCOS could play a pivotal role in the propagation of gingival inflammation. Akcali et al. (12) observed an increase in the salivary levels of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* in PCOS patients when compared to healthy controls. Active progesterone and oestrogen was found to supply crucial nutrients needed for bacterial growth. In light of the accumulation of these hormones in periodontal tissues, the author concluded that hormonal alterations in PCOS may influence the salivary levels of putative periodontal pathogens and/or their systemic antibody responses. This is especially true in the presence of gingival inflammation (26).

Our results were in agreement with the study conducted by Porwal et al. (27) that observed an increase in the BOP in the newly diagnosed PCOS group when compared to the healthy group. These results indicate the impact of PCOS on the gingival inflammation.

Young female individuals between 18 to 35 years of age are generally not prone to periodontitis (28), but the results of our present study showed a comparatively high level of putative periodontal pathogens in PCOS patients which subsequently may lead to an increase in the inflammatory burden. This points us the fact that the presence of PCOS in young female individuals can predispose them to periodontal diseases. Hence, PCOS can be considered a viable risk factor in the initiation and progression of periodontal diseases in the young healthy female individuals. To our knowledge, this is the first study to assess the levels of suspected periodontal pathogens in dental plaque samples from non-obese women with PCOS. The limitations of the present study include a small sample size and site-specific based assessment of oral microbiota. Insulin resistance of patients wasn't considered in the present study and thereby could be a confounding factor. Future longitudinal studies with larger sample size, are needed to evaluate the influence of PCOS on the subgingival microbial environment and also, to investigate probable relationships between PCOS and periodontal disease.

Conclusion

The persistent polymicrobial challenge to the local host tissues often results in the progressive destruction of soft and hard tissues which is considered as the hallmark of periodontitis. Pathogenic consortium which causes these responses include the red complex bacteria such as *P. gingivalis*, *T. denticola* and *T. forsythia*. Additionally, higher concentration of possible periodontopathogens such as *Fusobacterium nucleatum*, *Prevotella species*, *Eikenella corrodens*, *Peptostreptococcus micros*, and *Campylobacter rectus* is found in deeper periodontal pockets. Oral health is often subjected to direct and indirect effects of the fluctuating levels of sex hormones during conditions like PCOS which can influence the

constituents of the oral microflora causing reverberation in periodontal health. Amongst the studied taxa in the present study, higher levels of *Fusobacterium. nucelatum* and *Porphyromonas. gingivalis* were observed in the subgingival plaque samples in patients with PCOS and periodontitis and patients with PCOS and gingivitis when compared to healthy individuals. Therefore, within the limitations of the study, it can be concluded that PCOS may quantitatively affect the constituents of oral microflora playing a pivotal role in subsequent gingival inflammation and periodontal health.

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Authors' Contributions

R.A.J.; Methodology, Software, Validation, Formal Analysis, Investigation, Resources, Data curation, Writing Original draft, Review, and Editing. S.A., M.S.; Conceptualization, Methodology, Validation, Writing, Review, Editing, Validation, Supervision, and Project administration. S.K.B.; Writing, Review, Editing, Validation, Supervision, and Project administration. All authors read and approved the final manuscript.

References

1. Legro RS, Arslanian SA, Ehrmann DA, Hoeger KM, Murad MH, Pasquali R, et al. Diagnosis and treatment of polycystic ovary syndrome: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2013; 98(12): 4565-4592.
2. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril. 2004; 81(1): 19-25.
3. Nidhi R, Padmalatha V, Nagarathna R, Amritanshu R. Prevalence of polycystic ovarian syndrome in Indian adolescents. J Pediatr Adolesc Gynecol. 2011; 24(4): 223-227.
4. Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. Sequence and expression of human estrogen receptor complementary DNA. Science. 1986; 231(4742): 1150-1154.
5. Mamalis A, Markopoulou C, Lagou A, Vrotsos I. Oestrogen regulates proliferation, osteoblastic differentiation, collagen synthesis and periostin gene expression in human periodontal ligament cells through oestrogen receptor beta. Arch Oral Biol. 2011; 56(5): 446-455.
6. Nebel D, Bratthall G, Ekblad E, Norderyd O, Nilsson BO. Estrogen regulates DNA synthesis in human gingival epithelial cells displaying strong estrogen receptor β immunoreactivity. J Periodontal Res. 2011; 46(5): 622-628.
7. Välimaa H, Savolainen S, Soukka T, Silvoniemi P, Mäkelä S, Kujari H, et al. Estrogen receptor-beta is the predominant estrogen receptor subtype in human oral epithelium and salivary glands. J Endocrinol. 2004; 180(1): 55-62.
8. Jönsson D, Andersson G, Ekblad E, Liang M, Bratthall G, Nilsson BO. Immunocytochemical demonstration of estrogen receptor beta in human periodontal ligament cells. Arch Oral Biol. 2004; 49(1): 85-88.
9. Kelly CC, Lyall H, Petrie JR, Gould GW, Connell JM, Sattar N. Low grade chronic inflammation in women with polycystic ovarian syndrome. J Clin Endocrinol Metab. 2001; 86(6): 2453-2455.
10. Ozçaka O, Nalbantsoy A, Buduneli N. Interleukin-17 and interleukin-18 levels in saliva and plasma of patients with chronic periodontitis. J Periodontal Res. 2011; 46(5): 592-598.
11. Tanguturi SC, Nagarakanti S. Polycystic ovary syndrome and periodontal disease: underlying links- a review. Indian J Endocrinol Metab. 2018; 22(2): 267-273.
12. Akcalı A, Bostanci N, Özçaka Ö, Öztürk-Ceyhan B, Gümüş P, Buduneli N, et al. Association between polycystic ovary syndrome, oral microbiota and systemic antibody responses. PLoS One. 2014; 9(9): e108074.
13. Saglam E, Canakci CF, Sebin SO, Saruhan N, Ingec M, Canakci H, et al. Evaluation of oxidative status in patients with chronic periodontitis and polycystic ovary syndrome: a cross-sectional study. J Periodontol. 2018; 89(1): 76-84.
14. Farook FF, Ng KT, MNM N, Koh WJ, Teoh WY. Association of periodontal disease and polycystic ovarian syndrome: a systematic review and meta-analysis with trial sequential analysis. Open Dent J. 2020; 13(1): 478-487.
15. Eke PI, Page RC, Wei L, Thornton-Evans G, Genco RJ. Update of the case definitions for population-based surveillance of periodontitis. J Periodontol. 2012; 83(12): 1449-1454.
16. Kugaji MS, Bhat KG, Joshi VM, Pujar M, Mavani PT. Simplified method of detection of Dialister invisus and Olsenella uli in oral cavity samples by polymerase chain reaction. J Adv Oral Res. 2017; 8(1-2): 47-52.
17. Kugaji MS, Muddapur UM, Bhat KG, Joshi VM, Kumbar VM, Peram MR, et al. Quantitative evaluation of porphyromonas gingivalis in indian subjects with chronic periodontitis by real-time polymerase chain reaction. J Adv Oral Res. 2019; 10(2): 137-144.
18. Yamaura M, Sato T, Echigo S, Takahashi N. Quantification and detection of bacteria from postoperative maxillary cyst by polymerase chain reaction. Oral Microbiol Immunol. 2005; 20(6): 333-338.
19. Hujoel PP, White BA, García RI, Listgarten MA. The dentogingival epithelial surface area revisited. J Periodontal Res. 2001; 36(1): 48-55.
20. D'Ercole S, Piccolomini R, Capaldo G, Catamo G, Perinetti G, Guida L. Effectiveness of ultrasonic instruments in the therapy of severe periodontitis: a comparative clinical-microbiological assessment with curettes. New Microbiol. 2006; 29(2): 101-110.
21. Dikilitaş A, Karaaslan F, Yig˘It U. The association between oral hygiene behavior and gingival health status with the stage and grade of periodontitis: a cross-sectional study. J Adv Oral Res. 2020; 11(2): 156-164.
22. Mariotti AJ. Estrogen and extracellular matrix influence human gingival fibroblast proliferation and protein production. J Periodontol. 2005; 76(8): 1391-1397.
23. Dursun E, Akalın FA, Güncü GN, Çınar N, Aksoy DY, Tözüm TF, et al. Periodontal disease in polycystic ovary syndrome. Fertil Steril. 2011; 95(1): 320-323.
24. Rahiminejad ME, Moaddab A, Zaryoun H, Rabiee S, Moaddab A, Khodadoustan A. Comparison of prevalence of periodontal disease in women with polycystic ovary syndrome and healthy controls. Dent Res J (Isfahan). 2015; 12(6): 507-512.
25. Abraham-Inpijn L, Polsacheva OV, Raber-Durlacher JE. The significance of endocrine factors and microorganisms in the development of gingivitis in pregnant women. Stomatologiia (Mosk). 1996; 75(3): 15-18. Russian.
26. Tanguturi SC, Nagarakanti S. Polycystic ovary syndrome and periodontal disease: underlying links- a review. Indian J Endocrinol Metab. 2018; 22(2): 267-273.
27. Porwal S, Tewari S, Sharma RK, Singhal SR, Narula SC. Periodontal status and high-sensitivity C-reactive protein levels in polycystic ovary syndrome with and without medical treatment. J Periodontol. 2014; 85(10): 1380-1389.
28. Shiau HJ, Reynolds MA. Sex differences in destructive periodontal disease: a systematic review. J Periodontol. 2010; 81(10): 1379-1389.

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Impacts of COVID-19 Pandemic on Three IVF Clinics of Jakarta, Indonesia: A Retrospective Qualitative and Quantitative Study

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Abstract

Background: Corona virus disease-19 (COVID-19) pandemic also led to a reduction or even the suspension of elective health services. These decisions affected *in vitro* fertilization (IVF) programs worldwide. Therefore, it is essential to map the readiness of IVF clinics in providing safety in this situation and in the future.

Materials and Methods: This is a retrospective qualitative and quantitative research done in 2021 that involved three IVF clinics of Jakarta, Indonesia. Those three clinics were government-owned, private-owned, and educational and training center. The qualitative data of each clinic's readiness towards COVID-19 was obtained from interviews with the clinics staff. The quantitative data were collected from the clinics patients' number and demographic data from 2019-2021 as well as from COVID-19 databases. Both data sets were analysed descriptively and only for the quantitative analysis Stata version 16 was used.

Results: There were changes in the domiciles and number of patients attending the three clinics. The ratio of patients from Jakarta increased while patients from outside Java Island decreased. There was a drop in annual patient numbers in 2020. However, from June 2020 to December 2021, the number of monthly IVF cycles increased significantly by 3.5 cycles per month (P=0.001). There was no association between IVF patients' attendance numbers and COVID-19 cases (P=0.785). One of the clinics had a negative pressure operating theatre, which made them more confident in treating patients with COVID-19 positive and made them even had higher IVF cycles started than the pre-pandemic period.

Conclusion: Those three clinics are prepared in facing COVID-19, as they complied with government regulations. As the COVID-19 pandemic progressed, the number of patients gradually returned to normal.

Keywords: COVID-19, Indonesia, *In Vitro* Fertilization

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Introduction

Since March 11th, 2020, the World Health Organisation (WHO) declared the Corona virus disease 2019 (COVID-19) a pandemic, healthcare endured significant changes, such as cancelling or postponing non-essential treatments (1, 2) that lead to consequences like a decrease in non-COVID-19 patients' quality of care (3, 4).

This situation has also impacted elective Gynaecological and *in vitro* fertilization (IVF) procedures (5). Also, the Indonesian Society of Obstetrics and Gynaecology, Perkumpulan Obstetri dan Ginekologi Indonesia, (POGI) released a recommendation which recommends any fertility treatment to be postponed. This recommendation was to minimise the risk of infection of COVID-19 (6, 7). However, delay in starting infertility treatment may cause a decrease in the success rate (8).

In Indonesia, along with the nationwide lockdown, there were regulations that suspended elective medical procedures, including infertility treatment. Therefore, infertile couples were affected in many aspects, including psychological (9). However, the relationship between the effects of the COVID-19 pandemic on the number and demographic of IVF patients has not been studied. This study aims to map any changes in patient numbers and demographics attending three different IVF clinics, before and after COVID-19, and clinic preparedness to face the pandemic situation.

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Materials and Methods

Ethical declaration for this study was approved by the University of Indonesia Medical Research Ethics Committee (MREC) in December 2021 (KET-1175/UN2. F1/ETIK/PPM/00/02/2021) and the Monash University Human Research Ethics Committee (MUHREC) (32625).

This research uses sequential exploratory mixed-methods study that included both qualitative and quantitative studies. The participants in this research were clinic management and staffs that were agreed to attended in this study. To map the clinic's preparedness in facing the COVID-19 pandemic, several questions were asked by the management concerning the changes in infectious disease precautions before and after the pandemic, such as its preventive measures, the clinic's sanitation regulations, and staff regulations. We used a semi-structured interview to obtain the primary data. The secondary data are monthly patient number and demographics were obtained from the patients' medical record from January 2019 to December 2022.

Results

This study was conducted from January 2019 to December 2021 at three clinics contains 2570 patients demographic and treatment data. Cohort characteristics are presented in Table 1.

Table 1: Characteristics of *in vitro* fertilization (IVF) Patients' Attendances from three clinics (n=2570)

Characteristics	n (%)
Age (Y)	
<35	1150 (45)
35-37	573 (22)
38-40	422 (16)
41-42	234 (9)
>42	191 (7)
Treatment	
Fresh embryo	1684 (66)
Frozen embryo transfer	886 (34)
Domicile	
Jakarta	1205 (47)
Greater Jakarta	787 (31)
Java Island	163 (6)
Outside Java	415 (16)

The ratio of patients from Jakarta increased during the COVID-19 pandemic, from 43 to 49%. In contrast, patients living outside Jakarta, including the patients from the rest of Java Island and outside of it, decreased in proportion, as can be seen in Figure 1.

Figure 2 presents an overview of the monthly numbers of patients undergoing IVF cycles between 2019 and 2021 in all three clinics, along with events affecting the number of IVF cycles. The events or factors that affected the number of IVF cycles started were ranging from travel restrictions to the Jakarta and two waves of COVID-19, with green and pink shaded areas on the graph. Due to COVID-19 early cases and national holiday combined, the travels were restricted in the 2020, while only national holiday restricted 2021 travels.

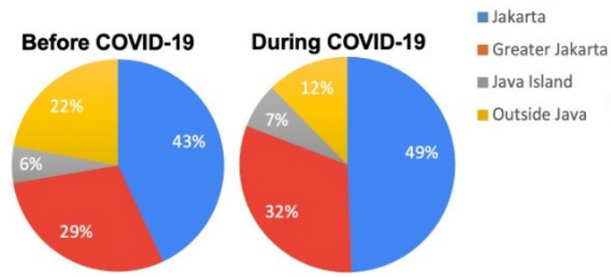

Fig.1: *In vitro* fertilization (IVF) patient origins proportion from overall three surveyed clinics.

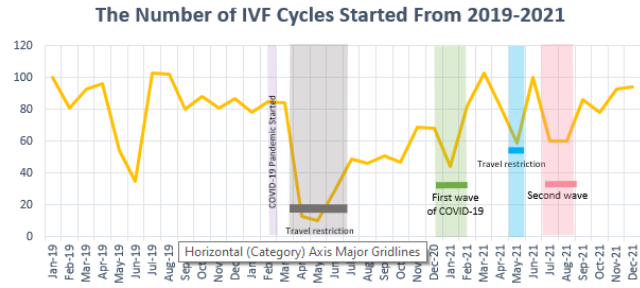

Fig.2: Monthly numbers of patients' attendance from 2019 to 2021 in all three *in vitro* fertilization (IVF) Clinics.

Using Stata version 16, the impact of the COVID-19 pandemic on the number of patients per month was analysed. The starting number of IVF cycles was 84 per month and the total number of IVF cycles appeared to be similar before March 2020 (-0.1, 95% CI −1.5 to 1.3, P=0.859). In the first month of the COVID-19 pandemic in Indonesia, which is March 2020, there was appeared to be a significant decrease in IVF cycles (-53.0, 95% CI −70.3 to -35.7, P<0.001), followed by a significant increase in the monthly trend of IVF cycles (relative to the pre-intervention trend) of 3.5 IVF cycles per month (95% CI 1.7 to 5.4, P=0.001), which is illustrated in Figure 3.

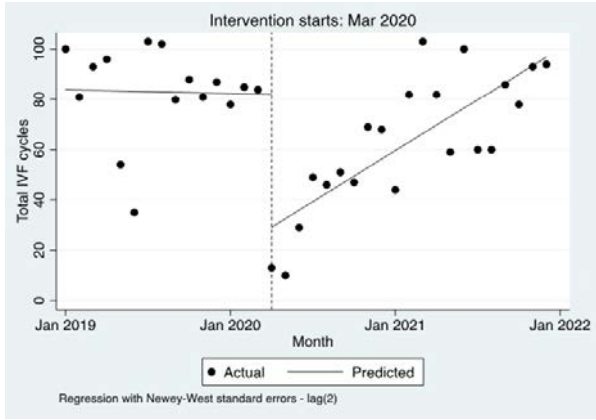

Fig.3: The impact of COVID-19 on total *in vitro* fertilization (IVF) cycles in all three hospitals, tested with regression with Newey-West standard errors.

A remarkably high number of IVF cycles done from January to March 2020, followed by a noticeable drop in April and almost no patients in May 2020. This drop was mainly caused by POGI's regulations to cancel the elective procedures, including IVF services. This regulation was relaxed in July 2020. In April 2021, there was a decrease in monthly IVF cycles, as there were the first and second wave peaks of COVID-19 cases in January 2021 and July-August 2021, respectively. All three clinics shared similar trends throughout the pandemic in 2020 and 2021. Overall clinics IVF patient number and COVID-19 cases comparison is illustrated in Figure 4.

Based on the linear regression result in the statistical software Stata, there was no association (P=0.785) between the monthly national COVID-19 cases and the monthly number of IVF cycles. There was also no association (P=0.640) between monthly Jakarta COVID-19 cases and the monthly number of IVF patients.

The emergence of COVID-19 forced the hospital to postpone or cancel various elective medical procedures, including IVF treatments and clinics. After two years of the pandemic, there were changes in numerous clinical and non-clinical protocols due to COVID-19. All different preparedness aspects from the interviews with clinics staffs are summarised in Table 2.

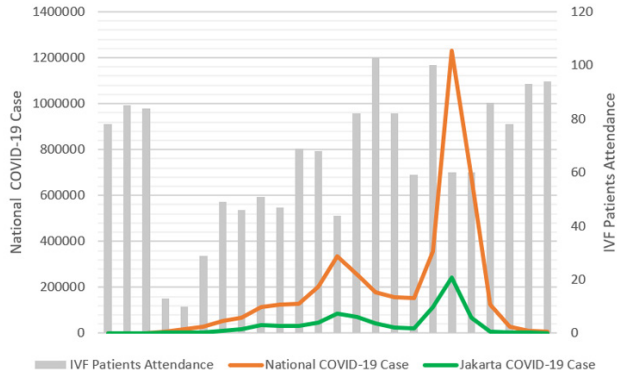

Fig.4: Monthly COVID-19 cases and monthly *in vitro* fertilization (IVF) cycle started in all three clinics.

Table 2: Summary of clinic preparedness in facing COVID-19

	Teratai	Yasmin	Melati
Preventive measures			
High-efficiency particulate absorbing (HEPA) filter air purifier	✓	✓	✓
Negative pressure OR	×	×	✓
Personal protective equipment is worn by staff	✓	✓	✓
COVID-19 testing for patients	✓	✓	✓
COVID-19 testing for staff	✓	✓	✓
Timetabling to reduce clinic traffic	✓	✓	✓
Partner restrictions to appointments	×	✓	✓
Keep safe distance	✓	✓	✓
Vaccine requirement to enter	✓	✓	✓
Prior to treatment COVID-19 exposure questionnaire	✓	✓	✓
Provide teleconsultation	✓	✓	✓
Sanitation regulation			
Operating Room is cleaned by the same staff	×	✓	✓
Electronic devices are covered with plastics	✓	✓	✓
Disinfected routinely	✓	✓	✓
Frequency	UV light exposure every day and disinfectant after every patient	×	After every patient
Regulations for non-medical and medical staff			
Hand washing	✓	✓	✓
Steps of wearing personal protective equipment	✓	✓	✓
Personal protective equipment level 1, 2	✓	✓	✓
Personal protective equipment level 3 (how to wear, how to take it off, and disposal)	✓	×	×
Individual meal breaks	✓	×	✓
Travelling staff monitoring	✓	×	×

Discussion

COVID-19 pandemic also led to a reduction or even the suspension of elective health services, including IVF treatment worldwide. IVF treatment, as well as its patient number, is highly affected by multiple factors, such as mobility and IVF clinics' readiness.

The increase of patients from Jakarta and Greater Jakarta was a consequence of mobility/ transport restrictions from the government. While the entry and exit points to and from Jakarta were blocked, the desire of IVF patients from outside Jakarta to travel to Jakarta was affected (10).

Based on the Indonesian Association for *In vitro* Fertilization (IAIVF) website registry, there was a gradual increase in the IVF cycle number from 2016 to 2019. This survey found that the annual patient numbers for three clinics decreased during 2020, the first year of the pandemic. This decrease might have been caused by POGI's regulations that restricted the elective procedures from May to July 2020. However, in 2021, the patient numbers had increased gradually, yet still lower compared to in 2019. Exceptionally, Melati IVF Clinic had a higher patient number in 2021 than the pre-pandemic period. Although, our results of these three clinics showed a lower patient number in comparison with the period before the pandemic period started, was contrary to a study done by Cutting et al. (11). They found an increase in patient load for IVF treatment after the lockdown period in the Indonesia by 25-50%. This contradiction may be because of considering only one clinic and mainly its location. Correlating with the lower desire of patients to travel to Jakarta, we assumed that patients prefer transferring to their nearer IVF clinics. Hence, there were IVF clinics outside Jakarta to have increased patient load, although IAIVF website registry data shows that most IVF clinics had declined trend during 2020 and 2021.

A study in Finland showed that there was an average 8% increase in waiting times of elective treatments in 2020, from 85.8 days before the pandemic to 92.6 days, which eventually led to decrease the patient number (12). This decreased in the patient load also happened worldwide, including in Indonesia (13). The result showed patients reduction in 2020, which indicates there were some procedural cancellations that led to delays in IVF treatment.

All three clinics recorded the lowest number of IVF cycles started in May for three years in a row, even before the pandemic occurred. This is because during the last three years, Ramadhan falls in May. Ramadhan is a sacred month for Muslims. During this month, Muslims are fasting from dusk till dawn, ranging from 12 to 19 hours (14). Almost 80% of the Indonesian population are Muslim and they tend to delay any IVF treatment during that period. Although, there is no significant effect found in the foetal growth, but the patients often feel dehydrated and uncomfortable to undergo any medical procedures (15). Those studies (16, 17) indicated there are some conditions, such as feeling dehydrated, and tendency to focus on praying that make patients have to postpone their treatment until after Ramadhan.

After POGI relaxed the elective treatment prohibition in July 2020, IVF patients seemed eager to start the treatment cycle, since both IVF patients and clinics are more prepared towards COVID-19. It was supposed that the infertility may be located at in the first line of stress for infertile couples rather than of the COVID-19 disease infection. According to other studies, stress levels in infertile couples are similar to those found in patients suffering from life-threatening illnesses (16-18). Vaughan et al. (19) stated that only 6% of the respondents agreed that assisted reproductive technology should be suspended during the pandemic.

Prior to the pandemic, from January 2019 to March 2020, the number of patients' attendance to those three IVF clinics higher compared to during the pandemic. During the first and second waves of the COVID-19 pandemic, there was a decline in IVF cycles in Indonesia, January 2021 and July-August 2021, respectively. However, the statistical analysis showed a gradual increase of IVF cycles, 3.5 cycles per month. This trend was due to the more prepared IVF clinics and patients feel more confident to visit IVF clinics.

Clinics tend to limit the patient load, as the pandemic indeed significantly caused impacts on healthcare professionals (20, 21). Although, it is causing patients backlog, a solution implemented among all IVF clinics surveyed in this research is by providing telehealth prior to face-to-face consultation in the clinic. This telehealth solution is in line with a study done by Hernández et al. (22), in which it was stated that telehealth might provide the obstetric gynaecologists better understanding of patient prognosis.

Due to the absence of a detailed and formal protocol, there was no standardised workflow within and between IVF clinics in Jakarta. The interviews showed that all three IVF clinics interviewed had followed the WHO guidelines, including providing personal protective equipment, training on using them, and hand hygiene education. Also, in line with WHO, routine screening was done on high-risk staff, which was extended to all staff when there was a significant increase in COVID-19 cases (23).

Concerning the high-exposure risk procedures, for instance, the ovum pick-up, all clinics required couples to have negative COVID-19 test results prior to the ovum pick up. Even though all clinics already had high-efficiency particulate absorbing (HEPA) filters in both operating and examining rooms, the only Melati IVF Clinic has equipped negative pressure operating theatre. Negative pressure operating rooms are involved in performing surgical procedures on patients who have airborne infectious illnesses (24). However, research done

by Rosenbaum et al. (25) reported that a portable HEPA filter is a sufficient alternative for the negative pressure individual rooms or units, which was already available in all three clinics, even though it is not suggested to be used for the COVID-19 positive patients.

Conclusion

To conclude, those three clinics are prepared in facing COVID-19, as they complied with government regulations. As the COVID-19 pandemic progressed, the number of patients gradually returned to normal, although there were any changes in the proportion of patients' domicile, the monthly number of patients and clinics' protocols due to the COVID-19 pandemic. Therefore, it is essential to create a formal regulation for IVF clinics worldwide to give the highest quality yet maintain everyone's safety.

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Authors' Contributions

F.A.; Data collection, Data processing, Manuscript conception, and Submission. M.P.; Supervision, Project development, and Manuscript editor. S.W.L.; Co-supervisor and Data collection. G.P.; Co-supervisor and Data collection. All authors read and approved the final manuscript.

References

1. Pignon B, Gourevitch R, Tebeka S, Dubertret C, Cardot H, Dauriac-Le Masson V, et al. Dramatic reduction of psychiatric emergency consultations during lockdown linked to COVID-19 in Paris and suburbs. Psychiatry Clin Neurosci. 2020; 74(10): 557-559.
2. Sparrow R, Dartanto T, Hartwig R. Indonesia under the new normal: challenges and the way ahead. Bull Indones Econ Stud. 2020; 56(3): 269-299.
3. Mafham MM, Spata E, Goldacre R, Gair D, Curnow P, Bray M, et al. COVID-19 pandemic and admission rates for and management of acute coronary syndromes in England. The Lancet. 2020; 396: 381-389.
4. Braithwaite J. Quality of care in the COVID-19 era: a global perspective. IJQHC Communications. 2021; 1(1): 1-3.
5. Alonso A, Deans R, Nesbitt-Hawes E, Yazdani A, McCormack L, Koh YY, et al. Gynaecological and IVF procedures billed through the Medicare Benefits Schedule (MBS) during the COVID-19 pandemic. Aust N Z J Obstet Gynaecol. 2021; 61(4): 585-590.
6. Ata B, Vermeulen N, Mocanu E, Gianaroli L, Lundin K, Rautakallio-Hokkanen S, et al. SARS-CoV-2, fertility and assisted reproduction. Hum Reprod Update. 2023; 29(2): 177-196.
7. Lawi GFK. Saran ahli, sebaiknya tunda program hamil selama pandemi Covid-19. 2020. Available from: https://lifestyle.bisnis.com/read/20200422/106/1231110/saran-ahli-sebaiknya-tunda-program-hamil-selama-pandemi-covid-19 (21 Sep 2021).
8. Balasch J, Gratacós E. Delayed childbearing: effects on fertility and the outcome of pregnancy. Fetal Diagn Ther. 2011; 29(4): 263-273.
9. Marom Haham L, Youngster M, Kuperman Shani A, Yee S, Ben-Kimhy R, Medina-Artom TR, et al. Suspension of fertility treatment during the COVID-19 pandemic: views, emotional reactions and psychological distress among women undergoing fertility treatment. Reprod Biomed Online. 2021; 42(4): 849-858.
10. Voice of Indonesia. Jakarta's entry and exit prohibition scheme is constrained by many problems at border points. 2021. Available from: https://voi.id/en/news/64085 (23 Sep 2021).
11. Cutting E, Catt S, Vollenhoven B, Mol BW, Horta F. The impact of COVID-19 mitigation measures on fertility patients and clinics around the world. Reprod Biomed Online. 2022; 44(4): 755-763.
12. Uimonen M, Kuitunen I, Paloneva J, Launonen AP, Ponkilainen V, Mattila VM. The impact of the COVID-19 pandemic on waiting times for elective surgery patients: a multicenter study. PLoS One. 2021; 16(7): e0253875.
13. Manusubroto W, Wicaksono AS, Tamba DA, Sudiharto P, Pramusinto H, Hartanto RA, et al. Neurosurgery services in Dr. Sardjito General Hospital, Yogyakarta, Indonesia, during the COVID-19 pandemic: experience from a developing country. World Neurosurg. 2020; 140: e360-e366.
14. Wahba KA. Effect of fasting of ramadan on infertile women undergoing in-vitro fertilization/intracytoplasmic sperm injection cycles: a prospective cohort study. Open J Obstet Gynecol. 2016; 06(03): 171-179.
15. Daley A, Pallan M, Clifford S, Jolly K, Bryant M, Adab P, et al. Are babies conceived during Ramadan born smaller and sooner than babies conceived at other times of the year? A Born in Bradford Cohort Study. J Epidemiol Community Health. 2017; 71(7): 722-728.
16. Marom Haham L, Youngster M, Kuperman Shani A, Yee S, Ben-Kimhy R, Medina-Artom TR, et al. Suspension of fertility treatment during the COVID-19 pandemic: views, emotional reactions and psychological distress among women undergoing fertility treatment. Reprod Biomed Online. 2021; 42(4): 849-858.
17. Harzif AK, Santawi VPA, Wijaya S. Discrepancy in perception of infertility and attitude towards treatment options: Indonesian urban and rural area. Reproductive BioMed Online. 2020; 114(3): 1-7.
18. Domar AD, Broome A, Zuttermeister PC, Seibel M, Friedman R. The prevalence and predictability of depression in infertile women. Fertil Steril. 1992; 58(6): 1158-1163.
19. Vaughan DA, Shah JS, Penzias AS, Domar AD, Toth TL. Infertility remains a top stressor despite the COVID-19 pandemic. Reprod Biomed Online. 2020; 41(3): 425-427.
20. Nashwan AJ, Mohamed AS, Kelly DR. Editorial: nursing education in the emergence of COVID-19. Open J Nurs. 2020; 10(6): 595-597.
21. Nashwan AJ, Abujaber AA, Mohamed AS, Villar RC, Al-Jabry MM. Nurses' willingness to work with COVID-19 patients: the role of knowledge and attitude. Nurs Open. 2021; 8(2): 695-701.
22. Hernández C, Valdera CJ, Cordero J, López E, Plaza J, Albi M. Impact of telemedicine on assisted reproduction treatment in the public health system. J Healthc Qual Res. 2020; 35(1): 27-34.
23. World Health Organization. Preventing and mitigating COVID-19 at work: policy brief, 19 May 2021. 2021; 1-21. Available from: https://www.who.int/publications/i/item/WHO-2019-nCoV-workplace-actions-policy-brief-2021-1.
24. Qian H, Zheng X. Ventilation control for airborne transmission of human exhaled bio-aerosols in buildings. J Thorac Dis. 2018; 10 Suppl 19: S2295-S2304.
25. Rosenbaum RA, Benyo JS, O'Connor RE, Passarello BA, Williams DR, Humphrey BD, et al. Use of a portable forced air system to convert existing hospital space into a mass casualty isolation area. Ann Emerg Med. 2004; 44(6): 628-634.

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The Effect of SARS-Cov2 Infection on The Spermogram: A Prospective Study

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Abstract

Background: During the Coronavirus disease 2019 (COVID-19) pandemic, there was always concern about damage to different organs of the body. In this study, we aimed to determine if coronavirus 2 (SARS-CoV-2) could influence the sperm parameters in inpatient adult men with COVID-19.

Materials and Methods: In this prospective study during 2021, 22 patients with COVID-19 diagnosed with polymerase chain reaction (PCR) test and clinical symptoms and history of admission and 19 volunteer healthy men as the control group participated. They were asked to provide semen samples at 2 and 6 months after hospital discharge and the same time for the control group. The following parameters were measured in all semen samples and beside the demographic data, they compared between the two groups: volume (mL), sperm concentration (10^6/mL), total motile sperm percentage, progressive percentage, normal morphology percentage, and DNA fragmentation index (DFI).

Results: The mean ± SD age of the participants in the COVID and control groups was 46.36 ± 9.94 and 45.84 ± 10.21 years, respectively (P=0.869). The mean ± SD body mass index (BMIs) of the participants in the COVID and control groups were 28.6 ± 5.460 and 29.6 ± 6.092, respectively (P=0.579). The mean ± SD number of children was 1.41 ± 1.054 in the COVID group and 1.47 ± 1.073 in the control group (P=0.847). All the sperm parameters were significantly impaired after 2 months in the COVID group in comparison with the control group (P<0.05). After 4 months from first sampling, all the parameters were improved significantly (except normal morphology) but had not yet reached the level of the control group.

Conclusion: SARS-CoV-2 affected semen parameters in patients admitted because of COVID-19, in the short term. It is expected that this will improve with time.

Keywords: Coronavirus SARS, Male Infertility, Semen Quality Analyses, Spermatogenesis

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Introduction

Coronavirus 2 (SARS-CoV2), as a severe acute respiratory syndrome, has infected millions of people since December 2019 that first reported from Wuhan, China (1). The Coronavirus disease 2019 (COVID-19) affects different body organs including the respiratory system, liver, kidney, heart, the gastrointestinal, hematological and nervous systems with a high rate of multi-organ failure and mortality (2).

Although, there are limited studies about andrological consequences of COVID-19, there is no consensus about its effect on semen quality (3, 4). Whether COVID-19 is detectable in semen fluid or not is debatable. In the evaluation of male patients with COVID-19 and those recovering from the disease, Li et al. (5) identified SARS-CoV-2 in semen fluid. In contrast, there are some other studies that do not confirm this finding (6, 7). COVID-19 may affect the testis parenchyma, changes the Sertoli

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and Leydig cells morphology, and causes lymphocytic inflammation (8). It may have a negative impact on semen quality and sex hormones, although it may be reversible (9- 12). There are limited studies about COVID-19 effects on the semen quality, therefore we designed the present study.

Material and Methods

The institutional board of research and Ethics Committee of Shahid Beheshti University of Medical Sciences confirmed the study protocol (IR.SBMU.RETECH.REC.1399.559). This study was conducted in accordance with the Declaration of Helsinki. All participants signed the informed consent.

Participants

Patients participating in this study were 20-60 years old male volunteers who had been diagnosed COVID-19 infection based on a nasopharyngeal SARS-CoV-2 real-time (RT) polymerase chain reaction (PCR) swab during their admission to Shohada-e-Tajrish or Taleghani Hospitals. They were divided into mild, moderate or severe groups depending on clinical characteristics and radiological findings (13, 14). Semen samples were collected from them at 2 and 6 months after hospital discharge. All patients were treated with the specified protocol (15). Their demographic data and the COVID-19 disease history were recorded.

In parallel, the semen fluid of 19 volunteered men which was obtained by masturbation was evaluated as the control group, at the beginning of the project. The control group was the men who live a normal life in society without any symptoms of COVID 19. They did not present any suspicious symptoms, including constitutional symptoms, fever, cough, gastrointestinal symptoms or any other symptoms that made us suspected of having COVID 19. All semen samples were collected by masturbation following sexual abstinence of 1-3 days. All semen analysis was evaluated according to WHO 2010 criteria (16) at the Andrology Laboratory of In Vitro Fertilization Center, Taleghani Hospital, Tehran, Iran. Participants who could not provide the second semen sample were excluded. Also, participants with orchitis or abnormal findings on scrotal examination including severe varicocele, history of vasectomy, testis torsion, single testis, testis trauma and any kind of scrotal -inguinal surgery were also excluded. All the participant characteristics including body mass index (BMI), age, history of smoking and the number of children were recorded.

Andrology assessment

The following parameters were measured in all semen samples (16) and compared between the two groups: volume (mL), sperm concentration (10^6/mL), total motile sperm, progressive percentage, normal morphology percentage (assessed by Strict criteria) and DNA fragmentation index (DFI, assessed by sperm chromatin dispersion). Sperm motility is the movement, or swimming of sperms. The progressive sperm motility defined as sperms that move along a straight line or around large circles which is necessary for pregnancy (17).

Statistical analysis

Quantitative data with normal distribution are shown as a mean ± standard deviation. To compare proportions and means, we used chi-square and Student's t tests, respectively. $P<0.05$ was considered statistically significant. SPSS software version 26 (IBM Corporation, Armonk, NY, USA) was used for statistical analysis.

Results

Totally, 41 men (22 patients with COVID-19 and 19 volunteers as the control group) participated in this study. Table 1 shows the demographic data of our groups.

Table 1: Participant's demographic characteristics

Characteristics	COVID group (n=22)	Control group (n=19)	P value
Age (Y)	46.36 ± 9.94	45.84 ± 10.21	0.869
Number of children	1.41 ± 1.054	1.47 ± 1.073	0.847
Smoking	11 (50)	11 (57.9)	0.756
BMI (kg/m^2)	28.6 ± 5.460	29.6 ± 6.092	0.579
COVID19 severity			
Mild	12		
Moderate	10		
Severe	0		

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

The sperm parameters including sperm motility, sperm concentration, progressive sperm percentage, normal morphology, and DFI were determined in each group and compared (Table 2). Six months after discharge, since the control group did not refer, we compared the patients' semen parameters to the baseline semen parameters of the same group through the paired t test. We also compared the second sperm analysis with the COVID-19 group after six months to the baseline sperm analysis of the control group that the results are shown in Table 2. As shown in Table 2, all the sperm parameters were impaired significantly in the COVID group at month two, in comparison with the control group. After a 6 months follow-up from discharge, all the parameters (sperm concentration: $P=0.005$, sperm motility: $P<0.001$, progressive sperm: $P=0.045$, DNA fragmentation index: $P=0.001$) were improved significantly except normal morphology ($P=0.066$), in the COVID group in comparison with themselves in the time points, 0 and 2 months after COVID-19 affection but it had not yet reached the level of the control group.

Table 2: Comparison sperm parameters between the COVID and control groups at 2 and 6 months

Sperm parameters	COVID group baseline	Control group	P value*
Sperm concentration (million/ml)	29.09 ± 14.46	63.74 ± 21.16	<0.001
Sperm motility	32.27 ± 11.61	60.05 ± 10.71	<0.001
Progressive motility	16.86 ± 10.27	46.32 ± 14.22	<0.001
Normal sperm morphology	0.66 ± 0.83	2.42 ± 1.21	<0.001
DNA fragmentation index	39.8 ± 15.28	14.4 ± 4.78	<0.001
	COVID group baseline	**COVID group after 6 months**	**P value****
Sperm concentration (million/ml)	29.09 ± 14.46	39.14 ± 16.84	0.005
Sperm motility	32.27 ± 11.61	49.05 ± 16.50	<0.001
Progressive sperm	16.86 ± 10.27	31.23 ± 14.12	0.045
Normal sperm morphology	0.66 ± 0.83	1.05 ± 0.95	0.066
DNA fragmentation index	39.8 ± 15.28	27.91 ± 12.35	0.001
	Control group	**COVID group after 6 months**	**P value***
Sperm concentration (million/ml)	63.74 ± 21.16	39.14 ± 16.84	<0.001
Sperm motility	60.05 ± 10.71	49.05 ± 16.50	0.015
progressive sperm	46.32 ± 14.22	31.23 ± 14.12	0.002
normal sperm morphology	2.42 ± 1.21	1.05 ± 0.95	<0.001
DNA fragmentation index	14.4 ± 4.78	27.91 ± 12.35	<0.001

Data are presented as mean± SD. *; Independent sample t test and **; Paired t test.

Discussion

The COVID19 and consequent severe acute respiratory syndrome has the ability to attack multiple organs and weaken overall immunity for months (18, 19). The man's fertility system may be affected by various infectious or viruses including mumps virus, influenza virus, human immunodeficiency virus (HIV), Zika virus and may even involve the testes tissues (16).

Pneumonia is the most common clinical manifestation of COVID-19, due to the presence of Angiotensin-converting enzyme 2 (ACE2)-containing cells among type 2 alveolar cells (20). The expression level of ACE2 in the Sertoli and Leydig cells, may be even higher than the alveolar type II cells. Testicles with ACE2 receptor may be vulnerable to the COVID-19 infection, which may become a target organ such as lungs (21).

A review study of evaluating biological implications of the COVID-19 on sexual transmissibility, fertility and viral presence, revealed that COVID-19 is potentially present in seminal fluid, but sexual transmission not reported (22). Previous epidemiologic studies show significant concerns regarding the long-term fertility capacity for patients who are their reproductive system affected by the Sars-CoV-2 (23). A systematic review for survey the potential impact of COVID-19 on male reproductive organs and male fertility concluded that, only one of the 28 studies had reported the presence of SARS-CoV-2 in the seminal fluid (24). They observed a decrease in semen quality in the patients with moderate infection rather than patients with mild infection and healthy controls. Impairment quality of seminal fluid may be related to inflammation and fever occurrence (5).

In our study, the seminal fluid analyses of the admitted patients with COVID-19 were compared between the period of disease and at least six months after discharge of hospital from COVID-19 and control groups. The long-term effect of COVID-19 on male fertility is a debate. A systematic review study compared parameters of seminal fluid in patients recovered from COVID-19 revealed seminal fluid volume, progressive sperm motility and sperm concentration were not significantly decreased after getting the disease. Mean sperm count and total sperm motility between the COVID-19 group and control group were statistically significant (25). Guo and colleagues found that the total sperm count, sperm concentration and progressive motility following recovery from the COVID-19 infection increased significantly after 84 days in comparison with the first 56 days of recovery (9). In another study, COVID-19 affected sperm parameters, as well (26). A prospective study of men who were recovered from the COVID-19 infection revealed that 25% of them were oligocrypto-azoospermic. From eleven men with seminal fluid impairment, eight were azoospermic and three were oligospermic. Thirty three patients (76.7%) showed pathological levels of IL-8 in seminal fluid. Oligo-crypto-azoospermia was significantly correlated with COVID-19 severity (27). All these studies approve our results about the effect of COVID19 on sperm quality.

Every febrile disease could lead to impaired semen quality and even azoospermia (28). Due to the nature of the spermatogenesis cycle, we evaluated the samples

two months and 6 months after hospital discharge in the COVID group to prevent the effect of febrile disease on sperm quality.

The studies that mentioned above and the present study showed that seminal fluid parameters and male fertility may be affected by COVID-19. The possibility of this fact that COVID-19 infection would affect the sexual function and male reproductive system and permanent effects on the human reproductive health should be evaluated long-term, multicenter, and prospective cohort studies with larger sample sizes. Here, we evaluated patients with a COVID-19 infection who needed to be hospitalized unlike most studies that considered outpatients. It seems that hospitalized patients experience severe degrees of illness than outpatients, what made our study differ of other published studies. Although, our present study is not free of some limitations, including low sample size, analyze other parameters such as sperm leukocyte count, sex hormones, anti-sperm antibodies which could provide useful information and long term follow up. Also, the follow-up semen analysis of the control group was impossible for us.

Conclusion

In this study, we concluded that SARS-CoV-2 affects semen parameters in patients who were admitted to the hospital because of COVID-19, in the short term. After the disease recovery, these parameters start to improve, but studies with larger sample size and longer follow up time are necessary to prove whether the parameters return to their previous level or not.

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Authors' Contributions

F.A., M.K., M.A., S.S., B.N., A.F., M.D.; Participated in study design and data collection. F.A., B.N., M.D.; Participated in statistical analysis and drafting. All authors read and approved the final manuscript.

References

1. Cascella M, Rajnik M, Aleem A, Dulebohn SC, Di Napoli R. Features, evaluation, and treatment of coronavirus (COVID-19). 2022. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2023.
2. Gupta A, Madhavan MV, Sehgal K, Nair N, Mahajan S, Sehrawat TS, et al. Extrapulmonary manifestations of COVID-19. Nat Med. 2020; 26(7): 1017-1032.
3. Vishvkarma R, Rajender S. Could SARS-CoV-2 affect male fertility? Andrologia. 2020; 52(9): e13712.
4. Ruan Y, Hu B, Liu Z, Liu K, Jiang H, Li H, et al. No detection of SARS-CoV-2 from urine, expressed prostatic secretions, and semen in 74 recovered COVID-19 male patients: a perspective and urogenital evaluation. Andrology. 2021; 9(1): 99-106.
5. Li D, Jin M, Bao P, Zhao W, Zhang S. Clinical characteristics and results of semen tests among men with coronavirus disease 2019. JAMA Netw Open. 2020; 3(5): e208292.
6. Pan F, Xiao X, Guo J, Song Y, Li H, Patel DP, et al. No evidence of severe acute respiratory syndrome-coronavirus 2 in semen of males recovering from coronavirus disease 2019. Fertil Steril. 2020; 113(6): 1135-1139.
7. Paoli D, Pallotti F, Colangelo S, Basilico F, Mazzuti L, Turriziani O, et al. Study of SARS-CoV-2 in semen and urine samples of a volunteer with positive naso-pharyngeal swab. J Endocrinol Invest. 2020; 43(12): 1819-1822.
8. Yang M, Chen S, Huang B, Zhong JM, Su H, Chen YJ, et al. Pathological findings in the testes of COVID-19 patients: clinical implications. Eur Urol Focus. 2020; 6(5): 1124-1129.
9. Guo TH, Sang MY, Bai S, Ma H, Wan YY, Jiang XH, et al. Semen parameters in men recovered from COVID-19. Asian J Androl. 2021; 23(5): 479-483.
10. Holtmann N, Edimiris P, Andree M, Doehmen C, Baston-Buest D, Adams O, et al. Assessment of SARS-CoV-2 in human semen-a cohort study. Fertil Steril. 2020; 114(2): 233-238.
11. Temiz MZ, Dincer MM, Hacibey I, Yazar RO, Celik C, Kucuk SH, et al. Investigation of SARS-CoV-2 in semen samples and the effects of COVID-19 on male sexual health by using semen analysis and serum male hormone profile: a cross-sectional, pilot study. Andrologia. 2021; 53(2): e13912.
12. Ma L, Xie W, Li D, Shi L, Ye G, Mao Y, et al. Evaluation of sex-related hormones and semen characteristics in reproductive-aged male COVID-19 patients. J Med Virol. 2021; 93(1): 456-462.
13. He X, Cheng X, Feng X, Wan H, Chen S, Xiong M. Clinical symptom differences between mild and severe COVID-19 patients in china: a meta-analysis. Front Public Health. 2021; 8: 561264.
14. Guler SA, Ebner L, Aubry-Beigelman C, Bridevaux PO, Brutsche M, Clarenbach C, et al. Pulmonary function and radiological features 4 months after COVID-19: first results from the national prospective observational Swiss COVID-19 lung study. Eur Respir J. 2021; 57(4): 2003690.
15. Stasi C, Fallani S, Voller F, Silvestri C. Treatment for COVID-19: An overview. Eur J Pharmacol. 2020; 889: 173644.
16. Khan I. Sperm quality parameters and oxidative stress: exploring correlation in fluoride-intoxicated rats. J Hum Reprod Sci. 2022; 15(3): 219-227.
17. Menkveld R, Wong WY, Lombard CJ, Wetzels AM, Thomas CM, Merkus HM, et al. Semen parameters, including WHO and strict criteria morphology, in a fertile and subfertile population: an effort towards standardization of in-vivo thresholds. Hum Reprod. 2001; 16(6): 1165-1171.
18. Ghahestani SM, Bonakdar Hashemi M, Yousefzadeh Kandevani N, Borumandnia N, Dadpour M, Sharifiaghdas F. Iranian urology association coronavirus disease 2019 (COVID-19) taskforce pamphlet (IUA-CTP) recommended practice based on national epidemiologic analysis. Urol J. 2020; 17(5): 543-547.
19. Abedi AR, Aliakbari F, Ghiasy S, Allameh F, Ghanbari MA. The impact of the first Covid 19 pandemic on urology residency training in Iran. Men's Health Journal. 2020; 4(1): e21.
20. Jiang F, Deng L, Zhang L, Cai Y, Cheung CW, Xia Z. Review of the clinical characteristics of Coronavirus disease 2019 (COVID-19). J Gen Intern Med. 2020; 35(5): 1545-1549.
21. Fan C, Li K, Ding Y, Lu W, Wang J. ACE2 expression in kidney and testis may cause kidney and testis damage after 2019-nCoV infection. medRxiv. 2020: 2020.02.12.20022418.
22. Tur-Kaspa I, Tur-Kaspa T, Hildebrand G, Cohen D. COVID-19 may affect male fertility but is not sexually transmitted: a systematic review. F S Rev. 2021; 2(2): 140-149.
23. Pike JFW, Polley EL, Pritchett DY, Lal A, Wynia BA, Roudebush WE, et al. Comparative analysis of viral infection outcomes in human seminal fluid from prior viral epidemics and Sars-CoV-2 may offer trends for viral sexual transmissibility and long-term reproductive health implications. Reprod Health. 2021; 18(1): 123.
24. He Y, Wang J, Ren J, Zhao Y, Chen J, Chen X. Effect of COVID-19 on male reproductive system - a systematic review. Front Endocrinol (Lausanne). 2021; 12: 677701.
25. Tiwari S, Kc N, Thapa S, Ghimire A, Bijukchhe S, Sah GS, et al. Semen parameters in men recovered from COVID-19: a systematic review and meta-analysis. Middle East Fertil Soc J. 2021; 26(1): 44.

26. Erbay G, Sanli A, Turel H, Yavuz U, Erdogan A, Karabakan M, et al. Short-term effects of COVID-19 on semen parameters: a multicenter study of 69 cases. Andrology. 2021; 9(4): 1060-1065.
27. Gacci M, Coppi M, Baldi E, Sebastianelli A, Zaccaro C, Morselli S, et al. Semen impairment and occurrence of SARS-CoV-2 virus in semen after recovery from COVID-19. Hum Reprod. 2021; 36(6): 1520-1529.
28. Carlsen E, Andersson AM, Petersen JH, Skakkebaek NE. History of febrile illness and variation in semen quality. Hum Reprod. 2003; 18(10): 2089-2092.

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Twelve Hours Post-Injection Serum Human Chorionic Gonadotropin and Body Mass Index Predicts *In Vitro* Fertilization Oocyte Maturation Rate: A Cross-Sectional Study

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Abstract

Background: *In vitro* fertilization (IVF) remains a main treatment for infertility cases. Post-injection human chorionic gonadotropin (hCG) level is an essential factor in determining oocyte maturation rate in IVF. This study aimed to determine the relationship between 12 hours post-injection serum hCG level and oocyte maturation rate among IVF participants.

Materials and Methods: A cross-sectional study on IVF participants was done at a tertiary hospital in Indonesia from January 2020 to December 2021. Subjects were injected with 250 µg of recombinant-hCG (r-hCG) subcutaneously. Twelve hours post-injection serum hCG levels and oocyte maturation rate data were retrieved and analyzed accordingly.

Results: Twenty-eight subjects were recruited into the study. Higher 12 hours post-injection serum hCG was related to oocyte maturation rate (P=0.046). The cut-off point of 12 hours post-injection serum hCG to predict better oocyte maturation rate was 90.15 mIU/mL (sensitivity 68.2%, specificity 83.3%). Oocyte maturation rate may be predicted using body mass index (BMI) and 12 hours post-injection serum hCG.

Conclusion: Higher 12 hours post-injection serum hCG was associated with a higher oocyte maturation rate in IVF subjects.

Keywords: Human Chorionic Gonadotropin Hormone, *In Vitro* Fertilization, Oocyte, Oocyte Maturation

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Introduction

In vitro fertilization (IVF) is the most commonly performed procedure in assisted reproduction for achieving pregnancy (1). The mean pregnancy rate in IVF ranges from 30 to 35% (2). The success of IVF is closely related to various factors, most of which are still unknown until the later cycles of therapy or even near the end of treatment. The failure to achieve pregnancy is thought to be caused by embryo quality and endometrial receptivity factors. However, there have been no proven indicators to predict oocyte maturation rate during IVF (3, 4).

Before picking the ovum, controlled ovarian stimulation was carried out starting on the second day of the menstrual cycle. Following controlled stimulation, injection of human chorionic gonadotropin (hCG) is administered for the later stages of follicle maturation. Due to the nearly similar (homologous) chemical chain structure of hCG and luteinizing hormone (LH), hCG administration is aimed to mimic the normal LH surge in ovulation. The injection of exogenous hCG is usually done 12 hours before ovum pick-up (OPU). Previous studies had contrasting results linking serum hCG levels with oocyte quality and number at the time of OPU (5). However, there has been no research regarding the direct relationship between 12 hours post-injection serum hCG levels and oocyte maturity in the IVF cycle. This study

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aimed to determine the relationship between 12 hours post-injection serum hCG level and oocyte maturation rate among IVF participants.

Materials and Methods

An observational study with cross-sectional design was done to investigate the relationship between 12 hours post-injection serum hCG and oocyte maturation rate. The study was done at Yasmin Clinic, Cipto Mangunkusumo National Kencana Hospital, Jakarta, Indonesia, from January 2020 to December 2021.

Subjects recruited for the study were all 20-40 years old IVF participants who had hCG injection as part of the protocol and willing to participate in the study. Meanwhile, the exclusion criteria were subjects with history of ovarian surgery, chemotherapy, or radiotherapy. Poor responder subjects (defined by those with anti-Mullerian hormone level of <1.5 ng/mL) were also excluded from the study.

This study utilized the standard protocol of IVF applied in Cipto Mangunkusumo National General Hospital. A total of 250 µg recombinant hCG (r-hCG) was injected as exogenous hCG source on all subjects before OPU. Serum hCG level was obtained 12 hours post-injection in each subject. Moreover, the OPU was done 36 hours following hCG injection. Mature oocyte was defined as the oocyte reaching metaphase II according to the oocyte cumulus–corona complex (OCCC) grading, which included the `sun-burst' appearance of the corona radiata and expanded cumulus (6). Meanwhile, oocyte maturation rate was defined in this study as the rate of mature oocyte divided by the total oocyte picked up at OPU. The cut-off for satisfactory oocyte maturation rate was set to be 75% (7).

The Research Ethics Committee had approved the protocol for human studies with ethical clearance letter number KET-967/UN2.F1/ETIK/PPM.00.02/2019. All subjects had given their informed consent before their inclusion in the study. Collected data of the subjects were analyzed using Statistical Package for Socials Sciences (SPSS) for Macintosh ver. 20 (IBM®, USA). The characteristics of patients were analyzed descriptively. Normally distributed numeric variables were presented in the form of mean ± standard deviation, while abnormally distributed numeric variables were presented in the form of median (minimum value-maximum value). Bivariate analysis was done using unpaired t test for variables with normal distribution or Mann-Whitney test for variables with abnormal distribution. In order to determine the ideal cut-off of 12 hours post-injection serum hCG level, receiver operating characteristics (ROC) curve would be used with satisfactory oocyte maturation rate as the state variable. Afterwards, multivariate analysis consisting of 12 hours post-injection serum hCG level and clinical characteristics would be done using the logistic regression method.

Results

A total of 32 subjects fulfilled the inclusion criteria during the study. However, 4 subjects were excluded due to not taking serum hCG level examination. Therefore, only 28 subjects were recruited and analyzed. The study recruitment flowchart can be found in Figure 1.

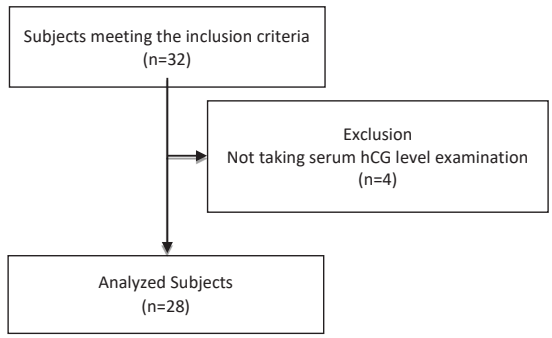

Fig.1: Recruitment flowchart of the study.

Following the recruitment of subjects, the subjects' clinical characteristics were analyzed. The results can be found in Table 1.

Table 1: Clinical characteristics of subjects

Characteristics	Frequency (n=28)
Age (Y)	33.32 ± 3.8
Body mass index (kg/m^2)	22.59 ± 2.9
Infertility duration (Y)	5.54 ± 2.7
Prior IVF history	
None	24 (85.7)
Once	2 (7.1)
Twice	2 (7.1)
Indication for IVF	
Infertility	27 (96.4)
Primary	24 (85.7)
Secondary	3 (10.7)
Social	1 (3.6)
Known etiology	
Male factors	9 (32.1)
Female factors	14 (50)
Uterine factor	10 (35.7)
Tubal factor	2 (7.1)
Ovarian factor	2 (7.1)
Female and male	2 (7.1)
Unknown	3 (10.7)
Basal AMH level (µg/mL)	3.29 (1.66-13.10)[#]
12 hours post-injection hCG level (mIU/mL)	101.81 ± 38.3
Oocyte maturation rate	
>75%	22 (78.6)
<75%	6 (21.4)
Oocyte retrieved per patient	11 (3-33)[#]

Data are presented as mean ± SD or n (%). IVF; *In vitro* fertilization, AMH; Anti mullerian hormone, hCG; Human chorionic gonadotropin, and [#]; Median (minimum value-maximum value).

Table 2: The relationship between 12 hours post-injection serum hCG level and oocyte maturation rate

Variable	Oocyte maturation rate	n	Mean ± SD	Mean diff	95% CI		P value
					Min	Max	
12 hours post-injection serum hCG level (mIU/mL)	≥75%	22	109.26 ± 38.0	34.78	0.60	68.95	0.046*
	<75%	6	74.48 ± 26.6				

hCG; Human chorionic gonadotropin, CI; Confidence interval, Min; Minimal, Max; Maximal, and *; Unpaired t test.

Table 3: Cut-off value for 12 hours post-injection serum hCG

AUC	12 hours post-injection serum hCG level (mIU/mL)	sn	sp	95% CI		P value*
				Min	Max	
78.8%	90.15	68.2	83.3	60.3%	94.0%	0.033

AUC; Aurea under the curve, Sn; Sensitivity, Sp; Specificity, CI; Confidence interval, Min; Minimal, Max; Maximal, and *; Unpaired t test

Furthermore, the analysis of relationship between 12 hours post-injection serum hCG level and oocyte maturation rate was also performed. The result of this analysis can be found in Table 2.

Based on the analysis, higher 12 hours post-injection serum hCG level was associated with a higher oocyte maturation rate (P=0.046). Moreover, the cut-off point for 12 hours post-injection serum hCG level was determined to predict a satisfactory oocyte maturation rate.

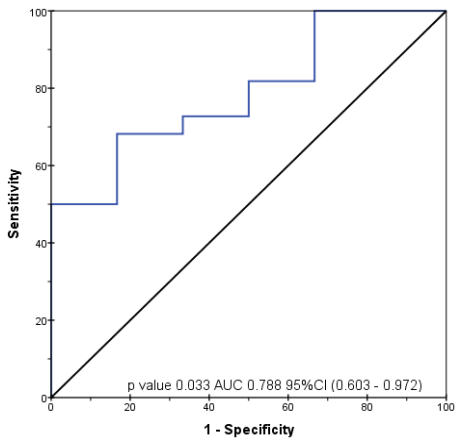

Fig.2: ROC curve of 12 hours post-injection serum hCG. ROC; Receiver operating characteristics, hCG; human chorionic gonadotropin, AUC; Aurea under the curve, and CI; Confidence interval.

Based on the analysis done in Table 3, it was found that the cut-off value for 12 hours post-injection serum hCG to predict satisfactory oocyte maturation rate was >90.15 mIU/mL. Furthermore, multivariate analysis was done. The result of the analysis can be found in Table 4.

Table 4: Multivariate analysis for predicting oocyte maturation rate

Variables	B	SE	Wald	P value	OR	95% CI	
						Min	Max
BMI	0.404	0.215	3.523	0.033	1.497	1.182	2.282
hCG	-0.032	0.018	3.140	0.048	1.012	1.005	1.030
Constant	-7.838	5.140	2.325	0.127			

BMI; Body mass index, hCG; Human chorionic gonadotropin, Min; Minimal, Max; Maximal, *; Logistic regression, B; Beta, S.E.; Standard of error, OR; Odds ratio, and CI; Confidence interval.

Based on the multivariate analysis, BMI and hCG level were the factors affecting oocyte maturation rate.

Discussion

Based on our analysis, higher 12 hours post-injection serum hCG was associated with higher oocyte maturation rate. The average age of subjects in our study was 33.32 ± 3.8 years, with most of the subjects having normal BMI. This value was similar to previous studies on IVF participants in Indonesia, with most subjects being aged over 30 years old (1, 2). In a previous study conducted in Jakarta, the best probability of IVF success was obtained when the participant's age was under 35 years old, with a success rate of 56.4% (1). Many factors are thought to be the cause of worse IVF success rate in older patients, some of which are disturbed hormonal factors and the existence of other comorbidities which increase along with aging (5). Other studies showed that subjects with older age would experience a higher rate of mitochondrial dysfunction associated with oxidative stress, resulting in worse oocyte quality (8). BMI is also known to be a determinant of oocyte maturity and clinical pregnancy in IVF through several mechanisms, one of which is lower hCG level in patients with higher BMI (1, 7). However, we could not generalize the result of this study as the number of included subjects in each body mass index group was not sufficient for adequate power in the analysis.

In this study, the level of oocyte maturity was assessed based on the rate of mature oocytes compared to the total number of oocytes obtained during oocyte pick up. The level of satisfactory oocyte maturation rate was determined to be more than 75%. Previous studies also used this value to predict clinical pregnancy in IVF patients (7, 8). In addition to this threshold, some studies determine other threshold values, depending on the agreement of the researchers (7, 9).

During the IVF procedure, hCG has a role to replace LH in triggering oocyte maturation in folliculogenesis. LH is required in the follicular phase to trigger paracrine signals between theca cells and granulosa cells, which are useful in triggering androgen and estrogen synthesis to achieve oocyte maturation. There are LH/choriogonadotropin (LH/CGR) receptors on the ovaries that can bind to both LH and hCG and play a major role in folliculogenesis. The role of hCG in folliculogenesis is largely related to its ability to bind to LH/CGR (10).

LH/CGR is required in the process of follicle maturation and ovulation. In the IVF procedure, hCG will replace the

role of the LH surge that should occur during ovulation by binding to the LH/CGR receptor. After the injection of r-hCG, it is estimated that a phenomenon similar to the LH surge will occur in a long time because the half-life is much longer, which is around 30 hours, so that a longer accumulation of effects will be obtained compared to physiological ovulation (10, 11).

Post-injection hCG levels in this study were assessed at 12 hours post-injection. Before the examination, all subjects were injected with r-hCG at a dose of 250 mg subcutaneously. This protocol was applied at the study site and was similar to the protocol used in the previous study (9). A previous study has shown that 250 mg r-hCG is superior than the 500 mg dose in preventing ovarian hyperstimulation syndrome (OHSS) (12). Although 12 hours post-injection hCG level in this study affected oocyte maturation, there were previous studies with contrasting results. One of the studies showing no correlation between hCG level and oocyte maturity nor embryo quality was the study performed by Gunnala et al. (13). However, Zhang et al. (14) recently stated that excessively high follicular fluid hCG levels would have a detrimental effect on oocyte maturation, fertilization, and embryonic development potential.

In addition to hCG levels, it was found that another factor influencing oocyte maturity in this study was body mass index. Previous studies indicated that IVF participants with higher body mass index are known to have worse oocyte maturation (1, 14). Previous studies suggested that larger doses of hCG should be injected in patients with a higher body mass index (12). However, this also carried a risk of increasing the likelihood of OHSS, so further research is needed to compare the benefits and impacts of the action (15, 16).

The main limitation of this study was its small sample size of the subjects. This limitation caused generalization to the general population unsuitable. Moreover, there was no data on the proportion of pregnancies, both biochemical, clinical, and pregnancy outcomes, so further analysis regarding the confounding factors of oocyte size could not be executed. The similarity of injected r-hCG dose among subjects also raised concerns due to body mass index being an influencing factors of oocyte maturity. Further studies with larger sample size and different r-hCG dose among the subjects may be considered.

Conclusion

Higher 12 hours post-injection serum hCG was associated with a higher oocyte maturation rate on IVF subjects. Furthermore, oocyte maturation rate might be predicted using BMI and 12 hours post-injection serum hCG.

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Authors' Contributions

B.W., E.S., V.S., A.K.H., G.P., K.S.; Conceptualization, Methodology, and Software. B.W., A.K.H., G.P., K.S., R.M., A.H.; Validation, Formal analysis, and Investigation. B.W., E.S., M.M., A.K.H., G.P., K.S.; Resources and Data Curation. E.S., B.W., M.M., A.K.H.: Writing- Reviewing and Editing. All authors read and approved the final manuscript.

References

1. Eskew AM, Jungheim ES. A History of developments to improve in vitro fertilization. Mo Med. 2017; 114(3): 156-159.
2. European IVF-monitoring Consortium (EIM); European Society of Human Reproduction and Embryology (ESHRE), Calhaz-Jorge C, De Geyter C, Kupka MS, de Mouzon J, et al. Assisted reproductive technology in Europe, 2013: results generated from European registers by ESHRE. Hum Reprod. 2017; 32(10): 1957-1973.
3. Xia Q, Li Y, Zhang Y, Tian F, Zhang Q, Yao Z. Identification of factors related to fertilization failure in in vitro fertilization-embryo transfer. Zhong Nan Da Xue Xue Bao Yi Xue Ban. 2020; 45(8): 960-965.
4. Franasiak JM, Alecsandru D, Forman EJ, Gemmell LC, Goldberg JM, Llarena N, et al. A review of the pathophysiology of recurrent implantation failure. Fertil Steril. 2021; 116(6): 1436-1448.
5. Tantitham C, Panunumpa S, Satirapod C. The effect of human chorionic gonadotropin on the in vitro development of immature to mature human oocytes: a randomized controlled study. J Hum Reprod Sci. 2020; 13(2): 133-137.
6. Gardner DK, Weissman A, Howles CM, Shoham Z. Textbook of assisted reproductive techniques. 5th ed. Boca Raton, FL: CRC Press; 2018; 96-121.
7. Nora H, Wiweko B, Muharam R, Rajuddin, Wangge G, Hestiantoro A, et al. Impact of serum human chorionic gonadotropin and luteinizing hormone receptor expression to oocyte maturation rate: a study of controlled ovarian stimulation. J Hum Reprod Sci. 2020; 13(1): 46-50.
8. Braga DPAF, Zanetti BF, Setti AS, Iaconelli A Jr, Borges E Jr. Immature oocyte incidence: contributing factors and effects on mature sibling oocytes in intracytoplasmic sperm injection cycles. JBRA Assist Reprod. 2020; 24(1): 70-76.
9. Abbara A, Clarke SA, Dhillo WS. Novel concepts for inducing final oocyte maturation in in vitro fertilization treatment. Endocr Rev. 2018; 39(5): 593-628.
10. Drakakis P, Loutradis D, Beloukas A, Sypsa V, Anastasiadou V, Kalofolias G, et al. Early hCG addition to rFSH for ovarian stimulation in IVF provides better results and the cDNA copies of the hCG receptor may be an indicator of successful stimulation. Reprod Biol Endocrinol. 2009; 7: 110.
11. Theofanakis C, Drakakis P, Besharat A, Loutradis D. Human chorionic gonadotropin: the pregnancy hormone and more. Int J Mol Sci. 2017;18(5): 1059.
12. Pakhomov SP, Orlova VS, Verzilina IN, Sukhih NV, Nagorniy AV, Matrosova AV. Risk factors and methods for predicting ovarian hyperstimulation syndrome (OHSS) in the in vitro fertilization. Arch Razi Inst. 2021; 76(5): 1461-1468.
13. Gunnala V, Melnick A, Irani M, Reichman D, Schattman G, Davis O, et al. Sliding scale HCG trigger yields equivalent pregnancy outcomes and reduces ovarian hyperstimulation syndrome: analysis of 10,427 IVF-ICSI cycles. PLoS One. 2017; 12(4): e0176019.
14. Zhang L, Wang H, Zhang R, Liang J, Liu C, Zhou Y, et al. Correlation of follicular fluid human chorionic gonadotrophin level with oocyte maturity and early embryonic development. Nan Fang Yi Ke Da Xue Xue Bao. 2014; 34(2): 260-264.
15. Lin Y, Yang P, Chen Y, Zhu J, Zhang X, Ma C. Factors inducing decreased oocyte maturation rate: a retrospective analysis of 20,939 ICSI cycles. Arch Gynecol Obstet. 2019; 299(2): 559-564.
16. Blumenfeld Z. The ovarian hyperstimulation syndrome. Vitam Horm. 2018; 107: 423-451.

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Forskolin Improves Male Reproductive Complications Caused by Hyperglycemia in Type 2 Diabetic Rats

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Abstract

Background: In many diabetic patients, spermatogenesis complications are frequent causing infertility problems. This study aimed to demonstrate the effect of Forskolin on male reproductive dysfunction caused by type 2 diabetes.

Materials and Methods: In this experimental study, type 2 diabetes was induced by a high-fat diet (HFD) for one month and then a low single dose injection (35 mg/kg) of streptozotocin (STZ) in Wistar rats. After 72 hours, rats with more than 200 mg/dl of blood glucose were considered type 2 diabetic rats. Forty rats (200-250 g) were divided into four groups (n=10) including group 1 (G1): rats with normal diet and buffer citrate (STZ solvent) injection, group 2 (G2): control type 2 diabetic rats with HFD and STZ injection, group 3 (G3): type 2 diabetic rats received phosphate buffer saline (PBS) as Forskolin solvent, and group 4 (G4): Forskolin treated diabetic rats (10 mg/kg) for 1 month.

Results: In comparison to control group, in diabetic groups (G2 and G3) some parameters are increased significantly: The blood glucose (P=0.00078), testicular malondialdehyde (MDA) level and body weight (P=0.00009) and *Bax* gene expression (P=0.00007). Unlike, some parameters are decreased significantly: The serum level of testosterone (P=0.0009), testicular superoxide dismutase (SOD, P=0.00007) and glutathione peroxidase (GPX) levels (P=0.00008), sperm concentration (P=0.00008), motility (P=0.00009), normal morphological sperm (P=0.00008) and *Bcl-2* gene expression (P=0.00009). However, in Forskolin treated group (G4) the parameters stayed close to control values that was significantly (P=0.00007) higher than in G2 and G3 groups. Therefore, treatment with Forskolin significantly improved these abnormal changes in Forskolin-treated group.

Conclusion: Our study demonstrates that Forskolin is an effective antidiabetic agent, which significantly improves sperm concentration, testosterone levels, and antioxidant activity in diabetic rats.

Keywords: Forskolin, Male Infertility, Oxidative Stress, Testicular Dysfunction, Type 2 Diabetes

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Introduction

Diabetes mellitus is an endocrine and metabolic disorder. There are two general types of diabetes: type 1 and type 2 diabetes. Type 2 diabetes accounts for almost 90% of all cases accompanying by hyperglycemia. Hyperglycemia is caused by the destruction of beta cells or insulin resistance of cells, which in general, causes complications such as inflammatory issues, oxidative stress, and obesity. Statistics show that there are more than 415 million diabetic people in the world, which is predicted to approximate 642 million by 2040 (1, 2). It is also more prevalent in men than women (3).

Complications of type 2 diabetes include retinopathy, nephropathy, neuropathy, cardiovascular problems, and reproductive disorders. Type 2 diabetes is often associated with decreased levels of testosterone (T) and gonadotropin-releasing hormones (GnRH) (4). Diabetic testicular disorders include decreased spermatogenesis, testicular germ cell destruction, decreased testosterone and estradiol synthesis, and abnormal semen parameters; they generally lead to diminished reproductive ability or even infertility. It has been shown that reactive oxygen species (ROS) are the main destructive players in hyperglycemia of type 2 diabetes. Prolonged hyperglycemia could produce

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large amounts of ROS, which can upset the balance of the antioxidant system in the body. Following the oxidative stress, testicular germ cells become apoptotic and the spermatogenesis is disrupted (5, 6). Therefore, one of the main therapeutic approaches in diabetic patients is reducing ROS using antioxidants. In this regard, the application of herbal and natural antioxidants has been well established (7). Forskolin, an herbal medicine, is extracted from an Indian plant called Coleus forskohlii. The chemical formula of Forskolin is $C_{22}H_{34}O_7$ and it has been used to treat and prevent cancers, cardiovascular diseases, asthma, glaucoma, obesity, and high blood pressure (8). Interestingly, Forskolin has also documented as an effective antidiabetic drug that increases insulin production from pancreatic beta cells and consequently decreases blood sugar levels (9). It has been demonstrated that cyclic AMP (cAMP) is the mediator that makes Forskolin a highly potent medication (10). In this regard, it has been reported that Forskolin-induced cAMP could reduce cytotoxicity and apoptosis. According to In vitro studies, Forskolin protects cells against H_2O_2 by increasing the antioxidant levels approximately two times (11). The antioxidant effect of Forskolin in different diseases has been documented (12), but its effect on the male reproductive disorders following type 2 diabetes has not been addressed yet. It seems clarifying the potential effects of Forskolin on hormonal status, spermatogenesis, and testicular tissue of diabetic patients might open new ways for the application of this herbal medicine. Therefore, the present study aims to evaluate the potential positive effects of Forskolin on diabetes-induced male reproductive dysfunction.

Materials and Methods

Animals and study design

All steps of the experiment were conducted according to the protocols and guidelines of the Ethical Committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1399.080). In this study, forty Wistar rats of 8 weeks' age and weight of 200 to 250 g were recruited. Animals were maintained under standard laboratory conditions including 50% humidity, 21-25°C temperature, and 12 hours. dark/light cycle. The animals were randomly divided into 4 groups including 10 animals in each group (n=10) as the following:

- Control group (G1): rats with a standard diet that were intraperitoneally treated with 0.5 ml of buffer citrate solution (0.05 M and pH=4.5) as Streptozotocin (STZ) solvent.
- Diabetic control group (G2): rats were fed a high-fat diet (HFD) for 4 weeks and then type 2 diabetes was induced with a single dose of 35 mg/kg STZ.
- Vehicle control group (G3): rats with type 2 diabetes that were treated with phosphate buffer saline (PBS), the solvent of Forskolin, using a gavage syringe.
- Diabetic treated group or experimental group (G4): rats with type 2 diabetes that received Forskolin (>96% pure, HPLC detected, Abcam. Cat. No. 66575-29-9) 10 mg/kg body weight/day for 30 days with a gavage syringe (13).

The HFD was continued for another 30 days after the first 4 weeks in G2, G3 and G4 groups to maintain the type 2 diabetic model. Forskolin was administrated orally to prevent other injuries such as peritoneal and hematologic infections as suggested by previous studies (9, 11, 13). The weight and blood sugar of animals in different groups were measured regularly after HFD, just before STZ injection, after induction of diabetes, just before starting the Forskolin treatment, and after Forskolin treatment.

Induction of type 2 diabetes

Groups 2, 3, and 4 were fed with an HFD consisting of 35% carbohydrates, 45% fat, and 20% protein for 4 weeks (14). At the end of the fourth week, the rats intraperitoneally received a single dose of STZ (35 mg/kg body weight) to induce type 2 diabetes (15). A single and low dose of STZ could affect some of the beta cells causing partial destruction of pancreatic beta cells which could result in type 2 diabetes development (16). The STZ solution was freshly prepared by dissolving STZ (Sigma, USA. Cat. No. S0130-1G) in 0.05 M citrate buffer solution (pH=4.5) (17, 18). To confirm diabetes development, the blood glucose was measured through tail vein 72 hours after STZ injection using a glucometer (Roche Diagnostics, Basel, Switzerland. Cat. No. 05213509001). The rats with stable blood sugar above 200 mg/dl were considered diabetic (15).

Measurement of blood glucose and body weight

In the present study, we measured the blood glucose four times: i. Before healthy rats grouping, ii. After HFD, iii. After STZ injection, iv. After treatment with Forskolin. In this way, we measured the fasting blood glucose using a glucometer (Roche Diagnostics, Basel, Switzerland. Cat. No. 05213509001). Also, we measured the body weight of rats 3 times: i. Before HFD, ii. After HFD and STZ injection, and iii. After treatment with Forskolin. The body weight was measured by digital scale (HL-200, Japan).

Sample collection

At the end of the drug-intervention period, fasting blood samples were collected from the animals, and then all animals sacrificed for sampling. During the animal sacrifice, for the minimum pain and stress in rats as well as the best sampling mode the blood samples were taken from the rats' hearts (cardiac puncture) under deep anesthesia 24 hours after the last treatment of Forskolin (Intraperitoneal, ketamine: 100 mg/kg and xylazine: 10 mg/kg) (19). Subsequently, serum samples were extracted from the blood by centrifugation (20 minutes at 3500 rpm) and kept at -80°C for antioxidant and hormonal analyses.

Semen evaluation

The caudal part of the rat epididymis was separated, chopped into smaller pieces and placed in 1.5 ml of PBS

(pH=7.2) for sperm counting (25). For this purpose, a drop of the solution containing the sperm was transferred to the Neubauer slide chambers (HBG, Germany) and the sperm were counted manually using a light microscope (Olympus CX31, Japan). The results of sperm count were reported as sperm/mL (20). To evaluate sperm motility, the number of motile sperms was counted under a light microscope (Olympus CX31, Japan) and the percentage of motile sperms (number of motile sperms/ the total number of counted sperms) calculated. Sperm motility was classified into three groups: progressive, non-progressive, and immotile (21). To examine sperm morphology, a sperm-contained droplet was placed on the slides to be air-dried and then fixed with 96% alcohol and stained with H&E. For each slide, 100 sperms were evaluated and the percentage of normal and abnormal sperms was determined (18, 22).

Hormonal measurements

Serum levels of luteinizing hormone (LH, mIU/mL), follicle-stimulating hormone (FSH, mIU/mL), and T (nmol/L) were measured by the Enzyme-Linked Immunosorbent Assay (ELISA) method using commercial kits (DRG Instruments GmbH, Marburg, Germany).

Biochemical analysis (lipid peroxidation and antioxidant enzymes)

Homogeneous testicular tissue samples were used to evaluate the levels of antioxidant enzymes and lipid peroxidation. Potassium chloride solution (1.5%) was applied to homogenized testicular tissue (26). To evaluate lipid peroxidation, the amount of malondialdehyde (MDA) was measured according to Nair and Turner's method using Biocore Diagnostik (ZellBio) MDA assay Kit. Briefly, the reaction of the testicular sample with the Thiobarbituric Acid (TBA) solution was used to measure MDA. The mixture was then centrifuged after heating, and the resulting supernatant was placed in a spectrophotometer to measure the amount of MDA (23).

To measure superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes commercial kits were used (Randox Laboratories, UK for SOD and Biocore Diagnostik, ZellBio for GPx). In brief, to measure SOD, a solution of hydrochloride, EDTA, and Triton X-100 was incubated with a homogeneous testicular sample and the amount of SOD enzyme measured as units/g protein with a spectrophotometer (24). To evaluate the amount of GPx enzyme, an enzymatic reaction with NaNO3, EDTA, glutathione reductase, cumene hydroperoxide, and NADPH was conducted. The resulting mixture was transferred to a spectrophotometer and the amount of enzyme obtained based on units/g protein (25).

Real-time quantitative polymerase chain reaction analysis

After Forskolin treatment, the samples were taken from the left testis of all rats, and the expression level of *Bax* and *Bcl-2* was evaluated. For this purpose, total RNA was extracted with TRIzol reagent solution (Invitrogen, Paisley, UK Cat. No. 15596026) (26). The RNA extraction was performed with the RNeasy Micro kit (Invitrogen Life Technologies, USA). We used DNase I enzyme to remove DNA contamination. After the determination of RNA density using an absorption ratio of 260 and 280 nm, the RNA was washed with RNase-free water and exactly adjusted to a concentration of 0.7 µg/ml. Then, the cDNA was synthesized in a volume of about 20 µl by applying a commercial Kit (Thermo Scientific, EU Cat. No. FERK1622) (27, 28).

The real-time quantitative polymerase chain reaction analysis (qRT-PCR) reaction was done in a 48-well plate. Each well contained 1 µl of each primer (forward and reverse primers), 1 µl of cDNA, 10 µl of SYBR Green, and 7 µl DNase/RNase free water (Sigma-Aldrich, Germany. Cat. No. 7732-18-5). For the thermal cycle, Biosystems (UK) sequence detection system was used according to the manufacturer's protocol. To determine the fold change expression of each gene, Pfaffl method ($2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct=\Delta Ct$ Sample-ΔCt Control) was utilized (27, 28).

Histological examination

The rats' testes were placed in Bowen's fixation solution for 48 hours and then embedded into paraffin blocks. Afterward, paraffin blocks with a thickness of 5 µm were sectioned and deparaffinized and the slides stained with H&E. A slide was taken from the top, middle, and bottom of each testicle, respectively. Then, the slides were observed under a light microscope (Olympus, Japan).

Ten round seminiferous tubules in each slide were randomly selected. Factors such as the Seminiferous Tubule Diameter (STD), the height of the germinal epithelium layer (HE), the overall shape, and internal spaces of the tubules were evaluated with image J software. To determine testicular damage, the process of spermatogenesis was examined histopathologically by the Mean Johnsen Score test (MJS) with scores from 1 to 10 (26).

Statistical analysis

The homogeneity test (Kolmogorov-Smirnov test), to check the normal distribution of data, was performed to ensure data normality. To analyze data, one-way and two-way ANOVA and also post hoc Tukey tests were performed. $P<0.05$ was considered statistically significant. Statistical analysis was performed using Prism GraphPad software (ver.7.0, Graph-Pad, San Diego, CA, USA). Data presented with mean ± SD.

Results

Blood sugar changes in different animal groups

Blood glucose was reported in the study groups in four time points, including: i. Before HFD, ii. After a HFD without STZ injection, iii. After HFD and STZ injection, and just before Forskolin treatment initiation, and iv.

After Forskolin treatment course. In G1 group, the rats had a normal blood glucose level in all four time points because of normal diet and the absence of any intervention (Fig.1A). In G2 group, a significant increase (P<0.00091) was observed in blood sugar after HFD which was noted as pre-diabetic status but not the diabetic phase. After the STZ injection, the blood sugar increased (P=0.00078) more than 200 mg/dl, and after a month, it continued to rise (P=0.00056, Fig.1A). In G4 or Forskolin treatment group, the blood glucose status was almost the same as in the G2 and G3 after HFD with slight increase (P=0.00086) but no in the diabetic range. After the STZ injection, the animals' blood sugar significantly rose again to higher than 200 mg/dl (P=0.00071). In this group, after one month of Forskolin treatment, a significant decrease was found (P=0.00016) in blood sugar which was although higher than the G1 group in the same time point, it was still very close to normal range; particularly when it was compared with the G2 at the same time that had blood sugar above 300 mg/dl (Fig.1A).

Effect of Forskolin on body weight in diabetic rats

The rats' weight was evaluated in three time points, including: i. Before HFD, ii. Three days after STZ injection (after induction of diabetes), and iii. After a one-month treatment of rats with Forskolin (after the treatment with Forskolin) (Fig.1B).

In the G1 group, there was no sharp change in the weight of rats at different time points and there was just a slight difference in the rats' weight after induction of diabetes compared to after the treatment (P=0.00032). After diabetes induction, a significant (P=0.00009) weight gain was observed in diabetic control group when compared to the healthy control group. In we also found significant weight gain (P=0.0008) after the induction of diabetes (before starting Forskolin treatment) the G4 group, but the weight of the animals decreased after treatment with Forskolin (P=0.00085) (Fig.1B).

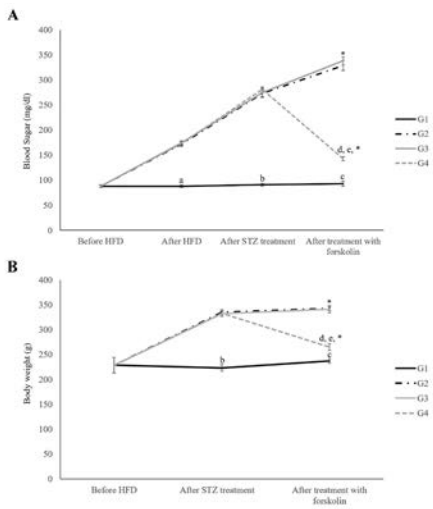


Fig.1: Effects of forskolin on blood sugar and body weight indexes in different time periods. Comparision of **A.** Blood sugar and **B.** Body weight in different experimental groups: control group (G1), diabetic control group (G2), vehicle control group (G3) and Forskolin-treated diabetic group (G4). There are ten rats in each group. One-way ANOVA test was used and all values were presented as mean ± SD. Values with various superscripts are significantly different. a; indicates significant difference between the G1 with G2, G3, and G4 groups after HFD (P<0.001), b; Indicates significant difference between the G1 with G2, G3, and G4 groups after STZ treatment (P<0.001), c; Indicates significant difference between the G1 with G2, G3, and G4 groups after Forskolin treatment (P<0.001), d; Indicates significant difference between the G4 with G2 after Forskolin treatment (P<0.001), e; Indicates significant difference between the G4 with G3 after Forskolin treatment (P<0.001), and *; Indicates significant difference among different treatment of the G2, G3, and G4 groups (P<0.001), HFD; High-fat diet, and STZ; Streptozotocin.

Effect of Forskolin on sperm parameters

Figure 2 shows the sperm parameters of different study groups. Sperm concentration (×10^6 /ml) (P=0.00008), percentage of normal morphology (P=0.00087) and percentage of sperm motility (P=0.00096) decreased in the diabetic group compared to the healthy control group. The treatment of diabetic rats with Forskolin could increase the sperm concentration, normal morphology, and motility when compared to the untreated group. The difference in sperm concentration of the G1 and G4 groups decreased but it was still significant (Fig.2A, P=0.00009). The comparison of morphology and motility in the G1 and G4 groups showed a decrease in the difference and a progression toward normalization, but the difference was still significant (P=0.00093, Fig.2).

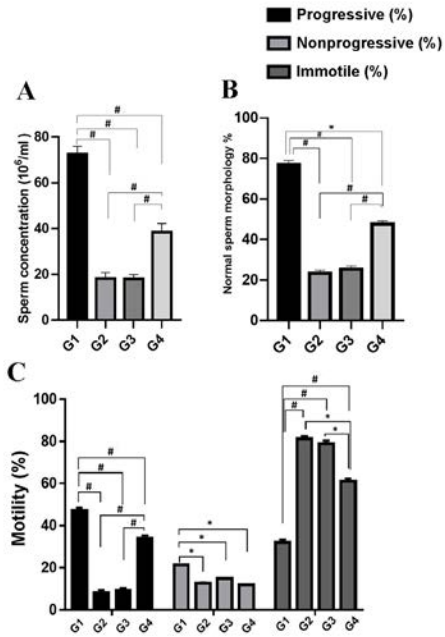


Fig.2: Effects of Forskolin on sperm parameters in different experimental groups. **A.** Sperm concentration (×10^6 /ml), **B.** Normal sperm morphology (%), and **C.** Sperm motility (%). Control group (G1), diabetic control group (G2), vehicle control group (G3) and Forskolin-treated diabetic group (G4). There were ten rats in each groups. One-way ANOVA test was used. Data represented as mean ± SD. Significant difference with *; P<0.001, #; P<0.0001, HFD; High-fat diet, and STZ; Streptozotocin.

Effect of Forskolin on serum levels of gonadotropins and testosterone

As shown in Figure 3, the serum levels of LH (P=0.0009), FSH (P=0.0008), and testosterone (P=0.0009) in the G2 group were significantly lower than the G1 group. administration of Forskolin in the G4 increased the levels of these hormones when comparing to the G2 group (P=0.0008). Moreover, we found significantly

higher levels of LH (P=0.0077) in serum of the G4 rats in comparison with the G1 group (Fig.3A). FSH and testosterone levels were significantly different between the G1 and G4 groups (Fig.3B, C, P=0.0009).

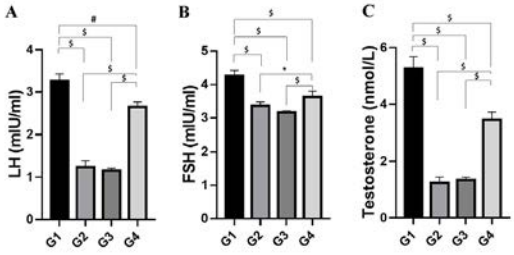


Fig.3: Effect of Forskolin on serum levels of gonadotropins and testosterone. Comparison of **A.** LH, **B.** FSH, and **C.** Testosterone hormones among different groups. Control group (G1), diabetic control group (G2), vehicle control group (G3) and Forskolin treated diabetic group (G4). There were ten rats in each groups. One-way ANOVA test was used. Data represented as mean ± SD. Significant difference with *; P<0.05, #; P<0.01, $; P<0.001, LH; Luteinizing hormone, and FSH; Follicle-stimulating hormone.

Effect of Forskolin on testicular levels of oxidative markers

The results are illustrated in Figure 4. Induction of type 2 diabetes (group G2) caused a significant decrease in the levels of SOD (P=0.0007) and GPx (P=0.0008) when compared to the control group (G1). On the other hand, Forskolin treatment significantly increased SOD and GPx enzymes levels when compared to the G2 (P=0.0009). Furthermore, the levels of MDA were higher in diabetic group (group G2) than the healthy group (P=0.0009). Forskolin treatment could significantly reduce MDA levels as we found significant difference in MDA levels between the G2 (P=0.0006) and G4 groups (P=0.0009, Fig.4C).

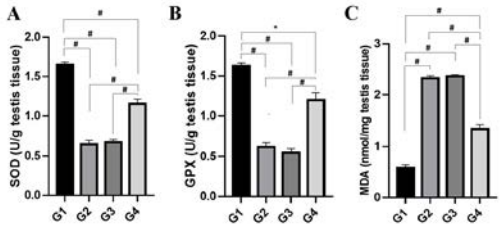
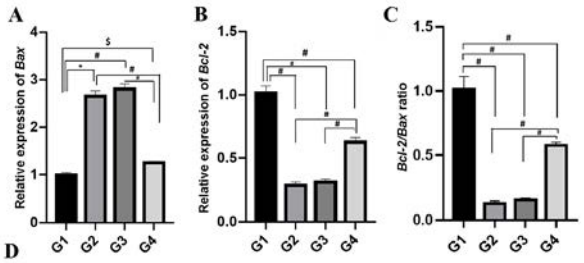


Fig.4: Effect of Forskolin on testicular levels of oxidative markers. Comparison of the **A.** SOD, **B.** GPx, and **C.** MDA levels in the testis tissue among study groups. Control group (G1), diabetic control group (G2), vehicle control group (G3) and Forskolin treated diabetic group (G4). There were ten rats in each groups. One-way ANOVA test was used. Data represented as mean ± SD. Significant difference with *; P<0.001, #; P<0.0001, SOD; Superoxide dismutase, GPx; Glutathione peroxidase, and MDA; Malondialdehyde.

Effect of Forskolin on the expression of *Bax* and *Bcl-2* genes

The effect of Forskolin on the expression of *Bax* (pro-apoptotic) and *Bcl-2* (anti-apoptotic) genes in testicular tissue of rats was evaluated by RT-PCR. Our results demonstrated that diabetes could cause a significant increase in mRNA levels of Bax when compared to the healthy groups (Fig.5A, P=0.00007). In addition, *Bax* expression was decreased in the G4 compared to the G2 group (P=0.0008). On the other hand, induction of type 2 diabetes reduced the expression level of *Bcl-2* gene when compared to healthy untreated rats (Fig.5B, P=0.0009). While treatment with Forskolin increased *Bcl-2* expression when compared to the diabetic rats (P=0.0009) and there was no significant difference between the G1 and G4 in the expression of *Bax* but there was significant difference between the G1 and G4 in the expression of *Bcl-2* (P=0.00007) genes.

Regarding the ratio of *Bcl-2* and *Bax* genes, the results show a significant decrease in the G2 (P=0.00007) and G3 (P=0.00007) groups, but with Forskolin treatment in the G4 group, an increase (P=0.00008) was observed in the ratio (Fig.5C). The primers sequences for *Bax*, *Bcl-2* , and *GAPDH* (housekeeping) genes are shown in Figure 5D (26).



D

Genes	Forward Primer (5'–3')	Reverse Primer (5'–3')	Annealing Temperature	Accession No	Product Size
BAX	TGGCAGCTGACATGTTTTCTGAC	TCACCCAACCACCCTGGTCTT	42°C	NM_017059	195 bp
Bcl-2	TCGCCCTGTGGATGACTGA	CAGAGACAGCCAGGAGAAATCA	42°C	NM_016993	134 bp
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG	42°C	NM_017008	160 bp

Fig.5: Effect of Forskolin on the expression of *Bax* and *Bcl-2* genes. The mRNA expression of **A.** *Bax*, **B.** *Bcl-2*, **C.** Their ratio in different groups as well as **D.** Applied primer sequences. Control group (G1), diabetic control group (G2), vehicle control group (G3) and Forskolin treated diabetic group (G4). There were ten rats in each groups. Expression level of genes (*Bax* and *Bcl-2*) were evaluated regarding the housekeeping gene (*GAPDH*). One-way ANOVA test was used. Significant difference with *; P<0.001, #; P<0.0001, and $; Non-significant.

Testicular histology and seminiferous tubular indices

Figure 6A shows histological structures of the testicular tissue in different study groups. Figure 6A1 is a testicular section from the control group demonstrating a quite regular lumen of tubules and a normal thickness of the germinal epithelium covering most diameter of the tubules. No congestion and edema were observed in this group. As shown in Figure 6B1, the tubular Lumina were irregular and the thickness of germinal epithelium was clearly declined in the diabetic control group. Figure 6A3, a section of vehicle control group, shows a reduced germinal epithelium thickness and vast tubular Lumina. The spaces between tubules indicates the presence of edema. Figure 6A4 is a section of Forskolin treated diabetic group. The thickness of the germinal epithelium and tubular Lumina in Forskolin treated group were almost similar to the control group. There was no edema and congestion between tubules.

Seminiferous tubular diameter (STD) are shown in Figure 6B. The STD in diabetic control group (G2) and vehicle control group (G3) were significantly lower

in comparison to the control group (G1, P<0.00067). In Forskolin treated diabetic group (G4), the STD was significantly lower than the control group (P=0.0091) and higher than the G2 and G3 groups (P=0.0074).

Figure 6B1 shows the thickness or height of the germinal epithelium (HE) in study groups. As the graph shows, HE in the G2 and G3 groups was significantly lower than the G1 (P=0.0009) and G4 (P=0.0008) groups. The HE in the G4 group was significantly lower than control group (P=0.0008). Figure 6B3 shows the Mean Johnsen Score (MJS) in study group. As the graph shows the MJS in the G2 and G3 groups was significantly lower than the G1 and G4 groups (P=0.0009). The MJS in the G4 group was slightly lower than the control group (P=0.043).

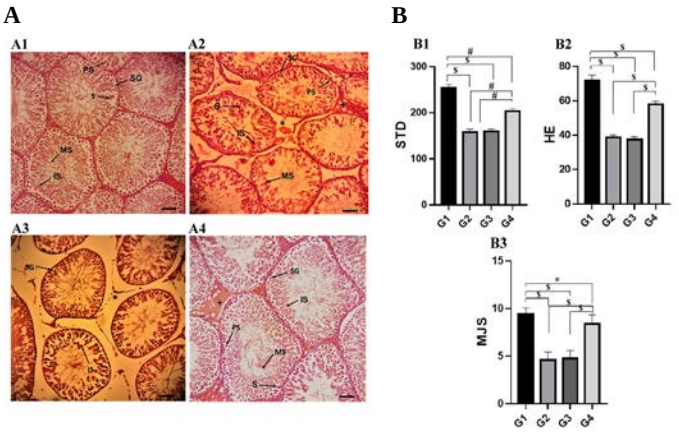


Fig.6: The histological finding in testicular sections and seminiferous tubular indices. **A.** Photomicrograph of testicular tissue sections showing morphological characteristics of seminiferous tubules in different groups. **A1.** Control group, **A2.** Diabetic control group, **A3.** Vehicle control group, and **A4.** Forskolin treated diabetic group. **B1.** Seminiferous tubule diameter (STD), **B2.** Height of germinal epithelium (HE), and **B3.** Mean Johnsen score (MJS)] in different groups. G1; Control group, G2; Diabetic control group, G3; Vehicle control group, G4; Forskolin treated diabetic group. There were ten rats in each group. One-way ANOVA test was used and data represented as mean ± SD. S; Sertoli cell, SG; Spermatogonia, PS; Primary spermatocyte, IS; Immature spermatid, MS; Mature spermatid, *; Space and +; Hemorrhage. H&E staining (scale bar: 50 µm, magnification: 400x). Significant difference with *; P<0.05, #; P<0.01 and $; P<0.001.

Discussion

To induce type 2 diabetes, rats were fed with a HFD and after a one-month feeding, low-dose STZ was injected. Diabetic rats showed: weight gain, hyperglycemia, and increased oxidative stress factors. The increase in body mass in diabetic rats was mostly due to fat accumulation of abdominal areas. Obesity and its problems is a type 2 diabetes complication (29). The body weight changes results in the present study are similar to a study conducted in 2019 that examined the therapeutic effect of Forskolin in diabetes models (30).

Diabetes-caused hyperglycemia leaves negative effects on testicular function such as decreased levels of testosterone and gonadotropins, the number of spermatogonia, Sertoli cells and Leydig, and finally reduced sperm production (31).

In a study by Singh et al. (30) by using Forskolin to evaluate its effect on the improvement of type 2 diabetic nephropathy, it was observed that Forskolin reduced blood sugar in therapeutic groups. Parallel to their study, a decrease in blood sugar levels was also observed in rats treated with Forskolin in the present study.

In another study in 2018, using Forskolin at a similar dose (10 mg/kg body weight/day) to the present study, in a diabetic model after one month with a report of blood sugar levels a significant reduction in blood sugar was reported, that is in line with the findings of the present study (9).

Type 2 diabetes-produced hyperglycemia causes an increase in ROS levels and then, inflammation through the polyol pathway. cAMP has been reported to be involved in regulating antioxidant, anti-inflammatory, and lipid metabolism activities. Increased production of cAMP due to the use of Forskolin, inhibits the activity of tumor necrosis factor (TNF)-α and nuclear factor-kappa B (NF-κB), which play role in causing tissue inflammation and ROS. NF-κB is a factor that is produced in almost all types of cells and its activation that will be due to different types of cellular stresses such as: hyperglycemia, oxidative stress, increased plasma fatty acids as a result of obesity, etc.

In the study, diabetic rats showed a significant decrease in sperm factors such as number, motility, and rapid increase in morphological abnormalities of sperm. In addition, in HFD/STZ diabetic rats, a significant reduction in the number of germ cells and destruction of the germinal layer of the tubules was observed.

Forskolin is very effective in sperm production and semen quality, in general, due to its antioxidant properties, scavenging properties of free radicals, and inhibition of lipid peroxidation. Girish and Reddy (13) found that the use of Forskolin (5 mg/kg bwt/day) as gavage in male Wistar rats of the infertility toxicity model induced by mancozeb for 65 days, increased sperm count, sperm viability, and motility.

Our studies showed that the induction of type 2 diabetes in rats significantly reduced the levels of testosterone, LH, and FSH hormones. Studies in mice with knocked out insulin receptor genes showed an association between fertility and insulin signaling. The linking mechanism behind is that the insufficient insulin in diabetic patients has a negative effect on the activity of the pituitary gland that reduces both the hormonal output of this gland and the levels of LH and FSH. The reduction of LH decreases the effect of LH on Leydig cells and consequently reduces the production of T from these cells and also reduces the production of FSH and less effect on Sertoli cell decreases the amount of sperm production (32). It has been found that one third of all men with type 2 diabetes have lower amounts than normal testosterone levels (33). Consistent with these findings, in animal models of type 2 diabetes induced by HFD/STZ and nicotinamide/STZ, the levels of LH, FSH, and testosterone hormones, sperm count, and motility were significantly reduced (34).

The positive effect of Forskolin on the process of spermatogenesis happens in two ways. First (indirect effect) is the healing effect of Forskolin on diabetes itself and the second (direct effect) is the positive effect on

the disorder in the spermatogenesis pathway caused by diabetes.

In the first case (indirect effect), Forskolin increases the level of insulin and eventually lowers blood sugar. In this pathway, by increasing the production of cAMP, due to Forskolin, cAMP probably increases the production of insulin from the protein kinase (PKA), guanine nucleotide, and beta cells viz. pathways (9).

In the second case (direct effect), the research has also shown that cAMP stimulates Leydig cells to produce testosterone hormone needless of LH hormone (35). Therefore, therapeutic use of Forskolin can change cAMP levels and thus, increase testosterone production. An investigation with the similar findings reported that the use of Forskolin increased the testosterone production in the mancozeb-induced toxicity model in rats (13).

Comparing rats in the diabetic group with rats in the healthy group revealed that the level of MDA in this group increased significantly and the amount of antioxidant enzymes (SOD and GPx) decreased sharply. The results in other studies showed the same changes (13, 36). It has been reported that the increased levels of ROS due to hyperglycemia can lead to infertility complications in diabetic males (37). When type 2 diabetic rats were treated with Forskolin, they showed an increase in the level of antioxidant enzymes (SOD and GPx) and a decrease in the level of MDA resulting in the process of spermatogenesis to resume naturally and increase in the production of testosterone. The results of the present study were similar to those research that reported the protective effect of Forskolin against ROS (13). Parallel to the present study Venkatachalapathi et al. (9) reported the similar changes in oxidative factors levels (SOD, GPx and MDA).

Hyperglycemia-produced ROS disturbs the balance of expression of pro- and anti-apoptotic genes (*Bax*, *Bcl-2*) (38). As a result, in male testes, ROS cause protein and gene damage, which causes cellular damage and subsequent cell apoptosis (39). Although our results showed a significant increase in *Bax* expression in the diabetic group when compared to the healthy control group, in the Forskolin-treated diabetic group, *Bax* levels were significantly lower than the untreated diabetic group. On the other hand, the expression of *Bcl-2* gene in the diabetic group decreased significantly when compared to the healthy group and in the treatment group with Forskolin, the expression of this gene increased compared to the untreated diabetic group.

According to the results, from a histopathological point of view, caused destructive changes in testicular tissue that can be recognized because of the disease. In G2 of diabetic rats, the diameter of the seminiferous tubules decreased sharply and their internal structure disrupted. There was also a significant decrease in mean Johnsen score for this group. These unhealthy tissue changes were greatly reduced by Forskolin treatment.

In fact, it is not possible to include all the items in one study, and according to the cases studied in this research, as well as comparing the results and data obtained from this study with that of other researches, cases such as: longer-term treatment with Forskolin, applying different doses of Forskolin, applying Forskolin in combination with other drugs, the study of specific genes involved in the process of spermatogenesis and etc. are among the items that should be considered in future studies related to the use of Forskolin in the field of spermatogenesis.

In general, type 2 diabetic rats (29, 31) showed advanced destructive tissue effects of testicular tissue and a sharp decrease in the number of spermatogonia following a decrease in the number of germ cells and a decrease in testosterone levels. Treatment of type 2 diabetic rats with Forskolin in 30 days resulted in a significant improvement in testicular tissue and sex hormones (LH, FSH and testosterone), especially testosterone, which were completely consistent with the results of other studies.

Conclusion

The present study showed that the use of Forskolin improves the sperm quality and sex hormones in diabetic rats. In addition, this study showed that Forskolin as a powerful antioxidant decreased the level of MDA and increased the level of antioxidant enzyme and antiapoptotic gene (*Bcl2*) also reduced the proapoptotic gene (*Bax*) in testis of type2 diabetic rats. Consequently, Forskolin can be considered an effective antidiabetic agent for improving sperm quality and fertility power of type2 diabetic rats.

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Authors' Contributions

M.N.; Designed the study, performed all experimental work, manuscript preparation, editing, and critical review. H.T.N., J.S.R.; Contributed extensively in interpretation of the data and the conclusion. M.S.Gh.F.; Contributed to all experimental work, and data analysis. D.M.; Supervised the study, performed acquisition, data and statistical analysis, and interpretation of data. A.G.; Monitoring the preparation and administration of drugs to animals. All authors read and approved the final manuscript.

References

1. Yu D, Zheng W, Cai H, Xiang YB, Li H, Gao YT, et al. Long-term diet quality and risk of type 2 diabetes among urban Chinese adults. Diabetes Care. 2018; 41(4): 723-730.
2. Ogurtsova K, da Rocha Fernandes J, Huang Y, Linnenkamp U, Guariguata L, Cho NH, et al. IDF diabetes atlas: global estimates for the prevalence of diabetes for 2015 and 2040. Diabetes Res Clin Pract. 2017; 128: 40-50.
3. Kautzky-Willer A, Harreiter J, Pacini GJEr. Sex and gender differences in risk, pathophysiology and complications of type 2 diabetes mellitus. Endocr Rev. 2016; 37(3): 278-316.

4. Maresch CC, Stute DC, Alves MG, Oliveira PF, de Kretser DM, Linn T. Diabetes-induced hyperglycemia impairs male reproductive function: a systematic review. Hum Reprod Update. 2018; 24(1): 86-105.
5. Halim M, Halim A. The effects of inflammation, aging and oxidative stress on the pathogenesis of diabetes mellitus (type 2 diabetes). Diabetes Metab Syndr. 2019; 13(2): 1165-1172.
6. Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. Causes and consequences of oxidative stress in spermatozoa. Reprod Fertil Dev. 2016; 28(1-2): 1-10.
7. Cakilcioglu U, Turkoglu I. An ethnobotanical survey of medicinal plants in Sivrice (Elazığ-Turkey). J Ethnopharmacol. 2010; 132(1): 165-175.
8. Srivastava S, Misra A, Mishra P, Shukla P, Kumar M, Sundaresan V, et al. Molecular and chemotypic variability of forskolin in coleus forskohlii Briq., a high value industrial crop collected from western himalayas (India). RSC Adv. 2017; 7(15): 8843-8851.
9. Venkatachalapathi A, Thenmozhi K, Karthika K, Ali MA, Paulsamy S, AlHemaid F, et al. Evaluation of a labdane diterpene forskolin isolated from Solena amplexicaulis (Lam.) Gandhi (Cucurbitaceae) revealed promising antidiabetic and antihyperlipidemic pharmacological properties. Saudi J Biol Sci. 2019; 26(7): 1710-1715.
10. Wagh VD, Patil PN, Surana SJ, Wagh KV. Forskolin: upcoming antiglaucoma molecule. J Postgrad Med. 2012; 58(3): 199-202.
11. Ríos-Silva M, Trujillo X, Trujillo-Hernández B, Sánchez-Pastor E, Urzúa Z, Mancilla E, et al. Effect of chronic administration of forskolin on glycemia and oxidative stress in rats with and without experimental diabetes. Int J Med Sci. 2014; 11(5): 448-452.
12. Rao DS, Rao VP, Rao KR. Pharmacological effects of forskolin isolated from coleus aromaticus on the lung damage rats. Int J Adv Pharm Sci. 2010; 1(1): 17-21.
13. Girish BP, Reddy PS. Forskolin ameliorates mancozeb-induced testicular and epididymal toxicity in Wistar rats by reducing oxidative toxicity and by stimulating steroidogenesis. J Biochem Mol Toxicol. 2018; 32(2): e22026.
14. Skovsø S. Modeling type 2 diabetes in rats using high fat diet and streptozotocin. J Diabetes Investig. 2014; 5(4): 349-358.
15. Reed MJ, Meszaros K, Entes LJ, Claypool MD, Pinkett JG, Gadbois TM, et al. A new rat model of type 2 diabetes: the fat-fed, streptozotocin-treated rat. Metabolism. 2000; 49(11): 1390-1394.
16. Lenzen S. The mechanisms of alloxan-and streptozotocin-induced diabetes. Diabetologia. 2008; 51(2): 216-226.
17. Farashah MS, Pasbakhsh P, Omidi A, Nekoonam S, Aryanpour R, Kashani IR. Preconditioning with SDF-1 improves therapeutic outcomes of bone marrow-derived mesenchymal stromal cells in a mouse model of STZ-induced diabetes. Avicenna J Med Biotechnol. 2019; 11(1): 35-42.
18. Shokoohi M, Gholami Farashah M, Khaki A, Khaki AA, Ouladsahebmadarek E, Aref Nezhad R. Protective effect of fumaria parviflora extract on oxidative stress and testis tissue damage in diabetic rats. Crescent J Med Biol Sci. 2019; 6(3): 355-360.
19. Wellington D, Mikaelian I, Singer L. Comparison of ketamine–xylazine and ketamine–dexmedetomidine anesthesia and intraperitoneal tolerance in rats. J Am Assoc Lab Anim Sci. 2013; 52(4): 481-487.
20. Ahangarpour A, Oroojan AA, Radan M. Effect of aqueous and hydro-alcoholic extracts of lettuce (Lactuca sativa) seed on testosterone level and spermatogenesis in NMRI mice. Iran J Reprod Med. 2014; 12(1): 65-72.
21. Bahmanzadeh M, Goodarzi MT, Rezaei Farimani A, Fathi N, Alizadeh Z. Resveratrol supplementation improves DNA integrity and sperm parameters in streptozotocin–nicotinamide-induced type 2 diabetic rats. Andrologia. 2019; 51(8): e13313.
22. Salimnejad R, Sazegar G, Mousavi SM, Borujeni MJ, Shokoohi M, Allami M, et al. Effect of Teucriumpolium on oxidative damages and sperm parameters in diabetic rat induced with streptozotocin. Int J Adv Biotechnol Res. 2017; 8(3): 1909-1917.
23. Nair V, Turner GA. The thiobarbituric acid test for lipid peroxidation: structure of the adduct with malondialdehyde. Lipids. 1984; 19(10): 804-805.
24. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1972; 247(10): 3170-3175.
25. Ozbal S, Ergur BU, Erbil G, Tekmen I, Bagrıyanık A, Cavdar Z. The effects of α-lipoic acid against testicular ischemia-reperfusion injury in rats. ScientificWorldJournal. 2012; 2012: 489248.
26. Shokoohi M, Shoorei H, Soltani M, Abtahi-Eivari SH, Salimnejad R, Moghimian M. Protective effects of the hydroalcoholic extract of Fumaria parviflora on testicular injury induced by torsion/detorsion in adult rats. Andrologia. 2018; 50(7): e13047.
27. Shoorei H, Khaki A, Khaki AA, Hemmati AA, Moghimian M, Shokoohi M. The ameliorative effect of carvacrol on oxidative stress and germ cell apoptosis in testicular tissue of adult diabetic rats. Biomed Pharmacother. 2019; 111: 568-578.
28. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29(9): e45.
29. Eckel RH, Kahn SE, Ferrannini E, Goldfine AB, Nathan DM, Schwartz MW, et al. Obesity and type 2 diabetes: what can be unified and what needs to be individualized? J Clin Endocrinol Metab. 2011; 96(6): 1654-1663.
30. Singh L, Rana S, Mehan S. Protective effect of forskolin on diabetes induced nephrophaty via antioxidant activity. Iran J Pharmacol Ther. 2019; 17(1): 1-8.
31. Omolaoye T, Du Plessis SS. Diabetes mellitus and male infertility. Asian Pac J Reprod. 2018; 7(1): 6-14.
32. Schoeller EL, Schon S, Moley KH. The effects of type 1 diabetes on the hypothalamic, pituitary and testes axis. Cell Tissue Res. 2012; 349(3): 839-847.
33. Al Hayek AA, Robert AA, Alshammari G, Hakami H, Al Dawish MA. Assessment of hypogonadism in men with type 2 diabetes: a cross-sectional study from Saudi Arabia. Clin Med Insights Endocrinol Diabetes. 2017; 10: 1179551417710209.
34. Ahangarpour A, Oroojan AA, Heidari H, Ghaedi E, Taherkhani R. Effects of hydro-alcoholic extract from arctium lappa l.(burdock) root on gonadotropins, testosterone, and sperm count and viability in male mice with nicotinamide/streptozotocin-induced type 2 diabetes. Malays J Med Sci. 2015; 22(2): 25-32.
35. Salvestrini V, Sell C, Lorenzini A. Obesity may accelerate the aging process. Front Endocrinol (Lausanne). 2019; 10: 266.
36. Long L, Qiu H, Cai B, Chen N, Lu X, Zheng S, et al. Hyperglycemia induced testicular damage in type 2 diabetes mellitus rats exhibiting microcirculation impairments associated with vascular endothelial growth factor decreased via PI3K/Akt pathway. Oncotarget. 2018; 9(4): 5321-5336.
37. La Vignera S, Condorelli R, Vicari E, D'Agata R, Calogero AE. Diabetes mellitus and sperm parameters. J Androl. 2012; 33(2): 145-153.
38. Li D, Ueta E, Kimura T, Yamamoto T, Osaki T. Reactive oxygen species (ROS) control the expression of Bcl-2 family proteins by regulating their phosphorylation and ubiquitination. Cancer Sci. 2004; 95(8): 644-650.
39. Agarwal A, Said TM. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. BJU Int. 2005; 95(4): 503-507.

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Evaluation of Predicting Factors Affecting Sperm Retrieval in Patients with Klinefelter Syndrome: A Prospective Study

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Abstract

Background: This study aimed to evaluate the predicting factors affecting sperm retrieval. We prospectively assessed the relationship between sonographic and microdissection testicular sperm extraction (mTESE) findings in Klinefelter syndrome (KS).

Materials and Methods: In this prospective study, 44 azoospermic men with 47, XXY karyotypes participated in this study. In order to evaluate the amount of blood supply in different parts of testicular tissue, a doppler ultra-sonographic was performed. Also, for the detection of sperm in this group mTESE technique was performed.

Results: The age average of positive mTESE and negative mTESE groups was 29.4 and 33.6 years, respectively. By comparing the testicle volume (based on the data obtained from the clinical examinations conducted by the urologist) it was determined that there is no significant difference between mTESE positive and negative groups. Follicle-stimulating hormone (FSH) levels in men with negative mTESE (P=0.03) and testosterone levels in men with positive mTESE significantly increased (P=0.017). The overall rate of testis vascularity was significantly higher in the positive mTESE group than in the negative mTESE group. The clinical pregnancy rate in positive mTESE men was 9% per cycle, 16.6% per embryos were transferred (ET), and 12.5% per cycle.

Conclusion: Totally, our observation indicated that there is not a significant relationship between sonographic and mTESE results in KS patients. However, more investigations with bigger sample Size can be useful to validate our results.

Keywords: Azoospermic, Klinefelter, Testicular Sperm Extraction, Ultrasonography

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Introduction

Infertility is a disease of the male or female reproductive system defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse. Causes of male infertility include disorder of spermatogenesis and sperm transfer, erectile dysfunction, impaired ejaculation, and intercourse (1). In many cases, there is no clear diagnosis of the cause of infertility. Although infertile men have normal masculine traits, the testicles are unable to produce sperm. About 90% of men's infertility is associated with a lack of/oligospermia or absence/azoospermia of sperm in an ejaculation (2). Many factors can cause oligospermia or azoospermia such as Hereditary factors, infectious agents, environmental pollutants, age, and obesity (3).

Approximately, 11% of male infertilities have been detected to oligospermia with an unknown cause (4). The majority of infertility cases of unknown causes are due to genetic defects. Genetic diseases such as Klinefelter syndrome (KS), Y chromosome microdeletion and cystic fibrosis play an important role in male infertility. KS is a chromosomal disorder in which there is an extra X chromosome in a person's genome. Various genotypes are observed in this disease, such as 49, XXXXY, 48, XXXY, 48, XXYY, but the most common is 47, XXY (5). The prevalence rate of this syndrome is reported 1 to 500-1000 per person. People with KS usually have small testis that does not produce an adequate amount of testosterone hormone. Decreased levels of this hormone can cause delayed or incomplete puberty, enlarged breasts

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(gynecomastia), decreased facial/body hair, and infertility. Since there is no cure for this syndrome; so timely diagnosis and early treatment of KS are very important (6). Sperm freezing before azoospermia is considered a way to treat infertility. It can even be recommended for a group of patients in their early teens. Testicular biopsy is also one of the ways to treat infertility. Although the majority of people with KS are azoospermic, sperm-producing foci have been observed in the testicular tissue in some cases. Briefly, there is no definite indicator that can predict the success of mTESE in these groups of patients (7).

Doppler ultrasound is a well-known method for evaluating TESE in non-obstructive azoospermia (NOA) men. It is based on the theory that testis structure in NOA men has several distinct points through which spermatogenesis takes place (8). On the other hand, there is a theory that relatively high blood flow is in these areas. Based on this evidence, it was concluded that the use of Doppler ultrasound is effective in predicting the presence of spermatogenesis in testis tissue (9). In the present study, we evaluated the relationship between sonographic and mTESE findings in KS patients.

Materials and Methods

This prospective study, was performed according to the guidelines of the Royan Institute Ethics Committee (IR.ACECR.ROYAN.REC.1398.166). Firstly, for infertile couples with male factor causes who were referred to the Royan institute, a complete history such as the history of treatment, used medications, and duration of infertility was taken. A spermogram test was requested for the patient, and the seminal fluid was examined for biochemical-cellular characteristics. The patients were examined for sexual secondary characteristics, testis, varicocele, and hernia. Then, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone hormones were monitored. In the absence of sperm in semen (Azoospermia) at least two semen analyses, increased levels of LH and FSH (two to three times) and decreased testosterone levels (less than 2.5-3 ng/ml), as KS is possible. In addition to the tests above, the karyotype and Y-chromosome AZF region's micro-deletions were requested by the physician. In this descriptive-analytical case-control study, 63 patients with 47, XXY karyotypes were included in this study, and 21 patients were excluded for various reasons. Finally, the current research was performed on 42 patients. After confirmation of KS, a Doppler ultrasound was requested to check the blood supply of testicular tissue. Doppler ultrasound was performed with Aloka ProSound Alpha and 10 MHz Linear probe. In this method, the testis was divided into 4 parts: lower medial (LM), lower lateral (LL), upper medial (UM) and upper lateral (UL).

Before performing the procedures, the informed consent form was completed by the patients. In mTESE method, the patient is under general anesthesia, and through scrotal incision testis delivered, the microtubule contents of the testis are examined under a microscope. A series of large, dilated, and opaque microtubules are biopsied for sperm. The sampling process continues to obtain 20-30 sperm. In the current study, diagnostic and therapeutic mTESE were performed. A diagnostic biopsy was performed on one or both testis based on the patient's clinical condition. Therapeutic mTESE was simultaneous with the day of the oocyte puncture in the patient's spouse. The mTESE was carried out from the same testis (or performed in contralateral tesis) that diagnostic mTESE has performed previously. If sperm is observed, they are frozen for intracytoplasmic sperm injection (ICSI). Fresh sperm samples are used for the ICSI technique; otherwise, frozen samples were used. Tukey's post hoc test was used to determine the differences between groups. The Pearson correlation coefficient was also used to evaluate the intensity of the relationship. Logistic regression analysis was used to control the effect of confounders on qualitative two-state variables and linear regression analysis was used on quantitative variables. A significance level of 5% ($P<0.05$) was considered.

Results

mTESE

Among the 42 infertile men with KS included in this study, the mTESE results of 18 men were positive (42.8%), and 24 men (57.2%) were negative. Out of a total of 51 performed mTESE surgeries (some performed more than once), 24 (47%) had a successful recovery. The results of 27 (53%) mTESE surgery (diagnostic or therapeutic) were negative. Among them, 21 cases (77.7%) were from the right testis and 6 cases (22.3%) were from the left testis. The success rate of mTESE in terms of sperm recovery was 63% in UL regions and 32% in LL regions (Fig.1).

Demographic information

The age average of positive mTESE and negative mTESE groups was 29.4 and 33.6 years, respectively. In this study, the age average of the positive group was significantly lower than the negative group (P=0.007). There was no significant difference between body mass index (BMI) in both groups (P=0.855, Table 1). By comparing the positive and negative mTESE groups, we observed that there is no significant difference between the levels of education between the two groups (Table 1).

Table 1: Comparison of two groups with successful and unsuccessful mTESE in demographics

Parameters	mTESE		P value
	+	-	
Age (Y)	29.47 ± 4.18	33.62 ± 5.21	0.007
Age wife (Y)	26.68 ± 11.17	31.17 ± 6.46	0.111
BMI (kg/m^2)	25.91 ± 6.49	26.26 ± 5.79	0.855
Education			0.935
Under diploma	9 (47.4)	12 (50)	
Diploma	6 (31.6)	8 (33.3)	
Educated	4 (21.1)	4 (16.7)	

Data are presented as mean ± SD or n (%). mTESE; Microdissection testicular sperm extraction and BMI; Body mass index.

Clinical information

At first, the normality test (Kolmogorov Smirnov) was performed, and since the distribution was not normal, non-parametric tests were used. By comparing the testicle volume (based on the data obtained from the clinical examinations conducted by the urologist) of the two mTESE positive and negative groups, it was determined that there is no significant difference between these two groups (P=0.855). The following graphs are a comparison between the average volume of the left and right testicles in two positive and negative groups (Table 2).

Table 2: Comparison of testicular size in positive and negative mTESE groups

mTESE	Number	Mean ± SD	P value
RT			
Positive	19	4.52 ± 2.29	0.158
Negative	24	3.70 ± 2.15	0.166
LT			
Positive	19	5.00 ± 2.53	0.194
Negative	23	4.08 ± 2.29	0.195

mTESE; Microdissection testicular sperm extraction, RT; Right testis, and LT; Left testis.

Hormonal profiles

Regarding the hormonal profile of the patients, the Kolmogorov Smirnoff test was also performed first; The distribution was not normal and as a result, non-parametric tests were used. Our results showed that FSH in the negative group (P=0.03) and testosterone in the positive group were significantly higher (P=0.017). Although LH level did not show a significant difference between the two groups, it was higher in the negative group (P=0.06, Table 3). The logistic regression analysis on the microtesse variable status by the age of LH, FSH, testosterone and overall ultrasound confirmed the obtained results (Table 4).

Table 3: Comparison of hormonal profiles in positive and negative mTESE groups

Hormone name	mTESE	Number	Mean ± SD	P value
LH	Positive	19	19.12 ± 9.96	0.060
	Negative	23	25.20 ± 8.77	
FSH	Positive	19	32.27 ± 16.07	0.030
	Negative	24	40.57 ± 15.02	
Testosterone	Positive	18	3.01 ± 1.76	0.017
	Negative	23	1.81 ± 1.34	

mTESE; Microdissection testicular sperm extraction, LH; luteinizing hormone, FSH; Follicle-stimulating hormone.

Doppler ultrasonography

Comparison of Doppler ultrasonography with mTESE results showed no significant relationship in the left testis (Fig.1), while in the LL and LM areas of the right testis, a positive significant relationship was observed. Furthermore, in the UM area, a negative significant relationship was observed (Fig.2).

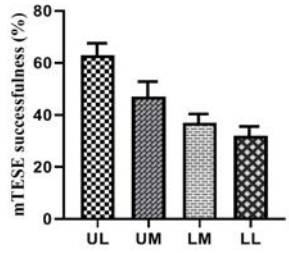

Fig.1: Comparison of vascularity rates of different testis regions in positive and negative mTESE groups of the right and the left testis. mTESE; Microdissection testicular sperm extraction, UL; Upper lateral, UM; Upper medial, LM; Lower medial, and LL; Lower lateral.

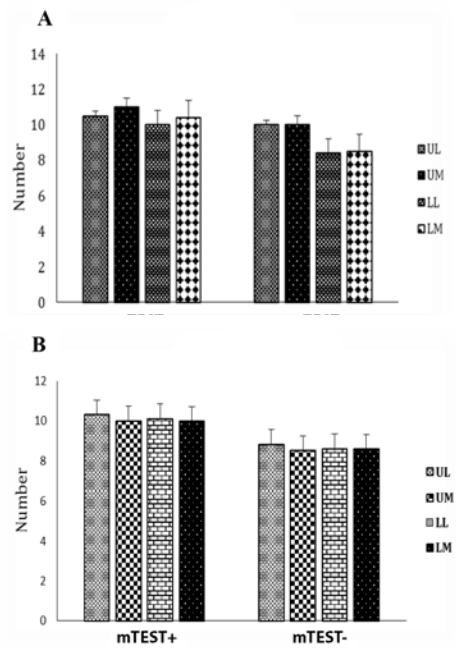

Fig.2: Comparison of vascularity rates in different testis regions in positive and negative mTESE groups of right testis. **A.** Right testis and **B.** Left testis. mTESE; Microdissection testicular sperm extraction, UL; Upper lateral, UM; Upper medial, LM; Lower medial, and LL; Lower lateral.

Table 4: Results of the logistic regression analysis on the mTESE variable status by the age of LH, FSH, testosterone and overall ultrasound

Parameters	Variables	Exp (β)	P value
Demographic	Age (Y)	0.836	0.022
	BMI (kg/m^2)	1.002	0.977
Testicular size	RT	1.205	0.206
	LT	1.198	0.188
Hormonal profiles	LH (ng/mL)	0.922	0.044
	FSH (ng/mL)	0.942	0.039
	Testesterone (ng/mL)	1.602	0.043
Ultrasonography	Total sono	1.951	0.008

Logistic regression data by considering the microtese variable as a dependent variable, the age of LH, FSH, testosterone and overall ultrasound had a significant relationship. Age, LH, and FSH have a significant negative relationship (because the value of exp (β) is less than 1) and testosterone and ultrasonography overall had a positive relationship with microtese. BMI; Body mass index, RT; right testis, LT; Left testis, LH; Luteinizing hormone, and FSH; Follicle-stimulating hormone. Exp (β) shows the superiority ratios for each of the predictor variables.

Assisted reproductive technology cycle

Among 18 patients with positive mTESE, 10 (55.5%) patients entered the assisted reproductive technology

(ART) cycle and a total of 11 cycles were performed. Sperm freezing was used for 7 patients and fresh sperm was used for 3 patients. For 5 patients no embryos were transferred (ET), however, for 5 other couples ET was performed (10 embryos in total) and finally, only one case was reported clinical pregnancy. The success rate of clinical pregnancy for this group of couples was estimated at 9% per cycle, 16.6% per ET, and 12.5% per embryo.

Discussion

KS is known as the most prevalent chromosomal abnormality among azoospermic patients, which can be along with some disorders such as extra gonadal germ cell tumours, cognition deficits, obesity, and gynecomastia (10, 11). It is stated that sonography outcomes can be used as a prognostic parameter in order to achievement in sperm recovery (10). Meanwhile, mTESE method has a capacity for sperm retrieval in NOA males (12). Hence, in the present study, we evaluated predicting factors affecting sperm parameters regarding these patients via finding a correlation between Doppler ultrasound and mTESE results. Regarding the effect of age on mTESE success rate, some studies have suggested that younger age has a positive effect on sperm recovery rate. Some researchers have observed that aging has a negative effect on sperm recovery rates (13, 14). The results of this study showed that age can be an influential factor in the success rate of sperm recovery in mTESE.

Studies on the effect of BMI on male fertility, semen parameters, and mTESE results are few and contradictory. The line with this, Pavan-Jukic et al. (15) by evaluating BMI effect on sperm recovery azoospermic subjects concluded that BMI cannot affect sperm recovery results in men with azoospermia. However, Kort et al. (16) revealed a dramatic and negative association between the whole number of normal sperm cells and BMI. This negative relationship is probably due to the imbalance between estradiol/testosterone hormones. Our results in this study did not show a significant difference in BMI between positive and negative mTESE groups.

Testis size is another effective criterion for sperm recovery rate, which was evaluated in this investigation. In this regard, Taha et al. (17) based on Doppler ultrasound findings provided a document that low sperm count in some fertility disorders may be significantly associated with reduced total testicular size. Here, the results of initial clinical examinations performed by a physician did not show a significant relationship between testis size and mTESE success rate. In accordance with our observation, Schill et al. (18) through the assessment of sonographic, clinical, and biochemical information before and after TESE in infertile men showed that TESE does not affect testis size in these patients. but our results by Doppler ultrasound showed a significant difference in testis size in positive and negative mTESE men. This emphasizes the importance of ultrasound examination of the testis in infertile men.

Hormone profile is another indicator used to predict mTESE results in KS men.

KS men possess LH and FSH higher than normal levels. According to the results of this study, FSH in the negative mTESE group and testosterone in the positive mTESE group are significantly higher. Although the LH level did not show a significant difference between the two groups, it was higher in the negative group. Also, our study showed that LH levels in positive mTESE men are lower than in controls. But according to evidence, mTESE in infertile males cannot change FSH, LH, and testosterone levels (18). On the contrary, Altinkilic et al. (9) reported significant differences in the levels of these hormones between negative and positive mTESE groups of patients with azoospermia.

Moreover, our results show that the echo-pattern of the right testis in mTESE group was negative. This parameter in about 71% of cases was heterogeneous, and in 29% of cases was homogeneous, which have significantly different from the positive group. These results are agreeing with the results previously reported by Rocher et al. (11), and testicular tissue in KS men showed a rough and knotted structure and in non-KS men showed a normal-striated pattern.

Having considered that the parameter of vascularity testicular has been reported as a predictor factor for spermatogenesis (19), the amount of vascularity in different areas of testicular tissue was examined by Doppler ultrasound. In the current study, vascularity was divided into three grades: normo-vascular, hypo-vascular, and hyper-vascular. Our study in terms of vascularity showed no significant difference between the positive and negative groups. The logistic regression analysis on the microtesse variable status by the age of LH, FSH, testosterone and overall ultrasound confirmed the obtained results.

The pathological examination of testicular can also be used to predict the outcome of mTESE. Based on this, it seems that there is a direct relationship between histopathological subtypes and mTESE success rate. But the point to note is that usually the sample sent to the embryologist and pathologist is not the same, and due to the heterogeneous nature of testicular tissue, it has often been observed that the results of embryological and histological examinations are inconsistent. In fact, Sousa's hypothesis can be used temporarily that there is no histological difference between the tissue sent to the pathologist and the embryologist. In this study, no significant difference was observed between the pathology results of positive and negativemTESEgroups. However, it is recommended that more studies on a larger population are needed to demonstrate our outcomes.

Conclusion

Totally, our observation indicated that there is not a significant relationship between sonographic and mTESE

results in KS patients. However, more investigations with bigger sample Sizes can be useful to validate our results.

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Authors' Contributions

F.A.; Participated in study design and writing the manuscript. N.T., F.R.-T., H.K, A.V.D.; Contributed to all experimental work, data collection, and evaluation. H.S., M.A.S.G.; Was responsible for project supervision, data analysis, and manuscript editing. M.M.; Contributed to data analysis and manuscript editing. All authors read and approved the final manuscript.

References

1. Durairajanayagam D. Lifestyle causes of male infertility. Arab J Urol. 2018; 16(1): 10-20.
2. Miyamoto T, Minase G, Shin T, Ueda H, Okada H, Sengoku K. Human male infertility and its genetic causes. Reprod Med Biol. 2017; 16(2): 81-88.
3. Babakhanzadeh E, Nazari M, Ghasemifar S, Khodadadian A. Some of the factors involved in male infertility: a prospective review. Int J Gen Med. 2020; 13: 29-41.
4. Aliakbari F, Taghizabet N, Azizi F, Rezaei-Tazangi F, Gelehkolaee KS, Kharazinejad E. A review of methods for preserving male fertility. Zygote. 2022; 30(3): 289-97.
5. Corona G, Pizzocaro A, Lanfranco F, Garolla A, Pelliccione F, Vignozzi L, et al. Sperm recovery and ICSI outcomes in Klinefelter syndrome: a systematic review and meta-analysis. Hum Reprod Update. 2017; 23(3): 265-275.
6. Samango-Sprouse CA, Counts DR, Tran SL, Lasutschinkow PC, Porter GF, Gropman AL. Update on the clinical perspectives and care of the child with 47,XXY (klinefelter syndrome). Appl Clin Genet. 2019; 12: 191-202.
7. Samango-Sprouse C, Lasutschinkow P, Powell S, Sadeghin T, Gropman A. The incidence of anxiety symptoms in boys with 47,XXY (Klinefelter syndrome) and the possible impact of timing of diagnosis and hormonal replacement therapy. Am J Med Genet A. 2019; 179(3): 423-428
8. Nariyoshi S, Nakano K, Sukegawa G, Sho T, Tsuji Y. Ultrasonographically determined size of seminiferous tubules predicts sperm retrieval by microdissection testicular sperm extraction in men with nonobstructive azoospermia. Fertil Steril. 2020; 113(1): 97-104. e2.
9. Altinkilic B, Pilatz A, Diemer T, Wolf J, Bergmann M, Schönbrunn S, et al. Prospective evaluation of scrotal ultrasound and intratesticular perfusion by color-coded duplex sonography (CCDS) in TESE patients with azoospermia. World J Urol. 2018; 36(1): 125-133.
10. Ekerhovd E, Westlander G. Testicular sonography in men with Klinefelter syndrome shows irregular echogenicity and blood flow of high resistance. J Assist Reprod Genet. 2002; 19(11): 517-522
11. Rocher L, Moya L, Correas JM, Mutuon P, Ferlicot S, Young J, et al. Testis ultrasound in Klinefelter syndrome infertile men: making the diagnosis and avoiding inappropriate management. Abdom Radiol (NY). 2016; 41(8): 1596-1603.
12. Franco G, Scarselli F, Casciani V, De Nunzio C, Dente D, Leonardo C, et al. A novel stepwise micro-TESE approach in non obstructive azoospermia. BMC Urol. 2016; 16(1): 20.
13. Emre Bakircioglu M, Erden HF, Kaplancan T, Ciray N, Bener F, Bahceci M. Aging may adversely affect testicular sperm recovery in patients with Klinefelter syndrome. Urology. 2006; 68(5): 1082-1086.
14. Okada H, Goda K, Yamamoto Y, Sofikitis N, Miyagawa I, Mio Y, et al. Age as a limiting factor for successful sperm retrieval in patients with nonmosaic Klinefelter's syndrome. Fertil Steril. 2005; 84(6): 1662-1664.
15. Pavan-Jukic D, Starc A, Stubljar D, Jukic T. Obesity with high body mass index does not influence sperm retrieval in males with azoospermia. Med Sci Monit. 2020; 26: e923060.
16. Kort HI, Massey JB, Elsner CW, Mitchell-Leef D, Shapiro DB, Witt MA, Roudebush WE. Impact of body mass index values on sperm quantity and quality. J Androl. 2006; 27(3): 450-452.
17. Taha EA, Abd El-Wahed SR, Mostafa T. Relation of color doppler parameters with testicular size in oligoasthenoteratozoospermic men with a varicocele. Hum Androl. 2012; 2(1): 6-11.
18. Schill T, Bals-Pratsch M, Küpker W, Sandmann J, Johannisson R, Diedrich K. Clinical and endocrine follow-up of patients after testicular sperm extraction. Fertil Steril. 2003; 79(2): 281-286.
19. Har-Toov J, Eytan O, Hauser R, Yavetz H, Elad D, Jaffa AJ. A new power doppler ultrasound guiding technique for improved testicular sperm extraction. Fertil Steril. 2004; 81(2): 430-434.

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Monosodium Glutamate Effect on The Expression of α7nACHR and α4nACHR Subunits in The Testicular Tissue

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Abstract

Background: Monosodium glutamate (MSG) is a popular food flavor enhancer, and a glutamate subset that induces different toxicities such as hepatotoxicity, neurotoxicity, reproductive toxicity, and nephrotoxicity. This study was conducted to assess the effects of MSG on the α7 and α4 nicotinic acetylcholine receptor (nACHR) protein subunits expression of adult rat testis and the safety role of vitamin C.

Materials and Methods: For this experimental research, 24 rats were haphazardly grouped into four equal groups (n=6) and orally gavaged for 30 days as follows: control group (distilled water gavage), MSG group (3 g/kg/b.w/day), vitamin C group (150 mg/kg/b.w/day), and MSG+vitamin C group (3 g/kg/b.w/day+150 mg/kg/b.w/day, respectively) that rats of all groups on the 30th day were anesthetized, and the left testes were used for of α4 and α7 nACHR protein subunit evaluation by immunohistochemistry (IHC). Statistical computations were performed using Graph Pad Prism software.

Results: The present study revealed a significant reduction in the expression and optical density (OD) of α7 nACHR and α4 nACHR in the seminiferous tubules and intertubular connective tissue in the MSG group compared to the control group. In the MSG+vitamin C group, the expression and OD of α7 nACHR and α4 nACHR increased in the seminiferous tubules and intertubular connective tissue but this improvement was not significant compared to the MSG group.

Conclusion: MSG decreased the expression level of nACHR protein subunits, α7 and α4, in the seminiferous tubules and interstitial testicular tissue. Vitamin C in the MSG+vitamin C group could not significantly improve the expression of α7 and α4 nACHR subunits in testicular tissue. Probably, MSG toxicity can be compensated with higher doses of vitamin C.

Keywords: Ascorbic Acid, Nicotinic Receptors, Sodium Glutamate, Testis

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Introduction

Nicotinic acetylcholine receptors (nACHR) are the main members of ligand-gated ion channels that respond to the acetylcholine binding. In the central nervous system (CNS), nACHRs are composed of various subunits. Subunits such as α7 and α4β2 are the most abundant subunits of nACHRs in the CNS (1). Although, some studies have reported different expressions of nACHRs subunits on germ cells and sertoli cells, they play roles in germ cell differentiation, proliferation, and sperm movement (2, 3).

Today, many food companies use flavor enhancers in their products, including canned vegetables, soups, and processed meats. The monosodium glutamate (MSG) is one of the most common food flavorings (4, 5). While, the glutamic acid is tasteless molecule, its combination with the sodium leads to glutamate receptors activation in the taste receptor cells. It causes a distinct sense of taste by sending signals to specific brain areas (6, 7).

Several pre-clinical studies have shown that the MSG is toxic to different tissue such as the CNS, testis, ovary, uterus, kidney, and liver (8-10). The MSG can decrease the germinal epithelial thickness, testosterone hormone level, leydig cell numbers and

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increase fibrosis, caspase-3, apoptotic cell numbers, and abnormal sperm morphology in the testis of male Wistar rats (5, 6, 11). One of the known mechanisms of these changes is oxidative stress induction follow of free radicals and hydrogen peroxide formation (7, 12). MSG's functional and morphological effects on the male reproductive system can also be due to decreased gonadotropin-releasing hormone (GnRH) secretion (8).

MSG directly influences the spermatogenic tubules glutamate transporter, which makes this molecule toxicity mechanism in the testis (8). Free glutamate derived from the MSG can create excitotoxicity and pathological alterations in peripheral tissues via acting on the glutamate receptors (13). The inevitable use of monosodium molecules in foods and their deleterious effects led to the requirement of using protective substances, especially antioxidants (8, 14). On the other hand, enzymatic and non-enzymatic antioxidants act as a defense mechanism by decreasing oxidative stress, lipid peroxidation, and pathological changes. Among these antioxidants, vitamin C molecules, ascorbic acid, is introduced as a protector of various cells and tissues against oxidative stress (15, 16). This study was conducted to assess the effects of the MSG molecules on the nACHR subunits, α7 and α4, expression of the adult rat testis and the protective role of the vitamin C molecule.

Materials and Methods

This experimental investigation was confirmed (IR.MUMS.fm.REC.1395.611) by the Ethics Committee of Mashhad University of Medical Sciences, Khorasan Razavi, Iran. Working with animals was also carried out on the basis of the guideline for the care and use of laboratory animals (NIH publication no. 86-23).

Animals

Twenty-four adult male wistar rats (body weight: 200-250 g, age: 6-8 week-old) were used for this experimental study. All animals were preserved under controlled conditions, 12 hours light/darkness cycle with free access to water and food. All rats were obtained from laboratory animals of the Mashhad University of Medical Sciences, Khorasan Razavi, Iran.

Experimental design

The animals were distributed into four equal groups (n=6). Administration of MSG ($C_5H_8NNaO_4$, Negin Tejarat Payam Co., Iran under the license of Huifenghe, China, Cat No; 142-47-2) (4, 5), vitamin C (Osve Co., Iran) (14), and distilled water was done with oral gavage for 30 days (once a day). On the 30th day, the rats were anesthetized by chloroform, and heart perfusion (17) was carried out for full formalin penetration. The left testes (12) were used for immunohistochemistry (IHC) evaluation (Table 1).

Tissue preparation

The testes were divided into several parts and fixed in 10% formalin for 5 days and after the tissue processes were embedded in paraffin. Serial sections of the tissue (5 µ thick) were taken by microtome and mounted on poly-L-lysine coated slides for IHC (12).

Table 1: Different experimental groups

Group name	Intervention
Control	Distilled water
MSG	MSG 3 g/kg/b.w/day
Vit. C	Vit. C 150 mg/kg/b.w/day
MSG+Vit. C	MSG 3 g/kg/b.w/day+Vit. C 150 mg/kg/b.w/day

MSG; Monosodium glutamate and Vit C; Vitamin C.

Immunohistochemistry

For immunohistochemical staining, the slides were deparaffinized by xylene (Mojallali, Iran), hydrated using a descending alcohol series (Mojallali, Iran), and washed in phosphate-buffered saline (PBS) for 10 minutes. The slides were kept in boiling PBS for 15 minutes to retrieve the antigen. Then the samples were treated with the bovine serum albumin (BSA, A4503, Sigma Aldrich, USA) at the room temperature. The sections were immersed in Hydrogen peroxide (H_2O_2-3%) for 15 minutes to block endogenous peroxidase and after that, incubated with normal goat serum (ab 7481, abcam, USA) for 25 minutes. In the next step, samples were covered with primary antibodies, rabbit anti-CHRNA4 antibody (Orb11095, Biorbyt, UK), and rabbit anti-CHRNA7 antibody (Orb11096, Biorbyt, UK) overnight at 4°C. After washing the slides, samples were treated with secondary antibody goat anti-rabbit HRP (1:1000, ab6721, Abcam, USA) for 1 hour at room temperature. After washing with PBS, we applied the diaminobenzidine solution (DAB, Sigma-Aldrich, USA) for 15 minutes in darkness. The slides were washed with water and counterstained with hematoxylin (05-M06004, Bio Optica, Italian) for 1 minutes (18).

Photomicrographs were taken by a bright-field microscope (BX51, Olympus, Japan) equipped with a camera (DP12, Olympus, Japan). The amount of brown color indicates the intensity of distribution of nACHR protein subunits, α7 and α4, expression in the testes parenchyma. ImageJ software (version 1.48, National Institutes of Health, Bethesda, MD, USA) was applied to measure the average staining intensity in the sections. The intensity of the brown color was converted into optical density (OD). The following formula was used to calculate OD using average staining intensity. The maximum intensity in RGB (Red-Green-Blue) figures is 255 (18, 19).

$$OD = \log\left(\frac{\text{Max intensity}}{\text{Mean intensity}}\right)$$

Statistical analysis

Statistical computations were performed using Graph Pad Prism software version 8 (GraphPad Prism Software Inc., San Diego, CA, USA). Statistical significance between different groups were compared using non-parametric Kruskal-Wallis test. Results were considered mean ± SEM with the significance level at $P<0.05$.

Results

Expression of the α4 nicotinic acetylcholine receptor in the seminiferous tubules

A significant reduction (P=0.0031) was observed in the expression of α4nACHR protein subunit in the seminiferous tubules in the MSG group in comparison with the control group. In the vitamin C treatment group, the distribution of α4nACHR protein subunit were significantly elevated in comparison with the MSG group (P=0.0080). In the vitamin C+MSG group, the augmented distribution of α4nACHR protein subunit was not remarkably different from the MSG group (P=0.9999).

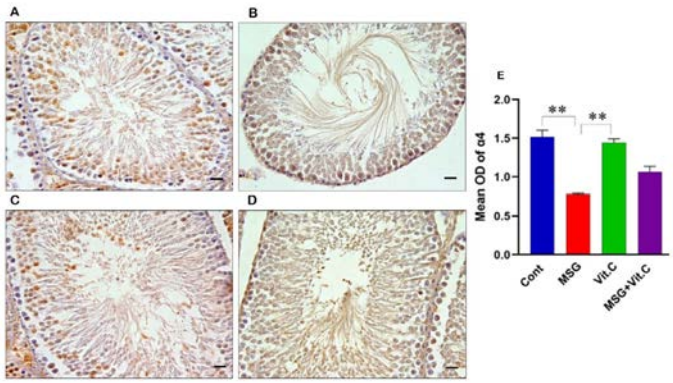

Fig.1: Expression of α4 nACHR in the seminiferous tubules in different groups. The brown color (dots and spots) indicates the immunoreactivity of α4 nACHR protein subunit expression in the seminiferous tubules of **A.** Control, **B.** MSG, **C.** Vit. C, and **D.** MSG+Vit. C groups. **E.** OD of α4 nACHR protein subunit in all groups. All data were considered as mean ± SEM values (Cont vs. MSG, **; P=0.0031, and Vit. C vs. MSG, **; P=0.0080) (scale bar: 100 µm). nACHR; Nicotinic acetylcholine receptor, MSG; Monosodium glutamate, Vit. C; Vitamin C, OD; Optical density, and Cont; Control.

Expression of the α7 nicotinic acetylcholine receptor in the seminiferous tubules

The results obtained in the current study showed that a significant decline in the expression and OD of the α7 nACHR protein subunit was recognized in the MSG group in comparison with the Control group (P=0.0017). In the vitamin C treatment group, expression and OD of the α7 nACHR protein subunit increased considerably in comparison with the MSG group (P=0.0385). In the vitamin C+MSG treatment group, the distribution and OD of α7nACHR protein subunit increased, but there was not a considerable difference between this group and the MSG group (P=0.5851, Fig.2, A-E).

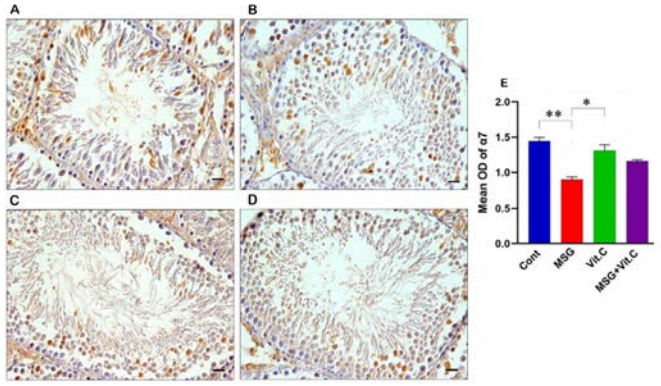

Fig.2: Expression of α7 nACHR in the seminiferous tubules. The brown color (dots and spots) demonstrates the immunoreactivity of α7 nACHR protein subunit expression in the seminiferous tubules of **A.** Control, **B.** MSG, **C.** Vit. C, and **D.** MSG+Vit. C groups. **E.** OD of α7 nACHR protein subunit in all groups. Results are presented as mean ± SEM values (Cont vs. MSG, **; P=0.0017, and Vit. C vs. MSG, *; P=0.0.0385) (scale bar: 100 µm). nACHR; Nicotinic acetylcholine receptor, MSG; Monosodium glutamate, Vit. C; Vitamin C, OD; Optical density, and Cont; Control.

Expression of the α4 nicotinic acetylcholine receptor in the intertubular connective tissue

According to Figure 3, the lowest distribution and OD of α4 nACHR protein subunit were indicated significantly in the MSG group compared to other groups (P=0.0009). In the MSG group, the distribution, and OD of α4 nACHR protein subunit elevated remarkably, and there was a meaningful difference between the vitamin C and MSG groups (P=0.0234). There was not a meaningful difference in the OD of the α4 nACHR protein between the MSG+vitamin C and MSG groups (P=0.9999, Fig.3E).

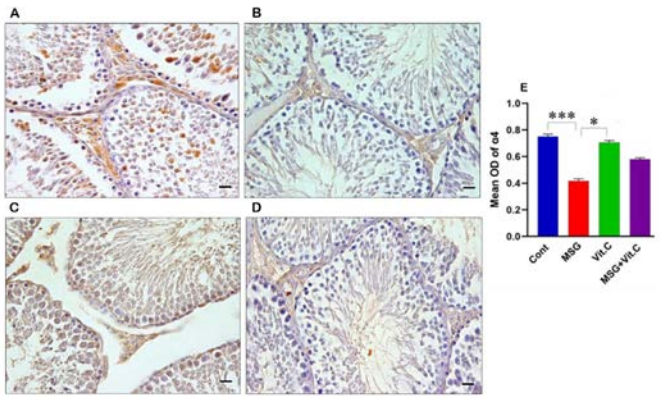

Fig.3: Expression of the α4 nACHR in the intertubular connective tissue. The brown color (dots and spots) between seminiferous tubules indicates the immunoreactivity of the α4 nACHR protein subunit of A. Control, B. MSG, C. Vit. C, and D. MSG+Vit. C groups. E. OD of α4 nACHR protein subunit in all groups. Results are presented as mean ± SEM values. (Cont. vs. MSG, ***; P=0.0009, and Vit. C vs. MSG, *; P=0.0234) (scale bar: 100 µm). nACHR; Nicotinic acetylcholine receptor, MSG; Monosodium glutamate, Vit. C; Vitamin C, OD; Optical density, and Cont; Control.

Expression of the α7 nicotinic acetylcholine receptor in the intertubular connective tissue

The control group had the highest distribution of the α7nACHR protein subunit, and the MSG group had the lowest distribution of the α7nACHR protein subunit (Cont. vs. MSG, P=0.0025). In the vitamin C treatment group, α7nACHR protein subunit expression significantly

increased in comparison with the MSG group (P=0.0097). There was no significant difference in the expression of the α7nACHR protein subunit in the MSG+vitamin C group in comparison with the MSG group (P=0.9999, Fig.4A-E).

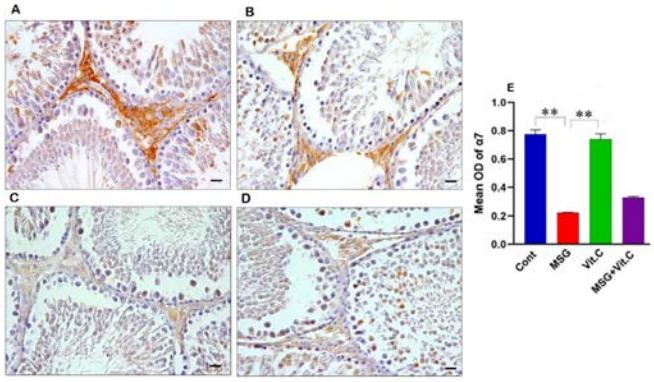

Fig.4: Expression of the α7 nACHR in the intertubular connective tissue. The brown color (dots and spots) between seminiferous tubules shows the immunoreactivity of the α7 nACHR protein subunit of **A.** control, **B.** MSG, **C.** Vit. C, and **D.** MSG+ Vit. C groups. E. α7 nACHR protein subunit in all groups. Results are presented as mean ± SEM values. (Cont. vs. MSG, **; P=0.0025, and Vit. C vs. MSG, **; P=0.0.0097) (scale bar: 100 µm). nACHR; Nicotinic acetylcholine receptor, MSG; Monosodium glutamate, Vit. C; Vitamin C, OD; Optical density, and Cont; Control.

Discussion

The present study evaluated the nACHR subunits, α7 and α4, protein expression in the testis of MSG treated rats, and also the vitamin C protective role. Our results revealed that expression these subunits of the nACHR protein in the seminiferous tubules and interstitial tissue of the MSG group were significantly lower than in our other groups. Also, the vitamin C was able to prevent the decrease in the expression of nACHRs protein subunits in our rat testis parenchyma, but not significantly.

The MSG has been shown to have destructive effects on the testicular tissue. It can reduce the weight of the testis (20, 21) and sperm motility, also induce oligozoospermia, testicular atrophy, and hemorrhage, and change the structure of the testis, sperm morphology, and sperm cell population (12, 21, 22). Also, the germinal epithelial thickness decreases in adult Wistar rats because of using MSG (11, 12, 21, 22). Of course, all of the complications mentioned above depend on the duration, course, dose, induction method of treatment with MSG, and species studied (23). It has been proven that testicular toxicity in the MSG group can be related to Ca^{2+} overloading (24), reactive oxygen species (ROS) production in the testis and decreased antioxidant activities (11, 12, 21, 22), increased malondialdehyde (MDA) and apoptosis (11,12, 25).

As we reported previously, the MSG caused testicular tissue germ cells apoptosis (12). It seems that the MSG probably reduces the number of testicular cells and subsequently the number of nicotinic receptors through apoptosis. In this line, John et al. confirmed that the MSG usage increases Ca^{2+} flow in the neuronal cells, enhances the level of inflammatory cytokines, ROS production, Bax, Bcl-2 and caspase-3 expression, and neuronal apoptosis in the brain tissue, which occurs through glutamate receptors (26). Testicular tissue, like the brain, has many glutamate receptors. Therefore, the MSG can affect the testis by the same mechanisms, apoptosis.

Furthermore, the MSG reduces the scavengers of free radicals, including antioxidant enzymes, glutathione, and ascorbic acid. These scavengers can protect cell membranes from lipid peroxidation and oxidative damages (11). Ismail El-Sawy et al. (27) showed that MSG decreases levels of testosterone and luteinizing hormone (LH) in the serum, and antioxidant activities in the testis, also disturb sperm profiles. Therefore, The destruction of cell membranes, apoptosis, and cells reduction in the seminiferous tubules and interstitial tissue can explain the decrease of nACHRs α7 and α4 subunits in the MSG administrated group.

Another explanation for the reduction of the nACHRs expression level in the MSG group is that it has a protective effect on the spermatogonia cell line and Leydig cells. Administered MSG can hyper-activate the glutamate receptors in spermatogonia cells, increasing intracellular Ca^{2+} flow, which lastly induces apoptosis and sloughing of spermatogenic cells into the seminiferous lumen (28). In such cases as a compensatory mechanism, nACHRs are likely to be reduced to prevent intracellular Ca^{2+} overload and induction of excessive apoptosis.

It is suggested that nACHRs can influence the differentiation, growth, maturation, and morphogenesis process of different types of testis cells (3). The α7 subunit of this protein deficiency in the mouse sperm induces impaired motility and reduced hyperactivation. On the other hand, studies reported that the MSG may alter normal sperm morphology and impair fertility (20, 21).

In this regard, we think that this phenomenon occurs through reducing nACHRs subunits, α7 and α4, expression.

Our study showed that these subunits, α7 and α4, expression reduced in the interstitial testicular tissue of the MSG-treated group. It can be related to apoptosis and reduction of Leydig cells in the interstitial tissue of the testis, as reported in 2019 by Shima (29). Therefore MSG probably through increased ROS and decreased testosterone can be stopped spermatogenic cell maturation and nACHRs subunits, α7 and α4, expression. This issue can be another explanation for the reduction of the mentioned receptors in the interstitial tissue. Inoue et al. showed the MSG intake increases intracellular and mitochondrial Ca^{2+} flow which induces the ROS production. ROS induces a lipid peroxidation process that damages the cell membranes and DNA in the Leydig cells (30). Also, ROS induces disruptions in the electron chain function of mitochondria. Lipid peroxidation process disarranges membrane permeability and ATP production, subsequently destroying the Leydig cells (29), it issue can lead to the reduction of nicotinic receptors in the testicular

interstitial tissue. Also, the loss of Leydig cells results in testosterone secretion decrease in the testis tissue (31). In addition to the testis, the glutamate receptors are abundant in the hypothalamus. ROS production in the hypothalamus decreases GnRH secretion and subsequently the LH secretion. These events disrupt Leydig cells' stimulation for secreting testosterone, and finally, reduce testosterone level (7, 32, 33). It has been proven that a testosterone level decrease can arrest the maturation of spermatogenesis and the protein expression of nACHRs (6, 11). Schirmer et al. (3) suggested the vital role of α4 and α7 subunits in the Leydig cells for androgen secretion.

In the present study, we observed that the vitamin C can prevent the harmful effects of MSG on the testicular tissue and increase nACh receptors, but these changes were not significant. Vitamin C is known as a powerful antioxidant, it is an electron donor, and this property causes all its known functions (34). Its double-bond electrons can transfer to oxidant molecules. Thus, vitamin C can regenerate active species involved in many diseases. It has been proven that MSG reduces the scavengers of free radicals, including ascorbic acid (11). Thus, vitamin C can compensate for this deficiency and reduce MSG complications. In contrast to the present results, Arzuaga et al. (35) and Rahayu et al. (33) stated that the composition of "Marsilea Crenata" can inhibit the MSG's harmful effects and preserve the Leydig and spermatogenic cells. They attributed this property to vitamin C existing in Marsilea Crenata. In our study, vitamin C in the MSG+vitamin C group probably can preserve the Leydig and spermatogenic cells. Thus, the expression of receptors improved in the MSG+vitamin C group (not meaningfully). In addition, vitamin C participates in the synthesis of L-carnitine. This compound plays a critical role in energy output by conveying fatty acids to the mitochondria (35, 36). Koohpeyma et al. (37) concluded that L-carnitine ameliorates MSG's renal damage through its antioxidant and anti-apoptotic effects. However, the results of this project showed that vitamin C had no significant protective effect against the MSG toxicity which is in agreement with Uzun et al. (38) and Zhou et al. (39) studies that reported that vitamin C could not improve testicular toxicity. Reasons such as choosing the correct dose of vitamin, the method of administration, and the route of vitamin absorption can be effective in this result. In addition, there is a possibility that MSG causes toxicity through pathways different from apoptosis and induction of oxidative stress and vitamin C cannot intervene in these pathways. However, these pathways need to be further investigated.

The strengths of this study can be mentioned in the investigation of nicotinic receptors which undergo changes during MSG consumption. One of the limitations of this study is the lack of testing of testosterone and LH serum levels. Also, if the treatment was continued for 64 days, we could check the sperms in the epididymis and get a more complete result. It is hoped that researchers will pay attention to the important role of nACh receptors in future studies.

Conclusion

Summarily, MSG decreased the expression levels of the nACHR subunits, α7 and α4, in the seminiferous tubules and interstitial testicular tissue, while the vitamin C administration increased the expression levels of these subunits in the testis tissue. The results of this study along with the results of other studies suggest being controlled and managing the use of MSG in the food industry.

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Authors' Contributions

R.B., N.Gh., N.A., F.R.A.; Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing draft, Review, and Editing. All authors read and approved the final manuscript.

References

1. Dineley KT, Pandya AA, Yakel JL. Nicotinic ACh receptors as therapeutic targets in CNS disorders. Trends Pharmacol Sci. 2015; 36(2): 96-108.
2. Wessler IK, Kirkpatrick CJ. Non-neuronal acetylcholine involved in reproduction in mammals and honeybees. J Neurochem. 2017; 142 Suppl 2: 144-150.
3. Schirmer SU, Eckhardt I, Lau H, Klein J, DeGraaf YC, Lips KS, et al. The cholinergic system in rat testis is of non-neuronal origin. Reproduction. 2011; 142(1): 157-166.
4. Hashem HE, El-Din Safwat MD, Algaidi S. The effect of monosodium glutamate on the cerebellar cortex of male albino rats and the protective role of vitamin C (histological and immunohistochemical study). J Mol Histol. 2012; 43(2): 179-186.
5. Iamsaard S, Sukhorum W, Samrid R, Yimdee J, Kanla P, Chaisiwamongkol K, et al. The sensitivity of male rat reproductive organs to monosodium glutamate. Acta Med Acad. 2014; 43(1): 3-9.
6. Alalwani AD. Monosodium glutamate induced testicular lesions in rats (histological study). Middle East Fertil Soc J. 2014; 19(4): 274-280.
7. Haddad M, Esmail R, Khazali H. Reporting the effects of exposure to monosodium glutamate on the regulatory peptides of the hypothalamic-pituitary-gonadal axis. Int J Fertil Steril. 2021; 15(4): 246-251.
8. Kayode OT, Rotimi DE, Kayode AAA, Olaolu TD, Adeyemi OS. Monosodium glutamate (MSG)-induced male reproductive dysfunction: a mini review. Toxics. 2020; 8(1): 7.
9. Kayode OT, Rotimi DE, Olaolu TD, Adeyemi OS. Ketogenic diet improves and restores redox status and biochemical indices in monosodium glutamate-induced rat testicular toxicity. Biomed Pharmacother. 2020; 127: 110227.
10. Kayode O, Kayode A, Mgbojikwe I, Rotimi D. Effect of ketogenic diet on monosodium glutamate-induced uterine fibroids in female wistar rats. J Babol Univ Med Sci. 2021; 23(1): 1-8.
11. Sarhan N. The ameliorating effect of sodium selenite on the histological changes and expression of caspase-3 in the testis of monosodium glutamate-treated rats: light and electron microscopic study. J Microsc Ultrastruct. 2018; 6(2): 105-115.
12. Anbarkeh FR, Baradaran R, Ghandy N, Jalali M, Nikravesh MR, Soukhtanloo M. Effects of monosodium glutamate on apoptosis of germ cells in testicular tissue of adult rat: An experimental study. Int J Reprod Biomed. 2019; 17(4): 261-270.
13. Gill SS, Mueller RW, McGuire PF, Pulido OM. Potential target sites in peripheral tissues for excitatory neurotransmission and excitotoxicity. Toxicol Pathol. 2000; 28(2): 277-284.
14. Husarova V, Ostatnikova D. Monosodium glutamate toxic effects and their implications for human intake: a review. JMED Research. 2013; 1-12.

15. Zhang X, Zhou C, Cheng W, Tao R, Xu H, Liu H. Vitamin C protects early mouse embryos against juglone toxicity. Reprod Toxicol. 2020; 98: 200-208.
16. Shen L, Tang X, Wei Y, Long C, Tan B, Wu S, et al. Vitamin E and vitamin C attenuate Di-(2-ethylhexyl) phthalate-induced blood-testis barrier disruption by p38 MAPK in immature SD rats. Reprod Toxicol. 2018; 81: 17-27.
17. Rahimi Anbarkeh F, Nikravesh MR, Jalali M, Sadeghnia HR, Sargazi Z. The effect of diazinon on cell proliferation and apoptosis in testicular tissue of rats and the protective effect of vitamin E. Int J Fertil Steril. 2019;13(2): 154-160.
18. Baradaran R, Anbarkeh FR, Delavar A, Khorasgani EM, Rahimian N, Abbasi Y, et al. Hippocampal asymmetry and regional dispersal of nAChRs alpha4 and alpha7 subtypes in the adult rat. J Chem Neuroanat. 2021; 116: 101977.
19. Ruifrok AC, Johnston DA. Quantification of histochemical staining by color deconvolution. Anal Quant Cytol Histol. 2001; 23(4): 291-299.
20. Nayanatara AK, Vinodini NA, Domadar G, Ahemed B, Ramaswamy CR, Shabarianth, et al. Role of ascorbic acid in monosodium glutamate mediated effect on testicular weight,sperm morphology and sperm count, in rat testis. J Chinese Clin Med. 2008; 3: 1-5.
21. Nosseir NS, Hassan M, Ali M, Ebaid HM. A histological and morphometric study of monosodium glutamate toxic effect on testicular structure and potentiality of recovery in adult albino rats. Res J Biol. 2012; 2(2): 66-78.
22. Zaheer K, Humayoun Akhtar M. An updated review of dietary isoflavones: Nutrition, processing, bioavailability and impacts on human health. Crit Rev Food Sci Nutr 2017; 57(6): 1280-1293.
23. Samuels A. Dose dependent toxicity of glutamic acid: a review. Int J Food Prop. 2020; 23(1): 412-419.
24. Bojanić V, Najman S, Veličkov A, Bojanić N, Milenković J. Diltiazem prevents monosodium glutamate toxicity in the rat testes. Acta Medica Median. 2020; 59(3): 20-26.
25. Barati E, Nikzad H, Karimian M. Oxidative stress and male infertility: current knowledge of pathophysiology and role of antioxidant therapy in disease management. Cell Mol Life Sci. 2020; 77(1): 93-113.
26. John E, Mishra B, Tripathi AS, Aleesha R. Protective effect of Celastrus paniculatus on cognitive function in glutamate-induced brain-injured mice by reducing the intracellular influx of Ca. Int J Dev Neurosci. 2022; 82(4): 331-338.
27. Ismail El-Sawy HB, Soliman MM, El-Shazly SA, Ali HAM. Protective effects of camel milk and Vitamin E against monosodium glutamate induced biochemical and testicular dysfunctions. Prog Nutr. 2018; 20(1): 76-85.
28. Hamza RZ, Al-Harbi MS. Monosodium glutamate induced testicular toxicity and the possible ameliorative role of vitamin E or selenium in male rats. Toxicol Rep. 2014; 1: 1037-1045.
29. Shima Y. Development of fetal and adult Leydig cells. Reprod Med Biol. 2019; 18(4): 323-330.
30. Inoue M, Baba T, Morohashi KI. Recent progress in understanding the mechanisms of Leydig cell differentiation. Mol Cell Endocrinol. 2018; 468: 39-46.
31. Ahmed RR, Abdul-Hamid M, Galaly SR, Hamdalla H. Monosodium glutamate-induced liver microscopic and biochemical changes in male rats, and the possible amendment of quercetin. Egypt J Zool. 2019; 71(71): 44-55.
32. von Rintelen K, Arida E, Häuser C. A review of biodiversity-related issues and challenges in megadiverse Indonesia and other Southeast Asian countries. Res Ideas Outcomes. 2017; 3: e20860.
33. Rahayu S, Annisa R, Anzila I, Christina YI, Soewondo A, Marhendra APW, et al. Marsilea crenata ethanol extract prevents monosodium glutamate adverse effects on the serum levels of reproductive hormones, sperm quality, and testis histology in male rats. Vet World. 2021; 14(6): 1529-1536.
34. Pehlivan FE. Vitamin C: an antioxidant agent. In: Hamza AH, editor. Vitam C. 2017; 23-35.
35. Arzuaga X, Smith MT, Gibbons CF, Skakkebæk NE, Yost EE, Beverly BEJ, et al. Proposed key characteristics of male reproductive toxicants as an approach for organizing and evaluating mechanistic evidence in human health hazard assessments. Environ Health Perspect. 2019; 127(6): 065001.
36. Foyer CH, Kyndt T, Hancock RD. Vitamin C in plants: novel concepts, new perspectives, and outstanding issues. Antioxid Redox Signal. 2020; 32(7): 463-485.
37. Koohpeyma F, Siri M, Allahyari S, Mahmoodi M, Saki F, Dastghaib S. The effects of L-carnitine on renal function and gene expression of caspase-9 and Bcl-2 in monosodium glutamate-induced rats. BMC Nephrol. 2021; 22(1): 162.
38. Uzun FG, Kalender S, Durak D, Demir F, Kalender Y. Malathion-induced testicular toxicity in male rats and the protective effect of vitamins C and E. Food Chem Toxicol. 2009; 47(8): 1903-1908.
39. Zhou J, Sun Q, Yang Z, Zhang J. The hepatotoxicity and testicular toxicity induced by arecoline in mice and protective effects of vitamins C and e. Korean J Physiol Pharmacol. 2014; 18(2): 143-148.

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The Relationship between Body Mass Index, Metal Elements, and Antioxidant Capacity of Semen on The Human Sperm Chromatin

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Abstract

Background: Sperm chromatin abnormalities are defects in nuclear maturation and DNA integrity. These defects originated from defective spermatogenesis due to a lack of DNA repair during chromatin remodeling. Changes in semen elements can cause damage to chromatin. There is little information about the relationship between changes in trace metal elements and total antioxidant capacity (TAC) with sperm chromatin damage. The present study was conducted to determine the relationship between Selenium (Se), Iron (Fe), Zinc (Zn), Copper (Cu) and the TAC of semen with the status of human sperm chromatin.

Materials and Methods: In this experimental study, semen samples (n=30) were collected from healthy men referred to Kermanshah Motazadi Hospital and stored in liquid nitrogen; after thawing and centrifugation, sperm were separated. The atomic absorption method was used to measure the concentration of metal elements. The TAC was evaluated using the ferric-reducing antioxidant capacity of the plasma method. Furthermore, the integrity of sperm chromatin was measured using the sperm DNA fragmentation (SDF) method.

Results: The status of sperm chromatin had a non-significant correlation with body mass index (BMI, P=0.25, r=0.21) and a non-significant negative correlation with sperm count (P=0.71, r=-0.71) and motility (P=0.75, r=0.61). In addition, there was no significant relationship between sperm chromatin and the TAC of semen (P=0.92, r=0.01). Additionally, there was no significant correlation between Se, Zn, or Cu concentration (P>0.05) and Fe concentration, which had a partially positive relationship with the chromatin state of sperm (P=0.24, r=0.20).

Conclusion: The trace metal elements in the seminal fluid did not play a significant role in the status of sperm chromatin.

Keywords: Antioxidant Capacity, DNA Fragmentation, Infertility, Semen, Sperm

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Introduction

Infertility is the inability of a couple to get pregnant with regular and unprotected intercourse after one year (1). Despite the development of medical science, infertility is still a significant problem; about 10-15% of couples have this problem. Infertility is divided into two primary and secondary groups; the prevalence of primary infertility is higher in most parts of the world. The causes of infertility vary from one population to another and are affected by socio-cultural differences and the prevalence of sexual transmission. In developed industrialized countries, about 15% of couples experience primary or secondary infertility, and almost half will never succeed in having the desired number of children (2, 3).

Disturbance in sperm production and function and damage in the process of spermatogenesis is one of the most common causes of male infertility. In addition, oxidative stress in the reproductive system affects the ability of sperm to fertilize. Furthermore, there is mounting proof that sperm function is considerably compromised by an increase in reactive oxygen species (ROS). Through pathways including the generation of DNA damage, apoptosis, and peroxidative damage to the sperm plasma membrane, these deficiencies have led to male infertility.

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To guarantee proper physiological activity and avoid pathological harm to the spermatozoa, ROS must be kept at the proper levels (4). Trauma and some therapeutic drugs disrupt sperm production and damage its DNA, leading to male infertility (3, 5). In addition, sperm exposure to environmental toxins and pollutants, drugs, ultraviolet rays, smoking, febrile illness, varicocele, and aging are also essential factors affecting sperm chromatin quality (6).

There is a certain amount of DNA breakage in men's sperm, and its level varies from one sperm to another. The integrity of sperm DNA affects the couple's fertility (7) and helps to predict the probability of pregnancy and its successful outcome. In addition, factors such as the concentration of metal elements can affect sperm chromatin damage (8). Metal elements such as Fe, Zn, Cu, and Se are present in very low amounts in the body, and due to their activity at the molecular level, they play an important role in the reproductive process of men (9).

Zn is the most abundant element in human tissues after Fe, and the World Health Organization (WHO) estimates that one-third of the world's population has Zn deficiency. Zn is secreted from the prostate gland, and its amount in seminal plasma usually indicates the secretory function of the prostate. A decrease in semen Zn concentration may be due to insufficient intake, decreased absorption, increased losses, or increased sperm excretion (9). Fe and its compounds are not toxic to humans and animals. Its numerous disorders can appear due to pathological conditions or long-term consumption of high doses of Fe in the regulating mechanism of its absorption. Fe has an essential role in vital biochemical activities (10), and its increase can negatively affect the morphology of sperm. It can also induce lipid peroxidation and inhibit sperm motility. These metals have negative health effects on people, including male infertility. Male fertility is severely impacted by exposure to harmful contaminants such as heavy metals, according to studies done in a variety of *in vitro* and *in vivo* models (11, 12).

Fe may mediate the effects of oxidative damage and play an essential role in spermatogenesis and male infertility. At the same time, Fe's absorption and metabolic function depend on the impact of many other elements, especially the antagonistic activity of cadmium, manganese, lead, Zn, and Cu. Therefore, although Fe and its compounds are not toxic to humans and animals, its overload can increase sperm DNA damage (13). Fe and Cu are necessary to maintain the function of living organisms. Excessive absorption of them causes oxidative damage to testicular tissue (14).

The increase of these ions can decrease the number and viability of sperm. Cu sulphate reduces the concentration of FSH, LH, and testosterone through physiological mechanisms and dysfunction of the gonads (15). Zn is present in sperm and seminal plasma, and its concentration is significantly higher than in the other body fluids. It directly affects the formation, motility, and morphology of sperm and may help fertility through its positive effect on spermatogenesis (16). Zn plays an important role in capacitation and may be a regulatory factor in spermatogenesis process. Its deficiency may lead to degenerative changes in the spermatogenesis lineage (17). This element affects the stability of sperm, also plays a role in forming free oxygen radicals, and can regulate the capacity and reaction of acrosomes (18). However, there is little information about the role of Zn in human semen.

Se has several important functions in human health and is an essential element for the normal function of sperm and the process of spermatogenesis. Its administration to infertile patients significantly increases sperm motility. It has a protective effect against oxidative damage to human sperm DNA (19). Antioxidants are compounds that can protect biological systems against the harmful effects of ROS. The increase in ROS production because of oxidative stress causes a decrease in the fertility capacity of sperm, as a result, damage to the cell membrane, motility, and morphological abnormalities such as twisted tail, and damage to the spermatozoa (20).

Oxidative stress in human sperm is associated with reduced its motility, viability, and weakness in sperm-to-ovum integration (21). Considering the concentration of metal elements influences the integrity of the sperm structure, on the other hand, the results of previous research have been contradictory in this connection. The present study was conducted to determine the relationship between Selenium (Se), Iron (Fe), Zinc (Zn), Copper (Cu) and the TAC of semen with the status of human sperm chromatin.

Materials and Methods

This experimental study was done after the approval of the Medical Ethics Committee of Kermanshah University of Medical Sciences (IR.KUMS.REC.1399.659) and obtaining informed consent and providing sufficient explanations to the patients, refers to 30 semen samples from men with normozoospermia. It was carried out at the infertility clinic of Motazadi Hospital in 2021. Inclusion criteria include age group 20-50 years, not having systemic diseases such as diabetes, blood pressure, heart and liver diseases. The participants in this study were anatomically healthy and did not intake any diet supplementation during the last three months, had both testicles, no history of smoking or drugs or intense exercise, without any special drugs, and refrained from sexual intercourse 2-3 days before giving the sample. Exclusion criteria included the absence of any of the items included in the study.

Semen preparation

Semen samples were collected in the clinic by self-ejaculation in sterile containers with wide openings. After liquefaction: their concentration, volume, and morphology were determined and centrifuged at 1500 rpm for 10 minutes to separate seminal plasma and sperm. Then, the supernatant was separated and divided into two parts, and

stored in a cryotube in liquid nitrogen. In addition, the sediment containing sperm, after washing twice with PBS (pH=7.4), was re-centrifuged at 400 rpm for 5 minutes and used for DNA fragmentation assay.

An atomic absorption device (VGA 77; Varian Inc., Mulgrave, Australia) measured the level of metal ions Fe, Zn, Cu, and Se elements of the samples. Samples were prepared by the Zanao et al. method (22). 200 µl of the sample was mixed with 800 µl of 2% nitric acid preparation solution and 5% Triton-X-100. Finally, 20 µl of the obtained solution and 10 µl of the moderator solution containing palladium 0.05% and magnesium nitrate 0.03% were injected into the device. The obtained result was expressed in mg/ml units.

FRAP assay

The ferric reducing antioxidant power (FRAP) method is used to determine the reducing ability of biological samples (total antioxidant capacity/TAC). In this method, colourless ferric complex (Fe^{3+} 2, 4, 6-tripyridyltriazine [TPTZ]) is reduced into a violent blue ferrous complex which indicates the reducing properties. Working FRAP reagent was prepared by mixing 2.5 ml TPTZ dissolved in HCl (40 mM), 2.5 ml ferric chloride (20 mM in water) and 25 ml acetate buffer (300 mM, pH=3.6). Then the mixture was heated to 37˚C for 10 minutes before use. Next 200 µl of samples were added to 1.5 ml of working FRAP solution and placed in a water bath (37˚C) for 30 minutes. The absorption at 593 nm was measured and recorded by spectrophotometer (Jenway 3620D, England). Standard solutions were used at concentrations of 0, 125, 250, 500, and 1000 µM from $FeSO_4$. $7H_2O$. Each sample was repeated three times (23).

DNA fragmentation test

The sperm DNA fragmentation (SDF) amount was checked with SDF kits manufactured by Jina Teb Company (Tehran, Iran). The sediment of the cellular part was washed with PBS and diluted with PBS to the amount of 5-10 million sperm per ml. Then the prepared samples were transferred into gel microtubes and kept in liquid nitrogen until the test was performed. Microtubes containing gel were placed at 95-100 degrees for 5 minutes until complete melting, and 50 µl of the sample was added to the microtube containing the gel and mixed completely. Then 20 µL was taken, placed on a special slide, and immediately covered with a coverslip. The prepared slide was incubated for 4 minutes in the temperature was kept at 2-8 degrees Celsius in the refrigerator.

Then the coverslip was slowly removed from the slide, and solution A of the kit was added to it and placed in the dark at room temperature for 7 minutes. Solution A was slowly drained, and solution B was added and kept at room temperature for 15 minutes. Next, solution B was slowly drained, and distilled water was added and placed at ambient temperature for 3 minutes. At least on each slide, a light microscope (1000× magnification) counted sperm. Finally, the number of sperms with a large or medium halo (normal) and with a small or no halo (broken) was reported as a percentage (24).

Statistical analysis

Data were analyzed with SPSS software (version 16, SPSS Inc., USA), their normality was assessed by Kolmogorov-Smirnov test and the results were presented as mean ± SD. Correlation between variables was determined using the Pearson correlation coefficient, and $P<0.05$ was considered significant.

Results

The participants in this study had an average age of 33.63 ± 1.84 (18-48) years and an average height and weight of 178.73 ± 60.1 cm and 79.9 ± 14.5 kg, respectively. The average body mass index (BMI) of these people was 25.01 ± 22.26. Sperm count and motility were 50 ± 30.3×106 and 22.83 ± 9.83 µM/seconds, respectively (Table 1). Furthermore, SDF and TAC were 21.33% ± 10.57 and 310.78 ± 215.36 µMol/L. The levels of Cu, Zn, Fe, and Se were shown in Table 1.

Table 1: Descriptive indicators of the study values are shown as mean ± SD

Parameter	Mean ± SD	Min-Max
Age (Y)	33.63 ± 1.84	18-48
Height (cm)	178.73 ± 60.1	160-192
Wieght (cm)	79.9 ± 14.55	60-115
BMI (kg/m^2)	25.01 ± 22.26	18.9-31.8
Sperm morphology (%)	7.3 ± 8.56	2-10
Sperm motility (µM/seconds)	22.83 ± 9.83	20-30
Sperm count (M/ml)	50 ± 30.3	20-100
SDF (%)	21.33 ± 10.57	5-50
TAC (µMol/L)	310.78 ± 215.36	129.6-521.7
Cu (µMol/L)	0.68 ± 0.001	0.1-1.5
Zn (µMol/L)	8552.63 ± 714.97	1951-16425
Se (µMol/L)	76.66 ± 50.62	31.98-141.24
Fe (µMol/L)	21.47 ± 18.42	4.06-72.54

BMI; Body mass index, SDF; Sperm DNA fragmentation, TAC; Total antioxidant capacity, Cu; Copper, Zn; Zinc, Se; Selenium, and Fe; Iron.

The relationship between the studied parameters is shown in Figure 1. There was a weak positive relationship between SDF and BMI, which was not significant (P=0.25, r=0.21) (Table 2). Furthermore, there was a moderate negative correlation between SDF and sperm count (P=0.71, r=-0.71), morphology, and motility (P= 0.75, r-0.61), but these correlations were not statistically significant. The data showed that there was no relationship between SDF and TAC (P=0.92, r=0.01) (Table 2). There is no significant correlation between SDF with the levels of semen Fe, Zn, Cu, and Se (P>0.05, Table 3).

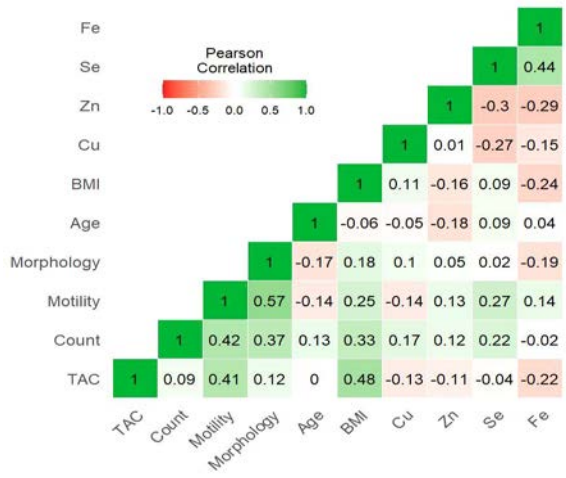

Fig.1: Statistical analysis using Pearson correlation test. The relationship between the parameters examined in this study shows the value of the correlation coefficient (r), while those that have reached statistical significance (P<0.05).

Table 2: SDF correlation with BMI, TAC, and sperm parameter

Parameter	Pearson correlation with SDF	P value	Number
BMI (kg/m^2)	0.21	0.25	30
TAC (µMol/L)	0.01	0.92	30
Sperm count (M/ml)	-0.71	0.71	30
Sperm motility (µM/seconds)	-0.61	0.75	30
Sperm morphology (%)	-0.57	0.60	30

Descriptive indicators of the study values are shown as mean ± SD. Data were analyzed by the Pearson correlation coefficient. SDF; Sperm DNA fragmentation, Fe; Iron, Se; Selenium, Zn; Zinc, and Cu; Copper.

Table 3: SDF Correlation with Fe, Cu, Zn, and Se

Parameter	Pearson correlation with SDF	P value	Number
Fe (µMol/L)	0.24	0.20	30
Se (µMol/L)	0.02	0.90	30
Zn (µMol/L)	-0.07	0.25	30
Cu (µMol/L)	0.02	0.76	30

Descriptive indicators of the study values are shown as mean ± SD. Data were analyzed by the Pearson correlation coefficient. SDF; Sperm DNA fragmentation, Fe; Iron, Se; Selenium, Zn; Zinc, and Cu; Copper.

Discussion

The present study investigated the relationship between sperm parameters, metal elements, and the total antioxidant capacity (TAC) of human semen with the state of sperm chromatin. There was a positive correlation between sperm chromatin and BMI, and a negative correlation with sperm count and motility, but there was no correlation with TAC. In addition, there was a weak correlation between this parameter with Fe concentration and no significant correlation with Se, Zn, and Cu concentration. There are conflicting results about the importance of trace elements and oxidative stress in semen. Our understanding in this field is still incomplete and often contradictory and needs to be completed.

In obese people, there is an increase in the permeability of metabolic endotoxemia in the intestine, which causes systemic inflammation and oxidative stress and damages the male reproductive system, thus leading to an increase in ROS production, oxidative stress, and reduction of sperm DNA integrity (24). A large study has investigated this relationship in 3 years in several centers, including 330 men in infertile couples. The uridine end labeling method showed that the amount of sperm DNA damage is increased in obese men (25). Our data showed that there is a negative correlation between SDF and the number and motility of sperm, this negative correlation was confirmed in previous studies, and the results obtained in the study are in line with previous studies because all samples are normozoospermic (26, 27).

In addition, our data showed that there is no significant relationship between SDF and TAC. Semen plasma TAC is measured by the level of enzymatic and non-enzymatic antioxidants. Low levels of TAC in semen may play an important role in male infertility. Although the amount of ROS is regulated by antioxidants, the excessive production of oxidative stress cannot be neutralized by the antioxidant system in infertile men. A major part of sperm DNA damage is related to oxidative stress. The imbalance between superoxide anion and peroxynitrite with antioxidant capacity of infertile men with abnormal sperm parameters is associated with higher SDF (28). The results of previous studies show a significant correlation between SDF and TAC (29-31), which does not match the results of the present study and is probably due to the difference between the sample of healthy people in our study and infertile people in their study.

A comparison of the SDF relationship with Fe, Cu, Zn, and Se showed that there was a weak correlation between Fe concentration and SDF. In addition, there was no significant relationship between Se, Zn and Cu concentration with SDF. Studies report trace elements' effects on human reproduction are ambiguous. A similar study tested the effect of lead (Pb), cadmium (Cd), Cu, Zn, and Se on SDF and showed that SDF is a dynamic process that increases with rising Pb levels in seminal plasma. The amount of Pb and Cd in the semen plasma of infertile men was higher compared to fertile men. The Zn, Cu, and Se level in semen plasma was higher in men with proven fertility than infertile men and had no significant effect on SDF dynamics. The level of Cd did not have a significant effect on the exacerbation of SDF (32). Due to the contradictory results obtained in this field, these studies should be expanded and continued in the future.

Conclusion

The results of this study showed that the metal elements measured in the seminal fluid of normozoospermic individuals did not show a noticeable role in sperm chromatin integrity.

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Authors' Contributions

Sh.R.; Participated in study design and evaluation. N.B.; Data collection. M.R.S.; Contributed extensively to interpreting the data and the conclusion. L.R., N.B.; Wrote the manuscript and editing. M.K.; Conceptualization and validation. All authors read and approved the final version of the manuscript.

References

1. Cui C, Wang L, Wang X. Effects of self-esteem on the associations between infertility-related stress and psychological distress among infertile chinese women: a cross-sectional study. Psychol Res Behav Manag. 2021; 14: 1245-1255.
2. van Roode T, Dickson NP, Righarts AA, Gillett WR. Cumulative incidence of infertility in a New Zealand birth cohort to age 38 by sex and the relationship with family formation. Fertil Steril. 2015; 103(4): 1053-1058. e2.
3. Winters BR, Walsh TJ. The epidemiology of male infertility. Urol Clin North Am. 2014; 41(1): 195-204.
4. Agarwal A, Virk G, Ong C, du Plessis SS. Effect of oxidative stress on male reproduction. World J Mens Health. 2014; 32(1): 1-17.
5. Wong WY, Thomas CM, Merkus JM, Zielhuis GA, Steegers-theunissen RP. Male factor subfertility: possible causes and the impact of nutritional factors. Fertil Steril. 2000; 73(3): 435-442.
6. Aydemir B, Kiziler AR, Onaran I, Alici B, Ozkara H, Akyolcu MC. Impact of Cu and Fe concentrations on oxidative damage in male infertility. Biol Trace Elem Res. 2006; 112(3): 193-203.
7. Rubes J, Selevan SG, Evenson DP, Zudova D, Vozdova M, Zudova Z, et al. Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. Hum Reprod. 2005 Oct;20(10):2776-83.
8. Abou-Shakra FR, Ward NI, Everard DM. The role of trace elements in male infertility. Fertil Steril. 1989; 52(2): 307-310.
9. Marzec-Wróblewska U, Kamiński P, Lakota P, Szymański M, Wasilow K, Ludwikowski G, et al. Zinc and iron concentration and SOD activity in human semen and seminal plasma. Biol Trace Elem Res. 2011; 143(1): 167-177.
10. Papanikolaou G, Pantopoulos K. Iron metabolism and toxicity. Toxicol Appl Pharmacol. 2005; 202(2): 199-211.
11. Al-Fartusie FS, Mohssan SN. Essential trace elements and their vital roles in human body. Indian J Adv Chem Sci. 2017; 5(3): 127-36.
12. Crocetto F, Risolo R, Colapietro R, Bellavita R, Barone B, Ballini A, et al. Heavy metal pollution and male fertility: an overview on adverse biological effects and socio-economic implications. Endocr Metab Immune Disord Drug Targets. 2023; 23(2): 129-146.
13. Perera D, Pizzey A, Campbell A, Katz M, Porter J, Petrou M, et al. Sperm DNA damage in potentially fertile homozygous beta-thalassaemia patients with iron overload. Hum Reprod. 2002; 17(7): 1820-1825.
14. Massányi P, Trandzik J, Nad P, Koreneková B, Skalická M, Toman R, et al. Concentration of copper, iron, zinc, cadmium, lead, and nickel in bull and ram semen and relation to the occurrence of pathological spermatozoa. J Environ Sci Health A Tox Hazard Subst Environ Eng. 2004; 39(11-12): 3005-3014.
15. Huang YL, Tseng WC, Lin TH. In vitro effects of metal ions (Fe^{2+}, Mn^{2+}, Pb^{2+}) on sperm motility and lipid peroxidation in human semen. J Toxicol Environ Health A. 2001; 62(4): 259-267.
16. Chia SE, Ong CN, Chua LH, Ho LM, Tay SK. Comparison of zinc concentrations in blood and seminal plasma and the various sperm parameters between fertile and infertile men. J Androl. 2000; 21(1): 53-57.
17. Andrews JC, Nolan JP, Hammerstedt RH, Bavister BD. Role of zinc during hamster sperm capacitation. Biol Reprod. 1994; 51(6): 1238-1247.
18. Bilaspuri GS, Babbar BK. Effect of albumin and zinc on capacitation and acrosome reaction of buffalo spermatozoa. Indian J Anim Sci. 2007; 77(8): 512-514.
19. Klein EA. Selenium: epidemiology and basic science. J Urol. 2004; 171(2 Pt 2): S50-S53.
20. Ribas-Maynou J, Yeste M. Oxidative stress in male infertility: causes, effects in assisted reproductive techniques, and protective support of antioxidants. Biology (Basel). 2020; 9(4): 77.
21. Alahmar AT. Role of oxidative stress in male infertility: an updated review. J Hum Reprod Sci. 2019; 12(1): 4-18.
22. Zanao RA, Barbosa Jr F, Souza SS, Krug FJ, Abdalla AL. Direct determination of selenium in whole blood by electrothermal atomic absorption spectrometry using W–Rh-coated platform and co-injection of Rh as thermal stabilizer. Spectrochimica Acta Part B: Atomic Spectroscopy. 2002; 57(2): 291-301.
23. Khazaei F, Ghanbari E, Khazaei M. Protective effect of royal jelly against cyclophosphamide-induced thrombocytopenia and spleen and bone marrow damages in rats. Cell J. 2020; 22(3): 302-309.
24. Pearce KL, Hill A, Tremellen KP. Obesity related metabolic endotoxemia is associated with oxidative stress and impaired sperm DNA integrity. Basic Clin Androl. 2019; 29: 6.
25. La Vignera S, Condorelli RA, Vicari E, Calogero AE. Negative effect of increased body weight on sperm conventional and nonconventional flow cytometric sperm parameters. J Androl. 2012; 33(1): 53-58.
26. Dupont C, Faure C, Sermondade N, Boubaya M, Eustache F, Clément P, et al. Obesity leads to higher risk of sperm DNA damage in infertile patients. Asian J Androl. 2013; 15(5): 622-625.
27. Boushaba S, Belaaloui G. Sperm DNA fragmentation and standard semen parameters in algerian infertile male partners. World J Mens Health. 2015; 33(1): 1-7.
28. Gill K, Jakubik J, Rosiak-Gill A, Kups M, Lukaszuk M, Kurpisz M, et al. Utility and predictive value of human standard semen parameters and sperm dna dispersion for fertility potential. Int J Environ Res Public Health. 2019; 16(11): 2004.
29. Park YS, Lee SH, Choi HW, Lee HS, Lee JS, Seo JT. Abnormal human sperm parameters contribute to sperm dna fragmentation in men with varicocele. World J Mens Health. 2018; 36(3): 239-247.
30. Khosravi F, Valojerdi MR, Amanlou M, Karimian L, Abolhassani F. Relationship of seminal reactive nitrogen and oxygen species and total antioxidant capacity with sperm DNA fragmentation in infertile couples with normal and abnormal sperm parameters. Andrologia. 2014; 46(1): 17-23.
31. Kamkar N, Ramezanali F, Sabbaghian M. The relationship between sperm DNA fragmentation, free radicals and antioxidant capacity with idiopathic repeated pregnancy loss. Reprod Biol. 2018; 18(4): 330-335.
32. Sheikh N, Amiri I, Najafi R, Goodarzi MT. The correlation between total antioxidant capacity and nitric oxide concentration in seminal plasma with sperm DNA damage. Afr J Biotechnol. 2010; 9(35): 5739-5745.

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The Correlation of Urine Bisphenol A with Semen Parameters in Men Referred to Infertility Centers: A Cross-Sectional Study

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Abstract

Background: Bisphenol A (BPA) is known as an endocrine disruptor that has harmful effects on general health. It is commonly used in various industrial products. In this study we tried to evaluate the amount of BPA in urine samples of the men referred to an infertility center.

Materials and Methods: The cross-sectional study population consisted of male partners of infertile couples, who were referred to infertility clinic in Mazandaran, a northern state of Iran. Questionnaires included demographic characteristics, medical history, lifestyle factors, physical examinations. A semen sample and a spot urine sample were taken from each participant. In the initial study group of 240 men, 3 groups were excluded, and 122 men remained for the analysis. High-performance liquid chromatography (HPLC) was applied to measure the amount of BPA in the urine samples.

Results: BPA was not detected in about half of the samples (53.3%). Multiple linear regression analysis showed that no significant relationship existed between the urine concentrations of BPA, semen parameters and male reproductive hormones. However, in a comparison with semen parameters in people with detectable urine BPA versus nondetectable ones, an inverse association was noticed with sperm concentration. In other parameters, differences were not significant. Smoking had no effects on sperm parameters, but body mass index (BMI) ≥25 reduced the percentage of normal sperm parameters.

Conclusion: In most participants, urinary BPA was not detected. Probably in this study low environmental exposure to BPA is the cause of lower urine BPA concentrations compared to other industrially developed countries. Therefore, no overall relationship was observed between BPA level and male infertility.

Keywords: Bisphenol A, Male Infertility, Semen Parameters

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Introduction

Endocrine disruptor chemicals (EDCs) are considered as exogenous chemicals, that interfere with hormonal actions (1, 2). Bisphenol A (BPA) is one of the EDCs that is suspected to have great adverse health consequences. BPA is an organic monomer that is widely used as the main and essential ingredient of epoxy resin and polycarbonate plastic. This substance enters the body through dietary and transdermal absorption. Continuous and extensive exposure to BPA, particularly in developed countries, has led to the presence of detectable levels of urine BPA, as certified by biomonitoring studies in humans. Therefore, the possibility of adverse effects of this substance, caused an increase in concerns about human health (3-5). The deferent acknowledge about biological effects of BPA are various. The larger portion of BPA molecule is less biologically active. Glucuronidation causes it to be metabolized into conjugated compounds in the liver, which is less water-soluble and quickly excreted in urine (6). One of the organs likely affected by BPA is the reproductive system. Exposure to BPA may cause poor semen quality (7). Even low dose exposures of this substance cause certain effects on the male reproductive system in animal studies (6).

Evidence shows that BPA affects the onset of meiosis (8, 9). Several recent studies in humans have evaluated BPA exposure and male fertility with inconsistent results. Some studies showed that BPA has negative effect on sperm, so that decrease sperm motility and sperm concentration, and increases abnormal sperm morphology (10-12). In contrast,

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Mendiola et al. (13) showed in 2010 that BPA cannot affect the semen quality. BPA can affect the hypothalamic-pituitary-gonadal axis, which causes hypogonadotropic hypogonadism (14). A study on male rats showed that even low doses of BPA exposure during the perinatal critical period of hypothalamic sexual differentiation caused a delayed onset of puberty (15). Furthermore, ongoing exposure to BPA and similar substances can change the structure of testicular tissue, thus modifying the endocrine glands in the male reproductive system (16). Oxidative stress produced by these chemicals affects the reproductive system and cause a negative effect on the testes and spermatogenesis in male rats (17).

Higher concentrations of BPA and its analogues BPA-B, BPA-F and BPA-S in the early development of male rats may lead to incorrect function of reproductive system (18). However, more studies on human exposure to such chemicals are needed to determine the relevance of these finding to human health. Since BPA is metabolized rapidly and is discarded through urine without evidence of collection in the body (19), we suppose that evaluation of BPA in urine sample prepare a better assessment of exposures than evaluation of these compounds in serum. Also, it has been previously shown that the half-life of BPA in serum or plasma is short and there is a possibility of contamination during sample collection and analysis. Studies that have investigated the role of BPA in male fertility are few and sometimes the outcomes turn out to be different. The present study tends to investigate the amount of urine BPA in infertile men referred to Fatemeh Zahra Infertility Clinic, in Mazandaran/Iran, and evaluate the correlation between urine BPA concentration and semen parameters and reproductive hormones such as testosterone, follicular-stimulating hormone (FSH), luteinizing hormone (LH), Prolactin, thyroid-stimulating hormone (TSH), and dehydroepiandrosterone sulfate (DHEA-S).

Materials and Methods

The cross-sectional study population consisted of male partners from infertile couples with unknown causes of infertility, attending our infertility clinic for diagnosis. The average sperm concentration of the samples from these men were >15 million/ml. These subjects were recruited from June 2020 through October 2020 (simultaneously with Covid-19 pandemic) in north of Iran, Mazandaran. In brief, all participants with no evidence of Covid-19 infection at the time of the interview prior to enrollment, signed a written informed consent form. To collect information, each participant filled a questionnaire that included demographic characteristics, medical history, and lifestyle factors. Also, physical examinations such as height, weight, waist and wrist circumferences of the men were measured. The lifestyle factors, including alcohol intake, smoking cigarette and the use of plastic containers in daily eating, were evaluated. Participants were given containers to collect urine samples at the beginning of the morning before any medical intervention. Also, each semen sample was obtained through masturbation. The obtained urine samples were transferred into special BPA-free containers in less than 1-hour post-collection and stored at -20ºC until analysis.

The tests to evaluate hormones such as FSH, LH, Prolactin, TSH, DHEA-S and testosterone, which would be performed routinely for male patients in this center, were used in this study. Of the 240 men who were originally interviewed for the study, 3 groups were excluded and were the limitations of our study: 1- having a history of a severe type of coronavirus (COVID-19) disease, that led to hospitalization in the last three months, 2- leaving an incomplete questionnaire with missing information, and 3- having a fear of getting coronavirus disease (COVID-19), during an interview. The remaining 122 eligible men were selected for the study and their data were collected for analysis.

Laboratory methods

To evaluated the optical density of LH, FSH and total testosterone, the wavelengths of 450 to 630 nm of an ELISA reader were used. Coefficients of variation of intra- and inter-assay contain 2.3 and 2.8% for LH, 3.9 and 4.5% for FSH and 5.6 and 6.6% for total testosterone, respecticely. Prolactin concentration was assessed by chemiluminescent immunoassays (normal range: 5-35 ng/mL), using a commercial kit (Shenzhen Yahuilong Biotechnology, Shenzhen, China). The thyroid function (normal range: 0.35-4.94 nmol/L) was determined through the evaluation of TSH. Chemiluminescent microparticle immunoassays was used for DHEA-S assessment.

Urinary Bisphenol A measurement

The urine samples that were stored in a freezer at -20°C were sent to a laboratory in the Department of Pharmaceutical Science Research Center of Babol University of Medical Science for analysis. BPA and NP >99% pure and some other chemicals were purchased from local commercial sources. The level of BPA in urine was determined using the method explained in Völkel et al. (20) study with some modification. Briefly, to measure the amount of BPA in the urine, 100 µl of the urine was transferred to a 5-ml vial. Then 100 µl of 0.01 M ammonium acetone buffer with pH=4.5 and 4 ml of a mixture of hexane and diethyl ether in a 70: 30 ratio were added to the vial. Then the mixture was vortexed for 30 seconds, then centrifuged at 12,000 rpm for 10 minutes. The organic layer was separated from the sample and placed in a refrigerator at 5°C to allow the solvent to evaporate Next, 400 µl of the mobile phase solvent was added to the vial and vortexed. The vial containing the sample was transferred to the refrigerator to reach the volume of the solvent and sample to 100 µl, and after reaching the desired volume, 20 microliters of it was injected into the HPLC. Total BPA (conjugated and free) in urine were measured with high-performance liquid chromatography (HPLC). Finally, the amount of urine BPA in the urine was measured using a standard chart.

The limit of detection (LOD) of urine BPA was 0.11 ng/mL that is in agreement with Adoamnei et al. (21).

Semen analysis

Briefly, after 2-5 days of sexual abstinence, participants gathered semen samples via masturbation into polypropylene containers. During the first hour after liquefaction, sperm concentration, motility and morphology was evaluated according to WHO, 2010 guidelines (22).

Statistical analysis

Data were presented as number (%) or mean ± SD. To assess the relationship among predictors the t test, one-way analysis of variance (ANOVA), Kruskal-Wallis test, and U Mann-Whitney test were used as appropriate according to the nature and distribution of the variables. Then, because of the abnormal distribution of urine BPA levels, log10-transforma was considered and a multiple linear regression model for log BPA was applied by all predictors in simple analysis. Regardless, the simple correlation of variables including BMI, smoking and alcohol history as well as location was significant. Based on the literature, some confounders in multiple regression models were determined. In linear regression, statistics is β and 95% confidence interval (CI) is related β. The urine BPA level was categorized to 4 quartiles, first percentile that is known as 25th percentile was below limit of detection, second percentile known as 50th percentile, that was between the 25th percentile to the median, third percentile named 75th percentile that was between 50th and 75th percentile, and finally fourth percentile, which was more than 75th percentile values. All quartiles were compared to the first quartile as a reference with the lowest concentration of BPA. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, version 20, SPSS Inc., USA) software. P<0.05 was considered as significant.

Ethical considerations

Ethical approval was obtained from the local Ethical Committee of the Iranian National Committee on Health Research Ethics (IR.IAU.SARI.REC.1399.153). All participants filled the informed written consent before participation.

Results

Demographic characteristics of all 122 males participants that were referred to infertility clinics for treatment are shown in Table 1. The compression of semen parameters with demographic variables showed that alcohol consumption and BMI ≥25 reduced the number of progressive motile sperms and changed the sperm morphology (P=0.01), but smoking and location had no effects on sperm parameters (P>0.05, Table 2).

BPA was not detected in about half of the samples (53.3%). In less than half of the samples (46.7%) the total urinary BPA concentrations were measured as a mean and median concentration of 0.9 and 0.04 ng/ml, respectively. First, participants were divided in two groups, including men who had no detectable urinary BPA and a BPA detectable group. A comparison was done between the two groups in terms of semen quality parameters and reproductive according to the type and distribution of the variables. In sperm concentration, there was a significant difference between the two groups P<0.01, while in semen volume, sperm morphology, progressive motile sperm differences were not significant. Then, a comparison was carried out among 4 quartiles of BPA in terms of semen quality parameters, among which no significant difference was observed (Table 3). Multiple linear regression analysis showed that there is no relationship between the urinary BPA and male reproductive hormones that are presented in Table 4. Also, there is no associations between semen quality parameters and urinary BPA concentration in men that are presented in Table 5.

Table 1: Demographic data from study population (n=122)

Characteristic	n (%)
Education	
Primary	75 (61)
High School	14 (11.5)
Higher	33 (27.5)
Location	
Urban	69 (56.6)
Rural	53 (43.4)
Smoking	
No	93 (76.2)
Yes	29 (23.8)
BMI (kg/m^2)	
<25	24 (19.6)
≥25	98 (76.2)
Mean ± SD	27.9 ± 4.7
Min-Max	17-48
Duration of couple's infertility (Y)	
1-2	64 (52.4)
2-3	11 (22)
3-5	24 (19.6)
>5	23 (18.8)
Age (Y)	
Mean ± SD	35.1 ± 23
Min-Max	20-50
Alcohol use	
None	107 (87.7)
<1 drink/week	6 (4.9)
1-3 drinks /week	7 (5.7)
Everyday	2 (1.6)

BMI; Body mass index.

Table 2: The comparison of quality sperm parameters based on certain demographic variables

Variables	Semen volume (mL)[a]	Sperm concentration (million/mL)[b]	Morphologically normal forms (%)[b]	Progressive motile (%)[a]
BMI				
<25	3.15 ± 1.28	60.00 (135.3)	5 (70.1)	48.66 ± 11.61
≥25	2.79 ± 1.14	40.00 (500.2)	4 (42.0)	48.64 ± 18.99
P value	0.17	0.11	0.01	0.62
Smoking user			3.00 (17.1)	
Yes	2.76 ± 1.16	40.00 (90.2)	4.00 (70.0)	49.00 ± 15.66
No	2.90 ± 1.18	50.00 (500.2)		46.43 ± 18.40
P value	0.57	0.07	0.35	0.50
Alchol user				
Yes	2.90 ± 0.96	30.00 (80.8)	4 (17.1)	52.86 ± 7.23
No	2.86 ± 1.20	50.00 (500.2)	4 (70.0)	46.22 ± 18.64
P value	0.91	0.23	0.53	0.01
Location				
Urban	2.83 ± 1.16	40.00 (50.2)	4 (70.0)	46.36 ± 18.24
Rural	2.91 ± 1.19	50.00 (80.5)	4 (8.0)	47.92 ± 17.24
P value	0.63	0.16	0.47	0.72

[a]; Based on Independent t test (mean ± SD), [b]; Based on U Mann-Whitney test t [median (IQR)], and BMI; Body mass index.

Table 3: The comparison of semen index among BPA quartiles

BPA quartiles	Semen volume (mL)[a]	Sperm concentration (million/mL)[b]	Morphologically normal forms (%)[b]	Progressive motile (%)[a]
1st quartile	2.83 ± 1.07	47.50 (2.500)	4 (0.70)	47.45 ± 17.98
2nd quartile	3.30 ± 1.87	55.00 (3.70)	4 (1.17)	41.40 ± 16.72
3rd quartile	2.25 ± 1.04	35.00 (3.70)	4 (2.7)	47.50 ± 12.58
4th quartile	3.32 ± 1.69	54.00 (10.60)	4 (1.24)	47.00 ± 22.42
P value	0.37	0.84	0.89	0.98

[a]; Based on one-way ANOVA (mean ± SD), [b]; Based on Kruskal-Wallis test [median (IQR)], and BPA; Bisphenol A.

Table 4: The comparison of semen index among BPA quartiles

BPA quartiles	T (nmol/L)	LH (IU/L)	FSH (pmol/L)	TSH (nmol/L)	DHEA-S (µg/dl)	PRL (ng/mL)
1st quartile	Reference	Reference	Reference	Reference	Reference	Reference
2nd quartile	1.82 (-1.62, 5.26)	-5.80 (-34.48, 22.83)	-0.14 (-3.49, 3.21)	-0.38 (-1.89, 1.74)	-1.67(-5.40,2.06)	4.74(-1.49,10.98)
P value	0.29	0.68	0.93	0.95	0.37	0.13
3rd quartile	1.01 (-3.98, 6.02)	-6.93 (-48.25, 34.38)	-0.32 (-5.15, 4.50)	-0.7 (-18.11, 13.66)	-0.38 (-5.80,5.04)	-4.35 (13.48,4.76)
P value	0.68	0.73	0.89	0.93	0.88	0.34
4th quartile	0.15 (-4.87, 5.18)	-1.09 (-42.57, 40.39)	0.87 (-3.96, 5.71)	1.24 (-0.55, 3.03)	-2.04(-7.45,3.36)	0.75(-8.46,9.97)
P value	0.15	0.95	0.71	0.55	0.45	0.87
Log BPA	0.54 (-2.18, 3.28)	5.65 (-16.89,28.19)	0.15 (-2.48, 2.79)	0.31 (-0.67, 1.30)	-1.10(-4.05,1.84)	2.11(-2.87,7.09)
P value	0.69	0.61	0.90	0.52	0.45	0.40

Data are presented as median (min-max). BPA; Bisphenol A, T; Testosterone, LH; Luteinizing hormone, FSH; Follicular-stimulating hormone, TSH; Thyroid-stimulating hormone, DHEA-S; Dehydroepiandrosterone sulfate, and PRL; Prplactin.

Table 5: The relationship between urinary BPA and semen parameters in men [considered as model coefficients (95% CI)].

BPA quartiles	Semen volume (mL)	Sperm concentration (million/mL)	Morphologically normal forms (%)	Progressive motile (%)
1st quartile	Reference	Reference	Reference	Reference
2nd quartile	0.38 (-0.56, 1.33)	-1.13 (-51.11, 48.28)	2.11 (-0.71, 4.95)	-2.48 (-16.11, 11.15)
3rd quartile	-0.79 (-2.14, 0.55)	-17.71 (-89.15, 53.72)	-0.33 (-4.51, 3.84)	1.54 (-18.11, 13.66)
4th quartile	0.1 (-0.83, 1.89)	5.4 (-0.66, 77.22)	4.87 (0.91, 8.83)	2.2 (-17.50, 21.90)
Log BPA	-0.41 (-0.71, 0.79)	-7.38 (-46.45, 31.69)	1.87 (-0.44, 4.01)	-4.71 (-15.38, 5.93)
P value (Log BPA)	0.72	0.16	0.88	0.32

Data are presented as median (min-max). Adjusted by BMI, smoking and alcohol history and geographic location. BPA; Bisphenol A, BMI; Body mass index, and CI; Confidence interval

Discussion

In this study, we found that in about half of the participants, urinary BPA was not detected. Sampling was done in Mazandaran province in northern Iran, which has a mild, semi-humid climate and is a non-industrialized area of Iran. Perhaps the simple lifestyle based on traditional agriculture, the non-industrial nature of the region and low environmental exposure to BPA are among the causes of finding lower urine BPA concentrations compared to relatively more developed industrial regions (12, 23). The level of BPA has been reported in several studies.

Li et al. (10) states that the median value in Chinese workers in a factory chosen for likely higher contamination is 38.7 ng/ml, while it is measured to be 0.4-20 ng/ml by Mendiola et al. (13) or 1.81- > 3.27 ng/ml by Pollard et al. (24), in environments, which have not been contaminated. We found that no adverse effects were observed with our detected BPA levels in the fourth quartiles and semen parameters and male reproductive hormones. Nevertheless, when comparing semen parameters in men who had non-detectable urinary BPA to those with detectable BPA levels, a significant inverse association was noticed with sperm concentration, while for other parameters, differences were not significant. Li et al. (10) showed in 215 factory workers that there was a reverse association between BPA concentration and total count, concentration, viability, and motility of sperm cells. However, the difference was not significant when comparing semen volume or sperm morphology. Interestingly, only in a creatinine-adjusted BPA subgroup a significant reduction in sperm concentration was observed. Pollard et al. (24) in a prospective pre-conception cohort study, in which 161 men in ages 18-40 with no known subfertility were participating, defined that high BPA exposure causes abnormal sperm tail morphology. However, the reports by Mendiola et al. (13) and Goldstone et al. (25) showed that there was no significant relationship between urinary BPA and semen parameters in male partners attending infertility clinics. Although both of these studies have been conducted in the US, their subjects were from regions that were not selected for contamination with BPA, but they may still be expected to be higher than a rural Iranian population in terms of PBA exposure. Also, a study conducted in Iran on women undergoing in vitro fertilization (IVF) treatment showed that the levels of BPA in follicular fluid had no negative effects on mature oocytes. However, it had adverse effects on degenerated oocytes and germinal vesicle (GV) (26).

Our study showed that there was no association between the urinary BPA and male reproductive hormones. There are a limited number of studies that have evaluated the endocrine-destroying effects of BPA on reproductive health, especially male hormones, and the results are somewhat inconclusive. For this reason, Mendiola et al. (13) assessed this relationship in 375 fertile men. Their findings were different from two previous cohort studies, as they found that BPA was lower [geometric mean=1.5 (0.8, 3.0) ng/mL], had positive association with sex hormone bonding globulin (SHBG), while presenting an inversed association with free androgen index (FAI) and FAI/LH. Also, in this population, there was no relationship between BPA levels and inhibin B, FSH, LH, free testosterone (fT) or testosterone (T). In the study by Hanaoka et al. (27) on Japanese men who were exposed to BPA with epoxy resin spray, it was shown that there was a relationship between urinary BPA and testosterone levels and plasma gonadotrophic. Although in this study two groups of exposed and un-exposed workers had the same levels of LH and free testosterone, but FSH levels in the 42 un-exposed workers were more than the occupationally exposed ones. BPA concentration in the exposed group was lower than that in BPA un-exposed group (median 1.06; not detected to 11.2 µmol/mol creatinine) and (median 0.5; not detected to 11.0 µmol/mol creatinine) respectively. Galloway et al. (28) showed that a higher level of urinary BPA causes a higher level of serum testosterone. But the same relationship was not seen between BPA, estradiol and (SHBG).

In the present study we showed that normal sperm morphology was reduced in men with BMI ≥25. There are contradictory results on the relationship between BMI ≥25 and sperm parameters among various studies. In a study by Jurewicz et al. (29), increased BMI causes semen volume reduction. Fejes et al. (30), on the other hand, showed that significant association was found between semen parameters and waist and hip circumferences. Chromatin-intact, normal-motile sperm, total sperm count and sperm motility per ejaculation were fewer in men with BMI ≥25 (31, 32). Whereas data from 31 meta-analysis studies revealed that increased BMI had no negative effects on semen parameters (33). We showed that alcohol users and smokers had lower semen values than reported in other studies. This study showed that sperm motility was lower, but not significantly, in people who drank alcohol. The existing data on alcohol and semen quality are sparse and sometimes contradictory in some studies. Data from Marinelli et al. (34) and Povey et al. (35). showed that moderate alcohol drinking has a definite protective effect on sperm parameters, may be caused by antioxidant effects of some alcoholic beverages. Also, authors in this study found that there was no evidence showing that cigarette smoking had reverse effects on sperm quality. while Sharma et al showed the opposite result in his study. They demonstrated that generally, semen parameters could be affected by cigarette smoking negatively (36). They emphasized that World Health Organization (WHO) laboratory procedure for the evaluation of semen parameter had a least effect on the value of effect size in their study, that supporting the adverse effects of cigarette smoking on conventional semen parameters.

Our study had some limitations: first; sample collection was the main limitation in our experiment because of the COVID-19 pandemic situation. Secondly; we did not examine urinary creatinine for daily assessment with

BPA, although urine dilution would have a better result with creatinine assessment. Thirdly; Due to the short half-life of urinary BPA, about 6 hours (37) it would have been better to collect 24-hour urine sample.

Conclusion

According to our findings, in about half of the participants, urinary BPA was not detected. Probably the low environmental exposure to BPA in this population lead to this less than detection levels. So, no adverse effects were observed between BPA levels in four urinary BPA quartiles and semen quality and male reproductive hormones.

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Authors' Contributions

M.M.R.A., H.S.; Participated in study design, data collection and visited the patients. M.G.T.; Designed and performed the experiments, analyzed data and wrote the first draft of the manuscript. L.M., A.A.; Contributed to designing the experiments and analyzed data. F.H., N.M.G., P.M.; Helped in sampling and filling out the questionnaires. All authors read and approved the final manuscript.

References

1. Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, et al. EDC-2: The endocrine society's second scientific statement on endocrine-disrupting chemicals. Endocr Rev. 2015; 36(6): E1-E150.
2. Zoeller RT, Brown TR, Doan LL, Gore AC, Skakkebaek NE, Soto AM, et al. Endocrine-disrupting chemicals and public health protection: a statement of principles from the endocrine society. Endocrinology. 2012; 153(9): 4097-4110.
3. Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. Environ Health Perspect. 2008; 116(1): 39-44.
4. Castellini C, Totaro M, Parisi A, D'Andrea S, Lucente L, Cordeschi G, et al. Bisphenol A and male fertility: myths and realities. Front Endocrinol (Lausanne). 2020; 11: 353.
5. Kasper-Sonnenberg M, Wittsiepe J, Koch HM, Fromme H, Wilhelm M. Determination of bisphenol a in urine from mother-child pairs-results from the duisburg birth cohort study, Germany. J Toxicol Environ Health A. 2012; 75(8-10): 429-437.
6. Cantonwine DE, Hauser R, Meeker JD. Bisphenol A and human reproductive health. Expert Rev Obstet Gynecol. 2013; 8(4): 10.1586/17474108.2013.811939.
7. Radwan M, Wielgomas B, Dziewirska E, Radwan P, Kałużny P, Klimowska A, et al. Urinary bisphenol a levels and male fertility. Am J Mens Health. 2018; 12(6): 2144-2151.
8. Brieño-Enríquez MA, Robles P, Camats-Tarruella N, García-Cruz R, Roig I, Cabero L, et al. Human meiotic progression and recombination are affected by Bisphenol A exposure during in vitro human oocyte development. Hum Reprod. 2011; 26(10): 2807-2818.
9. Brieño-Enríquez MA, Reig-Viader R, Cabero L, Toran N, Martínez F, Roig I, et al. Gene expression is altered after bisphenol A exposure in human fetal oocytes in vitro. Mol Hum Reprod. 2012; 18(4): 171-183.
10. Li DK, Zhou Z, Miao M, He Y, Wang J, Ferber J, et al. Urine bisphenol-A (BPA) level in relation to semen quality. Fertil Steril. 2011; 95(2): 625-630. e1-4.
11. Meeker JD, Ehrlich S, Toth TL, Wright DL, Calafat AM, Trisini AT, et al. Semen quality and sperm DNA damage in relation to urinary bisphenol A among men from an infertility clinic. Reprod Toxicol. 2010; 30(4): 532-539.
12. Lassen TH, Frederiksen H, Jensen TK, Petersen JH, Joensen UN, Main KM, et al. Urinary bisphenol A levels in young men: association with reproductive hormones and semen quality. Environ Health Perspect. 2014; 122(5): 478-484.
13. Mendiola J, Jørgensen N, Andersson AM, Calafat AM, Ye X, Redmon JB, et al. Are environmental levels of bisphenol a associated with reproductive function in fertile men? Environ Health Perspect. 2010; 118(9): 1286-1291.
14. Wisniewski P, Romano RM, Kizys MM, Oliveira KC, Kasamatsu T, Giannocco G, et al. Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic-pituitary-testicular axis. Toxicology. 2015; 329: 1-9.
15. Oliveira IM, Romano RM, de Campos P, Cavallin MD, Oliveira CA, Romano MA. Delayed onset of puberty in male offspring from bisphenol A-treated dams is followed by the modulation of gene expression in the hypothalamic-pituitary-testis axis in adulthood. Reprod Fertil Dev. 2017; 29(12): 2496-2505.
16. Ullah A, Pirzada M, Jahan S, Ullah H, Turi N, Ullah W, et al. Impact of low-dose chronic exposure to bisphenol A and its analogue bisphenol B, bisphenol F and bisphenol S on hypothalamo-pituitary-testicular activities in adult rats: a focus on the possible hormonal mode of action. Food Chem Toxicol. 2018; 121: 24-36.
17. Ullah A, Pirzada M, Jahan S, Ullah H, Shaheen G, Rehman H, et al. Bisphenol A and its analogs bisphenol B, bisphenol F, and bisphenol S: Comparative in vitro and in vivo studies on the sperms and testicular tissues of rats. Chemosphere. 2018; 209: 508-516.
18. Ullah A, Pirzada M, Jahan S, Ullah H, Razak S, Rauf N, et al. Prenatal BPA and its analogs BPB, BPF, and BPS exposure and reproductive axis function in the male offspring of Sprague Dawley rats. Hum Exp Toxicol. 2019; 38(12): 1344-1365.
19. Völkel W, Colnot T, Csanády GA, Filser JG, Dekant W. Metabolism and kinetics of bisphenol a in humans at low doses following oral administration. Chem Res Toxicol. 2002; 15(10): 1281-1287.
20. Völkel W, Kiranoglu M, Fromme H. Determination of free and total bisphenol A in urine of infants. Environ Res. 2011; 111(1): 143-148.
21. Adoamnei E, Mendiola J, Vela-Soria F, Fernández MF, Olea N, Jørgensen N, et al. Urinary bisphenol A concentrations are associated with reproductive parameters in young men. Environ Res. 2018; 161: 122-128.
22. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization; 2010.
23. Amin MM, Ebrahim K, Hashemi M, Shoshtari-Yeganeh B, Rafiei N, Mansourian M, et al. Association of exposure to bisphenol A with obesity and cardiometabolic risk factors in children and adolescents. Int J Environ Health Res. 2019; 29(1): 94-106.
24. Pollard SH, Cox KJ, Blackburn BE, Wilkins DG, Carrell DT, Stanford JB, et al. Male exposure to bisphenol A (BPA) and semen quality in the home observation of periconceptional exposures (HOPE) cohort. Reprod Toxicol. 2019; 90: 82-87.
25. Goldstone AE, Chen Z, Perry MJ, Kannan K, Louis GM. Urinary bisphenol A and semen quality, the LIFE study. Reprod Toxicol. 2015; 51: 7-13.
26. Poormoosavi SM, Behmanesh MA, Janati S, Najafzadehvarzi H. Level of Bisphenol A in follicular fluid and serum and oocyte morphology in patients undergoing IVF treatment. J Family Reprod Health. 2019; 13(3): 154-159.
27. Hanaoka T, Kawamura N, Hara K, Tsugane S. Urinary bisphenol A and plasma hormone concentrations in male workers exposed to bisphenol A diglycidyl ether and mixed organic solvents. Occup Environ Med. 2002; 59(9): 625-628.
28. Galloway T, Cipelli R, Guralnik J, Ferrucci L, Bandinelli S, Corsi AM, et al. Daily bisphenol A excretion and associations with sex hormone concentrations: results from the InCHIANTI adult population study. Environ Health Perspect. 2010; 118(11): 1603-1608.
29. Jurewicz J, Radwan M, Sobala W, Ligocka D, Radwan P, Bochenek M, et al. Lifestyle and semen quality: role of modifiable risk factors. Syst Biol Reprod Med. 2014; 60(1): 43-51.
30. Fejes I, Koloszár S, Szöllosi J, Závaczki Z, Pál A. Is semen quality

affected by male body fat distribution? Andrologia. 2005; 37(5): 155-159.
31. Kort HI, Massey JB, Elsner CW, Mitchell-Leef D, Shapiro DB, Witt MA, et al. Impact of body mass index values on sperm quantity and quality. J Androl. 2006; 27(3): 450-452.
32. Sekhavat L, Moein MR. The effect of male body mass index on sperm parameters. Aging Male. 2010; 13(3): 155-158.
33. MacDonald AA, Herbison GP, Showell M, Farquhar CM. The impact of body mass index on semen parameters and reproductive hormones in human males: a systematic review with meta-analysis. Hum Reprod Update. 2010; 16(3): 293-311.
34. Marinelli D, Gaspari L, Pedotti P, Taioli E. Mini-review of studies on the effect of smoking and drinking habits on semen parameters. Int J Hyg Environ Health. 2004; 207(3): 185-192.
35. Povey AC, Clyma JA, McNamee R, Moore HD, Baillie H, Pacey AA, et al. Modifiable and non-modifiable risk factors for poor semen quality: a case-referent study. Hum Reprod. 2012; 27(9): 2799-2806.
36. Sharma R, Harlev A, Agarwal A, Esteves SC. Cigarette smoking and semen quality: a new meta-analysis examining the effect of the 2010 World Health Organization Laboratory methods for the examination of human semen. Eur Urol. 2016; 70(4): 635-645.
37. Vagi SJ, Azziz-Baumgartner E, Sjödin A, Calafat AM, Dumesic D, Gonzalez L, et al. Exploring the potential association between brominated diphenyl ethers, polychlorinated biphenyls, organochlorine pesticides, perfluorinated compounds, phthalates, and bisphenol A in polycystic ovary syndrome: a case-control study. BMC Endocr Disord. 2014; 14: 86.

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Awareness of Polycystic Ovary Syndrome among Schoolgirls and Their Mothers: A Cross-Sectional Study

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Abstract

Background: One of the most common endocrine disorders of reproductive age women is polycystic ovary syndrome (PCOS). Women with PCOS are at risk for infertility, endometrial cancer, metabolic disorders, and cardiovascular disease. Awareness of the signs and symptoms of PCOS can be effective in diagnosing early stage PCOS and promoting quality of life (QoL). This study assesses the awareness of PCOS in schoolgirls and their mothers.

Materials and Methods: This cross-sectional study was carried out on 1580 high school girls and 480 of their mothers in Tehran (capital of Iran) in 2017 and 2018. We used the stratified sampling method and divided Tehran into five geographic regions: north, south, east, west, and central. Schools were randomly selected from each of these regions. Students and their mothers separately answered a self-administered questionnaire that pertained to their knowledge of PCOS. Statistical analyses were carried out with SPSS, version 22 (Inc. Chicago, IL, USA) and R version 3.2.1.

Results: Students had an average age of 16.97 ± 0.84 years and their mothers' average age was 45.19 ± 5.03 years. The average body mass index (BMI) of the students was 22.01 ± 5.54 kg/m^2. The results of this study showed that only 48 students (3.2%) and 148 mothers (27%) had acceptable knowledge about PCOS. The knowledge of students about PCOS was positively related to their mothers' knowledge about PCOS (P<0.001).

Conclusion: The level of PCOS awareness in Iranian women is insufficient and this may affect their QoL. Therefore, the health authorities should implement educational programs to challenge women's incorrect beliefs about PCOS and increase their awareness of this disease.

Keywords: Polycystic Ovary Syndrome, Knowledge, Schoolgirls

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most common and complex endocrine disorders that affects 5-10% of reproductive-aged women (1). The range of PCOS prevalence is 8 to 13% and depends on the type of diagnostic criteria, race, and ethnicity of the studied population (2). For instance, the prevalence of PCOS in the United States ranges from 5 to 7% (3); however, it is at least 2.2% in China (4) and as high as 14.1% in Iran (5). The results of a study conducted in the United Kingdom indicated that women in South Asia were significantly more likely to hyperandrogenic symptoms in comparison with Caucasian women (6). These differences in phenotypes have been mentioned in other populations, including African Americans and Hispanics (7, 8). Differences in the reported prevalence of PCOS are probably be due to differences in diagnostic criteria (9). For instance, Tehrani et al. (10) have reported a prevalence of PCOS in women ages 18-45 years of 7.1% according to National Institutes of Health (NIH) criteria, 11.7% by Androgen Excess Society (AES) criteria, and 14.6% according to Rotterdam criteria. Sayehmiri et al. (11) reported a prevalence of

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PCOS among 10-54 years old females of 6.8% (NIH), 19.5% (Rotterdam), and 41.4% (ultrasound) criteria. In addition, the prevalence of PCOS may vary according to age. Koivunen et al. (12) reported a higher prevalence of PCOS in young women compared to women over 35 years of age. PCOS seems to have a genetic source that is affected by environmental factors of eating habits, lifestyle, and social status. It is characterised by a wide range of signs and symptoms that are associated with dysfunction in the reproductive organs, endocrine functions, and metabolic systems (13, 14). The signs and symptoms of PCOS in women are painful and uncomfortable. Barnard et al. (13) have reported that women with PCOS had poor quality of life (QoL), high levels of depression, emotional disturbance, obesity, infertility, acne, menstrual symptoms, and hirsutism. Also, PCOS is a leading cause of anovulatory infertility, a risk factor for endometrial dysfunction and uterine cancer, which is a serious lifelong health concern (15, 16). Therefore, awareness of the signs, symptoms, and complications of early stage PCOS could be of tremendous importance in improving QoL in these women (1).

Sunanda and Nayak (17) reported that, in recent years, the number of PCOS patients increased by 30% in India. Lack of knowledge and lifestyle changes among the women appeared to be two important factors that led to increased numbers of PCOS patients. Hajivandi et al. (18) reported decreased consumption of healthy foods (e.g., fibre, meat, beans, fish, seafood, and dairy products) among adolescent girls with PCOS. The results of studies indicate that weight loss and lifestyle modification are two, initially important steps for treatment of PCOS (19-21). The actual cause of PCOS is unclear and symptoms vary from case to case (22). PCOS is caused by an imbalance of female sex hormones, which may lead to changes in the menstrual cycle, multiple cysts in the ovaries, failure to conceive, and other health issues (17). Symptoms of PCOS begin after onset of the menstrual cycle in women. The most common symptom is irregularity in the menstrual cycle along with symptoms of obesity, menstrual abnormalities (amenorrhea, oligomenorrhea, and other menstrual irregularities), hirsutism, acne, sleep apnoea, depression, and infertility (23).

Diagnosis of the early symptoms of PCOS is possible in individuals with late puberty and early adolescence, both of which are commonly correlated with lifestyle and environmental changes. Early diagnosis of PCOS is necessary for timely intervention in order to reduce both the initial and chronic complications of PCOS (24).

Obesity and insulin resistance are important risk factors of metabolic syndrome in PCOS patients. The high risks associated with such metabolic disorders call for rapid and timely identification of PCOS. In this regard, the first step is to increase women's awareness of PCOS (25) because an accurate diagnosis can improve QoL in these women (26). Awareness of PCOS symptoms is of great importance for both treatment and prevention of complications (27). Haq et al. (28) have reported that although the prevalence of signs and symptoms of PCOS is increasing, there is still a substantial lack of awareness of the symptoms among females. According to Rahmanpour et al. (25), awareness of women with PCOS in terms of symptoms, complications (obesity, insulin resistance, infertility, etc.) and ways to prevent PCOS progression that include lifestyle changes and decreased consumption of fast foods can play an important role in the future.

To the best of our knowledge, this is the first study to evaluate the level of awareness towards PCOS among high school girls and their mothers in Iran. The results of this study can help to change the approach of the education system in Iran. Therefore, educating high school girls about the symptoms, risk factors, and prevention of diseases (such as PCOS) can be a significant help in improving women's community health in the future.

Materials and Methods

This was a cross-sectional study performed on 1580 schoolgirls who attended randomly chosen schools from 19 school districts in Tehran (capital of Iran) and 480 of their mothers. The study was conducted in 2017 and 2018. Of note, the school principals of these schools invited all mothers to participate in a meeting, but only 480 of them attended.

The Ethical Committee of Royan Institute, Tehran Iran (IR.ACECR.ROYAN.REC.1396.45) approved this study. The study aim and data confidentiality were clearly explained for all participants along with ethical issues, which were written above the questionnaires. Each schoolgirl and her mother who wished to participate were included in this study. Each completed questionnaire was considered to be that participant's written informed consent.

A review of available sources and texts was conducted by researchers on different sites and no similar questionnaire was located. Therefore, in order to achieve the main goal of the study, we designed this questionnaire. Initially, similar articles about PCOS were extracted by searching various databases and locating the available sources and texts. This was a knowledge-based questionnaire; therefore, content validity was confirmed via thorough review and revision by 15 experts and specialists (obstetricians and gynaecologists, midwives and nurses). Face validity was also used for the spoken items in the questionnaire. The designed questionnaire included ten positive and negative questions about PCOS. Participants who answered more than half of these questions were considered to "have knowledge". Demographic and general information questions that included region of school and grade, age, weight, height, birth order, type of delivery (vaginal or caesarean section), nutrition in infancy (breast milk, powdered milk, or both) were included. The mother's questionnaire included demographic information that pertained to

age, birth order, type of delivery, nutrition in infancy, occupation, and education, etc… A yes/no general question about self-perception of PCOS awareness was also included.

Any clinical or laboratory diagnostic methods were not included in this study.

We used a stratified sampling method to divide Tehran into five geographic regions: north, south, east, west, and central, from which the schools were randomly selected. In the next step, with the coordination of the school principals, we conducted a conference entitled "Awareness of PCOS for Girls and Their Mothers". Students and their mothers answered a self-administered questionnaire of their knowledge about PCOS prior to the conference.

Based on this method, estimating the sample size, 95% confidence level, 0.025 (two-way) confidence interval, and proportional probabilities of the three-stage clustering method were used. In the first stage, with equal probability, regions and schools were randomly selected with an equal probability within the clusters. Finally, we selected 40 students from each school. Due to the structure of the data collection and the random number of participants in the third stage of the surveys, we selected the minimum sample size available to decrease the possibility of insufficiency of the sample at this stage (Table S1, See supplementary Online Information at www.ijfs.ir).

Statistical analysis

The descriptive characteristics of the students and their mothers are reported as mean ± standard deviation or frequency (percentage). The McNemar test was performed to estimate the relation between mothers and daughters' knowledge about PCOS. The Chi-square test of independence or Fisher's exact test was used to assess the association of the PCOS categorical variables. Type I error was considered to be 0.05. Statistical analyses were conducted using SPSS, version 22 (Inc. Chicago, IL, USA) and R version 3.2.1.

Results

School girls had a mean age and body mass index (BMI) of 16.97 ± 0.84 years and 22.01 ± 5.54 kg/m^2, respectively. Most students were from the east region (29.4%) and most were the first child in their family (52.4%). A total of 71% were born by caesarean section and 63.8% were completely breastfed during their childhood. Table 1 lists the demographic characteristics of the students.

Table 2 lists the demographic characteristics of the mothers. The mothers had an average age of 45.19 ± 5.03 years, 62.7% of them were born by vaginal delivery, and 71.9% were completely breastfed. The majority of mothers who were housewives with a diploma degree had more information about PCOS compared to the other mothers.

Table 1: Demographic characteristics of the schoolgirls

Variables	Mean ± SD or frequency (%)
Age (Y)	16.97 ± 0.84
BMI (Kg/m^2)	22.01 ± 5.54
Region	
South	192 (11.5)
East	492 (29.4)
North	219 (13.1)
West	426 (25.5)
Center	251 (15)
Birth order	
1	779 (52.4)
2	502 (33.7)
3	160 (10.8)
4	40 (2.7)
5	6 (0.4)
7	1 (0.1)
Type of delivery	
Vaginal	575 (29)
Caesarian	898 (71)
Nutrition in infancy	
Breast	927 (63.8)
Powder	108 (7.4)
Both	418 (28.8)

BMI; Body mass index.

Table 2: Demographic characteristics of the mothers

Variables	Mean ± SD or frequency (%)
Age (Y)	45.19 ± 5.03
Number of daughters	1.53 ± 0.78
Number of sons	0.67 ± 0.71
Number of family members	2.21 ± 0.99
Occupation	
Housewife	421 (88.8)
Employee	53 (11.2)
Education	
High school	85 (18)
Diploma	288 (61)
Graduated	99 (21)
Nutrition in infancy	
Breast	339 (71.9)
Powder	54 (11.3)
Both	85 (17.8)
Type of delivery	
Vaginal	301 (62.7)
Caesarian	179 (37.3)

The awareness rate of PCOS in schoolgirls was 3.2% (n=48), and 22.91% (n=11) had PCOS. There was a history of PCOS in their mothers (n=5, 10.41%) and sisters (n=6, 12.5%). The awareness rate of PCOS among mothers was 27% (n=128).

In terms of self-perception of awareness, 6.45% (n=102) of the students considered themselves to have knowledge of PCOS compared to 44.58% (n=214) of the mothers.

Sources of information about PCOS for schoolgirls included their mothers (31.25%), the Internet (25%), health educators and radio /television (each one 18.75%), their friends and study about PCOS (each one 14.58%).

Table 3 shows a significant positive relation between awareness of the schoolgirls about PCOS and their mothers' knowledge about PCOS (P<0.001).

Table 3: The relationship between schoolgirls' awareness of PCOS and their mothers' knowledge

Awareness of PCOS	Mothers		McNemar test
	Yes	No	P value
Schoolgirls			<0.001
Yes	9 (2.3)	12 (3.1)	
No	97 (24.9)	271 (69.7)	
Total	106 (27.2)	283 (72.8)	

Data are presented as n (%). PCOS; Polycystic ovary syndrome.

The result of our study showed that awareness of PCOS in mothers and their daughters was significantly related to the level of mothers' education (P=0.002). In addition, PCOS awareness among mothers was significantly related to their educational level (P<0.001, Table 4).

We observed a significant correlation between mothers' occupations and their awareness of PCOS (P=0.01). However, there was no correlation between mothers' occupations and their daughters' awareness of PCOS (P=0.06, Table 5).

Table 4: The relationship between mothers' education with awareness of PCOS for themselves and their daughters

Awareness of PCOS in	Mothers' education			Total	P value
	Under diploma	Diploma	Uper diploma		
Schoolgirls					0.002
Yes	9 (2.3)	12 (3.1)	0 (0)	21 (5.4)	
No	59 (15.2)	233 (60.2)	74 (19.1)	366 (94.6)	
Total	68 (17.6)	245 (63.3)	74 (19.1)	387 (100)	
Mothers					<0.001
Yes	6 (1.3)	82 (17.6)	40 (8.6)	128 (27.4)	
No	79 (16.9)	203 (43.5)	57 (12.2)	339 (72.6)	
Total	85 (18.2)	285 (61)	97 (20.8)	467 (100)	

Data are presented as n (%). PCOS; Polycystic ovary syndrome.

Table 5: The relationship between mothers' occupation to awareness of their PCOS and their daughters

Awareness of PCOS in	Mothers' education		Total	P value
	Housewife	Employee		
Schoolgirls				0.06
Yes	21 (5.4)	0 (0)	21 (5.4)	
No	321 (82.7)	46 (11.9)	367 (94.6)	
Total	342 (88.1)	46 (11.9)	388 (100)	
Mothers				0.01
Yes	103 (22)	21 (4.5)	124 (26.4)	
No	313 (66.7)	32 (6.8)	345 (73.6)	
Total	416 (88.7)	53 (11.3)	469 (100)	

Data are presented as n (%). PCOS; Polycystic ovary syndrome.

Table 6: The association between awareness of PCOS and demographic variables of the participants

Demographic variables		Mothers' awareness			Students' awareness			
		Yes	No	P value		Yes	No	P value
Birth order[a]	1.00	54 (24.7)	165 (75.3)	<0.001*	1.00	25 (3.2)	754 (96.8)	0.24
	2.00	46 (37.1)	78 (62.9)		2.00	15 (3)	487 (97)	
	3.00	6 (15.4)	33 (84.6)		3.00	8 (5)	152 (95)	
	≥4	0 (0)	7 (100)		≥4	0 (0)	47 (100)	
Nutrition in infancy[b]	Breast	86 (25.5)	251 (74.5)	0.01*	Breast	26 (2.8)	901 (97.2)	0.71
	Powder	9 (16.7)	45 (83.3)		Powder	3 (2.8)	105 (97.2)	
	Both	31 (37.8)	51 (62.2)		Both	10 (2.4)	408 (90)	
Type of delivery[b]	Vaginal	80 (26.6)	221 (73.4)	0.81	Vaginal	26 (4.5)	549 (95.5)	0.02*
	Caesarian	48 (27.6)	126 (72.4)		Caesarian	21 (2.3)	876 (97.7)	
Age[b] (Y)	<42	28 (18.9)	120 (81.1)	<0.001*	≤15	0 (0)	29 (100)	0.23
	42-45	41 (46.6)	47 (53.4)		16	10 (2.8)	342 (97.2)	
	45-48	18 (18.2)	81 (81.8)		17	22 (3.1)	680 (96.9)	
	>48	32 (29.6)	76 (70.4)		18	16 (4)	380 (96)	
Region[b]	South	15 (20.8)	57 (79.2)	0.01*	South	2 (1.1)	184 (98.9)	0.01*
	East	35 (21.9)	125 (78.1)		East	23 (5.2)	423 (94.8)	
	North	12 (30.8)	27(69.2)		North	8 (3.7)	211 (96.3)	
	West	24 (31.6)	52 (68.4)		West	6 (1.5)	388 (98.5)	
	Center	42 (32.8)	86 (67.2)		Center	9 (3.6)	238 (96.4)	

Data presented as frequency (%). [a]; Fisher Exact test, [b]; Chi Square test, *; Significant, and PCOS; Polycystic ovary syndrome.

Our results showed that the level of awareness towards PCOS among students was significantly related to two variables: maternal delivery type (P=0.02) and district of residence (P=0.01). In this regard, students who were delivered by caesarean section and lived in the east region had more information of PCOS compared to other students. Table 6 shows that mothers who had higher awareness of PCOS compared to the other participants had the following characteristics: first child (P<0.001), age range: 42–45 years (P=0.01), nutrition in infancy (P<0.001), and residence in the central region of the capital (P=0.01).

Discussion

The first step in managing PCOS is awareness and accurate diagnosis, which can both improve QoL in a patient (1). This study was conducted to assess the knowledge of PCOS among schoolgirls and their mothers. The results of our study showed that awareness of PCOS in schoolgirls and their mothers was 3.2% (n=48) and 27% (n=148), respectively. Among them, 22.91% (n=11) of girls and 10.41 % (n=5) of mothers were diagnosed with PCOS.

Gul et al. (29) reported that only 20 out of 177 (11.3%) Pakistani women who participated in their study had inadequate information about PCOS, and 11 participants had degrees in the Medical Sciences. In another study in Pakistan, Haq et al. (28) reported that 72.5% of the women were unaware of PCOS. Awareness of PCOS in Saudi women was 56.7% of which 15.3% of these women had PCOS (27). Additionally, Shammi (26) showed that 66.94% of female students had the least knowledge and 2.20% had high awareness of PCOS, 30.85% had no knowledge of PCOS, and 22.25% were diagnosed with PCOS. Upadhye and Shembekar (1) conducted a study in India and reported that 72% of girls (medical students) had knowledge of PCOS, and 6% had PCOS. The high level of awareness of girls in their study might be due to their medical school education.

In a study conducted by Alessa et al. (27), 72.9% of females were university graduates and 1.2% had only primary school education. They found that a significantly greater level of awareness of PCOS among women with higher education levels; most of the women with knowledge of PCOS had a history of medical and health education. Aminrad et al. (30) reported that an increase in age and level of education resulted in an increase in awareness and attitude of individuals towards environmental issues. In our study, the average age of high school girls was 16.97 ± 0.84 years. Upadhye and Shembekar (1) reported that 62.5% of participants were young adults (20-24 years) and 37.5% were adolescents (18-19 years); only 28% were unaware of PCOS. Sills et al. (31) found that participants in the age group of 26-34 years had significantly higher information about PCOS than others. Alessa et al. (27) reported a relatively high awareness level (56.7%) towards PCOS among Saudi women (18-50 years). Due to less experience and study levels among younger age groups, they might receive less information about PCOS than older age groups; however, Strong (32) believes that people who live longer will have a better chance of learning. In other words, as young girls become older, their level of awareness and curiosity increase with respect to female-related illnesses, which could be due to the exposure of students, friends, and relatives to relative illnesses and symptoms.

In the present study, the most common source of information for girls was from their mothers (31.25%), followed by the Internet (25%), health educators and radio /television (each one 18.75%), and their friends and study about PCOS (each one 14.58%). Alessa and colleagues (27) reported that among women who had knowledge of PCOS, 15.3% had PCOS and they received information about their disease from the Internet (21.3%), other patients (10.4%), doctors (10.8%), and books (3.1%). In another study, 82.02% of the students with PCOS had access to information via doctors (17.98%), media (11.24%), the Internet, and physiotherapists (1.1%) (26). Sills et al. (31) reported that women with PCOS, regardless of age, received more information on PCOS from their doctors. Upadhye and Shembekar (1) indicated that 33% of young girls and teenagers received their information about PCOS from teachers, friends (19%), physicians (11.5%), newspapers (3.5%), and the Internet (5%).

The results of our study showed that schoolgirls who attended high schools in east Tehran had the highest knowledge about PCOS and students in the south had the least awareness of PCOS. The east parts of Tehran appear to be some of the older, original regions, and had less migration than the other regions. Also, the quality of education in this area is higher than the south of Tehran, with a more native and marginal tissue. Shokouhi (33) believes that one of the consequences of uncontrolled migration to large cities, such as Tehran, is marginalization that has expanded over the past few decades.

Mothers' knowledge about PCOS significantly affects the awareness of their daughters. In addition, mothers who are the first-born in a family have more awareness of PCOS. It seems that mothers are more sensitive and spend more time to train and educate their first-born children. Mothers usually obtain their information through consultation with friends, doctors, radio and television, reading books, and the Internet. If a mother has PCOS, she can inform her daughter of its symptoms and side effects. Therefore, this awareness can lead to a faster diagnosis and prevention of complications.

Abdel Azim Mohamed (34) concluded that educating woman about PCOS could play a significant role in increasing their awareness. They reported that prior to the establishment of educational sessions, most students (84.4%) had poor knowledge about PCOS. After the educational sessions, there was an increase in the knowledge score of PCOS. Nivetha and Suganya (24) reported that educating women about all aspects of PCOS would increase community knowledge about the impact

of PCOS on women and their loved ones. Jakhar et al. (35) believes that screening of PCOS in schools/colleges/ universities is extremely important and awareness of PCOS could enhance a woman's lifestyle and her future reproductive life.

The limitations of our study include the lack of suitable cooperation by schools, students, and their mothers with researchers. The allotted time for students and mothers to complete the questionnaires was 8 am to 2 pm that majority of mothers were housewives. The exclusion of diagnostic analysis; and the criterion for PCOS was self-reported (mother or student).

Conclusion

The level of PCOS awareness in Iranian women is insufficient and this affects their QoL. Adolescent girls should receive knowledge about PCOS because it can affect their health and future pregnancies since PCOS is associated with foetal and maternal complications. A mother's awareness about PCOS can be a tremendous assistance to her daughter's knowledge of PCOS.

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Authors' Contributions

A.M., R.O.S.; Study design and conception, responsible for the overall supervision, and editing of the manuscript. F.M.; Study design contributed to the interpretation of the data and writing manuscript. B.N., M.M.; Data collection and manuscript writing. M.M.; Data analysis and interpretation and manuscript writing. All authors approved the final version of the manuscript for submission.

References

1. Upadhye JJ, Shembekar CA. Awareness of PCOS (polycystic ovarian syndrome) in adolescent and young girls. Int J Reprod Contracept Obstet Gynecol. 2017; 6(6): 2297-2301.
2. Teede HJ, Misso ML, Costello MF, Dokras A, Laven J, Moran L, et al. Recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome. Fertil Steril. 2018; 110(3): 364-379.
3. Lizneva D, Suturina L, Walker W, Brakta S, Gavrilova-Jordan L, Azziz R. Criteria, prevalence, and phenotypes of polycystic ovary syndrome. Fertil Steril. 2016; 106(1): 6-15.
4. Chen X, Yang D, Mo Y, Li L, Chen Y, Huang Y. Prevalence of polycystic ovary syndrome in unselected women from southern China. Eur J Obstet Gynecol Reprod Biol. 2008; 139(1): 59-64.
5. Rashidi H, Ramezani Tehrani F, Bahri Khomami M, Tohidi M, Azizi F. To what extent does the use of the Rotterdam criteria affect the prevalence of polycystic ovary syndrome? A community-based study from the Southwest of Iran. Eur J Obstet Gynecol Reprod Biol. 2014; 174: 100-105.
6. Mani H, Davies MJ, Bodicoat DH, Levy MJ, Gray LJ, Howlett TA, et al. Clinical characteristics of polycystic ovary syndrome: investigating differences in White and South Asian women. Clin Endocrinol (Oxf). 2015; 83(4): 542-549.
7. Legro RS, Myers ER, Barnhart HX, Carson SA, Diamond MP, Carr BR, et al. The pregnancy in polycystic ovary syndrome study: baseline characteristics of the randomized cohort including racial effects. Fertil Steril. 2006; 86(4): 914-933.
8. Zhao Y, Qiao J. Ethnic differences in the phenotypic expression of polycystic ovary syndrome. Steroids. 2013; 78(8): 755-760.
9. Lujan ME, Chizen DR, Pierson RA. Diagnostic criteria for polycystic ovary syndrome: pitfalls and controversies. J Obstet Gynaecol Can. 2008; 30(8): 671-679.
10. Tehrani FR, Simbar M, Tohidi M, Hosseinpanah F, Azizi F. The prevalence of polycystic ovary syndrome in a community sample of Iranian population: Iranian PCOS prevalence study. Reprod Biol Endocrinol. 2011; 9: 39.
11. Sayehmiri F, Kiani F, sayehmiri K, Maleki F, Ahmadi M, Shohani M. Prevalence of polycystic ovary syndrome in Iranian women: a systematic review and meta-analysis. IJOGI. 2014; 17(115): 11-21.
12. Koivunen R, Laatikainen T, Tomás C, Huhtaniemi I, Tapanainen J, Martikainen H. The prevalence of polycystic ovaries in healthy women. Acta Obstet Gynecol Scand. 1999; 78(2): 137-141.
13. Barnard L, Ferriday D, Guenther N, Strauss B, Balen AH, Dye L. Quality of life and psychological well being in polycystic ovary syndrome. Hum Reprod. 2007; 22(8): 2279-2286.
14. Jones GL, Palep-Singh M, Ledger WL, Balen AH, Jenkinson C, Campbell MJ, et al. Do south asian women with PCOS have poorer health-related quality of life than Caucasian women with PCOS? A comparative cross-sectional study. Health Qual Life Outcomes. 2010; 8: 149.
15. Wild RA. Long-term health consequences of PCOS. Hum Reprod Update. 2002; 8(3): 231-241.
16. Wild S, Pierpoint T, Jacobs H, McKeigue P. Long-term consequences of polycystic ovary syndrome: results of a 31 year follow-up study. Hum Fertil (Camb). 2000; 3(2): 101-105
17. Sunanda B, Nayak S. A study to assess the knowledge regarding PCOS (polycystic ovarian syndrome) among nursing students at NUINS. NUJHS. 2016; 6(3): 24-26.
18. Hajivandi L, Noroozi M, Mostafavi F, Ekramzadeh M. Food habits in overweight and obese adolescent girls with Polycystic ovary syndrome (PCOS): a qualitative study in Iran. BMC Pediatr. 2020; 20(1): 277.
19. Abd Elmenim SO, Mohamed Emam AM. Effect of lifestyle changes on symptoms of polycystic ovarian syndrome in obese girls. IOSR JNHS.2016; 5(3): 1-10.
20. Kite C, Lahart IM, Afzal I, Broom DR, Randeva H, Kyrou I, et al. Exercise, or exercise and diet for the management of polycystic ovary syndrome: a systematic review and meta-analysis. Syst Rev. 2019; 8(1): 51.
21. Vilmann LS, Thisted E, Baker JL, Holm JC. Development of obesity and polycystic ovary syndrome in adolescents. Horm Res Paediatr. 2012; 78(5-6): 269-278.
22. Colwell K, Lujan ME, Lawson KL, Pierson RA, Chizen DR. Women's perceptions of polycystic ovary syndrome following participation in a clinical research study: implications for knowledge, feelings, and daily health practices. J Obstet Gynaecol Can. 2010; 32(5): 453-459.
23. Shorakae S, Boyle J, Teede H. Polycystic ovary syndrome: a common hormonal condition with major metabolic sequelae that physicians should know about. Intern Med J. 2014; 44(8): 720-726.
24. Nivetha M, Suganya SG. Survey of poly cystic ovarian disease (pcod) among the girl students of bishop heber college, Trichirapalli, Tamil Nadu,India. IOSR-JNHS. 2016; 5(4): 44-52.
25. Rahmanpour H, Jamal L, Mousavinasab SN, Esmailzadeh A, Azarkhish K. Association between polycystic ovarian syndrome, overweight, and metabolic syndrome in adolescents. Pediatr Adolesc Gynecol J. 2012; 25(3): 208-212.
26. Shammi MN. A Study on knowledge and awareness of polycystic ovarian syndrome among female students of public and Private Medical Colleges in Dhaka. Presented for the B. Pharm., Dhaka. East West University. 2017.
27. Alessa A, Aleid D, Almutairi S, AlGhamdi R, Huaidi N, Almansour E. Awareness of polycystic ovarian syndrome among Saudi females. Int J Med Sci Public Health. 2017; 6(6): 1013-1019.
28. Haq N, Khan Z, Riaz S, Nasim A, Shahwani R, Tahir M. Prevalence and knowledge of polycystic ovary syndrome (PCOS) among female science students of different public universities of Quetta, Pakistan. Imp J Interdiscip Res.2017; 3(6): 385-392.
29. Gul S, Zahid SA, Ansari A. PCOS: symptoms and awareness in urban Pakistani women. Int J Pham Res Health Sci. 2014; 2 (5): 356-360.
30. Aminrad Z, Sayed Zakaria SZB, Hadi AS. Influence of age and level of education on environmental awareness and attitude: case study on Iranian students in Malaysian Universities. Soc Sci. 2011; 6(1): 15-19.
31. Sills ES, Perloe M, Tucker MJ, Kaplan CR, Genton MG, Schattman

GL. Diagnostic and treatment characteristics of polycystic ovary syndrome: descriptive measurements of patient perception and awareness from 657 confidential self-reports. BMC Womens Health. 2001; 1(1): 3.
32. Strong CA. The impact of environmental education on children's knowledge and awareness of environmental. Mark Intel Plan.1998; 16: 349-355.
33. Shokouhi H. Social geography of cities, social ecology of cities. 8th ed. Tehran: Jihad Daneshgahi Publishing Organization; 2013.
34. Abdel Azim Mohamed H. Effect of educational program on the level of knowledge regarding polycystic ovarian syndrome among adolescent girls. J Nurs Educ Pract. 2016; 6(10): 80-81.
35. Jakhar R, Dewanji Sen E, Dutt R. Awareness of polycystic ovarian syndrome among college going females in Gurgaon: a cross-sectional study. Ann Natl Acad Med Sci. 2022; 58(03): 149-156.

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Joint Modeling of *In Vitro* Fertilization Outcomes among A Population of Iranian Infertile Couples: A Historical Cohort Study

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Abstract

Background: Women who undergo *in vitro* fertilization (IVF) cycles should successfully go via multiple stages (i.e., clinical pregnancy, no abortion under 12 weeks, no abortion under 20 weeks, and delivery) to achieve a live birth. In this study, data from multiple IVF cycles and its multiple stages were reanalyzed to illustrate the success factors associated with various stages of IVF cycles in a population of Iranian infertile women.

Materials and Methods: This historical cohort study includes 3676 assisted reproductive technology (ART) cycles. Covariates take into account in this study were women's age, type of infertility (primary, secondary), body mass index (BMI), cause of infertility, history of abortion, duration of infertility, number of oocytes, number of embryos, fertilization rate, semen factors (Spermogram) and having polycystic ovarian syndrome (PCOS) during IVF cycles. Joint modeling was fitted to apply informative cluster size.

Results: Increasing age un women was associated with an increase in the BMI and a positive history of abortion and PCOS, and also, an increase in the number of treatment cycles, while in men was associated with the negative spermogram. With the increase in the number of treatment cycles, the result of the IVF success decreased, but with the increase in the number of embryos, fertilization rate and also, quality and / or quantity parameters of spermogram, we encountered with an increase in the IVF success rate.

Conclusion: It seems that a joint model of the number of treatment cycles and the result of IVF is a valuable statistical model that does not ignore the significant effect of cycle numbers, while this issue is ignored usually in the univariate models.

Keywords: Cluster Analysis, Infertility, *In Vitro* Fertilization

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Introduction

According to the clinical and epidemiological definitions of the World Health Organization (WHO), the prevalence of primary infertility in Iran is 20.2 and 12.8%, in order. Furthermore, the secondary infertility rate is 4.9% (1). In recent years, lifestyle factors have been shown to play an important role in reducing fertility and increasing the use of assisted reproductive techniques (ART) (2). Since infertility can change demographic patterns and have economic, social, and health consequences, different groups of sociologists, epidemiologists, and medical researchers have focused on it (3). The increasing fame of ART, the factor influencing its outcome and the matter of success rate, has led researchers to model the success rate of ART and recognize the factors affecting it in different ways (4-6).

One of the first methods of ART was the "*in vitro* fertilization (IVF)" approach. The process of *in vitro* fertilization (IVF) includes retrieving the oocytes from the female and the sperm from the male and allowing the sperm to fertilize the eggs in laboratory conditions. Then, the embryo(s) is (are) transferred to the uterus,

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and hormones are administered to take place, an implantation (7). A successful IVF must go through several stages successfully [such as chemical pregnancy, clinical pregnancy, non-spontaneous abortion (SAB), and successful delivery] to lead a live birth.

It seems that, success at each stage of IVF can be a predictive tool of the success likelihood of the next stage. In addition, a woman's different cycle relates to pregnancy outcomes, and the female fertility outcome in the present cycle is affected by the outcome of the previous ART cycle. Thus, instead of just considering current cycle data, we also need to consider previous cycle data (8).

ART data analysis methods range widely, from simple binomial tests for intricate models, particularly IVF data. Most ART data studies examine only parts of the data of infertile women (9-12), while it is better to consider the results of the treatments before this treatment. Pregnancy outcomes are often related to a woman's clinical characteristics, making it more likely that women who have previously experienced negative pregnancy outcomes (such as preterm birth, stillbirth, or SAB) will also experience negative fertility outcomes in their current pregnancy. Consideration of early reproductive outcomes, as opposed to just those connected to known pregnancies, is necessary because of the vast range of reproductive outcomes that can demonstrate such intra-woman grouping. A method based on the principles of discrete survival analysis of IVF data with many cycles and various failure kinds for each individual was published by Maity et al. (13). Additionally, the informative cluster size-a measure of the number of cycles an infertile woman completes-relates to the success or failure of the IVF outcome. So it is better to consider it in the analysis to get more accurate results.

The size of the informative cluster is not taken into account in the model of Maity et al. (13). In the present study, a joint modeling of logistic (for outcome of IVF) and Exponentiated exponential geometric regression (EEGR) (for cluster size) was employed to predict the variables affecting the binary outcome of success or failure at various IVF cycle phases while managing the informative cluster size.

Materials and Methods

The Ethics Board of Shahid Beheshti University of Medical Science (Tehran, Iran) approved the present study (IR.SBMU.RETECH.REC.1401.517) and Royan Institute (EC/90/1086). All subjects provided informed consent before the initiation of the treatment. Subjects received assurances that no personal information would be revealed.

Study patients and design

On 3676 cycles of infertile couples who were engaged in ART therapy at the Royan Institute, (Tehran, Iran), a referral infertility center, between April 2011 and March 2015, a historical cohort study was carried out. Only women who had experienced embryo transfer were included in this study. Trained nurses retrieved all the study's variables from the participants' medical records. At each of the four stages, the result variable was success or failure: i. Clinical pregnancy (attendance of an intrauterine gestational sac), ii. Abortion under 12 weeks iii. Abortion under 20 weeks, and iv. Delivery (live birth).

Extracted data of women included age, number of treatment cycles, body mass index (BMI), cause of infertility, history of abortion, duration of infertility, number of oocytes in the last cycle, number of embryos transferred in the last cycle, presence of polycystic ovarian syndrome (PCOS) during IVF cycles, fertilization rate in the last and spermogram (one score sperm-related factor) were all taken into account as covariates in this study.

Statistical analysis

The outcome of each stage, including chemical pregnancy, clinical pregnancy, SAB and delivery, was taken into account as a binary response variable that signifies the success or failure of each stage. The probability of success occurring at a specific stage of the ART cycle could be related to the stage, cycle number, and covariates of interest. Also, the next response variable, which is the number of cycles, is counted and because cycle numbers with disproportionately many ones, we minus 1 in all them and use zero inflated exponentiated EEGR for them. At first, a univariate model was used, and then significant variables were entered into a multiple model (for logistic and EEGR). Finally, the significant variables of multiple models entered into the joint model. The models were applied in accordance with Maity et al. (13) model to determine the impact of covariates on the binary and count outcomes as well as to calculate odds ratios (OR) and 95% confidence intervals (CI). The Statistical Analysis Software (SAS) program version 9.4 "nlmixed" procedures were used.

Results

Demographic data

This study comprised 3636 ART cycles. Following one to three cycles, women were underwent embryos transfer. We present the demographic data of all participants in Table 1.

Univariate models

Table 2 shows the univariate results about logistics and EEGR models. As you can see in this Table, in logistic model, cycle number had a positive significant effect on the failure in each stage after ART [odds ratio (OR) confidence interval (CI 95%): 1.017 (0.988-1.047)]. Duration of infertility had also a positive significant effect [OR (CI 95%): 1.025 (0.004-1.057)]. Although, variables such as number of oocyte, fertilization rate and spermogram had negative significant effect on the failure of each stage of the cycle [OR (95% CI): 0.976 (0.940-1.012), 0.117 (0.061-0.296), 0.134 (0.088-0.282), respectively].

Table 1: Demographics data of our participants

Variable	Frequency (%) or mean ± SD
Age (Y)	31.04 ± 5.02
BMI (Kg/m^2)	26.91 ± 4.10
Infertility type	
Primary	2587 (70.4)
Secondary	893 (24.3)
Duration of infertility (Y)	5.83 ± 4.26
Number of previous treatment	1.67 ± 1.75
History of abortion	0.23 ± 0.63
PCOS	1193 (18.9)
Cause of infertility	
Female	724 (20)
Male	1873 (51.8)
Both	357 (9.9)
Unknown	548 (15.2)

BMI; Body mass index and PCOS; Polycystic ovarian syndrome.

Table 2: The results of univariate logistic and EEGR models

Variable	Logistic model	EEGR model	
	OR (95% CI)	e^{β} (95% CI)	
		Count process	ZI Part
Age (Y)	1.017 (0.988-1.047)	1.519 (1.194-2.323)	0.996 (0.945-1.050)
Cycle number	1.505 (1.061-2.179)		
Type of infertility			
Primary	Ref		
Secondary	1.093 (0.812-1.474)	0.529 (0.280-1.158)	1.519 (0.994-2.323)
BMI (kg/m^2)	1.007 (0.975-1.040)	0.995 (0.002-55.15)	0.347 (0.130-0.664)
Cause of infertility			
Female	Ref		
Male	0.874 (0.616-1.237)	1.169 (0.000-7847)	0.989 (0.818-1.195)
Both	1.172 (0.720-1.909)	0.142 (0.073-1.684)	1.615 (1.269-2.056)
Unknown	0.611 (0.384-0.971)	4.490 (0.168-16.314)	5.226 (2.750-9.933)
History of abortion	1.030 (0.847-1.252)	3.219 (3.040-9.815)	2.278 (1.988-2.611)
PCOS	1.122 (0.804-1.565)	1.721 (1.602-1.864)	0.029 (0.002-0.383)
Duration of infertility	1.025 (0.994-1.057)	1.026 (0.171-6.128)	0.928 (0.909-0.947
Number of oocyte	0.976 (0.940-1.012)	0.221 (0.013-3.760)	1.006 (0.981-1.031)
Number of embryos	0.971 (0.921-1.022)	0.970 (0.932-1.008)	0.881 (0.546-1.002)
Fertilization rate	0.117 (0.061-0.296)	0.220 (0.110-3.220)	0.848 (0.740-0.972)
Spermogram	0.134 (0.088-0.282)	0.785 (0.637-0.968)	0.946 (0.857-1.046)

EEGR; Exponentiated exponential geometric regression, OR; Odds ratio, CI; Confidence interval, ZI; Zero Inflated, and BMI; Body mass index.

Age is directly associated with the cycle number [OR (CI 95%): 1.519 (1.194-2.323)]. As well, the history of abortion and PCOS had the same effect as age on cycle number [OR (95% CI): 3.219 (3.04-9.815), 1.721 (1.602-1.864), respectively]. However, Spermogram had a negative effect on cycle number 0.785 (0.637-0.968).

In zero inflated parts of number cycle, BMI, PCOS, duration infertility and fertilization rate had an opposite effect on having only one cycle number. Moreover, the history of abortion and the number of oocytes were resulted as positively responsible variables than having only one cycle number [2.278 (1.988-2.611), 1.026 (0.981-1.031), respectively].

Multiple models

The results of multiple model are shown in Table 3. In this model, cycle number and duration of infertility had a positive effect on failure in ART [OR (95% CI): 1.141 (1.071-1.282), 1.015 (0.976-1.054), in order], however number of oocytes, fertilization rate and Spermogram had an opposite effect [OR (95% CI): 0.995 (0.940-1.052), 0.333 (0.147-0.769), 0.900 (0.840-1.113), respectively].

In the count part, age, history of abortion and PCOS had a positive effect and Spermogram had a negative effect of the cycle number. In zero-inflated part, history abortion and the number of oocytes had a direct influence, although BMI, PCOS, duration of infertility and fertilization rate had a reverse influence.

Table 3: The result of multiple Logistic and EEGR model

Variable	e^{β} (95% CI)
Logistic model	
Cycle number	1.141 (1.071-1.282)
Duration of infertility	1.015 (0.976-1.054)
Number of oocyte	0.995 (0.940-1.052)
Fertilization rate	0.333 (0.147-0.769)
Spermogram	0.900 (1.008-1.113)
EEGR model	
Count process	
Age (Y)	1.370 (1.170-1.548)
History of abortion	1.569 (1.346-1.720)
PCOS	1.103 (0.995-1.304)
Spermogram	0.140 (0.030-0.985)
ZI Part	
BMI (kg/m^2)	0.465 (0.141-0.651)
History of abortion	1.576 (1.203-1.783)
PCOS	0.090 (0.023-0.142)
Duration of infertility	0.938 (0.910-0.967)
Number of oocyte	1.049 (0.990-1.112)
Fertilization rate	0.882 (0.733-1.062)
EEGR parameter	12.511 (4.608)
Random intercept standard deviation	Estimate (SE)
Logistic part	0.022 (0.012)
Count part	0.237 (0.106)
Zero inflated part	0.166 (0.073)

EEGR; Exponentiated exponential geometric regression, PCOS; Polycystic ovarian syndrome, BMI; Body mass index, and CI; Confidence interval.

Joint modeling

Table 4 shows that estimations of all models that point in the same direction. Parameters, including, age, history of abortion and PCOS history, had a positive effect and Spermogram had a negative effect on the cycle number in count part of EEGR model. According to the results, both parameter, abortion history and oocyte number, had a direct relation and BMI, PCOS, duration of infertility and fertilization rate had a reverse relation with the cycle number in the ZI part. Also, in logistic part of our joint model, cycle number and duration of infertility had a positive effect on the ART failure and the number of oocytes in the last cycle. In addition, fertilization rate and Spermogram had a negative effect on them.

Table 4: The result of joint modeling

Variable	e^{β} (95% CI)	
Logistic submodel[a]		
Cycle number	1.282 (1.089-1.654)	
Duration of infertility	1.009 (0.987-1.030)	
Number of oocyte	0.403 (0.272-0.735)	
Fertilization rate	0.217 (0.104-0.545)	
Spermogram	0.535 (0.108-0.861)	
EEGR submodel		
Count process[b]		
Age (Y)	1.367 (1.172-1.570)	
History of abortion	1.876 (1.404-2.234)	
PCOS	2.904 (2.185-3.211)	
Spermogram	0.137 (0.032-0.428)	
Zero inflation[c]		
Intercept	0.0009 (0.0003-1.395)	
BMI (kg/m^2)	0.265 (0.140-1.090)	
History of abortion	1.075 (0.405-3.607)	
PCOS	0.076 (0.023-1.702)	
Duration of infertility	0.555 (0.320-0.966)	
Number of oocyte	1.049 (0.990-1.112)	
Fertilization rate	0.882 (0.731-1.061)	
EEGR parameter	12.511 (4.608)	
Parameter	Estimate (SE)	Wald statistic (P value)
RISD: Logistic part	0.049 (0.009)	
RISD: Count part	0.322 (0.041)	
RISD: Zero inflated part	0.204 (0.023)	
Correlation between a and b	0.421 (0.034)	12.38 (<0.001)
Correlation between a and c	0.650 (0.101)	6.43 (<0.001)
Correlation between c and b	0.512 (0.045)	11.37 (<0.001)

RISD; Random intercept standard deviation, PCOS; Polycystic ovarian syndrome, CI; Confidence interval, [a]; Logistic submodel, [b]; Count process, and [c]; Zero inflation.

The estimation of random intercepts in the models is relatively high which implies the use of mixed model. The correlation among the random intercepts of logistic submodel and the count process was 0.421 (standard error: 0.034). This means that cases with more number of cycles are more prone to experience failure in delivery at some stages, from clinical pregnancy to delivery. The correlation between the logistic and zero inflation sections was 65% that shows a direct association between having only one cycle number and failure in delivery. We observed a positive and significant association between the random effects of count process and zero inflation section.

Discussion

There are several methods for modelling IVF data that contain numerous cycles with various failure categories (11). One way to obtain better estimates of the covariate effects can be obtained by proposing the entire set of IVF data for each woman as opposed to the conventional method, which simply takes into account the first cycle or models each IVF outcome independently.

Studies on this type of data, use informative cluster sizes since it is thought that each infertile woman's cycle count is related to the success or failure of IVF outcomes. Joint modelling was used in this study. The number of cycles and the odds that an IVF procedure will fail were found to have strong positive relationships in this historical cohort study on Iranian infertile women as well, indicating the presence of informative cluster size (14).

Based on the joint modeling, our results show that, the older a woman is, the more cycles are needed to conceive. That is, pregnancy occurs earlier at younger ages due to healthier eggs. In 2019, Ubaldi et al. (15), pointed out in their article that the success of IVF decreases after the age of 35, because maternal age is related to a decline in both ovarian reserve and oocyte qualification. Previous studies have found a strong correlation between women's age and fertility (16-20) which is in agreement with our finding. Also, the history of abortion had a positive relationship with the number of cycles of IVF. This result has been proven in other studies. For example, in endometriosis patients (21), and other types of patients undergoing an IVF cycle (22-25). Having a history of abortion, which may be due to genetic causes or different diseases such as endometriosis, may lead to more IVF treatment cycles.

PCOS is one of the causes of infertility. According to our findings, women with PCOS usually needed more IVF treatment cycles to have the desired number of children. Women with PCOS are more likely to miscarry both after spontaneous and induced ovulation (12). Studies in different years had results consistent with our study (26-29). Of course, some studies had opposite results (8, 30).

In our study, we concluded that Spermogram, i.e. sperm parameters, have a positive relationship with treatment success. The reason is that the healthier sperm, will be formed the healthier embryo, and the pregnancy will be positive as a result. In another study, we see the same result (31, 32).

About BMI, had positive relation to the number of cycles. That means, overweight women need more cycles of IVF

treatment to reach a successful result, although there are some studies have reported opposite results. Rittenberg et al. (33) did a systematic review and meta-analysis in 2011 and also showed that BMI has a conflicting role in the IVF outcome and in specific, there is inadequate evidence to define how BMI affects live birth rates. But Veleva et al. (34) concluded in 2008 that being underweight and being overweight increases the chance of miscarriage in IVF. Also, in 2022, Bellver (35) concluded that the IVF result outcome and, overall successful pregnancies were lower in the obese women than non-obese of them. In 2021, Chen et al. (36) linked BMI to gestational diabetes mellitus (GDM) and gestational hypertension but not embryo transfer outcomes following fresh embryo transfer in women receiving their first IVF/ICSI treatment.

Data from multiple IVF treatment cycles were used in this study, along with information about their relationships. Due to the lack of a national registry, past cycles that infertile women may have completed at different infertility clinics were not included in this study.

Conclusion

In this study, we come to the conclusion that the number of cycles or cluster size is informative and has a direct effect on the treatment result.

Acknowledgements

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Authors' Contributions

M.M., A.K., T.M.; Conceptualization. M.M., A.K., P.A., A.G.; Methodology and Software. M.M., P.A.; Data curation, Writing-Original draft preparation. M.M.; Writing-Reviewing and Editing. All authors read and approved the final manuscript.

References

1. Akhondi MM, Ranjbar F, Shirzad M, Behjati Ardakani Z, Kamali K, Mohammad K. Practical difficulties in estimating the prevalence of primary infertility in Iran. Int J Fertil Steril. 2019; 13(2): 113-117.
2. Jesse DE, Walcott-McQuigg J, Mariella A, Swanson MS. Risks and protective factors associated with symptoms of depression in low-income African American and Caucasian women during pregnancy. J Midwifery Womens Health. 2005; 50(5): 405-410.
3. Thoma ME, McLain AC, Louis JF, King RB, Trumble AC, Sundaram R, et al. Prevalence of infertility in the United States as estimated by the current duration approach and a traditional constructed approach. Fertil Steril. 2013; 99(5): 1324-1331. e1.
4. Smith JF, Eisenberg ML, Millstein SG, Nachtigall RD, Sadetsky N, Cedars MI, et al. Fertility treatments and outcomes among couples seeking fertility care: data from a prospective fertility cohort in the United States. Fertil Steril. 2011; 95(1): 79-84.
5. McLernon DJ, Steyerberg EW, Te Velde ER, Lee AJ, Bhattacharya S. Predicting the chances of a live birth after one or more complete cycles of in vitro fertilisation: population based study of linked cycle data from 113 873 women. BMJ. 2016; 355: i5735.
6. Güvenir HA, Misirli G, Dilbaz S, Ozdegirmenci O, Demir B, Dilbaz B. Estimating the chance of success in IVF treatment using a ranking algorithm. Med Biol Eng Comput. 2015; 53(9): 911-920.
7. Van Voorhis BJ. Clinical practice. In vitro fertilization. N Engl J Med. 2007; 356(4): 379-386.
8. Ghaheri A, Rasekhi A, Omani Samani R, Hajizadeh E. Modeling in vitro fertilization data considering multiple outcomes observed among Iranian infertile women. Int J Fertil Steril. 2018; 12(1): 27-30.
9. Durairaj M, Thamilselvan P. Applications of artificial neural network for IVF data analysis and prediction. JEC&AS. 2013; 2(9): 11-15.
10. Roberts SA, Stylianou C. The non-independence of treatment outcomes from repeat IVF cycles: estimates and consequences. Hum Reprod. 2012; 27(2): 436-443.
11. Missmer SA, Pearson KR, Ryan LM, Meeker JD, Cramer DW, Hauser R. Analysis of multiple-cycle data from couples undergoing in vitro fertilization: methodologic issues and statistical approaches. Epidemiology. 2011; 22(4): 497-504.
12. Gray KE. Prediction modeling for live birth in in vitro fertilization. Presented as Ph.D., Washigton. University of Washigton. 2004.
13. Maity A, Williams PL, Ryan L, Missmer SA, Coull BA, Hauser R. Analysis of in vitro fertilization data with multiple outcomes using discrete time-to-event analysis. Stat Med. 2014; 33(10): 1738-1749.
14. Seaman S, Pavlou M, Copas A. Review of methods for handling confounding by cluster and informative cluster size in clustered data. Stat Med. 2014; 33(30): 5371-5387.
15. Ubaldi FM, Cimadomo D, Vaiarelli A, Fabozzi G, Venturella R, Maggiulli R, et al. Advanced maternal age in IVF: still a challenge? The present and the future of its treatment. Front Endocrinol (Lausanne). 2019; 10: 94.
16. Sneed ML, Uhler ML, Grotjan HE, Rapisarda JJ, Lederer KJ, Beltsos AN. Body mass index: impact on IVF success appears age-related. Hum Reprod. 2008; 23(8): 1835-1839.
17. Omani-Samani R, Ranjbaran M, Mohammadi M, Esmailzadeh A, Sepidarkish M, Maroufizadeh S, et al. Impact of unintended pregnancy on maternal and neonatal outcomes. J Obstet Gynaecol India. 2019; 69(2): 136-141.
18. Preutthipan S, Amso N, Curtis P, Shaw RW. Effect of maternal age on clinical outcome in women undergoing in vitro fertilization and embryo transfer (IVF-ET). J Med Assoc Thai. 1996; 79(6): 347-352.
19. Yiğit P, Bener A, Karabulut S. Comparison of machine learning classification techniques to predict implantation success in an IVF treatment cycle. Reprod Biomed Online. 2022; 45(5): 923-934.
20. Li NJ, Yao QY, Yuan XQ, Huang Y, Li YF. Anti-müllerian hormone as a predictor for live birth among women undergoing IVF/ICSI in different age groups: an update of systematic review and meta-analysis. Arch Gynecol Obstet. 2023; 308(1): 43-61.
21. Duan H, Li X, Hao Y, Shi J, Cai H. Risk of spontaneous abortion after antibiotic therapy for chronic endometritis before in vitro fertilization and intracytoplasmic sperm injection stimulation. Fertil Steril. 2022; 118(2): 337-346.
22. Bates GW Jr, Ginsburg ES. Early pregnancy loss in in vitro fertilization (IVF) is a positive predictor of subsequent IVF success. Fertil Steril. 2002; 77(2): 337-341.
23. Kupka MS, Dorn C, Richter O, Felberbaum R, van der Ven H. Impact of reproductive history on in vitro fertilization and intracytoplasmic sperm injection outcome: evidence from the German IVF Registry. Fertil Steril. 2003; 80(3): 508-516.
24. Wang Y, Sun Y, Di W, Kuang YP, Xu B. Association between induced abortion history and later in vitro fertilization outcomes. Int J Gynaecol Obstet. 2018; 141(3): 321-326.
25. Zou L, Hong M, Dai Z, Zhu J, Peng Q, Wang W. The association between previous induced abortion and in vitro fertilization outcomes: A retrospective cohort study in Hefei, China. Eur J Obstet Gynecol Reprod Biol. 2021; 262: 124-128.
26. Baxi SR, Patel H, Rao Gedela D, Verma PJ, Shah A. Comparison of oocyte and embryo quality and reproductive outcome in PCO and normoresponder patients undergoing IVF-A retrospective case-control study. IJHRRD. 2021; 1(1): 39-45.
27. Diamanti-Kandarakis E, Dunaif A. Insulin resistance and the polycystic ovary syndrome revisited: an update on mechanisms and implications. Endocr Rev. 2012; 33(6): 981-1030.
28. Dewailly D, Lujan ME, Carmina E, Cedars MI, Laven J, Norman RJ, et al. Definition and significance of polycystic ovarian morphology: a task force report from the androgen excess and polycystic ovary syndrome society. Hum Reprod Update. 2014; 20(3): 334-352.
29. Gremeau AS, Andreadis N, Fatum M, Craig J, Turner K, McVeigh E, et al. In vitro maturation or in vitro fertilization for women with polycystic ovaries? A case-control study of 194 treatment cycles. Fertil Steril. 2012; 98(2): 355-360.
30. Shapiro BS, Daneshmand ST, Desai J, Garner FC, Aguirre M,

Hudson C. The risk of embryo-endometrium asynchrony increases with maternal age after ovarian stimulation and IVF. Reprod Biomed Online. 2016; 33(1): 50-55.
31. Harada Y, Fukunaga E, Shiba R, Ikeda M, Okano S, Kinutani M. Relationship between sperm movement characteristics assessed by a sperm motility analysis system (SMAS) and normal fertilization rates in vitro. Hum Reprod. 2022; 37 Supplement 1: deac107. 021.
32. Safari H, Anbari F, Ghasemi-Esmailabad S, Maleki B, Dehghan Marvast L, Reza Talebi A. Relationship between sperm quality and total fertilization failure in intracytoplasmic sperm injection and in vitro fertilization cycles: a cross-sectional study. Int J Reprod Biomed. 2022; 20(5): 413-422.
33. Rittenberg V, Seshadri S, Sunkara SK, Sobaleva S, Oteng-Ntim E, El-Toukhy T. Effect of body mass index on IVF treatment outcome: an updated systematic review and meta-analysis. Reprod Biomed Online. 2011; 23(4): 421-439.
34. Veleva Z, Tiitinen A, Vilska S, Hydén-Granskog C, Tomás C, Martikainen H, et al. High and low BMI increase the risk of miscarriage after IVF/ICSI and FET. Hum Reprod. 2008; 23(4): 878-884.
35. Bellver J. BMI and miscarriage after IVF. Curr Opin Obstet Gynecol. 2022; 34(3): 114-121.
36. Chen H, Li J, Cai S, Zeng S, Yin C, Kuang W, et al. Impact of body mass index (BMI) on the success rate of fresh embryo transfer in women undergoing first in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment. Int J Obes (Lond). 2022; 46(1): 202-210.

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1. Title page should contain the title, name of the author/coauthors, their academic qualifications, designation & institutions they are affiliated with, mailing address for future correspondence, email address, phone, and fax number.
2. Text of manuscript and References prepared as stated in the "guide for authors" section.
3. Tables should be in a separate page. Figures must be sent in color and also in GIF or JPEG format with 300 dpi resolutions.
4. Cover Letter should be uploaded with the signature of all authors.
5. An ethical committee letter should be inserted at the end of the cover letter.

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