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# Contents

Systematic Review

# Urocortin Expression in Endometriosis: A Systematic Review

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## Abstract

Urocortin (UCN) is a neuropeptide that belongs to the corticotrophin-releasing hormone family and is expressed by eutopic and ectopic human endometria. The past years, this expression has been thoroughly investigated in the field of endometriosis. The objective of this systematic review is to accumulate current evidence related to the expression of UCN in tissue and blood samples of patients suffering from endometriosis. Literature search was designed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and primarily conducted using the Medline (1966-2018), Scopus (2004-2018), EMBASE (1947-2018) and Clinicaltrials.gov (2008-2018) databases, along with the reference lists of electronically retrieved full-text papers. Overall, eight studies were retrieved. Current evidence suggests that the expression of UCN is increased in patients with ovarian endometriomas and that its levels may correlate with the severity of the disease. The diagnostic efficacy of UCN1 plasma levels was evaluated in three studies. Two of them suggested that the sensitivity and specificity of the method may reach, and even exceed, 80%. However, the wide variation in outcome reporting and outcome reporting measures in endometriosis among the included studies precludes meta-analysis of available data. Therefore, although UCN seems to be a promising biomarker for the identification and follow-up of patients that suffer from endometriosis, more studies are needed to reach firm conclusions with respect to its predictive accuracy.

***Keywords:*** Endometrioma, Endometriosis, Urocortin

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## Introduction

Endometriosis is a benign inflammatory gynecological disease that manifests in women of reproductive age and is defined as the presence of stromal and viable endometrial glands outside the uterine cavity. It has an estimated prevalence of up to 10% in the general population and is associated with chronic pelvic pain, dysmenorrhea and infertility (1, 2). The pathogenic mechanisms, however, still remain unclear and the aetiology of the disease is believed to be multifactorial. Various endocrinological and immunological factors have been investigated and are considered to significantly contribute to its pathophysiology (3, 4).

During the past few years, several novel serum biomarkers have been proposed for the early diagnosis of endometriosis. The most consistently studied molecule is cancer antigen 125 (CA-125), a glycoprotein that has been established as a biomarker for the follow-up of patients with epithelial ovarian cancer. Similarly, in endometriosis, CA-125 can only be used as a prognostic rather than a diagnostic marker as it is accompanied by a significant amount of false negative results (5).

Recently, urocortin (UCN) has been extensively investigated in the field of endometriosis. UCN is a neuropeptide that belongs to the corticotrophin-releasing hormone (CRH) family and is expressed by eutopic and ectopic human endometria and is thought to play a role during decidualization (6, 7). Because of its paracrine and immunomodulatory nature, UCN is thought to contribute to the pathogenesis of endometriosis. To date, three different isoforms have been described (UCN1, UCN2 and UCN3), all of which exert their biological action by activating corticotropin-releasing hormone (CRH) receptors 1 and 2. Their main difference relies in the fact that UCN1 binds to both CRH1 and CRH2, whereas UCN2 and UCN3 bind selectively to CRH2 (8).

To date, it remains unclear whether UCN may be used as a screening and/or prognostic biomarker of endometriosis. The objective of this systematic review is to accumulate current evidence related to the expression of UCN in tissue and blood samples of patients suffering from endometriosis and provide directions for future research.

## Materials and Methods

### Study design

The present systematic review was designed according

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to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (9). Eligibility criteria were assessed by the authors. Briefly, date and language restrictions were avoided during the literature search. All observational studies (both prospective and retrospective) that presented data relevant to the expression of UCN in tissue and blood samples of patients with endometriosis were included in this systematic review. Studies that defined their controls as either women with no pathology or women with other benign pathology were evaluated and included. Review articles, animal studies and case reports were excluded from the present review. The selection process took place in three consecutive stages. Firstly, the titles and abstracts of all electronic articles were screened to assess their eligibility. Subsequently, the articles that met or were presumed to meet the criteria were retrieved as full texts. In the final stage references of articles that were retrieved in full text were evaluated to identify studies that might have been overlooked during the electronic search. Any discrepancies in the methodology, retrieval of articles and statistical analysis were resolved through the consensus of all authors.

## Literature search and data collection

Literature search was primarily conducted using the Medline (1966-2018), Scopus (2004-2018), EMBASE (1947-2018) and Clinicaltrials.gov (2008-2018) databases, along with the reference lists of electronically retrieved full-text papers. Additional sources were identified through the Google Scholar (2004-2018) database. Our last search was on the 04 February 2018. The search strategy included the words "endometriosis and urocortin" and is schematically presented in the PRISMA flow diagram (Fig.1).

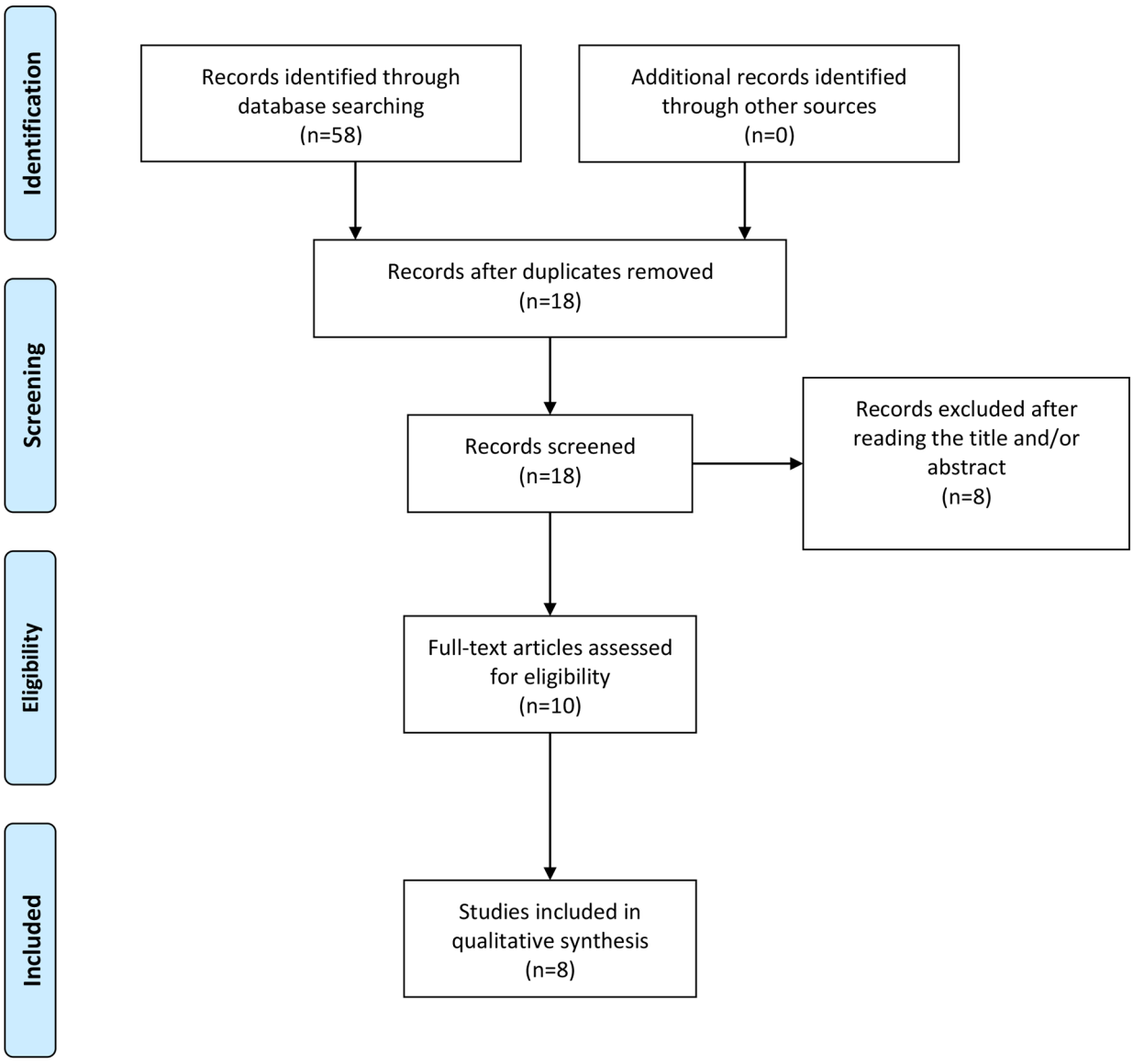


**Fig.1:** Search plot diagram.

## Results

Overall, 8 studies were included in the present systematic review and outcomes from a total of 567 women were assessed (10-18). The methodological characteristics of included studies are presented in Table 1. One study was excluded from the present systematic review as it presented preliminary data that were solely based on immunohistochemistry (13).

### *UCN*, *UCN2*, *UCN3* in utopic endometrium and endometriotic lesions

Kempuraj et al. (13) investigated in this pilot study the expression of *UCN* in biopsies from 10 patients with endometriosis and 3 patients that did not have endometriosis. Although they did not perform statistical analysis on the observed differences, they found that endometriotic lesions had increased *UCN* expression compared with healthy peritoneum and normal endometrium. Two studies evaluated *UCN* transcript expression (10, 18) and one study assessed *UCN1* and *UCN2* transcript expression in eutopic endometrium and in endometriotic lesions. While *UCN2* expression did not seem to differ between eutopic endometrium and endometriomas (16), the expression levels of *UCN* and *UCN3* in the endometriotic foci (ectopic lesions) were significantly higher compared with those in eutopic endometrium of the same women (10, 16, 18). Vergetaki et al. (18) showed an almost 3.4-fold increase in the expression levels of *UCN* transcripts when ectopic and eutopic endometrium samples were compared (0.9566 ± 0.136 a.u vs. 0.2826 ± 0.075 a.u respectively).

The extent, depth of invasion and location of endometriotic lesions were shown to be associated with *UCN* transcript levels. Specifically, Carrarelli et al. (10) observed that deep infiltrating endometriosis (DIE) is associated with higher levels of *UCN* than ovarian endometriomas (OMA) (10).

Novembri et al. (15) studied *UCN*, *UCN2* and *UCN3* gene expression in eutopic endometrium of healthy women and women with endometriosis during the menstrual cycle. In women suffering from endometriosis, the expression of *UCN*, *UCN2* and *UCN3* transcripts was the same in the secretory and the proliferative phases, whereas in healthy women *UCN* levels differed between the two phases. Specifically, *UCN2* expression had peak values during the early proliferative phase, while *UCN3* expression was at its maximum in the secretory phase (15, 16). Both *UCN2* and *UCN3* expression levels were significantly lower in women with endometriosis when compared with healthy women (16).

### Effect of UCN on the decidualization process

Decidualization is a process of endometrial remodeling that occurs in the secretory phase of the menstrual cycle and is essential for early pregnancy. Current knowledge suggests that this process is initiated by progesterone and is mediated by various molecules such as UCN. Novembri et al. (15) showed that women with endometriosis have decreased levels of CRH and UCN, and suggested that this could negatively affect decidualization.

**Table 1:** Methodological characteristics of included studies

| Author | Patient | Country | Inclusion criteria | Tissue examined | UCN form | Method of assessment |
|---|---|---|---|---|---|---|
| Maia et al. (14) | 59 vs. 38 n=97 | Brazil | Consecutive list of patients that undergone laparoscopy for endometriosis | Plasma | Protein | Enzyme Immunoassay |
| Carrarelli et al. (10) | 22 vs. 26 n=48 | France | Women with endometriosis* | OMA, DIE, endometrium | RNA, IHC | qRT-PCR |
| Chmaj-Wierzchowska et al. (11) | 48 vs. 38 n=86 | Poland | Consecutive list of patients that undergone laparoscopy for endometriosis or ovarian teratoma | Plasma | Protein | ELISA |
| Vergetaki et al. (18) | 10 vs. 16 n=26 | Greece | Women that undergone surgery and hysteroscopy for endometriosis | DIE, endometrium | RNA, IHC | RT-PCR |
| Tokmak et al. (17) | 46 vs. 42 n=88 | Turkey | Consecutive list of patients that undergone laparoscopy for OMA vs benign cysts | Plasma | Protein | ELISA |
| Novembri et al. (16) | 41 vs. 39 n=80 | Italy | Women that undergone surgery for OMA | OMA, Endometrium | RNA, IHC | RT-PCR |
| Novembri et al. (15) | 36 vs. 26 n=62 | Italy | Women that undergone surgery for OMA | OMA, Endometrium | RNA, IHC | RT-PCR |
| Florio et al. (12) | 40 vs. 40 n=80 | Italy | Women that undergone surgery for OMA or OMA and peritoneal endometriosis | Plasma | Protein | ELISA |

UCN; Urocortin, OMA; Ovarian endometrioma, DIE; Deeply infiltrating endometriosis, *; Patients with both OMA and DIE lesions were excluded, IHC; Immunohistochemistry, RT-PCR; Real-time polymerase chain reaction, and ELISA; Enzyme-linked immunosorbent assay.

**Plasma UCN as a diagnostic marker of endometriosis**

Two studies evaluated preoperative plasma levels of UCN as a diagnostic factor that would help differentiate patients with endometriosis from patients with non-endometriotic, benign ovarian cysts (12, 17). Specifically, Florio et al. (12) reported that plasma UCN levels were two-fold higher in women with endometriomas (median 49 pg/mL, interquartile range 41-63 pg/mL) compared with controls (19 pg/mL, P<0.001). The receiver operating characteristic (ROC) analysis showed that UCN detected 88% of cases that had endometriomas with a specificity of 90% and an area under the curve (AUC) equal to 0.961 ± 0.021 (cut-off value 33 pg/ml). Positive and negative likelihood ratios for UCN were 8.8 and 0.14 respectively. On the contrary, Tokmak et al. (17) reported no difference in the expression of UCN between patients with endometriomas and the control group (4.8 ± 1.00 ng/ml vs. 4.5 ± 1.03 ng/ml, P=0.21). When the cut-off point was set at 4.16 ng/ml, the sensitivity of the UCN protein in detecting endometriosis was 76.2%, the specificity was 45.7% and the positive predictive value was 56.1%.

Chmaj-Wierzchowska et al. (11) compared UCN levels between patients with endometriomas and patients with mature teratomas. The expression of UCN was not significantly different between the two groups (252.37 ± 348.77 pg/ml vs. 256.03 ± 353.92 pg/ml, P=0.0727).

Maia et al. (14) studied plasma levels of UCN1 as a diagnostic biomarker of endometriosis among symptomatic patients. Compared with no-lesion patients (median 34 pg/ml, interquartile range 22-43 pg/ml), patients with endometriosis showed elevated UCN1 plasma levels (median 59 pg/ml, interquartile range 48-107 pg/ml). The ROC analysis identified plasma UCN1 concentration of 46 pg/mL as the best cut-off point to differentiate women with endometriosis from those with no lesions, with 76% sensitivity, 88% specificity and an AUC equal to 0.827. However, an optimal cut-off that would distinguish endometriosis from other benign pathology (including benign ovarian cysts, ovarian teratoma, hydrosalpinx, salpingitis, ectopic pregnancy, uterine leiomyoma and ovarian cancer) was not identified.

## Discussion

Current evidence suggests that UCN may be a promising factor for the identification and follow-up of patients that suffer from endometriosis. However, the methodological heterogeneity of these studies in terms of the reported measures precludes firm conclusions. The expression of UCN has been investigated post-transcription both at the transcription and protein levels. Three studies suggested that the expression of *UCN* transcripts is significantly higher in endometriotic lesions compared with eutopic endometrium of endometriotic women (10, 16, 18). Its correlation with the severity of the disease also implies that it might become a useful tool for the classification of endometriosis (10). On the other hand, data related to the plasma levels of the protein were conflicting. Specifically, two studies reported significant differences between patients with endometriosis and controls (12, 14), whereas another two reported that *UCN* levels did not differ between patients with endometriomas and patients with other benign cysts (11, 17). Maia et al. (14) suggested that the diagnostic accuracy of the expression at the protein level is promising, however, further evidence is needed to confirm these findings.

Despite the fact that our study is based on a meticulous review of current literature, certain limitations preclude definitive conclusions. Firstly, the wide variation in outcome reporting and outcome reporting measures of UCN expression in endometriosis precludes meta-analysis of current data. Furthermore, the correlation between the expression of UCN in endometriotic lesions and peripheral blood remains to be investigated. Its actual value as a minimally invasive diagnostic method therefore, remains to be elucidated. Future studies should also clarify whether UCN levels are elevated not only in endometriomas but also in non-ovarian endometriosis and in early stage disease. The presence of a biomarker with high sensitivity and specificity in these cases is particularly important for the differential diagnosis since, to date, minimally invasive methods are non-existent (19). Moreover, to standardise the measurement of UCN levels in daily clinical practice, further investigation is necessary to determine whether the peptide concentration is affected by factors including menstrual cycle, obesity, exercise, stress, diabetes mellitus and other chronic diseases (20). Accordingly, research should also focus on potential confounders that may affect the plasma levels of this protein, including diseases that may trigger chronic inflammation. To date, there is insufficient evidence to suggest that UCN acts as a mediator of inflammation, as its expression might have been an effect rather than a cause of inflammation (21).

However, this interesting point deserves further investigation. Moreover, previous studies have also shown that UCN is produced by the liver and kidney of healthy animals (22) but evidence in humans is still lacking. Therefore, the evaluation of UCN in patients with hepatic and/or liver dysfunction needs to be elucidated to help exclude these diseases as confounders. It would also be prudent to perform multivariate analyses to determine the impact of the various factors that were mentioned in this section potentially affecting UCN levels. Finally, cut-off values should be introduced to investigate the predictive efficacy of UCN. These cut-offs should be based on previous proposed values that were mentioned in this systematic review to evaluate consistency of results. Optimal cut-offs should also be reported to help future research in this field.

## Conclusion

Current evidence suggests that UCN may be a promising factor for diagnosis and/or prognosis of patients that suffer from endometriosis. However, available data are scarce and the findings of currently published studies remain to be validated. Specifically, future studies should

examine whether plasma and tissue levels of UCN correlate in patients. Moreover, evaluation of the predictive accuracy of UCN with the use of pre-specified cut-off values, mentioned in the present systematic review, is needed to assess the reproducibility of previous findings in the field.

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## Authors' Contributions

V.P.; Conceived the idea, formed the tables and performed the meta-analysis. N.M.T.; Collected the data, tabulated data and wrote the manuscript. A.T., O.K., A.N.; Collected the data and wrote the manuscript. P.T.; Conceived the idea and wrote the manuscript. All authors read and approved the final manuscript.

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Original Article

# The Association between *TNF-alpha* Gene Polymorphisms and Endometriosis in An Iranian Population

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## Abstract

**Background:** Tumor necrosis factor-alpha (*TNF-α*) is an important cytokine in acute inflammatory response to infective factors. Based on investigation in different populations, it is thought that this response increases in patients with endometriosis due to the presence of cytokines such as *TNF-α*. This study aimed to examine the association of four *TNF-α* polymorphisms, namely -238G/A, -308G/A, -857C/T and -863C/A, with susceptibility to endometriosis in an Iranian population.

**Materials and Methods:** We recruited 150 women with endometriosis and 150 women without endometriosis in this case-control study and collected 4 ml of blood from all subjects. After DNA extraction, the polymorphisms were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

**Results:** The allele frequency of *TNF-α* -863C/A in the case and control groups showed a significant difference [odds ratios (OR)=0.64, 95% confidence interval (CI)=0.41-0.99, P=0.047] but the result is not significant when Adjusting for multiple testing (P=0.188). No significant difference in the allele frequencies of -238G/A (OR=1.07, 95% CI=0.51-2.25, P=0.862), -308G/A (OR=0.79, 95% CI=0.43-1.45, P=0.438) and -857C/T (OR=1.03, 95% CI=0.66-1.61, P=0.887) was observed. We adjusted all four polymorphism genotypes by age and body mass index (BMI), however, no significant difference was detected. There was an association between the case and control and BMI when adjusting by age (OR=1.082, 95% CI=1.009-1.162, P=0.028).

**Conclusion:** For the first time the association of the four polymorphisms in the promoter region of the *TNF-α* gene with endometriosis has been conducted in women of Iranian origin. The present research reveals the -863 A allele may play a role in incidence of endometriosis among Iranian women. Development of endometriosis among those people with -863 A allele seems low. According to the results, the current study indicates that there might be a correlation between BMI and progression of endometriosis.

***Keywords:*** Body Mass Index, Endometriosis, Polymorphisms, Restriction Fragment Length Polymorphism, *TNF-α*

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## Introduction

Endometriosis is developed as a result of endometrial tissue exposing outside the uterine cavity. Studies have reported the pelvic and the peritoneum as the most common sites of replacement (1, 2). This highly prevalent disease can be really enervating (about 30% in infertile and 10% in fertile women) (3). Approximately, 25-50% of infertile women develop endometriosis while 30-50% of women with endometriosis are infertile (4).

This polygenic disease with its complex genetic background (5, 6) occurs as a result of interactions between genetically determined factors and environment. The genetic component of endometriosis has been shown through studying the kinship of patients (7, 8). To date, the most common method for investigating genetic factors underlying complex diseases is the hypothesis-based candidate gene studies (8). One of the most important factors in endometriosis is mutations in cytokine genes. The tumor necrosis factor-alpha (*TNF-α*) is an important cytokine in acute inflammatory response to infective factors and is genetically variable. Based on multiple studies, *TNF-α* is thought to be a molecular indicator for gynecological-related diseases. It is suggested that the inflammatory response in endometrio-

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sis increases because of cytokines such as *TNF-α* (8, 9).

Studies on patients diagnosed with endometriosis have highlighted that *TNF-α* is a likely factor in developing endometriosis as suggested by elevated levels of *TNF-α* in peritoneal fluid and the up-regulation of *TNF-α* in peritoneal macrophages and peripheral blood monocytes (10, 11). However, the exact role which *TNF-α* plays in endometrial tissue is ambiguous (12). Thus far, some polymorphisms in the promoter region of the *TNF-α* gene have been examined in patients with endometriosis (12-20). Therefore, for the first time, we aimed to examine the relationship of *TNF-α* -238G/A, -308G/A, -857C/T and -863C/A polymorphisms with risk of developing endometriosis in Iranian women.

## Materials and Methods

### Subjects

This case-control study enrolled a total of 150 Iranian women with endometriosis who had referred to Avicenna Infertility Clinic and Tehran Clinic Hospital, Tehran, Iran. Diagnostic laparoscopy was performed in all patients. The severity of endometriosis was determined using the revised American Society for Reproductive Medicine (ASRM) classification (stages I-IV of disease). The control group consisted of 150 women without endometriosis. Only women who underwent laparoscopy for non-endometriosis infertility and showed absence of endometriosis were included as controls. Stages I and II of endometriosis are commonly found in asymptomatic women (21). The exclusion criteria in our study were the following: having a history of rheumatoid arthritis, diabetic retinopathy and Behcet's disease. Approval from the Avicenna Research Institute Ethics and Human Rights Committee was obtained for using blood samples and the designed protocol. Written informed consent was obtained from all patients with inclusion criteria to take part in the study.

### DNA extraction and genotyping

Blood was collected in tubes with 200 µl EDTA (0.5 M), as an anti-clotting factor, and stored at -20ºC until DNA extraction. Genomic DNA was extracted by salting out method from peripheral blood samples. Genotyping of the -238G/A (rs361525), -308G/A (rs1800629), -857C/T (rs1799724) and -863C/A (rs1800630) polymorphisms in the 5'-untranslated region of *TNF-α* was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Details on primers and restriction enzymes are presented in Table 1.

The PCR reactions carried out in final volume of 25 µl containing: 10X PCR Buffer (Roche, Germany), 1.5 mM $MgCl_2$ (Roche, Germany), 0.4 µM of each dNTP (Fermentas, Germany), 5 pmol of each primer, 50 ng template DNA, 1 U Taq DNA polymerase (Roche, Germany) and sterile distilled water up to 25 µl. Amplification conditions start with an initial denaturation step of 5 minutes at 94ºC, followed by 30 cycles of 30 seconds denaturation (94ºC), 30 seconds annealing (63ºC) for -238G/A, -857C/T and -863C/A and 30 seconds annealing (66ºC) for -308G/A and 30 seconds extension (72ºC), ended by a final extension for 5 minutes (72ºC) and finally cooling to 4ºC.

Polymerase chain reaction products were electrophoresed on a 1.5% agarose gel in 1X TAE and stained with ethidium bromide and visualized by ultraviolet light. After reviewing the PCR products, they were treated with restriction enzymes (Hpa II and NcoI at 37ºC and TaiI at 65ºC) overnight. The digestion products were subjected to 10% polyacrylamide gel electrophoresis and stained with silver nitrate (Fig.1).

### Statistical analyses

Results were analyzed by SPSS 24.0 software (IBM SPSS Statistical Software, USA). The analysis of age and body mass index (BMI) in the study groups were performed using t test. The allele frequencies were compared using the Chi-squared test. Genotype distributions in the case and control groups were also analyzed. Age and BMI were considered as potential confounders. The analyses were performed and adjusted in terms of age and BMI using logistic regression. $P<0.05$ was considered statistically significant. The P value corrected using Bonferroni method for the multiple testing. Logistic regression was used to predict the odds of developing a given disease based on observed characteristics of the patients. In our study, the criterion variable was the logistic regression of disease and no-disease. To perform the statistical analysis using SPSS, we considered the two case and control groups as dependent variables. Age and BMI were considered as covariants and genotype selected as the basis of categorical covariant.

**Table 1:** Information about primers and restriction enzymes used

| Gene | Variation | Primers (5´-3´) | Size (bp) | Restriction enzyme | Allele | Cutting product (bp) |
|---|---|---|---|---|---|---|
| *TNF-α* | -238G/A | F: AGAAGACCCCCCTCGGAACC<br>R: CTCATCTGGAGGAAGCGGTA | 165 | Hpa II (New England BioLabs) | G | 136<br>19 |
| | -308G/A | F: AGGCAATAGGTTTTGAGGGCCAT<br>R: TCCTCCCTGCTCCGATTCCG | 107 | NcoI (New England BioLabs) | G | 87<br>20 |
| | -857C/T | F: GGCTCTGAGGAATGGGTTAC<br>R: CCTCTACATGGCCCTGTCTAC | 128 | TaiI (New England BioLabs) | C | 107<br>21 |
| | -863C/A | F: GGCTCTGAGGAATGGGTTAC<br>R: CTACATGGCCCTGTCTTCGTTACG | 125 | TaiI (New England BioLabs) | A | 101<br>24 |

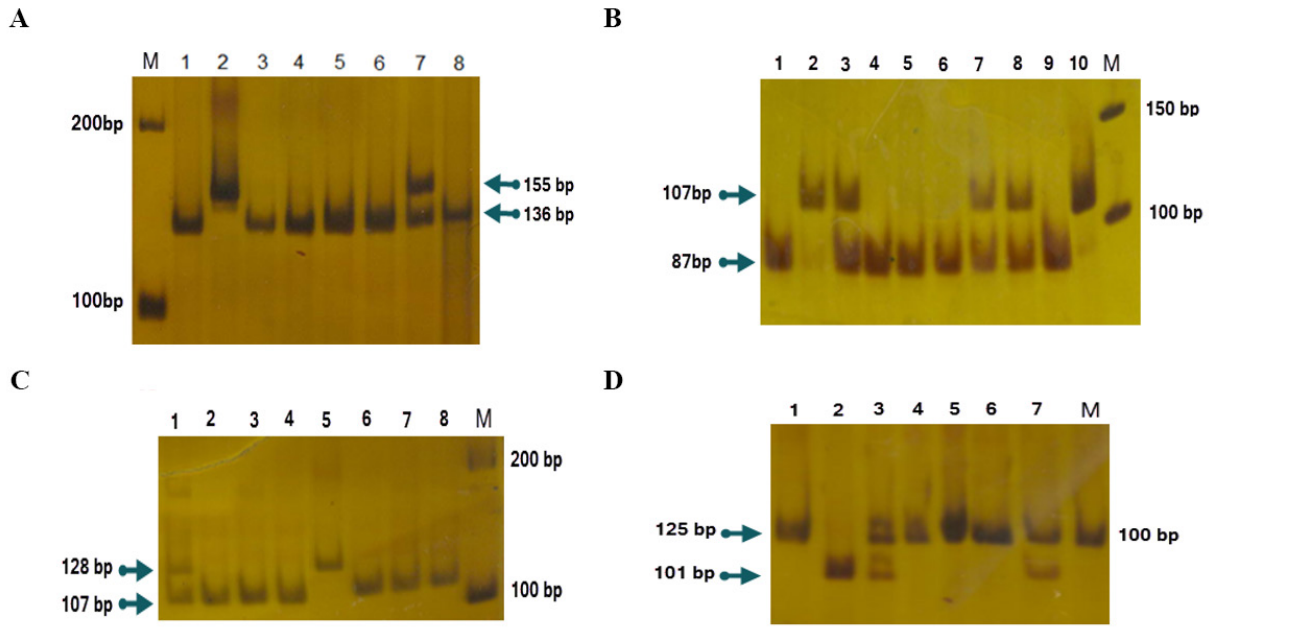


**Fig.1:** Representative gel pictures of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) results. **A.** The -238G/A polymorphism PCR-RFLP result. Lane M; Ladder 100 bp, No. 1, 3-6 and 8; Homozygote (GG), No. 2; Homozygote (AA), No. 7; Heterozygote (GA), **B.** The -308G/A polymorphism PCR-RFLP result. Lane M; Ladder 50 bp, No.1, 4-6 and 9; Homozygote (GG), No. 3, 7 and 8; Heterozygote (GA), No.2; Homozygote (AA), and No. 10; Undigested PCR product as the control, **C.** The -857C/T polymorphism PCR-RFLP result. Lane M; Ladder 100 bp, No. 2-4 and 6-8; Homozygote (CC), No. 1; Heterozygote (TC) and No. 5; Homozygote (TT), and **D.** The -863C/A polymorphism PCR-RFLP result. Lane M; Ladder 100 bp, No.1 and 2; Heterozygote (AC) and No. 3-5; Homozygote (CC).

For interaction analysis, the STRING online server (http://string-db.org/) was used to acquire the network of protein-protein interactions for *TNF-α*.

## Results

According to the analysis of descriptive variables, the age range was from 19 to 50 years (mean=31, SD=6.1) in the patients, and from 19 to 44 years (mean=29.2, SD=5.2) in the control group. The mean BMI (Kg/m$^2$) in the case and control groups were 25.2 (SD=3.7) and 26.2 (SD=4) respectively. Genotypes of the *TNF-α* -238G/A, -308G/A, -857C/T and -863C/A polymorphisms were obtained in 150, 150, 148, 150 patients and 149, 150, 143, 150 control samples respectively. Genotype frequencies of the *TNF-α* -238G/A, -308G/A, -857C/T and -863C/A polymorphisms in the case and control groups were in Hardy-Weinberg equilibrium. Genotype and allele frequencies for the *TNF-α* -238G/A, -308G/A, -857C/T and -863C/A are shown in Table 2.

The *TNF-α* -863C/A allele A frequency between case and control groups represented a significant difference (P=0.047) but the result is not significant when adjusting for multiple testing (P=0.188). However, no significant difference was observed in the allele frequencies of the -238G/A (P=0.862), -308G/A (P=0.438) and -857C/T (P=0.878) polymorphisms in *TNF-α* between the case and control groups. We adjusted all four polymorphism genotypes by age and BMI but according to the results, no significant difference was discovered between the groups. But there was an association between the case and control and BMI when adjusting by age (OR=1.082, 95% CI=1.009-1.162, P=0.028).

*TNF-α* interacts with 10 other proteins according to STRING (Fig.2). In specific, they are i. TGF-beta activated kinase 1/MAP3K7 binding protein 2 *(TAB2)*, ii. Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 *(NFKB1)*, iii. TNF receptor-associated factor 2 *(TRAF2)*, iv. TNFRSF1A-associated via death domain *(TRADD)*, v. Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta *(IKBKB)*, vi. Receptor interacting serine-threonine kinase 1 *(RIPK1)*, vii. Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma *(IKBKG)*, viii. Baculoviral IAP repeat containing 2 *(BIRC2)*, ix. Tumor necrosis factor receptor superfamily, member 1A *(TNFRSF1A)*, and x. Tumor necrosis factor receptor superfamily, member 1B; *(TNFRSF1B)*.

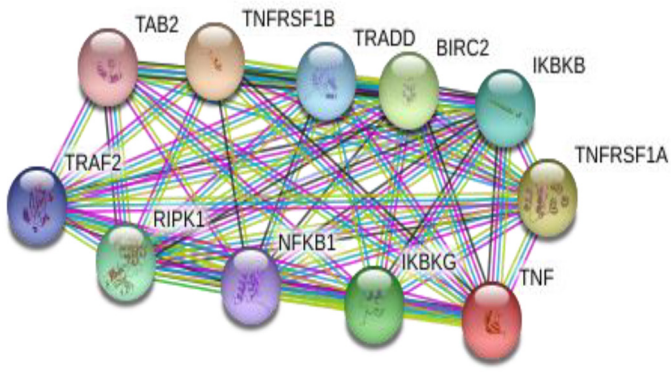


**Fig.2:** *TNF-α* protein-protein interactions network obtained from STRING (htpp://string-db.org/).

**Table 2:** Genotype and allele frequencies of the four polymorphisms in the promoter region of TNF-α in patients with stage I-IV of endometriosis and controls

| Polymorphisms* | | | Cases | Controls | OR | 95% CI | P value | Corrected P value** |
|---|---|---|---|---|---|---|---|---|
| The -238 (rs361525) | Genotype | GG | 137 (91.3%) | 135 (90.6%) | | | | |
| | | GA | 11 (7.3%) | 14 (9.4%) | 0.65 | 0.24-1.71 | 0.381 | 0.762 |
| | | AA | 2 (1.3%) | 0 (0.0%) | NA | NA | NA | NA |
| | Allele | G | 285 (95.0%) | 284 (95.3%) | | | | |
| | | A | 15 (5.0%) | 14 (4.7%) | 1.07 | 0.51-2.25 | 0.862 | 1 |
| The -308 (rs1800629) | Genotype | GG | 131 (87.3%) | 127 (84.7%) | | | | |
| | | GA | 18 (12.0%) | 21 (14.0%) | 0.8 | 0.35-1.84 | 0.604 | 1 |
| | | AA | 1 (0.7%) | 2 (1.3%) | 0 | NA | 0.999 | 1 |
| | Allele | G | 280 (93.3%) | 275 (91.7%) | | | | |
| | | A | 20 (6.67%) | 25 (8.3%) | 0.79 | 0.43-1.45 | 0.438 | 1 |
| The -857 (rs1799724) | Genotype | CC | 102 (68.9%) | 102 (71.3%) | | | | |
| | | CT | 43 (29.1%) | 36 (25.2%) | 1.62 | 0.86-3.04 | 0.137 | 0.274 |
| | | TT | 3 (2.0%) | 5 (3.5%) | 0.46 | 0.05-4.35 | 0.499 | 0.998 |
| | Allele | C | 247 (83.5%) | 240 (83.9%) | | | | |
| | | T | 49 (16.5%) | 46 (16.1%) | 1.03 | 0.66-1.61 | 0.887 | 1 |
| The -863 (rs1800630) | Genotype | CC | 114 (76.0%) | 99 (66.0%) | | | | |
| | | CA | 32 (21.3%) | 44 (29.3%) | 0.66 | 0.35-1.27 | 0.215 | 0.43 |
| | | AA | 4 (2.7%) | 7 (4.7%) | 0.68 | 0.19-2.47 | 0.557 | 1 |
| | Allele | C | 260 (86.7%) | 242 (80.67%) | | | | |
| | | A | 40 (13.3%) | 58 (19.3%) | 0.64 | 0.41-0.99 | 0.047 | 0.188 |

**OR; Odds ratios, CI; Confidence interval, BMI; Body mass index, NA; No answer, *; The effect of genotypes were adjusted by age and BMI, and **; The P value corrected using Bonferroni method for the multiple testing.**

**Table 3:** *TNF-α* promoter polymorphisms studies

| SNP name | Association with susceptibility | |
|---|---|---|
| | **Association**<br>**Population/(number of cases and controls)** | **No-association**<br>**Population/(number of cases and controls)** |
| -1031T/C | Japanese (123, 165) (Teramoto et al.) (20)<br>Japanese (130, 185) (Asghar et al.) (12)<br>Iranian (135, 173) (Saliminejad et al.) (15)<br>Iranian (65, 65) (Abutorabi et al.) (16) | Australian (958, 959) (Zhao et al.) (19) |
| -863C/A | Japanese (123, 165) (Teramoto et al.) (20)<br>Iranian (150, 150) (This study)*, £ | Japanese (130, 185) (Asghar et al.) (12) |
| -857C/T | Japanese (123, 165) (Teramoto et al.) (20)<br>Iranian (148, 143) (This study)£ | Japanese (130, 185) (Asghar et al.) (12)<br>Australian (958, 959) (Zhao et al.) (19)<br>Iranian (148, 143) (This study)** |
| -308G/A | Iranian (150, 150) (This study)£ | Taiwanese (120, 106) (Hsieh et al.) (13)<br>Korean (70, 202) (Lee et al.) (17)<br>Austrian (92, 69) (Wieser et al.) (18)<br>Japanese (130, 185) (Asghar et al.) (12)<br>Australian (958,959) (Zhao et al.) (19)<br>Chinese (76,87) (Lu et al.) (14)<br>Iranian (65, 65) (Abutorabi et al.) (16)<br>Iranian (150, 150) (This study)** |
| -238G/A | Iranian (150, 150) (This study)£ | Korean (70, 202) (Lee et al.) (17)<br>Austrian (92, 69) (Wieser et al.) (18)<br>Japanese (130, 185) (Asghar et al.) (12)<br>Australian (958, 959) (Zhao et al.) (19)<br>Iranian (65, 65) (Abutorabi et al.) (16)<br>Iranian (149, 150) (This study)** |

**BMI; Body mass index, *; Allele frequencies, **; Genotype adjusted by age and BMI, and £; case and control and BMI adjusted by age.**

## Discussion

Endometriosis is a multifactorial disease with both genetic and environmental components (8). Studies have scanned the genome and specific candidate genes to determine the genetic aetiology of this disease (22), reporting on endometriosis and related genes involved in "detoxification, galactose metabolism, steroid hormone production and inflammation" (15, 21, 22). Studies have also shown that any change in function and number of immune cells as well as high levels of inflammatory cytokines may lead to endometriosis (23). The present study aimed at examining the association of four *TNF-α* polymorphisms, -238G/A, -308G/A, -857C/T and -863C/A with endometriosis in an Iranian sample.

The effects of polymorphisms on cytokines genes have been examined by many studies (22) as well as the possible association of *TNF-α* polymorphisms with augmented endometriosis risk (12, 13, 15, 17-20). So far, many polymorphic variants have been examined in different populations, leading to various results (24). A number of polymorphisms have been associated with the disease in many populations, however, in some studies no association with the disease was observed. The reason for these observed differences may include different diagnostic criteria in selection of patients and controls, distinct living setting, number of samples, and varying populations and ethnicities (24, 25).

All in all, endometriosis has been reported to be linked with a number of polymorphisms of TNF-α. The study by Asghar et al. (12) and two other investigations by Lee et al. (17) concluded that -238G/A, -308G/A, -857C/T and -863C/A polymorphisms in the promoter region of *TNF-α* had no impact on women developing endometriosis. In contrast, these studies and that by Saliminejad et al. (15) showed that the frequency of the -1031C allele in the *TNF-α* gene in patients suffering severe endometriosis was significantly lower than that of their control group. Additional genetic studies on promoter polymorphisms in *TNF-α* by Wieser et al. (18) (-238 G/A and -308G/A), and Hsieh et al. (13) and Lu et al. (14) (-308G/A) found no association with endometriosis in the Asian population. Another study by Zhao et al. (19) in Australia reported that the -863C/A polymorphisms in *TNF-α* had no effect on patients with endometriosis. Moreover, Abutorabi et al. (16) found a positive association between the -1031 T/C polymorphism with endometriosis. However, no significant association was observed between the -238 G/A and -308 G/A polymorphisms with the disease. A similar study by Teramoto et al. (20) discovered an overrepresentation between the TNF-U01 haplotype (-1031T, -863C and -857C) and endometriosis in Japanese women.

According to the studies mentioned, *TNF-α* -238G>A has been inspected in three studies, -308G>A in five articles, -857C>T in three articles and -863C>A in four articles, all of which showed compatible findings where no significant association was reported between these four polymorphisms and endometriosis in any of the models. Our study reported an association between the -863C/A polymorphism in the promoter region of *TNF-α* and endometriosis. We also observed a direct relationship between the case and control and BMI when adjusting by age (Table 3).

It has been shown that the presence of polymorphism in promoter regions can affect gene expression (26). STRING showed that *TNF-α* interacts with 10 other molecules. These interactions are likely to be functionally important, especially those with proteins involved in cell survival and apoptosis such as *TRAF2*, which regulates activation of *NF-Kappa-B* and *JNK* and has a central role in the regulation of cell survival and apoptosis (27, 28). Also, the interaction with *TNFRSF1B* (receptor with high dependency for *TNFSF2/TNF-α*) is essential for mediating most of the metabolic efficacy of *TNF-α* (27, 29).

This study has some limitations in spite of its strengths. The limitations of our study on endometriosis were not only the difficulty in choosing the controls, but also in recruiting patients. This is because laparoscopy should be undertaken to confirm the disease and its steady state that was a matter of time to collect samples.

## Conclusion

We investigated the association of four polymorphisms in the promoter region of *TNF-α* in Iranian women with endometriosis (stages I-IV of disease). *TNF-α* -863 A allele was significantly lower in women with endometriosis than controls, suggesting that the -863 A allele may play a role in incidence of endometriosis among Iranian women. Development of endometriosis among those people with -863 A allele seems low although it should be noted that the calculations show is not significant when adjusting for multiple testing. According to the results, the current study indicates that there might be a direct relationship between BMI and progression of endometriosis.

## Acknowledgements

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## Authors' Contributions

A.A., H.R.K.K., F.S.; Participated in the conception and design of the study. A.A.; Was responsible for overall supervision of the project, revised statistical analysis and data interpretation. B.B.; Collected the data, conducted molecular and statistical analyses, interpreted the data and wrote the first draft of the manuscript. H.B.-F., Undertook literature search and contributed to data analysis and statistical analysis. All authors performed editing and approved the final version of this manuscript prior to submission.

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Original Article

# The State of Peripheral Blood Natural Killer Cells and Cytotoxicity in Women with Recurrent Pregnancy Loss and Unexplained Infertility

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## Abstract

**Background:** The prognostic value of peripheral natural killer (pNK) cells, as a screening test in women with recurrent pregnancy loss (RPL) and unexplained infertility, is still a matter for discussion. The purpose of this study was to compare the percentage of circulating $CD56^+$NK cells, CD69 and perforin markers between women with unexplained infertility and RPL with the healthy control group.

**Materials and Methods:** In this case-control study, the percentage of $CD56^+$NK cells and activation markers (CD69 and perforin levels) in the peripheral blood were measured in 25 women with unexplained infertility, 24 women with idiopathic RPL and 26 women from the healthy control group, using specific monoclonal antibodies by flow cytometry.

**Results:** The percentage of $CD56^+$NK cells was significantly higher in patients with infertility in comparison with the healthy control group (P=0.007). There were not significant differences either in the total number of $CD56^+$ cells between the RPL group and the control group (P=0.2) or between the RPL group and the infertile group (P=0.36). The percentage of $CD69^+$ lymphocytes in RPL group was significantly higher than in the infertility group (P=0.004). There was a statistically significant difference in Perforin levels between RLP and control (P=0.001) as well as RPL and infertile (P=0.002) groups.

**Conclusion:** An increased percentage of $CD56^+$NK cells in patients with unexplained infertility, an elevated expression of CD69 on NK cells in patients with RPL and infertility and a high level of perforin on $CD56^+$ cells in the RPL group might be considered as immunological risk factors in these women.

***Keywords:*** $CD56^+$, Infertility, Perforin, Peripheral Natural Killer Cell, Recurrent Miscarriage

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## Introduction

Infertility is defined as the failure of a couple to get pregnant after 12 months or more of having regular unprotected intercourse. Unexplained infertility is idiopathic and its cause remains unclear when the standard investigation of both male and female partner has made other infertility diagnoses impossible. Recurrent pregnancy loss (RPL), a heterogeneous circumstance often idiopathic, is described as three or more sequential miscarriages occurring before 20 weeks of gestation (1). However, the American Society of Reproductive Medicine (ASRM) has lately defined again RPL as two or more failed pregnancies and the American College of Obstetrician and Gynecologists has stated that the causes of recurrent fetal losses are similar in women who have had two or more miscarriages in comparison with women with three losses (2). The causes of RPL could be chromosomal abnormalities, uterine anomalies, endometrial infections, endocrine etiologies. antiphospholipid syndrome inherited thrombophilias, and alloimmune factors.

Among these suggested causes, only chromosomal abnormalities, antiphospholipid syndrome and uterine anatomic abnormalities are universally approved (3). One of these causes can be seen in about 50% of patients. How-

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ever, in the remainder the cause is unknown (1, 3).

There is increasing evidence that these cases of unexplained infertility and RPL might have an immunological background. Natural killer (NK) cells which are present in the endometrium at the time of implantation and during early pregnancy seem to play a role in this regard. NK cells are a section of the innate immune system, and constitute 5-10% of peripheral blood lymphocytes (PBL) and 70-90% of uterine lymphocytes. There are two clearly different subgroups of human NK cells identified by cell surface density of CD56 (D56bright or CD56dim). Although both peripheral NK (pNK) and uterine NK (uNK) cells show the surface CD56, pNK cells differ from uNK cells in both phenotype and function and the fact that 10% of pNK cells are similar to uNK cells (4). Moreover, 90% of pNK cells are CD-56dim and $CD16^{+}$, while only 80% of uNK cells are CD56bright and CD16. CD56dim cells have a more toxic activity, although the CD56bright part is the most important source of NK cell-derived immunoregulatory cytokines (5). Obviously uNK cells are important for the success and continuance of pregnancy.

One study reported that pNK cell levels show changes in decidual NK cell levels (6). Whereas some other reports have shown that the assessment of peripheral blood NK cells would not determine the events in the uterus (7, 8). Several studies have tried to find out the relationship between altered pNK cell parameters and RPL. Some case-control studies have discovered a relationship between pNK cell numbers (9, 10) and activity (11-13) with RPL. On the other hand, some studies have shown no difference in pNK cells levels between RPL and controls (14-16). Similarly, there isn't much information on the relationship between marker of CD69 and RPL. Two different studies reported that NK cells from patients with RPL showed more CD69 than NK cells from controls (9, 17).

A recent study has not indicated any significant difference in CD96 marker between RPL and controls (13). Also there is not consistency in the association of pNK cells with infertility. Some studies have shown a relationship between pNKcells and infertility (9, 18-20), while some have not (21). A systemic review in 2011 (22) and a large cohort study in 2013 (23) have shown that the prognostic value of analyzing pNK or uNK cell parameters remains doubtful and more researches are needed to accept or deny the role of NK cell measuring as a predictive test for screening women possibly benefiting from immunotherapy. There are few studies which have compared pNKcell numbers and cytotoxicity level at the same time in women with RPL and idiopathic infertility and there are not any studies to measure perforin level in pNK cell in these women. So we decided to determine whether there was a remarkable difference in pNK percentage, CD69 marker and perforin level between women with a history of recurrent miscarriage or unexplained infertility and healthy control women.

## Materials and Methods

All the samples were taken from patients who came to the clinic of Amir Al-Momenin Hospital, Semnan, Iran from June 2011 to December 2013 for the evaluation of RPL or infertility in a case control study. The Research Council and Ethical Committee of Semnan University of Medical Sciences provided us with the ethical approval and later the informed written consents were collected from patients for this case-controlled study. Seventy five women were included in three age match group in this study (24 with a history of unexplained RPL, 25 with unexplained infertility and 26 healthy women with no history of pregnancy problem, convenient sampling). In the infertility group, women had an infertility history of more than 1 year, normal serum prolactin (PRL) and thyroid function tests (T4 and TSH), documented patent tubes by hysterosalpingography, and had no other infertility factor, and the male partner had a normal sperm count, motility and morphology according to the World Health Organization (WHO 2010) standards. Women with RPL had a history of at least two sequential spontaneous miscarriages.

Unexplained RPL was defined as a history of $\geq$2 sequential miscarriages in which all the following results were normal: parental karyotypes, thyroid function, fetal bovine serum (FBS), anti-cardiolipin antibodies, antiphospholipid antibodies, lupus anticoagulant, follicle-stimulating hormone (FSH), prolactin, progesterone, estrogen, testosterone, free androgen index, prothrombotic risk factors including activated protein-C resistance, factor V Leiden and prothrombin mutations, pelvic ultrasonography and hysterosalpingogram. Twenty six healthy parous women had at least one live birth and had no history of miscarriage, preeclampsia, ectopic pregnancy or preterm delivery.

Sampling: 5 ml of heparinized peripheral blood was taken in mid luteal phase and in women with RPL, at least 2 months after the last abortion. The blood samples were immediately taken to the Immunology Laboratory of Semnan University of Medical Sciences. The whole blood sample was separated into peripheral blood mononuclear cells (PBMC) by ficoll separation and then PBMCs were labeled and kept in freezing condition medium: (RPMI1640+10%FCS+10%DMSO) at the -70°C freezer until all patient samples were collected.

### Flow cytometry analysis

After sampling was completed, the stored cells were thawed and subsequently surface and intracellular staining were performed. Surface markers were determined by flow cytometry, using fluorochrome-conjugated monoclonal antibodies, anti CD3, CD69, CD19, CD56, and perforin using permabilization buffer for permabilizing cell membrane to facilitate antibody entry into cells. Antibodies were bought from BD Biosciences (San Jose, CA, USA) or ebioscience. Appropriate concentrations of antibodies in addition to isotype matched control were added to the cells ($5\times10^5$ cells/tube) in 100 µL staining buffer

and incubated for 25 minutes at 4°C in the dark. Analysis were done by using PARTEC, CyFlow® Space device and FlowMax software. At least 50,000 lymphocyte-gated cells were obtained and analyzed for $CD56^+CD19^+$, $perforin^+$ cells. The criteria for positive staining were set at a fluorescent intensity displayed by <0.5% of the cells stained by the appropriate fluorochrome-conjugated isotype control monoclonal antibodies (mAb). The results and graphs were analyzed using Flowjo version 10A software (Flowjo, USA).

### Statistical analysis

The Kolmogorov-Smirnov test was used to examine the normality of the distributions. A one-way analysis of variance and Tukey's range test for normally distributed data and Kruskal-Wallis analysis for data with non-normal distribution were used to compare study groups. The results were reported to be statistically significant if the P value was<0.05.

## Results

Mean age of the study population was 29.2 ± 3.4 (mean ± SD) years in infertile group, 28.9 ± 3.2 (mean ± SD) years in RPL group and 28.8 ± 3.3 (mean ± SD) years in control group. There were no significant differences in age distribution among them (P=0.6).

Mean percentage of $CD56^+$ cells in infertile, RPL and control groups were respectively: 18.36 ± 7.9, 15.97 ± 5.1, 13.26 ± 5.02. The Mean percentage of peripheral $CD56^+$ cells in the infertile group was remarkably higher (P=0.007) than that of the control subjects. There were not significant differences in the total number of $CD56^+$ cells between the RPL group and the control group (P=0.2) and neither between the RPL group and the infertile group (P=0.36, Table 1, Fig.1).

The median percentage of $CD69^+$ cells were: 4.5 (1.5-8)% in infertile group, 8 (6-10)% in RPL group, and 6 (4-11)% in control group. The Manne-Whitney analysis between groups showed a significantly higher percentage of $CD69^+$ cells in RPL group than the infertile group (P=0.004). However, there were no significant differences between the infertile group (P=0.11) and the RPL group (P=0.1) with the control group. The Perforin positive cells median percentage in the control group was 6.4 (4-8)%, in RPL was 16.5 (9-31)% and in the infertile group was 8 (5-14)%. The Perforin positive cells in infertile group were significantly higher than others (Table 1).

The results showed that 15.8% ± 5.9 of total CD56 cells in patients with RPL and 32% ± 14.4 in the infertile group expressed CD69 as compared with 10.6% ± 5.01 in control group.

**Table 1:** The percentage of peripheral NK cells (%) and expression of CD69 and perforin levels on these cells in RPL, infertile and controls groups

| Cell population | Control | RPL | Infertile | P1 | P2 | P3 |
|---|---|---|---|---|---|---|
| $CD56^+$ | 13.26 ± 5.02 | 15.97 ± 5.1 | 18.36 ± 7.9 | 0.2 | 0.007 | 0.36 |
| $CD69^+$ | 6 (4-11) | 8 (6-10) | 4.5 (1.5-8) | 0.1 | 0.11 | 0.004 |
| $Perforin^+$ | 6.4 (4-8) | 16.5 (9-31) | 8 (5-14) | 0.001 | 0.07 | 0.002 |
| $CD56^+CD69^+$ | 10.6 ± 5.01 | 15.8 ± 5.9 | 32 ± 14.4 | 0.001 | 0.001 | 0.001 |
| $CD56^+Perforin^+$ | 7 (4-12) | 16 (9-23) | 8 (5-9) | 0.001 | 0.6 | 0.001 |
| $CD69^+Perforin^+$ | 6 (4-10) | 10 (6-16) | 6.5 (3-9.5) | 0.02 | 0.7 | 0.01 |

**Values are presented as mean ± SD and median (interquartile range). NK; Natural killer, RPL; Recurrent pregnancy loss, P1; P value: for the difference between mean value in the RPL group and control group, P2; P value: for the difference between mean value in the infertile group and control group, and P3; P value: for the difference between mean value in the infertile group and RPL group.**

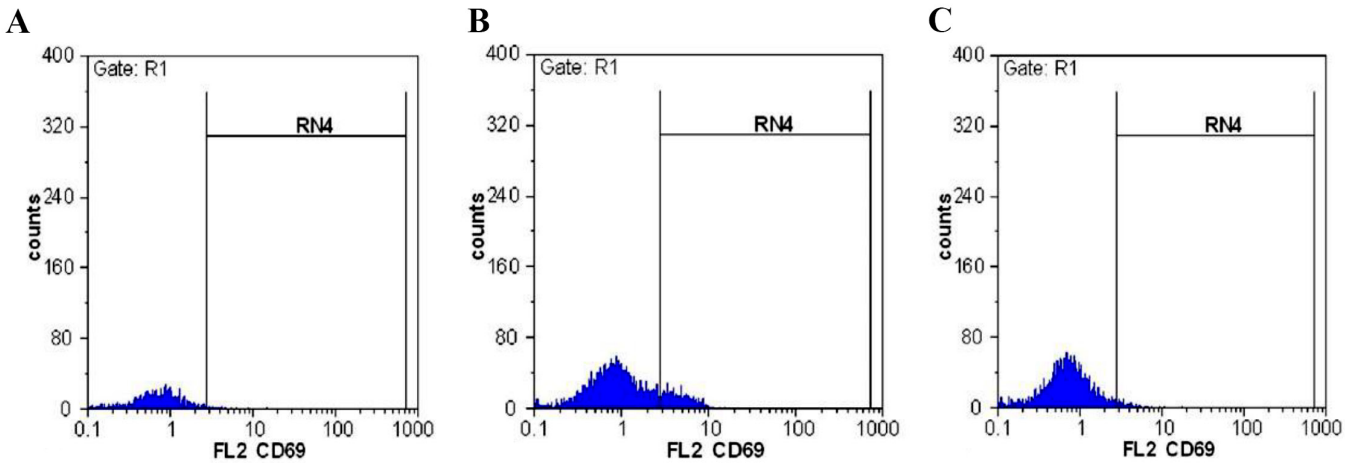


**Fig.1:** CD69 positive population in $CD56^+$ gated cells. **A.** Control group, **B.** Recurrent pregnancy loss (RPL) patients, and **C.** Infertile.

There was a statistically significant difference in the expression of CD69 in $CD56^+$ cells between the control group and RPL group (P=0.001), the infertile group (P=0.001) and between RPL and infertile group (P=0.001, Table 1). This study showed that 16 (9-23)% of total CD56 cells in patients with RPL and 8 (5-9)% in the infertile group expressed perforin as compared with 7 (4-12)% in control group. There was a statistically significant difference in the expression of perforin in $CD56^+$ cells between the RPL group compared with the control group (P=0.001) and the infertile group (P=0.001). However, there was not a significant difference in the expression of perforin in $CD56^+$ cells the infertile group and the control group (P=0.6).

The triple staining results showed the $CD69^+Perforin^+$ population in control group was 6 (4-10)% in RPL group was 10 (6-16)% and in the infertile group was 6.5 (3-9.5)%. The statistical analysis showed a significant difference between RPL and infertile group and control (P=0.01, P=0.02) without a significant difference between control and Infertile groups (P=0.7).

## Discussion

The findings of this study showed that the levels of $CD56^+$ T cells were remarkably higher in infertility group than the control group. But there were no important differences in the total levels of $CD56^+$ cells between the RPL group and the other two groups. Moreover, there was a significant increase in the display of CD69 on $CD56^+$ cells in the RPL group and the infertile group compared with the control group. We also showed that the level of perforin on $CD56^+$ cells significantly increased in the RPL group compared with the other two groups. Findings of this study were similar to those of case-control studies of Emmer et al. (14), Souza et al. (15) and Wang et al. (16). They were also unable to discover a significant difference in pNK parameters between women with RPL and controls. On the other hand, Ntrivalas et al. (9) and Yamada et al. (10) showed a relationship between pNK cell numbers and RPL. Aoki et al. (11) and Shakhar et al. (12) also showed an increased pNK cell activity, using both standard and whole blood assays in women with RPL.

King et al. (13) showed that in women with RPL, the NK percentage was significantly higher and CD56bright to CD56dim ratio was significantly lower than controls. They also noticed that an NK percentage of 18% was very particular for women with RPL and thus described 12.5% of women with RPL as having high NK percentage, in comparison with 2.9% of controls. Katano et al. (23) in a cohort study on 552 patients with a history recurrent miscarriages showed that high pNK cell activity was found not to be a nondependent risk factor for the next miscarriages. They suggested the clinicians should not consider the NK activity as a systematic RPL investigation, since its clinical importance has not been established yet.

There are also contradictory reports regarding the association of pNK cells with infertility. Some studies have shown a relationship between pNK cells and infertility (18-20). In 1996, Beer et al. (18) showed that women with unexplained infertility and several former *in vitro* fertilization (IVF) failures showed significantly increased levels of $CD56^+$ PBL than normal fertile controls and also reported that the pregnancy rate was much better in those with $CD56^+$ levels less than 12%. Matsubayashi et al. (19) also showed a significantly higher NK-cell activity by using a chromium-51 release cytotoxicity assessment in 94 infertile women who, despite the treatment, failed to get pregnant for 6 or more months in comparison with the control group. They continued their study with 77 patients out of 94 who were watched for 2 years, 28 of whom had conceived but 49 had not. They observed that the peripheral NK activity of the group which had got pregnant was significantly lower than that of non-conception group (20). However, Thum et al. (24) and Baczkowski and Kurzawa (25), in two separate studies, compared the percentage of pNK cell in patients with IVF failure with successfully treated IVF cases from the control group.

They noticed no difference in percentage NK cell and NK cell subpopulation in infertile women who were unable to get pregnant and those who became pregnant after assisted reproductive technology. Tang et al reported a systemic review and came to this conclusion that there was no association between the subsequent pregnancy result and either pNK or uNK cell activity in women with RPL and infertility (22). Recently Seshadri in a meta-analysis showed remarkably higher NK cell numbers or percentages in women with RPL in comparison with the controls. They also noticed that the number of peripheral NK cells was significantly higher in infertile women versus fertile controls. On the other hand, the meta-analysis of studies where uNK cells were measured showed no significant difference in women with RPL versus controls.

They recommended that more research should be conducted before NK cell assessment can be suggested as a diagnostic method in the area of female infertility or RPL. There is no clear reason why the results are different when the information for NK cells is shown as numbers or a percentage. So, they suggested that NK cell measuring and immune therapy should not be recommended except in the area of clinical research (26). CD69 is one of the earliest particular markers of NK cell activity (27, 28). The elevated NK cell CD69 presentation is closely linked with higher cytotoxic activity and target cell lysis (29, 30). In the present study, the expression of CD69 on $CD56^+$ cells in the RPL group and infertile group were remarkably higher than the control group. In normal pregnancy, compared to an embryonic pregnancy, NK cell cytotoxicity decreases, suggesting that activated CD69 expressing NK cells have a significant role in controlling trophoblast growth and placental development (31). Ntrivalas et al. (9) showed that women with a history of recurrent miscarriage or unexplained infertility had a significant increase in CD69 expression on CD56 NK cells in comparison with that of normal controls.

In a comparative study of activation and inhibition markers of circulating NK cells, Coulam and Roussev (32) also reported that infertile women had a remarkably more increased expression of NK cell activation markers of CD69C and CD161C than fertile women.

Ghafourian et al. (33) reported that the percentage of NK cells and the expression of CD69, CD94 and CD161 surface markers on $CD56^+$NK cells were remarkably more elevated in patients with RPL and in women who had a history of IVF failure than the healthy multiparous and successful IVF control groups. However, Baczkowski and Kurzawa (25) reported there was no difference in CD69 expression on PBL subpopulations including T and B and NK cells among the fertile control group, infertile women who got pregnant and those who did not get pregnant after intracytoplasmic sperm injection. It is a well-known fact that NK cells release both perforin and serine proteases such as granzyme B upon target cell contact (34, 35). It has been hypothesized that granzyme B induces apoptosis of the target cell in the presence of perforin (36, 37). In this study we showed that the level of perforin on $CD56^+$ cells significantly increased in the RPL group compared with the other two groups. Yamada et al. (38) showed a small increase in perforin-positive uNK cells in human spontaneous miscarriage with a normal fetal chromosomal karyotype. On the other hand, Nakashima showed that the number of granulysin-positive CD56bright uNK cells was remarkably higher in the decidua basalis in spontaneous miscarriage than in normal pregnancy, although he did not notice any difference in the numbers of perforin-positive and granzyme B-positive cells (39).

## Conclusion

The findings of this study showed a significant increase in the percentage of $CD56^+$ pNK cells among the infertility group and also a significantly higher level of CD69 expression on $CD56^+$ NK cells in women with RPL and unexplained infertility in comparison with healthy control group. We also showed that the level of perforin on $CD56^+$ cells significantly increased in the RPL group compared with the other two groups. Although it can be considered as immunological risk markers in these women, the prognostic value of PNK number assessment or activity remains still doubtful. So because of many arguments in this field, further researches are needed to accept or deny the role of NK cell evaluation as a predictive test for screening women with infertility or RPL.

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## Authors' Contributions

A.A., Y. M.; Participated in study design, patient sample collection and preparation of the first draft of the manuscript. M.S.B., M.B.; Contributed in sample analysis by flow cytometry, data collection and statistical analysis. P.K.; Participated in study design, data interpretation and correction of final version of manuscript. All authors read and approved the final manuscript.

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Original Article

# Effect of Vitamin D Supplementation on Intracytoplasmic Sperm Injection Outcomes: A Randomized Double-Blind Placebo-Controlled Trial

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## Abstract

**Background:** Despite numerous studies indicating an imperative role for reproduction, however, the role of Vitamin D supplementation on outcomes of assisted reproductive techniques remains controversial. This clinical trial was performed to evaluate the effect of Vitamin D supplementation 6 weeks prior to intracytoplasmic sperm injection (ICSI) on fertility indices.

**Materials and Methods:** The present study was a double-blind clinical trial conducted on infertile women was randomly allocated into two groups: Vitamin D supplementation (42 participants) and placebo (43 participants). Serum Vitamin D was measured before and six to eight weeks after treatment, on the day of ovum pick up. Results were analyzed using SPSS16 and fertility indices were compared between the two groups.

**Results:** No significant difference was observed between the intervention and control groups regarding the mean number of oocytes retrieved, percentage mature oocyte, fertilization rate and the rate of good quality embryos (all P>0.05). But, percentages of the individual with suitable endometrium (7-14 mm thickness) were significantly higher in the Vitamin D compared to control group (P=0.011). The rate of chemical (47.6 vs. 25.5%, P=0.013) and clinical pregnancy rate (38.1 vs. 20.9%, P=0.019) were also significantly higher in the Vitamin D compared to control group.

**Conclusion:** The present study reveals that consuming Vitamin D for 6 weeks prior to ICSI improves quality of endometrium, rate of chemical and clinical pregnancy (Registration Number: IRCT2015111124999N1).

***Keywords:*** Assisted Reproductive Techniques, Infertility, Pregnancy, Vitamin D

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## Introduction

Infertility is multifactorial in its origin and is affected by different factors including lifestyle, eating habits or nutrition. Numerous studies have shown that reduce exposure to sunlight and poor eating habits have led to Vitamin D insufficiency and/or Vitamin D deficiency, even in sunny countries among men and women of reproductive age (1) and this phenomenon is considered as one etiology for infertility (2-7).

Vitamin D is a fat-soluble vitamin and is considered as an essential nutrient required for our health. One of the main functions of Vitamin D is to help with the absorbance of calcium and phosphate, and helps building bones and keeps them strong and healthy. It also blocks the release of the parathyroid hormone involved in reabsorption of bone tissue, which makes bones thin and brittle. Considering these functions of Vitamin D, it plays a central role in calcium and phosphate hemostasis and in turn is-needed for the normal mineralization of bone, muscle contraction, nerve conduction, and general cellular function in all cells of the body including cell growth.

Vitamin D receptor (VDR) is a member of nuclear receptor family of transcription factors. It forms a heterodimer with a retinoid-X receptor and binds to hormone response elements on DNA to regulate expression of specific gene products. At post transcriptional level Vitamin D regulates gene expression through microRNA-directed mechanisms (7). VDR is present throughout reproductive axis including endometrial epithelial cells, granulosa, fallopian tube epithelial cells and cells of cumulus oophorus in ovaries (8). Therefore, the reproductive axis is considered as one of the target organs for Vitamin D (9). In this regard, some studies have advocated Vitamin D plays role

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in the biosynthesis of sex hormones (estrogen and progesterone) and also post fertilization in the process such as implantation (10) and production of human chorionic gonadotropin (hCG) (11). Considering roles of Vitamin D in reproductive biology, numerous studies have shown the association between Vitamin D insufficiency and deficiency with fertility or poor pregnancy outcomes (12, 13). In this regard, Somigliana et al. (14) showed the time to pregnancy is longer in women with Vitamin D insufficiency. However, it is also important to note, contrary reports also exist in the literature (15).

According to the aforementioned role of Vitamin D in reproduction, researchers have tried to assess the association between serum Vitamin D concentrations and assisted reproductive outcomes. In this regard, Pacis et al. (16) in their systematic review titled "Vitamin D and assisted reproduction: should Vitamin D be routinely screened and supplemented prior to assisted reproductive techniques (ART) state that Rudick et al. (17) and Garbedian et al. (18) have confirmed the effect of Vitamin D on improvement of assisted reproductive treatment outcomes, but Aleyasin et al. (19) in their study showed that Vitamin D had no significant effect on outcomes of assisted reproductive treatments. Pacis et al. (16) also stated that in contrary to several reports stating beneficial effect of Vitamin D supplementation on ART outcomes "cost-benefit analysis for a single ART cycle involving fresh single blastocyst embryo transfer suggests that screening and supplementing vitamin D prior to ART might significantly decrease societal cost per ongoing pregnancy by implementing a simple intervention, if the magnitude of the observed effect was confirmed in future studies". Surprisingly the study by Anifandis et al. (20) conducted on women who were candidates for assisted reproductive treatments showed that the increase in serum level of Vitamin D was associated with decrease in the quality of embryos and the rate of achieving biochemical and clinical pregnancy. Therefore, taking into consideration the controversial results about the effect of Vitamin D supplementation and according to Vanni study (21) stating that the effect of Vitamin D on ART outcomes is not clear and should be evaluated in different populations by randomized controlled trial and cohort studies, hence current trial is very important and valuable.

## Materials and Methods

The clinical trial study was approved by Ethical Committee of Isfahan University of Medical Sciences and was registered in Iranian registry for the clinical trial (IRCT2015111124999N1) and was designed to be carried out at infertile couples that referred to Isfahan Fertility and Infertility Center from March 2016 to June 2016 and candidate of ICSI. Female with age ranging from 18 to 38 years who had Vitamin D level below 30 ng/ml without symptom of Vitamin D deficiency participated in the study.

Based on the ethical committee, initially individuals were questioned regarding clinical symptom of Vitamin D deficiency and individuals with these symptoms were excluded from the study, as Vitamin D treatment was mandatory for these individuals. Additionally, to roll out effects of male factor infertility and advance maternal age, couples with abnormal semen parameter based on WHO (2010) and/or female age greater than 38 were also excluded from the study. Other exclusion criteria were: secondary female infertility, polycystic ovarian disease, endometriosis, congenital or acquired uterine malformations, drugs consumption that would affect metabolism and Vitamin D absorption such as Carbamazepine and Phenobarbital Phenytoin, body mass index of lower 18 or higher 30 kg/m$^2$ and hypothyroidism. Couples at risk of ovarian hyper stimulation syndrome or poor endometrium (less than 7 mm or grater 14 mm) were also excluded during the course of the study, since all the embryos for these case were vitrified

Initially 159 couples were interviewed based on Vitamin D level below 30 ng/ml. Fifty one couples were excluded based on exclusion criteria. All of the participants entered the study after giving written informed consent and were allowed to leave the study at any desired time.

Six couples were also excluded for other reasons including declining to participate. The remaining 108 couples were randomly divided into Vitamin D and placebo groups based computer-generated or random allocation software with one block (Fig.1). Participants or Vitamin D group received a weekly dose of 50000 units of Vitamin D supplementation or placebo for six weeks as pearls orally. Boxes containing Vitamin D and Placebo peals were labeled based on random allocation number, except the two individuals allocating the Vitamin D and placebo, participants, clinician and *in vitro* fertilization (IVF) laboratory personnel were all blind to the study. Administration of Vitamin D or placebo started on the second day of the last menstrual period (LMP) prior to ICSI cycle and continued to day of hCG administration which was around 6 weeks. Vitamin D (50000 units) and placebo pearls were purchased from Zahravi (Tabriz, Iran).

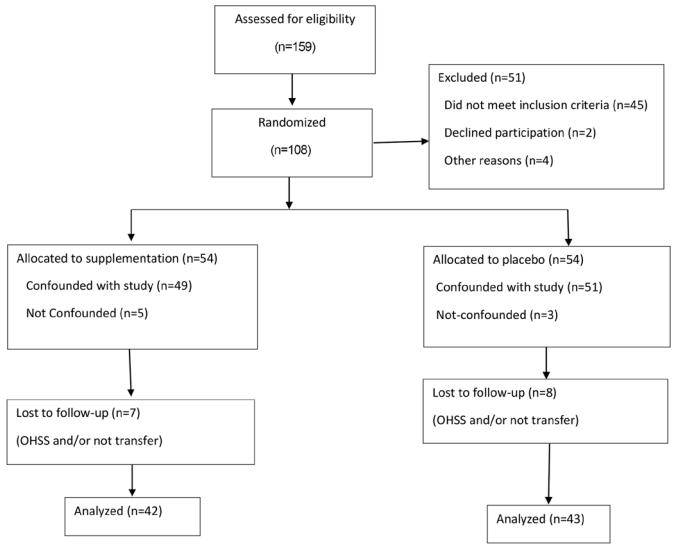


**Fig.1:** Flow diagram of the progress through the phases of a 2-group parallel randomized trial.

Serum Vitamin D was assessed by high-performance liquid chromatography and defined based on couple's information before starting the trial and also six to eight weeks after treatment, on the day of ovum pick. All the Vitamin D assessment was carried out at a single laboratory. The codes were unraveled after completion of data. Semen parameters, including volume, sperm density, percentage motility and normal morphology were also defined based on WHO (2010) manual.

Ovulation induction: all the participants received a combination of recombinant follicle-stimulating hormone (FSH) and human menopausal gonadotrophins (hMG) and were followed by sequential vaginal ultrasound. Gonadotropin releasing hormone (GnRH) antagonist was administered when size of dominant follicles was around 12-14 mm and continued until the day of hCG administration. On the day of hCG administration, number of follicles greater than 12 mm and type of endometrium were also defined and recorded. Type of endometrium was defined according to study by Zhao et al. (22), briefly: cycles were divided into 3 groups depending on the thickness (group 1: ≤7 mm; group 2: >7 mm to ≤14 mm; group 3: >14 mm). Each group was subdivided into three groups according to the endometrial pattern as follows: pattern A (a triple-line pattern consisting to a central hyperechoic line surround by two hypoechoic layers); pattern B (an intermediate isoechogenic pattern with the same reflectivity as the surrounding myometrium and a poorly defined central echogenic line); and pattern C (homogenous, hyperechogenic endometrium). Based on exclusion criteria individuals with endometrium thickness of less than 7 mm and grater 14 mm were excluded from the study. Induction of ovulation was induced with administration of 10000 IU hCG when dominant follicles reached size of 17-18 mm. vaginal ultrasound ovum pick up was performed 36 hours post hCG administration. Standard ICSI program was carried out using G-V series (VitroLife, Guttenberg).

Numbers of oocytes were recorded on the day of oocyte retrieval. All the couples underwent ICSI based Isfahan Fertility and Infertility policy. Fertilization rate was calculated based on the number of 2PN observed over the number of injected oocytes. On day 3, embryos were scored for the number of blastomeres, blastomere regularity and percentage cytoplasmic fragmentation. Embryos were considered as "good quality" that had between 6-8 blastomeres with even size and less than 25% fragmentation. These outcomes were taken as primary outcomes.

β-hCG greater than 20 IU was considered as chemical pregnancy and clinical pregnancy was defined as pregnancy diagnosed by ultrasound through visualization of one or more gestational sac. Of note, multiple gestational sacs were considered as one clinical pregnancy. Therefore, clinical pregnancy rate was defined as the number of clinical pregnancy per 100 embryo transfer. These outcomes were considered as secondary outcomes.

### Statistical analysis

Gathered data were analyzed using SPSS for Windows (version 16, SPSS Inc., Chicago, IL, USA). Continuous variables between two groups were compared with the independent t test, and categorical variables were compared with the chi-square test.

## Results

In the present study, the mean age of women in the intervention group was 31.9 ± 4.2 years and in the control group was 30.8 ± 4.4 years. The mean of body mass index (BMI) in the intervention group was 23.9 ± 2.1 and in the control group was 23.8 ± 1.9 and statistical analysis showed no significant difference between the demographic characteristics and the BMI of the intervention and the control group (P>0.05, Table 1). No statistical difference was observed for male age, educational and duration of infertility and number of previous ART cycles. Therefore, these data suggest that the samples were randomly allocated into the two groups and both groups were similar. We also assess semen parameters between the two groups and no statistical difference was observed between the two groups. Comparison of semen parameters including semen volume, sperm concentration, motility and morphology revealed no statistical differences between the two groups (data not shown).

**Table 1:** Comparison of basal and clinical characteristics of couples in Vitamin D and Placebo groups

| Variable | Vitamin D | Placebo | P value |
|---|---|---|---|
| Female age (Y)* | 31.9 ± 4.2 | 30.8 ± 4.4 | 0.29 |
| Male age (Y)* | 35.26 ± 5.2 | 35.1 ± 4.7 | 0.98 |
| Duration of infertility (month)* | 77.4 ± 22.1 | 68.1 ± 19.3 | 0.28 |
| Number of previous ART cycle* | 1.9 ± 1.2 | 2.3 ± 1.5 | 0.37 |
| Female education (%) | | | |
| High school | 12 | 10 | 0.45 |
| Diploma | 19 | 19 | |
| Master | 11 | 15 | |
| Body mass index (kg/m$^2$)* | 23.9 ± 2.1 | 23.8 ± 1.9 | 0.65 |

ART; Assisted reproductive techniques and *; Data are presented as mean ± SD.

The primary serum Vitamin D levels of the intervention and the control group were 14.4 ± 6.6 ng/ml and 12.7 ± 6.4 ng/ml, respectively. The differences between the two groups were insignificant. Six weeks after treatment with Vitamin D or placebo, the level of Vitamin D significantly raised to 37.1 ± 7.7 ng/ml in the Vitamin D group while it remained low (13.6 ± 6.6 ng/ml) in the placebo group (Fig.2). Unlike in the Vitamin D group, in the placebo group the difference before and after 6 was insignificant.

Regarding the ICSI primary outcomes, Table 2 showed that the mean number of retrieved oocytes in the intervention or Vitamin D group was 9.42 ± 4.4 and in the control group was 8.72 ± 5, and their

difference was not statistically significant (P>0.05). Percentage of type A endometrium on the day of hCG injection was 81% and 55.8% in Vitamin D and placebo groups, respectively and the difference between the two groups was statistically significant (P<0.05). The rate of fertilization in the Vitamin D group was 68.80% and in the control group was 68% and the difference was not statistically significant. The rate of good quality embryo on day3 in the Vitamin D group was 59.9 and in the control group was 53.59% and the difference was not statistically significant (P=0.36, Table 2). We also categorize the individuals based on vitamin D deficiency (<10 ng/ml) and insufficiency (10-30 ng/ml) and compared the primary outcomes in the two categories and except for type endometrium, no difference was observed between the two groups for primary outcomes.

**Table 2:** Comparison of ICSI outcomes in Vitamin D and placebo groups

| Variable | Vitamin D | Placebo | P value |
|---|---|---|---|
| Endometrium (%) | | | |
| Type A | 81 | 55.8 | 0.01 |
| Type B | 19 | 39.5 | |
| Type C | 0.0 | 04.7 | |
| Mean number of oocyte (Mean ± SD) | 9.42 ± 4.4 | 8.72 ± 5 | 0.55 |
| Fertilization rate (%) | 68.8 | 68 | 0.88 |
| Mean number of embryo (Mean ± SD) | 5 ± 2.5 | 4.6 ± 3.3 | 0.53 |
| Good quality embryo (%) | 59.9 | 53.59 | 0.36 |

ICSI; Intracytoplasmic sperm injection.

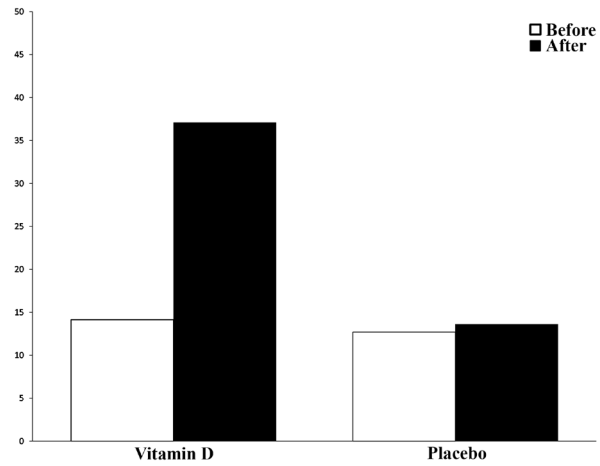


**Fig.2:** Comparison of serum Vitamin D levels in Vitamin D and placebo groups before and after intervention.

According to the results, chemical pregnancy was defined by positive β-hCG in the intervention and control groups were 47.6 and 25.5%, respectively, and the difference between both groups was statistically significant (P=0.013, Fig.3). The rate of clinical pregnancy in the intervention group was 38.1% and in the control group was 20.9% and statistical analysis revealed a significant difference between both groups (P=0.019, Fig.4).

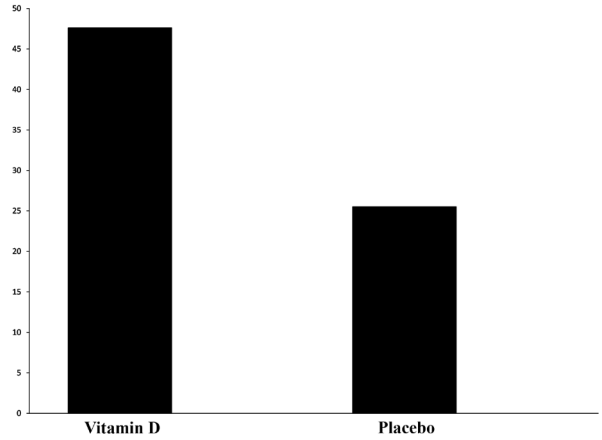


**Fig.3:** Comparison of chemical pregnancy rate assessed by beta-human chorionic gonadotrophin (β-hCG) in Vitamin D and placebo group.

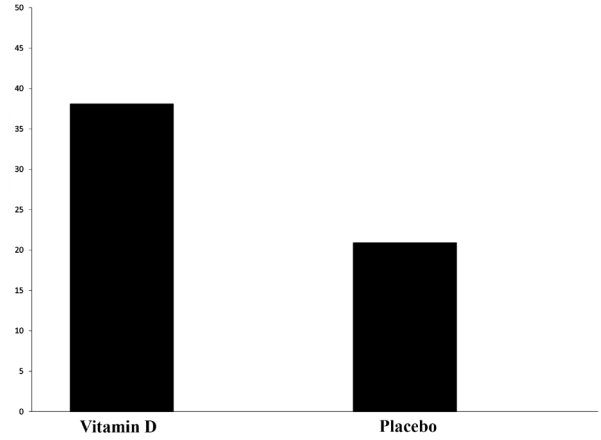


**Fig.4:** Comparison of clinical pregnancy rate in Vitamin D and placebo groups.

## Discussion

Based on background studies Vitamin D plays an imperative role in reproduction and therefore, assessment of Vitamin D and thereby Vitamin D supplementation is becoming part of daily practice. However, role of Vitamin D supplementation during assisted reproductive management remains controversial and there appear to be more room for further study and to evaluate which parameters are most affected by Vitamin D deficiency and thereby supplementation. Part of these controversies may be related to confounding factors affecting both Vitamin D levels and assisted reproductive outcome. An example of these confounding factors is the seasonal effect on Vitamin D level (6), therefore, in this study; the effort was taken so that sampling, measurement of serum Vitamin D level and supplementation took place during spring and early summer.

The results of this study showed that despite similar demographic and fertility characteristics between the two groups, Vitamin D supplementation significantly improves serum Vitamin D level in comparison to placebo group and this observation is in line with previous studies in this filed (23-25).

Comparing the mean value of serum Vitamin D between both groups before intervention revealed no sig-

nificant difference between the two groups and 50000 units of Vitamin D supplementation per week for 6 weeks based on the previous study by Diamond et al. (24) resulted in significant increase in serum Vitamin D level compared to before treatment and also compared to placebo group. Indicating that the level of Vitamin D increased to higher than 30 ng/l, the cut of value for Vitamin D deficiency. The outcome of the study is in accordance with previous report Aflatoonian et al. (23) and Spedding et al. (25), indicating that this dosage of Vitamin D supplementation was effective in improving the level of serum Vitamin D.

Our results also reveal that the improved Vitamin D level is also associated with significant difference observed in type of endometrium but no difference was observed between other assessed parameters, including percentage of mature oocytes, fertilization rate and embryo quality. These observations are in concordance with previous report by Asadi et al. (26) and Rudick et al. (17). In this regard, Kinuta et al. (27) show that VDR null mice present uterine hypoplasia. This phenomenon has been related to regulation of expression P450 aromatase activity through *CYP19* gene containing a Vitamin D element in its promoter. These authors state that "the action of Vitamin D on estrogen biosynthesis was partially explained by maintaining calcium homeostasis. However, direct regulation of the expression of the aromatase gene should not be neglected". But, since, the endometrium in individual undergoing ovarian hyperstimulation is confronted with high level of estrogen in both groups, and the difference in endometrial quality might be due to altered calcium homeostasis in the uterus, but this proposition needs further exploration and validation. It is important to note that as one of the shortcomings of this study, was lack of assessment of estrogen level, but it is also important to consider that we, like others (28) did not observe any difference in the number of follicle and number of oocyte retrieved between the two groups.

Assessment of ICSI outcome in accordance with literature showed that improved Vitamin D has no effect on fertilization and embryo quality on day 3. In contrary to our results and similar studies in this filed, only one study suggest that high concentration Vitamin D reduces embryo quality score following ICSI (20). These authors suggest that glucose provides an essential substrate for cumulus-oocyte complex (COC) and propose that Vitamin D may have a physiological effect on insulin and glucose metabolism in a manner that remains to be elucidated. They believe increase follicular Vitamin D level decreases the availability of glucose to the COC and they state that this proposition may account for negatively correlation with embryo quality and FF Vitamin D levels which opposes our findings and findings of Polyzos et al. (29), Ozkan et al. (28), and Rudick et al. (17) that believe the deleterious effect of Vitamin D deficiency is mediated via on endometrial receptivity rather than reduced embryo quality due to high Vitamin D level. It is important to note the based on their figures number of individuals presenting lower than 15 and 40 ng/ml Vitamin D are very small.

Another major finding of the present study was the difference observed in rates of chemical and clinical pregnancies. In this study rates of chemical and clinical pregnancy rates relative to control group was improved by 10.7% (47.6 vs. 25.5) and 82% (38.1 vs. 20.9), respectively. These results are in accordance with several previous studies, suggests that probably Vitamin D improves ICSI in term of both chemical and clinical pregnancy rates (28-30). Based on the literature and transfer of embryos from donor cycle, it appears that improved effect is very likely related to the improved quality of the endometrium, as also was observed in this study and by other authors (17, 26, 29).

These improved effect has been postulated to be related to mechanisms including i. Miss regulation of NK cell activity, ii. Immunomodulatory role during implantation and recurrent miscarriage, iii. Regulation of cross talk involved between embryos and endometrium which consequently regulates of HOXA10 involved in embryo implantation. It has been shown that endometrial HOX10A expression increase in parallels that Vitamin D receptor around time of implantation, at the time of maximal endometrial differentiation (16, 31). Indeed, increase quality of endometrium, which is reported to be lower in Vitamin D deficient individuals is also related to proper differentiation of endometrial cells (17).

## Conclusion

Results of the present study showed that consuming Vitamin D supplementation could be effective in improving the clinical outcome of ICSI. Based on literature this effect is very likely to be attributed to local effect of Vitamin D on endometrium.

## Acknowledgements

The authors would like to thank the personnel of Isfahan Fertility and Infertility Center and all of the participants in the present study, whom without their cooperation, conducting this study was not possible. We are also thankful for the financial support of the research council of Isfahan University of Medical Sciences (Grant No.393794). There is no conflict of interest in this study.

## Authors' Contributions

S.A; Participated in study design, data collection and drafting the manuscript. M.T.; Contributed to the study design, evaluation, interpretation and editing the manuscript. M.H.N.H.; Conducted all experimental work and prepared oocytes for ICSI pertaining to this component of the study, contributed extensively in interpretation of the data revising and editing the manuscript. All authors read and approved the final manuscript.

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Original Article

# The Effects of Maternal and Paternal Body Mass Index on Live Birth Rate after Intracytoplasmic Sperm Injection Cycles

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## Abstract

**Background:** We designed the present study to evaluate the simultaneous effect of obesity in couples on *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) outcomes.

**Materials and Methods:** In this cross-sectional study, performed at Royan Institute between January 2013 and January 2014, we evaluated the recorded data of all patients during this time period. The study population was limited to couples who underwent ICSI or IVF/ICSI cycles with autologous oocytes and fresh embryo transfers. We recorded the heights and weights of both genders and divided them into groups according to body mass index (BMI). Multilevel logistic regression analysis was used to determine the odds ratio for live births following ICSI or IVF/ICSI.

**Results:** In total, 990 couples underwent IVF/ICSI cycles during the study period. Among the ovulatory women, a significant difference existed between the BMI groups. There was a 60% decrease [95% confidence interval (CI): 0.11-0.83] in the odds of a live birth among overweight subjects and 84% (95% CI: 0.02-0.99) decrease among obese subjects. Among the anovulatory women, the association between the BMI and live births presented no clear tendencies. We did not observe any significant relationship between male BMI and live birth rate. The results demonstrated no significant association between the couples' BMI and live birth rate.

**Conclusion:** Based on the present findings, increased female BMI independently and negatively influenced birth rates after ICSI. However, increased male BMI had no impact on live births after ICSI, either alone or combined with increased female BMI.

***Keywords:*** Body Mass Index, Female, Intracytoplasmic Sperm Injections, Live Birth, Male

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## Introduction

Obesity is an important risk factor for health problems and is deemed to be 1 of the 10 global diseases that contributes to an increased health burden. There is a rapidly increasing incidence of this complication in many industrialized countries, particularly the United States, and in developing Asian countries (1).

In numerous studies, researchers evaluated the effects of obesity on assisted reproductive technology (ART) cycle outcomes in women (2-13) and reported inconsistent results. Koning et al. (14) in a review article, reported that there were limited data despite 14 available studies in this area and concluded that further studies were needed to achieve an accurate insight.

Currently, there is no evidence to indicate that obesity increases the risk for ART complications; however, some researchers have reported the negative effects of obesity on pregnancy rates (14). In contrast, a review article published by Rittenberg et al.(15) reported an association between obesity and excess weight in women with poor pregnancy outcomes. This finding included reduced rates for clinical pregnancy and live births. Luke et al. (7) concluded that obesity had a negative impact on clinical pregnancy and live birth rates along with ART cycles with autologous oocytes. They emphasized that this risk could be brought under control by the use of donor oocytes.

The mechanism of the effects of female obesity on ART outcomes is controversial. The impact of obesity on ART outcomes in men is less studied (1, 16, 17) with conflicting results. A systemic review and meta-analysis by MacDonald et al. (18) published in 2010, has found no evidence of a relationship between increased body mass index (BMI) and semen parameters. Thus, further studies would be

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warranted in this field. Petersen et al. (19) reported that maternal and paternal BMI, both independently and combined, exerted negative effects on live birth rates after *in vitro* fertilization (IVF) cycles, but this association in intracytoplasmic sperm injection (ICSI) cycles was less obvious. In light of the current evidence, we designed the present study to assess the impacts of obesity in a couple on ART outcomes. This study sought to answer the question of whether obesity simultaneously in a couple has a negative effect on ICSI cycle outcomes in comparison to couples who have normal BMIs.

## Materials and Methods

This was a cross-sectional study performed at Royan Institute between January 2013 and January 2014. The Review Board and Ethics Committees of Royan Institute approved the study protocol. All participating couples provided ethical permission at their initial visit for the use of their treatment outcomes. Participant confidentiality for all participants was assured during the research and written informed consent was obtained from them.

### Subjects

We evaluated the data recorded during the study period from all of the study participants. The study population was limited to patients who underwent ICSI or IVF/ICSI cycles that resulted in the transfer of 2 or 3 fresh embryos. Height and weight were recorded for all couples. Couples whose female partner was ≤39 years of age and the male partner was <55 years of age (17) at the time of the treatment cycle onset were enrolled to minimize the effect of age as a confounding factor. We excluded all cases with uterine factor, severe male factor, severe endometriosis, and gamete or embryo donor recipients (Fig.1).

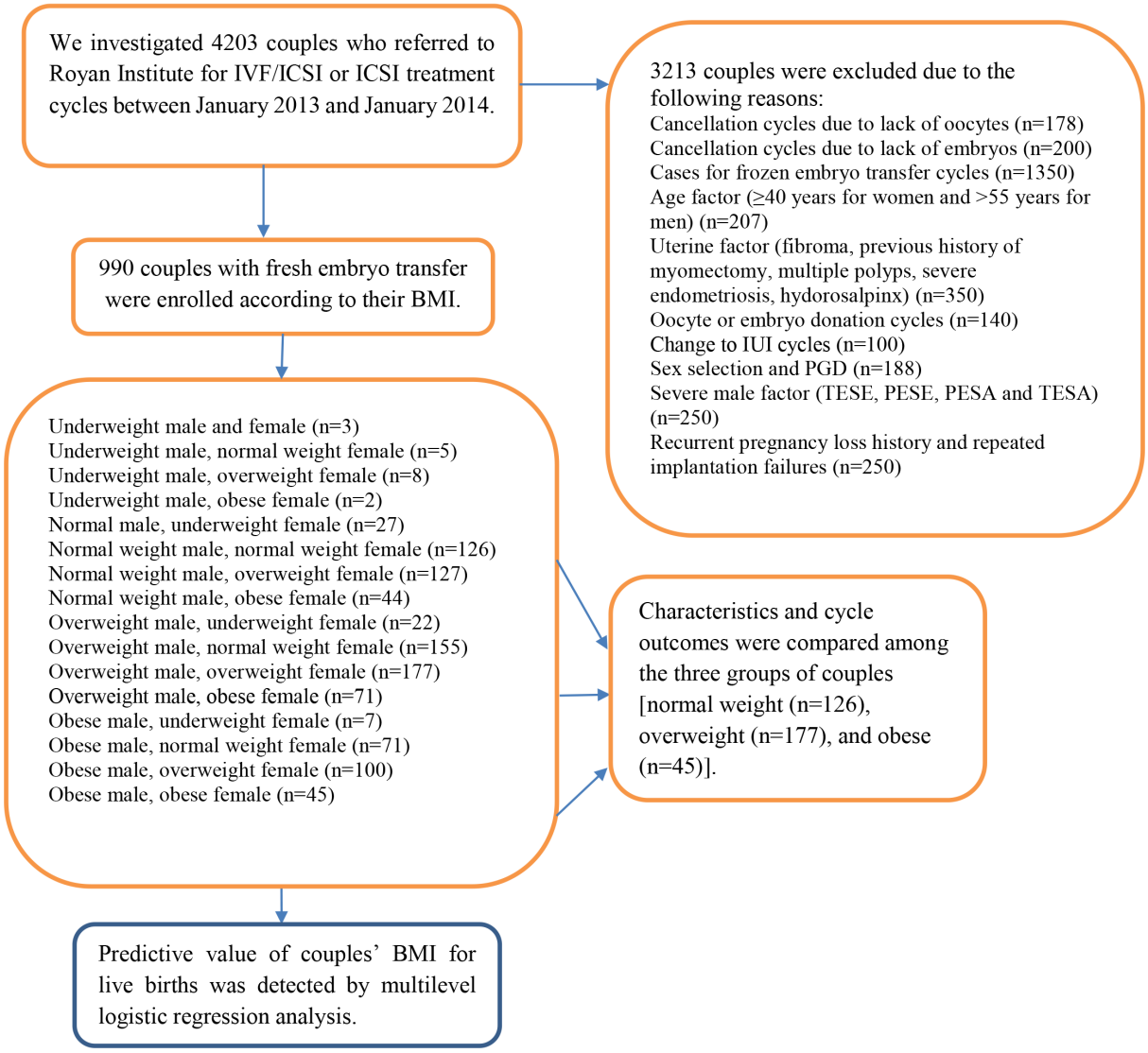


**Fig.1:** Sampling procedure and the distribution of the couples according to their BMI. IVF; *In vitro* fertilization, ICSI; Intra-cytoplasmic sperm injection, BMI; Body-mass index, IUI; Intrauterine insemination, PGD; Pre-gestational diagnosis, TESE; Testicular sperm extraction, PESE; Percutaneous epididymal sperm extraction, PESA; Percutaneous epididymal sperm aspiration, and TESA; Testicular sperm aspiration.

The patients' age (years) was recorded at the beginning of treatment. At the onset of treatment, we classified participants as smokers or non-smokers according to the number of cigarettes smoked per day. The diagnosis of infertility was determined according to the 10th revision of the International Classification of Diseases (11). Accordingly, women participants were categorized as ovulatory or an ovulatory. Standard ovarian stimulation protocols were performed according to routine clinical practice. In brief, suppression of the endogenous luteinizing hormone surge was performed with either gonadotropin-releasing hormone agonists or antagonists. Controlled ovarian stimulation was performed with recombinant follicle-stimulating hormone (rFSH) and/or human menopausal gonadotropin (hMG); trans-vaginal ultrasound guided ovum pickup was performed 34-36 hours after administration of human chorionic gonadotropin (hCG). ICSI for retrieved MII oocytes, with or without insemination, was performed in accordance with standard general recommendations.

We defined normal fertilization as the appearance of the 2nd polar body at 16-19 hours after insemination or microinjection. In our institute, embryo quality is graded as A, B, C, and D, with "A" defined as the best quality and "D", the worst, according to cell numbers, percentage of fragmentation, and cell symmetry. All embryo transfers were performed with a Labotect catheter (Labotect, Germany) by experienced gynaecologists and embryologists on day 3 after IVF/ICSI. Luteal phase support was provided by administration of 400 mg of vaginal progesterone twice a day until the day of the β-hCG test. Luteal support with progesterone was prescribed until the observation of foetal heart activity and subsequently tapered until week 8 of gestation. The main outcomes were fertilization, implantation, clinical pregnancy, and live birth rates. The implantation rate was denoted as the number of visualized intrauterine gestational sacs divided by the number of transferred embryos. A clinical pregnancy was documented by ultrasound observation of an intrauterine gestational sac with foetal cardiac activity. We defined spontaneous abortion as the loss of clinical pregnancy prior to 20 weeks gestation. Trained nurses routinely measured height and weight in participants of both genders prior to the onset of the treatment cycle. The balance scale for the measurement of weight was calibrated daily and verified by a one kg counterweight. We used the World Health Organization's definition of BMI ($kg/m^2$) to classify male and female participants as underweight ($<18.5$ $kg/m^2$), normal (18.5-24.9 $kg/m^2$), overweight (25-29.9 $kg/m^2$), or obese ($\geq 30$ $kg/m^2$) (20). The small number of underweight couples precluded their inclusion in the couples' analysis. We divided the couples into 3 groups based on male and female BMI results: group 1 (normal weight), group 2 (overweight), and group 3 (obese). The main outcomes were compared among the three groups.

**Statistical analysis**

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS), version 20.0 (SPSS Inc., Chicago, IL, USA). The study population's characteristics were compared according to the couples' BMI (normal, overweight, and obese) using one-way analysis of variance (ANOVA), Kruskal-Wallis nonparametric analysis of variance, and the chi-square test when appropriate. Multilevel logistic regression analysis was applied to determine the odds of live births following ICSI cycles. The analysis was conducted according to the female and male BMI groups. Normal-weight patients were considered to be the reference group. Analysis of female BMI was adjusted for age and duration of infertility. Likewise, analysis of the male BMI was adjusted for age, duration of infertility, and smoking status.

A multilevel logistic regression analysis was used to detect the predictive factors for live births after ICSI cycles. All possible factors that affected the live birth rate, which included female and male ages, couples' BMI ($<25$ $kg/m^2$ and $\geq 25$ $kg/m^2$), male smoking status, cause and duration of infertility, ovarian stimulation protocol [long gonadotropin-releasing hormone (GnRH) agonist and GnRH antagonist protocols], and number and quality of transferred embryos were incorporated into the model. The results of the multilevel logistic regression analysis have been presented as adjusted odds ratios (ORs) with 95% confidence intervals (CIs). P values$<0.05$ were considered statistically significant.

**Results**

In total, there were 4203 ART cycles during the study period. A total of 990 eligible women and their husbands underwent 927 ICSI and 63 ICSI with insemination (IVF/ICSI) cycles according to the inclusion criteria. The sampling procedure and distribution of the couples according to their BMI has been illustrated in (Fig.1). According to BMI, of the 990 women participants, there were 59 (6%) underweight, 357 (36%) with normal weight, 412 (41.6%) overweight, and 162 (16.4%) obese participants. Of the 990 men evaluated, there were 18 (1.8%) underweight, 325 (32.7%) normal weight, 425 (43%) overweight, and 223 (22.5%) obese participants.

The characteristics of the study population according to gender and BMI have been presented in (Table 1). The distribution of smoking in the males significantly differed among the BMI groups (P=0.006). The majority of females (n=786, 79.3%) had normal menses and ovulation. As expected, there were more anovulatory cases in the obese group than in the other groups (P=0.003).

Anovulatory cases in the present study consisted of participants with PCOS (n=153, 75%) and age factor (over 37 to 39 years, n=51, 25%).

**Table 1:** Basic characteristics of the studied population according to gender and body mass index

| Characteristics | Underweight | Normal weight | Overweight | Obese | P value |
|---|---|---|---|---|---|
| | n=59 | n=357 | n=412 | n=162 | |
| Women | | | | | |
| Age (Y) | 29.0 ± 3.9 | 30.0 ± 4.5 | 30.7 ± 4.8 | 32.2 ± 4.9 | <0.001 |
| Anovulatory cases | 11 (18.6) | 59 (16.5) | 84 (20.4) | 50 (30.9) | 0.003 |
| | n=45 | n=297 | n=425 | n=223 | |
| Men | | | | | |
| Age (Y) | 35.1 ± 6.7 | 34.8 ± 5.6 | 35.0 ± 5.7 | 36.0 ± 6.4 | 0.081 |
| Smoking cases | 21 (35.5) | 58 (17.7) | 88 (21.3) | 69 (42.5) | 0.006 |

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

We separately evaluated the impact of female and male BMI on the live birth rate in ICSI. The results of the multilevel regression analysis according to female and male BMI has been shown in (Table 2). Among the ovulatory women, there was a significant difference between the BMI groups, with a 60% (95% CI: 0.11-0.83) decrease in the odds for live birth among overweight individuals and 84% (95% CI: 0.02-0.99) decrease in the odds for live birth among obese individuals. Trend analysis showed a significant reduction of 9% (95% CI: 0.83-0.99) with each one unit increase in BMI (P=0.04). Among anovulatory women, the association between BMI and live births presented no clear tendencies, even though the ORs indicated lower probabilities for live births among overweight and obese anovulatory women. The 95% CIs were not significant. Among anovulatory women, the trend analysis showed a significant reduction of 15% (95% CI: 0.72-0.98) with every one unit increase in BMI (P=0.02). In both ovulatory and anovulatory underweight women, we observed a significant elevation in the odds of live births of 6.5 times (95% CI: 2.1-20.65) and 7.3 times (95% CI: 0.99-55.1), but the CIs were too wide because of the low sample size. The results for men participants presented no significant relationship between BMI and live births.

The comparison of the three groups of couples according to BMI has been demonstrated in (Table 3). The overweight (P=0.01) and obese (P<0.001) couples were significantly older than normal weight couples. The results indicated that the three groups were comparable in terms of type, cause of infertility, number of previous ART cycles, and stimulation outcomes. There were no significant differences between the three groups in terms of fertilization, implantation, clinical pregnancy, multiple pregnancy, miscarriage, and live birth rates.

**Table 2:** Multilevel logistic regression analyses of the probability for live births following all ICSI or IVF/ICSI cycles according to gender and stratified by BMI

| Variables | n (%) | OR[a] | 95% CI | P value[b] |
|---|---|---|---|---|
| Female[b] BMI, (n=990 ICSI cycles among 990 women) | | | | |
| Ovulatory | | | | |
| Underweight | 48 (6.1) | 6.5 | (2.1-20.65) | 0.001 |
| Normal weight | 298 (37.9) | Reference group | - | - |
| Overweight | 328 (41.0) | 0.30 | (0.11-0.83) | 0.021 |
| Obese | 112 (14.2) | 0.14 | (0.02-0.99) | 0.049 |
| Trend | 786 (100) | 0.91 | (0.83-0.99) | 0.045 |
| Anovulatory | | | | |
| Underweight | 11 (5.3) | 7.30 | (0.99-55.1) | 0.050 |
| Normal weight | 59 (28.9) | Reference group | - | - |
| Overweight | 84 (41.1) | 0.50 | (0.10-2.4) | 0.392 |
| Obese | 50 (24.5) | 0.14 | (0.009-2.4) | 0.186 |
| Trend | 204 (100) | 0.85 | (0.72-0.98) | 0.021 |
| Male[b] BMI, (n=990 ICSI cycles among 990 men) | | | | |
| Underweight | 45 (4.5) | 0.295 | (0.02-3.96) | 0.352 |
| Normal weight | 297 (30.0) | Reference group | - | - |
| Overweight | 425 (42.9) | 0.07 | (0.0-10.71) | 0.360 |
| Obese | 223 (22.5) | 0.012 | (0.0-21.89) | 0.294 |
| Trend | 990 (100) | 1.01 | (0.95-1.09) | 0.647 |

**OR; Odds ratio, CI: Confidence interval, BMI; body mass index, IVF; *In vitro* fertilization, ICSI; Intra-cytoplasmic sperm injection, [a]; ORs with 95% CIs and P values from Wald tests, and [b]; Female analyses adjusted for age and duration of infertility. Male analyses adjusted for age, duration of infertility, and smoking status.**

**Table 3:** Comparison of study population characteristics and cycle outcomes among three groups of couples according to BMI

| Variable | Normal weight couples n=126 | Overweight couples n=177 | Obese couples n=45 | Test | P |
|---|---|---|---|---|---|
| Female age (Y) | 30.0 ± 4.7 | 30.5 ± 4.6 | 32.3 ± 4.7 | ANOVA | 0.018[a] |
| Male age (Y) | 33.4 ± 4.7 | 35.1 ± 5.9 | 37.9 ± 5.4 | ANOVA | <0.001[b] |
| Female FSH (IU/l) | 6.8 ± 3.6 | 6.1 ± 3.0 | 6.9 ± 3.4 | ANOVA | 0.160 |
| Female LH (IU/l) | 4.7 ± 3.3 | 4.6 ± 4.1 | 4.6 ± 4.7 | ANOVA | 0.986 |
| Female AMH (ng/ml) | 2.3 ± 1.7 | 2.4 ± 1.6 | 2.0 ± 1.4 | Kruskal-Wallis | 0.646 |
| Female TSH (mIU/l) | 2.1 ± 1.8 | 2.3 ± 1.9 | 2.3 ± 1.7 | ANOVA | 0.658 |
| Type of infertility | | | | Chi-square | 0.426 |
| Primary | 107 (84.9) | 157 (88.7) | 37 (82.3) | | |
| Secondary | 19 (15.1) | 20 (11.3) | 8 (17.7) | | |
| Cause of infertility | | | | Chi-square | 0.150 |
| Ovulatory | 105 (83.3) | 32 (71.1) | 147 (83.1) | | |
| Anovulatory | 21 (16.7) | 13 (28.9) | 30 (16.9) | | |
| Male factor infertility cases | 89 (70.6) | 30 (66.7) | 131 (74) | Chi-square | 0.577 |
| Infertility duration (Y) | 5.1 ± 3.4 | 6.39 ± 4.7 | 7.08 ± 4.7 | ANOVA | 0.011[c] |
| Number of previous ART cycles | 0.4 ± 0.9 | 0.3 ± 0.6 | 0.4 ± 0.9 | ANOVA | 0.583 |
| Stimulation duration (days) | 10.7 ± 2.0 | 10.6 ± 2.1 | 10.9 ± 2.4 | ANOVA | 0.664 |
| Total amount of rFSH dose (IU) | 1932.6 ± 724.0 | 1810.1 ± 821.8 | 1839.9 ± 844.4 | Kruskal-Wallis | 0.176 |
| Total dose of used gonadotropins (IU) | 2086.1 ± 1005.4 | 2087.9 ± 903.8 | 2251.6 ± 1206.3 | ANOVA | 0.582 |
| Stimulation protocol | | | | Chi-square | 0.564 |
| Long agonist | 108 (85.7) | 40 (88.9) | 149 (84.2) | | |
| Antagonist | 18 (14.3) | 5 (11.1) | 28 (15.8) | | |
| Total number of retrieved oocytes | 8.9 ± 4.3 | 8.3 ± 3.4 | 8.0 ± 3.52 | ANOVA | 0.327 |
| Total number of embryos | 5.1 ± 2.7 | 5.0 ± 2.7 | 5.0 ± 2.50 | ANOVA | 0.974 |
| Number of transferred embryo | 2.4 ± 0.56 | 2.4 ± 0.5 | 2.4 ± 0.55 | ANOVA | 0.978 |
| Endometrial thickness on transfer day (mm) | 9.9 ± 1.4 | 9.9 ± 1.6 | 9.5 ± 1.9 | ANOVA | 0.293 |
| Quality of transferred embryos (ET)[*] | | | | Chi-square | 0.675 |
| Good | 82 (65.1) | 111 (62.7) | 32 (71.1) | | |
| Fair | 11 (8.7) | 20 (11.3) | 2 (4.4) | | |
| Poor | 33 (26.2) | 46 (26) | 11 (24.4) | | |
| Fertilization rate | 0.74 ± 0.23 | 0.73 ± 0.24 | 0.78 ± 0.21 | ANOVA | 0.523 |
| Implantation rate | 0.28 ± 0.173 | 0.31 ± 0.2 | 0.33 ± 0.16 | ANOVA | 0.593 |
| Clinical pregnancy rate | 40 (78.4) | 68 (89.5) | 21 (87.5) | Chi-square | 0.407 |
| Blighted ovum | 9 (17.6) | 6 (7.9) | 2 (8.3) | Chi-square | 0.231 |
| Ectopic pregnancy rate | 2 (3.9) | 2 (2.6) | 1 (4.2) | Chi-square | 0.936 |
| Multiple pregnancy rate | 7 (19.4) | 15 (27.3) | 5 (27.8) | Chi-square | 0.664 |
| Miscarriage rate | 2 (5.3) | 9 (14.1) | 2 (10) | Chi-square | 0.362 |
| Live birth rate | 36 (94.7) | 55 (85.9) | 18 (90) | Chi-square | 0.345 |

**Data are presented as mean ± SD or n (%). BMI; Body mass index, FSH; Follicle stimulating hormone, LH; Luteinizing hormone, AMH; Anti-Müllerian hormone, TSH; Thyroid stimulating hormone, ART; Assisted reproductive technology, rFSH; Recombinant follicle-stimulating hormone, ANOVA: One-way analysis of variance, [a]; Obese couples vs. overweight couples (P=0.015), normal BMI vs. obese couples (P=0.040) according to Tukey's test, [b]; Normal BMI vs. overweight couples (P=0.013), normal BMI vs. obese couples (P<0.001), overweight vs. obese couples (P=0.086) according to Tukey's test, [c]; Normal BMI vs. overweight couples (P=0.032), normal BMI vs. obese couples (P=0.021) according to Tukey's test, [*]; Good quality embryos-all ET were A, B, or AB, Fair-half of ET were good quality (AC, BC), Poor quality-all ET were C, D, or CD.**

The results of the multilevel logistic regression model for the detection of the predictive factors for the live birth rate showed that none of the included variables remained in the final model as significant factors. The results also revealed no significant association between the couples' BMI and live births (Table 4).

**Table 4:** Multilevel logistic regression analysis for detection of predictive factors for live birth after ICSI or IVF/ICSI cycles in the studied population

| Combined BMI (kg/m²) | | | Live birth per ICSI cycle | | |
|---|---|---|---|---|---|
| **Women** | **Men** | **n (%)** | **OR** | **95% CI** | **P value** |
| <25 | <25 | 161 (16.3) | 1 | Reference group | - |
| <25 | ≥25 | 255 (25.8) | 1.03 | 0.5-1.9 | 0.914 |
| ≥25 | <25 | 181 (18.3) | 0.6 | 0.3-1.2 | 0.262 |
| ≥25 | ≥25 | 393 (39.7) | 0.8 | 0.4-1.9 | 0.864 |

**ICSI; Intracytoplasmic sperm injection, IVF; *In vitro* fertilization, BMI; Body mass index, OR; Odds ratio, and CI; Confidence interval.**

## Discussion

Previous studies separately evaluated the effects of both genders' BMI on ART outcomes. The synergistic effects of obesity in couples were reported in limited studies (8, 19). We have excluded the main confounding factors that affect live birth rates in order to accurately assess the independent effects of a couple's obesity on ART outcomes. Our results revealed that a couple's BMI had no effect on the outcomes of ICSI with fresh cleavage-stage embryo transfer cycles.

Our results supported those published in 2013 by Petersen et al. (19), who reported that the combined increased maternal and paternal BMI had no significant effect on live birth rate in ICSI cycles. However, the authors have presented the negative impacts of increased female and male BMI, both individually and combined, on live births in IVF cycles. In our institute, treatment cycles with only IVF are uncommon and the majority of treatment cycles include ICSI or IVF/ICSI procedures. Therefore, we could not evaluate these subjects according to IVF cycles.

The effects of female BMI on ART outcomes were evaluated in several studies. Our findings showed that among ovulatory women, BMI had a negative impact on live births. In anovulatory women, we observed a tendency for less odds of live births in the obese group, which was not statistically significant. Therefore, our results agreed with some recent studies where female BMI negatively impacted ART outcomes (8, 11, 13, 15, 20). On the other hand, previous studies indicated no negative effect of female BMI on ART outcomes (2, 12, 21-23). Petersen et al. (19) demonstrated that the female BMI had a negative impact on live birth rates in IVF cycles, but this was less clear in ICSI cycles. A prospective study conducted by Chavarro et al. (24) evaluated 170 women who underwent 233 ART cycles and found an association between overweight and obese women with decreased live birth rates. Moragianni et al. (25), in a retrospective research of 4609 patients, found that obesity had significant negative effects on ART outcomes, with up to 68% lower odds of live births following the first ART cycle. Rittenberg et al. (15), in a meta-analysis of 47967 IVF/ICSI cycles, reported that an increased female BMI was aligned with adverse pregnancy outcomes in IVF/ICSI treatment cycles and this effect was observed in both overweight and obese women. Since the earlier investigations did not categorize their findings according to type of treatment cycle (IVF or ICSI ), a more adverse influence of increased BMI in IVF compared to ICSI might have been concealed and the intensity of the BMI impact on IVF/ICSI possibly relied on the IVF and ICSI cycle distributions in the sample size (19). Although a number of multiparous women are obese, a negative association of obesity with women's reproductive health has been reported (26). Because of the conflicting results reported by studies, the mechanism action of maternal obesity on IVF or IVF/ICSI outcomes remains unclear (27). Although a number of oocyte donation studies have suggested negative effects of obesity on the endometrium (6, 28), others have not (2, 21, 29). Endocrine changes related to obesity such as hyperandrogenism and insulin resistance as well as alterations in the local insulin-like growth factors (IGFs), cytokines, and leptin levels may play a major role in the adverse effects of an increased BMI on ART outcomes (4). According to previous studies (13), the mechanism of action of obesity in anovulatory cases, especially PCOS women, is different and depends on the intensity of the endocrine changes.

The influence of male BMI on ART outcomes has been less studied. The existing literature contains only 7 studies on this subject (1, 8, 11, 16, 17, 19, 30). The first study, published in 2011 by Bakos et al. (1), reported an association between high paternal BMI with significantly reduced clinical pregnancy and live birth rates after ART. Two recent studies presented that male BMI was associated with a negative impact on clinical pregnancy and live birth rates after IVF, but not after ICSI. Additionally, the previous studies in this field reported that ICSI might overcome the negative impact of obesity on sperm-oocyte interaction (16, 19). On the other hand, a prospective study conducted by Colaci et al. (17) evaluated 114 couples who underwent 172 ICSI cycles and concluded that male obesity was associated with decreased odds for live births after ICSI. Our results indicated that the male BMI had no effect on live birth rates after ICSI. The deleterious effects of male obesity could be due to an altered hormonal profile and decreased semen quality related to increased leptin and E2 levels, and disturbance in spermatogenesis (19, 31). However, a systematic review with meta-analysis found no evidence of an association between an increased BMI and semen parameters (32). A systematic review by Campbell et al. (33) in 2015 reported that the rate of births per ART cycle was reduced by 35% in obese men. The salient weak point in the previous studies and our study was the use of BMI as a marker

of body fat in men. In view of these conflicting results, we suggest that prospective studies should evaluate the effects of male and female abdominal obesity on reproductive parameters and ART outcomes via other anthropometric measurements (waist and hip circumferences). Currently, the role of the male BMI in ART processes and outcomes is partly understood. Further investigations are needed to arrive at reliable conclusions (13).

We analysed the couples and found no synergistic negative impact of increased female and male BMI on live births after ICSI cycles. This finding agreed with studies by Petersen et al. (19) and Schliep et al. (34). Some studies assessed the effects of combined male and female BMI on ART outcomes (4, 8, 10, 19). Petersen et al. (19) evaluated the effects of parental BMI on live birth rates after ART cycles. They reported that increased combined female and male BMI had a negative impact on live birth rates after IVF cycles; however, its effects in terms of ICSI were less clear. Schliep et al. performed a prospective assessment of 721 couples and found no influence of the couples' weight status on IVF outcomes (34). In contrast, a recent study by Wang and colleagues retrospectively investigated 12061 first fresh IVF/ICSI cycles and reported that female obesity exerted negative effects on live births after IVF; nonetheless, there was no evidence of a negative impact by the parental BMI on ICSI outcomes (4). In contrast, Umul et al. (10) found that couples' obesity had a negative impact on clinical pregnancy rates and live birth rates following ICSI cycles. In the present study we meticulously analysed the characteristics of the couples in ICSI cycles and adjusted the impact of confounding factors on our results. Recent data have confirmed the findings of those previous studies that reported no significant influence of the parental BMI on ICSI success. In view of the conflicting results, we suggest that more research be undertaken to shed sufficient light on this issue.

The present study has some limitations. There was no data about the specific hormonal profile and android or gynoid distribution of fat in anovulatory and ovulatory women, and no data about semen analysis parameters to compare among different BMI groups. We propose that these parameters should be considered in future studies.

## Conclusion

Based on the current findings, an increased maternal BMI independently influenced negatively live birth rate after ICSI cycles, whereas increased paternal BMI separately and in combination with maternal BMI did not show this affect.

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## Authors' Contributions

A.A, M.A., M.H.; Participated in study design, data collection, drafting and writing the manuscript. M.H., A.A.; Contributed extensively to data interpretation and the conclusion. Z.Z.; Conducted the data collection and analysis. All authors participated in the finalization of the manuscript and approved the final draft.

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Original Article

# Diagnostic Accuracy of Body Mass Index and Fasting Glucose for The Prediction of Gestational Diabetes Mellitus after Assisted Reproductive Technology

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## Abstract

**Background:** The aim of the present study was to determine the maternal pre-pregnancy body mass index (BMI), first-trimester fasting blood sugar (FBS), and the combination of (BMI+FBS) cut-points for at-risk pregnant women conceived by assisted reproductive technology (ART) to better predict the risk of developing gestational diabetes mellitus (GDM) in infertile women.

**Materials and Methods:** In this nested case-control study, 270 singleton pregnant women consisted of 135 (GDM) and 135 (non-GDM) who conceived using ART were assessed. The diagnosis of GDM was confirmed by a one-step glucose tolerance test (O-GTT) using 75 g oral glucose. BMI was classified base on World Health Organization (WHO) criteria. The relationship between BMI, FBS, and BMI+FBS with the risk of GDM development was determined by logistic regression and adjusted for confounding factors. Receiver operating characteristic (ROC) curve analysis was performed to assess the value of BMI, FBS, and BMI+FBS for the prediction of GDM.

**Results:** The GDM group had significantly higher age, BMI, family history of diabetes, and history of polycystic ovary syndrome in comparison with the non-GDM group ($P<0.05$). Overweight and obese women had 3.27, and 5.14 folds increase in the odds of developing GDM, respectively. There was a 17% increase in the risk of developing GDM with each 1 mg/dl increase in fasting glucose level. The cut points for FBS 84.5 mg/dl (72.9% sensitivity, 74.4% specificity), BMI 25.4 kg/m$^2$ (68.9% sensitivity, 62.8% specificity), and BMI+FBS 111.2 (70.7% sensitivity, 80.6% specificity) was determined.

**Conclusion:** The early screening and high-quality prenatal care should be recommended upon the co-occurrence of high FBS (≥84.5 mg/dl) in the first-trimester of the pregnancy and the BMI (≥25.4 kg/m$^2$) in pre-pregnancy period in women undergone ART. The combination of BMI and FBS is considered a better prediction value.

***Keywords:*** Assisted Reproductive Technology, Body Mass Index, Gestational Diabetes Mellitus

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## Introduction

Gestational diabetes mellitus (GDM) is one of the main obstetrics complications among pregnant women with a history of fertility problem (1), particularly in mothers receiving infertility treatment by assisted reproductive technology (ART) (2). Recent studies reported the association between ART treatment and GDM risk (3). Moreover, pregnancies complicated with GDM can result in adverse maternal and perinatal consequences (4). Genetic predisposition, ethnicity, and age are the most significant risk factors for GDM; furthermore, maternal obesity is consistently proposed as a major and modifiable risk factor (5). Along with obesity accretion rate, there is an increase in the number of obese infertile women seeking infertility treatments through ART (6).

In general terms, GDM is detected at mid-pregnancy (24$^{th}$-28$^{th}$ weeks of gestation) by oral glucose tolerance test (OGTT). Nevertheless, there is evidence suggesting that

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GDM occurs in all trimesters of pregnancy (7). However, high-risk mothers are assessed in the first-trimester for hyperglycemia in pregnancy. Lately, several studies proposed evaluating the first-trimester prediction of GDM based on maternal characteristics (8). Previously, researchers found that body mass index (BMI) (9) and fasting blood sugar (FBS) level (10, 11) were independent predictors of GDM in normal pregnancy and pregnancies in women with a prior history of polycystic ovary syndrome (PCOS) (12). Recent investigation showed that age, BMI and mode of ART were independent risk factors for GDM in patients undergoing ART (13).

The national institute for health and care excellence (NICE) guidelines (2013) recommended determining the cut-off points for BMI among different populations to help prevent diabetes and the other chronic conditions (14). Furthermore, BMI cut-off as an indicator of GDM was diverse in compliance with race and ethnicity (15). Recent systematic review and meta-analysis evaluated the predictive accuracy of the different combination of GDM risk factors in high-risk women in spontaneous pregnancy (16).

However, there is no consensus about GDM diagnosis regarding screening time, method and, the optimal cut points. Also, there is no direct evidence concerning the cut-off levels for pre-pregnancy BMI and fasting glucose to predict the risk of developing GDM in infertile women as a high-risk population. Accordingly, the present study was designed to evaluate the predictive values of maternal BMI and FBS to predict GDM risk, and then to determine the cut-points for BMI, FBS, and the combination of two biomarkers (BMI+FBS) for the diagnosis of at-risk pregnant women conceived using ART to target clinical surveillance in a more effective manner.

## Materials and Methods

This nested case-control study was conducted between October 2016 and June 2017. The data from 270 women with singleton pregnancies (135 GDM and 135 non-GDM women) conceived by ART treatment referred to Royan Institute were studied. ART was defined as being conceived by intracytoplasmic sperm injection (ICSI) and/or *in vitro* fertilization (IVF). Prior to data collection, the protocol of the study was approved by the institutional review board and Ethics Committee of Iran University of Medical Science (Project number: 25469). Clinical records of the participants were reviewed. Consent form was obtained and completed by participants. Data on maternal history and demographic characteristics as well as the records of the first-trimester para-clinical evaluations were collected from the documents. The target population was defined as women with singleton pregnancy via ART and aged between 20-42 years. The exclusion criteria were pre-gestational diabetes; chronic diseases (consisted of hypertension, cardiovascular diseases, untreated thyroid disease, liver diseases, renal diseases, autoimmune diseases, and connective tissue disorders); corticosteroids usage, and incomplete records. Pre-gestational diabetes was defined when the first-trimester FBS was above 125 mg/dl. GDM was confirmed by an OGTT using 75 g oral glucose at the first-trimester (for high-risk subjects) or 24-28 weeks of gestation (for non-GDM subjects). The results of OGTT were interpreted by American diabetes association (ADA) criteria (17). The diagnosis of gestational diabetes was based on FBS ≥92 mg/dl, 1 hour OGTT ≥180 mg/dl mg/dl, and 2 hour OGTT ≥153 mg/dl. Women with high-risk GDM were screened by OGTT on their first antenatal visit. High-risk subjects were defined as individuals with a history of GDM, obesity, impaired glucose metabolism, and history of PCOS. Non-GDM women were screened by OGTT at the 24$^{th}$ to 28$^{th}$ weeks of gestation. Women who had normal OGTT at the 24$^{th}$ to 28$^{th}$ weeks of pregnancy were considered the non-GDM (control) group and individuals with abnormal OGTT were considered the GDM group.

The data pertaining to the characteristics of patients and infertility treatment cycle were collected as previously described in details (18). Pre-pregnancy weight (weight) and height were measured before the initiation of ART cycles by trained nurses. BMI was calculated as the weight in kilograms was divided by the square of height in meters. According to world health organization (WHO), diagnostic criteria (19) women were categorized as normal weight (BMI <25 kg/m$^2$), overweight (BMI 25.0 -29.9 kg/m$^2$), and obese (BMI≥30.0 kg/m$^2$).

### Statistical analysis

Data were analyzed using the statistical package for the social sciences (SPSS) software for Windows (version 20, Chicago, IL, USA). Descriptive data were presented as the mean ± standard deviation (SD) or number (%) where appropriate. The independent sample t test was used to compare quantitative data with normal distribution between the two groups. Chi-square test was applied to compare the qualitative variables. The logistic regression analysis was performed to calculate the relationship between BMI, FBS, and BMI+FBS with the risk of GDM after ART cycles. The result of the analysis was expressed as odds ratio (OR) and 95% confidence intervals (CIs). ORs were presented either as crude or adjusted values for confounding variables (age, gravidity, PCOS diagnosis, and family history of diabetes). The patients with BMI<25 kg/m$^2$ were considered the reference group. The Hosmer-Lemeshow test was used for the goodness of fit in logistic regression models and the Pearson's chi-square was calculated. The Nagelkerke Pseudo-R$^2$ was determined to quantify predictive ability or model performance.

The receiver operating characteristic (ROC) curve analysis was done by MedCalc statistical software to measure the diagnostic accuracy of BMI, FBS, and BMI+FBS, as well as the optimal cut-point value as predictors for GDM. The DeLong method was used to compare the area under individual and paired ROC curves (AUC). Youden's index and associated cut-off points were used to measure the overall diagnostic effectiveness. The level of significance was set at P<0.05.

## Results

The clinical and biochemical baseline characteristics of participants are presented in Table 1. The mean maternal age was significantly higher in the GDM group (32.15 ± 5.07 vs. 30.28 ± 4.89, P=0.003). There were significant differences in terms of gravidity, pre-pregnancy weight, BMI, history of diabetes in first relative degree, FBS, and PCOS diagnosis between the two groups (P<0.001 for all variables). There were no significant differences between the two groups in terms of parity, systolic and diastolic blood pressure, maternal education and infertility cause. The incidence of overweight (48.9 vs. 32.5%) and obesity (23.7 vs. 10.9%) was significantly higher in the GDM group (P<0.001).

**Table 1:** Clinical and biochemical baseline characteristics of women conceived via ART with and without GDM

| Variable | Non-GDM group n=135 | GDM group n=135 | P value |
|---|---|---|---|
| Maternal age (Y) | 30.28 ± 4.89 | 32.15 ± 5.07 | 0.003 |
| Gravidity (=1, primigravida) | 100 (74.0) | 79 (58.5) | 0.001 |
| Parity (=0, nulliparous) | 116 (85.9) | 114 (84.4) | 0.184 |
| Weight (kg) | 64.34 ± 10.18 | 69.77 ± 10.45 | <0.001 |
| BMI (kg/m$^2$) | 24.57 ± 3.89 | 27.38 ± 3.91 | <0.001 |
| BMI (kg/m$^2$) | | | <0.001 |
| <25 | 73 (56.6) | 37 (27.4) | |
| 25.0-29.9 | 42 (32.5) | 66 (48.9) | |
| ≥30.0 | 14 (10.9) | 32 (23.7) | |
| History of diabetes in first relative degree | 21 (15.5) | 62 (45.9) | <0.001 |
| Maternal education | | | 0.636 |
| Lower secondary | 93 ( 68.9) | 95 (70.1) | |
| Upper secondary | 42 (31.1) | 40 (29.9) | |
| FBS (mg/dl) | 80.81 ± 5.45 | 90.66 ± 10.24 | <0.001 |
| PCOS diagnosis | 11 (8.1) | 35 (25.9) | <0.001 |
| Systolic blood pressure (mmHg) | 104.26 ± 8.50 | 106.26 ± 9.77 | 0.078 |
| Diastolic blood pressure (mmHg) | 65.66 ± 7.05 | 66.70 ± 7.58 | 0.248 |
| Infertility cause | | | 0.714 |
| Ovulatory factor | 41 (30.3) | 48 (35.8) | |
| Male factor | 65 (48.2) | 59 (44.1) | |
| Tubal factor | 8 (6.0) | 9 (6.7) | |
| Unexplained | 21 (15.5) | 18 (13.4) | |

**Data are presented as mean ± SD or n (%). ART; Assisted reproductive technology, GDM; Gestational diabetes mellitus, BMI; Body mass index, FBS; Fasting blood sugar, and PCOS; Polycystic ovary syndrome.**

**Table 2:** Crude and adjusted odds ratios of BMI categories and FBS for development of GDM

| Variable | OR crude (95% CI) | OR adjusted (95% CI) (Model 1) | OR adjusted (95% CI) (Model 2) | OR adjusted (95% CI) (Model 3) |
|---|---|---|---|---|
| BMI ( Kg/m$^2$) | | | | |
| <25 | Reference | Reference | Reference | Reference |
| 25-29.9 | 3.10 (1.78,5.39) | 2.26 (1.10,4.6) | 2.79 (1.37,5.68) | 3.27 (1.61,6.66) |
| ≥30.0 | 4.51 (2.15,9.47) | 2.27 (0.649,7.96) | 3.58 (1.05,12.20) | 5.14 (1.53,17.26) |
| Nagelkerke R² | 0.118 | 0.126 | 0.119 | 0.116 |
| Hosmer and Lemeshow Test | | | | |
| Chi-square | 1 | 2.003 | 4.08 | 4.16 |
| P value* | 0.01 | 0.981 | 0.855 | 0.842 |
| FBS (mg/dl) | 1.171 (1.12-1.20) | 1.56 (1.28-1.90) | 1.71 (1.41-2.07) | 1.4 (1.26-1.56) |
| Nagelkerke R² | 0.364 | 0.400 | 0.429 | 0.422 |
| Hosmer and Lemeshow Test | | | | |
| Chi-square | 15.46 | 11.87 | 12.613 | 12.67 |
| P value* | 0.051 | 0.157 | 0.126 | 0.124 |

**BMI; Body mass index, FBS; Fasting blood sugar, CI; Confidence interval, GDM; Gestational diabetes mellitus, OR; Odds ratio, and PCOS; Polycystic ovary syndrome. Data are presented as OR (95% CI), Model 1; Adjusted by age and gravidity, Model 2; Adjusted by age, gravidity and PCOS diagnosis, Model 3; Adjusted by age, gravidity, PCOS diagnosis and family history of diabetes, and *; The P value is related to the Hosmer and Lemeshow test- which is not significant- it shows goodness of fitting the model.**

Logistic regression analysis illustrated that both FBS level and BMI were significant and independent risk factors for development of GDM after adjustment for confounding variables (age, gravidity, PCOS and family history of diabetes). The results of logistic regression showed that overweight and obese women had a 3.27-fold [adjusted OR (a-OR) 3.27, 95% CI, (1.61, 6.66), P<0.002] and 5.14-fold [aOR 5.14, 95% CI, (1.53, 17.26), P<0.002] higher odds for GDM than that of normal weight women, respectively. There was an approximately 17% increase in the odds of developing GDM with each 1 mg/dl increase in FBS level [OR 1.17, 95% CI, 1.17 (1.12-1.20), P<0.001] (Table 2).

Our result presents that Nagelkerke Pseudo-$R^2$ are 0.118 (BMI) and 0.364 (FBS). It means that our model is stable and can predict the results. The Chi-square of Hosmer-Lemeshow test is the interpreter the goodness of fit test for logistic regression and shows this model is fit for our data.

The ROC curves illustrate the ability of FBS, BMI, and BMI+FBS to predict GDM development (Fig.1). The ROC curve analysis showed the predictive values of 0.69, 0.79, and 0.83 for BMI, FBS, and BMI+FBS, respectively.

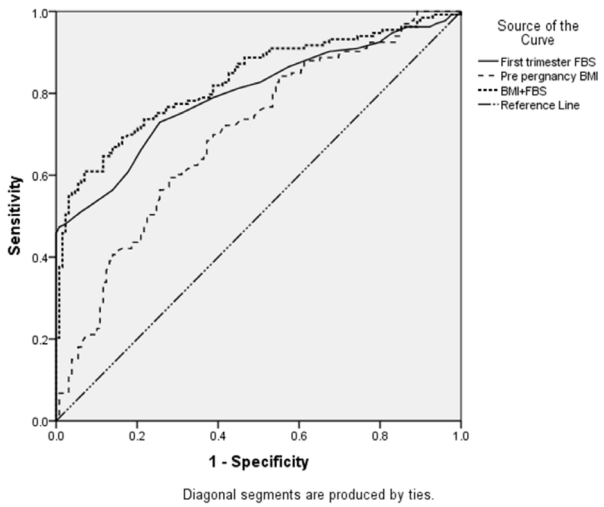


**Fig.1:** Receiver operating characteristics (ROC) curve analysis for the ability of the first-trimester fasting blood sugar (FBS), pre-pregnancy body mass index (BMI), and BMI+FBS to predict gestational diabetes mellitus (GDM) in women conceived via assisted reproductive technology (ART).

The values of BMI, FBS, and BMI+FBS for the prediction of GDM and overall diagnostic effectiveness of each factor were presented in Table 3. On the basis of the ROC curves, the best cut-off point for FBS was 84.5 mg/dl, with a sensitivity of 72.9% (95% CI: 64.5-80.3) and specificity of 74.4% (95% CI: 66.0-81.7). Regarding BMI, the best cut-off point was obtained as 25.4 kg/$m^2$ with a sensitivity of 68.9% (95% CI: 60.4-76.6), specificity of 62.8% (95% CI: 53.8-71.1). The combination of two biomarkers (BMI+FBS) has a better AUC value (0.83). The best cut-off point for BMI+FBS was 111.2 with a sensitivity of 70.7% (95% CI: 62.2-78.2) and specificity of 80.6% (95% CI: 72.7-87.0), separately.

**Table 3:** The values of BMI, FBS and BMI+FBS for the prediction of GDM and their overall diagnostic effectiveness

| ROC index | BMI | FBS | BMI+FBS |
|---|---|---|---|
| AUC | 0.69 | 0.79 | 0.83 |
| 95% CI of AUC | 0.63-0.76 | 0.74-0.85 | 0.78-0.88 |
| P value* | <0.0001 | <0.0001 | <0.0001 |
| Youden index J | 0.304 | 0.473 | 0.513 |
| Cut-off criterion | 25.4 | 84.5 | 111.2 |
| Sensitivity (%) | 68.8 | 72.9 | 70.7 |
| 95% CI of sensitivity | 60.4-76.6 | 64.5-80.3 | 62.2-78.2 |
| Specificity | 62.79 | 74.42 | 80.62 |
| 95% CI of specificity | 53.8-71.1 | 66.0-81.7 | 72.7-87.0 |
| Positive likelihood ratio | 1.85 | 2.85 | 3.65 |
| Negative likelihood ratio | 0.5 | 0.36 | 0.36 |

**BMI; Body mass index, FBS; Fasting blood sugar, GDM; Gestational diabetes mellitus, ROC; Receiver operating characteristic, AUC; Under individual ROC curves, CI; Confidence interval, and *; P< 0.05 was significant.**

The AUC of three ROC curves is compared in Table 4. The results indicate that there are significant differences among pairwise groups. The combination of BMI+FBS significantly improves the predictive ability of FBS or BMI alone for GDM development.

**Table 4:** The pairwise comparison of the area under the ROC curves between BMI, FBS, and BMI+FBS

| Variable | BMI vs. FBS | BMI vs. BMI+FBS | FBS vs. BMI+FBS |
|---|---|---|---|
| Difference between areas | 0.09 | 0.13 | 0.03 |
| Standard error | 0.041 | 0.031 | 0.013 |
| 95% confidence interval | 0.016-0.18 | 0.070-0.19 | 0.0069-0.060 |
| z statistic | 2.35 | 4.20 | 2.46 |
| Significance level | P=0.02* | P<0.0001* | P=0.01* |

**ROC; Receiver operating characteristic, BMI; Body mass index, FBS; Fasting blood sugar, *; P<0.05 was significant.**

## Discussion

In the present study, the predictive values of first-trimester FBS, pre-pregnancy BMI, and the combination of two biomarkers for the development of GDM in pregnant women after ART treatment were determined. The results of this study demonstrated that overweight and obese women had approximately 3 and 5 folds increase in the odds of developing GDM, respectively. The cut-off point of 84.5 mg/dl for FBS had a sensitivity of 72.9% and specificity 74.4%, while the cut-off point of 25.4 kg/$m^2$ for BMI had a sensitivity of 68.8% and specificity of 62.8%. However, the combination of BMI and FBS significantly improves the predictive ability for GDM development (BMI+FBS cut point: 111.2 with 70.7% sensitivity, 80.6% specificity).

Current evidence indicates that obesity has a negative effect on female reproductive health including ovulatory dysfunction, infertility problems, and poorer outcomes

after infertility treatment. Moreover, obesity is associated with impaired ovarian responsiveness to IVF treatment, a lower rate of oocyte fertilization, poor embryo quality, and higher abortion rates (20).

In our study, a higher incidence of overweight (48.9 vs.32.5%) and obesity (23.7 vs.10.9%) was observed in GDM compared to that of the non-GDM group in the ART population. Provost et al. (21) reported a higher rate of overweight (22.9%) and obesity (17.8%) among women undergoing ART.

Our data show a significant association between BMI and GDM in the ART population. Overweight and obese women had approximately 3 and 5 folds increase in odds of GDM, compared with normal BMI women. Consistent with our findings, Torloni et al. (5), in a meta-analysis of 70 studies, reported that the risk of developing GDM in overweight and obese women in natural pregnancy was almost 2 and 4 folds higher in comparison to normal-weight women. Furthermore, they showed an approximately 0.92% increase in the risk of developing GDM with each 1 kg/m$^2$ BMI increase in women with a BMI>25 kg/m$^2$, (95% CI: 0.73-1.10). Another study performed by Ogonowski et al. (22), showed that the risk for GDM is increased in parallel with rising in pre-pregnancy BMI not only in overweight but also in normal-weight women. BMI is a strong predictor for GDM requiring insulin therapy. A recent large population-based study revealed the association between BMI and diabetes in pregnancy among women of various ethnicities (23). Moreover, overweight women had a 2.37-fold and obese women had a 5.88-fold increase in the risk of diabetes in pregnancy. Similar to our results, Nishikawa et al. (23) showed that applying a BMI cut-off of 25 kg/m$^2$ would identify 68% of South Asian women with diabetes in pregnancy.

Anyway, there is controversy on GDM definition, screening time and method, and threshold values. Previously, a risk factor for GDM was defined as obese women who have BMI above 30 kg/m$^2$ (24). ADA indicates BMI 25kg/m$^2$ or less as the low-risk group (25). The present study concludes that women with BMI≥ 25.4 kg/m$^2$ are at high risk of GDM development after ART.

Recently, Cai et al. (26) demonstrated that IVF pregnancies are associated with a higher rate of GDM along with elevated fasting and 2-hour OGTT blood glucose levels in the late second-trimester, particularly in overweight and obese mothers; however, the first-trimester FBS was not measured. Previously, Szymanska et al. (27), in a retrospective study, compared 36 singleton pregnant women with GDM who have undergone IVF with 137 non-IVF women with GDM and reported higher levels of FBS in pregnant women with GDM undergone IVF. Similarly, we found that GDM women undergone ART had higher levels of FBS in the first-trimester of pregnancy compared with the non-GDM subjects, but FBS levels were observed within the normal range (FBS <92 mg/dl). Conversely, Sacks et al. (28) found that the measurement of FBS at the first-prenatal visit is not an efficient method for screening of GDM in natural pregnancies because of poor specificity (high false positive rate). Our findings show an approximately 17% increase in the risk of developing GDM with each 1 mg/dl increase in FBS level. In addition, we found a cut-off point of 84.5 mg/dl for FBS with sensitivity 73% and specificity 74%. Riskin-Mashiah et al. (10) reported a cut-off point of 79 mg/dl for FBS using 100 g OGTT with sensitivity 80% and specificity 53% for GDM diagnosis in natural pregnancies. In addition, they found that with each 5 mg/dl increase in fasting glucose or 3.5 kg/m$^2$ increase in BMI, the risk of developing GDM increases 1.5-fold among young fertile women.

As there is controversy about GDM screening and diagnosis, recent studies focused on the first-trimester prediction of GDM based on the maternal and clinical characteristics (8, 29, 30) or biomarkers (31) during the natural pregnancy. Hence, in order to predict GDM risk, it is suggested considering multi-parametric models according to maternal clinical risk factors and biomarkers in the first-trimester in pregnancy. Our results showed that the combination of two biomarkers (BMI+FBS) had a cut-point of 111.2 with 70.7% sensitivity and 80.6% specificity to predict GDM development. Moreover, the combination of BMI+FBS significantly improved the predictive ability of FBS or BMI alone for GDM development. Similar to our data, Hao and Lin (30) in a retrospective study carried out on 820 Chinese pregnant women who naturally conceived reported that appropriate fasting plasma glucose (FPG) cut-off value for predicting GDM was 4.6 mmol/L (82.8 mg/dl) with a sensitivity of 53.89% and specificity of 70.90%. The BMI cut-off value was 23.5 kg/m$^2$ with a sensitivity of 48.50% and specificity of 73.05%. They found that the combination of these two indices could occupy a larger area under the curve for GDM prediction. Therefore, high levels of FBS or BMI in the first-trimester, especially when combined with each other, should be noticed by health care providers to screen GDM in women who conceived using ART.

On the basis of the authors' knowledge, this is the first report in ART subjects. The current research has some limitations. We did not evaluate infertility-related factors (hormonal and environmental factors), habits, physical activity, pre-pregnancy waist and hip circumferences, and the dietary regimen of the participants.

## Conclusion

Pre-pregnancy BMI and the first-trimester FBS are independent predictors of GDM in pregnant women conceived by ART. The co-occurrence of high FBS and obesity increases the risk of GDM dramatically in pregnant women conceived by ART.

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## Authors' Contributions

A.K.; Contributed to the design of the study, drafting and revising the manuscript. M.E.K.; Contributed to the conception of the study, drafting and revising the manuscript. A.M., R.P., A.A.; Contributed to the data acquisition, drafting and revising the manuscript. Z.Z.; Contributed to the data analysis and revising the manuscript. R.H.; Contributed to the conception of the study, interpretation of data and drafting and revising the manuscript. H.R.B.; Contributed to the supervision and critical revision of the manuscript for important intellectual content. All authors approved the final copy of the manuscript.

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Original Article

# *In Vitro* Maturation of Oocytes in Women at Risk of Ovarian Hyperstimulation Syndrome-A Prospective Multicenter Cohort Study

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## Abstract

**Background:** *In vitro* maturation (IVM) is an artificial reproductive technology in which immature oocytes are harvested from the ovaries and subsequently will be matured *in vitro*. IVM does not require ovarian hyperstimulation (OH) and thus the risk of ovarian hyperstimulation syndrome (OHSS) is avoided. In this study, we assessed the live birth rate per initiated IVM cycle in women eligible for *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) and at risk for OHSS. Furthermore, we followed women who were not pregnant after IVM and committed to a conventional IVF/ICSI procedure.

**Materials and Methods:** In this multicenter prospective cohort study, we started 76 IVM cycles using recombinant follicle stimulating hormone (rFSH) priming in 68 patients. There were 66 oocyte retrievals, in which a total of 628 oocytes were collected. We incubated the immature oocytes for 24-48 hours and fertilized those that reached metaphase II by ICSI.

**Results:** Three hundred eighty six (61% oocytes) achieved metaphase II. The fertilization rate was 55%. We performed 59 embryo transfers (1.9 embryos per transfer) in 56 women, including 3 frozen embryo transfers. There were four ongoing pregnancies (5.3% per initiated cycle) leading to the birth of a healthy child at term. None of the patients developed OHSS. The ongoing pregnancy rate of the first conventional IVF/ICSI cycle after an unsuccessful IVM cycle was 44%, which was unexpectedly high.

**Conclusion:** We concluded that IVM led to live births but with low effectiveness in our study. Earlier reported IVM success rates are higher which can be caused by a more extended experience in these centers with the intricate laboratory process. However, a possible selection bias in these studies cannot be ruled out. Furthermore, IVM might have a beneficial effect on further IVF/ICSI treatments due to its "ovarian drilling" effect.

***Keywords:*** *In Vitro* Maturation, Polycystic Ovarian Syndrome, Prospective Study

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## Introduction

*In vitro* maturation (IVM) is an artificial reproductive technology which involves the retrieval of immature oocytes from the ovaries. Subsequently, these oocytes are matured *in vitro*. Since IVM does not require ovarian hyperstimulation (OH), the risk of ovarian hyperstimulation syndrome (OHSS) is avoided. Other potential benefits of IVM may include patient friendliness and reduced costs when compared to a conventional *in vitro* fertilization (IVF) treatment.

The first report of a pregnancy and childbirth after IVM was published in 1991 (1). It was estimated in 2012 that over 3,000 children were born after IVM worldwide. Clinical pregnancy rates per embryo transfer considerably vary from 4-53% (2-4). Also, different IVM techniques are applied with regard to the administration of human chorionic gonadotropin (hCG) and recombinant follicle stimulating hormone (rFSH) priming. Furthermore, indications for IVM treatment have expanded in recent years including oocyte donation and fertility preservation (5-9).

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It is hard to value the success rates of the published cohort studies so far. In most studies, the criteria for patient selection are not clarified and no information is given on previous infertility treatments in selected patients (10, 11). Furthermore, studies often report pregnancy rates per oocyte pickup or even per embryo transfer and not per started cycle. It is therefore hard to counsel future patients. Will they benefit from IVM? Or will they stand a better chance with another medically assisted reproductive technique?

In this study, we aimed to establish the live birth rate with IVM in a well-defined and prospectively registered population of women with an indication for IVF or intracytoplasmic sperm injection (ICSI) that were at risk of OHSS. Furthermore, the follow up of the children born after IVM is described and the follow up of patients with regard to their subsequent fertility treatments.

The goal of this study was to introduce IVM as a novel technique in the Netherlands and to continue with a randomized trial comparing pregnancy rates for IVM and IVF.

## Materials and Methods

This multicenter prospective cohort study was performed in three non-academic hospitals in the Netherlands: Jeroen Bosch Hospital, St Elisabeth Hospital, and Isala Clinics. All participants provided written informed consent. The study was approved by the Central Committee on Research involving Human Subjects (CCMO NL29051.000.09) and by the board of each participating hospital. All initiated IVM cycles were registered prospectively.

### Subjects

Patients were eligible when at least one previous IVF cycle had been complicated by OHSS or cancelled because of imminent OHSS. Also, PCOS patients with an indication for IVF because of failure to achieve an ongoing pregnancy after regular treatments of ovulation induction (OI) with clomiphene citrate, laparoscopic ovarian drilling and rFSH with or without intrauterine inseminations (IUI) could enter the study. PCOS was diagnosed according to the Rotterdam criteria (12, 13). Patients had to be between 18 and 38 years of age.

### Introduction of *in vitro* maturation technique

To prepare the staff for implementing the IVM technique two clinicians and two embryologists visited the Väestöliitto (The Family Federation of Finland) Fertility Clinic, Helsinki, Finland and the Biogenesi Reproductive Medicine Centre, Istituti Clinici Zucchi, Monza, Italy. Protocols were studied and discussed, and all different treatment steps were practiced. Next, mock cycles with immature oocytes of regular IVF and ICSI cycles were practiced in our Dutch laboratories. Then, the proof of principle cycles was started with consenting couples.

The prospective cycles reported in this study were not started before we proved to be able to achieve ongoing pregnancies.

### Cycle monitoring and oocyte retrieval

Cycle monitoring and oocyte retrieval were based on the 'Monza-protocol' (7). At the start of the treatment, a baseline ultrasound was performed on cycle day 2, 3 or 4. In patients with severe oligomenorrhoea (cycle length >42 days) or amenorrhoea, a withdrawal bleeding was induced with 7 days 10 mg progestagen (Provera®, Pfizer) orally. The cycle was excluded if at baseline ultrasound the endometrial thickness exceeded 4 mm or an ovarian cyst larger than 12 mm was present.

Subsequently, ovarian priming was performed by the administration of 150 IU rFSH s.c. (Puregon®, Merck Sharp & Dohme) on cycle day 3 to 5. The second ultrasound was scheduled at cycle day 6 to 8 and thereafter at one- or two-day intervals until the identification of a dominant follicle. A dominant follicle was defined as a follicle that had grown to at least 8 mm (but not larger than 12 mm) accompanied by a thickening of the endometrial lining to 5 mm or more (14). Subsequently, 10.000 IU of hCG (Pregnyl®, Merck Sharp & Dohme) were administered subcutaneously and the oocyte retrieval was scheduled 38 hours later (15). A cycle was cancelled when there was a follicle larger than 14 mm or an endometrium less than 5 mm. Oocytes were retrieved by transvaginal ultrasound-guided needle aspiration at a vacuum pressure of 80-100 mmHg with a 16 gauge needle (Origio).

### *In vitro* maturation and embryo culture

In the fertility laboratory, the oocytes were isolated from the punctate using a cell strainer filter and transferred to IVM culture medium (Medicult IVM®, Origio). This medium was supplemented with 100 mIU/ml hCG, 75 mIU/ml FSH, and 10% protein solution (GPO, Sanquin, the Netherlands).

Subsequently, the collected oocytes were incubated (at 36.8 Celsius and 5.2% $CO_2$) in this medium for 24-48 hours to induce final oocyte maturation. Oocytes reaching the metaphase II stage were fertilized using ICSI. In cases in which a metaphase II oocyte already was present at oocyte retrieval, this oocyte was injected with the husband's sperm the same day (16). After ICSI, the oocytes were transferred to Human Tubal Fluid (HTF, Lonza, Belgium) with 8,8% protein solution (GPO, Sanquin, the Netherlands) and incubated at 36.8-37.0°C and 5.0-5.2% $CO_2$. Embryo morphology was assessed daily from day 1 up to day 5, based on the cell number and overall appearance of the embryo (good, average, poor) considering fragmentation, equality of the blastomeres and multinucleation. A good embryo has 0-20% fragmentation, equal blastomeres, and no multinucleation. An average embryo has more than 20% fragmentation but not 50%, or unequal blastomeres, but no multinucleation. A poor embryo has more than 20% fragmentation and unequal blastomeres or more than 50% fragmentation or is multinucleated. For

embryo transfer, the best available embryo or embryos were selected based on the above-mentioned criteria.

### Luteal support and embryo transfer

At the day of oocyte retrieval, luteal support was started with Estradiol (Progynova®, Bayer) 2 mg orally three times a day and Progesterone (Utrogestan®, Besins International) daily vaginally 600 mg started on the day after the oocyte retrieval. In cases with a positive pregnancy test, oestrogen and progesterone supplementation were continued until ten weeks of gestation.

Embryo transfer was conducted on day three or day four after ICSI and a maximum of two embryos were transferred per cycle. Whenever an embryo was available, it was transferred, without again determining endometrial thickness or endocrine parameters. Remaining embryos were selected for cryopreservation according to the standard IVF/ICSI procedures and criteria.

### Outcome

The primary endpoint of the study was the live birth rate per started cycle. Secondary endpoints of the study were antral follicle count at the start of the IVM treatment cycle, number of retrieved oocytes per cycle, number of metaphase II oocytes at retrieval, maturation rate of oocytes, fertilisation rate of mature oocytes, number and quality of embryos, clinical pregnancy rate, live birth rate per oocyte retrieval and per embryo transfer, and the number and nature of adverse events during or following IVM/ICSI. Further endpoints were the health and development of IVM/ICSI children during a two-year follow-up program and the ongoing pregnancy rate of women who continued with IVF after unsuccessful IVM.

Live birth rate was defined as the birth of a living child beyond 24 weeks of gestation. Clinical pregnancy was defined by the ultra-sonographic presence of a gestational sac, four weeks after embryo transfer. Ongoing pregnancy was defined by the ultra-sonographic presence of a vital embryo eight to ten weeks after embryo transfer.

### Paediatric follow up

To monitor the safety of the IVM technique, children were evaluated after birth and at ages of 6 months, 1 and 2 years. Follow up consisted of an evaluation on the following domains using internationally accredited and validated tests: motor development, cognitive development, and behaviour [Alberta Infant Motor Scale (AIMS), Bayley scale of infant development III (BSID III), Movement ABC-II, Wechsler Preschool and Primary Scales of Intelligence].

### Statistical analysis

We calculated the percentage of ovum pick-ups, embryo transfers, pregnancies, and live birth per cycle; both for the IVM group, as well as for the subsequent IVF group. Continuous variables were presented as the mean, median (including range) or percentage where appropriate. Differences in the variables of IVF treatments prior to and after IVM were analysed with the Wilcoxon Signed Rank Test. Analyses were performed using the Statistical Package for the Social Sciences 22.0 software for Windows.

### Sample size

This cohort study was designed as a pilot for starting a randomized controlled trial of IVM versus conventional controlled ovarian hyperstimulation (COH)/IVF or COH/ICSI. Therefore, we had to establish the live birth rate of IVM in our selected population. We expected the live birth rate of conventional COH/IVF or COH/ICSI in this group to be 15% per started cycle. We argued that IVM, to be a reasonable alternative to the conventional techniques and worthwhile to stand in a direct comparison in a trial comparing 2 IVM cycles to one conventional IVF or ICSI cycle, should at least have a mean live birth rate of 7.5% in 75 started cycles.

## Results

### Participants

Between May 2010 and October 2011, we included 68 subfertile women. In these women, we conducted 76 IVM cycles. The mean female age was 29.8 ± 3.9 years (mean ± SD) and the mean duration of subfertility was 2.7 ± 1.6 years. The main primary diagnoses were PCOS (n=32) and male subfertility (n=29, Table 1).

**Table 1:** Baseline characteristics (n=68)

| Variable | n |
|---|---|
| Age (Y) | 29.8 ± 3.9 |
| BMI (kg/m$^2$) | 24.6 ± 4.74 |
| Subfertility couple | |
| Primary | 44 (65) |
| Secondary | 24 (35) |
| Duration subfertility (Y) | 2.7 ± 1.6 |
| Primary diagnosis | |
| Cycle disorder | 32 (47) |
| Male subfertility | 29 (43) |
| Other | 7 (10) |
| Previous fertility treatment | |
| None | 5 |
| Ovulation induction with anti-estrogens and gonadotropins | 18 |
| IVF or ICSI cycles | |
| 1 | 22 |
| 2 | 15 |
| 3 or more | 8 |

Data are presented as mean ± SD or n (%). BMI; Body-mass index, IVF; *In vitro* fertilization, and ICSI; Intracytoplasmic sperm injection.

### Previous fertility treatments

Most women had previously received assisted reproductive therapy (n=63, 91%). There were 18 women who

had undergone OI with or without IUI, while 45 women had at least one previous IVF or ICSI cycle (Table 2). They started a total of 81 IVF cycles of which 41 were cancelled. Of these 41 cancelled cycles, 32 cycles were cancelled because of imminent OHSS and 9 cycles were cancelled because of insufficient follicle growth, mainly following a previous cancelled cycle of imminent OHSS. The remaining 40 cycles were completed but did not result in pregnancy.

**Table 2:** Cycle and laboratory data of 76 *in vitro* maturation cycles in 68 women

| Variable | n or n (%) |
|---|---|
| Started cycles | 76 |
| Oocytes collections | 66 |
| Antral follicle count (mean) | 30 (range 9-80) |
| Dominant follicle size (mean) | 11 (range 8-14) mm |
| Endometrial thickness at the time of oocyte collection (mean) | 8 (range 4-15) mm |
| Oocytes retrieved | 628 |
| Retrieved oocytes per oocyte collection (mean) | 9.5 (range 0-29) |
| Mature oocytes at the time of oocyte collection (%) | 34 (5) |
| Mature oocytes available and inseminated (%) | 386 (61) |
| Fertilized eggs (%) | 212 (55) |
| Embryos | 197 |
| Embryos per retrieval (mean) | 3 |

Five patients were treatment-naive when entering the study. These were patients with a combined diagnosis of PCOS and severe male subfertility leading to an indication for ICSI (Fig.1).

### *In vitro* fertilization treatments

The mean antral follicle count at the start of the IVM cycle was 30. Of 76 started IVM cycles, 10 cycles were cancelled because of inadequate endometrial response, meaning an endometrial thickness of less than 5 mm at the time that follicular dominance was seen at the transvaginal ultrasound. In the remaining 66 cycles, a total of 628 oocytes were collected (range 0-29 oocytes per oocyte retrieval). Of these, 5% already were at metaphase II at oocyte retrieval, 56% reached metaphase II after 24-28 hours of maturation. In total 61% (386 oocytes) achieved metaphase II (range 0-17 oocytes per oocyte retrieval). The fertilization rate was 55% (212 embryos). 59 embryo transfers (mean of 1.9 embryos per transfer) were performed, including 3 frozen embryo transfers (Table 2). The quality of the transferred embryos is described in Table 3.

Six pregnancies occurred, of which four were ongoing (5.3% per initiated cycle). One of the ongoing pregnancies developed from a frozen embryo transfer. All ongoing pregnancies led to the birth of a healthy child at term. None of the patients developed OHSS. The live birth rate per OPU was 6%, the live birth rate per embryo transfer was 10%.

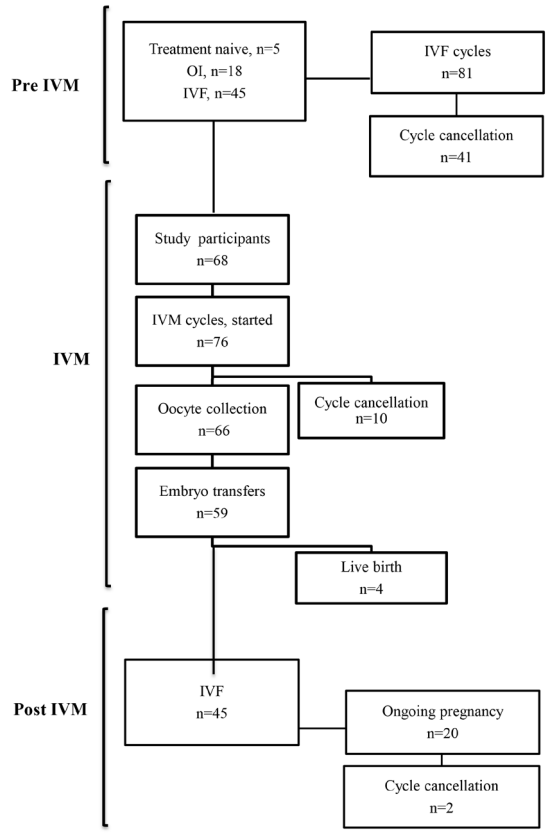


**Fig.1:** Representative scheme showing the patient flow, including previous and subsequent treatment cycles. IVM; *In vitro* maturation, IVF; *In vitro* fertilization, and OI; Ovulation induction.

**Table 3:** Embryo transfer, embryo quality, and clinical outcome

| Variable | n |
|---|---|
| Embryo transfers | 59 (3 frozen embryo transfers) |
| Transferred embryo's (mean) | 1.9 |
| Embryo quality at transfer (%) | |
| Good | 51 |
| Average | 40 |
| Poor | 9 |
| Positive β-hCG tests | 6 |
| Clinical pregnancies | 5 |
| Live births | 4 |
| Live birth rate per started cycle (%) | 5.3 |
| Multiple pregnancies | 0 |

**β-hCG; Human chorionic gonadotropin.**

### Subsequent *in vitro* fertilization cycle

Of 64 patients with an unsuccessful IVM treatment, 45 proceeded to an IVF cycle. The treatment protocol consisted of a gonadotropin-releasing hormone (GnRH) agonist or antagonist scheme, and a daily dose of rFSH of 75 to 225 IU, followed by hCG triggering (10.000 IU) and luteal support with progesterone. The number of cancelled cycles was two (4%). OHSS was the reason for cancelling one cycle. In total, 44% (20/45) of these patients achieved an ongoing singleton pregnancy in their first IVF treatment after IVM. Of them, 36% (16/45) was pregnant after a fresh embryo transfer and four pregnancies resulted from cryo-transfers of embryos from the first IVF cycle after IVM (Fig.1).

**Table 4:** Cycle and laboratory characteristics of conventional IVF/ICSI cycles before and after the studied IVM cycles

| Variable | IVF before IVM n=23 | IVF after IVM n=20 | P value |
|---|---|---|---|
| Stimulation protocol (n) | | | |
| Agonist | 19 | 14 | |
| Antagonist | 3 | 2 | |
| Other[a] | | 4 | |
| Insemination (n) | | | |
| Conventional | 10 | 9 | |
| ICSI | 13 | 11 | |
| Starting dose gonadotrophin (IU, median, range) | 150 (75-225) | 125 (75-225) | NS |
| Total gonadotrophin (IU, median, range) | 1316.5 (750-4900) | 1200 (600-2250) | NS |
| Number of dominant follicles (≥12 mm, median, range) | 18 (1-42) | 12 (2-25) | 0.003 |
| Number of collected oocytes (median, range) | 9 (3-24) | 7 (3-21) | NS |
| Number of embryos (median, range) | 6 (2-16) | 3 (1-16) | NS |
| Number of transferred embryos (median, range) | 1.0 (1-2) | 1.0 (1-2) | NS |

IVF; *In vitro* fertilization, ICSI; Intracytoplasmic sperm injection, IVM; *In vitro* maturation, NS; Not significant, and [a]; Clomiphene citrate.

Of these patients, we aimed to compare the cycle characteristics of the IVF cycle before and after the IVM treatment (Table 4). Details of gonadotropin dosage and the number of follicles were available in 23 patients. Gonadotropin dosage was determined according to the local protocol which allowed dose adjustments considering an observed response to earlier ovarian stimulation. Overall, there was a small but insignificant difference in the starting dose and the total dose of gonadotropins. The number of dominant follicles was lower in IVF cycles performed after IVM (18 vs. 12, P=0.003) but that did not result in a lower number of collected oocytes.

Furthermore, there were three cases of spontaneous conceptions among the remaining women with polycystic ovary syndrome (PCOS)-related anovulation. In these patients, spontaneous ovulation and conception occurred within four months after IVM treatment.

### Follow up of children

All four IVM children were delivered at term (Table 5). One was large for gestational age, and the other three had an appropriate birth weight. No congenital malformations were present. The follow up showed normal physical growth for all children. Two children were in the normal to high range on the various motor, cognition, and behaviour scales. Motor development was slow in two children (AIMS <5$^{th}$ percentile and BSID-II development index of <55, respectively).

**Table 5:** Obstetric outcomes of children born after IVM treatment

| Gestational age (weeks) | Mode of delivery | Sex | Birth weight (g and P value*) |
|---|---|---|---|
| 42+3 | Caesarean section | Male | 4870 (>p97.7) |
| 41+3 | Vacuum extraction | Male | 4105 (p80-p84) |
| 39 | Spontaneous | Female | 3290 (p20-p50) |
| 41 | Caesarean section | Male | 4200 (p84-p90) |

*; The Netherlands Perinatal Registry Birth weight centiles and SD, www.perinatreg.nl., and IVM; *In vitro* maturation.

## Discussion

We were able to introduce IVM in the Netherlands, accomplishing successful pregnancies and the birth of healthy newborns. We selected a group of patients with increased risk of OHSS and in none of the IVM cycles OHSS occurred. The live birth rate per started cycle was limited to 5.3%. This percentage is low compared to the results previously published. In other studies, however, pregnancy rates per started cycle are not always available, mostly pregnancy rates per oocyte retrieval or embryo transfer are reported. Also, the maximum number of embryos per transfer in our study was limited to two, while other studies report transfers of up to four embryos per cycle (16). Furthermore, as already mentioned in the introduction section, in most previous studies patient characteristics and the previous fertility treatments are not revealed. Therefore, it is difficult to compare the results in our group of patients with the results of others. Possibly, the a priori chance of pregnancy was reduced in our patients as the majority already underwent several unsuccessful treatment cycles of OI, IUI and/ or IVF.

Apart from differences in patient characteristics, there is a lot of variation in how IVM is performed. IVM can vary from not using any gonadotropins at all, using FSH priming, triggering with hCG, or using both FSH and hCG. Nowadays, most research groups use FSH priming as it improves the yield of competent oocytes. The use of hCG triggering, however, is more controversial, as it may lead to maturation of oocytes *in vivo*. This can result in a mix of immature and also mature oocytes at oocyte retrieval. Some argue, therefore, that using hCG is not compatible with the true definition of IVM. Accordingly, cycles with hCG-use should be distinguished and renamed as "natural cycle IVF" or "truncated IVF" (17, 18).

Indeed, in our study, with FSH priming and the administration of hCG 38 hours prior to ovum pick up a small

proportion of 5% metaphase II oocytes were retrieved. It is not likely that this had a large influence on our success rate. A recent systematic review could not find a significant difference in live birth rates in hCG versus non-hCG IVM cycles (19).

In two recent cohort studies, a comparison between IVM and IVF in PCOS patients was made. Walls et al. (4) showed a lower clinical pregnancy rate in the IVM group. There were significantly fewer live births resulting from IVM treatment for both fresh and cumulative cycle outcomes. However, there was no difference in live birth rates resulting from frozen embryo transfers between IVM and IVF treatment. Das et al. (20) compared IVM with a more novel GnRH agonist trigger IVF protocol. The latter protocol has been shown to result in lower OHSS rates than IVF protocols with hCG triggering (21). Both IVM and IVF with GnRH-antagonist protocol seem to be effective treatment regimens in women with PCOS. Although IVM was associated with a lower risk of OHSS, the live birth rate was significantly higher in IVF with GnRH agonist triggering. Also, Gremeau and colleagues reported the higher implantation and pregnancy rates in conventional IVF using a long GnRH agonist protocol (22).

At the time of our study, the GnRH agonist protocol was the prevailing method in the Netherlands. In the present study, the follow-up data of subsequent IVF treatments of 44% ongoing pregnancies per started cycle were highly favourable. This is remarkable considering the poor results of the IVF cycles in these patients prior to the IVM treatment cycles, which were characterized by very high cancellation rates. On the one hand, this can be explained by FSH dose adjustments. However, in our study, the mean starting dose and total dose of FSH of the cycle following IVM treatment was not significantly different from the dose in the IVF cycle preceding IVM. On the other hand, the oocyte retrieval in IVM may induce a change in the ovaries comparable to the result of laparoscopic ovarian drilling. This is compatible with our finding of significantly fewer follicles $\geq$ 12 mm in the IVF cycle following IVM. Furthermore, we have described spontaneous cycle restoration and spontaneous pregnancies in a case series of three patients following IVM treatments (23). Also others have reported improved outcomes in IVF cycles that were preceded by IVM (24, 25). Findings of transient but significant changes in ovarian endocrine parameters after the retrieval of immature oocytes in patients with PCOS could be a possible biological plausibility for this (26, 27).

The limitations of our study were in the cohort study design and the number of studied patients. However, the sample size of the study was comparable with other previous studies (2, 11). Also, an overlap of patients in some of the previous reports cannot be excluded.

As IVM is considered a novel technique in the Netherlands, a learning curve has been probably interplayed with the final study results, although the maturation rates and fertilization rates were comparable with other IVM studies. Thus far, only a small number of research groups seem to achieve high pregnancy rates (3, 28). These research groups may be able to harvest and process oocytes faster, which sustains viability. Also, subtle adjustments in culture protocols optimizing culture media, incubator temperature or $CO_2$ concentration can play a role.

Strengths of our study were the inclusion of a well-defined patient group. Although we included patients with different fertility diagnoses, they all were characterized by an increased risk of OHSS and eligible for IVF or ICSI. All patients and all IVM cycles including cancelled cycles were recorded prospectively in a central study register. Also, this is the first study in which all data are reported on fertility treatments prior to and after the IVM study cycle, as well as the follow up of the IVM-children up to two years of age.

In this study, no malformations were found. Although all children were thriving, in two of four children, motor development was slow on validated tests. In several reviews, the authors have proposed that IVM is not associated with an increase in numbers of congenital malformations (29, 30). However, more subtle developmental differences cannot be ruled out. A French cohort of IVM children, for example, showed a higher mean weight in girls at one year of age (31). We should consider that data on IVM children are limited, both in the number and duration of the follow-up (32). Further monitoring of these infants outcomes is required.

## Conclusion

We concluded that IVM led to live births but with low effectiveness in our study. Based on the data presented in this article, the national medical ethical committee did not allow IVM to be continued in the Netherlands and we had to cancel our plans to conduct an a randomized controlled trial (RCT) of IVM versus IVF. According to a Cochrane review, there is still no evidence from randomized controlled trials upon which to base any practice recommendations regarding IVM before IVF or ICSI for women with PCOS. Thus randomized control trials comparing IVM with IVF are needed for a more exact estimate of the effectiveness of IVM in specific groups of patients. Finally, we would like to encourage other IVM researchers to reveal data on patient selection criteria in future publications.

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## Authors' Contributions

S.C.B.; Acquisition of data, analysis, and interpretation

of data, drafting the manuscript. J.P.B., D.C., M.H.J.M.C.; The concept and design, acquisition of data, the analysis and interpretation of data, drafting the manuscript. B.W.J.M, C.J.C.M.H., B.J.C., J.M.J.S., S.R.; Concept and design, revising the manuscript for important intellectual content. All authors performed editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript and approved the final draft.

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Original Article

# Comparing The Effects of *Glycyrrhiza glabra* Root Extract, A Cyclooxygenase-2 Inhibitor (Celecoxib) and A Gonadotropin-Releasing Hormone Analog (Diphereline) In A Rat Model of Endometriosis

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## Abstract

**Background:** The purpose of this study was to compare the effects of *Glycyrrhiza glabra* (Licorice), a cyclooxygenase-2 inhibitor (Celecoxib) and a gonadotropin-releasing hormone analog (Diphereline®), with a control group on endometrial implants in rats.

**Materials and Methods:** In this experimental study, endometriosis was induced in rats by auto transplantation and after confirmation, the rats were divided into 4 groups that were treated for 6 weeks with normal saline (0.5 ml/day, orally), licorice extract (3000 mg/kg/day, orally), celecoxib (50 mg/kg, twice a day, orally) or diphereline (3 mg/kg, intramuscularly). At the end of treatments, the mean area, volume, histopathology and hemosiderin-laden macrophage (HLM) counts of the endometrial implants were evaluated and compared among the four groups.

**Results:** The mean area, volume and HLM counts of the implants in the licorice group were significantly lower than those of the control group ($P<0.001$). The histopathologic grades of endometrial implants were significantly decreased by licorice compared to the control group ($P<0.001$). There was no significant change in the mentioned parameters in rats treated with celecoxib compared to the control group. Diphereline was the most potent agent for suppressing the growth of endometrial implants in terms of all of the above-mentioned parameters.

**Conclusion:** Licorice decreased the growth and histopathologic grades of auto-transplanted endometrial implants. However, while celcoxib had no significant effect, diphereline showed the highest potency for decreasing the endometrial growth. Licorice may have the potential to be used as an alternative medication for the treatment of endometriosis.

***Keywords:*** Celecoxib, Cyclooxygenase-2 Inhibitor, Endometriosis, *Glycyrrhiza glabra*, Gonadotropin Releasing Hormone

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## Introduction

Endometriosis, an estrogen-dependent inflammatory disease affecting 10-25% of women, is associated with significant reductions in fertility and is one of the most common benign gynecological diseases. Retrograde menstruation with subsequent adhesion formation, invasion, and neovascularization are believed to give rise to the occurrence of endometriosis lesions. The most common locations for endometrial implants are the ovaries, fossa ovarica, uterosacral ligaments, and posterior cul-de-sac (1).

Although different medications are used to control endometriosis, their adverse effects following long-term use and recurrence of disease after discontinuation of therapy limit their applications. Additionally, these medications are not useful for endometriosis-associated infertility (2). Regarding the fact that no ideal medical treatment is

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available to control endometriosis, introducing new medical agents with minimal side effects and improved effectiveness for infertility treatment, are required.

Gonadotropin releasing hormone agonists (GnRHa) such as diphereline, as standard medications for the treatment of endometriosis, are able to induce inactivation and degeneration of endometrial implants via suppression of hypothalamic-pituitary-gonadal axis and ovarian estrogen production (3). GnRHa not only induces amenorrhea, but also may cause hot flush, depression, headache, hair loss, musculoskeletal stiffness, vaginal dryness and bone loss (4).

It is known that in women with endometriosis, the growth of endometrial cells within the peritoneal cavity is induced by inflammatory mechanisms (5); so, anti-inflammatory drugs are suggested to control endometriosis growth. Cyclooxygenase enzymes (COXs), known as prostaglandin-endoperoxide synthase, are responsible for formation of inflammatory mediators such as prostaglandins. COX-1 is expressed in almost all cells for maintenance of cell. COX-2 is produced at sites of inflammation, angiogenesis, and estrogenic cellular processes. Pharmacological inhibition of COX-2 was able to reduce the survival and growth of endometrial tissues at ectopic sites (6). NSAIDs (non-steroidal anti-inflammatory drugs) such as celecoxib, inhibit cyclooxygenase isoforms and induce gastrointestinal side effects (7).

Using herbal medicine has always played a significant role in Iranian culture and civilization and some of these herbs have been recommended for treatment of infertility-related diseases. Licorice (*Glycyrrhiza glabra*), is one of the most widely used herbal drugs in Iranian traditional medicine. Licorice root contains triterpene, saponins, flavonoids, isoflavonoids, hydroxycoumarins, steroids and volatile oil. Licoricidin, is a potent compound isolated from licorice root (8). Studies showed that licoricidin is a selective COX-2 inhibitor and inhibits phospholipase A2 activity that is a critical enzyme involved in numerous inflammatory processes (9, 10). Licorice root with its anti-inflammatory/anti-platelet, antiviral, antifungal and mineralocorticoid functions has been used for the treatment of gastric ulcers, cough and bronchitis since the ancient times. Licorice is not recommended to be used for more than 6 weeks. Complications such as hypokalemia, hypernatremia, edema, hypertension and cardiac complaints are associated with long-term time use of licorice (8).

We hypothesized that licorice or celecoxib might be good candidates for treatment of endometriosis as an inflammatory condition. In the present study, we compared the effects of licorice, celecoxib and diphereline on the growth of endometrial implants in rats.

## Materials and Methods

In this experimental study, 48 mature female Sprague-Dawley rats (almost 8 weeks old, weighting 220 ± 20 g) were purchased from the Center of Comparative and Experimental Medicine at Shiraz University of Medical Sciences (SUMS), Shiraz, Iran. The animals were kept on 12 hours light: 12 hours dark cycles at a controlled temperature with free access to water and food. The animal experiments were performed according to the principles of the care and use of laboratory animals established by the National Institutes of Health, Bethesda, MD, USA, and approved by the Institutional Animal Ethics Committee at SUMS (No. 92-01-01-6869). These animal experiments were performed in the animal house of Shiraz University of Medical Sciences. To select the rats with normal estrous cycle, daily vaginal smears were taken and evaluated by a light microscope. Rats with three normal estrous cycles were used in the next steps.

### Preparation of licorice extract

Licorice roots were purchased from herbal stores in Shiraz, Iran). *Glycyrrhiza glabra* was preserved in herbarium after authentication by a botanist (Voucher No. PM 684). L. Licorice roots were air-dried, powdered and an alcoholic extract was produced by using ethanol (80%) and percolation method. Then, solvent was completely removed by drying under reduced pressure in a rotary evaporator. The extract was stored at 4°C until use.

### Induction of endometriosis

Endometriosis was induced surgically using the method described by Vernon and Wilson with little modifications (11) (Fig.1). It should be mentioned that as the growth of endometriosis is estrogen-dependent, if induction of endometriosis is performed in an ovariectomized animal, estrogen supplementation is mandatory (11, 12). However similar to the previous researches, since in our study adult intact rats were used, we did not use exogenous estrogen for induction of endometriosis (11-13).

Briefly, all the female rats were anesthetized using ketamine hydrochloride 10% (100 mg/kg, Alfasan, Netherlands) and xylazine 2% (10 mg/kg, Alfasan, Netherlands). Then, rats' abdomen was opened through a 4 cm midline incision starting from 1 cm below the xiphoid. The left horn of uterus was ligated at both the uterotubal and cervical junction ends and removed. A longitudinal cut was made through the uterine horn. By a punch biopsy, 4 round pieces of the distal part of uterine tissue were excised (4×4×1 mm) and placed in warm sterile saline 0.9%. Two implants were sutured with proline 5-0, one on the left and the other on the right side of the peritoneal cavity on the areas of well-visible vasculature with endometrial surface facing the peritoneum. Finally, the abdominal muscles, fascia and skin were sutured. Then, chlortetracycline (Cyclo Spray, Eurovet, UK) was sprayed on the incisions site and animals were allowed to recover from anesthesia. Two rats died due to hemorrhage at this stage. Six weeks after the first surgery, a second look laparotomy was performed and the viability of endometrial implants was confirmed by observation of good vascular supply and pinkish colored tissue in contrast to necrosis and fibrosis seen in two rejected cases as showed in Figure 1.

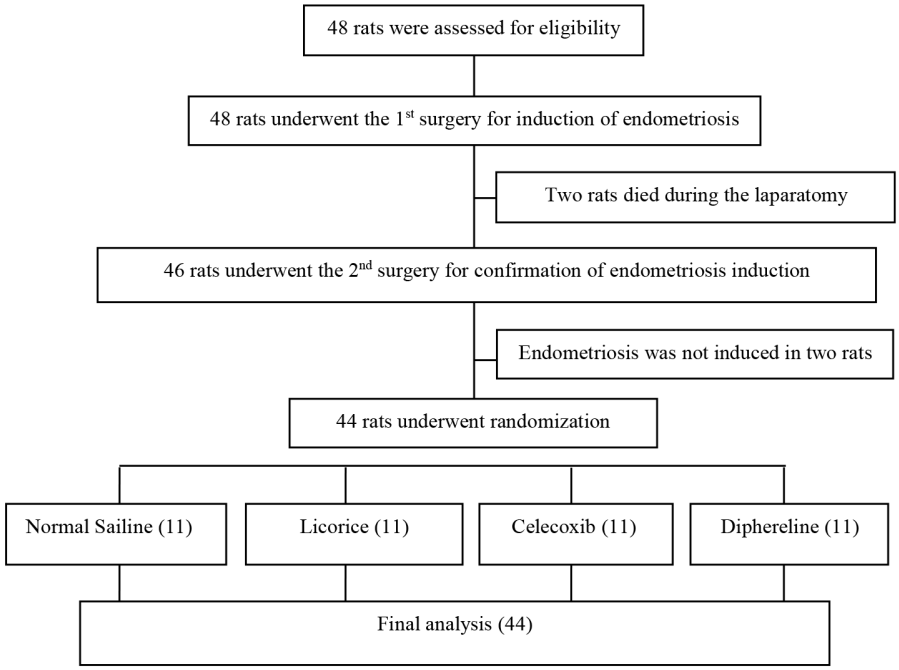


**Fig.1:** The flow diagram of the study.

## Treatments

At this stage, 44 female rats were divided into 4 groups (11 rats in each group). The control group was treated by 0.5 ml of saline 0.9%/day, the second group by licorice root extract (3000 mg/kg/day) and the third group took celecoxib (Damloran Razak Pharmaceutical Co., Iran, 50 mg/kg, twice a day, dissolved in 0.5 ml of saline 0.9%) for the next 6 weeks. All the treatments of these three groups were administered by oral gavage. The fourth group received a single IM injection of diphereline S.R. 11.25 mg (3 mg/kg, Ipsen, France). Six weeks after the treatments the rats were sacrificed and endometrial implants were evaluated as shown in Figure 2.

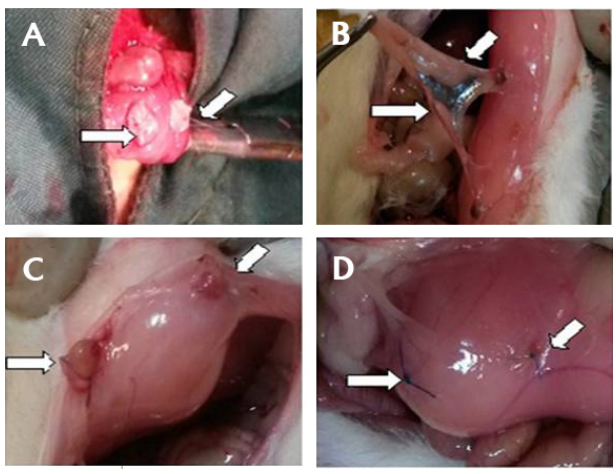


**Fig.2:** Endometrial implants in different times and groups. **A.** The first laparotomy: auto-transplant of endometrial implants on the peritoneum, **B.** The second look surgery: shows the adhesion bands and endometrial implants six weeks after induction of endometriosis, **C.** The third surgery: necropsy of a rat in the control group showing growth of implanted lesions of endometriosis, and **D.** The third surgery: necropsy of a rat in diphereline group showing regression of the implants.

## Measuring area and volume of endometrial implants

The length, width and height of each implant were carefully measured using collis rulers by one researcher who was blinded to the treatment arms. The area of the endometrial implants in four groups was measured by multiplying length by width), and the volume was calculated by ellipsoid volume formulation ($\pi/6\times length\times width\times height$).

## Pathologic scoring of implants

All of the endometrial implants were fixed in formalin, placed in paraffin, cut into 5 μm sections, stained with hematoxylin-eosin and evaluated by the same pathologist. Photographs were taken by a digital camera (Sony, Japan). To classify the persistence of epithelial cells in grafts, the scoring system applied by Keenan et al. (14) was used with score 0 showing no epithelial layer, and scores 1, 2 and 3 show poorly, moderately and well-preserved epithelial layers, respectively. The percentage of hemosiderin-laden macrophages (HLMs) was also measured in all of the sections. The surgeon, pathologist, and the lab technicians were blinded to the groupings, medications, and specimens.

## Statistical analysis

For statistical analysis, the software SPSS 15 (SPSS Inc., Chicago, USA) was employed. To compare the mean area and the mean volume, ANOVA followed by Tukey HSD test was performed. To compare the histopathologic scoring, Kruskal-Wallis test and Mann-Whitney U test with Bonferroni correction were applied. A $P<0.05$ was considered significant.

**Table 1:** The mean area, volume and pathologic scores of implants in control, licorice, celecoxib and diphereline groups

| Groups | Area ($cm^2$) | Volume ($cm^3$) | Pathologic score | Hemosiderin-laden macrophages |
|---|---|---|---|---|
| Control | 42.94 ± 11.76 | 125.90 ± 11.69 | 2.5 ± 0.70 | 51.00 ± 9.90[c] |
| Licorice | 27.57 ± 17.84[a] | 90.86 ± 19.32[a] | 1.90 ± 1.04 | 1.20 ± 1.07[d] |
| Celecoxib | 39.87 ± 13.57 | 121.03 ± 7.08 | 2.44 ± 0.88 | 41.80 ± 6.4 |
| Diphereline | 8.60 ± 2.53[b] | 11.00 ± 2.56[b] | 0.54 ± 0.68[b] | 1.2 ± 1.00 |

**Data was shown as mean ± SD. P<0.05 were considered statistically significant. [a]; Statistically significant differences between licorice and the control group, [b]; Statistically significant differences between diphereline and the control group, [c]; Statistically significant differences between control and other groups, and [d]; Statistically significant differences between Licorice, diphereline and celecoxib group.**

## Results

Two rats died during laparotomy due to hemorrhage and in two other rats the implants did not grow. The remaining 44 rats were divided into 4 groups and treated. In licorice group, the mean area and volume values of endometrial implants were significantly lower than those of the control group (P=0.042 and P<0.001, respectively) (Table 1). The mean area and volume of endometrial implants in the celecoxib group were lower compared to the control group, but the differences were not statistically significant (P= 0.953 and P=0.818, respectively). The mean area and volume of diphereline group were significantly lower compared to the control group (P<0.001 and P<0.001, respectively). The pathologic scores of the licorice and celecoxib groups were lower than those of the control group, but the differences were not statistically significant (P=0.221 and P=0.960, respectively). Poorly preserved epithelial layers were observed in diphereline group and the mean pathological score in this group, was significantly lower compared to the control group (P<0.001, Fig.3).

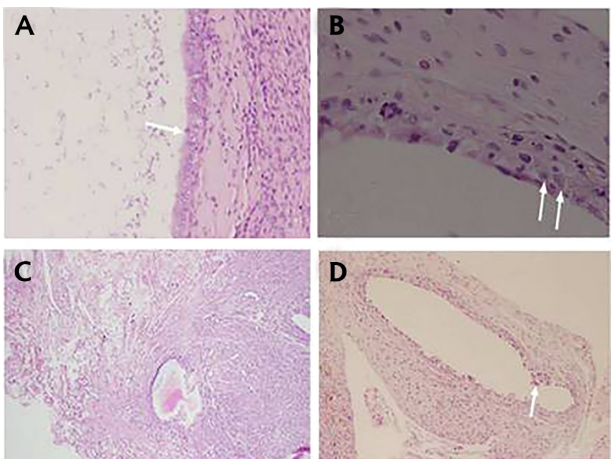


**Fig.3:** Specimens of the treated groups stained by hematoxylin-eosin. **A.** Well preserved epithelial layer of endometrial implants in the control group (grade 3) (scale bar: 50 µm), **B.** Poorly preserved epithelial layer of endometrial implants in the licorice group (grade 1) (scale bar: 50 µm), **C.** Moderately preserved epithelial layer of endometrial implants in licorice group (grade 2) (scale bar: 100 µm), and **D.** Poorly preserved epithelial layer of endometrial implants in diphereline group (grade 1) (scale bar: 100 µm). Arrows demonstrate epithelial layer of endometrial implants.

The percentage of HLMs in endometrial implants of rats in celecoxib, licorice and diphereline group was significantly lower than that of the control group (P=0.004, P=0.000 and P=0.000, respectively). Also, the percentage of HLMs was significantly lower in licorice and diphereline group compared to celecoxib group (P<0.001 and P<0.001, respectively). The percentage of HLMs was not different between licorice and diphereline group (P=1.000, Fig.4).

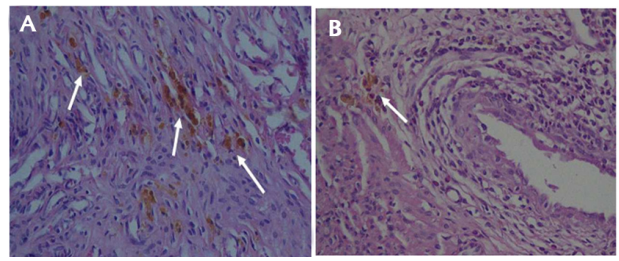


**Fig.4:** Hemosiderin-laden macrophages (HLMs) in the specimens of different groups. **A.** Control group and **B.** Licorice group (scale bar: 10 µm). Arrows indicate hemosiderin - laden macrophages.

## Discussion

We compared the effects of licorice, celecoxib, and diphereline in a rat model of endometriosis induced by auto-transplantation of endometrium on the peritoneal surface as a well-established method (11). Licorice decreased the growth of endometrial implants; celecoxib had no significant effect and diphereline had the highest potency in suppression of the endometrial growth. According to our knowledge, this is the first study on the effect of licorice on the endometrial implants.

Previous studies showed that glycyrrhetinic acid as a constituent of licorice extract, inhibits thrombin-induced platelet aggregation (9, 15) and has steroid-like anti-inflammatory effects similar to glucocorticoids (16, 17). Park and colleagues showed that administration of hexane/ethanol extract of *Glycyrrhiza uralensis* to mice decreases cell proliferation, inhibits the expression of angiogenic and inflammatory proteins and induces cell cycle arrest or apoptosis (18). Also, they observed that licoricidin reduces macrophages number and tumor growth in the tumor microenvironment. In another study, it was shown that licoricidin inhibits the metastatic and invasive capacity of malignant prostate cancer cells *in vitro* (19). La et al. (20) reported that licoricidin suppresses the production of inflammatory cytokines. The anti-inflammatory property of licoricidin is due, in part, to the inhibition of phospholipase A2 activity, resulting in inhibition of cyclooxygenase activity and prostaglandin formation (9, 16, 17). Licoricidin also inhibits an isomer of platelet-activating factor and acetyltransferase resulting in an anti-inflammatory activity (21).

COX-2 overexpression has been detected in both eutopic and ectopic endometrium, and also in peritoneal macrophages derived from women with endometriosis (22, 23). In the family of selective COX-2 inhibitors, rofecoxib and valdecoxib, are no longer used because of their side effects but celecoxib with lower gastrointestinal problems is still used.

Histhopathologic slides prepared from endometriosis showed higher counts of HLMs that serve as an indirect evidence for diagnosis of endometriosis. As shown in our study, endometrial implants had normal growth with intact endometrial lining and more scattered foci of HLMs after treatment with celecoxib or normal saline. However, after taking licorice or diphereline, growth of endometrial implants were highly limited with lower HLMs. These findings are in favor of the potential therapeutic effect of licorice in suppression of endometrial implants growth.

In this study, celecoxib did not significantly reduce the growth of endometrial implants that was against our primary hypothesis. There are studies that showed that celecoxib, dexketoprofen trometamol or rofecoxib were able to cause regression and atrophy of endometriosis lesions (7, 21, 24). However, Hull et al. (25) showed that subcutaneous injection of nimesulide, a COX-2 inhibitor did not reduce the size and number of the endometriosis lesions that is in agreement with our results. We believe that ineffectiveness of celecoxib in our study might have been because of two reasons. First, it was previously shown that COX-2 immunostaining density was greater in ovarian endometrioma than in peritoneal implants and it was concluded that celecoxib might influence ovarian endometrioma more than peritoneum (26). Second, the celecoxib brand that we used was different from that of other studies.

Since endometriosis is a chronic disease and needs long-term treatment, complications of prolonged use of licorice such as hypokalemia, hypernatremia, edema, hypertension and cardiac complaints should be kept in mind before human application. It should be considered that the maximum permitted dosage of licorice root is 5 to 15gr/day and the duration of treatment should not exceed 6 weeks in humans (8). Studies on all components and fractions of licorice are also needed to discover its active component(s) and exact mechanism(s) of action to introduce a safe pharmacological agent with targeted effects and without adverse effects.

As a limitation of our study, we did not evaluate inflammatory markers such as white blood cells counts, nor interleukin-6 (IL-6), vascular endothelial growth factor (VEGF) and tumor necrosis factor-alpha (TNF-α) levels in the peripheral blood and peritoneal fluid before and after interventions to assess the anti-inflammatory properties of licorice.

We believe that licorice might have the potency to be used as a novel and excellent alternative in the management of endometriosis after in-depth investigations in animals and humans.

## Conclusion

Licorice decreased the growth and histopathologic grades of auto-transplanted endometrial implants. However, celecoxib had no significant effect and diphereline had the highest potency in reduction of the endometrial growth.

## Acknowledgements

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## Authors' Contribution

B.N.J., M.E.P., F.F., N.T., P.V.K.; Designed the study. B.N.J., F.F., S.A., N.T.; Participated in data collection, evaluation and statistical analysis. P.V.K.; Participated in pathological scoring of endometrial implants. B.N.J., S.A.; Prepared the manuscript. All authors read and approved the final manuscript.

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Original Article

# Protective Effect of N-Acetyl Cysteine on Chlorpyrifos-Induced Testicular Toxicity in Mice

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## Abstract

**Background:** Chlorpyrifos (CPF), an organophosphate pesticide, is widely used in farms in order to preserve crops and fruits. Previous studies have shown that CPF exposure might cause chronic toxicity in male genital system. The present study investigated the protective effect of N-Acetyl Cysteine (NAC), a potent antioxidant against testicular toxicity of CPF in male mice.

**Materials and Methods:** In this experimental study, 42 adult male mice were divided into seven groups, CPF low (0.5 mg/kg.b.w) and high (5 mg/kg.b.w) doses groups, NAC group (35 mg/kg.b.w), NAC+CPF 0/5 mg/kg.b.w, NAC+CPF 5 mg/kg.b.w, dimethyl sulfoxide (DMSO, 0.75% solution mg/kg.b.w) and control group. All treatment were done intraperitoneally. Treatment was conducted for four consecutive weeks (five days each week). However NAC was injected to NAC+CPF groups five days before initiation of the treatment procedure. One week after the last injection, mice were sacrificed using anesthetic gas to evaluate alterations in testicular histology and sperm parameters.

**Results:** Seminiferous tubules area and diameter were significantly diminished in the group treated with 5 mg/kg CPF ($P<0.05$). CPF also statistically reduced sperm parameters (count and motility) and damaged sperm morphology) at both doses ($P<0.05$). However, NAC significantly improved spermatogonia, spermatocytes, spermatid cell counts as well as sperm parameters in mice treated with both CPF concentrations ($P<0.05$).

**Conclusion:** According to our results, NAC may significantly ameliorate CPF-induced damages to spermatogonia, spermatocytes, spermatids cell counts and sperm parameters.

***Keywords:*** Chlorpyrifos, N-acetylcysteine, Protective, Sperm

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## Introduction

As reported by the World Health Organization (WHO), unsuccessful pregnancy has been globally increased. Researchers found that 48.5 million couples worldwide were unable to have a child after five years of unprotected regular sexual intercourse (1).

Almost all people working on agricultural fields are exposed to various toxins that may cause reproductive toxicity. Pesticides are widely used for eliminating pests to protect corps and fruits. Organophosphate pesticides are regarded as dangerous types of pesticides for the environment as they can affect humans and animals health (2). Chlorpyrifos (O,O-Diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate), is an organophosphate pesticide which can cause adverse effects on the reproductive system of both males and females (3). For instance, seminiferous tubules were significantly degenerated in chlorpyrifos (CPF)-treated mice (4). In addition, sexual hormones disturbance and also defects in sperm production have been reported following CPF exposure (5). CPF was also shown to increase DNA impairment (6, 7) and induce harmful effects in different organs such as the thyroid (8) and lung (9). CPF permanently binds acetylcholinesterase and inhibits deactivation of acetylcholine in the synapses. So, acetylcholine signaling may last longer. This process is irreversible unless new acetylcholinesterase enzymes are synthesized. It has been reported that CPF also induces oxidative stress (8, 10).

Acetylcysteine, also known as N-acetyl cysteine (NAC) is widely used in management of acetaminophen overdose, cystic fibrosis and also chronic obstructive pulmo-

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nary disease. NAC may be useful in toxins treatments, since it can escalate glutathione levels and prevent further injuries caused by lipid peroxidation (11). Furthermore, a significant improvement of sperm motility and morphology were observed by NAC treatment in varicocele and also other models induced by synthetic drugs such as paracetamol (12, 13). Moreover, NAC may protect male genital system against strong toxins such as arsenic trioxide in (14). It has been observed that NAC has more marked effects compared to vitamin C in improvement of sperm parameters (15). Therefore, this study was conducted to investigate the protective effect of NAC on histopathology of testis and sperm parameters in CPF-treated mice.

## Materials and Methods

### Chemicals

Chlorpyrifos (99%) and NAC (99%) technical grade were purchased from Sigma-Aldrich (St. Louis, MO, USA) [lot No. LC13116V and 616-91-1, respectively]. Also dimethyl sulfoxide (DMSO) was provided from Sigma too (St. Louis, MO, USA) [Lot No. 67-68-5].

### Experimental design

Forty-two healthy adult BALB/c mice (6-8 weeks old) were obtained from the Animal Research Unit, Babol Medical University, Babol. Animal care and handling was done based on Animal Research Unit and following approval of Ethics Committee (MUBABOL.HRI.REC.1395.73). The animals were habituated to laboratory conditions for 1 week before initiation of the experiment. Mice were maintained on 12 hours light-dark cycle at 21-24°C with 50-60% humidity. Mice had free access to normal diet and water, ad libitum. The animals were divided into seven groups: group I (control group) received normal saline, group II (sham group) received DMSO (0.75% solution), group III received NAC 35 mg/kg.b.w, group IV (high CPF) received CPF 5 mg/kg. b.w, group V (low CPF) received CPF 0.5 mg/kg.b.w, and group VI and VII received CPF at low (0.5 mg/kg.b.w) and high (5 mg/kg.b.w) doses, respectively along with NAC on a daily basis. In groups VI and VII, NAC was given intraperitoneally from five days before the experimental timeline, in order to acclimate mice with this antioxidant. All groups were treated intraperitoneally except the control group. Treatment was conducted for 4 weeks and injections in all groups were administrated on five consecutive days each week. One week after the last injection, mice were sacrificed using anesthetics to evaluate sperm parameters and testis histopathological alterations.

### Chemical solution preparation

Here, 15 µL DMSO was added to 1985 µL distilled water to prepare 2 ml DMSO solution to be administered to the sham group. Also, 1 mL DMSO was added to CPF powder vial (1 mg) in order to prepare CPF stoke solution (1mg/1mL). Afterward, 15 µL CPF was added to 135 µL distilled water and after pipetting, the whole solution was added to 1850 µL distilled water to prepare 2 ml High CPF (5 mg/kg.b.w) solution. Eventually, 200 µL of high CPF solution was added to 1800 µL distilled water to prepare low CPF (0.5 mg/kg.b.w) solution. NAC was dissolved in water at 35 mg/kg.b.w. It should be noted that fresh CPF solutions were daily prepared.

### Sperm motility, count and morphology assessment

Seven days after the last day of treatment, mice were anaesthetized via an inhalation induction chamber and sacrificed. Right testis of each animal was excised and put in 10% formalin solution for histopathological evaluations. Afterward, the caudal of left epididymis of each animal was excised and put in petri dish containing 3 mL Ham's F10 (St. Louis, MO, USA) [Lot No. 87120401]. According to diffusion method (16), for assessment of sperm parameters, epididymis was tattered to smaller pieces using sterile needle syringe and kept in a $CO_2$ incubator at 37°C for almost 30 minutes. Then, sperm parameters including sperm count, motility and morphology were evaluated under light microscopy.

From semen samples prepared by diffusion method, almost 50 µL semen from each mouse was smeared by a pipette on a slide. Afterwards, maximum 100 sperms were observed on right upper quarter of each slide to examine sperm count, motility and morphology. Sperm normality percentage for each mouse was easily calculated using a counter by knowing about mice sperm abnormalities (16).

It was very important that well-mixed semen sample was spread at appropriate thickness on each slide to evaluate sperm parameters. During sperm assessment, room temperature was maintained at 21-24°C because increased temperature may enhance semen degeneration speed.

### Histopathological examinations

Testis specimens were kept in 10% neutral buffered formalin. For testis histopathological evaluations, 5 µm sections were prepared from each testis, stained with haematoxylin and eosin (H&E) and observed under a light microscope. Images were captured by Olympus optical microscope equipped with a Canon HD camera at magnifications ×4, ×10 and also ×40 at four random points. Afterwards, data were evaluated on a proper personal computer using Motic software instruction (17). Numbers of spermatogonia, spermatocytes and spermatid cells and also seminiferous tubules area and diameter were observed by using Motic histomorphometric utility options.

### Statistical analysis

Data were presented as mean ± standard error (SE). Statistical analysis was performed in SPSS (version 22, SPSS Inc., Chicago, IL) using one-way analysis of variance (ANOVA) followed by Tukey as the post hoc test.

**Table 1:** Effect of NAC on sperm parameters in CPF-induced mice

| | Control | DMSO | NAC | Low CPF | High CPF | Low CPF+NAC | High CPF+NAC |
|---|---|---|---|---|---|---|---|
| Sperm motility (%) | 75.83 ± 0.83 | 71.66 ± 1.66 | 75.83 ± 0.83 | 45 ± 2.23[a] | 33.33 ± 4.77[a] | 75 ± 0.1[b] | 57 ± 4.03[b] |
| Sperm normality (%) | 66 ± 2.73 | 62.50 ± .17 | 63.66 ± 1.02 | 54 ± 1.98[a] | 30.66 ± 0.7[a] | 59.16 ± 1.60[b] | 62.83 ± 1.85[b] |
| Sperm count (sperm cell concentration/ml) | 87.5 ± 3.09 | 90 ± 4.47 | 93.33 ± 4.21 | 55 ± 2.23[a] | 65.83 ± 5.23[a] | 82.5 ± 3.59[b] | 70 ± 2.58 |

**The data are presented as mean ± SE (n=6). Sperm count is expressed as number×10⁶ per caudal epididymis. [a]; Indicates a significant difference as compared to control group (P<0.05), [b]; Indicates a significant difference as compared to CPF group (P<0.05), NAC; N-Acetyl Cysteine, CPF; Chlorpyrifos, and DMSO; Dimethyl sulfoxide,**

# Results

## Morbidity and mortality

Male mice that received CPF (0.5 and 5 mg/kg.b.w/day) for 35 days showed signs of toxicity such as salivation, diarrhea and tremor. No death was recorded throughout the study period.

## Sperm characteristics

According to our data, no significant differences were found in sperm characteristics between DMSO and control group (Table 1, P>0.05). Administration of CPF 0.5 mg/kg.b.w/day (low CPF) showed significant decreases in sperm motility, count and also morphology (P<0.0001). In addition, sperm characteristics considerably decreased following administration of CPF 5 mg/kg.b.w/day as compared to control group (P<0.0001). Treatment with NAC alone made no significant changes to motility, counts and morphology. However, NAC treatment in combination with low CPF caused significant increases in motility and counts and markedly improved sperm morphology as compared to control CPF-induced groups (P<0.0001). NAC also caused significant increases in motility and improvements in morphology when co-administered with high CPF (P<0.0001), while sperm count showed no significant increases.

## Histomorphometry

Average count of spermatogonia, spermatocytes and spermatid in DMSO group slightly decreased but it was not significant; however, a significant increase was observed in NAC group (P<0.001, Fig.1). It was demonstrated that mean number of spermatogonia cells significantly decreased in low CPF group (P<0.04). Meanwhile in High CPF group, spermatogonia, spermatocytes and spermatids were considerably decreased (P<0.0001). Treatment of CPF groups with NAC resulted in significant increases in the average number of spermatocytes and spermatids (P<0.001 and P<0.007, respectively). However, mean of spermatogonia cells counts in high CPF+NAC group had no significant increase (P<0.05).

Based on data given in Figures 2 and 3, there was no significant increase in mean seminiferous tubules area and diameter in DMSO group compared to control (P>0.05); but, NAC showed a significant increase in both variables (P<0.001). While high CPF treatment significantly diminished seminiferous tubules, low CPF treatment (0.05 mg/kg.b.w) caused no considerably damage in seminiferous tubules shape. NAC could not ameliorate the effects caused by high CPF (P>0.05).

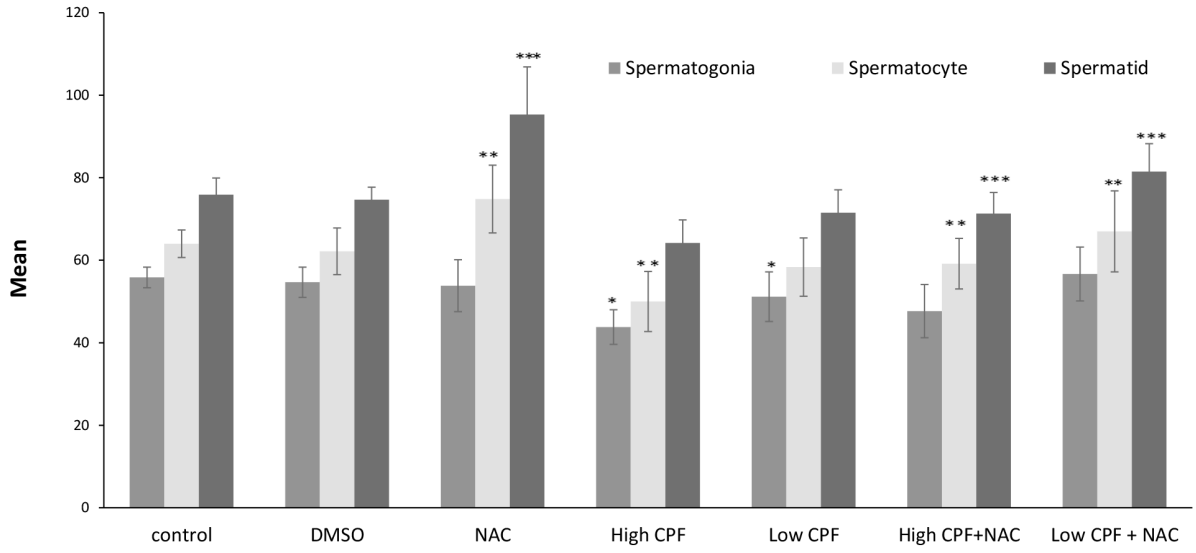


**Fig.1:** Bars presents mean ± SE of spermatogonia, spermatocytes and spermatid cells counts in different groups. *; Indicates a significant difference in spermatogonia counts as compared to control group (P<0.05), **; Indicates a significant difference in spermatocytes counts as compared to control group (P<0.05), ***; Indicates a significant difference in spermatids counts as compared to control group (P<0.05), NAC; N-Acetyl Cysteine, CPF; Chlorpyrifos, and DMSO; Dimethyl sulfoxide.

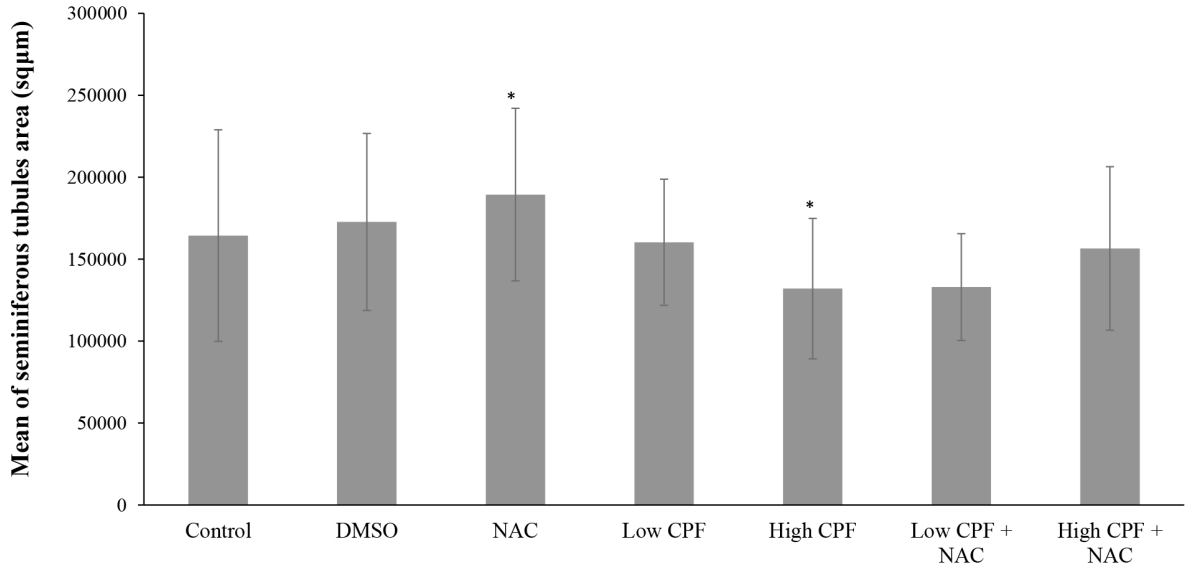


**Fig.2:** Bars presents mean ± SE of seminiferous tubules area in different groups. *; Indicates a significant difference as compared to control group (P<0.05), NAC; N-Acetyl Cysteine, CPF; Chlorpyrifos, and DMSO; Dimethyl sulfoxide,

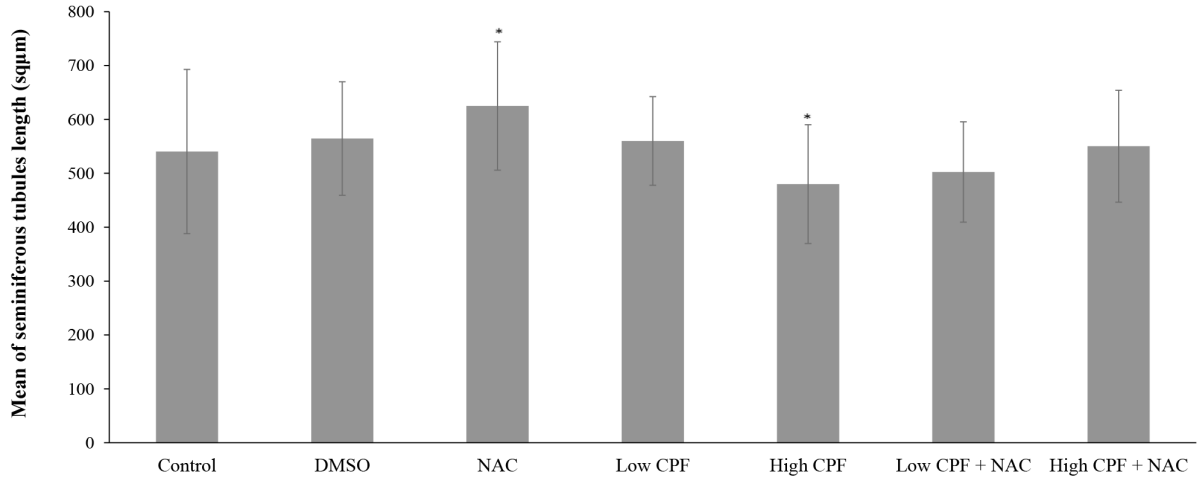


**Fig.3:** Bars presents mean ± SE of seminiferous tubules diagonal length in different groups. *; Indicates a significant difference as compared to control group (P<0.05), NAC; N-Acetyl Cysteine, CPF; Chlorpyrifos, and DMSO; Dimethyl sulfoxide.

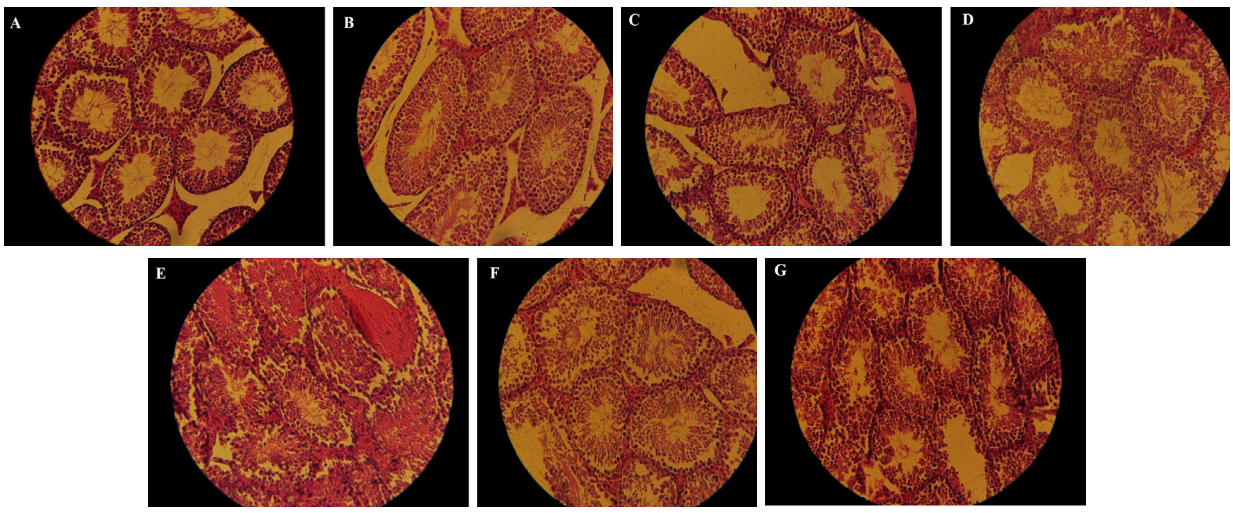


**Fig.4:** Histopathological difference is shown between experimental groups. It is demonstrated a massive destruction in CPF groups. However NAC considerably improved histopathology of testis. **A.** Control, **B.** DMSO, **C.** NAC, **D.** Low CPF, **E.** High CPF, **F.** Low CPF+NAC, and **G.** High CPF+NAC. NAC; N-Acetyl Cysteine, CPF; Chlorpyrifos, and DMSO; Dimethyl sulfoxide.

Based on data given in Figure 4, there was no significant increase in mean seminiferous tubules area and diameter in DMSO group compared to control (P>0.05); but, NAC showed a significant increase in both variables (P<0.001). While high CPF treatment significantly diminished seminiferous tubules, low CPF treatment (0.05 mg/kg.b.w) caused no considerably damage in seminiferous tubules shape. NAC could not ameliorate the effects caused by high CPF (P>0.05).

## Discussion

In CPF-exposed mice, a considerable reduction in sperm parameters was found. Meanwhile, NAC could not significantly improve sperm motility, morphology and count. NAC in combination with CPF 5 mg/kg.b.w. considerably prevented further damages to sperm motility and morphology; However, NAC could not improve sperm counts caused by CPF-induced toxicity. In a similar study on CPF reproductive toxicity, a significant decrease in sperm motility and counts was observed by CPF gavage at 20 mg/kg.b.w (18).

In addition, CPF considerably decreased the level of antioxidant enzymes and glutathione in plasma. *Nigella sativa* oil can act like NAC as a potent protective agent which statistically improved sperm parameters, antioxidant enzymes activity and testosterone level (18). According to our results, adverse effects of CPF is likely irreversible and has negative effects on genitalia systems.

For decades, CPF devastating effects on spermatogenesis process was unclear. Other scientists investigated various pesticides at different doses for their negative effects on spermatogenesis process (19). Organophosphates or any other chemicals, such as different toxins, that have adverse effects on tissues and cells may be inadvertently absorbed through the skin or digestion system. In order to confront these harmful effects, consumption of antioxidant substances like syrup of Malva sylvestris (20) along with hydroalcoholic extract of *Fumaria parviflora* (21) or products such as propolfenol (22) is highly recommended to protect against damages induced by toxins, particularly against those cause in the genital system. However, some antioxidant materials such as catechin and quercetin did not have significant influences in this regard (9). Nevertheless, natural nutrients such as ginger and cinnamon could be effective on male genital dysfunction due to their anti-oxidant efficacy (23).

Vitamin C and E have been widely used in previous studies and were introduced as beneficial protective materials to compensate damages induced by organophosphates such as malathion, a broad spectrum organophosphate pesticide that could decrease sperm parameters and induce histopathological alteration (24). Although vitamin C and E are potent protective materials against various toxins, a lower dose of intraperitoneal NAC possibly has a more marked impact on sperm parameters based on our findings but use of food or fruits overfilled with these vitamins is suggested for people who are daily exposed to pesticides (25). Another study showed that vitamin C only resulted in a significant improvement of sperm motility (26).

The present study indicates that NAC at 35 mg/kg.b.w somehow significantly increased seminiferous tubules area, diagonal diameter, and spermatogonia, spermatocytes and spermatids counts. Meanwhile CPF 0.5 mg/kg.b.w could not considerably reduce seminiferous tubules area and diagonal diameter. Furthermore, CPF 5 mg/kg.b.w significantly diminished seminiferous tubules.

Based on these findings, NAC in combination with CPF ameliorates the pesticide's adverse effects on testis. It seems that intraperitoneal injection of NAC, even at a low dose has a more marked effect on sperm parameters than gavage administration (15). It has been proven that NAC can affect lipid peroxidation (LPO) (27). Therefore, NAC might decrease ROS elevation caused by CPF. However, in the present study, NAC exact effects on sexual hormones or anti-oxidant enzymes such as superoxide dismutase, catalase, or glutathione in treated groups, were not evaluated. But considering significant reductions in testis germinal cells, oxidative stress level was probably elevated by CPF and NAC ameliorated the adverse effect of CPF on the testis.

According to our results, seminiferous tubules area and diagonal diameters were not affected by CPF. It suggests that resting times at the end of each week and also seven days after the last injection of CPF might provide a chance for the immune system to recover and regenerate genital and possibly other tissues. Therefore we did not expect NAC to protect these two unaffected variables. Meanwhile, we assume that CPF at the dose of 0.5 mg/kg.b.w could not significantly diminish seminiferous tubules area following four-week administration. Maybe by longer treatment periods, CPF could induce more destructive effects at the dose of 0.5 mg/kg.b.w It is clear that NAC is able to confront negative effects of CPF toxicity in male genital system but what if we could use NAC at doses higher than 35 mg/kg.b.w? In this case, we probably observe NAC protective effects against CPF typical tissue toxicity. Further *in vivo* studies using intraperitoneal injections, are highly recommended to affirm our data.

## Conclusion

Both low and high doses of CPF can decrease sperm parameters. Also, this pesticide at 5 mg/kg.b.w dose significantly diminishes the length and diagonal diameter of seminiferous tubules. NAC significantly improved CPF adverse effects on sperm parameters and spermatogenesis cells except spermatogonia. However, this antioxidant could not statistically ameliorate the histopathological alterations of seminiferous area induced by CPF.

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## Authors' Contributions

S.G.A.J.; Contributed to conception and built an ideal design. R.K.; Contributed to all experimental work, wrote the manuscript, and also performed statistical analysis. A.A.M, N.N.; Contributed to pharmacological and chemical approaches, respectively. F.F.; Contributed to histopathology part of this study and revised the manuscript. All authors read and approved the final manuscript.

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Original Article

# The Effects of Olive Leaf Extract on The Testis, Sperm Quality and Testicular Germ Cell Apoptosis in Male Rats Exposed to Busulfan

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## Abstract

**Background:** Busulfan (BU) has a destructive effect on the male reproductive system. The goal of this study was to assess the effects of olive leaf extract (OLE) as a source of antioxidants and phenolic compounds, on BU-induced damages in rat testes.

**Materials and Methods:** In this experimental study, 40 male Wistar rats were randomly divided into 5 groups. The control group (CTL) received a single intraperitoneal (i.p.) injection of dimethyl sulfoxide (DMSO), followed by oral administration of distilled water for 5 weeks. In BU group, BU (10 mg/kg) was administrated i.p. once. In co-treatment groups, first, received BU (10 mg/kg, a single i.p. injection) then, OLE was administrated orally at different doses of 250 mg/kg (BU+OLE 250), 500 mg/kg (BU+OLE 500) and 750 mg/kg (BU+OLE 750), for 5 weeks. Next, blood and sperm samples were collected. The left testis was removed to investigate testicular parameters and apoptosis by using H&E and TUNEL staining, respectively. All data were analyzed by SPSS software and a $P<0.05$ was considered significant.

**Results:** There was a significant decline in sperm viability ($P=0.017$), number of primary spermatocyte (PS) ($P=0.001$) and Leydig cells ($P=0.023$) in the BU group versus the CTL group. OLE at three doses could repair these defects versus BU group. Increases in apoptotic spermatogonia cells (SG) due to BU were significantly reduced by OLE 250 and 500 mg/kg ($P<0.01$). A reduction in germinal epithelium height and an increase in apoptotic SG were observed in BU+OLE 750 group vs. other groups ($P<0.01$) and alkaline phosphatase (ALP) was at the highest level, also Aspartate aminotransferase (AST) increased markedly vs. CTL ($P=0.024$).

**Conclusion:** Oral administration of OLE at the doses of 250 and 500 mg/kg could be helpful in ameliorating BU-induced toxicity in rat testes, while OLE 750 mg/kg not only did not cause positive effects, but also could exacerbate the harmful effects.

***Keywords:*** Apoptosis, Busulfan, Olive Extract, Spermatogenesis, Testicular Germ Cell

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## Introduction

The use of anti-cancer drugs has been increased. Busulfan (BU) is one such anti-cancer drug that is used to treat lymphoma, chronic leukemia, and ovarian cancer. It is also used as a part of a regimen administered before bone marrow transplantation. However, studies showed that this drug has side effects on many organs such as the male reproductive system (1). The negative effects of BU on the male reproductive system include decreasing testis weight (2), increasing abnormal sperm parameters (motility and morphology) (3), oligo-azoospermia, destroying almost all testicular germ cells (4), and causing temporary or permanent sterility.

Since BU is an alkylating agent with oxidative properties (5), therefore, it is believed that antioxidant therapy may be helpful in reducing its harmful effects. Several animal studies have reported an ameliorating effect of plant extracts possessing antioxidant properties on the male reproductive system following exposure to BU (1, 6).

Olive (*Olea europaea* L.) is commonly used as a part of traditional herbal medicine to treat disease in the Mediterranean area (7). Olive leaf is rich in antioxidant phenolic

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compounds such as oleuropein, verbascoside, ligstroside, as well as flavonoid compounds like tyrosol and hydroxytyrosol (7, 8). Oleuropein scavenges harmful free radicals and prevents oxidative damage (9). It was reported that treatment with olive leaf extract (OLE) improved total antioxidant capacity (TAC) level in rat testicular tissue. Also, it was shown that OLE can improve sperm parameters and testis antioxidant conditions in rats exposed to rotenone (10).

With increasing prevalence of cancer, the number of individuals being treated with BU has significantly increased. Noteworthy, most of these BU-treated subjects are in childbearing ages and it is not possible to restore fertility following BU exposure. Therefore, research on new agents and/or herbal extracts which can reduce these adverse effects on the male reproductive system is essential. For the first time, in this study, OLE which contains phenolic compounds and exerts antioxidant properties, was given to different groups of BU-treated animals to investigate the effect of oral administration of OLE on testis structure, sperm parameters and apoptosis in rat testes. To evaluate the safety profile of the extract, we measured levels of liver enzymes to assess possible toxic effects of different doses of OLE on the liver as an important organ that in involved in drug absorption and elimination.

## Materials and Methods

The present experimental study was approved by the Ethics Committee of Kerman University of Medical Sciences, Kerman, Iran (IR.KMU.REC.1394.641).

### Olive leaves extract preparation

Olive leaves were collected from the olive tree farms from Kazeroon, Iran, authenticated by an expert and kept at the herbarium of pharmacognosy department, faculty of pharmacy, Kerman University of medical sciences, Kerman, Iran. The leaves were washed and dried at room temperature. Dried leaves (500 g) were milled and passed through a sieve (mesh 300). Plant extraction was performed using warm maceration with ethanol 80% for 72 hours. Obtained extract was concentrated under vacuum and finally dried in an oven at 40°C for 24 hours. The extract was stored at -20°C for subsequent experiments. The extract was dissolved in distilled water before use (11).

### Determination of total phenol content of olive leaf extract

Total phenolic content of OLE was determined by Folin-Ciocalteu assay. Gallic acid was used for calibration. A stock solution of gallic acid (1000 ppm) was prepared; next, 0.1 ml stock solution was added to 0.4 ml sodium carbonate, 0.5 ml Folin reagent and 3 ml distilled water after 40 minutes incubation at room temperature. Absorbance was measured at 765 nm. The calibration curve was plotted for gallic acid based on 7 serial dilutions. After measurement of absorbance, calibration curve was plotted. By determination of extract absorbance as mentioned above and using the curve equation, total phenolic content was expressed as mg gallic acid equivalents per g of the extract (12). Each experiment was done in triplicate.

### Estimation of total flavonoid content of olive leaf extract

Total flavonoid content was measured by the aluminum chloride colorimetric assay. Rutin as the major flavonoid compound of the plant, was assessed for standardization using thin layer chromatography. First, 1 ml rutin (50 ppm) was added to 1 ml aluminum chloride 2%. After 30 minutes of incubation at room temperature, absorbance was recorded at 200-400 nm and maximum wavelength was 275 nm. Calibration curve was prepared using different dilutions of rutin. As mentioned above , total flavonoid content of the plant was expressed as mg rutin equivalents per g of the extract (12). Each experiment was done in triplicate.

### Animals and chemicals

Adult male Wistar rats (8-10 weeks old) were obtained from animal house of the university. Animals were kept in a temperature-controlled room (at 22˚C) with 12 hours/12 hours light/dark cycles. Food and water were readily available. All chemicals were purchased from Sigma-Aldrich, unless otherwise noted.

### Experimental design

Forty adult rats were randomly divided into 5 groups of control (n=8), BU (n=9) and BU co-administrated with three doses of OLE 250 mg/kg (n=8), 500 mg/kg (n=6) and 750 mg/kg (n=8) (BU+OLE 250, BU+OLE 500 and BU+OLE 750, respectively). In this study, BU was diluted in dimethyl sulfoxide (DMSO) and distilled water (D.W.) as solvent. The OLE was dissolved in the D.W. The animals in the control group (CTL) received a single intraperitoneal (i.p.) injection of BU solvent (i.e. DMSO+D.W.) and then D.W. was administrated orally by gavage for 5 weeks. The BU group received a single i.p. injection of BU (10 mg/kg) (13), the BU+OLE 250 group received OLE (250 mg/kg) orally for 5 weeks after receiving a single BU injection (10 mg/kg, i.p.), the BU+OLE 500 group received OLE (500 mg/kg) orally for 5 weeks after receiving a single BU injection (10 mg/kg, i.p.), the BU+OLE 750 group received OLE (750 mg/kg) (14) orally for 5 weeks after receiving a single BU injection (10 mg/kg, i.p.). Some rats died during the study, especially in the BU+OLE 750 group. At the beginning and end of the experiment, all rats were weighted.

### Sample collection

After the end of the treatment period, the rats were deeply anesthetized by chloral hydrate (400 mg/kg) (15). After making an incision in their chests, the heart blood samples were collected from the left ventricle for biochemical and hormone analysis. The left testis and vas

deferens were removed and separated from surrounding tissue. The testes' weight and diameter were recorded and then tissues were fixed in formalin 10% (16) for histologic analysis. Left vas deferens was dissected and sperms were collected.

**Serum testosterone and liver enzyme levels measurement**

The blood (1.5 ml) collected from the heart was centrifuged at 3000 rpm for 30 minutes. Serum was carefully separated from plasma and immediately stored in a freezer at -20˚C until analyzed. The level of serum testosterone and liver enzymes including alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured in duplicate samples by an enzyme-linked immunosorbent assay (ELISA) using IBL kit (IBL company, Germany) and Biorex kit (Biorex company, UK) , respectively, according to the manufacturer's instructions.

**Assessment of sperm parameters**

Under sterile conditions, the inferior part of rat abdomen was incised, the left vas deferens was removed and placed in a petri dish containing pre-warmed alpha MEM medium (2 ml), supplemented with 10% bovine serum albumin. It was dissected into several fragments and then, incubated at 37°C with $CO_2$ 5% in humidified air for 30 minutes to permit the migration of all spermatozoa from the reproductive duct to supplemented medium (15). Thereafter, the medium containing spermatozoa was collected and sperm quality was evaluated in terms of sperm motility, count, morphology and viability according to WHO guideline (17).

**Sperm motility**

Immediately, 10 µl of sperm suspension (including the supplemented medium and spermatozoa) was placed on a slide and covered by a coverslip. Sperm motility was classified as fast progressive motility, slow progressive motility and immotile according to WHO guideline (17) and expressed as the percent of each one per total sperm number in at least 10 fields (300 sperms).

**Sperm count**

First, 10 µl of sperm suspension was added into 10 µl of fixative solution (formalin/sodium bicarbonate). Next, 10 µl of this mixture was placed on Neubauer haemocytometer and covered by a coverslip. The counting chamber was then placed on the light microscope stage (Nikon TS-100, Japan) at ×200 magnification, and sperms were counted in four large squares. The average of counted sperms was multiplied and was expressed as million/ml of suspension (18).

**Sperm viability**

Sperm viability was assessed using eosin-nigrosin staining. Sperm suspension (5 µl) was added to eosin-nigrosin stain (5 µl). Smear was then prepared and at least 200 spermatozoa were randomly counted under a light microscope (at ×400 magnification). Sperm with red or pink head considered dead sperm and non-stained sperm, with white head, considered alive (17). The percentages of live spermatozoa were noted.

**Measurement of body and testis weight**

Body weights of rats were noted prior to start of experiment and 24 hours after the final day of treatment. The left testis was weighted by using a digital balance.

**Evaluation of testis histology**

Left testis diameter, length and width were recorded by using standard digital calipers. In order to assess the alterations in spermatogonia cell (SG) population, histological evaluation of the rat testis was carried out (eight testis samples in each group). After testis fixation in formalin 10%, testis was dehydrated in increasing concentrations of ethanol (70, 90 and 100%) and embedded in paraffin. Five-micron thick sections of testis (at 50 µm interval) were prepared using microtome, mounted carefully and stained with hematoxylin and eosin (H&E). The slides were examined under a light microscope (Olympus/BX51, Japan). In each section, 15 seminiferous tubules were randomly examined. Two perpendicular diameters of each seminiferous tubule (from the basement membrane to lumen) were calculated via calibrated linear scale of the Analysis software in the 10X eyepiece of Olympus microscope (18). The average of these diameters was reported. Also, thickness of the germinal epithelium layer was measured. The number of SG, primary spermatocyte (PS) cells and also Leydig cell were recorded from 10 microscopic fields at ×400.

**Evaluation of testicular apoptotic cells by using TUNEL assay**

Terminal deoxynucleotidyl-transferase-mediated DNA nick end-labelling (TUNEL) assay is a valuable method to detect apoptotic cells by labeling the terminal end of nucleic acids. TUNEL staining was done by an in situ cell death detection kit, POD (Roche-11684817910 version 14, Germany), according to the manufacturer's instructions.

First, the testicular slides were deparaffinized by incubation at 60˚C for 30 minutes, and then rehydrated in xylene (for 30 minutes) and increasing concentrations of ethanol 70, 90, and 100%, respectively (each one for 6 minutes). Slides were washed twice with distilled water.

Next, the slides were incubated in proteinase K (20 µg/ml in 10 mM Tris buffer) at 37˚C for 30 minutes and washed three times with phosphate buffered saline (PBS). Afterwards, the slides were incubated in hydrogen peroxide solution ($H_2O_2$) 3% at room temperature for 10 minutes in the dark and re-washed three times with PBS. Immediately, TUNEL reaction mixture (enzyme solution 50 IU and label solution 450 IU) was prepared. The sections were incubated

in a moist chamber containing a TUNEL reaction mixture at 37˚C temperature for 60 minutes. After three-time washing by PBS, the slides were incubated in POD (anti-fluorescein antibody, FAB fragment from sheep, conjugated with peroxidase) intra a moist chamber for 30 minutes at 37ºC. The sections re-washed three times with PBS. 3,3′-Diaminobenzidine (DAB) substrate was added to the slides and incubated at room temperature for 10 minutes in the dark. After washing with PBS (once), the slides re-washed carefully with distilled water. Next, sections were stained with hematoxylin at room temperature for 30 seconds. After washing the slide with distilled water and dehydration by ascending degrees of ethanol (70, 90 and 100% respectively), the slides were mounted using Entellan. TUNEL-positive cells per tubule in at least 20 tubules from the testes were counted under a light microscope (Olympus/BX51, Japan) (18).

### Statistical analysis

Statistical analysis was carried out by using Statistical Package for the Social Sciences software, version 21 (SPSS, Chicago, IL, USA). All data were expressed as means ± standard errors of the mean (SEM). At first, one-sample Kolmogorov-Smirnov test was used to check the normality of variables. Next, the differences in normal-distributed variables among five experimental groups were analyzed by using one-way ANOVA test followed by Tukey post hoc test. For nonparametric variables, non-parametric Kruskal-Wallis test (TUNEL SG, germinal epithelium height, alive and fast progressive sperm, and length of testis) was used. The level of significance was set at $P<0.05$.

## Results

### Assessment of flavonoids and phenolic content of olive leaf extract

The total flavonoid content of the OLE, calculated using calibration curve of rutin ($R^2$=0.9635) was 1.43 g ruin equivalent/g plant extract. Total phenolic content of the plant, calculated from gallic acid standard curve ($R^2$=0.9857) was 1.44 g gallic acid equivalents in 1000g OLE (Fig.1).

### Testosterone and liver enzymes assay

There was no significant difference in blood levels of testosterone among different groups. Analysis of the liver enzyme level showed that ALP level increased significantly in BU+OLE 750 compared to control ($P<0.001$), BU ($P<0.001$), BU+OLE 500 ($P=0.003$) and BU+OLE ($P=0.030$). Also, ALP level in BU+OLE 250 was significantly elevated versus the control ($P=0.011$). Compared to the BU group, AST and ALT enzyme levels did not vary significantly between BU+OLE 250 and BU+OLE 500 groups. Furthermore, AST level in rats treated with 750 mg/kg OLE showed a significant increase as compared to BU ($P=0.005$) and CTL ($P=0.024$, Table 1).

### Rat body and testis weights measurement and morphological assessments

There were no significant differences in testis weight and changes in rat body weight in any group ($P>0.05$). The testis width, length and diameter in OLE-treated animals (all doses) remained unchanged and were similar to those of the control and BU-exposed animals ($P>0.05$) (data not shown).

### Sperm characteristics

The effects of BU and different doses of OLE on sperm count, motility, morphology, and viability are summarized in Table 2. Comparing sperm count among 5 groups by using one way-ANOVA test, showed that although exposure to BU could non-significantly decrease the sperm count (3.23 ± 0.62, $P=0.086$) as compared to that of CTL (5.75 ± 0.82), all doses of OLE caused an increase in sperm count as compared to BU group. Fast progressive motility decreased non-significantly in BU group (4.74 ± 1.33) vs. the CTL (11.67 ± 4.97) ($P=0.19$).

Oral administration of OLE at different doses of 250 (17.91 ± 2.85), 500 (28.75 ± 5.86) and 750 (20 ± 4.10) mg/kg could significantly improve the proportion of sperms to the fast progressive motility versus BU group ($P=0.002$, $P=0.009$, $P=0.003$, respectively).

The percentage of viable sperms in the BU group was significantly lower than that of the CTL ($P=0.017$). Compared with BU group, a significant change was observed viable sperm percentage in all OLE-treated groups (250 mg/kg, $P<0.001$, 500 mg/kg, $P=0.003$, and 750 mg/kg, $P<0.001$) (Table 2).

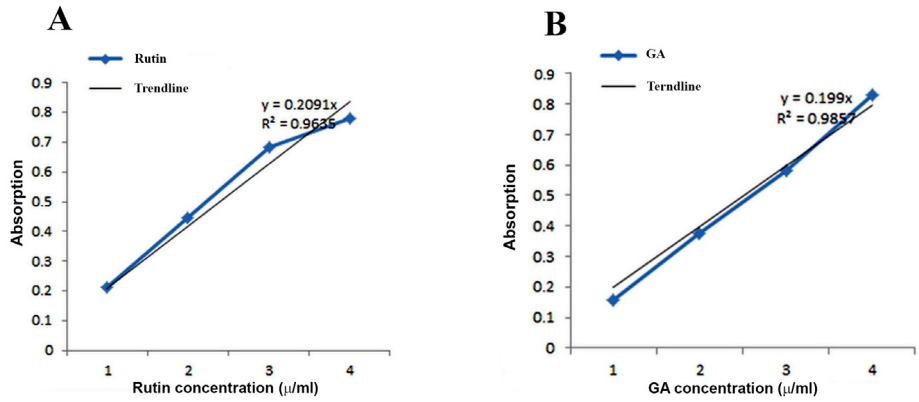


**Fig.1:** The standard curves of phenols (gallic acid equivalents) and flavonoids (rutin equivalents) by drawing adsorption against concentration. **A.** Standard curves of rutin and **B.** Standard curve of gallic acid. The results have been resulted from triplicate experiments.

**Table 1:** Effect of busulfan (BU) and different doses of OLE (250,500 and 750 mg/kg) on liver enzymes and testosterone hormone levels after 5 weeks of treatment

| Group | ALP (U/l) | AST (U/l) | ALT (U/l) | Testosterone (ng/ml) |
|---|---|---|---|---|
| CTL | 340 ± 41.33 | 155.50 ± 24.85 | 58.50 ±7.62 | 3.714 ± 1.08 |
| BU | 522.83 ± 70.19 | 133.25 ± 5.59 | 72.17 ± 5.59 | 3.129 ± 0.77 |
| BU+OLE 250 | 648.67 ± 68.16[a] | 162.80 ± 9.98 | 76 ± 9.98 | 1.63 ± 0.67 |
| BU+OLE 500 | 549.20 ± 61.00 | 165.40 ± 10.23 | 73.20 ± 10.23 | 2.54 ± 0.89 |
| BU+OLE 750 | 932.60 ± 64.46[c] | 220.83 ± 6.67[ab] | 86.67 ± 6.67 | 2.75 ± 0.63 |

**Results are expressed as mean ± SEM. Significant differences (P<0.05) are indicated by [a]; vs. control group in the same column, [b]; vs. BU group, [c]; BU+OLE 750 group vs. another groups in the same column, ALP; Alkaline phosphatase, ALT; Alanine aminotransferase, AST; Aspartate aminotransferase, OLE; olive leaf extract, and CTL; Control group.**

**Table 2:** Effect of busulfan (BU) and different doses of OLE (250,500 and 750 mg/kg) on testis histology and sperm parameters

| Group | Control | BU | BU+OLE 250 | BU+OLE 500 | BU+OLE 750 |
|---|---|---|---|---|---|
| Spermatogonia number | 37.74 ± 2.93 | 33.67 ± 1.82 | 44.35 ± 2.17[b] | 42.11 ± 3.47 | 32.91 ± 1.93[c] |
| Primary spermatocyte number | 174.19 ± 16.66[b] | 75.52 ± 8.62[a] | 161.46 ± 7.80[b] | 168.42 ± 18.63[b] | 128.75 ± 21.56 |
| Leydig cell number | 5.47 ± 0.43[b] | 2.32 ± 0.32[a] | 5.8 ± 0.92[b] | 5.97 ± 1.148[b] | 4.23 ± 0.71[b] |
| Seminiferous tubules diameter (mean D and d) (µm) | 299.21 ± 7.68 | 300.48 ± 6.11 | 299.68 ± 2.88 | 305.66 ± 10.96 | 275.28 ± 7.2 |
| Germinal epithelium height (µm) | 86.29 ± 3.36 | 86.88 ± 2.72 | 131.77 ± 43.91 | 90.89 ± 3.63 | 76.66 ± 2.98[bce] |
| Alive sperm (%) | 48.67 ±7.51 | 23.61 ± 3.54[a] | 57.72 ± 5.60[b] | 56.25 ± 1.44[b] | 61.72 ± 6.16[b] |
| Sperm count (×10$^6$/ml) | 5.76 ± 0.82 | 3.23 ± 0.62[cf] | 7.97 ± 0.76 | 6.12 ± 0.55 | 7.83 ± 0.74 |
| Fast progressive sperm (%) | 11.66 ± 4.96 | 4.74 ± 1.33 | 17.91 ± 2.85 | 28.75 ± 5.86[ab] | 20.00 ± 4.10[b] |
| Slow progressive sperm (%) | 43.53 ± 6.30 | 39.32 ± 4.94 | 35.25 ± 4.75 | 25.52 ± 5.82 | 38.00 ± 4.19 |
| Immotile (%) | 45.30 ± 3.65 | 53.94 ± 4.68 | 46.67 ± 3.69 | 45.60 ± 2.16 | 41.84 ± 3.04 |

**Results are expressed as mean ± SEM. Significant differences (P<0.05) are indicated by [a]; vs. control group in the same row, [b]; vs. BU group in the same row, [c]; vs. BU+OLE 250 group in the same row, [e]; vs. BU+OLE 500 group in the same row, [f]; vs. BU+OLE 750 group in the same row, D; Long diameter, d; Short diameter, and OLE; Olive leaf extract.**

## Testis histological study

### Spermatogenesis assessment

Spermatogenesis in CTL testis was normal (Table 2). Although the mean number of SG in the BU-treated testis was numerically lower than the CTL, it did not reach a significant level (P=0.747). The BU+OLE 250 group showed a significant increase in the mean number of SG when compared to the BU group (P=0.026). There was a significant difference between BU+OLE 750 and BU+OLE 250 group with respect to SG number (P=0.013).

A statistically significant difference was observed in the number of the PS (P=0.001) and Leydig cells (P=0.023) between the BU and CTL. Compared to the BU group, OLE 250 (P=0.004) and 500 (P=0.003) provided a significant increase in the number of PS following BU-exposure. No statistically significant difference was observed in the average number of PS and Leydig cells between groups treated with different doses of OLE and CTL. There was a significant difference between control and BU groups with regard to the number of Leydig cells (P=0.023). The number of testicular Leydig cells in OLE 500 group was higher than that of the BU group (P=0.013).

The results showed that all doses of OLE, following BU exposure, can repair spermatogenesis to varying extents (Fig.2).

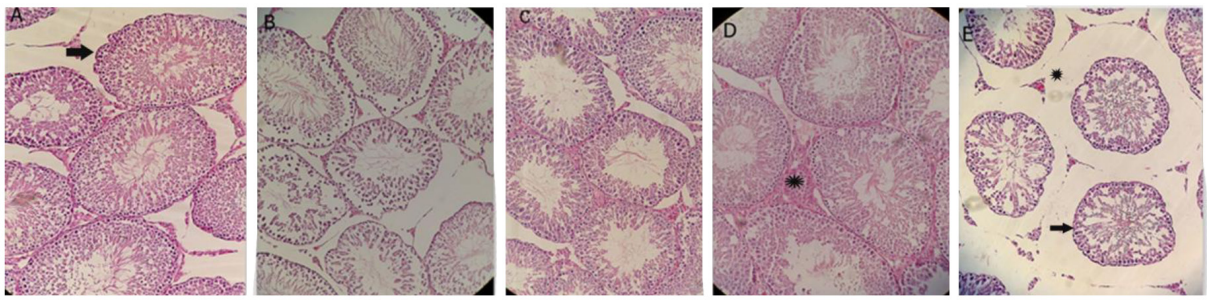


**Fig.2:** Light micrographs of rat testis (H&E staining, ×200 magnifications). **A.** Seminiferous tubules showing normal structure and active spermatogenesis in the control group, **B.** The most of spermatogonia and primary spermatocytes cells are destroyed in busulfan group, **C.** Photomicrographs of testis from rats that treated with 250 mg/kg, **D.** 500 mg/kg, and **E.** 750 mg/kg olive leaf extract (OLE) demonstrated that both 250 and 500 mg/kg OLE caused a normal and regular structure of seminiferous tubules and obvious improvement in spermatogenesis, while 750 mg/kg OLE reduced cell lines and height of seminiferous epithelium (→) and destroy interstitial space (*).

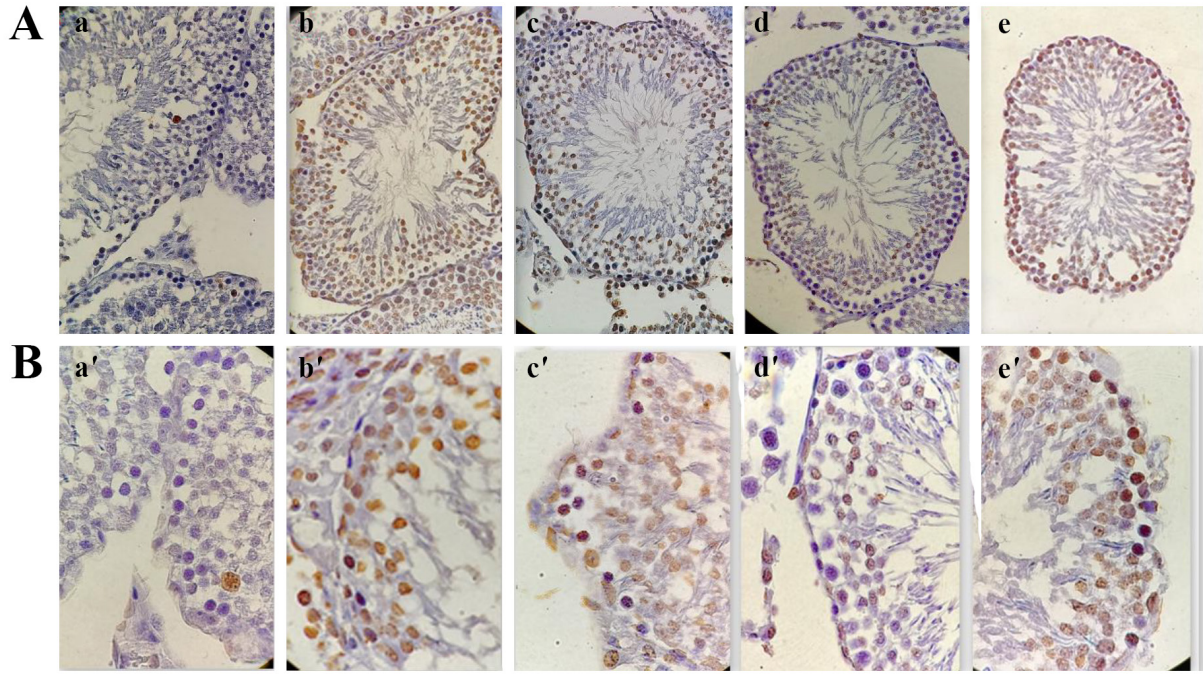


**Fig.3:** Immunohistochemical staining of the rat testis tissue in experimental groups. **A.** Light microscopy of TUNEL-stained rat testicular sections (×400 magnifications), **a.** Apoptotic cells are seen brown color. Apoptosis is extremely low in control testes, **b.** The most of testicular germ cells is undergoing apoptosis in busulfan testes, **c.** Although TUNEL-positive germ cells are still visible in testicular sections from rats that treated with olive leaf extract (OLE) at dose of 250 mg/kg, **d.** 500 mg/kg OLE caused a marked decrease in apoptotic testicular germ cells, **e.** High level of apoptotic cells was observed in testes of rats that treated with OLE at dose of 750 mg/kg and **B.** ×1000 magnification.

### Seminiferous tubule morphometry

No statistically significant difference was observed in the mean diameter of seminiferous tubules among different groups (P>0.05). Comparison of the mean of germinal epithelium thickness between BU-treated testis and control, BU+OLE 250 and BU+OLE 500 testes showed no significant differences (P>0.05). However, significant decreases in thickness of the germinal epithelium of BU+OLE 750 group were observed as compared to the BU (P =0.033) and OLE 250 and 500 (P=0.019) treatment groups (Table 2).

### Germ cell apoptosis assessment

In the present study, apoptotic germ cells were distinguished by TUNEL staining (Fig.3). The apoptotic index was calculated as follows: TUNEL positive nuclei (dark brown cells) /number of total germ cells. As summarized in Figure 4, a significant increase in the mean number of apoptotic SG (P<0.001) and PS (P=0.029) cells was observed in rats exposed to BU as compared the CTL. However, BU-mediated increase in apoptotic SG was significantly reduced by administration of OLE 250 and 500 mg/kg (P<0.001). This decline was markedly higher in the group administrated with OLE 500 mg/kg compared to the other doses of OLE (P<0.001), and nearer to that of the CTL. In contrast, the percentage of apoptotic SG was enhanced in rats which received OLE 750 mg/kg, compared to the other groups (P<0.001). Also, the TUNEL-positive PS counts in the seminiferous tubules showed that OLE 250 mg/kg could not decrease the level of apoptotic PS cells significantly compared to BU group. Interestingly, only a small number of apoptotic cells were observed in the testicular sections of OLE 500 group, which was significantly different from that of the BU (P=0.029) and the other OLE groups (P=0.029). However, this reduction was more marked compared to the CTL. In contrast, the group which received OLE 750 mg/kg was shown to have significantly higher levels of apoptotic PS cells, in comparison to OLE 500 and CTL (P=0.029).

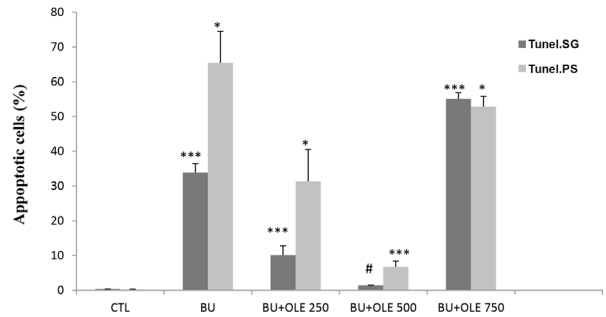


**Fig.4:** Effect of OLE treatment after busulfan exposureon percentage of apoptotic testicular germ cells. Significant differences (P<0.05) are indicated by ***; vs. all groups, *; vs. control and BU+OLE 500 groups, and #; vs. all groups except control. Values are expressed as % mean ± SEM. CTL; Control group, BU; Busulfan, PLE; Olive leaf extract, SG; Spermatogonia, and PS; Primary spermatocye cells.

## Discussion

The present study showed that administration of a single dose of BU to Wistar rats, leads to a significant reduction in sperm and testicular parameters (i.e. sperm viabil-

ity and the number of PS and Leydig cells). Furthermore, our results demonstrated that BU could increase the rate of apoptotic SG and PS in the rat testis. However, it was shown that OLE administration at two doses of 250 and 500 mg/kg to rats that received BU, could significantly improve the afore-mentioned parameters in testis following BU -induced toxicity. Oral administration of OLE at 750 mg/kg has a negative effect in many cases (i.e. the thickness of germinal epithelium, spermatogenesis lineage cells, and apoptosis), and leads to increased levels of liver enzymes.

These findings are in line with previous reports showing toxic effects of BU in rat testis, including changes in sperm parameters and spermatogenesis along with pro-apoptotic BU potential in murine male germ cells (3, 13, 19). BU could induce oxidative damage to the testis (20). In addition to, BU is an alkylating agent that by attaching to double strand DNA could prevent DNA replication and RNA transcription leading to stem cell death. These could explain inhibition of spermatogenesis process in the present study. In the present study, sperm motility decreased non-significantly in BU-exposed rats, and also sperm tail abnormality was higher than those of the other groups. ROS can attack and damage bio-molecules such as DNA and lipids. As the sperm plasma membrane has a high content of polyunsaturated fatty acids, sperms are highly susceptible to oxidative stress. Oxidative stress induced by BU could affect the polyunsaturated fatty acids in the tail membrane of the sperm cell, disturb its fluidity and lead to a reduction in sperm motility (21). Also, previous studies showed that lenght of the sperm flagella reduces in rats that received BU, leading to decreased sperm motility (13).

In agreement with our data, Anjamrooz et al. (3) reported that sperm count, viability, and motility markedly decline after exposure to BU even at its lowest dose (10 mg/kg) and exposure period (four weeks) when compared to CTL. The destructive effect of BU on germ cell vitality was also observed. Apoptosis is programmed cell death characterized by some distinct changes in cell morphology and genetic material.

This process could occur under normal conditions of organs or abnormal situations such as chemical-induced cell death. Under normal conditions, apoptosis could happen during normal spermatogenesis to balance the ratio of germ cells and sertoli cells number in testicular tissue (22). This appropriate rate of apoptosis is most commonly seen in SG (A2, A3 and A4 stages). Abnormal conditions such as administration of cytotoxic agents, for example BU, could cause abnormal rate of apoptosis in germ cells, spermatocyte, spermatid cells and SG (23) and lead to pathological condition.

This study, in accordance with another study (22), showed that after BU administration, the number of apoptotic SG and spermatocyte cells in rat testis tissue significantly increase. This cytotoxic agent could induce germ cell apoptosis afterward direct BU-induced damage to the germ cells by reduction of the expression level of c-kit as a survival factor, in SG (24) or indirectly via inducing apoptosis in sertoli cells (25) by increasing ck18 level (a death factor) in these testicular supporting cells. However, due to marked dependence of the germ cells on the function of the sertoli cells, the apoptosis of these supporting cells can also endanger the germ cells vitality. Also, it was suggested that BU increases malondialdehyde (MDA, a marker oxidative stress and lipid peroxidation) level. Therefore, BU-induced oxidative stress might be a reason of germ cell death, spermatogenesis disturbance and infertility (2). With increasing incidence of cancer throughout the world, the use of cytotoxic and anti-cancer drugs are increased. Therefore, many studies today focus on increasing fertility potential after exposure to cytotoxic agents.

Nowadays, plant extracts as sources of antioxidants and phenolic compounds have attracted considerable attention. Several studies reported the improving effect of various plant extracts on BU-induced testis toxicity (1). In this study, for the first time, we examined at the effects of different doses of OLE in BU-treated animals.

OLE contains different types of polyphenolic compounds including simple phenols such as gallic acid, flavonoids such as rutin and secoiridoids such as oleuropein at different concentrations (26). Among phenolic compounds present in OLE, oleuropein, luteolin and hydroxytyrosol have powerful antioxidant activities (27). It was reported that administration of 300 mg/kg OLE markedly decreases testis MDA level and improves sperm parameters (10). In the present study, administration of OLE 250 and 500 mg/kg might cause a reduction in BU-induced ROS production, lipid peroxidation and stress oxidative in testis and therefore markedly modulated or repaired the sperm and testicular defects. Similarly, Sarbishegi et al. (10) reported that OLE 150 and 300 mg/kg (not 75 mg/kg), improve the sperm quality and testis oxidative stress after rotenone exposure. Oleuropein, one of the phenolic constituents of olive leaf, was shown to exert ameliorating effects on alcohol-induced oxidative stress in male rat testis and improve sperm parameters (5). Inconsistent with our study, another study showed that administration of olive fruit extract to rats have a negative effect on sperm parameters (28). This might be explained by different dosage and duration of treatment.

In the current study, a decrease in the number of apoptotic germ cells also observed when OLE 250 and 500 mg/kg were administrated to Wistar rats treated with a single dose of BU. OLE acts as an anti-apoptotic agent via decrement of the expression level of caspase 3, a death factor that could initiate apoptotic DNA fragmentation and promote apoptosis. It could also reduce the BAX/BCL2 ratio. Therefore, it seems that OLE inhibits the apoptotic pathway via reduction of pro-apoptotic proteins and improves cell vitality (29). As another mechanism, it was suggested that OLE increases antioxidant capacity due to high content of flavonoids and phenols therefore could directly scavenge free radicals (30) and/or diminish oxi-

dative stress via increasing superoxide dismutase (SOD) and decreasing MDA levels (31). The demotion of these oxidative markers improved spermatogenesis and fertility potential.

However, OLE 750 mg/kg (the highest dose used in the present study) did not show a markedly higher efficacy compared to the other doses. Although all doses of OLE could significantly improve the number of PS and Leydig cells when compared to BU, but the numbers of spermatogenic cells and Leydig cells in the testes of the BU+OLE 750 group were not higher than those of the other doses of OLE. Also, the epithelium height in BU+OLE 750 group was the lowest. Furthermore, administration of OLE 750 mg/kg produced high apoptotic germ cell counts, even higher than BU. This data demonstrated that OLE 750 mg/kg not only failed to attenuate BU-induced testicular apoptosis but also worsened the BU impact. Similarly, several studies have shown that higher doses of herbal extracts may have adverse effects on organs. It was reported that OLE has a negative effect at high doses (0.75 and 0.50%) on rat liver tissue (32). Wang et al. (33) showed that administration of OLE (250, 500 and 1000 mg/kg) improves apoptosis ratio on lead-induced damage in brain cortex. Unlike our data, they demonstrated that the highest dose of OLE (1000 mg/kg) was the most effective. However, further molecular and antioxidant studies are needed in order to determine the exact mechanism underlying the effects of different doses of OLE on BU-induced toxicity.

Traditional herbs usage for therapeutic purposes has never guaranteed the safety of these plant. The liver is one of the most important organs in the uptake, metabolism, and elimination of drugs; therefore, in this study, in order to monitor possible toxicity of different doses of this extract on the liver, liver enzymes were studied. In the present study, in line with toxic effects of OLE 750 mg/kg on the testis, an increase in liver enzyme levels in rats that received OLE 750 mg/kg, indicated liver damage (34) and suggested that this dose of OLE could be toxic. In addition, inflammation was observed in the liver of rats that received OLE 750 mg/kg. However, such changes are not seen following administration of other doses of OLE.

Al-Attar and Abu Zeid (14) reported that diazinon could increase liver enzyme levels; in addition, administration of OLE 400 mg/kg to male mice exposed to diazinon could decline the levels of the mentioned enzymes but it did not reach the levels of the controls. In the current study, no statistically significant difference was observed in liver enzymes levels between rats which received OLE 500 mg/kg for five weeks and the control and BU groups. However, some studies, in agreement with our data, showed that administration of olive extract at high doses may be associated with liver damage. Omer-Sawsan showed that OLE at high concentrations (0.9%) increased ALP, LDH, and AST enzymes after seven and 14 weeks of treatment. They showed that this effect of OLE is dose-dependent (35). Arantes-Rodrigues et al. (32) demonstrated that different concentrations OLE (0.5 and 0.75% m/m) may have negative effects on liver function and even cause liver cirrhosis.

Previous studies demonstrated that the level of testosterone is different between control and BU groups (13). On the Contrary, in this study, there was no significant difference in total testosterone level among all groups. However, in this study, destruction rate of Leydig cells was higher in BU-treated than the control testes, while the number of Leydig cells increased significantly by all three doses OLE compared to BU-exposed testes. On the basis of our findings, we cannot attribute increased Leydig cell count in OLE-treated rats to the unchanged testosterone level.

## Conclusion

This study, for the first time, showed that administration of two doses of OLE (250 and 500 mg/kg), to Wistar rats could improve BU-impaired spermatogenesis and sperm quality without inducing liver damage. However, OLE 750 mg/kg not only had no ameliorating effect on testis and sperm parameters in BU-exposed animals, but also increased apoptosis rate in the germ cell and enhanced liver enzymes that indicate a liver damage and probable dysfunctions of other important organs.

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## Authors' Contributions

S.G.H.; Performed data collection and manuscript writing. F.S.; Contributed to plant standardization. T.H.; Performed data analysis and manuscript editing. A.B.; Contributed to study design. S.H.E-V.; Contributed to study design and co-edited the article. All authors read and approved the final manuscript.

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Original Article

# The Relationship between Perceived Stress and Marital Satisfaction in Couples with Infertility: Actor-Partner Interdependence Model

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## Abstract

**Background:** Infertility, one of life's great stressors, may adversely affect marital satisfaction. No studies have investigated the relationship between perceived stress and marital satisfaction at the dyadic level. The current study assessed the actor and partner effects of perceived stress on marital satisfaction in husband-wife dyads using an innovative dyadic analysis approach, the Actor-Partner Interdependence Model (APIM).

**Materials and Methods:** In this cross-sectional study, we recruited a total of 141 infertile couples. Marital satisfaction and stress were assessed using the ENRICH Marital Satisfaction Scale (EMS Scale) and Perceived Stress Scale-4 Item (PSS-4), respectively. Dyadic data have been analysed by the APIM approach, with distinguishable dyads. In this approach, actor effect is the impact of a person's perceived stress on his/her own marital satisfaction. Partner effect is the impact of a person's perceived stress on the partner's marital satisfaction.

**Results:** Both men and women's perceived stress exhibited an actor effect on their marital satisfaction ($\beta$=-0.312, P<0.001, $\beta$=-0.405, P<0.001, respectively). Women's perceived stress had a negative relationship to the marital satisfaction of their partner ($\beta$=-0.174, P=0.040). Although the partner effect of men's perceived stress on woman's marital satisfaction was not significant ($\beta$=-0.138, P=0.096), women whose husbands had higher levels of stress were more likely to have poorer marital satisfaction. Both actor and partner effects of perceived stress on marital satisfaction were similar among men and their wives.

**Conclusion:** The findings of this study have highlighted that marital satisfaction in patients with infertility was influenced by not only their own perceived stress, but also their spouses' perceived stresses. Therefore, psychological interventions that target a reduction in perceived stress and enhancement of marital satisfaction in the context of infertility should treat the couple as a unit.

***Keywords:*** Actor-Partner Interdependence Model, Infertility, Marital Satisfaction, Stress

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## Introduction

Infertility is medically defined as "the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (1). It is a public health concern that affects 9% of reproductive-aged couples worldwide (2). Infertility has been ranked as one of the great stressors in life and has a considerable impact on a person's quality of life (3, 4). Infertility is negatively related to personal and marital health among infertile couples since it signifies one's loss of ability to achieve parenthood (5). Infertile people experience more stress related to both infertility as a disease and its treatments when compared to fertile people (6). In addition, numerous researches have shown a negative association of stress with marital satisfaction (7, 8) and a relationship to a range of adverse health outcomes (9). For these reasons, this concept has received increased attention in marital studies in recent years. Studies have focused on different types of stress (e.g., internal vs. external, minor vs. major, and chronic vs. acute) and two key theoretical models (family and couples' stress models). They have indicated that the role of stress is detrimental to the quality and longevity of a relationship (10).

Many of the phenomena studied by scientists in social and behavioural sciences are dyadic in nature and include research on man-woman dyads and parent-child dyads. The observations that arise from such designs are interdependent rather than independent; however, in this case, independence refers to independence from dyad to dyad (11,

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12). Statistically, conventional parametric statistics developed for independent individuals are not appropriate for non-independent observations. Instead, the non-independence due to the dyadic nature of data must be taken into account when relationships are examined. An example of non-independence is the characteristic or behaviour of one person that affects his or her partner's outcomes; therefore, an analysis that takes non-independence into account is required. The Actor-Partner Interdependence Model (APIM), an innovative dyadic analysis approach, simultaneously estimates the effects an individual's characteristics and the partner's characteristics on an outcome variable. The APIM approach uses the dyad, not the individual, as the sampling unit. This approach provides separate, but simultaneous estimates of actor and partner effects (12). The actor effect measures the degree to which one's own characteristics impacts his/her own outcomes, whereas the partner effect measures the degree to which one individual is influenced by the other individual or the partner.

Most studies that investigate the relationships between psychological distress and marital satisfaction in couples with infertility use the individual as the unit of analysis. Although valuable, these researches fail to show the impact that partner distress has on individual marital satisfaction. Since infertility is a shared problem, it is particularly relevant to examine the impact of partner distress (13). Perceived stress by the husband or wife does not only affect his/her own marital satisfaction, but also their partner's marital satisfaction. Therefore, the current study has aimed to examine whether differences existed in the levels of perceived stress and marital satisfaction between men and women dyads with infertility. We also used the APIM approach to elucidate and differentiate actor effects and partner effects of perceived stress on marital satisfaction.

## Materials and Methods

### Participants and study design

This was a cross-sectional study of a sample of couples with infertility from Tehran, Iran. Patients were recruited from the Infertility Treatment Centre of Royan Institute, a referral centre for infertility treatment in Tehran, Iran (14). The data were collected using the convenience sampling method between February and May 2017. Couples who met the following criteria were included in the present study: i. Married and in a heterosexual relationship, ii. Willingness to participate in the study, iii. Presence of fertility problems, iv. Age >18 years, and v. Ability to read, write, and comprehend Persian. The couples with infertility were asked to fill out the questionnaires separately from each other and refrain from discussing their answers. In total, 141 couples with infertility agreed to participate and completely filled out the questionnaires (response rate: 82.3%).

### Ethical consideration

The Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran, approved this study. The participants were informed of the aim of the study and were assured of confidentiality. After signing a consent form and agreement to participate, the couples with infertility completed the questionnaires.

### Questionnaires

#### Ten item ENRICH Marital Satisfaction Scale (EMS Scale)

The ENRICH Marital Satisfaction Scale (EMS Scale) is a 10-item self-report instrument designed to measure marital satisfaction (15). Each item is scored on a 5-point Likert scale as follows: 1 (strongly disagree), 2 (moderately disagree), 3 (neither agree nor disagree), 4 (moderately agree), and 5 (strongly agree). Total scores range from 10 to 50; higher scores are indicative of greater marital satisfaction. The Persian version of the EMS Scale has been shown to have good psychometric properties (16). For this study, the Cronbach's alpha coefficient of the EMS Scale was 0.771.

#### Perceived Stress Scale-4 item (PSS-4)

The Perceived Stress Scale-4 item (PSS-4) is a short form of the PSS that measures the degree to which situations in one's life over the last month are appraised as unpredictable, uncontrollable, and overloaded. Each item is scored on a 5-point Likert scale that ranges from 0 (never) to 4 (very often). Total scores range from 0 to 16, with higher scores indicating higher levels of stress (17). The Persian version of PSS has been shown to have good psychometric properties (18, 19). For this study, the Cronbach's alpha coefficient of the PSS-4 was 0.572.

### Statistical analysis

Comparison of demographics characteristics, perceived stress, and marital satisfaction for husbands and wives were made using the McNemar test and paired sample t test. Pearson's correlation coefficient was used to examine the correlation among the study variables.

We used the APIM with distinguishable dyads to determine the impact of husbands' and wives' perceived stresses on their own marital satisfaction, as well as their spouse's marital satisfaction (12). Figure 1 depicts the APIM of a husband-wife dyad in which there are two variables from each in the dyad: perceived stress (independent variable) and marital satisfaction (outcome variable). The husband's level of marital satisfaction is affected by his own level of perceived stress (actor effect, $A_m$) and by his wife's perceived stress (partner effect, $P_{mf}$). Similarly, the wife's level of marital satisfaction is influenced by her own perceived stress (actor effect, $A_f$) and her husband's perceived stress (partner effect, $P_{fm}$). There are two important correlations in the model. The curved line that connects the independent variables indicates how similar the partners are on the predictor variables and the correlation between the error or residual terms ($E_m$ and $E_f$), which represents the non-independence that is not explained by the APIM.

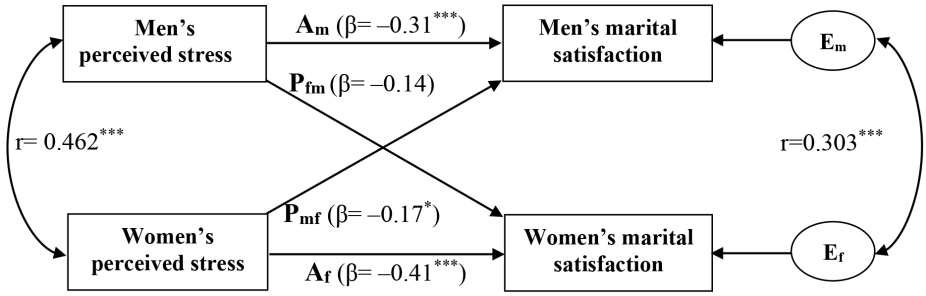


**Fig.1:** Actor-Partner Interdependence Model (APIM) of perceived stress and marital satisfaction.
$A_m$; Actor effect of husband's perceived stress on his own marital satisfaction, $A_f$; Actor effect of wife's perceived stress on her own marital satisfaction, $P_{fm}$; Partner effect of the husband's perceived stress on his wife's marital satisfaction, $P_{mf}$; Partner effect of the wife's perceived stress on the husband's marital satisfaction, $E_m$ and $E_f$; Residual errors on marital satisfaction for men and women, respectively, *; P<0.05, and ***; P<0.001.

Three different methods can be used to estimate the APIM: pooled regression modelling, multilevel modelling, and structural equation modelling (SEM). According to Kenny et al. (12), SEM with distinguishable dyads is the simplest data analytic method to estimate the APIM. The SEM approach involves estimating the APIM parameters as they appear in the model presented in Figure 1. Based on the dyad-level structure, there are two linear equations:

$$Y_m=A_m X_m+P_{mf} X_f+E_m,$$
$$Y_f=A_f X_f+P_{fm} X_m+E_f,$$

where $Y_m$ is the husband's marital satisfaction, $Y_f$ is the wife's marital satisfaction, Xm is the husband's perceived stress, and $X_f$ is the wife's perceived stress. In the first equation, $A_m$ refers to the effect of the husband's perceived stress on his own level of marital satisfaction (actor effect) and the partner effect, $P_{mf}$, is the effect of the wife's perceived stress on her partner's marital satisfaction. Since the dyad is the unit of analysis, the sample size in this analysis is the number of couples (n=141).

A useful attribute of SEM approach is that it allows model constraints to be placed and tested in the APIM framework. For example, this approach can test whether the husband's actor effect is equal to the wife's actor effect ($A_m=A_f$) and subsequently measure the degree to which this constraint significantly worsens the model fit (12, 20). The equality constraint test has been used to compare actor effects for men and women by examination of the chi-square difference test. If the chi-square difference test is statistically significant, it indicates that the actor effects for men and women cannot be the same.

In order to compute a chi-square difference test, the difference of the chi-square values of the two models (constrained and unconstrained) in question is taken as well as the difference of the degrees of freedom.

$$\chi^2_{diff}=\chi^2_{constrained}-\chi^2_{unconstrained}$$
$$df_{diff}=df_{constrained}-df_{unconstrained}$$

In the current study, all preliminary analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA). APIM analysis was performed using Mplus software version 6.12 (Muthén and Muthén, Los Angeles, CA, USA).

## Results

### Characteristics of dyads for men and women

The demographic and clinical characteristics of the men and women dyads are presented in Table 1. On average, husbands were 5.10 years older than their wives ($t_{(140)}$=12.88, P<0.001) and they had a similar education level as their wives ($\chi^2_{(1)}$=2.56, P=0.109). The mean duration for marriage was 7.37 ± 4.40 years and for infertility, it was 4.85 ± 3.76 years. The causes of infertility were as follows: male factor (36.2%), female factor (21.3%), both (19.1%), and unexplained (23.4%). The majority of the couples had primary infertility (72.3%) and no history of abortion (76.6%). Half experienced at least one failure in previous ART treatments.

### Marital satisfaction and perceived stress in dyads for men and women

As presented in Table 2, the marital satisfaction scores for the husbands and their wives were similar ($t_{(140)}$=0.09, P=0.925), but the women had greater perceived stress compared to their husbands ($t_{(140)}$=2.06, P=0.042).

Perceived stress in husbands was correlated with both their own marital satisfaction (r=-0.393, P<0.001) and their wives' marital satisfaction (r=-0.325, P<0.001). Perceived stress in wives was also correlated with both their own marital satisfaction (r=-0.469, P<0.001) and the husband's marital satisfaction (r=-0.319, P<0.001) (Table 3).

**Table 1:** Demographic and clinical characteristics of the men and women dyads (n=141 couples)

| Variable | Men | Women | Test statistic | P value |
|---|---|---|---|---|
| Age (Y) | 34.92 ± 6.35 | 29.82 ± 6.00 | $t_{(140)}$=12.88 | <0.001 |
| Educational level | | | $\chi^2_{(1)}$=2.56 | 0.109 |
| Non-academic | 96 (68.1) | 85 (60.3) | | |
| Academic | 45 (31.9) | 56 (39.7) | | |
| Duration of marriage (Y) | 7.37 ± 4.40 | - | | |
| Duration of infertility (Y) | 4.85 ± 3.76 | - | | |
| Cause of infertility | | | | |
| Male factor | 51 (36.2) | - | | |
| Female factor | 30 (21.3) | - | | |
| Both | 27 (19.1) | - | | |
| Unexplained | 33 (23.4) | - | | |
| Failure of previous treatment | | | | |
| No | 71 (50.4) | - | | |
| Yes | 70 (49.6) | - | | |
| History of abortion | | | | |
| No | 108 (76.6) | - | | |
| Yes | 33 (23.4) | - | | |
| Type of infertility | | | | |
| Primary | 102 (72.3) | - | | |
| Secondary | 39 (27.7) | - | | |

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

**Table 2:** Comparisons in marital satisfaction between men and women, and perceived stress (n=141 couples)

| Variable | Men | Women | $t_{(140)}$ [a] | P value |
|---|---|---|---|---|
| Perceived stress | 5.83 ± 2.80 | 6.33 ± 2.81 | 2.06 | 0.042 |
| Marital satisfaction | 39.31 ± 6.56 | 39.26 ± 6.70 | 0.09 | 0.925 |

**[a]; Test statistic. Values are presented as mean ± SD.**

**Table 3:** Correlations among predictors and outcomes in dyads for men and women (n=141 couples)

| Variable | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 Perceived stress in males | 1 | | | |
| 2 Marital satisfaction in males | -0.393 | 1 | | |
| 3 Perceived stress in females | 0.462 | -0.319 | 1 | |
| 4 Marital satisfaction in females | -0.325 | 0.423 | -0.469 | 1 |

**All correlations were significant at the 0.001 level.**

## Impact of perceived stress on marital satisfaction at the dyadic level

According to Table 4 the results for the APIM indicated that the husband's perceived stress as well as the wife's perceived stress exhibited an actor effect on their marital satisfaction (β=-0.312, P<0.001, β=-0.405, P<0.001, respectively). With regard to partner effects, only the woman's perceived stress had a partner effect on the husband's marital satisfaction (β=-0.174, P=0.040). Although the partner effect of the husband's perceived stress on the wife's marital satisfaction was not significant (β=-0.138, P=0.096), women whose husbands had higher levels of stress were more likely to have poorer marital satisfaction.

We used the equality constraint tests to compare actor effects as well as partner effects for men and women by examination of the chi-square difference test. Constraining the actor effects to be equal did not significantly worsen the model fit ($\chi^2_{(1)}$=0.60, P=0.437), which indicated that the actor effects of perceived stress on marital satisfaction were similar for men and women. The same findings were also observed in the partner effects ($\chi^2_{(1)}$=0.07, P=0.795).

**Table 4:** Actor and partner effects of perceived stress on marital satisfaction in couples with infertility (n=141)

| | Men | | | Women | | |
|---|---|---|---|---|---|---|
| | β (95% CI) | t[a] | P value | β (95% CI) | t[a] | P value |
| Actor's stress | -0.31 (-0.48, -0.15) | 3.77 | <0.001 | -0.41 (-0.56, -0.25) | 5.22 | <0.001 |
| Partner's stress | -0.17 (-0.34, -0.01) | 2.05 | 0.040 | -0.14 (-0.30, 0.02) | 1.67 | 0.096 |

**[a]; Test statistic.**

## Discussion

To the best of our knowledge, this study is the first of its kind to use the APIM to examine the impact of actor and partner stress on marital satisfaction in a sample of couples with infertility. Although the majority of researches that have examined psychological distress and marital satisfaction both in infertile and fertile couples assessed the actor effect of stress on marital satisfaction, there are increasing calls to investigate the partner effect of these variables. Since infertility is a shared problem within the couple, both men and women need to be involved and considered as a dyad.

As expected, perceived stress among the wives was higher than their husbands, which suggested that women tend to perceive stressful life events as less controllable than men and generally seem to be more affected in terms of negative life consequences. Another explanation for this difference could be that generally, particularly in Iran or Middle Eastern countries, childbirth is considered the women's duty and infertility is considered a disease in women. The burden of infertility is mostly on women. This result has supported the findings of previous studies (21, 22). Consistent with a study by Peterson et al. (22), marital satisfaction was unrelated to gender. In a study conducted among couples with infertility in Poland, women had worse marital satisfaction than men (23).

The current study has found the actor effect of perceived stress on marital satisfaction. In other words, the greater level of stress that is perceived by either men or women contributes to lower marital satisfaction for themselves. This is in line with a study of patients with infertility in France, in which the predictive effects of infertility-related stress on both emotional and marital distress have been confirmed (21). Additionally, in a review based on 24 empirical studies, different types of stressors were associated with marital satisfaction and its longevity (10).

The most important finding of the current study was the link between an individual's perceived stress and their partner's marital satisfaction. In accordance with our expectation, we found that a woman's perceived stress negatively impacted the man's marital satisfaction. Contrary to our expectation, our study did not confirm a strong partner effect of a man's perceived stress on marital satisfaction, although the impact of the husband's perceived stress on his wife's marital satisfaction was marginal.

Our results indicated that the actor effects and partner effects of perceived stress on the marital satisfaction were similar for both men and women. Although the levels of perceived stress differed between men and women, the associations between stress and marital satisfaction were not substantially different between them. This finding might indicate that both members of couples with infertility share a similar mechanism through which perceived stress influences marital satisfaction.

This study has several limitations that should be considered when interpreting the results. First, the generalizability of the results might be affected by the fact that it was a single-centre study with a relatively small sample size. Second, because of the cross-sectional nature of the study design, causal inferences could not be made. In addition, this study relied on self-reported data that might be prone to social desirability bias. Despite these limitations, this study has provided valuable information regarding the actors and partner effects of perceived stress on marital satisfaction in men-women dyads that experience infertility.

## Conclusion

The findings demonstrate that partner effects are present in couples with infertility and support the idea that a person's perceived stress can impact his or her partner's marital satisfaction. Psychological interventions that target a reduction of perceived stress and enhancement of marital satisfaction in the context of infertility should treat the couple as a unit.

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## Authors' Contributions

S.M., M.H.; Study design and conception, data analysis and interpretation, and manuscript writing. A.R.F.; Study design and conception, manuscript editing. R.O.-S.; Study design and conception, data acquisition, data interpretation, and manuscript editing. P.A.; Study design and conception, data acquisition, and manuscript editing. All authors approved the final version of the manuscript for submission.

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Original Article

# Impact of Endometriosis-Related Adhesions on Quality of Life among Infertile Women

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## Abstract

**Background:** Endometriosis is considered the most common cause of pelvic adhesions in women. Endometriosis-associated adhesions could result in the formation of fibrous bands, which contain endometriotic glands, stroma and scarring. The aim of this study was to identify the impact of endometriosis-related adhesions on quality of life among infertile women.

**Materials and Methods:** This descriptive study was conducted at Endoscopic Unit, in Zagazig University Hospitals, Egypt. Oral consent for participation in this study was taken from 109 women who were candidates for laparoscopy as infertile cases and were diagnosed with endometriosis. They were classified into two groups namely, group I (n=41) who had endometriosis with adhesions and group II (n=68) who had endometriosis without adhesions. A structured interviewing form, adhesion scoring method of the American Fertility Society, and Global Quality of Life Scale were used to collect required information.

**Results:** The prevalence of adhesions resulted from endometriosis was 37.6%. Demographic characteristics of the women with endometriosis-related adhesions were not significantly different from those of women without endometriosis-related adhesions. The most common location for endometriotic adhesions was adnexal adhesion (51.2%) followed by adhesion of anterior abdominal wall (24.4%). Quality of life was significantly impacted by endometriosis-related adhesions (P=0.002).

**Conclusion:** A high percentage of studied patients had a moderate degree of adhesions. Adhesions caused by endometriosis had an impact on quality of life of the studied women.

***Keywords:*** Adhesions, Endometriosis, Impact, Infertility, Quality of Life

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## Introduction

Endometriosis is a chronic gynecological condition in which, endometrial tissue is found outside the uterine cavity. It is a relatively common disorder among women of reproductive-age and is associated with marked pain and morbidity (1). The definite cause of endometriosis is unknown, but the theory of retrograde menstruation has received the most attention. It causes endometrial tissue to reflux through the uterine tubes into the peritoneum (2).

Endometriosis is considered one of the main underlying causes of the development of adhesions unrelated to a previous operation (3). Adhesions are bands of connective tissue, which connect two different tissues that are normally separated thus, interfering with the function of the organs that are affected (4).

Local inflammation associated with endometriosis is viewed as an important element in the formation of adhesions. Adhesions may form as a result of endometrial implants bleeding into the surrounding area and causing an inflammatory reaction which leads to the formation of a band between two organs. Adhesions correlated with endometriosis have different types (i.e. thin, filmy and transparent or thick, dense, and opaque). In severe cases, adhesions found within the pelvis, could cause a fatal condition called "frozen" pelvis (5).

There are many complications associated with endometriosis-related adhesions such as dyspareunia, and rectal constriction which leads to constipation. Also, in this sense, adhesions correlated with endometriosis, are responsible for infertility, chronic pelvic pain, and bowel obstruction (6).

Women with pelvic adhesions should be informed about the risk associated with endometriosis-related adhesions

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and instructed how to deal effectively with this condition (7). There are several studies reporting endometriosis-related adhesions that affected women's life physically, mentally and socially in the form of social withdrawal, fatigue, lack of enthusiasm for their work, decreased libido, negative self-image, pessimistic attitude and worthlessness (8).

The study aimed to identify the impact of endometriosis-related adhesions on quality of life among infertile women.

## Materials and Methods

The present descriptive study was conducted to identify the impact of adhesions associated with endometriosis on quality of life among infertile women. The study was performed at Endoscopic Unit, in Zagazig University Hospitals in Egypt. This research was conducted after getting permission from the director of Faculty of Nursing-Zagazig University and director of Endoscopy Unit.

These cases were chosen from more than 756 cases from December 2016 to March 2018. Among 756 cases, only 109 women were candidates for laparoscopy due to infertility issues and were diagnosed with endometriosis. According to the revised American Society for Reproductive Medicine (r-ASRM) classification of endometriosis (9), we recruited all the patients with stage III (moderate) endometriosis. Women were eligible for recruitment in this study if they met the following criteria: married women with primary or secondary infertility, women diagnosed with endometriosis with adhesion based on laparoscopy and also, women who desired to participate in this study.

### Exclusion criteria

i. Existence of adhesions due to other reasons such as pelvic inflammatory disease (PID) or myoma or adenomyosis, ii. Existence of chronic pelvic pain that defined as pelvic pain which is constant or cyclical in nature for 6 months or more, iii. Women with previous surgery in the abdomen or pelvis (cesarean section or appendectomy), and iv. Women with autoimmune and/or allergic disease.

Oral consent was taken from women who desired to participate in this research. All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee as well as the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Zagazig University-Faculty of Nursing Ethical Committee with the ethical code ZU.NUR/25/22-8-2016.

After revising the laparoscopic report details for recruited women (n=109), participants were grouped into group I (n=41) with subjects who had endometriosis with adhesions and group II (n=68) with subjects who had endometriosis without adhesions.

The tool used for data collection in this research was a structured interview form which was designed by the researchers in order to gather the following data: demographic characteristics as; age (years), body mass index (BMI) (Kg/m$^2$), educational level and occupational status. Also, using this tool, the following variables were recorded: duration of the marriage (years), menarche age (years), contraception type and family history of endometriosis.

Adhesions detected during laparoscopy were divided regarding the locality into adenxal adhesion, anterior abdominal wall adhesion, vesico-uterine adhesion, uterus to abdominal wall adhesion, and frozen pelvis.

Adhesions were classified in terms of severity by grading using adhesion scoring method of the American Fertility Society (AFS) (10): i. Mild adhesions, which are thin, filmy, vascular, and transparent adhesions that are easily cut with blunt dissection and subsequently easily freeing adherent organs, ii. Moderate adhesions, which are opaque, possess moderately thick layers, exert moderate degrees of vascularity and bleed minimally on dissection, and iii. Severe adhesions, which are very thick, opaque, and highly vascular and bleed much on dissection.

Validated language version of the Global Quality of Life Scale (GQOL) was used to measure the quality of life for women with adhesions related to endometriosis. The Global Quality of Life Scale is a single scale that directly evaluates the quality of life by patients themselves by using a scale between 0 (='no quality of life') and 100 (='perfect quality of life'). Also, in this scale, each patient was asked to describe her life quality by writing a number between 0 and 100 (11). Global quality of the life scale is the person's overall judgment of his/her life quality, so we particularly used this tool to assess the impact of adhesions on the women's quality of life who were not able to return to hospital again after performing the laparoscopic procedure. The tool was tested for content validity and reliability by five professors in the field of Obstetrics and Gynecological Medicine.

Data were gathered and outcome measures were coded, entered into and analyzed by Microsoft Excel program. Statistical analysis was done with IBM SPSS Statistics V. 20 (SPSS Inc., Chicago, IL, USA). According to the type of data, qualitative data were presented as number and percentage, continues quantitative data were represented as mean ± SD; also, the following tests were utilized to test differences between groups; difference and association of qualitative variables were tested by Chi-square test ($X^2$) and differences between quantitative independent groups were assessed by t test. A $P<0.05$ was regarded as statistically significant. Also, cases who received a score ≤40 in global quality of life scale were considered the cases whose quality of life was affected and cases with a score ≥ 45 in global quality of life scale, were considered the cases whose quality of life was not affected. We considered in our study score 40 or less to be impacted on quality of life, according to classification suggested by Hyland and Sodergren (11).

**Table 1:** Demographic characteristics of women with and without endometriosis-related adhesions

| Variables | Group I (cases with endometriosis-related adhesions) (n =41) n (%) or (mean ± SD) | Group II (cases without endometriosis-related adhesions) (n=68) n (%) or (mean ± SD) | T test | P value |
|---|---|---|---|---|
| Age (Y) | 32.1 ± 5.6 | 31.7 ± 5.8 | 0.34 | 0.7 |
| Menarche age (Y) | 12.2 ± 1.4 | 12.6 ± 1.3 | 1.2 | 0.1 |
| Duration of marriage (Y) | 5.8 ± 1.5 | 5.6 ± 1.7 | 0.48 | 0.5 |
| BMI (Kg/m²) | 30.2 ± 4.5 | 29.5 ± 4.8 | 0.737 | 0.4 |
| | | | $X^2$ | |
| Educational level | | | | |
| Elementary education | 12 (29.3) | 19 (27.9) | 0.18 | 0.9 |
| Secondary education | 12 (29.3) | 18 (26.5) | | |
| College education or above | 17 (41.4) | 31 (45.6) | | |
| Occupational status | | | | |
| Worked | 10 (24.4) | 16 (23.5) | 0.01 | 0.9 |
| Not worked | 31 (75.6) | 52 (76.5) | | |
| Family history of endometriosis | | | | |
| No | 12 (29.3) | 21 (30.9) | 0.03 | 0.8 |
| Yes | 29 (70.7) | 47 (69.1) | | |
| Contraceptive type | | | | |
| None | 31 (75.6) | 40 (58.8) | 3.8 | 0.1 |
| Oral | 6 (14.6) | 21 (30.9) | | |
| IUD | 4 (9.8) | 7 (10.3) | | |

**BMI; Body mass index, IUD; Intrauterine device, T test; Independent samples t test, MCP; P value based on Mont Carlo exact probability, and $X^2$; Chi-square test.**

## Results

In this study, 109 cases with endometriosis were enrolled at the time of laparoscopy. Among them, 41 participants were found to have adhesions with endometriosis and 68 cases had no adhesions with endometriosis; thus, the prevalence of endometriosis-related adhesions was 37.6%.

The mean and the standard deviation (mean ± SD) of age, BMI, menarche age and duration of marriage of the women with and without endometriosis-related adhesions are presented in Table 1.There was no statistically significant difference in the afore-mentioned factors between the women with adhesions and the women without adhesions (P=0.7, 0.4, 0.1 and 0.5, respectively).

Also, based on data shown in Table 1, we found no correlations between the two groups regarding educational level, occupational status, family history of endometriosis and contraceptive type (P=0.9, 0.9, 0.8 and 0.1, respectively).

Table 2 reveals that the greater part of the studied women (51.2%) had adnexal adhesion followed by anterior abdominal wall adhesion 10 (24.4%) and vesico-uterine adhesion 5 (12.2%). Few patients (2.4%) had frozen pelvis. Concerning the severity of adhesions resulted from endometriosis, Table 2 indicates that almost all of the participated women had a moderate degree of severity 19 (46.3%) and only nine cases (22.0%) showed a severe degree of adhesions.

**Table 2:** Locations and grading of pelvic adhesions (n=41)

| Parameters | n (%) |
|---|---|
| Locations of adhesion | |
| Adnexal adhesion | 21 (51.2) |
| Anterior abdominal wall adhesion | 10 (24.4) |
| Vesico-uterine adhesion | 5 (12.2) |
| Uterus to abdominal wall adhesion | 4 (9.8) |
| Frozen pelvis | 1 (2.4) |
| Grading of the pelvic adhesion | |
| Mild | 13 (31.7) |
| Moderate | 19 (46.3) |
| Severe | 9 (22.0) |

Based on Table 3, it was found that the quality of life was significantly impacted by adhesions (P=0.002). Chi-square test ($X^2$) showed a significant association between impacted quality of life and adhesions that were resulted from endometriosis as 34.1% of cases with adhesions had a negatively influenced life while only 10.3% of cases without adhesions reported a negatively impacted quality of life.

**Table 3:** Association between adhesions related to endometriosis and quality of life (n=109)

| Variable | Group I (cases with adhesions related to endometriosis) (n=41) | Group II (cases without adhesions related to endometriosis) (n=68) | $X^2$ | P value |
|---|---|---|---|---|
| Quality of life | | | | |
| Not impacted | 27 (65.9) | 61 (89.7) | 9.3 | 0.002* |
| Impacted | 14 (34.1) | 7 (10.3) | | |

**Data are presented as n (%). $X^2$; Chi-square test, MCData P; P value based on Mont Carlo exact probability, and *; P<0.05 (significant).**

## Discussion

Endometriosis is a condition in which a multiple interplay between the shed endometrial tissue, the peritoneal environment, and the peritoneal lining occurs. When the peritoneum cannot remove the endometrial tissue in time, these tissues will have the chance to adhere to the peritoneal lining which finally leads to this disease (12). Formation of adhesions is a major complication of surgical treatment of endometriosis. Also, recent researches suggest that peritoneal inflammation, which may cause adhesions, occurs in the presence of active endometriosis (13).

Adhesions and endometriosis are connected together because endometriosis is an adhesiogenic disease. The nature of recurrence of endometriosis means that repeated surgical operations are usually performed, which in turn increase the chance of adhesion formation. So, the current research was performed to understand the impact of adhesions with endometriosis on the women's quality of life.

The prevalence of adhesion among women with endometriosis in the current study, was 37.6%. While, the study conducted by Parker et al. (14) showed that 74.0% of women had pelvic adhesions at the first surgery, and 82.0% of cases had adhesions at the time of second surgery. Of the 8 cases without a previous endometriosis surgery, 6 (75.0%) had adhesions at the first operation.

Concerning the demographic characteristics of patients, the two groups with and without adhesions were similar in age, occupational status, level of education and body mass index. Thus, these variables did not influence the frequency of adhesions in each group, nor the risk factors analyzed in the current study.

On the other hand, our study did not include any women with an extremely high BMI; mean and the standard deviation (mean ± SD) of BMI were 30.2 ± 4.5 in patients with adhesions associated with endometriosis and 29.5 ± 4.8 in the group of women without adhesions. The above-mentioned results were coinciding with those reported by Stocker et al. (15).

The majority of studies to date, have reported that early menarche (<11 years) increases the danger of endometriosis, but our results did not find any significant dif¬ference between the age of menarche and endome-triosis and the formation of adhesion. Peterson et al. (16) reported that there was no relation between endometriosis and history of menstrual cycle (i.e. length of the cycle, frequency of menstrual cycles per month, and age at menarche).

Use of contraception, as oral contraceptive pills (OCPs) and intrauterine contraceptive device (IUD), is also known to affect menstrual flow. If retrograde menstruation is involved in induction of endometriosis, usage of IUD (a common reason of menorrhagia) would be expected to increase the risk of the disease. Hughes et al. (17) mentioned that the use of IUD not influence the development of endometriosis. In other studies, OCPs exposure was associated with a lower risk of endometriosis (18). Our results indicate no significant difference between usage of contraceptive methods and adhesions with endometriosis in infertile women.

Adnexal area, anterior abdominal wall, bladder and uterus were the most common locations of adhesions in our research. These findings are different from several reports showing that adhesions are more often found in the omentum (19-21). Noticed difference can be due to the point that all cases in our study were women with endometriosis and the most affected areas of endometriosis are the ovaries followed by Douglas' pouch, uterosacral ligament, vesico-uterine pouch, serosal surface of the uterus, fallopian tubes, round ligament and rectovaginal septum (22).

In the current study, concerning the associations between endometriosis-related adhesions and quality of life, the results showed that the quality of life is significantly impacted by adhesions. In our study, it was noticed that all the cases with a severe degree of adhesions presented with poor quality of life. This finding was consistent with previous studies (23-25) which discussed that endometriosis and adhesions have significant negative effects on sexual intercourse, educational, social, and familial and professional aspects of the daily life of the patient. The pain, as well as mental and social dysfunction subsequently impairs the quality of life and lowers productivity of working women. Absolute cause or cure is not identified, so this disorder is considered to be chronic and recurrent. The suspected impact of such diseases on sexual relation and fertility has a persistent negative effect on patient's partnership.

This research had some limitations such as a decrease in the number of participants and samples' readiness in participating. Pain was not discussed in the present study, although it might affect the quality of life as we focused on the adhesions in cases of endometriosis. No long-term follow-up was done in this research as many of patients did not return to the hospitals after doing the procedure of laparoscopy.

## Conclusion

Based on the findings of the present study, it can be concluded that the prevalence of adhesions associated with endometriosis was 37.6%. Also, an association between adhesions related to endometriosis and quality of life among infertile women was found. Further researches might be conducted to study the same problems in larger populations of the women with long-term follow-up.

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## Authors' Contributions

A.I.A.E-K.; Attended all laparoscopic procedures and contributed extensively in interpretation of the data and the conclusion. M.L.M.; Performed all the laparoscopic procedure for participated women, data gathering, statistical data analysis, and interpretation of data. A.S.G., S.L.M.; Conducted the overall supervision and provided the first draft of the manuscript before its publication, participated in all the steps of research. All authors revised and approved the final manuscript.

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Short Communication

# Analysis of *PRM1* and *PRM2* Polymorphisms in Iranian Infertile Men with Idiopathic Teratozoospermia

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## Abstract

Single nucleotide polymorphisms (SNPs) in a number of genes involved in sperm maturation are considered as one of the main factors for male infertility. The aim of the present case-control study was to examine the association of SNPs in protamine1 (*PRM1*) and protamine2 (*PRM2*) genes with idiopathic teratozoospermia. In this case-control study, some SNPs in PRM1 (c.49 C>T, c.102 G>T and c.230A>C) and *PRM2* (rs545828790, rs115686767, rs201933708, rs2070923 and rs1646022) were investigated in 30 idiopathic infertile men with teratozoospermia (case group) in comparison with 35 fertile men (controls). Genotyping of SNPs was undertaken using polymerase chain reaction (PCR)-direct sequencing. For PRM1, c.230A>C, as a synonymous polymorphism, was detected in both teratozoospermic men (heterozygous n=26, homozygous minor n=1) allele frequency C(48) A(52) and controls (heterozygous n=15, homozygous minor n=4). All cases and controls were genotyped for rs545828790 in *PRM2*, a missense polymorphism, as well as rs115686767 and rs201933708, both of which synonymous variants. The findings showed an intronic variant in *PRM2* (rs2070923) was also present in both groups. Also, rs1646022, a missense polymorphism, occurred in teratozoospermic men (heterozygous n=10, homozygous minor n=5) and controls (heterozygous n=13, homozygous minor n=2). However, there were no significant differences in SNPs of *PRM1* and *PRM2* between the two groups, however, for c.230A>C, the frequency of the CA genotype was significantly higher in infertile men with teratozoospermia (P=0.001). We demonstrate that *PRM2* G398C and A473C polymorphisms were associated with the teratozoospermia and its genetic variation was in relation to semen quality, sperm apoptosis, and morphology in the Iranian population. This study is a preliminary study and presenting data as part of a future comprehensive study to clinically establish whether these gene polymorphisms are biomarkers for susceptibility to teratozoospermia.

***Keywords:*** Single Nucleotide Polymorphisms, Sperm, Teratozoospermia

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Genetic factors are responsible for 50% or more of male infertility etiology and nearly 7% of men suffer from infertility worldwide (1, 2). It is generally accepted that abnormalities in sperm chromatin and DNA are one of the main factors affecting pregnancy rates. Sperm DNA packaging, which occurs during spermiogenesis, is a unique process because of histone-protamine replacement.

Two types of protamines are known to exist in humans, amely protamine1 (PRM1) and protamine 2 (PRM2). The expression of this two proteins in the sperm nucleus is almost equal (3). The protamine proteins are characterized by an arginine-rich core and cysteine residues (4). Protamine has specialized in sperm for several reasons including more chromatin condensation, faster spermatozoa, effective oocyte fertilization, protecting the maternal genome from nuclease and toxins, permitting oocyte to reprogramme the paternal genome, and for having an imprinting pattern and reactivation upon fertilization (5).

Therefore, due to the importance of protamines in male fertility, many studies have shown altered expression of protamines in several groups of infertile men (3, 6-8). *PRM1* and *PRM2* are located on chromosome 16 and both genes contain a single intron (9). A number of reports have verified different variations in *PRM1* and *PRM2* sequences (NM_002761.2 and NM_002762.3) in humans with various associations with male infertility (10-13). Infertile men with high levels of abnormal sperm morphology are considered to teratozoospermia (14). There is some evidence that protamine mutations or polymorphisms may induce conformational changes the protein level, altering their incorporation into sperm chromatin thus leading to sperm defects. PRM deficiency causes sperm morphology defects, motility reduction, and infertility as a result of haploinsufficiency in mice (15, 16). *PRM1* variant rs35576928 is a single nucleo-

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tide polymorphism (SNP) that is present at a significantly higher frequency in infertile patients with non-obstructive azoospermia and altered morphology of the spermatozoa (17). Also, variants of *PRM1* and *PRM2* have been shown to be associated with male infertility and abnormal sperm morphology. A polymorphism in the *PRM1* promoter (−190 C→A) is known to increase *PRM1* to *PRM2* ratio (18-20). The aim of our study was to examine the association of SNPs in *PRM1* and *PRM2* with idiopathic teratozoospermia.

In this case-control study, semen samples were collected from 35 fertile men (control group) and 30 infertile men (case group) referred to our Andrology Lab at the Research and Clinical Center for Infertility of Yazd. Sperm samples were obtained after informed consent from the participants. A comprehensive evaluation was undertaken to identify the etiology of infertility including physical examination, smoking history, and reproductive hormonal assays. The infertile man was defined as a man who had no child after a period of unprotected intercourse for more than one year. The control group included fertile donors with one naturally conceived child during the past 12 months who also had normal semen parameters according to the recommendations of the World Health Organization (WHO, 2010). Heavy smokers (more than one pack of cigarettes per day during the past year), drug addicts, alcohol consumers, men with a history of varicocele and those aged more than 45 years were excluded from the study. Theliquefiedsemen of each man was evaluated for sperm parameters according to to WHO 2010 (14). Sperm morphology was evaluated using the strict criteria of Kruger et al. (21) and at least 200 cells were examined per slide. To determine the genetic status of protamine genes, the DNA sample was extracted using the salting out method from the peripheral blood of each individual (22). This study was approved by the Ethics Committee of the Yazd Research and Clinical Center for Infertility.

Genomic DNA isolation from peripheral blood samples was performed using the protease and phenol purification protocol (23). Each polymerase chain reaction (PCR) reaction consisted of 1-2 µl (100 ng) of DNA, 0.5-1 µl (0.2 µM) of each specified primer (Pishgam co., Iran) (Table 1) (11) and 12.5 µL of the 2X mastermix (amplicon) in a total volume 25 µL. The cycling conditions for PCR were an initial denaturation of DNA at 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 66°C for *PRM1* and 69°C for *PRM2* for 45 seconds, 72°C for 30 seconds, and a final extension of 10 minutes at 72°C. To verify fragment lengths, 2 µl of each PCR fragment was electrophoresed on a 1.5% agarose gel stained with SYBR DNA Safe Stain (Invitrogen, USA).

**Table 1:** Two primer pairs for amplification of the *PRM1* and *PRM2* genes

| Gene | Sequence primer (5'-3') | Product size (bp) |
|---|---|---|
| *PRM1* | F: CCCCTGGCATCTATAACAGGCCGC<br>R: TCAAGAACAAGGAGAGAAGAGTGG | 558 |
| *PRM2* | F: CTCCAGGGCCCACTGCAGCCTCAG<br>R: GAATTGCTATGGCCTCACTTGGTG | 599 |

After PCR, all of the PCR products were purified and sequenced on an Applied Biosystems 3730 XL DNA analyzer according to the manufacturer's instructions. Using designed primers (forward and reverse), the amplified products with sizes of 557 nucleotides (from -42 to 515) for *PRM1* and 599 nucleotides (from 49 to 648) for *PRM2* were sequenced. Chromatograms were 3 analyzed using Chromas 2 (Technelysium Pty. Ltd., South Brisbane, QLD, Australia).

In this study, we used SPSS 20 (SPSS Inc., Chicago, IL, USA) for all statistical analyses. The frequency of SNPs in *PRM1* and *PRM2* in case and control groups were compared using logistic regression. Differences between groups were examined using one-way ANOVA (followed by Turkey test) for sperm characteristics.

A total of 65 semen samples were examined in two groups. The mean age of participants was 35.21 ± 5.5 vs. 33.71 ± 4.5 years in the case and control groups respectively. In the case and control group, we observed three SNPs in *PRM1* and five SNPs in *PRM2*, namely C230A, G102T, and C49T in *PRM1*, and C288T, C401T, C248T, G398C, A473C, and G271C in *PRM2*. The PCR products were verified on agarose gels (Fig.1).

The frequency of these SNPs differed in groups of fertile and infertile men. The three SNPs G102T and C49T in *PRM1*, and C248T in *PRM2* were not observed in either group. Other SNPs were found in non-coding regions (Table 2).

Table 3 shows the association of the most frequent genotypes (three SNPs) with seminal characteristics of participants (Table 3).

Abnormal morphology, as well as sperm apoptosis (TUNEL+), was significantly elevated in the GG genotype compared with other genotypes in rs1646022 and rs2070923 in *PRM2*. However, in *PRM1* rs737008, the highest percentage of abnormal morphology, apoptotic sperms, and abnormal motility belonged to the AA genotype. There was no difference between genotypes regarding sperm protamine deficiency and sperm concentrations for all SNPs in *PRM1* and *PRM2* (Table 4).

In this study, infertile men with a history of defects at sperm head morphology and stretch of this region (tapered head) were analyzed for *PRM1* and *PRM2* polymorphisms and compared with fertile men. The findings showed an intronic variant in *PRM2* (rs2070923) which was also present in both groups. Also, rs1646022, a missense polymorphism, occurred in teratozoospermic men (heterozygous n=10, homozygous minor n=5) and controls (heterozygous n=13, homozygous minor n=2). However, there were no significant differences in SNPs of *PRM1* and *PRM2* between the two groups for c.230A>C, the frequency of the CA genotype was significantly higher in infertile men with teratozoospermia.

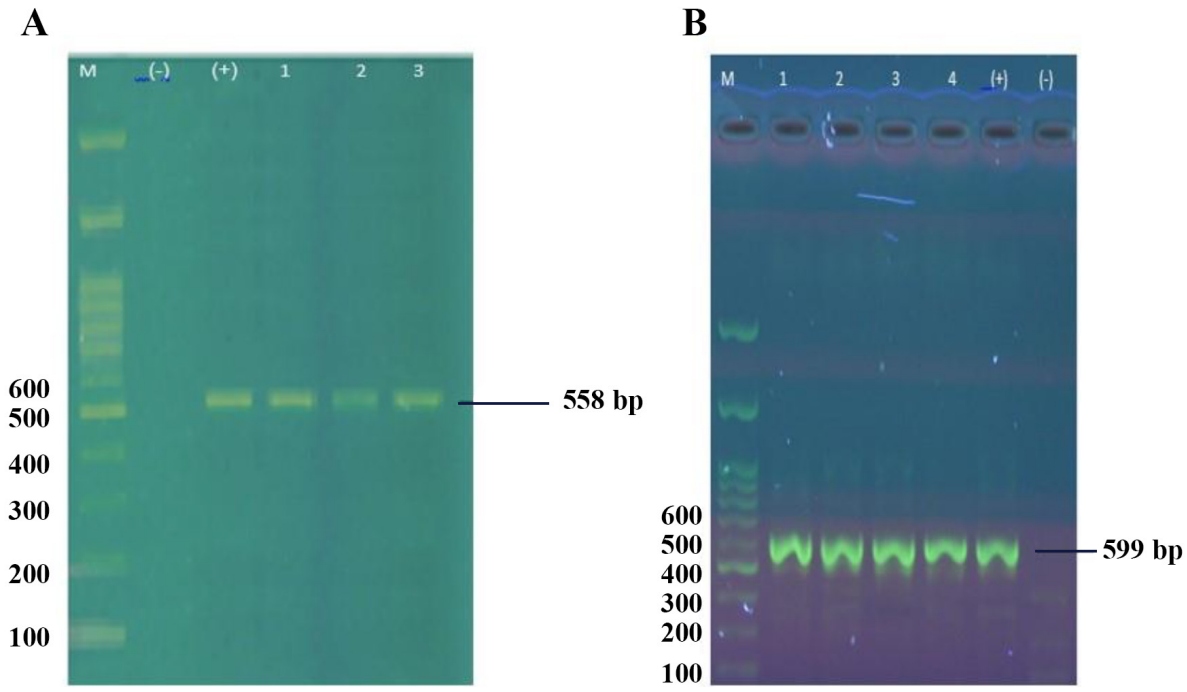


**Fig.1:** Polymerase chain reaction (PCR) product of genomic DNA using specific primers. **A.** *PRM1* with 557 bp and **B.** *PRM2* with 599 bp length was detected. M; Molecular marker (100 bp), (+); Positive control, and (-); Negative control.

**Table 2:** The frequency of single nucleotide polymorphisms (SNPs) in *PRM1* and *PRM2* in case and control groups

| Gene | Nucleotide change | Region | AA change | NCBI ID | Infertile | | Fertile | | P value |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Genotype frequency (%) | Allele frequency | Genotype frequency (%) | Allele frquency | |
| *P1* | C230A | Exon | None | rs737008 | CC: 0.0<br>CA: 96<br>AA: 4 | C(48)<br>A(52) | CC: 26<br>CA: 57<br>AA: 17 | C(54.5)<br>A(45.5) | 0.000<br>0.002 |
| | G102T | Exon | R→S | - | GG: 100<br>GT: 0.0<br>TT: 0.0 | G(100)<br>T(0) | GG: 100<br>GT: 0.0<br>TT: 0.0 | G(100)<br>T(0) | NS<br>NS |
| | C49T | Exon | R→C | - | CC: 100<br>CT: 0.0<br>TT: 0.0 | C(100)<br>T(0) | CC: 100<br>CT: 0.0<br>TT: 0.0 | C(100)<br>T(0) | NS<br>NS |
| *P2* | C288T | Intron | None Coding | rs115686767 | CC: 100<br>CT: 0.0<br>TT: 0.0 | C(100)<br>T(0) | CC: 94.11<br>CT: 5.89<br>TT: 0.0 | C(97.05)<br>T(2.95) | NS<br>NS |
| | C401T | Intron | None Coding | rs545828790 | CC: 100<br>CT: 0.0<br>TT: 0.0 | C(100)<br>T(0) | CC: 100<br>CT: 0.0<br>TT: 0.0 | C(100)<br>T(0) | NS<br>NS |
| | C248T | Exon | E-Q | - | CC: 100<br>CT: 0.0<br>TT: 0.0 | C(100)<br>T(0) | CC: 100<br>CT: 0.0<br>TT: 0.0 | C(100)<br>T(0) | NS<br>NS |
| | G398C | Intron | None Coding | rs1646022 | GG: 44.44<br>GC: 38.86<br>CC: 16.7 | G(63.87)<br>C(36.13) | GG: 31.25<br>GC: 62.5<br>CC: 6.25 | G(62.5)<br>C(37.5) | 0.004<br>0.012 |
| | A473C | Intron | None Coding | rs2070923 | AA: 33.33<br>AC: 33.34<br>CC: 33.33 | A(50)<br>C(50) | AA: 43.75<br>AC: 39.59<br>CC: 16.66 | A(63.55)<br>C(36.45) | 0.073<br>0.007 |
| | G 271C | Intron | None Coding | rs201933708 | GG: 100<br>GC: 0.0<br>CC: 0.0 | G(100)<br>C(0) | GG: 93.75<br>GC: 6.25<br>CC: 0.0 | G(96.87)<br>C(3.13) | NS<br>NS |

**Logistic Regression Modeling was used for statistical analysis. All P values were two-sided and considered significant at the 0.05 level and showed the comparisons between the allele frequencies in case and control groups. *P1; PRM1, P2; PRM2,* AA; Amino acid R; Arginine, S; Serine, C; Cysteine, Q; Termination codon, E; Glutamic acid , and NS; No significant.**

**Table 3:** Association of C230A polymorphism in *PRM1* with sperm characteristics

| Genotype | Apoptosis | Protamine deficiency | Abnormal motility | Concentration | Abnormal morphology |
|---|---|---|---|---|---|
| CC (n=6) | 45.66 ± 16.23 | 35.6 ± 12.34 | 41.85 ± 6.89 | 64.83 ± 58.67 | 5.26 ± 3.49 |
| CA (n=55) | 30.8 ± 12.45 | 27.33 ± 9.67 | 45.33 ± 7.34 | 77.47 ± 60.5 | 48.48 ± 29.78 |
| AA (n=4) | 56 ± 18.96 | 29.66 ± 10.56 | 23.5 ± 3.45 | 49.25 ± 32.75 | 1.4 ± 0.32 |
| P value | <0.001* | 0.157 | <0.001* | 0.602 | <0.001* |

**Values are presented as a mean ± standard deviation. Tukey's test was used for statistical analysis. *; The P<0.05 were considered to indicate statistical significance. Evaluating the sperm parameters was according to the World Health Organization (WHO, 2010).**

**Table 4:** Association of *PRM2* G398C and A473C polymorphisms with human sperm characteristics

| Genotype | Apoptosis | Protamine deficiency | Abnormal motility | Concentration | Abnormal morphology |
|---|---|---|---|---|---|
| rs1646022 | | | | | |
| GG (n=6) | 43.4 ± 18.3 | 28.75 ± 9.25 | 47 ± 8.45 | 74.4 ± 34.5 | 2.33 ± 2.13 |
| GC (n=55) | 29.9 ± 9.94 | 30.9 ± 15.45 | 57.54 ± 12.36 | 52.54 ± 28.75 | 28.7 ± 18.5 |
| CC (n=4) | 12.25 ± 3.88 | 37.75 ± 17.72 | 60 ± 14.87 | 74.5 ± 38.65 | 27.75 |
| P value | <0.001* | 0.63 | 0.12 | 0.11 | 0.003* |
| rs2070923 | | | | | |
| AA (n=16) | 24.83 ± 8.25 | 27.33 ± 6.85 | 55.66 ± 10.76 | 84.83 ± 64.23 | 33.33 ± 23.32 |
| AC (n=34) | 13.5 ± 3.97 | 26 ± 11.83 | 63.33 ± 8.69 | 102.66 ± 85.43 | 37 ± 30.54 |
| CC (n=15) | 41 ± 14.28 | 26.66 ± 12.35 | 44.66 ± 10.73 | 54.66 ± 47.23 | 5 ± 4.55 |
| P value | <0.001* | 0.92 | 0.02* | 0.12 | <0.001* |

***; The P<0.05 were considered to indicate statistical significance. Data are presented as mean ± SD. Post-hoc test to ANOVA was used for statistics evaluating the sperm parameters was according to the World Health Organization (WHO, 2010).**

Protection and support of the sperm genome are the main functions of protamines. It is shown that incomplete protamination of sperm DNA causes high susceptibility of the genome to nucleases, endogenous and exogenous free radicals and mutagens (24). Studies have also DNA damage (25, 26). Consistent with our results, Aoki et al. (27) analyzed 15 SNPs such as G102T and C203A in *PRM1* and observed similar frequencies between an infertile population and normal controls. But, in contrast to Aoki et al. (27), C203A( rs737008) was different between two groups in the present study. The frequency of C49T, located in the exonic region, was the same between groups whereas these results were different in study by Jodar et al. (28). They found that infertile men with normal sperm count but with both abnormal motility and morphology had a nonsynonymous substitution at position C49T (R17C). A recent review article demonstrated that C230A variant had a higher frequency infertile men compared with controls (19) which were also in agreement with our results. In addition, Tanaka et al. (11) reported 5 gene polymorphisms in *PRM1* and 3 polymorphisms in *PRM2* in infertile men. Cho et al. (15) found one stop-gained variant, which converted glutamic acid to a stop codon (known as C248T), in one individual from an infertile azoospermic group. We identified G398C (rs1646022) and A473C (rs2070923) in our samples, however, Tanaka et al. (11) did not show the above-mentioned SNPs in azoospermic and oligospermic patients. Another study conducted by Jiang al. (19) showed that G398C is present in infertile men, which was in line with our findings.

Although Aoki et al. (27) identified15 SNPs, however, given that their frequencies in cases were almost identical to controls, they did not consider them as the underlying genetic cause of abnormalities in the expression of *PRMs* in infertile couples. In this study, the C281T substitution was selected and evaluated among other SNPs and as in Aoki et al. (27) results, we did not observe C281T in either group. It is likely that the variation in results of these studies is due to differences in study populations, which in fact shows that most of these SNPs were at variable frequencies in different populations, indicating that the distribution of genotypes related to different polymorphisms of *PRM1* and *PRM2* genes have ethnic variation. We also detected significant associations between the frequencies of GG and CC genotypes of *PRM2* rs1646022 and rs2070923 respectively with apoptosis, morphology and total motility. Interestingly, these genotypes were the most frequent genotypes in infertile men with taper head spermatozoa. In contrast, there was no association between genotype AA at rs737008 with male infertility. In our previous study, we reported sperm protamine deficiency, lower rates of normal sperm parameters, and apoptosis in infertile men with idiopathic teratozoospermia compared to the controls.

We saw that the concentration of sperm cells was lower in the case group than controls. Also, total motility and sperm morphology were significantly lower in the case group than in the control group. Furthermore, we showed significantly higher rates of protamine deficiency as well as sperm apoptosis in patients with tapered sperm compared with the fertile group using CMA3 florescent stain-

ing and TUNEL assay respectively (29). The findings of the present survey are in agreement with the mentioned previous study, demonstrating the probable relationship between male infertility in patients with tapered head sperms and *PRM2* polymorphisms as well as sperm protamine deficiency, apoptosis rate, and morphology. These damages may affect the quality of the ejaculated spermatozoa and decrease their fertility potential in natural conception or ART cycles. Furthermore, a recent study reported that *PRM2* G398C is associated with the pathogenesis of male infertility in idiopathic infertile men from Chinese Han population, which was in line with our results despite having studied different populations (18). Also, another recent study reported that the c.−190 C>A transversion may be involved in the susceptibility for oligozoospermia and could be used as a non-invasive molecular marker for genetic diagnosis of idiopathic oligozoospermia (30). Finally, a more recent study showed that c.-9C>T and c.368A>G polymorphisms of *H2BFWT* may be genetic risk factors for male infertility so we suggested these polymorphisms investigated in the teratospermia group (31).

We show that *PRM2* G398C and A473C polymorphisms are associated with male infertility in men with teratozoospermia and sperm parameters including semen quality, sperm apoptosis, and morphology in the Iranian population. This study is a preliminary study of a larger comprehensive research program aiming to identify clinically relevant polymorphisms as biomarkers for susceptibility to teratozoospermia.

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## Authors' Contributions

A.R.T., F.D.; Participated in study design and contributed extensively in the interpretation of the data and the conclusion. F.F.; Performed semen specimen analysis, contributed extensively in the interpretation of the data and the conclusion, and conducted molecular experiments and PCR analysis. E.Z.M., S.M.M. A.H.; Contributed to conception and design, all experimental work, data and statistical analysis, and interpretation of data, drafted the manuscript. All authors performed editing and approved the final version of this paper for submission.

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Case Report

# Double Cervix with Normal Uterus and Vagina - An Unclassified Müllerian Anomaly

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## Abstract

Müllerian anomalies are very common, and a frequent cause of infertility. The most used classification system until now, proposed by the American Society for Reproductive Medicine in 1988, categorizes comprehensively uterine anomalies but fails to classify defects of the cervix or vagina. This is based on a developmental theory that postulates that müllerian duct fusion is unidirectional, beginning caudally and extending cranially, which does not account for isolated cervical or vaginal defects. More recently, the European Society of Human Reproduction and Embryology has developed a consensus, which allows for independent cervical anomalies. We present a case of a 39-year-old woman with secondary infertility, found to have a cervical duplication in an anteroposterior disposition, which puts into question the principles of embryology formerly known, but supports the theory that development happens in a segmentary fashion.

***Keywords:*** Female Infertility, Female Tract, Müllerian Anomaly, Urogenital Abnormalities

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## Introduction

Female genitourinary tract malformations are extremely common, being found in around 5.5% of the general population and 8% of infertile women, specifically affecting as many as 25% of women with infertility due to miscarriage (1). The real prevalence may be even higher, considering most of them will go undiagnosed either for being asymptomatic or because of having no access to methods for accurate diagnosis. The spectrum of these malformations is enormous, and although there have been several attempts to catalogue them-the most utilized until recently being the classification by the American Society for Reproductive Medicine (ASRM) from 1988 (2), which included mostly uterine anomalies-it is still necessary to extend the list for a more complete record. Over the years reports of types of malformations have arose, not included in this classification system, predominantly of associated or isolated cervical and vaginal anomalies. We present a case of a cervical duplication with a normal uterus and normal vagina, but with an anteroposterior disposition of the cervix, which supports the theory that isolated segment defects may occur. This case questions the embryology theory that has supported the ASRM classification for decades.

## Case Report

A 39 year-old woman was referred to our institution due to secondary infertility. Menarche was at 14 years of age, with regular cycles and slight dysmenorrhoea. She had experienced a term caesarean section 8 years prior due to failure to progress, and had been trying to get pregnant for 3 years. Her past medical history was unremarkable. On gynaecological examination external genitalia and vagina were normal; two cervical orifices in an anteroposterior disposition were clearly visualized (Fig.1)-this was confirmed with curetting of the posterior canal, which revealed "normal endocervical mucosa", excluding other pathologies such as uterovaginal/cervicovaginal fistulae. Menstrual blood was observed exiting both cervical orifices. Hysterosalpingography (HSG) revealed a normal uterine cavity and tubes, although contrast was visualized exiting the posterior endocervical canal (Fig.2). Transvaginal ultrasound revealed a normal retroverted uterus, with one internal cervical OS and two endocervical canals diverging from it in an anteroposterior arrangement (Fig.3). Because both these exams did not suggest a uterine cavity defect, we chose not to pursue with further tests such as magnetic resonance imaging (MRI) or hysteroscopy, having to subject the patient to bothersome and invasive

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testing that would not alter clinical conduct. Consent form was obtained and completed by participant.

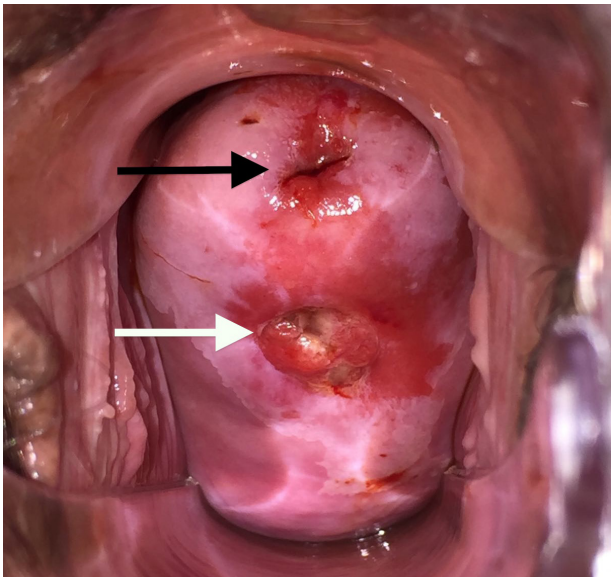

**Fig.1:** Speculum examination showing anterior (black arrow) and posterior (white arrow) cervical OS.

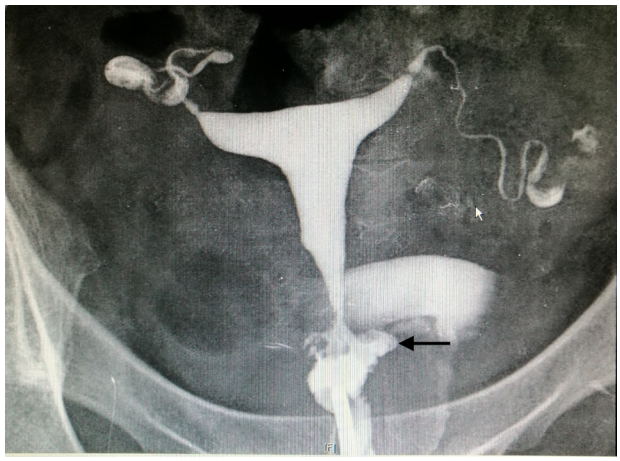

**Fig.2:** Hysterosalpingogram showing a normal uterine cavity with contrast extravasation through a posterior cervical canal (black arrow).

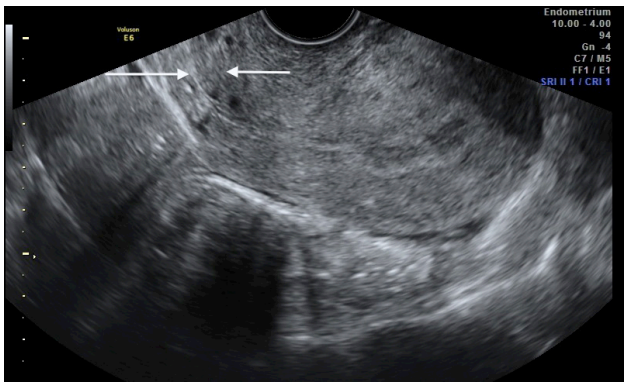


**Fig.3:** Transvaginal ultrasound displaying one internal cervical os (black arrow) and two cervical canals (white arrows).

## Discussion

An extremely rare müllerian malformation is described, which questions classical knowledge of developmental embryology. An extensive literature search was conducted revealing only a few similar cases (3, 4), one in a 4-month infant with other multiple malformations, and another with a side-by-side disposition of the cervix. Only one other case anatomically similar to ours is depicted in the literature (5).

The aetiology for most of the congenital anomalies of the female genital tract is unknown. The importance of normal embryological development lays in its reproductive consequences, and also in concomitant urological abnormalities (which are more common with more proximal defects) and in quality of life (for possible dysmenorrhoea or dyspareunia in obstructive defects). In 1988 the ASRM attempted to classify these anomalies (2), however it documents only the more common uterine anomalies, not including rarely occurring cervical or vaginal defects (6). Nevertheless, until recently it has been the most commonly used classification for congenital anomalies.

Two main theories, both described in 1960s, are the foundations for the classification system of the ASRM. The first theory, described by Crosby and Hill (7), suggests that uterine development is a result of müllerian duct fusion between the 11$^{th}$ and the 13$^{th}$ weeks of embryonic life, beginning caudally and progressing cranially; this process is then followed by septal reabsorption, which begins at any point of fusion and moves in either or both directions. The downside of this unidirectional theory is that it does not account for lower segment defects with normal upper segments, as is the case of vaginal/cervical duplications with normal uteri. The second theory, argues that müllerian duct fusion is initiated in the middle portion, at the uterine isthmus, and proceeds simultaneously in a cranial and caudal direction, and that the septal reabsorption follows a similar bidirectional pattern, with complete uterus formation independently from the formation of cervix and vagina (8). This theory, which seems to encompass defects not explained by the first, still does not justify the existence of a middle segment isolated defect, as in our case.

Acién et al. (8) advocated that in fact the müllerian ducts do not contribute to the formation of the vagina, instead the vaginal walls are formed by cells from the wolffian ducts and are then covered by cells of the müllerian tubercle. Therefore the processes of fusion and reabsorption of the müllerian ducts may affect: i. Both converging and diverging portions (superior and inferior uterine segments), ii. Just one of them, or iii. Even just a small specific area, giving rise to segmentary defects.

More recently, the European Society of Human Reproduction and Embryology (ESHRE) developed an anatomy-based consensus on congenital anomalies of the female genital tract and its related clinical significance, in a comprehensive and accessible system. Here, cervical and vaginal anomalies are categorized into independent sub-classes (9), and a normal uterus can be associated with either an abnormal cervix or an abnormal vagina, or both. Therefore, this system encompasses "segmentary

defects", and supports Acién's theory. Yet another classification system, this time by El Saman et al. (10), attempts to classify müllerian duct anomalies based on both embryological development and a "treatment-based" categorization, also including segmentary defects, allowing for a more comprehensive approach to congenital anomalies.

## Conclusion

Embryology of the female genital tract is not completely understood, as the mechanism of müllerian development is more complex than previously described. Acién's segmentary theory is currently the one that best explains segment malformations as the one presented here. This theory puts into question our decade-long understanding of embryological development of the female reproductive system, and supports the classification system of müllerian anomalies of ESHRE. The fact that in our case the two cervices were displayed in an anteroposterior fashion also calls into question the fact that development may not always occur in a side-by-side manner.

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## Authors' Contributions

I.L.A., C.T., I.B.; Contributed to conception and design. I.L.A.; Drafted the manuscript, which was revised by J.L.M., A.Q. and P.S.M., who were also responsible for data acquisition. All authors read and approved the final manuscript.

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**Letter to The Editor**

# Perinatal Mental Health: A Public Health Concern

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Psychiatric disorders are prevalent throughout the world and based on the latest survey on the prevalence of mental disorders among Iranians, these disorders were found in 23.6% of the population and were more prevalent in women than men (1). Pregnancy, childbirth and a year after delivery is considered "the perinatal period" which is a critical period for mothers, babies and the entire family. The prevalence of psychiatric disorders in the perinatal period is 15-29% (2) with depressive and anxiety disorders being the most common conditions (3). Also, these disorders are more prevalent in low-income and middle-income countries (LMICs) than high-income countries (HIC) and they might have a robust effect on the new baby and the wellbeing of the family specially that of the other children in the family (4).

Almost one-third of pregnancies in Iran are unwanted and considerable proportion of them are seen in women using contraceptives (5). Therefore, as it is common that the patients and the physicians are unaware of a probable pregnancy, the physicians should take all perinatal considerations in practice for childbearing-aged women and educate these women to run an effective pregnancy prevention program.

The 2015 Mothers and Babies: Reducing Risk through Audits and Confidential Enquiries (MBRRACE) introduced psychiatric disorders as one of the main causes responsible for maternal death. In addition, mental issues lead to about one-quarter of maternal deaths from six weeks to one year following pregnancy; also, 14% of mortalities were suicidal deaths and drugs and alcohol contributed to 9% of deaths (6). To the best of our knowledge, in Iranian maternal death report, there are no mental health-related findings. So, in-depth investigation in this field is highly required to have a better insight into Iranian women's status and take more proper actions to reduce probable risk factors.

As mentioned above, the perinatal psychiatric disorders are responsible for maternal morbidity and mortality; also, they might cause neonatal problems like premature delivery, low birth weight, etc. Moreover, these disorders may have an association with subsequent psychiatric disorders (e.g. depression and anxiety disorders) in childhood and adolescence. Additionally, the mothers' perinatal mental problems can disturb attachment formation to insecure attachment in the child, specially disorganized mother-infant attachment. Attachment formation is so important for parenting functioning and in case of failure, child maltreatment and other emotional and behavioral consequences may occur (7).

To make sure, public screening and referral to treatment are vital for mothers and the children. Fortunately, since 2016, the Integrated Health System Network called SIB (the Persian abbreviation for the system) has been established to register health information of the community and the perinatal mental health problems and child developmental disturbances have been screened.

However, there are many obstacles in the way to the treatment; it is known that a high percentage of women with perinatal mental disorders does not receive proper treatments (2); so, research for investigating barriers to help seeking and to take proper treatment should be encouraged. Also, there is lack of national guidelines for management and treatment of psychiatric disorders in perinatal period; in this regard, based on a 2018 requirement from the UK Medicines and Healthcare products Regulatory Agency (MHRA), Valproate was contraindicated in women and girls with childbearing potential unless they are on a proper pregnancy prevention program (8). However, it is still frequently used in the absence of a contraception program; so, national guidelines and regulatory rules are needed to rectify the current practice. Furthermore, in case of sever psychiatric disorders, admission of the mother is inevitable which unfortunately lead to mother and baby separation which has an adverse effect on attachment as well as the disorder itself. So, Mother and Baby Units are needed for such conditions. Evidence showed that the Mother and Baby Units have positive effects on mothers and babies and the Mother and Baby Units can facilitate the holistic approach to the attachment of mothers and babies and parenting issue as well as psychiatric therapies (9).

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Finally, the authors suggest Iranian policymakers and academic society to promote research in perinatal field and maternal morbidity and mortality with special focus on mental health, extend the screening programs, develop national perinatal mental health care guidelines, educate physicians and encourage them to practice all perinatal considerations for childbearing-aged women, enhance public awareness for warning signs and construct facilities for perinatal mental health care services like perinatal mental health clinics and Mother and Baby Units.

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## Authors' Contributions

F.H., E.S., S.S.; Contributed in the design of study and interpretations of data. F.H.; Contributed in writing the manuscript and data gathering. S.S., E.S.; Contributed in editing the manuscript. The authors read and approved the final manuscript.

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

**Aims and Scope: International Journal of Fertility & Sterility** is a quarterly English publication of Royan Institute of Iran. The aim of this journal is to disseminate information through publishing the most recent scientific research studies on Fertility and Sterility and other related topics. **Int J Fertil Steril** has been certified by Ministry of Culture and Islamic Guidance since 2007. It has also been accredited as a scientific and research journal by HBI (Health and Biomedical Information) Journal Accreditation Commission since 2008. **This open access journal holds the membership of the Committee on Publication Ethics (COPE).**

**1. Types of articles**

The articles in the field of Fertility and Sterility can be considered for publications in Int J Fertil Steril. These articles are as below:

**A. Original articles** are scientific reports of the original research studies. The article consists of English Abstract (structured), Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, Authors' Contributions, and References **(Up to 40).**

**B. Review articles** are the articles written by well experienced authors and those who have excellence in the related fields. The corresponding author of the review article must be one of the authors of at least three published articles appearing in the references. The review article consists of English Abstract (unstructured), Introduction, Conclusion, Authors' Contributions, and References **(Up to 70).**

**C. Systematic Reviews**

Systematic reviews are a type of literature review that collect and critically analyzes multiple research studies or papers. The Systematic reviews consist of English Abstract (unstructured), Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, Authors' Contributions, and References **(Up to 70).**

**D. Short communications** are the articles containing new findings. Submissions should be brief reports of ongoing researches. The short communication consists of English Abstract (unstructured), the body of the manuscript (should not hold heading or subheading), Acknowledgements, Authors' Contributions, and References **(Up to 30).**

**E. Case reports** are short discussions of a case or case series with unique features not previously described which make an important teaching point or scientific observation. They may describe novel techniques or use equipment, or new information on diseases of importance. It consists of English Abstracts (Unstructured), Introduction, Case Report, Discussion, Acknowledgements, Authors' Contributions, and References **(Up to 30).**

**F. Editorial** should be written by either the editor in chief or the editorial board.

**G. Imaging in reproductive medicine** should focus on a single case with an interesting illustration such as a photograph, histological specimen or investigation. Color images are welcomed. The text should be brief and informative.

**H. Letter to the editors** are welcome in response to previously published **Int J Fertil Steril** articles, and may also include interesting cases that do not meet the requirement of being truly exceptional, as well as other brief technical or clinical notes of general interest.

**I. Debate.**

**2. Submission Process**

It is recommended to see the guidelines for reporting different kinds of manuscripts here. This guide explains how to prepare the manuscript for submission. Before submitting, we suggest authors familiarize themselves with **Int J Fertil Steril** format and content by reading the journal via the website (www.ijfs.ir). The corresponding author ensures that all authors are included in the author list and agree with its order, and they must be aware of the manuscript submission.

**A. Author contributions statements**

It is essential for authors to include a statement of responsibility in the manuscript that specifies the contribution of every one of them. This participation must include conception and design of the manuscript, data acquisition or data analysis and interpretation, drafting of the manuscript and/or revising it for critically important intellectual content, revision and final approval of the manuscript and statistical analysis, obtaining funding, administrative, technical, or material support, or supervision. Authors who do not meet the above criteria should be acknowledged in the **Acknowledgments Section**.

**B. Cover letter**

Each article should be accompanied by a cover letter, signed by all authors specifying the following statement: “The manuscript has been seen and approved by all authors and is not under active consideration for publication. It has neither been accepted for publication nor published in another journal fully or partially (except in abstract form). I hereby assign the copyright of the enclosed manuscript to **Int J Fertil Steril**. The corresponding author must confirm the proof of the manuscript before online publishing. Also, is it needed to suggest three peer reviewers in the field of their manuscript.

**C. Manuscript preparation**

Authors whose first language is not English encouraged to consult a native English speaker in order to confirm his manuscripts to US or British (not a mixture) English usage and grammar. It is necessary to mention that we will check the plagiarism of your manuscript by iThentical Softwere. The manuscript should be prepared in accordance with the “International Committee of Medi-

cal Journal Editors (ICMJE)". Please send your manuscript in two formats (word and Pdf). The abstract and text pages should have consecutive line numbers in the left margin beginning with title page and continuing through the last page of the written text. Each abbreviation must be defined in the abstract and text when they are mentioned for the first time. Avoid using abbreviation in title. Please use the international and standard abbreviations and symbols.

It should be added that an essential step toward the integration and linking of scientific information reported in published literature is using standardized nomenclature in all fields of science and medicine. Species names must be italicized (e.g., Homo sapiens) and also the full genus and species written out in full, both in the title of the manuscript and at the first mention of an organism in a paper.

It is necessary to mention that genes, mutations, genotypes, and alleles must be indicated in italics. Please use the recommended name by consulting the appropriate genetic nomenclature database, e.g., HUGO for human genes. In another word; if it is a human gene, you must write all the letters in capital and italic (e.g., OCT4, c-MYC). If not, only write the first letter in capital and italic (e.g., Oct4, c-Myc). **In addition, protein designations are the same as the gene symbol but are not italicized.**

**Of note, Int J Fertil Steril** will only consider publishing genetic association study papers that are novel and statistically robust. Authors are advised to adhere to the recommendations outlined in the STREGA statement (http://www.strega-statement.org). The following criteria must be met for all submissions:

**1.** Hardy-Weinberg Equilibrium (HWE) calculations must be carried out and reported along with the P-values if applicable [see Namipashaki et al. 2015 (Cell J, Vol 17, N 2, Pages: 187-192) for a discussion].

**2.** Linkage disequilibrium (LD) structure between SNPs (if multiple SNPs are reported) must be presented.

**3.** Appropriate multiple testing correction (if multiple independent SNPs are reported) must be included.

Submissions that fail to meet the above criteria will be rejected before being sent out for review.

Each of the following manuscript components should begin in the following sequence:

**Authors'** names and order of them must be carefully considered (full name(s), highest awarded academic degree(s), email(s), and institutional affiliation(s) of all the authors in English. Also, you must send mobile number and full postal address of the corresponding author).

**Changes to Authorship** such as addition, deletion or rearrangement of author names must be made only before the manuscript has been accepted in the case of approving by the journal editor. In this case, the corresponding author must explain the reason of changing and confirm them (which has been signed by all authors of the manuscript). If the manuscript has already been published in an online issue, an erratum is needed.

**Title** is providing the full title of the research (do not use abbreviations in title).

**Running title** is providing a maximum of 7 words (no more than 50 characters).

**Abstract** must include Background, Materials and Methods, Results, and Conclusion (no more than 300 words).

**Keywords,** three to five, must be supplied by the authors at the foot of the abstract chosen from the Medical Subject Heading (MeSH). Therefore; they must be specific and relevant to the paper.

The following components should be identified in the abstract:

**Introduction:** The Introduction should provide a brief background to the subject of the paper, explain the importance of the study, and state a precise study question or purpose.

**Materials and Methods:** It includes the exact methods or observations of experiments. If an apparatus is used, its manufacturer's name and address should be stipulated in parenthesis. If the method is established, give reference but if the method is new, give enough information so that another author can perform it. If a drug is used, its generic name, dose, and route of administration must be given. Standard units of measurements and chemical symbols of elements do not need to be defined.

**Statistical analysis:** Type of study and statistical methods should be mentioned and specified by any general computer program used.

**Ethical considerations:** Please state that informed consent was obtained from all human adult participants and from the parents or legal guardians of minors and include the name of the appropriate institutional review board that approved the project. It is necessary to indicate in the text that the maintenance and care of experimental animals complies with National Institutes of Health guidelines for the humane use of laboratory animals, or those of your Institute or agency.

**Clinical trial registration:** All of the Clinical Trials performing in Iran must be registered in Iranian Registry of Clinical Trials (www.irct.ir). The clinical trials performed abroad, could be considered for publication if they register in a registration site approved by WHO or www.clinicaltrials.gov. If you are reporting phase II or phase III randomized controlled trials, you must refer to the CONSORT Statement for recommendations to facilitate the complete and transparent reporting of trial findings. Reports that do not conform to the CONSORT guidelines may need to be revised before peer reviewing.

**Results:** They must be presented in the form of text, tables, and figures. Take care that the text does not repeat data that are presented in tables and/or figures. Only emphasize and summarize the essential features of the main results. Tables and figures must be numbered consecutively as appeared in the text and should be organized in separate pages at the end of article while their location should be mentioned in the main text.

**Tables and figures:** Tables should have a short descriptive heading above them and also any footnotes. Figure's legend should contain a brief title for the whole figure and continue with a short explanation of each part and also the symbols used (no more than 100 words). All figures must be prepared based on cell journal's guideline in color (no more than 6 Figures and Tables) and also in GIF or JPEG format with 300 dpi resolutions.

**Supplementary materials** would be published on the online version of the journal. This material is important to the understanding and interpretation of the report and should not repeat material within the print article. The amount of supplementary material should be limited. Supplementary material should be original and not previously published and will undergo editorial and peer review with the main manuscript. Also, they must be cited in the manuscript text in parentheses, in a similar way as when citing a figure or a table. Provide a legend for each supplementary material submitted.

**Discussion:** It should emphasize the present findings and the variations or similarities with other researches done by other researchers. The detailed results should not be repeated in the discussion again. It must emphasize the new and important aspects of the study.

**Conclusion:** It emphasizes the new and important aspects of the study. All conclusions are justified by the results of the study.

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

**Conflict of Interest:** Any conflict of interest (financial or otherwise) and sources of financial support must be listed in the Acknowledgements. It includes providers of supplies and services from a commercial organization. Any commercial affiliation must be disclosed, regardless of providing the funding or not.

**References:** The references must be written based on the Vancouver style. Thus the references are cited numerically in the text and listed in the bibliography by the order of their appearance. The titles of journals must be abbreviated according to the style used in the list of Journals Indexed in PubMed. Write surname and initials of all authors when there are six or less. In the case of seven or more authors, the names of first six authors followed by "et al." must be listed. The reference of information must be based on the following order:

**Article:**

Surname(s) and first letter of name & middle name(s) of author(s). Manuscript title. Journal title (abbr).publication date (year); Volume) Issue(: Page number.

Example: Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U, et al. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. Biol Reprod. 1995; 52(4): 864-867.

**Book:**

Surname(s) and first letter of name & middle name(s) of author(s). Book title. Edition. Publication place: publisher name; publication date (year); Page number.

Example: Edelman CL, Mandle CL. Health promotion throughout the life span. 2nd ed.ST Louis: Mosby; 1998; 145-163.

**Chapter of book:**

Surname(s) and first letter of name & middle name(s) of author(s). Chapter title. In: Surname(s) and first letter of name &middle name(s) of editor(s), editors. Book title. Edition. Publication place: publisher name; publication date (year); Page number.

Example: Phillips SJ, Whisnant JP. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York: Raven Press; 1995; 465-478.

**Abstract book:**

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

**Thesis:**

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

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

**Conferences:**

Name(s) of editor(s). Conference title; Holding date; Holding place. Publication place; Publisher name; Publication date (year).

Example: Harnden P, Joffe JK, Jones WG, editors. Germ cell tumors V.Proceedings of the 5th Germ Cell Tumors Conference; 2001 Sep 13-15; Leeds,UK. New York: Springer; 2002.

**Internet References**

**Article:**

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

Example: Jahanshahi A, Mirnajafi-Zadeh J, Javan M, Mohammad-Zadeh M, Rohani M. Effect of low-frequency stimulation on adenosineA1 and A2A receptors gene expression in dentate gyrus of perforant path kindled rats. Cell J. 2008; 10 (2): 87-92. Available from: http://www.celljournal.org. (20 Oct 2008).

**Book:**

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

**Law:**

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

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**3. General information**

**A.** You can send your article via online submission system which is available at our website: **http://www.ijfs.ir.** If the article is not prepared according to the format of **Int J Fertil Steril**, it will be returned to authors.

**B.** The order of article appearance in the Journal is not demonstrating the scientific characters of the authors.

**C. Int J** Fertil Steril has authority to accept or reject the articles.

**D.** The received articles will be evaluated by one epidemiologist. Then associate editor will determine its reviewers. If three reviewers pass their judgments on the article, it will be presented to the editorial board of **Int J Fertil Steril.** If the editorial board has a positive judgment about the article, reviewers' comments will be presented to the corresponding author (the identification of the reviewers will not be revealed). The executive member of journal will contact the corresponding author directly within 7-8 weeks by email. If authors do not receive any reply from journal office after the specified time, they can contact journal office. Executive manager will respond promptly to authors' message.

**The Final Checklist**

The authors must ensure that before submitting the manuscript for publication, they have to consider the following parts:

**1.** Title page should contain 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.** Covering Letter

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