INTERNATIONAL JOURNAL OF FERTILITY AND STERILITY (Int J Fertil Steril)

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**Printing Company:**
Naghsh e Johar Co.
NO. 103, Fajr alley, Tehranpars Street, Tehran, Iran

INTERNATIONAL JOURNAL OF FERTILITY AND STERILITY

*Int J Fertil Steril, Vol 16, No 4, October-December 2022, Pages: 251-319*

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Original Article

# Growth Hormone: A Potential Treatment of Patients with Refractory Thin Endometrium: A Clinical Trial Study

**Soghra Hosseini Aghdam, M.D.[1, 2], Alyeh Ghasemzadeh, M.D.[1, 2*], Laya Farzadi, M.D.[1, 2], Kobra Hamdi, M.D.[1, 2], Nazli Navali, M.D.[1, 2], Parvin Hakimi, M.D.[1, 2], Marayam Baradaran-Binazir, Ph.D.[3], Mohammad Nouri, Ph.D.[4], Amir Fattahi, Ph.D.[1, 4*], Ralf Dittrich, Ph.D.[5]**

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## Abstract

**Background:** Growth hormone (GH) is a potential treatment in the assisted reproductive technology (ART) to improve endometrial receptivity and thickness. In the current study, we investigated the effect of the intrauterine administration of GH on the endometrial thickness (EMT) and ART outcomes in the patients with refractory thin endometrium.

**Materials and Methods:** In this clinical trial study, women with a refractory thin endometrium and a history of one or more frozen embryo transfer (FET) cancellation who were referred to the infertility center of the Tabriz Al-Zahra hospital (Tabriz, Iran) and Milad Infertility Clinic (Tabriz, Iran) received intrauterine injections of GH every other day from day 14 of the menstrual cycle until the EMT reached ≥7 mm in addition to the routine endometrium preparation protocol. EMT was evaluated during the treatment and in the cases with EMT ≥7 mm, biochemical/clinical pregnancy was evaluated after embryo transfer.

**Results:** Thirty-one women aged 35.29 ± 6.21 years were included in this study. The mean amount of EMT was significantly increased following the GH treatment (7.03 ± 1.23 mm) vs. before treatment (5.14 ± 1.1 mm, P<0.001). The EMT reached ≥7 mm in the 65% patients (20/31). Also, the embryo transfer resulted in pregnancy in the patients, biochemical pregnancy: 9/20 (45%) and clinical pregnancy: 7/20 (35%). There was a positive correlation between EMT on the day 13 of cycle (before the treatment) and the maximum EMT (r=0.577 and P=0.001). The EMT was statistically different on the embryo transfer day between clinically pregnant and non-pregnant women (7.18 ± 0.56 vs. 6.21 ± 0.72 mm, P=0.007).

**Conclusion:** The intrauterine administration of GH could be an appropriate therapeutic strategy for patients with refractory thin endometrium. This treatment could significantly increase the EMT as well as implantation and pregnancy rates in these patients (registration number: IRCT20210220050429N1).

***Keywords:*** Assisted Reproductive Technology, Growth Hormone, Implantation, Pregnancy

**Citation:** Hosseini Aghdam S, Ghasemzadeh A, Farzadi L, Hamdi K, Navali N, Hakimi P, Baradaran-Binazir M, Nouri M, Fattahi A, Dittrich R. Growth hormone: a potential treatment of patients with refractory thin endometrium: a clinical trial study. Int J Fertil Steril. 2022; 16(4): 251-255. doi: 10.22074/IJFS.2022.541389.1210.


## Introduction

Although assisted reproductive technology (ART) has greatly advanced in recent years (1), clinical studies show that even with the application of comprehensive chromosome screening of embryos, the ongoing pregnancy rate with euploid embryo transfer (ET) is about 45% (2, 3). This means that factors other than chromosomal abnormalities are responsible for more than 50% of ART failures. More recently, attention has been directed to the endometrium in an attempt to optimize the chance of embryo implantation. The embryo implantation can be occurred in the window of implantation from day 22 to 24 of a 28-day cycle (4).

The thin endometrium, the thickness <7 mm, with the incidence of about 1% to 2.5% is one of the common issues that can cause cycle cancelation or implantation failure (5). It has been shown that the recovery of endometrium thickness in patients with thin endometrium could improve endometrial receptivity, implantation, and live birth rates (6, 7). Currently, several therapeutic strategies have been applied to restore endometrial thickness (EMT) and receptivity in patients with refractory thin endometrium, including administration of Tamoxifen, Pentoxifylline, a high dose of estradiol, vitamin E, low dose of human chorionic gonadotropin, low dose Aspirin, L-Arginine, acupuncture and neuromuscular electrical stimulation, Nitroglycerin patches, intrauterine infusion of granulocyte

Received: 22/October/2021, Accepted: 06/February/2022
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Royan Institute
International Journal of Fertility and Sterility
Vol 16, No 4, October-December 2022, Pages: 251-255

colony-stimulating factor (GCSF), and stem cells (8, 9). However, the above-mentioned therapeutic methods have not been able to produce very good results, especially in patients with refractory thin endometrium; therefore, novel treatments are required to improve the endometrial thickness as well as the pregnancy rate in these patients.

Growth hormone (GH) is used as an adjuvant treatment in the ART. Studies have demonstrated that this hormone and its receptors are expressed in the endometrium and might involve in the EMT and endometrial receptivity (10-12). There are contradictory results regarding the effectiveness of intravenous GH administration on the EMT in the ART cycles (13, 14). The mechanism through which GH improves the EMT and *in vitro* fertilization (IVF) outcomes are almost unknown; however, different molecules have been suggested to be involved in this process, including insulin-like growth factor (IGF), leukemia inhibitory factor (LIF), integrin, and home box containing transcription factors (15). Since the local administration of GH may be more effective on the EMT and endometrial receptivity, for the first time, Yu et al. (11) evaluated the intrauterine perfusion of GH for the treatment of human thin endometrium. This study demonstrated that intrauterine administration of GH could positively affect EMT and endometrial receptivity.

Given the potential of GH to improve endometrial status as well as pregnancy outcome and also, lack of sufficient data on the effect of the intrauterine administration of GH, the present study aimed to evaluate the effect of intrauterine administration of GH on the EMT and ART outcomes in the patients with refractory thin endometrium.

## Materials and Methods

### Ethical considerations

This study was conducted in accordance with the Declaration of Helsinki and all procedures were approved by the Ethical Committee of Tabriz University of Medical Sciences, Tabriz, Iran (IR.TBZMED.REC.1399.1039). Moreover, signed informed consent was obtained from each participant before entering the study. The study has been registered in the Iranian Registry of Clinical Trials (IRCT20210220050429N1).

### Study population

In this clinical trial study, the participants were recruited from patients who were referred to the infertility center of the Tabriz Al-Zahra hospital (Tabriz, Iran) and Milad Infertility Clinic (Tabriz, Iran), for frozen ET (FET) in the hormonal replacement cycle due to reduced ovarian reserve the recruitment procedure is detailed in the Figure 1. All participants had a history of one or more ET cancellations due to EMT <7 mm after standard hormone replacement therapy (HRT). The previous HRT treatment included estradiol valerate tablets with a constant dose of 6 mg per day for 7 days and increasing the dose of estradiol valerate, up to 8mg/day for four more days in patients with EMT<7 mm.

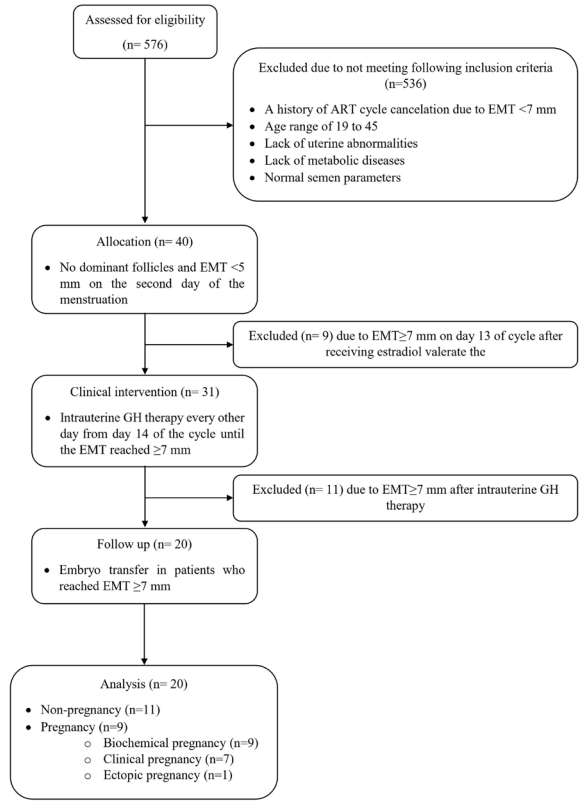


**Fig.1:** Consolidated standards of reporting trials (CONSORT) flow diagram. EMT; Endometrial thickness.

Inclusion criteria were as follows: i. Age range of 19 to 45 years, ii. EMT <7 mm at the end of estrogen priming day in the frozen embryo cycle in the previous cycle(s), and iii. No obvious abnormality during hysteroscopy examination within the past 6 months. Patients with a history of cancer, cardiovascular disease, uterine abnormalities (e.g. Asherman's syndrome, fibroid, polyp, and adenomyosis), any medical contraindication for GH treatment such as having diabetes, hyperlipidemia, metabolic diseases, and thyroid disorders were excluded from the study. Moreover, we excluded couples with abnormal semen analysis (possibility of male infertility).

### Treatments and ultrasound assessment

After collecting some demographic data (weight, height, and age) of patients, the HRT in the FET cycle was started after confirmation of no dominant follicles in the ovaries and EMT <5 mm on the second day of the menstruation period by using ultrasound. The EMT was measured by ultrasonography (Micromaxx, Sonosite.inc, USA) in the median sagittal plane at the thickest three-line pattern part. In the HRT, the endometrium was prepared by estrogen. In this regard, on the second day of the cycle, all patients received estradiol valerate tablets (2 mg, Aburaihan CO., Tehran, Iran) with a constant dose of 6 mg per day for 7 days (days 2 to 8 of the cycle) to prevent follicular recruitment. After the one-week treatment (day 9 of the cycle), the second ultrasound evaluation was performed. If the EMT was <7 mm at the thickest part of the uterine longitudinal axis, the dose of estradiol valerate

was increased up to 8 mg/day for four more days. Then, the ultra-sonography evaluation was repeatedly done two times and the refractory thin endometrium was approved in patients (n=31) when the EMT was still less than 7 mm. These patients received intrauterine injections of GH (CinnaTropin®, CinnaGen, Tehran, Iran) every other day from day 14 of the cycle until the EMT reached ≥7 mm (maximum of five times injection). The GH solution was prepared by dilution of 1.5 ml recombinant GH (5 mg/1.5 ml, CinnaTropin®, CinnaGen, Tehran, Iran) with 0.3 ml of 0.9% saline (Iranian Parenteral and Pharmaceutical Company (IPPC), Tehran, Iran). For intrauterine GH therapy, cervical mucus was wiped out using a cotton swab (Deltalab, Barcelona, Spain) and then 0.6 ml diluted GH solution (contained 5 mg GH) was slowly injected into the endometrial cavity at the bottom of the 0.5 cm-1. 0 cm at the distance, by a soft catheter (Labotec, Gottingen, Germany) and then let the patient rest at 15-30 degrees of hip elevation position for 15 minutes. In cases whose EMT did not reach 7 mm, the FET cycle was canceled.

In the cases with EMT ≥7 mm, serum estrogen levels were measured after 48 hours using competitive chemiluminescent immunoassay and the patients received 100 mg intramuscular progesterone (50 mg/ml Amp, Aburaihan, Tehran, Iran) 3-5 days before ET depending on the stage of the embryo. After transfer of 2-3 high-quality embryos, progestin supplementation was done until two weeks. If the pregnancy was achieved it was continued till 12 weeks of pregnancy. The biochemical pregnancy was confirmed when serum beta human chorionic gonadotropin (β-hCG) levels reached >20 IU/L two weeks after the ET. The clinical pregnancy was defined when the gestational sac was observed four weeks after the ET by ultrasonography examination. Ongoing pregnancy was defined as a ≥12 weeks of gestation.

### Statistical analysis

Data were statistically analyzed by SPSS (version 20, Chicago, USA). We demonstrated mean ± standard deviation (SD) of numerical data and the categorical data was shown as a number and percentage. The independent t test was used to compare the body mass index (BMI), EMT, and blood estrogen levels between the pregnant and non-pregnant groups. The EMT before and after the treatment was compared using the paired-samples t test. To compare the frequency of GH injection between pregnant and non-pregnant groups, the Chi-Square test was used. Moreover, the association between quantitative factors was evaluated by the Pearson coefficient correlation test. The statistical significance was considered as $P<0.05$.

## Results

Thirty-one patients with a mean age of 35.29 ± 6.21 years and BMI of 28.4 ± 3.65 kg/m$^2$ were included in this study. Before and after the GH treatment, the mean amount of EMT was 5.14 ± 1.1 mm and 7.03 ± 1.23 mm, respectively, that shows a statistically significant increase in the EMT following the treatment ($P<0.001$). Despite the significant increase in the EMT following GH administration, the ET was canceled in the 11 (35.5%) patients since the EMT did not reach 7 mm. There was a significant positive correlation between the EMT on the menstrual cycle day 13 (before starting the treatment) and the maximum amount of the EMT ($r=0.577$ and $P=0.001$). However, we found no significant correlation among the EMT of pre- or post-treatment with age, BMI, and estradiol levels ($P>0.05$).

Following the ET in the 20 patients with EMT ≥ 7 mm, we observed 17 pregnancies occurrence: 9 (45%) biochemical pregnancy and 7 (35%) clinical pregnancy, and also, one (5%) ectopic pregnancy. The EMT was not statistically different on the day of ET between biochemically pregnant and non-pregnant women ($P=0.266$, Fig.2A). However, we found a significant difference in the EMT on the day of ET between clinically pregnant and non-pregnant women (7.18 ± 0.56 vs. 6.21 ± 0.72 mm, $P=0.007$, Fig.2B). The maximum EMT amount between pregnant (biochemically or clinically) women with non-pregnant ones was not significantly different (7.89 ± 0.57 vs. 7.68 ± 0.57 mm, $P=0.432$ and 8.07 ± 0.49 vs. 7.66 ± 0.55 mm; $P=0.126$, respectively). Moreover, we found no significant difference in the EMT on the menstrual cycle day 13 (before the GH treatment), BMI, age, and estrogen levels among pregnant (biochemically or clinically) with non-pregnant women ($P>0.05$).

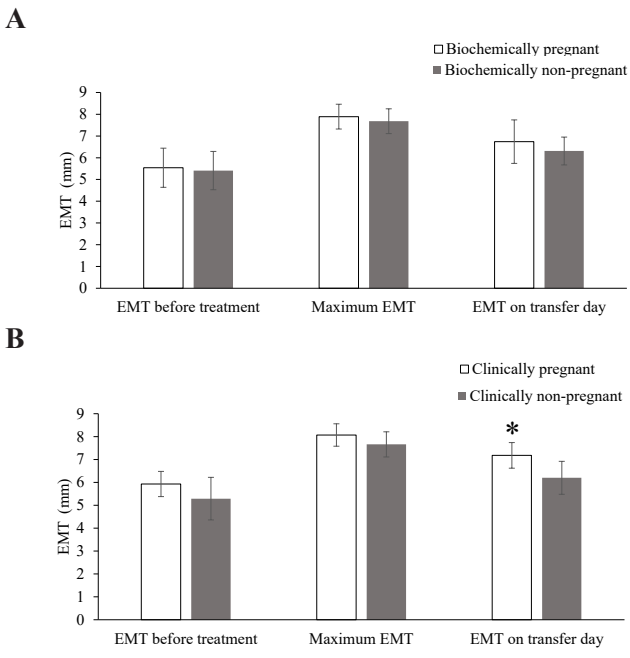


**Fig.2:** The endometrial thickness (EMT) of pregnant and non-pregnant women. **A.** Biochemically pregnant women (serum beta human chorionic gonadotropin >20 IU/L) vs. non-pregnant women and **B.** Clinically pregnant women (existing of gestational sac) vs. non-pregnant women. *; Significant differences ($P<0.007$) by using independent t test.

## Discussion

The refractory thin endometrium is currently an unresolved clinical problem which its underlying

mechanism is not very clear (5). However, it has been suggested that the endometrial stem cell damage and subsequent impairment of endometrial tissue repair can be the possible reason for the non-response thin endometrium (11). Since thin endometrium is one of the reasons for ART cycle cancellation, in the current study, we investigated the potential of intrauterine GH administration in the improvement of EMT and preparation of these patients for the FET cycle.

Our results demonstrated that intrauterine administration of GH could significantly increase EMT. In this regard, the EMT of 64.5% of our patients who had the refractory thin endometrium reached ≥ 7 mm. Previous studies consistently reported a positive effect of subcutaneous (SQ) injection of the GH on the EMT in the infertile women with repeated implantation failure (RIF) and thin endometrium (16-18). In a meta-analysis study, it has been also documented that GH could enhance the EMT in the women with thin endometrium [odds ratio (OR)=10.62, 95% confidence interval (CI) (2.97, 38.00)] (19); however, this effect of GH was not confirmed by others (13, 14). Such controversial findings regarding the effect of the GH on the EMT could be due to the differences in the doses of GH, starting time and duration of GH treatment, EMT evaluation method as well as the patient selection. In this respect, it has been observed that starting GH treatment earlier in the menstrual cycle could improve better the EMT (13). Moreover, in contrast to this study, GH was systematically administrated by the subcutaneous (SQ) or intravenous (IV) or intraperitoneal (IP) injection, and as far as we know there is only one report on the intrauterine administration of GH in the only five patients with thin endometrium (11). In this regard, they indicated that intrauterine administration of GH at 8-12 days after menstruation every other day could significantly increase the EMT in the patients with refractory thin endometrium. The current study also confirmed the effectiveness of the intrauterine perfusion of GH in the increasing EMT of 31 patients. It seems local administration (intrauterine) of the GH could be more beneficial in comparison with the systematic treatment (SQ, IV, and IP) due to i. Higher effect on the endometrial cells because of the direct delivery of the GH to the cells, ii. Application of a lower dose in comparison with the systemic administration, and iii. Lack or negligible side effect of the GH on the body. Regarding the latter reason, it has been mentioned that the GH may induce malignancy and metabolic disorder in the individuals without GH deficiency (20). Moreover, it has been documented that the GH can negatively affect insulin resistance and glucose tolerance (21).

The mechanism(s) by which the GH can increase the EMT has not been completely described. However, it has been shown that this hormone can induce vascularization, glandularization, and stromal loosen in the endometrium via interacting with its receptor and IGFs. Moreover, the GH stimulates the expression of inflammatory cytokines such as integrin and LIF, and consequently mitosis of endothelial cells and the endometrial blood flow (22). Since the vascular endothelial growth factor expression, vascularization, and glandular epithelium growth are decreased and the uterine artery blood flow is decreased in the thin endometrium (23, 24), GH can promote EMT amount by the above mentioned mechanisms.

We found that the transfer of embryos in the patients with an EMT score ≥7 mm after GH administration, resulted in 45% biochemical pregnancies and 35% clinical pregnancies which are almost satisfying rates among patients with refractory thin endometrium. Moreover, it has been seen that the EMT score was significantly higher among patients who got clinically pregnant in compared to those who did not. These findings can confirm the positive effect of the GH on the endometrial preparation and receptivity and consequently the chance of pregnancy in addition to increasing its thickness. Several studies have also demonstrated a beneficial effect of the GH on the embryo implantation and clinical pregnancy in the infertile women , RIF affected as well as refractory thin endometrium patients (12-14, 16-18). For example, Cui et al. (16) reported that administration of the 4.5 IU GH since the day of progesterone administration of the ET day, every alternate day, could significantly increase the EMT amount and subsequent implantation rate and clinical pregnancy rate in patients with thin endometrium. However, some studies observed a lack of beneficial effects of the GH on the pregnancy rate (10, 25). Previous studies have revealed positive associations between the EMT with implantation and pregnancy rates (26, 27). It has been also found that women with thicker endometrium on the day of hCG injection had a higher pregnancy rate than those who had thinner ones (28, 29). Therefore, it can be postulated that one of the mechanisms of the GH that increases the chance of pregnancy may be promoted the EMT amount. Moreover, the GH induces production of different factors by the endometrium such as LIF, vascular endothelial growth factor (VEGF), IGFs, matrix metalloproteinase-9 (MMP-9), and tissue inhibitors of matrix metalloproteinase-1 (TIMP-1) which can positively affect endometrial receptivity and subsequent pregnancy outcome (16, 30); nevertheless, we did not evaluate the molecular mechanisms underlying the positive effect of the GH on the implantation and pregnancy and further studies are required to shed more light on this issue. Moreover, some confounding factors might be able to affect our results, particularly the implantation and pregnancy rates, such as genetic abnormalities of the embryos, the difference in the stage of transferred embryos (cleave or blastocyst) also the relatively small sample size.

## Conclusion

This study showed that intrauterine administration of the GH every other day from day 14 of the menstrual cycle could be an appropriate therapeutic strategy for the patients with refractory thin endometrium. This treatment could significantly increase the EMT as well as implantation and pregnancy rates in the patients with refractory thin endometrium. Intrauterine perfusion of

the GH in comparison with the systemic administration of GH can have negligible side-effects, while we did not observe any adverse effects in our patients.

## Acknowledgements

We appreciate the patients that contributed to this research project. We thank the staff of Tabriz Al-Zahra Hospital (Tabriz, Iran) and Milad Infertility Clinic (Tabriz, Iran) for providing the patients. This study was financially supported by the Women's Reproductive Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. The authors declare that they have no competing interests.

## Authors' Contributions

A.Gh., S.H.A.; Involved in the conception and design of the study. S.H.A, L.F., K.H., M.B.-B.; Participated in the acquisition, planning of the analysis, and data interpretation. S.H.A., A.F., R.D., M.N.; Conducted statistical analysis, critical revisions, and drafted the manuscript. R.D., A.F., A.G., M.N.; Revised the manuscript critically. N.N., P.H.; Provided samples, follow upped patients, and gave clinical advises. All authors read and approved the final version of the manuscript.

## References

1. Toner JP, Coddington CC, Doody K, Van Voorhis B, Seifer DB, Ball GD, et al. Society for assisted reproductive technology and assisted reproductive technology in the United States: a 2016 update. Fertil Steril. 2016; 106(3): 541-546.
2. Cimadomo D, Soscia D, Vaiarelli A, Maggiulli R, Capalbo A, Ubaldi FM, et al. Looking past the appearance: a comprehensive description of the clinical contribution of poor-quality blastocysts to increase live birth rates during cycles with aneuploidy testing. Hum Reprod. 2019; 34(7): 1206-1214.
3. Munné S, Kaplan B, Frattarelli JL, Child T, Nakhuda G, Shamma FN, et al. Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: a multicenter randomized clinical trial. Fertil Steril. 2019; 112(6): 1071-1079.
4. Kim SM, Kim JS. A review of mechanisms of implantation. Dev Reprod. 2017; 21(4): 351-359.
5. Eftekhar M, Tabibnejad N, Tabatabaie AA. The thin endometrium in assisted reproductive technology: an ongoing challenge. Middle East Fertil Soc J. 2018; 23(1): 1-7.
6. Zadehmodarres S, Salehpour S, Saharkhiz N, Nazari L. Treatment of thin endometrium with autologous platelet-rich plasma: a pilot study. JBRA Assist Reprod. 2017; 21(1): 54-56.
7. Kim H, Shin JE, Koo HS, Kwon H, Choi DH, Kim JH. Effect of autologous platelet-rich plasma treatment on refractory thin endometrium during the frozen embryo transfer cycle: a pilot study. Front Endocrinol (Lausanne). 2019; 10: 61.
8. Garcia-Velasco JA, Acevedo B, Alvarez C, Alvarez M, Bellver J, Fontes J, et al. Strategies to manage refractory endometrium: state of the art in 2016. Reprod Biomed Online. 2016; 32(5): 474-489.
9. Keyhanvar N, Zarghami N, Bleisinger N, Hajipour H, Fattahi A, Nouri M, et al. Cell-based endometrial regeneration: current status and future perspectives. Cell Tissue Res. 2021: 384(2): 241-254.
10. Bassiouny YA, Dakhly DMR, Bayoumi YA, Hashish NM. Does the addition of growth hormone to the in vitro fertilization/intracytoplasmic sperm injection antagonist protocol improve outcomes in poor responders? A randomized, controlled trial. Fertil Steril. 2016; 105(3): 697-702.
11. Yu H, Gao S, Tang H, Chen H, Deng Z, Yang L, et al. Growth hormone intrauterine perfusion combined with replacement cycle in the treatment of non-response thin endometrium: report of 5 cases. Int J Clin Exp Med. 2016; 9(6): 11982-11989.
12. Du Xf, Yang Xh, Li J, Hao M, Guo Yh. Growth hormone co-treatment within a GnRH agonist long protocol improves implantation and pregnancy rates in patients undergoing IVF-ET. Arch Gynecol Obstet. 2016; 294(4): 877-883.
13. Xue-Mei W, Hong J, Wen-Xiang Z, Yang L. The effects of growth hormone on clinical outcomes after frozen–thawed embryo transfer. Int J Gynaecol Obstet. 2016; 133(3): 347-350.
14. Yang JY, Li H, Lu N, Li L, Sun XX. Influence of growth hormone supplementation in patients with thin endometrium undergoing frozen embryo transfer. Reprod Dev Med. 2019; 3(1): 49.
15. Karizbodagh MP, Rashidi B, Sahebkar A, Masoudifar A, Mirzaei H. Implantation window and angiogenesis. J Cell Biochem. 2017; 118(12): 4141-4151.
16. Cui N, Li AM, Luo ZY, Zhao ZM, Xu YM, Zhang J, et al. Effects of growth hormone on pregnancy rates of patients with thin endometrium. J Endocrinol Invest. 2019; 42(1): 27-35.
17. Chen Y, Liu F, Nong Y, Ruan J, Guo Q, Luo M, et al. Clinical efficacy and mechanism of growth hormone action in patients experiencing repeat implantation failure. Can J Physiol Pharmacol. 2018; 96(9): 929-932.
18. Altmäe S, Mendoza-Tesarik R, Mendoza C, Mendoza N, Cucinelli F, Tesarik J. Effect of growth hormone on uterine receptivity in women with repeated implantation failure in an oocyte donation program: a randomized controlled trial. J Endocr Soc. 2018; 2(1): 96-105.
19. Liu Y, Zhancai W, Xuehong Z. Meta-analysis of the effectiveness of growth hormone on patients with endometrial dysplasia during in vitro fertilization-embryo transfer. Prog Obstet Gynecol. 2014; 23(7): 560-563.
20. Boguszewski MC, Cardoso-Demartini AA, Boguszewski CL, Chemaitilly W, Higham CE, Johannsson G, et al. Safety of growth hormone (GH) treatment in GH deficient children and adults treated for cancer and non-malignant intracranial tumors—a review of research and clinical practice. Pituitary. 2021: 24(5): 810-827.
21. Kim SH, Park MJ. Effects of growth hormone on glucose metabolism and insulin resistance in human. Ann Pediatr Endocrinol Metab. 2017; 22(3): 145-152.
22. Liu FT, Wu Z, Yan J, Norman RJ, Li R. The potential role of growth hormone on the endometrium in assisted reproductive technology. Front Endocrinol. 2020; 11: 49.
23. Yanping X, Zhao G, Miao J, Tan J. Changes in vimentin and vascular endothelial growth factor expression in a rat model of thin endometrium established by 95% ethanol. Chin J Tissue Eng Res. 2016; 20(5): 718-722.
24. Alfer J, Happel L, Dittrich R, Beckmann MW, Hartmann A, Gaumann A, et al. Insufficient angiogenesis: cause of abnormally thin endometrium in subfertile patients? Geburtshilfe Frauenheilkd. 2017; 77(07): 756-764.
25. Eftekhar M, Aflatoonian A, Mohammadian F, Eftekhar T. Adjuvant growth hormone therapy in antagonist protocol in poor responders undergoing assisted reproductive technology. Arch Gynecol Obstet. 2013; 287(5): 1017-1021.
26. Khan MS, Shaikh A, Ratnani R. Ultrasonography and Doppler study to predict uterine receptivity in infertile patients undergoing embryo transfer. J Obstet Gynaecol India. 2016; 66(1): 377-382.
27. Zhang T, Li Z, Ren X, Huang B, Zhu G, Yang W, et al. Endometrial thickness as a predictor of the reproductive outcomes in fresh and frozen embryo transfer cycles: a retrospective cohort study of 1512 IVF cycles with morphologically good-quality blastocyst. Medicine. 2018; 97(4).
28. Fang R, Cai L, Xiong F, Chen J, Yang W, Zhao X. The effect of endometrial thickness on the day of hCG administration on pregnancy outcome in the first fresh IVF/ICSI cycle. Gynecol Endocrinol. 2016; 32(6): 473-476.
29. Ma NZ, Chen L, Dai W, Bu ZQ, Hu LL, Sun YP. Influence of endometrial thickness on treatment outcomes following in vitro fertilization/intracytoplasmic sperm injection. Reprod Biol Endocrinol. 2017; 15(1): 1-7.
30. Xiang Yg, Tan L, Dong Fl. Effect of GH on the Expressions of VEGF, LIF, MMP-9 and TIMP-1 in Endometrium of Mouse. Reprod Contracept. 2007; 27(10): 639.

Original Article

# Fresh or Frozen Embryo Transfer in The Antagonist *In Vitro* Fertilization Cycles: A Retrospective Cohort Study

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## Abstract

**Background:** Gonadotropin-releasing hormone antagonist (GnRH-ant), widely adopted protocol, is more in line with the physiological processes, and induces a shorter and more cost-effective ovarian stimulation. In order to assess the success rate of embryo transferring (ET) in the antagonist *in vitro* fertilization (IVF) cycles, we compared the fresh ET with the frozen ET outcomes.

**Materials and Methods:** In this retrospective cohort study, one hundred five cases of ET of the infertility clinic of the Besat hospital (Kurdistan, Iran) between March 2014 to March 2020 that were treated with antagonist cycle (both fresh and frozen) were analyzed. The difference between the two groups in baseline data and reproductive outcomes were evaluated using Independent sample t test, Mann-Whitney U test, Chi-squared test, and Fisher's exact test in SPSS software (version 22).

**Results:** Out of 105 cases, 48 and 57 were in the fresh and frozen ET groups, respectively. The participants age was 35.75 ± 4.9 Y. In the fresh ET group, and 33.98 ± 5.1 Y in the frozen ET group. The percentage of chemical pregnancy was 12 (25%) in the fresh ET group and 15 (26.3%) in the frozen ET group (P=0.8); Clinical pregnancy rate was 11 (22.9%) in the fresh ET group and 11 (19.3%) in the frozen ET group (P=0.6); the rate of abortion in the fresh ET group was 3 (6.3%, P=0.2), and in the frozen ET group was 8 (14%, P=0.2); and the live birth rate was 9 (18.8%) in the fresh ET group, in comparison with 7 (12.3%) in the frozen ET group (P=0.3).

**Conclusion:** Not statistically significant, the percentage of chemical pregnancy and abortion were higher in the frozen ET group. The percentage of clinical pregnancy and live birth were higher in the fresh ET group.

***Keywords:*** Assisted Reproductive Technology, Embryo Transfer, *In Vitro* Fertilization

**Citation:** Seyedoshohadaei F, Rahmani Kh, Allahveisi A, Rezaei M, Rezaie MJ, Zandvakili F, Soufizadeh N, Honarbakhsh Y. Fresh or frozen embryo transfer in the antagonist in vitro fertilization cycles: a retrospective cohort study. Int J Fertil Steril. 2022; 16(4): 256-262. doi: 10.22074/IJFS.2022.538452.1181.


## Introduction

Infertility can be defined as the failure to achieve a pregnancy within one year of regular unprotected intercourse (1, 2). Infertility is affecting 8-12% of couples worldwide (3). Couples undergo infertility treatments due to male factor, female factors or unexplained infertility (4). Female factor accounts for 33-41% of infertility cases, male factor accounts for 25-39% of the cases and 9-39% are due to a combination of both male and female factors (5). The variability in patient characteristics and response to assisted reproductive technology (ART) dictate the need for proven, personalized diagnostic and therapeutic approaches to optimize efficacy and safety of treatment (6). Under a standard infertility treatment algorithm (SITA), couples who do not become pregnant with ovulation induction, undergo assisted reproductive techniques such as *in vitro* fertilization and embryo transfer (IVF-ET). Although, a fresh ET is still routine practice in the IVF cycles, elective frozen ET has emerged as an important method that can influence IVF outcomes (7).

After 40 years of development of IVF and ET, many IVF-ET cycles are failing and no signs of embryo implantation or the production of human chorionic gonadotropin (hCG) are achieved (8). One possible cause of the unsuccessful implantation rate is reduced endometrial receptivity despite of high quality transferred embryos (9). Poor endometrial receptivity is a major factor that leads to recurrent implantation failure. However, the traditional method cannot accurately evaluate endometrial receptivity (10). Endometrial receptivity is reduced during ovulation cycles, including in both gonadotropin-releasing hormone [GnRH agonists (GnRH-a) and GnRH antagonist (GnRH-ant) cycles], and is lower in patients who undergo GnRH-ant

Received: 08/September/2021, Accepted: 08/January/2022
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Royan Institute
International Journal of Fertility and Sterility
Vol 16, No 4, October-December 2022, Pages: 256-262

protocol cycles than in those who receive the conventional GnRH-a long protocol cycles (11-13). Endometrial receptivity should be assessed before transferring embryos. Endometrial thickness (EMT) can be measured by transvaginal ultrasonography (TVU). Several studies suggest that an EMT <8 mm is associated with implantation failure in both fresh and frozen ET cycles (14-16).

In evaluating the success rate in each cycle, we should consider the expenses, treatment side effects, patient satisfaction, and complications in mothers and fetuses. It is necessary to focus on finding important indicator for making decisions and should be considered as a key point in defining the success of assisted reproductive technology (ART) treatment. This not only reflects the outcome of an embryo transfer, such as pregnancy rate, abortion rate, but also evaluates the potency of all embryos after one oocyte retrieval cycle (17). A successful fertilization depends on the synchronic cytoplasmic and nuclear maturation (18). In recent years, there have been many reports on the pregnancy outcomes of fresh blastocyst transfer (BT) and frozen-thawed BT, but the conclusions are controversial and incomplete (19).

GnRH antagonists have been widely used for prevention of premature luteinizing hormone (LH) surges during controlled ovarian stimulation (COS) before IVF-ET (20). Simple method, short medication duration, and low incidence of ovarian hyperstimulation syndrome are some advantages of the GnRH-ant protocol (21). GnRH antagonists are also not associated with acute induction of gonadotropins, which may induce cyst formation. GnRH antagonists (GnRH-ant) does not result in profound hypo-oestrogenemia observed with GnRH agonists (GnRH-a) therefore no hot flushes are observed with GnRH-ant (22). Patients with high risk of polycystic ovarian syndrome, and poor responders are some of the main applications of antagonist IVF cycles. The overall cumulative live birth rate (CLBR) of poor ovarian responders (POR) is extremely low. In studies, some poor responders were retrospectively identified after some forms of conventional ovarian stimulation. Patients with advanced age or abnormal ovarian reserve tests [such as high follicle-stimulating hormone (FSH) or low anti-mullerian hormone (AMH) levels], are more appropriately defined as expected poor responders (23). Due to the increasing application of antagonistic cycles, in this single-center retrospective cohort study, we aimed to analysis the fertility rate and ART outcome of fresh ET and frozen ET in the antagonist IVF cycles, to close the better chance of ET with higher success rates. Many studies have compared the results of fresh versus frozen ET in IVF cycles (both agonist and antagonist), but there are not many research studies that compare the ART outcome in antagonist IVF cycles alone. Here, we focused on this to find a better understanding of the factors affecting their outcomes.

## Materials and Methods

### Ethical considerations

Patients of the infertility clinic of the Besat Hospital (Kurdistan, Iran) between March 2014 to March 2020, who received antagonist IVF cycle treatment were invited to this study. They were informed that only the outcome of their clinical process will reanalyze and targeted for research purposes. Then, the records of whom that provided written informed consent used in this study. This study was conducted after approval by the Ethics Committee of the Kurdistan University of Medical Sciences, Kurdistan, Iran (IR.MUK.REC.1399.042).

### Participants

The inclusion criteria for this study consisted of infertile women, in their reproductive age, referred to the infertility clinic of the Besat Hospital, admitted from March 2014 to March 2020, and being treated with an antagonistic IVF cycle. Patients with incomplete hospital records, that we were unable to obtain the necessary information, patients with no retrieved oocytes, and also patients who did not complete their antagonist cycle and embryo transfer, were excluded from the study.

### Study design

We considered two groups for this study. Fresh ET group, and frozen ET group. Fresh ET group includes patients undergoing antagonistic IVF cycle who received fresh embryo(s). The frozen ET group included the frozen embryo(s) transfer. The demographic data and other required clinical and paraclinical data were collected from patients' records.

ET was performed on the third day of fertilization when the embryos were at the 8 cell stage (cleavage-stage embryos). The embryos were graded into four categories according to their fragmentation index: grade A: equal size blastomeres and less than 10% fragmentation; grade B: slightly unequal blastomeres with up to 20% fragmentation; grade C: unequal sized blastomeres, up to 50% fragmentation and large granules; and grade D: unequal blastomeres with significant fragmentation (>50%) and large granules (24, 25). Due to low implantation potential of human embryos with greater than 25% fragmentation, have a (25), we only transferred embryos grade A and B.

We did not transfer embryos that were arrested in 2 cell stage, 4 cell stage, and 6 cell stage, as these factors can be considered confounding variables. In this study, we only transferred grade A, and B embryos. The criteria we considered for ET included: being equal in size, low fragmentation percent, and the accordance of embryo growth to fetal age. We used vitrification technique. The Kitazato vitrification kit (VT-601, Kitazato, Japan) was used, and we followed Kitazato kit protocol; i.e. fifteen minutes in the equilibration solution (ES), and the last one minute in the vitrification solution (VS).

In the frozen group, all embryos have been frozen, and with an interval of more than 2 months, the embryos were

transferred in one of the following methods: i. Suppression with the gonadotropin agonist, Diphereline, with half of a 3.75 ampoule, one week before menstruation, and initiation of the Estradiol valerate, the dose of which was determined individually for each patient, ii. Starting the cycle without suppression, starting with Estradiol valerate from the second day of the cycle, iii. Cycle stimulated with Clomiphene or Letrozole, and injection of hCG during follicle maturation, and subsequent embryo transfer, and iv. Patients' own normal cycle and stimulation with hCG.

After the EMT reached above 8 mm, 100 mg of Progesterone (Fertigest, 50 mg Amp*2, Aburaihan Company, Iran) was given daily for 2 to 4 days, and frozen embryos were transferred according to the patient's condition. We performed a Beta-HCG laboratory test to assess chemical pregnancy, and ultrasound evaluation of the patients to determine clinical pregnancy. If the pregnancy was confirmed, patients were followed by phone calls, clinic visits, and also obtaining information from their medical records, to record any abortion, or continuation of the pregnancy, or any other possible consequences. We also contacted patients and reviewed their hospital records, to obtain any information regarding unwanted events.

### Measurements

Demographic information of the patients, including age, and body mass index (BMI), was collected from patients' records. We also gathered information regarding the type of infertility, and the reason they were selected for the antagonist IVF cycle. BMI of the patients was divided into five categories: i. Underweight (BMI<18.5), ii. Normal (BMI: 18.5-24.9), iii. Overweight (BMI 25-29.9), iv. Obese (BMI 30-34.9), and v. Extremely obese (BMI>35). The type of infertility was divided into two groups: i. Primary infertility ii. Secondary infertility. The reason for choosing antagonist IVF cycle was categorized into three reasons: i. Polycystic ovarian syndrome (PCOS), ii. Poor responders, and iii. Failure of the previous agonist cycle. During this study, we assessed and compared the number of follicles, number of degenerated oocytes, mature oocytes, immature oocytes, injected oocytes, fertilized oocytes, number of transferred embryos, and quality of transferred embryos, in both groups. After completing the antagonist cycle, we studied cases leading to chemical pregnancy and clinical pregnancy, which were determined using the β-hCG test, and ultrasound results, respectively. Among the pregnant cases, we studied the number of miscarriages, twin, and live birth. In both groups, complications were also recorded and compared based on hospital records and specialist reports.

### Data analysis

The collected data were analyzed using SPSS software (version 22, SPSS Inc., Chicago, IL, USA). In the data description section, descriptive statistical methods such as mean, standard deviation, frequency, and relative frequency as well as the related tables were used to summarize the results. The difference between the two study groups were evaluated using Independent sample t test, Mann-Whitney U test, Chi-squared test, and Fisher's exact test. The significance level of the tests was considered 0.05.

## Results

According to the number of available records and to increase the accuracy of the study, 105 patients were studied, including 48 patients in the fresh group, 57 patients in the frozen group. The sample size was calculated using alpha error of 0.05, and beta error of 0.20, and assuming 40% difference in outcome indices in the two groups, using R software.

We compared the reason for choosing antagonist IVF cycle and no statistically significant difference was found (Table 1).

**Table 1:** Comparing the reason for choosing antagonist IVF cycle and type of infertility between the two groups

| Variables | Fresh | Frozen | P value |
|---|---|---|---|
| Reason for antagonist cycle | | | 0.3* |
| PCOS | 14 (29.2) | 19 (33.3) | |
| Poor responder | 20 (41.7) | 16 (28.1) | |
| Previous failure of agonist cycle | 14 (29.2) | 22 (38.6) | |
| Type of infertility | | | 0.4* |
| Primary | 39 (81.3) | 43 (75.4) | |
| Secondary | 9 (18.8) | 14 (24.6) | |

Data are presented as n (%). *; Chi-squared test, IVF; *In vitro* fertilization, and PCOS; Polycystic ovarian syndrome. P≤0.05 was considered significant.

Using an independent t test, we did not observe a significant difference of age, and BMI, between our groups. Also, no statistically significant difference was found in the other parameters such as Immature GV (Table 2).

### The quality of transferred embryos

Considering the quality of transferred embryos in fresh and frozen ET groups, can be concluded that the most common type of embryo transferred in both groups was grade "A". After grade A, the "both grades A & B" group and the "grade B" groups were the most frequent qualities used. Type C embryos were not used in any of the patients in our study. Out of 48 patients in the fresh ET group, 41 (85.4%) received the grade "A" quality embryos, 2 (4.2%) received the grade "B" quality embryos, and in 5 (10.4%) patients, "both grade A and grade B" embryos were transferred. Out of 57 patients in the FET group, 50 (87.7%) received "A" quality embryos, 4 (7%) received "B" quality embryos, and 3 (5.3%) received both grade A and grade B. In this study, no grade "C" embryos were transferred to any of the patient groups (Table 2).

**Table 2:** Comparing age, BMI, embryogenic factors, and quality of transferred embryos in our groups

| Variable | Fresh (n=48) | Frozen (n=57) | P value |
|---|---|---|---|
| Age (Y) | 35.75 ± 4.9 | 33.98 ± 5.1 | 0.07£ |
| BMI (kg/m$^2$) | 26.71 ± 3.8 | 27.52 ± 4.3 | 0.6£ |
| Follicles/oocytes | 6.63 ± 4.83 | 7.58 ± 6.02 | 0.3£ |
| Degenerated oocytes | 0.56 ± 0.82 | 0.56 ± 1.01 | 0.9£ |
| Immature GV | 0.27 ± 0.70 | 0.40 ± 1.05 | 0.4£ |
| Immature M1 | 0.48±0.82 | 0.51 ± 0.98 | 0.8£ |
| Mature M2 | 5.29±3.74 | 6.18 ± 5.68 | 0.3£ |
| Injected oocytes | 5.65±4.02 | 6.68 ± 5.69 | 0.2£ |
| Fertilized oocytes 2PN | 4.75±3.16 | 5.37 ± 4.45 | 0.4£ |
| Embryos | 4.65±3.21 | 5.35 ± 4.48 | 0.3£ |
| Grade of transferred embryo | | | |
| A | 41 (85.4) | 50 (87.7) | 0.5¶ |
| B | 2 (4.2) | 4 (7) | |
| A and B | 5(10.4) | 3 (5.3) | |
| C | 0 | 0 | |

Data are presented as mean ± SD or n (%). £; Independent sample t test, ε; Mann-Whitney U test, ¶; Fisher's exact test, BMI; Body mass index, SD; Standard deviation, GV; Germinal vesicle, M1; Metaphase 1, M2; Metaphase 2, and 2PN; Two-pronuclear zygote. P≤0.05 was considered significant.

Comparing the frequency of chemical pregnancies, a positive serum β-HCG in the fresh ET group with the frozen ET group, was non significantly lower (Table 3). Comparing the frequency of clinical pregnancies detected by a first trimester ultrasonography, in the fresh ET group with the frozen ET group reveals that the percentage of clinical pregnancy is higher in the group of fresh ET, but this difference is not statistically significant. The abortion frequency in the fresh ET group in comparison with the frozen ET group, was non significantly higher in the frozen ET group. Comparison of the frequency of twins in the fresh ET group with the frozen ET group, confirms that the rate of twins in the group of fresh ET is non significantly higher. The live birth frequency in the fresh ET group in comparison with the frozen ET group shows the nonsignificant higher rate (Table 3).

**Table 3:** Comparing the final results between fresh vs. frozen embryo transfer groups

| Variable | Treatment group | Yes | No | P value |
|---|---|---|---|---|
| Chemical pregnancy | Fresh | 12 (25) | 36 (75) | 0.8* |
| | Frozen | 15 (26.3) | 42 (73.7) | |
| Clinical pregnancy | Fresh | 11 (22.9) | 37 (77.1) | 0.6* |
| | Frozen | 11 (19.3) | 46 (80.7) | |
| Abortion | Fresh | 3 (6.3) | 45 (93.8) | 0.2¶ |
| | Frozen | 8 (14) | 49 (86) | |
| Twin | Fresh | 2 (4.2) | 46 (95.8) | 0.5¶ |
| | Frozen | 1 (1.8) | 56 (98.2) | |
| Live birth | Fresh | 9 (18.8) | 39 (81.3) | 0.3* |
| | Frozen | 7 (12.3) | 50 (87.7) | |

Data are presented as n (%). *; Chi-squared test and ¶; Fisher's exact test. P≤0.05 was considered significant.

## Unwanted side effects

In this study, three types of unwanted side effects were observed and recorded during the treatment period in our groups. These unwanted events included: 1. Ovarian hyperstimulation syndrome, 2. Ectopic pregnancy, and 3. Loss of a fetus in a twin pregnancy (Table 4).

**Table 4:** Comparing the unwanted adverse events between the two groups

| Type of adverse event | Fresh | Frozen | P value |
|---|---|---|---|
| Severe ovarian hyper stimulation syndrome | 4 (8.3) | 3 (5.3) | 0.48¶ |
| Ectopic pregnancy | 0 (0) | 1 (1.8) | |
| Loss of one embryo in twin pregnancy | 1 (2.1) | 0 (0) | |
| No adverse events | 43 (89.6) | 53 (93) | |

Data are presented as n (%). ¶; Fisher's exact test. P≤0.05 was considered significant.

Overall, adverse events happened in 5 patients (10.4%) in the fresh group, and 4 patients (7.1%) in the frozen group (P=0.48). Fortunately, 43 patients (89.6%) in the fresh group, and 53 patients (93%) in the frozen group did not experience any type of adverse events (P=0.48, Table 4).

## Effect of quality of transferred embryos on final results

Using fisher's exact test, and chi-squared test, we assess the different quality of embryos that we transferred in both groups and their effect on our results. We observed that the highest rate of chemical and clinical pregnancy, in both groups, was in "grade A" embryo transfer, but this difference was not statistically significant. And also, the highest percentage of abortions was seen in the frozen ET group to which "grade A" embryos were transferred, but this difference was not statistically significant. Three cases of twins were observed, all cases from "grade A" embryo group, 2 cases in the fresh group, and 1 case in the FET group. It was seen that the most live births belonged to the group that received "grade A" embryos, but this difference was not statistically significant.

# Discussion

The Gonadotropin Releasing Hormone antagonist (GnRH-ant) protocol is widely used as a convenient and cost-effective treatment for patients undergoing IVF (26). Currently, there is no consensus whether fresh ET versus frozen one, could improve IVF outcomes in GnRH-ant cycles. In this retrospective cohort study, we reviewed the treatment process and analyzed data from one hundred five patients treated with antagonistic IVF cycles in two groups of fresh and frozen ET.

Impaired endometrial receptivity has been suggested as an etiology of reduced pregnancy rates in the fresh embryos transferred ARTs (27). Endometrial receptivity can affect implantation rate, and decrease the chance of the embryo to implant (28). Frozen ET cycles are performed in a physiological uterine environment, and this may be the reason that some studies observed better IVF outcomes following the frozen ET than after fresh

ET (19, 27, 29). In a systematic review and meta-analysis performed by Roque et al. (30), they compared the outcomes in the fresh ET versus frozen ET in IVF cycles. They concluded that IVF outcomes may be improved by performing frozen ET (FET) compared with fresh ET.

The progress in embryo cryopreservation techniques has made freeze-all strategy more acceptable. Freeze all strategy has its advantages and disadvantages. No clinical data supports the use of freeze-all strategy for all patients (31). Dieamant et al. (32) conducted a meta-analysis to evaluate whether the freeze-all strategy can improve the outcomes when compared to the fresh ET in patients undergoing an ART cycle in accordance with the mean number of oocytes collected. They concluded that the freeze-all strategy could be favorable when high numbers of oocytes are collected, signaling an association between higher ovarian stimulation and consequent impairment of endometrial receptivity. However, when the mean number of oocytes collected is <15, the freeze-all strategy does not appear to be advantageous. In our study, the mean number of collected oocytes was 6.6 in the fresh ET group, and 7.5 in the frozen ET group, and the ART outcome was not significantly different between the two groups, and therefore, the results matched with "freeze-all strategy" study.

Similar results have been reported in other studies. Basirat et al. (33) observed in their study population that there was no significant difference in the pregnancy rate following ICSI treatment between fresh ET and frozen ET groups. Seyedoshohadaei et al. (34) reported that fresh ET versus frozen ET in their patients who underwent intracytoplasmic sperm injection (ICSI) had no significant effect on the final ART outcomes. Although, they did not study antagonist cycles specifically, they concluded that no statistically significant difference was found in the chemical and clinical pregnancy between frozen ET and fresh ET methods. In the current study, we could not find a significant difference in the chemical and clinical pregnancy between the two groups as well.

However, some other investigations have reported different results. Roque et al. compared IVF outcomes between fresh ET and frozen ET (the "freeze-all" policy) (35). Five hundred thirty patients underwent a gonadotropin-releasing hormone-antagonist protocol, and cleavage-stage, day-3 ET. The ART outcomes were significantly better in the freeze-all group in comparison with the fresh ET. Their results suggested that endometrial receptivity may have been impaired by COS, and outcomes may be improved by using the freeze-all policy, which is different from the results obtained in our study. Liu et al. (36) conducted a retrospective cohort study to compare frozen ET versus fresh ET in GnRH antagonist cycle in women with 3-10 oocytes retrieved. They concluded that the pregnancy rate was significantly higher in the frozen ET group than the fresh ET group (63.70% vs. 54.50%, P<0.001), which is different with the results in our study.

Pregnancies following ART are at higher risk of antenatal complications, and poor neonatal outcomes. This can result from not only a higher incidence of multiple pregnancy, but also the manipulation involved in ART processes (37). The high twinning rate is directly linked to the number of embryos transferred (38). Particularly at risk are young women who have good quality embryos. Single embryo transfer (SET) can decrease the incidence of multiple pregnancies, including twin pregnancies, after assisted reproduction. Among our study population, we had 2 twin pregnancies (4.2%) in the fresh ET group and 1 twin pregnancy (1.8%) in the frozen ET group. In a recent study, Stormlund et al. (39) compared the ongoing pregnancy rate (OPR) between a freeze-all strategy and a fresh transfer strategy in ART treatment in women with regular menstrual cycles. They had 223 patients in the freeze-all group and 230 in the fresh transfer group, no twin pregnancies occurred in either of the groups in their study, that is lower than the twin rate in our study, probably due to fewer number of embryos transferred. In another study performed by Ashrafi et al. (40), the factors affecting the outcome of a frozen ET cycle were assessed. The number of singletons in their study was 45 (78.9%), and multiple pregnancies were observed in 21.1% (17.6% twins and 3.5% triplets), twin percentage was higher compared to our study, this can be explained by different number of embryos transferred.

Application of a proper embryo scoring system has many potential benefits such as; i. Accurate selection of embryos prior to transfer, ii. Reduction of the risk of multiple pregnancies, iii. Assessment of different culture media, and iv. Comparison of embryo quality between patient cycles. Quality assessment of cleavage stage embryos is a common method in embryo quality assessment accepted by numerous embryologists. For this aim, some morphological features have been suggested. The most important qualities to consider are: fragmentation rate (Fr), blastomeres irregularities, multinucleation and blastomere number (25).

Also, there were studies in the past, which evaluated the ART outcome of fresh ET and frozen ET, but present study focused on the patients who received an antagonist IVF cycle. As these patients are usually poor responders, older in age, or polycystic ovary syndrome (PCOS) cases, and therefore a much harder group to achieve pregnancy. This study had its limitations. It was a single-center research project with limited study population; therefore, we suggest performing same studies on a larger study population, prospective, or multi centric.

## Conclusion

In order to have a better chance of ET with higher success rates, we studied the fertility rate and ART outcome of fresh ET and frozen ET in antagonist IVF cycles. Currently, there is no consensus whether fresh ET versus frozen one, could improve IVF outcomes in GnRH-ant cycles. GnRH antagonists have been widely used recently as a convenient and cost-effective treatment for patients undergoing IVF, and has many advantages including: prevention of premature luteinizing hormone

(LH) surges during COS before IVF-ET, simple method, short medication duration, and low incidence of ovarian hyperstimulation syndrome. Moreover, no cyst formation, and no hot flushes are observed. Patients with high risk of polycystic ovarian syndrome, and poor responders are some of the main applications of antagonist IVF cycles, which are harder groups of patients to achieve pregnancy. Therefore, it is worthwhile to study and analyze the factors determining success rate and ART outcomes in GnRH-ant IVF cycles. Although not statistically significant, the percentage of chemical pregnancy and abortion was higher in the frozen ET group. The percentage of clinical pregnancy and live birth was higher in the fresh ET group.

## Acknowledgements

We appreciate the patients of our clinic that contributed to this research project by giving us consent to use their medical records for scientific and research purposes. The authors would like to thank all of the team members in the Infertility Clinic and IVF Center of The Besat Hospital, Kurdistan, Iran. This study was financed by the Kurdistan University of Medical Sciences, Kurdistan, Iran. The authors declare no conflict of interest.

## Authors' Contributions

F.S.; Contributed to the conception and design, methodology, and ART specialist. Kh.R.; Provided data analysis and statistical consultant. A.A., M.J.R.; Chief embryologist, preserving and handling oocytes and embryos. M.R.; ART specialist, planning and performing IVF cycles, and supervision. F.Z., N.S.; Interpretation and supervision. Y.H.; Contributed in acquisition of data, data analysis, and writing original draft. All authors read and approved the final manuscript.

## References

1. Carson SA, Kallen AN. Diagnosis and management of infertility: a Review. JAMA. 2021; 326(1): 65-76.
2. Vander Borght M, Wyns C. Fertility and infertility: definition and epidemiology. Clin Biochem. 2018; 62: 2-10.
3. Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S, Stevens GA. National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. PLoS Med. 2012; 9(12): e1001356.
4. Glujovsky D, Pesce R, Sueldo C, Quinteiro Retamar AM, Hart RJ, Ciapponi A. Endometrial preparation for women undergoing embryo transfer with frozen embryos or embryos derived from donor oocytes. Cochrane Database Syst Rev. 2010; (1): CD006359.
5. Wasilewski T, Łukaszewicz-Zając M, Wasilewska J, Mroczko B. Biochemistry of infertility. Clinica Chimica Acta. 2020; 508: 185-190.
6. Fauser BC, Diedrich K, Devroey P. Predictors of ovarian response: progress towards individualized treatment in ovulation induction and ovarian stimulation. Hum Reprod Update. 2008; 14(1): 1-14.
7. Roque M, Valle M, Guimarães F, Sampaio M, Geber S. Freeze-all cycle for all normal responders? J Assist Reprod Genet. 2017; 34(2): 179-185.
8. Gerber RS, Fazzari M, Kappy M, Cohen A, Galperin S, Lieman H, et al. Differential impact of controlled ovarian hyperstimulation on live birth rate in fresh versus frozen embryo transfer cycles: a Society for Assisted Reproductive Technology Clinic Outcome System study. Fertil Steril. 2020; 114(6): 1225-1231.
9. Kliman HJ, Frankfurter D. Clinical approach to recurrent implantation failure: evidence-based evaluation of the endometrium. Fertil Steril. 2019; 111(4): 618-628.
10. Zhao Y, He D, Zeng H, Luo J, Yang S, Chen J, et al. Expression and significance of miR-30d-5p and SOCS1 in patients with recurrent implantation failure during implantation window. Reprod Biol Endocrinol. 2021; 19(1): 138.
11. Zhang D, Han M, Zhou M, Liu M, Li Y, Xu B, et al. Down-regulation of S100P induces apoptosis in endometrial epithelial cell during GnRH antagonist protocol. Reprod Biol Endocrinol. 2021; 19(1): 99.
12. Yeh JS, Steward RG, Dude AM, Shah AA, Goldfarb JM, Muasher SJ. Pregnancy rates in donor oocyte cycles compared to similar autologous in vitro fertilization cycles: an analysis of 26,457 fresh cycles from the Society for Assisted Reproductive Technology. Fertil Steril. 2014; 102(2): 399-404.
13. Orvieto R, Meltzer S, Rabinson J, Zohav E, Anteby EY, Nahum R. GnRH agonist versus GnRH antagonist in ovarian stimulation: the role of endometrial receptivity. Fertil Steril. 2008; 90(4): 1294-1296.
14. Chan JM, Sukumar AI, Ramalingam M, Ranbir Singh SS, Abdullah MF. The impact of endometrial thickness (EMT) on the day of human chorionic gonadotropin (hCG) administration on pregnancy outcomes: a 5-year retrospective cohort analysis in Malaysia. Fertil Res Pract. 2018; 4: 5.
15. Basir GS, O WS, So WW, Ng EH, Ho PC. Evaluation of cycle-to-cycle variation of endometrial responsiveness using transvaginal sonography in women undergoing assisted reproduction. Ultrasound Obstet Gynecol. 2002; 19(5): 484-489.
16. Dessolle L, Daraï E, Cornet D, Rouzier R, Coutant C, Mandelbaum J, et al. Determinants of pregnancy rate in the donor oocyte model: a multivariate analysis of 450 frozen-thawed embryo transfers. Hum Reprod. 2009; 24(12): 3082-3089.
17. Yang J, Zhang X, Ding X, Wang Y, Huang G, Ye H. Cumulative live birth rates between GnRH-agonist long and GnRH-antagonist protocol in one ART cycle when all embryos transferred: real-word data of 18,853 women from China. Reprod Biol Endocrinol. 2021; 19(1): 124.
18. Pereira N, Neri QV, Lekovich JP, Palermo GD, Rosenwaks Z. The role of in-vivo and in-vitro maturation time on ooplasmic dysmaturity. Reprod Biomed Online. 2016; 32(4): 401-406.
19. Yang M, Lin L, Sha C, Li T, Gao W, Chen L, et al. Which is better for mothers and babies: fresh or frozen-thawed blastocyst transfer? BMC Pregnancy Childbirth. 2020; 20: 559.
20. Luo X, Pei L, Li F, Li C, Huang G, Ye H. Fixed versus flexible antagonist protocol in women with predicted high ovarian response except PCOS: a randomized controlled trial. BMC Pregnancy Childbirth. 2021; 21(1): 348.
21. Xia M, Zheng J. Comparison of clinical outcomes between the depot gonadotrophin-releasing hormone agonist protocol and gonadotrophin-releasing hormone antagonist protocol in normal ovarian responders. BMC Pregnancy Childbirth. 2021; 21(1): 372.
22. Depalo R, Jayakrishan K, Garruti G, Totaro I, Panzarino M, Giorgino F, et al. GnRH agonist versus GnRH antagonist in in vitro fertilization and embryo transfer (IVF/ET). Reprod Biol Endocrinol. 2012; 10: 26.
23. Liu Y, Su R, Wu Y. Cumulative live birth rate and cost-effectiveness analysis of gonadotropin releasing hormone-antagonist protocol and multiple minimal ovarian stimulation in poor responders. Front Endocrinol (Lausanne). 2020; 11: 605939.
24. Halvaei I, Khalili MA, Esfandiari N, Safari S, Talebi AR, Miglietta S, et al. Ultrastructure of cytoplasmic fragments in human cleavage stage embryos. J Assist Reprod Genet. 2016; 33(12): 1677-1684.
25. Nasiri N, Eftekhari-Yazdi P. An overview of the available methods for morphological scoring of pre-implantation embryos in in vitro fertilization. Cell J. 2015; 16(4): 392-405.
26. Wang R, Lin S, Wang Y, Qian W, Zhou L. Comparisons of GnRH antagonist protocol versus GnRH agonist long protocol in patients with normal ovarian reserve: a systematic review and meta-analysis. PLoS One. 2017; 12(4): e0175985.
27. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C, Thomas S. Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: a prospective randomized trial comparing fresh and frozen-thawed embryo transfer in normal responders. Fertil Steril. 2011; 96(2): 344-348.
28. Shah MS, Caballes M, Lathi RB, Baker VL, Westphal LM, Milki AA. In vitro fertilization outcomes after fresh and frozen blastocyst transfer in South Asian compared with Caucasian women. Fertil Steril. 2016; 105(6): 1484-1487.
29. Roque M, Valle M, Sampaio M, Geber S. Obstetric outcomes after fresh versus frozen-thawed embryo transfers: a systematic review and meta-analysis. JBRA Assist Reprod. 2018; 22(3): 253-260.
30. Roque M, Lattes K, Serra S, Solà I, Geber S, Carreras R, et al. Fresh embryo transfer versus frozen embryo transfer in in vitro fertilization cycles: a systematic review and meta-analysis. Fertil Steril. 2013; 99(1): 156-162.
31. Ding X, Yang J, Li L, Yang N, Lan L, Huang G, et al. Fertility outcomes in women after controlled ovarian stimulation with gonadotropin releasing hormone agonist long protocol: fresh versus frozen embryo transfer. BMC Pregnancy Childbirth. 2021; 21(1): 207.
32. Dieamant FC, Petersen CG, Mauri AL, Comar V, Mattila M, Vagnini LD, et al. Fresh embryos versus freeze-all embryos - transfer strategies: nuances of a meta-analysis. JBRA Assist Reprod. 2017; 21(3): 260-272.
33. Basirat Z, Adib Rad H, Esmailzadeh S, Jorsaraei SG, Hajian-Tilaki K,

Pasha H, et al. Comparison of pregnancy rate between fresh embryo transfers and frozen-thawed embryo transfers following ICSI treatment. Int J Reprod Biomed. 2016; 14(1): 39-46.
34. Seyedoshohadaei F, Rezaei M, Allahveisi A, Rahmani K, Amirkhani Z. Effect of fresh and frozen embryo transfer method on fertility success in assisted reproduction: a comparative study. J Postgrad Med Inst. 2019; 33(2).
35. Roque M, Valle M, Guimarães F, Sampaio M, Geber S. Freeze-all policy: fresh vs. frozen-thawed embryo transfer. Fertil Steril. 2015; 103(5): 1190-1193.
36. Liu X, Bai H, Shi W, Shi J. Frozen-thawed embryo transfer is better than fresh embryo transfer in GnRH antagonist cycle in women with 3-10 oocytes retrieved: a retrospective cohort study. Arch Gynecol Obstet. 2019; 300(6): 1791-1796.
37. Zhu L, Zhang Y, Liu Y, Zhang R, Wu Y, Huang Y, et al. Maternal and live-birth outcomes of pregnancies following assisted reproductive technology: a retrospective cohort study. Sci Rep. 2016; 6: 35141.
38. Karlström PO, Bergh C. Reducing the number of embryos transferred in Sweden-impact on delivery and multiple birth rates. Hum Reprod. 2007; 22(8): 2202-2207.
39. Stormlund S, Sopa N, Zedeler A, Bogstad J, Prætorius L, Nielsen HS, et al. Freeze-all versus fresh blastocyst transfer strategy during in vitro fertilisation in women with regular menstrual cycles: multicentre randomised controlled trial. BMJ. 2020; 370.
40. Ashrafi M, Jahangiri N, Hassani F, Akhoond MR, Madani T. The factors affecting the outcome of frozen–thawed embryo transfer cycle. Taiwan J Obstet Gynecol. 2011; 50(2): 159-164.

Original Article

# Assessing The Role and Accuracy of Ultrasonographic Imaging in The Diagnosis of Deep Infiltrating Endometriosis: A Cross-Sectional Study

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## Abstract

**Background:** Deep infiltrating endometriosis (DIE) is described as an endometriotic tissue that penetrates more than 5 mm under the peritoneal surface. It's suggested that trans vaginal sonography (TVS) is 79% sensitive and 94% specific in the assessment of intestinal DIE. Considering the possibility that DIE ultrasonography (rectal and\or vaginal ultrasonography) might be more accurate, we designed this study to assess this study to evaluate the accuracy of DIE ultrasonography.

**Materials and Methods:** In this retrospective cross-sectional study, we designed and conducted this study from 2019 to 2020 on patients suspected of severe endometriosis. Our patients underwent ultrasonographic imaging and based on the results became candidates for surgery. We compared histopathological results with sonographic findings using crosstabulation and chi-square tests were used to measure accuracy. $P<0.05$ were considered statistically significant.

**Results:** Following pathological assessments of 109 cases, 97 cases had ovarian endometrioma, 42 cases had intestinal involvement and 56 cases had uterosacral DIE. The results for accuracy were as the following; uterosacral ligament (USL) involvement SE: 96.4% and SP: 59.1%; intestinal involvement SE: 97.6% and SP: 73.8%; and Cul de sac involvement with SE: 100% and SP: 50.8%. With regards to ovarian endometrioma, ultrasonographic imaging was 99.0% sensitive and 84.6% specific. With regards to intestinal involvement, ultrasonography performed a reliable overall diagnosis (97.6% sensitive and 73.8% specific). However, the results showed lower accuracy regarding the level of intestinal involvement. The accuracy for other sites and cavities was low except for ovarian endometrioma.

**Conclusion:** The results of the present study demonstrated that pre-operative TVS and Transrectal ultrasound (TRUS) can be a helpful paraclinical tool in the assessment and diagnosis of DIE and endometriosis in general and particularly with adnexal and bowel lesions, it can have some shortcomings with respect to cul de sac and USLs.

***Keywords:*** Laparoscopic Surgery, Ovarian Endometrioma, Ultrasonographic Imaging

**Citation:** Asgari Z, Farzadi S, Hosseini R, Hadizadeh A, Mortezazadeh M. Assessing the role and accuracy of ultrasonographic imaging in the diagnosis of deep infiltrating endometriosis: a cross-sectional study. Int J Fertil Steril. 2022; 16(4): 263-267. doi: 10.22074/IJFS.2021.535199.1167.


## Introduction

Endometriosis is described as the presence of endometrial tissue in a space outside of the uterus and endometrial cavity. This disease affects almost 10% of women of reproductive age and is usually diagnosed with clinical history as most of the cases complain of chronic pelvic pain (1, 2). The average interval between the start of symptoms and surgical diagnosis is 10.4 years (3). Beyond the clinical symptoms and physical examination, imaging is the modality for the initial assessment of these patients. Imaging techniques currently used to diagnose endometriosis are magnetic resonance imaging (MRI) and ultrasonography with a preference for sonography in recent years (4).

However, the combination of transvaginal sonography (TVS) and MRI is not recommended for a more accurate diagnosis (5). But still, other causes such as fibroma, corpus luteum, cystadenoma, tubo-ovarian abscess, teratoma, and carcinoma are needed to be ruled out (6-8). Identification of the endometriotic nodules and their correct localization enables complete lesion mapping before surgery and prevents unexpected plan changes in surgery (1, 6, 9, 10).

Deep infiltrating endometriosis (DIE) is recognized as

Received:03/August/2021, Accepted: 11/December/2021
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Royan Institute
International Journal of Fertility and Sterility
Vol 16, No 4, October-December 2022, Pages: 263-267

the most severe form of endometriosis has a complex clinical approach; it is described as a lesion that penetrates >5 mm under the peritoneal surface (11). DIE accounts for 15 to 30% of all endometriosis cases of which 90% are characterized by chronic pelvic pain and infertility, and 25% are accidentally discovered during laparoscopy or laparotomy (12, 13). DIE nodules infiltrate mostly the uterosacral ligaments (USL), rectosigmoid, vaginal fornix, rectovaginal septum, and/or bladder (14).

Intestinal endometriosis comprises a spectrum from simple adhesions between the intestine and cervix to nodular lesions that might involve serous membrane to the mucosa. These kinds of severe involvements require simultaneous cooperation between the colorectal and the gynecology surgeons. Due to various diameters and involvement stages, several surgical approaches have been proposed and used (1, 9). While smaller, less invasive, lesions are removed using stapled trans-anal resection, the larger and more invasive ones need segmental resection (6, 15).

A precise consensus on the definition and severity of endometriosis isn't reached yet but the most frequently used classification is the American Society of Reproductive Medicine (ASRM) classification; however, it fails to completely represent DIE's characteristics (16, 17). It's suggested that TVS is 79% sensitive and 94% specific in the assessment of the extent of DIE (2) meanwhile, it is proposed that DIE pelvic ultrasonography, which includes rectal and\or vaginal ultrasonographic imaging, is more accurate regarding the extent and severity (1, 2, 6, 11).

We designed and conducted this cross-sectional study to assess the accuracy of DIE ultrasonography and to do so, we compared the results with pathological and surgical findings, particularly with results of rectal involvement. It's suggested that TVS is 79% sensitive and 94% specific in the assessment of intestinal DIE. In this study, we assessed the accuracy of DIE ultrasonography (rectal and\or vaginal ultrasonography) which is thought to be more accurate.

## Materials and Methods

We designed and conducted this cross-sectional study on patients with severe endometriotic symptoms who were a candidate for laparoscopic surgery and their disease was later confirmed histologically from December 2019 to December 2020. Our patients who were suspected of DIE were assessed in regards to the following characteristics and variables: age, body mass index (BMI) category, confirmed DIE or ovarian endometrioma (OMA), and the respective location and the level of involvement. Our patients were 35.41 years old on average with a standard deviation of 5.94. The symptoms included pelvic pain, dysmenorrhea, dyspareunia, infertility, abnormal uterine bleeding (AUB), and dysphasia. The patients were enrolled from the laparoscopic office of Arash hospital at Tehran university of medical sciences. Our exclusion criteria included the patients who were pregnant, menopausal, or had a non-endometrial mass in adnexa, or other malignancies. We also excluded any patients who had any contraindications from the surgery. The patients who were of reproductive age and had a typical medical history compatible with endometriosis were also assessed, and if their imaging and pathological findings were consistent, they were included in the study. All patients included in the study provided informed consent. In this study we considered pathologically approved surgical results as our gold standard; thus, all our data was compared and tested with surgical findings confirmed by pathology. All patients were assessed by both the attending professor and the fellowship trainees, and all data relating to endometriosis such as pelvic pain, dysmenorrhea, dyspareunia, infertility, and AUB dysphasia were collected.

All the features and data gathered from ultrasonographic imaging along with surgical and pathological findings were collected, recorded, and analyzed. The patient's intestinal involvement was scored from 0 to 3 (0 being no involvement and 3 being full mucosal involvement). Other anatomical sites and areas such as adnexa, cul de sac, USLs, and the salpinx were also assessed and compared. We also gathered general body statistics of the patients and assessed the accuracy using the aforementioned data.

Based on the assumption from previous studies that DIE ultrasonography is up to 96% sensitive we calculated that our minimum cases should include 70 patients (Cochrane's sample size formula). In total, 109 cases were chosen for the study, and the data were analyzed using IBM's SPSS v26 software (IBM, USA). Our primary goal was to assess the sensitivity, specificity, and positive predictive value of DIE ultrasonographic examination particularly in the extent of intestinal involvement. We also used cross tabulation and chi-square tests to assess the significance of the tests.

This study was ethically approved by the Ethical Committee of the Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1399.936) and all patients had signed informed consent forms.

## Results

In total, 150 cases with symptoms were chosen and 109 cases had either DIE or OMA, and 41 were not chosen due to no findings in ultrasonography. As reported by the pathology laboratory, there were 97 cases of pathologically confirmed ovarian endometrioma, 42 cases had intestinal involvement, 56 had uterosacral DIE, 19 cases had uterus adenomyosis and 9 cases were diagnosed with myoma. We also asked the patients to evaluate and score their symptoms from 0 to 10 and on average; The main symptoms that patients complained of were pelvic pain (80.3%), dysmenorrhea (85.3%), dyspareunia (48.6%), dysphasia (43.1%), AUB (29.4%) and infertility (29.4%) respectively (Fig.1). The symptoms were scored as follows; scored as the following dysmenorrhea at 6.74, dyspareunia at 3.36, and dysphasia at 2.72 respectively.

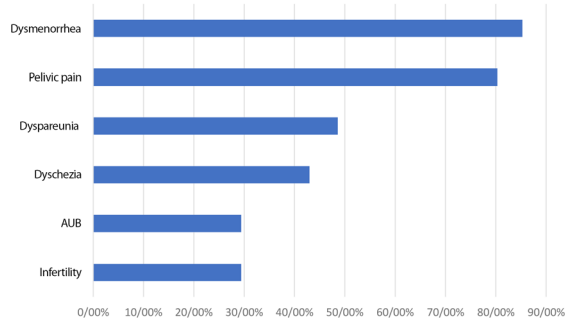


**Fig.1:** Proportion of each symptom felt by patients.

In regards to the accuracy of ultrasonography imaging in the diagnosis of intestinal DIE, which was our primary outcome, we found that ultrasonographic imaging performed excellently in overall diagnosis since it was 97.6% sensitive and 73.8% specific. However, laparoscopic evaluation was far more diagnostic (97.6% sensitive and 97.2% specific). As for the levels of involvement in the intestine, we compared the ultrasonographic imaging findings with pathologic results and the results showed lower accuracy; 55.6, 50.0, 66.7% sensitive, and 72.0, 85.6, 91.5% specific for serous membrane, muscular layer and mucus membrane respectively. The average BMI was 24.7 and most of the cases were in the normal range (46.3%) (Tables 1, 2, Fig.2).

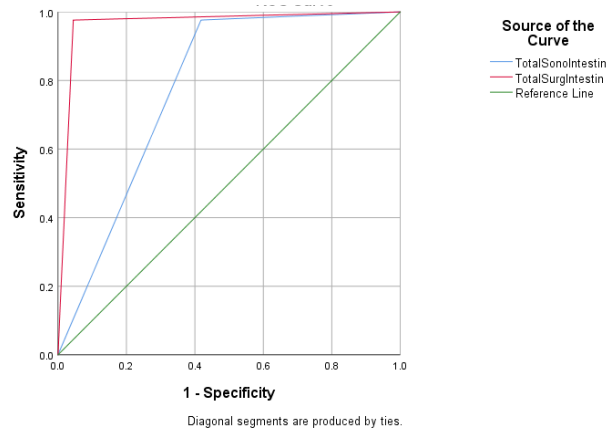


**Fig.2:** ROC curve diagrams showcasing the accuracy of DIE sonography and laparoscopy in regards to intestinal involvement. ROC; Receiver operating characteristic and DIE; Deep infiltrating endometriosis.

We also assessed the effect of obesity and weight on US imaging; we compared the results of US imaging of the intestine in 4 BMI brackets as follows: underweight (BMI<18.5), normal range (18.5 to 24.9), overweight (25 to 29.9), and obese (BMI>30). The results showed that both sensitivity and specificity were negatively affected. These results were statistically significant except for the underweight BMI bracket, which we believe was due to the small sample size (Table 3).

**Table 1:** Diagnostic accuracy of DIE ultrasonography and laparoscopy in diagnosis of DIE and endometrial lesions

| Accuracy assessment | Sensitivity (%) | | Specificity (%) | | PPV (%) | | NPV (%) | |
|---|---|---|---|---|---|---|---|---|
| Lesion location | DIE US | Laparoscopy | DIE US | Laparoscopy | DIE US | Laparoscopy | DIE US | Laparoscopy |
| Intestine | 97.6 | 97.6 | 73.8 | 97.2 | 59.4 | 93.2 | 97.1 | 95.5 |
| OMA | 99.0 | 99.0 | 84.6 | 88.0 | 88.0 | 78.0 | 97.1 | 88.0 |
| Cul de sac | 100 | 100 | 50.8 | 100 | 25.0 | 100 | 58.0 | 100 |
| Uterosacral ligament | 96.4 | 85.7 | 59.1 | 84.9 | 58 | 85.7 | 84.9 | 84.9 |

**DIE US; Ultrasonographic imaging, PPV; Positive predictive value, NPV; Negative predictive value, and OMA; Ovarian endometrioma.**

**Table 2:** Diagnostic accuracy of ultrasonographic imaging in regards to intestinal level of involvement

| BMI (kg/m$^2$) | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Chi square exact (P value) | Frequency n (%) | Fisher's exact test (P value) |
|---|---|---|---|---|---|---|---|
| BMI group I | 100.0 | 80.0 | 75.0 | 100.0 | 0.071 | 8 (7.3) | 0.071 |
| BMI group II | 94.7 | 70.0 | 66.7 | 95.5 | <0.001 | 49 (45) | <0.001 |
| BMI group III | 93.8 | 61.0 | 55.6 | 87.5 | 0.037 | 35 (32.1) | 0.037 |
| BMI group IV | 100 | 66.7 | 62.5 | 100 | 0.028 | 14 (12.8) | 0.028 |

**PPV; Positive predictive value, NPV; Negative predictive value, and BMI; Body mass index.**

**Table 3:** The effect of body mass composition on accuracy of ultrasonographic imaging

| Accuracy for assessment bowel layer | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | P value |
|---|---|---|---|---|---|
| Serous | 55.6 | 72.0 | 39.5 | 83.1 | 0.009 |
| Muscular | 50.0 | 85.6 | 15.0 | 97.8 | 0.014 |
| Mucus | 66.7 | 91.5 | 40.0 | 93.3 | 0.003 |

**PPV; Positive predictive value and NPV; Negative predictive value.**

We also assessed the accuracy with respect to OMA and the DIE that infiltrates cul de sac and USLs. The results indicated that ultrasonographic imaging was 99.0% sensitive and 84.6% specific. The data of accuracy show that the examination for cul de sac was 100% sensitive, and 50.8% specific while it was 96.4% sensitive and 59.1% specific in assessing USLs. Although imaging is quite sensitive, it can be inaccurate regarding cul de sac and USL assessment since their positive predictive value was 25.0 % and 58% respectively; however, the results for ovarian assessment showed 92.3% PPV (Table 1).

## Discussion

In our study, we identified that overall diagnostic accuracy was 97.6% sensitive and 73.8% specific. However laparoscopic evaluation was found to be far more accurate (97.6% sensitive and 97.2% specific). DIE was also found to lack accuracy in regard to the extent of involvement. It was also not accurate with respect to assessing cul de sac and USL. Imaging has always been an important tool in both the diagnosis and surgical approach to endometriosis. A thorough evaluation can help diagnosis and the entire approach and planning. Thus, it's of utmost importance that the data pertaining to the lesion is both accurate and reproducible, therefore we aimed to assess DIE ultrasonographic imaging as a complementary and multi-perspective imaging approach. DIE pelvic ultrasonography consists of vaginal and\or rectal US imaging (18). In a study conducted by S. Alborzi et al. (19), it was stated that ultrasonographic imaging (transvaginal or transrectal) is as accurate as MRI in the detection of lesions.

In our study, the diagnostic accuracy of ultrasonographic imaging in the identification of intestinal lesions, which was our primary outcome, was almost as high as laparoscopic evaluation. Therefore, we suggest that, in the overall diagnosis of DIE in the intestine, this procedure could be useful. In a multicenter prospective and retrospective cohort study conducted in the royal college of obstetrics and gynecology the accuracy of the preoperative ultrasound-based endometriosis staging system (UBESS) regarding the complexity of surgery was assessed; this study showed that US-based imaging can be utilized to plan the surgery (16).

We also assessed the accuracy of DIE US imaging with respect to other pelvic cavities and sites; in regards to ovarian endometrioma, we concluded that DIE ultrasonography can be a very efficient and accurate tool (99.0% sensitive and 84.6% specific) and as manifested by several other studies such as the study conducted by Holland et al. (16) can distinguish between different pathologies. Their study showed that TVS is an accurate assessment tool for the severity of pelvic endometriosis and the results are mostly in accord with laparoscopic findings. Meanwhile, we also studied the accuracy of DIE ultrasonographic imaging in the diagnosis of lesions located at USLs and cul de sac and concluded that even though sensitivity for these lesions was high (100% and 96.4% sensitive for cul de sac and USLs respectively) the tests can be inaccurate as their PPV and specificity were low.

There were some limitations in our study that reduced the diagnostic accuracy of ultrasonography in DIE patients. We believe that ultrasound imaging accuracy could be hampered as poor bowel preparation can limit ultrasound wave penetration. On the other hand, the procedure itself (TRUS) is painful. These two can both limit the time required for investigation. Another reason that has led to lower accuracy could be the fact that linear nodules could be missed during laparoscopic surgery, particularly in cul de sac. we lacked sufficient samples for specific groups such as the patients with obese body composition.

In regards to the body composition of the subjects, we concluded that with higher BMI values the efficacy of US imaging plummets. As described by Bushberg et al. (20), due to fat impedance, 94% of the original sound wave is attenuated particularly in patients with more than 8 cm of subcutaneous fat before it even reaches the peritoneal cavity; hence, this phenomenon affects the acuity of ultrasonographic imaging.

## Conclusion

Our study showed that while DIE pelvic ultrasonographic imaging can be a helpful paraclinical tool in the assessment and diagnosis of DIE and endometriosis in general and particularly with adnexal and bowel lesions, it can have some shortcomings with respect to cul de sac and USLs. We also suggest that in overweight patients these procedures should be performed more meticulously and probably in conjunction with other imaging methods such as MRI.

## Acknowledgements

There is no financial support and conflict of interest in this study.

## Authors' Contributions

Z.A., S.F.; Contributed to developing the research idea, composing, and revising the manuscript. R.H.; Contributed to composing and revising the manuscript. A.H., M.M.; Contributed to statistical analysis developing the research idea and revising the manuscript. All authors read and approved the final manuscript.

## References

1. Parasar P, Ozcan P, Terry KL. Endometriosis: epidemiology, diagnosis and clinical management. Curr Obstet Gynecol Rep. 2017; 6(1): 34-41.
2. Berek JS. Berek and Novak's gynecology essentials. 1st ed. USA: Lippincott Williams and Wilkins; 2020.
3. Hadfield R, Mardon H, Barlow D, Kennedy S. Delay in the diagnosis of endometriosis: a survey of women from the USA and the UK. Hum Reprod. 1996; 11(4): 878-880.
4. Young SW, Groszmann Y, Dahiya N, Caserta M, Yi J, Wasson

M, et al. Sonographer-acquired ultrasound protocol for deep endometriosis. Abdom Radiol (NY). 2020; 45(6): 1659-1669.
5. Bazot M, Daraï E. Diagnosis of deep endometriosis: clinical examination, ultrasonography, magnetic resonance imaging, and other techniques. Fertil Steril. 2017; 108(6): 886-894.
6. Halis G, Mechsner S, Ebert AD. The diagnosis and treatment of deep infiltrating endometriosis. Dtsch Arztebl Int. 2010; 107(25): 446-455.
7. Turocy JM, Benacerraf BR. Transvaginal sonography in the diagnosis of deep infiltrating endometriosis: a review. J Clin Ultrasound. 2017; 45(6): 313-318.
8. Kinkel K, Frei KA, Balleyguier C, Chapron C. Diagnosis of endometriosis with imaging: a review. Eur Radiol. 2006; 16(2): 285-298.
9. Menakaya U, Reid S, Lu C, Bassem G, Infante F, Condous G. Performance of ultrasound-based endometriosis staging system (UBESS) for predicting level of complexity of laparoscopic surgery for endometriosis. Ultrasound Obstet Gynecol. 2016; 48(6): 786-795.
10. Menakaya UA, Adno A, Lanzarone V, Johnson NP, Condous G. Integrating the concept of advanced gynaecological imaging for endometriosis. ANZJOG. 2015; 55(5): 409-412.
11. Tosti C, Pinzauti S, Santulli P, Chapron C, Petraglia F. Pathogenetic mechanisms of deep infiltrating endometriosis. Reprod Sci. 2015; 22(9): 1053-1059.
12. Ahn SH, Monsanto SP, Miller C, Singh SS, Thomas R, Tayade C. Pathophysiology and immune dysfunction in endometriosis. Biomed Res Int. 2015; 2015: 795976.
13. Bulun SE. Endometriosis. N Engl J Med. 2009; 360(3): 268-279.
14. Raiza LCP, Bianchi P, Cordioli E, et al. Prevalence of sonographic signs of deep infiltrative endometriosis amongwomen submitted to routine transvaginal sonography. J Minim Invasive Gynecol. 2016; 23(7): S27.
15. Chapron C, Fauconnier A, Dubuisson JB, Barakat H, Vieira M, Bréart G. Deep infiltrating endometriosis: relation between severity of dysmenorrhoea and extent of disease. Hum Reprod. 2003; 18(4): 760-766.
16. Holland T, Hoo W, Mavrelos D, Saridogan E, Cutner A, Jurkovic D. Reproducibility of assessment of severity of pelvic endometriosis using transvaginal ultrasound. Ultrasound Obstet Gynecol. 2013; 41(2): 210-215.
17. Holland TK, Cutner A, Saridogan E, Mavrelos D, Pateman K, Jurkovic D. Ultrasound mapping of pelvic endometriosis: does the location and number of lesions affect the diagnostic accuracy? A multicentre diagnostic accuracy study. BMC Womens Health. 2013; 13: 43.
18. Park SB, Kim JK, Cho KS. Sonography of endometriosis in infrequent sites. J Clin Ultrasound 2008; 36(2): 91-97.
19. Alborzi S, Rasekhi A, Shomali Z, Madadi G, Alborzi M, Kazemi M, et al. Diagnostic accuracy of magnetic resonance imaging, transvaginal, and transrectal ultrasonography in deep infiltrating endometriosis. Medicine (Baltimore). 2018; 97(8): e9536.
20. Bushberg JT, Scibert JA, Leidholdt EM, Boone JM. The essential physics of medical imaging. 3th ed. USA; Lippincott Williams and Wilkins; 2011.

Original Article

# The Effect of High Intensity Intermittent and Combined (Resistant and Endurance) Trainings on Some Anthropometric Indices and Aerobic Performance in Women with Polycystic Ovary Syndrome: A Randomized Controlled Clinical Trial Study

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## Abstract

**Background:** Overweight and obesity are associated with cardiometabolic risk in polycystic ovary syndrome (PCOS). Lifestyle adjustment, such as increasing physical activity, is a first-line strategy to treat PCOS. The current study aims to compare and examine the effect of high intensity intermittent training (HIIT) and combined (COM) training on some anthropometric indices and aerobic performance in PCOS females.

**Materials and Methods:** This randomized controlled clinical trial was conducted on 45 women with PCOS divided into three groups receiving HIIT (n=15), COM interventions (n=15) or control group (n=15) for eight weeks. Some anthropometric indices factors including weight, body mass index (BMI), waist to hip ratio (WHR), body fat percent (FP), and visceral adipose tissue (VAT) as well as $VO_{2max}$ were measured at the baseline at the eighth week. Data were analyzed by one-way ANOVA test. Tukey post hoc tests were used to compare the pair differences.

**Results:** After eight-week intervention, weight, BMI, WHR, FP, and VAT decreased significantly in both groups of COM and HIIT ($P<0.05$) relative to the control group. There were no differences between HIIT group and COM group in terms of these variables ($P>0.05$). $VO_{2max}$ increased significantly after COM and HIIT interventions relative to the control group ($P=0.001$); however, HIIT was statically more effective than COM ($P=0.011$).

**Conclusion:** The current study revealed that both HIIT and COM trainings could be beneficial in improving some anthropometric indices in addition to aerobic capacity, although HIIT was more effective on aerobic performance (registration number: IRCT20130812014333N143).

***Keywords:*** Body Composition, Endurance Training, High Intensity Intermittent Training, Polycystic Ovary Syndrome, Resistance Training

**Citation:** Nasiri M, Monazzami A, Alavimilani S, Asemi Z. The effect of high intensity intermittent and combined (resistant and endurance) trainings on some anthropometric indices and aerobic performance in women with polycystic ovary syndrome: a randomized controlled clinical trial sudy. Int J Fertil Steril. 2022; 16(4): 268-274. doi: 10.22074/IJFS.2022.551096.1279.



## Introduction

Polycystic ovary syndrome (PCOS) is distinguished as the most frequent endocrine disease with 5 to 10 percent patient among females in reproductive age (1). Multiple factors have been reported to be involved in its pathogenesis in which the main one is long-standing lack of ovulation results from hyperandrogenism. Its clinical appearance is various and may exist in anovulation, oligoovulation as well as hyperandrogenism (2). Diverse elements like dysregulation of mitochondria, inflammatory pathways, oxidative stress and change in hormones, are seen in PCOS (3). Furthermore, abdominal obesity, abnormal lipid and glucose metabolism, insulin resistance (IR), and hypertension (4, 5) are related to PCOS development which increase the danger of cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) incidence (5).

In addition to genetic involvement (6), environmental aspects such as obesity are found to affect the progression of PCOS or even to make patients' clinical condition worse. Diet (7) or physical activity (8) are recommended as the first treatments for PCOS cure (9). Metabolic comorbidities and hyperandrogenism have been reported to be ameliorated by regular physical activity leading to the treatment of anovulation and in turn fertility restoration.

**Received: 28/March/2022, Accepted: 21/June/2022**
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**Royan Institute**
**International Journal of Fertility and Sterility**
**Vol 16, No 4, October-December 2022, Pages: 268-274**

The aerobic training with the intensity of moderate or high benefits PCOS via ameliorating metabolic and fertility related expressions in PCOS individuals. This type of exercise can also positively affect anovulation, IR, obesity and cardio metabolic indices (10).

Nate less, the literature is restricted to sparse randomized controlled trials (RCTs) limiting to some general advise of training instead of developing a clear exercise guideline for PCOS ameliorating (11). For instance, diet and exercise interconnection, duration, appropriate intensity, or modality of exercise have not been clear yet. Also training has positive functions on elevating energy consumption, hormones like cortisol, growth related, insulin, sex related catecholamine, etc. as well as lowering fat accumulation (12). Previous works have indicated that high-intensity interval training (HIIT), containing alternative phases of intensity, may increase the total metabolic capacity and ameliorate metabolic diseases such as diabetes mellitus and obesity which are both important in PCOS progression (13). Sprung et al. (14) revealed that, aerobic exercise interferences, three times a week, 30-60% heart rate reserve, 20-45 minutes, improve endothelial function and an adaptation associated with reduced CVD risk independent of changes in body weight and body composition. Besides, it has been reported that resistance training improves insulin resistance, obesity and metabolic factors (15). Thus, it could be reasonable to prescribe resistance exercise to patients with PCOS. Resistance training inhibits osteoporosis and benefits of the musculoskeletal system. Additionally, resting metabolic rate, glucose homeostasis and insulin resistance, as well as body fat could be positively affected by this form of exercise (16).

Moreover, fat hypertrophy, as an increase in cell size resulting in a reduction in blood perfusion, eventually causes hypoxia. This additionally leads to cell apoptosis, resulting in a greater macrophage cell infiltration and improves the secretion of pro-inflammatory cytokines. Consequently, extra fat tissue can reflect an etiological issue in the pathogenesis of PCOS (17).

To our knowledge, there is no evidence comparing the effects of HIIT and combined (COM) (resistance and endurance) training concerning the improvement of cardiopulmonary fitness, body composition or weight loss in women with PCOS. Moreover, there is a wide variety of training structures used in previous studies. Thus, it is not feasible to advise a favorable type of training in PCOS patients. Therefore, the purpose of study was to evaluate the effects of these two well-known types of exercises on improving PCOS.

## Materials and Methods

### Study design

This study was carried out as a randomized clinical trial from April 2020 to December 2020 on the patients referring to the gynecological clinic of Imam Reza Hospital, Kermanshah, Iran. Rotterdam criteria was applied for PCOS diagnosis (18). Patients were between the ages of 18 and 40. The exclusion criteria included taking oral contraceptives, taking hormone drugs affecting total testosterone levels over the past three months. As the flowing diagram in (Fig.1) illustrates, 51 women with PCOS joined in this study; however, 6 individuals were not eligible based on the inclusion criteria. Finally, 45 participants were randomly allocated in 3 groups called HIIT training group; COM training group; and the control group 15 in each group for eight weeks. All of the patients completed the study. Randomization was done from a computer-generated sequence, concealed in sequentially numbered, sealed, opaque envelopes, and kept by the gynecological clinic physician.

At the beginning of this study, all individuals were asked to sustain a normal diet during the project. To increase the cooperation of patients, a short message was sent on their mobile phone to confirm the time to attend the gym.

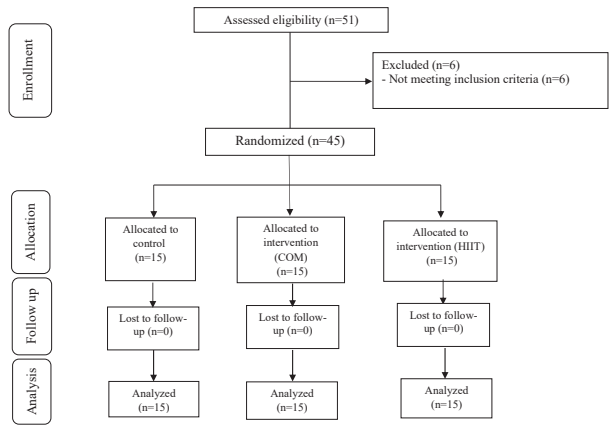


**Fig.1:** Summary of patients' flow diagram.

### Exercise protocols

#### Combined (RT+MICT) and high intensity intermittent training protocols

In the COM training group, the resistance training program consisted 24 sessions of selected resistance exercises during eight weeks holding 3 sessions each week. The subjects performed different exercises including, Bench press, Barbell curl, lying triceps press, lat pull down, leg press، leg extension, lying leg curl, and standing calf raise (19-23). Each training session included a warming-up phase (5 minutes), a resistance training phase [3 sets, 50-70% of one maximum repetition (1 RM), 10-16 repetitions] and the cooling-down phase (5 minutes). The whole training session lasted 30 to 40 minutes. Brzycki formula was applied to measure 1RM as follow:

$$1\ RM = \frac{\text{Weight of displaced}}{[1.0278 - (\text{Repeat to fatigue} * 0.278)]}$$

**Table 1:** Combined training program (endurance and strength training) in eight weeks

| Training programs | Weeks of training | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | First | Second | Third | Fourth | Fifth | Sixth | Seventh | Eighth |
| Endurance training | | | | | | | | |
| Intensity (HRR), % | 60 | 60 | 65 | 65 | 60 | 65 | 70 | 70 |
| Duration (minutes) | 25 | 30 | 35 | 30 | 35 | 40 | 35 | 40 |
| Strength training | | | | | | | | |
| Intensity (1 RM), % | 50 | 50 | 60 | 50 | 60 | 70 | 60 | 70 |
| SET | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Repetition | 16 | 16 | 14 | 16 | 14 | 10 | 14 | 10 |
| Rest (minutes) | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 | 1-3 |

**HRR; Heart rate reserve and RM; Repetition maximum.**

The duration and intensity of training program was 30 minutes and 50% in the first week and reached to 40 minutes and 70% in the eighth week (Table 1). Immediately after resistance training, the subjects were asked to perform endurance training which consisted 24 sessions of running on treadmill with 60 to 70% of the target heart rate (THR), which was measured using the Carvonen formula as below:

Reserve heart rate=resting heart rate-maximum heart rate

Target heart rate=resting heart rate+(60-70% reserve heart rate)

A Beurer pulse digital monitoring were used for monitoring subject's heart rate (made in Germany, model PM80) during training. The duration and intensity of training was 25 minutes and 60% in the first week and reached to 40 minutes and 70% in the eighth week (Table 2).

The subjects in the control group were also enquired to do only their normal daily routines and avoid doing any physical activities throughout the training program (19-23).

The high-intensity interval training program included warming up for each session (including 15 minutes of standard warm-up), starting with a low-intensity run (50% of maximum aerobic speed) and then 3 repetitions of sprint running for 30 seconds followed by 30. Seconds of slow running and 5 minutes of dynamic stretching were performed. The high-intensity interval training program for the first week included intermittent running for 30 seconds with intensity of 100% maximum aerobic speed (MAV),4×30-seconds HIIT interspersed with 4×30-seconds of active recovery with 5 minutes of passive recovery between the repetitions. The number of sets and laps for HIIT program changed according to Table 2 for the following weeks.

## Outcomes measurements

At baseline and the eight weeks, participants were tested for 1 RM to determine muscle strength in the COM and control groups. In the COM training group, the resistance training was performed with (50-70% 1 RM) and aerobic training (running, 60-70% HRR) programs were performed three times weekly for eight consecutive weeks.

Multi stage fitness test (MSFT) was carried out to determine aerobic power on the treadmill. The speed of the subjects started from 8.5 km/h for one minute. In each stage the patients' speed increased by 0.5 km/h. Aerobic power was calculated applying the following formula:

$$VO_{2max}=6[\text{measured speed (km/h)}]-22.4$$

This speed was considered as maximum aerobic velocity (MAV). HIIT program included intermittent running with 100% MAV and 50% MAV. The patients in the control group was asked not to do any exercises during the program and do only their normal activities (19-23).

**Table 2:** HIIT Training program

| Training program | Weeks of training | | | |
|---|---|---|---|---|
| | First, Second | Third, Fourth | Fifth, Sixth | Seventh, Eighth |
| Repetitions | 4 | 4 | 4 | 4 |
| Intervals | 4 | 6 | 6 | 6 |
| Exercise/rest (seconds) | 30:30 | 30:30 | 30:30 | 30:30 |
| Exercise: rest intensity (MAV%) | 100:50 | 100:50 | 110:50 | 110:50 |
| Rest (minutes) | 5 | 5 | 5 | 5 |

**HIIT; High Intensity intermittent and MAV; Maximum aerobic velocity.**

Anthropometric quantities were weighed via a professional technician at the clinic at the starting point and the end of the trial. Height was measured by automatic stadiometer (Aneascale, Iran). Weight, body fat percent (FP), visceral adipose tissue (VAT) and body mass index (BMI) were determined by 3D body scanner (Anea 3D, Iran) (24). To measure the waist to hip ratio (WHR), waist circumference and pelvic circumference (cm) were calculated from the lateral view.

## Statistical analysis

The shapiro-wilk test was used to define the normality of data. Two-way ANOVA and Bonferroni post hoc tests were applied to compare the differences in each group. One-way ANOVA test was used to assess treatment effects (pre-test and post-test in terms of Delta, Δ changes) on study outcomes and comparison among groups. Tukey post hoc tests were used to compare the pair differences.

Calculations were performed by SPSS software version 23 (SPSS Inc., Chicago, Illinois, USA) and the significance level of the tests was considered as P<0.05.

### Ethical considerations

The current study has been approved by the Iranian website of clinical trials registration with IRCT number: IRCT20130812014333N143. The protocol of this work was validated by the Ethics committee of Kermanshah University of Medical Sciences, Kermanshah, Iran (IR.KUMS.REC.1398.1186). Paper-based illuminated consent was also attained from all participants.

## Results

The general characteristics of patients have been presented in Table 3. As shown, there were no significant variances among the participants in terms of age (P=0.64), height (P=0.91), BMI (P=0.66) and weight (P=0.42) at baseline.

**Table 3:** General characteristics of the participants

| Variables | Control group | COM group | HIIT group | P value* |
|---|---|---|---|---|
| Age (Y) | 23.1 ± 5.1 | 24.4 ± 5.7 | 24.9 ± 5.4 | 0.64 |
| Height (m) | 162.6 ± 5.5 | 163.1 ± 4.5 | 162.3 ± 5.3 | 0.91 |
| Weight-baseline (kg) | 84.1 ± 6.3 | 80.7 ± 12.1 | 78.6 ± 13.9 | 0.42 |
| BMI-baseline (kg/m$^2$) | 30.7 ± 3.7 | 29.9 ± 4.3 | 29.3 ± 4.3 | 0.66 |

*; Obtained from Anova test, COM; Combined Training, HIIT; High intensity intermittent training, and BMI; Body mass index.

After eight weeks of COM intervention, weight (80.7 ± 12.1 to77.8 ± 12.2, P<0.001), BMI (29.9 ± 4.3 to 28.8 ± 4.2, P<0.001), WHR (0.93 ± 0.02 to 0.91 ± 0.03, P<0.001), FP (29.7 ± 2.1 to 28.6 ± 2.1, P<0.001), VAT (120.4 ± 17.8 to 117.9 ± 18.2, P=0.014) significantly decreased compared with pre-test. The result also revealed that after eight weeks of HIIT intervention, weight (78.6 ± 13.9 to 74.8 ± 13.9, P<0.001), BMI (29.3 ± 4.3 to 28.2 ± 4.3, P<0.001), WHR (0.91 ± 0.04 to 0.89 ± 0.04, P=0.005), FP (29.4 ± 2.4 to 27.7 ± 2.1, P<0.001), and VAT (121.5 ± 16.1 to 118.8 ± 16.7, P=0.007) significantly decreased compared with pre-test .VO2max significantly increased in COM (30.3 ± 1.9 to 31.8 ± 1.8, P<0.001) and HIIT (30.8 ± 2.3 to 34.1 ± 2.4, P<0.001) interventions after eight weeks compared with pre-test (Fig.2). The result of delta change (Δ) through one-way ANOVA revealed that weight, BMI, WHR, FP, and VAT significantly decreased in both groups of COM and HIIT compared with the control (P<0.05). The post-hoc tukey test indicated that there were no differences between HIIT group and COM group in terms of these variables (P>0.05, Fig.2). VO2max increased significantly after COM and HIIT interventions compared with control group (P<0.001). Moreover, the data from tukey test showed there was statistically significant difference between two groups of COM and HIIT as HIIT was more effective than COM (P<0.001, Fig.2).

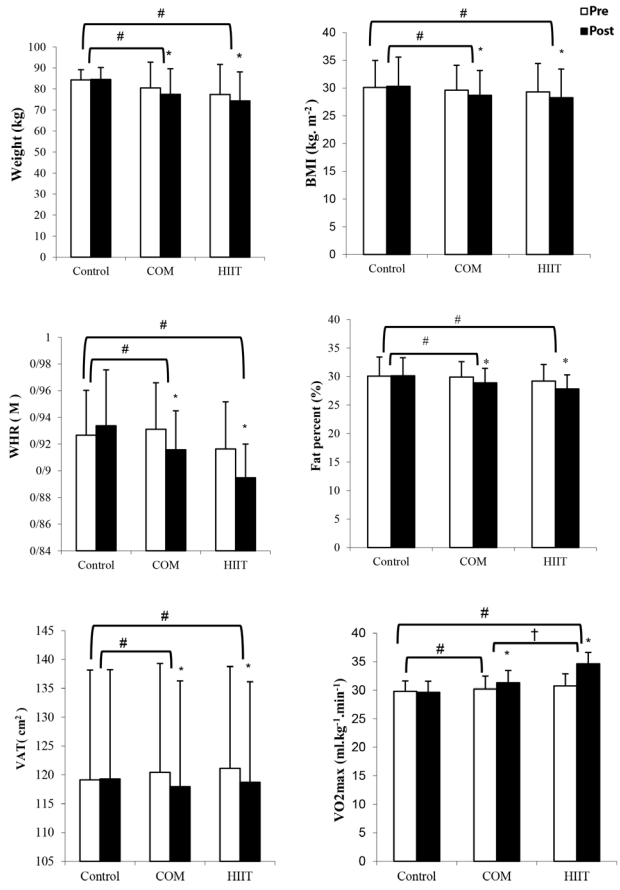


**Fig.2:** Anthropometrics and fitness variables at baseline and after 8-week intervention in patients with PCOS used Paired t test, One-way ANOVA and Tukey post hoc tests. BMI; Body mass index, WHR; Waist to hip ratio, VAT; Visceral adipose tissue, $VO_{2max}$; Maximal oxygen consumption, *; Compare to pre-test (P<0.05), #; Compare with the change (Δ) of the control group (P<0.05), and †; Compare with the combined training group (P<0.05).

## Discussion

This trial aimed to indicate a comparison between the efficacy of two various exercise training programs in the treatment of PCOS. In this trial, we analyzed the effects of two different exercise protocols on anthropometrics and fitness variables including weight, BMI, FP, WHR, VAT, and $VO_{2max}$ in women with PCOS. The findings revealed that both training programs had beneficial effects on these variables in the patients during eight weeks. There was no statistically significant difference among COM and HIIT groups in BMI, FP, WHR, and VAT, although HIIT was more operative only in $VO_{2max}$ compared to COM group.

In current study, it was reported that COM (resistant and endurance) training could get $VO_{2max}$ significantly increased. Two recent works on overweight and PCOS women reported significant decreased amounts of $VO_{2max}$ than healthy controls. A study by patten et al. (9), suggested that exercises can improve $VO_{2max}$ in this population. In addition, recent evidence showed that the resistance training in young and elderly individuals led to an elevation in $VO_{2max}$ (25, 26).

Decreased $VO_{2max}$ seems to be caused naturally by age; however, inactivity is its main reason. An elevation

in $VO_{2max}$ can improve oxygen consumption, leading to a 15% reduction in the risk of CVD related mortality (25, 26) . Moreover, Low $VO_{2max}$ has been related with elevated risk and mortality of chronic diseases. More consequences of decreased $VO_{2max}$ are impaired capability to exercise, impairment in daily activity and quality of life. Sabag et al. (27) also proved an association between cardiopulmonary fitness (CRF) and cardiometabolic health indices in type 2 diabetic patient. Thus, enhancing $VO_{2max}$ of patients may be beneficial for the treatment of PCOS.

We also found reductions in weight, FP, VAT, WHR and BMI in COM group, telling that resistant workout in combination with aerobic training can lead to advantages in some anthropometric indices in women with PCOS. WAT is thought to be a superior free predictor of obesity-associated disorders than BMI (28). Indeed, central adiposity plays a main role in the progression IR and T2DM, even in the individuals with standard BMI. Similar to our work, Miranda-Furtado et al. (8), indicated that in PCOS patients, WHR decreased after 4 months of strength training. Moreover, Zhang et al. (29) investigation observed that daily physical activities can improve weight and BMI dramatically in addition to metformin. In contrast, an investigation reported that resistance training (three times weekly for 1 month) did not change BMI or metabolic parameters, although it can ameliorate hyperandrogenism and reproduction in PCOS patients (30).

In a recent study (30) examined WAT as an operative sense for central adiposity, while Almenning et al. (31) employed FP to determine overall body fat quantity. As FP is the greatest assessment for total obesity, although central adiposity can estimate the risk for chronic disease more efficiently, we measured both FP and WAT that were measured in the present study. Kogure et al. (30) reported that visceral fat in overweight women with sedentary life decreased after a resistance training intervention. This point should be considered that resistance training programs have been different in details such as repetition, administration, and intensity leading to different results. However, each of these resistance training protocols has led to decreased amounts of body fat in women with PCOS basically owing to decreased abdominal adiposity.

Based on recent data, endurance training with moderate intensity diminishes the risk factors of cardio-metabolism in PCOS (7). In a recent work, endurance training improved symptoms in PCOS women by decreasing total testosterone level and WAT. Aerobic training could reduce heart rate, the levels of total cholesterol and LDL, and WHR. Yilmaz et al. (6) indicated regular endurance training ameliorated anthropometric variables as well as hyperandrogenism in PCOS cases.

Literature evaluating the effects of combination of resistance and endurance trainings in PCOS women is very limited. A 20-week investigation evaluated the impact of endurance and endurance-resistance training programs joined with an energy-controlled high protein diet on metabolic as well as reproductive parameters in overweight/obese women with PCOS. The findings from this study reveal that weight and FP reduced in both groups, but had no effect on cardiometabolic, hormonal, and reproductive factors compared to diet alone (32).

Aerobic training benefits some anthropometric indices and several cardiometabolic risk factors free of weight reduction in obese people (32). Resistance training is also efficacious for ameliorating insulin sensitivity and body composition as well as reserving lean tissue in energy-controlled diet, improving declines in resting metabolic rate after weight loss. Combining endurance and resistance exercise training programs has been observed to be more effective in insulin sensitivity reduction, glycemic management, and abdominal fat loss in obese population. Reduced BMI, and particularly abdominal fat in PCOS patients, has a key involvement in lowering risk factors for infertility leading to amelioration of hormonal and clinical outcomes. Decreased body fat can also result in improved insulin sensitivity and total cholesterol in these patients (33).

In current study, it was reported that eight-week intervention with HIIT led to an improvement in $VO_{2max}$ in PCOS women which was greater than the effect of COM intervention. Previous studies suggested significant improvements in $VO_{2max}$ following HIIT interventions in obesity, cardiometabolic disease and PCOS (34). Likewise, a cross-over study evaluated the effect of HIIT along with group counselling periods on anthropometry and cardio-respiratory health in women with PCOS (35). The result showed a decline in waist circumference and BMI as well as an elevation in $VO_{2max}$. Previously, Daussin et al. (35) observed elevated maximal stroke volume and cardiac variables result from 2 months of interval training, but not aerobic exercise, in low-active individuals. Moreover, Perry et al. (36), indicated the effect of HIIT training on fat and carbohydrate metabolism capacity. They showed that these types of training resulted in an 18 to 29% increase in the content of several mitochondrial proteins and an increase in fatty acid transporters. They resulted that HIIT training increased not only mitochondrial enzymes and fatty acid transporters in the short period but also lipid oxidation also.

Decreased amounts of body fat percentage and visceral adipose index were observed after HIIT intervention for eight weeks. Similar to our study, a recent trial found a decrease in body FP after high intensity training. This was similarly stated in other previous studies. For illustration, in a recent randomized controlled trial PCOS women were divided to take high intensity interval training, or strength training, for three times per week. The results showed that HIIT for ten weeks enhanced body FP and deprived weight loss in women with PCOS (31). In consistent to our results, Hutchison et al. (37) showed decreased visceral fat after HIIT in obese women with PCOS. A meta-analysis study indicated that HIIT was more productive in lowering total body

adiposity, whereas lower intensities had a better impact on abdominal and visceral fat bulk (38).

Recent evaluations demonstrated that HIIT is potential to elevate cardiopulmonary fitness and ameliorate insulin sensitivity. Moreover, multiple works have indicated no significant weight decline after HIIT compared with continuous exercises (39, 40).

In a recent meta-analysis study seven trials with training intensity among 90% and 95% of the maximum heart rate, 3 times/week, no less than 10 weeks, were included. Results indicated that HIIT alone is beneficial for lowering weight and BMI in females with PCOS (39). In another human study, obese patients with PCOS received AHIIT+ metformin, or metformin (control group). The exercises were performed in three sessions for 12 weeks. After 12 weeks, no significant changes were seen in parameter of WHR, but BMI and fat mass remarkably lowered and clinical parameters were improved (40). Finally, the use of an expert instructor to design and monitor the training program and habitual physical activity changes in all training sessions and monitoring the dietary change of the subjects are considered as the strengths of this research. Indirect meausres of $VO_{2max}$ and non-gold standard measures of body composition are the limitations of the study that might have affected the results.

## Conclusion

The findings from the clinical trial showed that both HIIT, COM aerobic and resistant training are successful in improving some anthropometric indices parameters including weight, BMI, WHR, FP, and VAT as well as $VO_{2max}$, as a cardiorespiratory element, in females with PCOS. Further investigations involving large clinical trials are needed to further determine health benefits and establish optimal therapeutic exercise dose in PCOS. Finally, if these two types of exercises are considered suitable and advantageous as a treatment policy for women with PCOS, additional trials with the aim of facilitating and removing the obstacles to exercise especial to women with PCOS is pivotal.

## Acknowledgements

The authors appreciated the cooperation of the patients of this research. This study was supported in part by a grant provided by the Department of Sports Physiology, Faculty of Sports Science, Razi University, and by a teaching and research scholarship from the Department of Sports Physiology. The authors declare no conflict of interest.

## Authors' Contributions

M.N., S.A.M.; Study concept and design. A.A.M., Z.A.; Analysis and interpretation of data. A.A.M.; Drafting of the manuscript, critical revision of the manuscript for important intellectual content, and statistical analysis. All authors read and approved the final manuscript.

## References

1. Ehrmann DA. Polycystic ovary syndrome. New Engl J Med. 2005; 352(12): 1223-1236.
2. Rosenfield RL, Ehrmann DA. The pathogenesis of polycystic ovary syndrome (PCOS): the hypothesis of PCOS as functional ovarian hyperandrogenism revisited. Endocr Rev. 2016; 37(5): 467-520
3. Barber TM, McCarthy MI, Wass JAH, Franks S. Obesity and polycystic ovary syndrome. Clin Endocrinol. 2006; 65(2): 137-145.
4. Ding T, Hardiman PJ, Petersen I, Wang FF, Qu F, Baio G. The prevalence of polycystic ovary syndrome in reproductive-aged women of different ethnicity: a systematic review and meta-analysis. Oncotarget. 2017; 8(56): 96351-96358.
5. Teede H, Deeks A, Moran L. Polycystic ovary syndrome: a complex condition with psychological, reproductive and metabolic manifestations that impacts on health across the lifespan. BMC Med. 2010; 8: 41.
6. Yilmaz B, Vellanki P, Ata B, Yildiz BO. Metabolic syndrome, hypertension, and hyperlipidemia in mothers, fathers, sisters, and brothers of women with polycystic ovary syndrome: a systematic review and meta-analysis. Fertil Steril. 2018; 109(2): 356-364. e32.
7. Crosignani PG, Colombo M, Vegetti W, Somigliana E, Gessati A, Ragni G. Overweight and obese anovulatory patients with polycystic ovaries: parallel improvements in anthropometric indices, ovarian physiology and fertility rate induced by diet. Hum Reprod. 2003; 18(9): 1928-1932.
8. Miranda-Furtado CL, Ramos FK, Kogure GS, Santana-Lemos BA, Ferriani RA, Calado RT, et al. A nonrandomized trial of progressive resistance training intervention in women with polycystic ovary syndrome and its implications in telomere content. Reprod Sci. 2016; 23(5): 644-654.
9. Patten RK, Boyle RA, Moholdt T, Kiel I, Hopkins WG, Harrison CL, et al. Exercise interventions in polycystic ovary syndrome: a systematic review and meta-analysis. Front Physiol. 2020; 11: 606.
10. Breyley-Smith A, Mousa A, Teede HJ, Johnson NA, Sabag A. The effect of exercise on cardiometabolic risk factors in women with polycystic ovary syndrome: a systematic review and meta-analysis. Int J Environ Res Public Health. 2022; 19(3): 1386.
11. Teede HJ, Misso ML, Costello MF, Dokras A, Laven J, Moran L, et al. Recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome. Fertil Steril. 2018; 110(3): 364-379.
12. Tsilchorozidou T, Honour JW, Conway GS. Altered cortisol metabolism in polycystic ovary syndrome: insulin enhances 5alpha-reduction but not the elevated adrenal steroid production rates. Clin Endocrinol Metab. 2003; 88(12): 5907-5913.
13. Lim SS, Norman RJ, Davies M, Moran L. The effect of obesity on polycystic ovary syndrome: a systematic review and meta-analysis. Obes Rev. 2013; 14(2): 95-109.
14. Sprung VS, Cuthbertson DJ, Pugh CJA, Aziz N, Kemp GJ, Daousi C, et al. Exercise training in polycystic ovarian syndrome enhances flow-mediated dilation in the absence of changes in fatness. Med Sci Sports Exerc. 2013; 45(12): 2234-2242.
15. Cheema BS, Vizza L, Swaraj S. Progressive resistance training in polycystic ovary syndrome: can pumping iron improve clinical outcomes? Sports Med. 2014; 44(9): 1197-1207.
16. Winett RA, Carpinelli RN. Potential health-related benefits of resistance training. Prev Med. 2001; 33(5): 503-513.
17. González F. Inflammation in polycystic ovary syndrome: underpinning of insulin resistance and ovarian dysfunction. Steroids. 2012; 77(4): 300-305.
18. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril. 2004; 81: 19-25.
19. Astinchap A, Monazzami A, Fereidoonfara K, Rahimi Z, Rahimi M. Modulation of fibroblast growth factor-21 and βklotho proteins expression in type 2 diabetic women with non-alcoholic fatty liver disease following endurance and strength training. Hepat Mon. 2021; 21(7): e116513.
20. Sharifi S, Monazzami A, Nikousefat Z, Heyrani A, Yari K. The acute and chronic effects of resistance training with blood flow restriction on hormonal responses in untrained young men: a comparison of frequency. Cell Mol Biol .2020; 66(1): 1-8.
21. Monazzami A, Momenpur R, Alipour E, Yari K, Payandeh M. Effects of eight-week combined resistance and endurance training on salivary interleukin-12, tumor necrosis factor, cortisol, and testosterone levels in patients with breast cancer. Int J Cancer Manag. 2021; 14(2): e109039.

22. Monazzami A, Rajabi H, Ghrakhanlou R, Yari K, Rahimi Z. Modulation of oxidative and glycolytic skeletal muscle fibers Na+/H+ exchanger1 (NHE1) and Na+/HCO3- co-transporter1 (NBC1) genes and proteins expression in type 2 diabetic rat (Streptozotocin + high fat diet) following long term endurance training. Cell Mol Biol. 2017; 63(5): 11-18.
23. Monazzami A, Rajabi H, Ghrakhanlou R, Yari K. Endurance training increases skeletal muscle Na/H+ exchanger1 (NHE1) and Na/HCO3 cotransporter1 (NBC1) gene and protein expressions in rats. Gene Rep. 2022; 26: 101469.
24. Tinsley GM, Moore ML, Dellinger JR, Adamson BT, Benavides ML. Digital anthropometry via three-dimensional optical scanning: evaluation of four commercially available systems. Clin Nutr. 2020; 74(7): 1054-1064.
25. Orio F Jr, Giallauria F, Palomba S, Cascella T, Manguso F, Vuolo L, et al. Cardiopulmonary impairment in young women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2006; 91(8): 2967-2971.
26. Keteyian SJ, Brawner CA, Savage PD, Ehrman JK, Schairer J, Divine G, et al. Peak aerobic capacity predicts prognosis in patients with coronary heart disease. Am Heart J. 2008; 156(2): 292-230.
27. Sabag A, Keating SE, Way KL, Sultana RN, Lanting SM, Twigg SM, et al. The association between cardiorespiratory fitness, liver fat and insulin resistance in adults with or without type 2 diabetes: a cross-sectional analysis. BMC Sports Sci Med Rehabil. 2021; 13(1): 40.
28. Lean ME, Han TS, Morrison CE. Waist circumference as a measure for indicating need for weight management. BMJ. 1995; 311(6998): 158-161.
29. Zhang J, Si Q, Li J. Therapeutic effects of metformin and clomiphene in combination with lifestyle intervention on infertility in women with obese polycystic ovary syndrome. Pak J Med Sci. 2017; 33(1): 8-12.
30. Kogure GS, Miranda-Furtado CL, Silva RC, Melo AS, Ferriani RA, De Sá MF, et al. Resistance exercise impacts lean muscle mass in women with polycystic ovary syndrome. Med Sci Sports Exerc. 2016; 48(4): 589-598.
31. Almenning I, Rieber-Mohn A, Lundgren KM, Shetelig Løvvik T, Garnaes KK, Moholdt T. Effects of high intensity interval training and strength training on metabolic, cardiovascular and hormonal outcomes in women with polycystic ovary syndrome: a pilot study. PLoS One. 2015; 10(9): e0138793.
32. Thomson RL, Buckley JD, Noakes M, Clifton PM, Norman RJ, Brinkworth GD. The effect of a hypocaloric diet with and without exercise training on body composition, cardiometabolic risk profile, and reproductive function in overweight and obese women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2008; 93(9): 3373-3380.
33. Anwar S, Shikalgar N. Prevention of type 2 diabetes mellitus in polycystic ovary syndrome: a review. Diabetes Metab Syndr. 2017; 11 Suppl 2: S913-S917.
34. Lionett S, Kiel IA, Røsbjørgen R, Lydersen S, Larsen S, Moholdt T. Absent exercise-induced improvements in fat oxidation in women with polycystic ovary syndrome after high-intensity interval training. Front Physiol. 2021; 12: 649794.
35. Daussin FN, Zoll J, Dufour SP, Ponsot E, Lonsdorfer-Wolf E, Doutreleau S, et al. Effect of interval versus continuous training on cardiorespiratory and mitochondrial functions: relationship to aerobic performance improvements in sedentary subjects. Am J Physiol Regul Integr Comp Physiol. 2008; 295(1): R264-2R72.
36. Perry CG, Heigenhauser GJ, Bonen A, Spriet LL. High-intensity aerobic interval training increases fat and carbohydrate metabolic capacities in human skeletal muscle. Appl Physiol Nutr Metab. 2008; 33(6): 1112-1123.
37. Hutchison SK, Stepto NK, Harrison CL, Moran LJ, Strauss BJ, Teede HJ. Effects of exercise on insulin resistance and body composition in overweight and obese women with and without polycystic ovary syndrome. J Clin Endocrinol Metab. 2011; 96(1): E48-E56.
38. Maillard F, Pereira B, Boisseau N. Effect of high-intensity interval training on total, abdominal and visceral fat mass: a meta-analysis. Sports Med. 2018; 48(2): 269-288.
39. Santos IKD, Nunes F, Queiros VS, Cobucci RN, Dantas PB, Soares GM, et al. Effect of high-intensity interval training on metabolic parameters in women with polycystic ovary syndrome: a systematic review and meta-analysis of randomized controlled trials. PLoS One. 2021; 16(1): e0245023.
40. Samadi Z, Bambaeichi E, Valiani M, Shahshahan Z. Evaluation of changes in levels of hyperandrogenism, hirsutism and menstrual regulation after a period of aquatic high intensity interval training in women with polycystic ovary syndrome. Int J Prev Med. 2019; 10: 187.

Original Article

# The Effect of Couples Coping Enhancement Counseling on Stress and Dyadic Coping on Infertile Couples: A Parallel Randomized Controlled Trial Study

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## Abstract

**Background:** The aim of this study was to determine the effect of couples coping enhancement counseling (CCEC) on stress and dyadic coping of infertile couples.

**Materials and Methods:** In this parallel randomized controlled trial study in 2020, seventy infertile couples were randomly divided into case and control groups. The intervention was performed in 7 sessions of couple counseling based on CCEC for the intervention group, no intervention was performed in the control group. Fertility Problem Inventory, Dyadic Coping Inventory and demographics questionnaires were completed by both couples separately before the intervention and 4 weeks after the last consultation session. Data were analyzed using IBM SPSS statistics 24 and statistical tests such as mean ± SD, frequency, percentage, Independent t test, Mann-Whitney test, Chi-square test or Fisher's exact test and Analysis of covariance. Significant level was considered less than 0.05.

**Results:** The mean stress scores of women in the intervention group before and after intervention decreased from (156.83 ± 23.57) to (139.43 ± 22.39) and the mean scores of dyadic coping increased from (126.83 ± 19.89) to (138.26 ± 16.92), these differences were statistically significant (P<0.001), also the mean stress scores of men in the intervention group before and after the intervention decreased from (143.80 ± 23.40) to (128.03 ± 22.24), the mean scores of dyadic coping increased (131.34 ± 20.67) to (136.40 ± 19.38), these differences were statistically significant (P<0.001).

**Conclusion:** Positive effects of CCEC were observed in reducing infertility stress and increasing dyadic coping in both women and men after the intervention, the effect of the intervention on women was greater than that of men. As a result, this intervention can play an important role in reducing stress and increasing the solidarity and support of infertile couples for infertility treatments (registration number: IRCT20120215009014N367).

***Keywords:*** Coping Skills, Counseling, Couples, Infertility Stress

**Citation:** Monirian F, Khodakarami B, Tapak L, Kimiaei Asadi F, Aghababaei S. The effect of couples coping enhancement counseling on stress and dyadic coping on infertile couples: a parallel randomized controlled trial study. Int J Fertil Steril. 2022; 16(4): 275-280. doi: 10.22074/IJFS.2022.540919.1203.


## Introduction

Infertility is the failure to become pregnant after 12 months of regular and unprotected sex (1). Worldwide, about 8 to 12% of reproductive age couples are affected by fertility problems (2). The prevalence of primary and secondary infertility in Iran was reported to be about 12.8% and 4.9% respectively in 2019 (3). Infertility may have many psychological consequences (4). Inflexible infertility treatment programs, long-term treatments, high treatment costs, constant worries about the outcome of treatment, the need for sex only for fertility, community pressure, family breakdown and loss of spouse interest puts a lot of stress on people and their spouses (5). Infertility stress is similar to post-traumatic stress disorder (6), results of a study by Roozitalab et al. (7) showed that 41.3% of infertile women had posttraumatic stress disorder symptoms. Stress and anxiety can affect the outcome of infertility treatment (8).

Increased stress due to infertility, leads to the activation of stress management in couples as a unit (9). As a result, the stress in couples is always considered a dual phenomenon, and coping with this stressful event, must include joint coping strategies (10, 11). Two major strategies for coping with stress include individual coping and dyadic coping (12). Dyadic coping includes perceived coping efforts by an individual (dyadic coping by the individual) and perceived coping efforts by the partner (dyadic coping by the partner) (13, 14). One of the counseling programs to increase dyadic coping skills is couples coping enhancement training (15), which improve stress management ability and increases the ability to cope as a couple, the couples' sensitivity to justice and mutual respect, improves the problem-solving skills of the couples (16). It is

Received: 14/October/2021, Accepted: 24/January/2022
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Royan Institute
International Journal of Fertility and Sterility
Vol 16, No 4, October-December 2022, Pages: 275-280

necessary to consider the strategies used to control and manage the consequences of infertility diagnosis (17).

Given that infertility stress studies have often been performed on infertile women and couples have been less studied, the present study aimed to determine the effect of CCEC on stress and dyadic coping of infertile couples.

## Materials and Methods

The present study was performed in 2020 as a parallel randomized controlled trial with trial registration number: IRCT20120215009014N367 on seventy infertile couples who were referred to the infertility center of Fatemieh Hospital in Hamadan city of Iran. Inclusion criteria included the desire of both couples to participate in the study, the presence of moderate stress in both couples (score 92-184) according to the fertility problem inventory, couples age between 20-45 years, first marriage and monogamy, having primary infertility, having at least one year of infertility, being literate, not attending other training programs, and being able to attend consecutive training sessions. Simultaneous participation in other treatment programs, the occurrence of stressful events during the counseling period, and positive pregnancy tests were the exclusion criteria. Sample size was calculates using the following equation:

$$n = \frac{\left(z_{1-\frac{\alpha}{2}} + z_{1-\beta}\right)^2 \left(\sigma_1^2 + \sigma_2^2\right)}{(\mu_1 - \mu_2)^2}$$

The sample size in each group was estimated to be at least 35 people, considering the confidence level of the test to be 95%, the test power of 90%, the common standard deviation of 34.76, the minimum significant difference between the two groups equal to 30 units and 10% probable loss of samples (12).

Sampling was initially done by availability method from the couples whom applied to and were eligible to participate in the study. Informed consent was obtained before participants who were recruited into the study by a colleague, then both couples completed the questionnaires and finally 70 couples were selected based on eligibility criteria and the score obtained from infertility stress test. The selected couples based on permutation block were divided into experimental and control groups, in this way, 4 blocks were considered as, ABAB, AABB, BAAB, ABBA, BABA, BBAA (A represents the experimental group and B represents the control group), then a list of the above blocks was randomly produced using the R software, so that 35 letter As and 35 letter Bs were produced. A total of 70 samples were assigned to one of the two groups of the test (A) and control (B), respectively, based on the list prepared (Fig.1).

Primary and secondary outcomes were measuring stress and dyadic coping before and after the intervention. In order to collect data, Dyadic Coping Inventory, the Fertility Problem Inventory, and demographics were completed by both couples separately. Dyadic Coping Inventory is a 37-item instrument designed to measure perceived communication and dyadic coping. It has 9 subscales (18). The Persian version of this questionnaire was approved by Fallahchai et al. (19) with a reliability coefficient of 0.84 and Cronbach's alpha of 0.939 for the whole scale. The Fertility Problem Inventory was designed by Newton et al. (20) to measure perceived infertility-related stress. This questionnaire has 46 questions and 5 subscales. Based on 6-choice Likert the minimum and maximum scores in this questionnaire will be 46 and 276 respectively. A score between 92 and 184 indicates a moderate level of infertility stress. The validity of this questionnaire was confirmed by Latifnejad Roudsari et al. (5) in Iran. The demographic information questionnaire also included information regarding age, occupation, level of education of women and their spouses, place of residence, history of illness, duration of the marriage, duration of infertility, duration and history of treatment and cause of infertility.

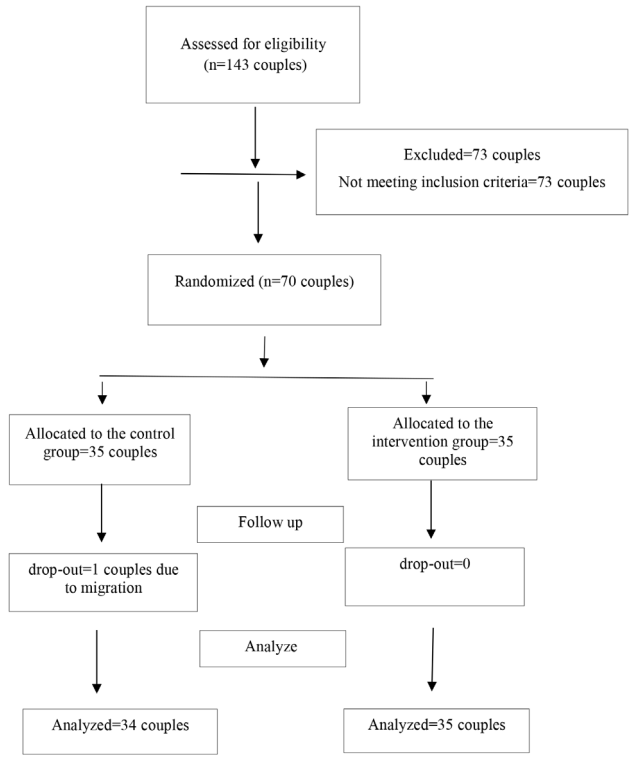


**Fig.1:** Consort flow diagram of study.

The intervention was performed by holding 7 counseling sessions based on CCEC with one session per week for the intervention group couples by the researcher under the supervision of an expert psychologist (13, 21), reassessment was performed in both groups 4 weeks after the end of the intervention. The couples in the control group did not receive any intervention until the end of the treatment. In order to observe ethical principles, at the end of the intervention with the coordination of the participants, two counseling sessions were held for the control group, and also all the necessary information was provided to them in the form of CDs and pamphlets. The content of the counseling sessions includes: Enhancing individual coping, enhancing dyadic coping, integrating fairness, equity, and boundaries, enhancing marital communication and problem-solving skills (Table 1).

**Table 1:** Content of counseling sessions

| Counseling sessions | Content of counseling sessions |
|---|---|
| First session | The concept of stress, causes, types and consequences of stress, cognitive assessment of stress, the relationship between stress and emotional reactions according to Lazarus and Folkman |
| Second session | Definition of coping and its types, stress prevention by predicting stressful conditions and preparation in advance, the role of planning, organizing activities and predicting the situation in stress prevention, methods of coping with unavoidable stress, relaxation training |
| Third session | Definitions and types of dyadic coping, the importance of dyadic coping in marital relationships, increasing understanding of partner stress, teaching dyadic coping skills, using the funnel and three-step method in dyadic coping and role playing |
| Fourth session | Reviewing the concepts of exchange, fairness and justice in marital relations, improving the awareness of couples about the importance of fair and reciprocal exchange in the field of marital confrontation, increasing sensitivity to personal needs and partner needs, intimacy in marital relationships |
| Fifth session | The importance of marital communication skills, negative and positive communication styles in marital relationships, improving speaking and listening skills, discovering inadequate communication behaviors and learning to overcome them |
| Sixth session | Teaching problem-solving steps, strengthening mutual problem-solving skills in couples |
| Seventh session | Summarize and review summary of past sessions |

## Statistical analysis

Data were analyzed using IBM SPSS Statistics 24 (IBM Corp., Armonk, New York, USA). Descriptive statistics such as mean/SD, Frequency and Percentage were used to describe the data, comparison of the two groups in terms of demographic and contextual variables was performed with independent t test or Mann-Whitney test if there was little data and with chi-square test or Fisher's exact test if it was qualitative. Analysis of covariance was used to compare the two groups after the intervention. Significant level in all statistical tests was considered less than 0.05.

## Ethical considerations

This master's thesis was approved by the Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1399.485). Necessary explanations were also given to the participants and the confidentiality of the information, and written consent was taken from them in the native language (Persian) before the study.

# Results

The mean ± SD age of the women was 29.37 ± 4.97 in the intervention group and 32.63 ± 5.43 in the control group, the two groups were not homogeneous (P=0.01). Other demographic characteristics in the two intervention and control groups were not significantly different and the two groups were homogeneous (P<0.05). The highest cause of infertility was related to women with 34.3% in the intervention group and related to men with 34.3% in the control group. The most reported treatment type in both groups was IVF (Table 2).

**Table 2:** Description of demographic and infertility variables of participants

| Variables | Intervention group (n=35) | Control group (n=34) | P value |
|---|---|---|---|
| Women's age (Y) | 29.37 ± 4.97 | 32.63 ± 5.43 | 0.01[c] |
| Men's age (Y) | 34.26 ± 3.7 | 36.23 ± 5.7 | 0.09[c] |
| Women's education | | | 0.82[a] |
| High school | 8 (22.9) | 9 (25.7) | |
| Diploma | 15 (42.8) | 12 (34.3) | |
| University | 12(34.3) | 14 (40.0) | |
| Men's education | | | 0.17[a] |
| High school | 18 (51.4) | 13 (37.1) | |
| Diploma | 6 (17.1) | 13 (37.1) | |
| University | 11(31.5) | 9 (25.8) | |
| Women's employment status | | | 0.61[b] |
| Employed | 1 (2.9) | 3 (8.6) | |
| Non employed | 34 (97.1) | 32 (91.4) | |
| Men's Employment status | | | 0.09[a] |
| Employed | 30 (85.7) | 23 (65.7) | |
| Non employed | 5 (14.3) | 12 (34.3) | |
| Residency | | | 1.00[b] |
| Urban | 25 (71.4) | 26 (74.3) | |
| Rural | 10 (28.6) | 9 (25.7) | |
| Economic situation | | | 0.92[a] |
| Good | 2 (5.7) | 2 (5.7) | |
| Medium | 14 (40.0) | 12 (34.3) | |
| Poor | 19 (54.3) | 21(60.0) | |
| Women's smoking | | | |
| Yes | 0 (0) | 0 (0) | |
| Men's smoking | | | 0.21[b] |
| Yes | 4 (11.4) | 9 (25.7) | |
| Women's history of physical illness | | | 0.25[b] |
| Yes | 6 (17.1) | 2 (5.7) | |
| No | 29 (82.9) | 33 (94.3) | |
| Men's history of physical illness | | | - |
| Yes | 2 (5.7) | 2 (5.7) | |
| Marriage duration (Y) | 7.8 ± 3.5 | 7.9 ± 3.1 | 0.91[c] |
| Duration of infertility (Y) | 5.45 ± 2.98 | 5.98 ± 4.76 | |
| Cause of infertility | | | 0.20[a] |
| Women | 12 (34.3) | 8 (22.8) | |
| Men | 7 (20.0) | 12 (34.3) | |
| Women and men | 10 (28.6) | 5 (14.3) | |
| Unknown | 6(17.1) | 10 (28.6) | |
| Treatment history | | | 0.33[a] |
| Yes | 17 (48.6) | 22 (62.9) | |
| No | 18 (51.4) | 13 (37.1) | |
| Type of treatment | | | 0.23[a] |
| Drug | 6 (17.1) | 6 (17.1) | |
| IUI | 5 (14.3) | 7 (20.0) | |
| IVF | 22 (62.9) | 20 (57.2) | |
| ICSI | 2 (5.7) | 2 (5.7) | |

Data are presented as mean ± SD or n (%). [a]; Chi-square, [b]; Fisher's Exact test, [c]; Independent-sample t test, IUI; Intrauterine insemination, IVF; *In vitro* fertilization, and ICSI; Intracytoplasmic sperm injection.

**Table 3:** Comparison of intervention and control groups in terms of fertility stress and dyadic coping after adjusting the impact of before intervention scores in women

| Variables | Group | Before intervention | Adjusted mean after intervention | P value | Effect size |
|---|---|---|---|---|---|
| Social concern | Intervention | 27.49 ± 7.36 | 22.00 ± 6.73 | <0.001[a, b] | 0.694 |
| | Control | 28.23 ± 7.24 | 27.71 ± 6.55 | | |
| Sexual concern | Intervention | 21.23 ± 7.81 | 17.17 ± 6.86 | <0.001[a, b] | 0.592 |
| | Control | 20.83 ± 7.33 | 21.11 ± 7.18 | | |
| Relationship concern | Intervention | 29.66 ± 7.57 | 24.34 ± 7.73 | <0.001[a, b] | 0.631 |
| | Control | 29.51 ± 7.86 | 29.11 ± 7.56 | | |
| Need for parenthood | Intervention | 46.46 ± 8.55 | 45.77 ± 8.57 | 0.04[a, b] | 0.297 |
| | Control | 44.14 ± 11.91 | 44.34 ± 11.81 | | |
| Rejection of childfree lifestyle | Intervention | 32.00 ± 6.45 | 30.14 ± 6.05 | <0.001[a, b] | 0.063 |
| | Control | 28.46 ± 7.65 | 28.83 ± 8.16 | | |
| Total score of stress | Intervention | 156.83 ± 23.57 | 139.43 ± 22.39 | <0.001[a, b] | 0.871 |
| | Control | 151.17 ± 26.55 | 151.11 ± 25.96 | | |
| Dyadic coping score | Intervention | 126.83 ± 19.89 | 138.26 ± 16.92 | <0.001[a, b] | 0.402 |
| | Control | 127.29 ± 18.06 | 131.77 ± 16.06 | | |

Data are presented as mean ± SD. [a]; Adjusted for age and before intervention scores and [b]; Analysis of covariance.

**Table 4:** Comparison of intervention and control groups in terms of fertility stress and dyadic coping after adjusting the impact of before intervention scores in men

| Variables | Adjusted mean after intervention | Before intervention | Group | Effect size | P value |
|---|---|---|---|---|---|
| Social concern | 20.26 ± 6.33 | 24.80 ± 7.13 | Intervention | 0.401 | <0.001[a, b] |
| | 25.69 ± 6.74 | 26.09 ± 6.75 | Control | | |
| Sexual concern | 14.60 ± 6.82 | 17.51 ± 7.86 | Intervention | 0.627 | <0.001[a, b] |
| | 20.54 ± 7.74 | 20.14 ± 8.04 | Control | | |
| Relationship concern | 24.71 ± 6.32 | 28.23 ± 6.83 | Intervention | 0.584 | <0.001[a, b] |
| | 28.26 ± 6.90 | 28.06 ± 7.09 | Control | | |
| Need for parenthood | 41.14 ± 10.33 | 42.94 ± 10.31 | Intervention | 0.242 | 0.03[a, b] |
| | 40.91 ± 12.15 | 41.34 ± 12.06 | Control | | |
| Rejection of childfree lifestyle | 27.31 ± 6.13 | 30.31 ± 6.45 | Intervention | 0.064 | <0.001[a, b] |
| | 27.57 ± 6.50 | 27.66 ± 6.47 | Control | | |
| Total score of stress | 128.03 ± 22.24 | 143.80 ± 23.40 | Intervention | 0.629 | <0.001[a, b] |
| | 142.97 ± 24.70 | 143.29 ± 25.61 | Control | | |
| Dyadic coping score | 136.40 ± 19.38 | 131.34 ± 20.67 | Intervention | 0.267 | <0.001[a, b] |
| | 130.66 ± 15.36 | 129.31 ± 16.38 | Control | | |

Data are presented as mean ± SD. [a]; Adjusted for before intervention scores and [b]; Analysis of covariance.

In order to control the variables before intervention and compare the effects of the intervention, analysis of covariance was used and the results are summarized in Tables 3 and 4. Based on the results, after the intervention in men and women in the intervention group compared to men and women in the control group, a significant decrease in all components of infertility stress, including social concern (P<0.001), sexual concern (P<0.001), relationship concern (P<0.001), rejection of childfree lifestyle (P<0.001), and the need for parenthood (women P=0.04, men P=0.03) was observed. Also, the overall stress score in both sexes in the intervention group had a significant decrease (P<0.001). Due to the intervention, the dyadic coping score in both sexes in the intervention group increased significantly compared to the control group (P<0.001, Tables 3, 4).

## Discussion

The aim of this study was to determine the effect of CCEC on stress and dyadic coping of infertile couples. Based on the results, CCEC was able to reduce all components of infertility stress, including social concern, sexual concern, relationship concern, rejection of childfree lifestyle, and the need for parenthood in both men and women in the intervention group compared with controls. In most studies, infertile women are usually studied (22-25), and in a few studies, infertile men were studied (26, 27), while in the present study, the focus was on both genders. In line with the present study, in the study of Ordoni Awal et al. (28), the score of all 5 dimensions of the infertility stress questionnaire decreased in the participants after the intervention, Karaca et al. (29) reported that cognitive-

behavioral group therapy intervention reduced the infertility-related psychosocial problems of infertile women. Similarly, in the study of Starabadi et al. (30), the effect of acceptance and commitment-based therapy in significantly reducing infertility stress in infertile couples was identified. Lukse (31) also reported the effect of group counseling in reducing the symptoms of grief experienced by some infertile couples. In contrast with the present study, Hammerli et al. (32), reviewed 21 controlled studies and concluded that psychological interventions were not associated with significant changes in mental status. Consistent with other studies and the present study, women felt more stress than men regarding infertility (33, 34).

According to the results of the study, CCEC significantly increased the dyadic coping score in men and women in the intervention group. The study of Sodani et al. (21) was conducted to determine the effectiveness of couple coping enhancement training on dyadic coping, conflict resolution style, ineffective dialogue, and intimacy security in couples. Based on the findings of this study, receiving training for strengthening couple confrontation could have an effect on couple confrontation variables. In the couple confrontation variable, the mean scores in the post-test had a significant increase compared to the pre-test. The results of a study by Omidian et al. (35) showed that couples coping enhancement training can improve the marital adjustment of wives in a sample of troubled couples in Shahr-e Kord city. Results of a study by Molgora et al. (36) showed that the adoption of positive coping styles by couples leads to increased marital adjustment and the success of ART treatment may be less in couples who do not have this type of reciprocal supportive behavior (36). In a randomized clinical trial conducted by Bodenmann et al. (37), coping-oriented couple therapy did not show better results compared to dyadic coping or relationship satisfaction, but it significantly improved the expression of emotions by partners.

The dyadic nature of dyadic coping style helps to reduce stress in couples (38). Male infertility can lead to infertility treatment problems and marital problems, hence, supportive and preventive measures are required to improve these conditions (39). The findings of a study by Chaves et al. (11), indicated the importance of male coping strategies for marital adjustment and men's emotional adjustment. Infertility is not only a medical issue but also a psychological crisis that threatens families and people's quality of life, and is identified as a health priority (24, 33, 39, 40). Therefore, during infertility treatments, it is necessary to pay attention to the burden of psychological changes caused by infertility diagnosis on couples that can be threatening infertility treatment and to take appropriate interventions to reduce these changes.

This study had several limitations including the self-reporting nature of the questionnaires that may introduce recall bias in the study, limited infertility treatment centers in the city of Hamadan, men's resistance to attend counseling sessions and the impossibility of more follow-ups due to time constraints.

## Conclusion

The results of this study showed that CCEC has been able to significantly reduce infertility stress and significantly increase dyadic coping in both women and men in the intervention group. As a result, training couples on this type of coping with stress can play an important role in reducing stress and increasing the solidarity and support of infertile couples for infertility treatments. These findings may be helpful in infertility psychological and counselling interventions.

## Acknowledegements

The authors would like to appreciate the Vice-chancellor for Research and Technology of Hamadan University of Medical Sciences of Iran, for financial support of the study (9907295350), and all people who participated in this study. No potential conflict of interest was reported by the authors.

## Authors' Contributions

F.M., S.A., L.T., B.Kh., F.K.A.; Conception and design of the study, drafting of the manuscript and statistical analysis. S.A.; Obtaining funding, administrative, technical, or material support, or supervision. All authors read and approved the final manuscript.

## References

1. Vander Borght M, Wyns C. Fertility and infertility: definition and epidemiology. Clin Biochem. 2018; 62: 2-10.
2. Inhorn MC, Patrizio P. Infertility around the globe: new thinking on gender, reproductive technologies and global movements in the 21st century. Hum Reprod Update. 2015; 21(4): 411-426.
3. Akhondi MM, Ranjbar F, Shirzad M, Ardakani ZB, Kamali K, Mohammad K. Practical difficulties in estimating the prevalence of primary infertility in Iran. Int J Fertil Steril. 2019; 13(2): 113-117.
4. Haririan HR, Mohammadpour Y, Aghajanloo A. Prevalence of depression and contributing factors of depression in the infertile women referred to Kosar infertility center, 2009. IJOGI. 2010; 13(2): 45-49.
5. Latifnejad Roudsari R, Rasolzadeh Bidgoly M, Mousavifar N, Modarres Gharavi M. The effect of collaborative counseling on perceived infertility-related stress in infertile women undergoing IVF. IJOGI. 2011; 14(4): 22-31.
6. Amini L, Ghorbani B, Sadeghi AvvalShahr H, Raoofi Z, Mortezapour Alisaraie M. The relationship between percieved social support and infertility stress in wives of infertile men. Iran Journal of Nursing. 2018; 31(111): 31-39.
7. Roozitalab S, Rahimzadeh M, Mirmajidi SR, Ataee M, Esmaelzadeh-Saeieh S. The relationship between infertility, stress, and quality of life with posttraumatic stress disorder in infertile women. J Reprod Infertil. 2021; 22(4): 282-288.
8. Terzioglu F, Turk R, Yucel C, Dilbaz S, Cinar O, Karahalil B. The effect of anxiety and depression scores of couples who underwent assisted reproductive techniques on the pregnancy outcomes. Afr Health Sci. 2016; 16(2): 441-450.
9. Martins MV, Peterson BD, Almeida V, Mesquita-GuimarÃ£es J, Costa ME. Dyadic dynamics of perceived social support in couples facing infertility. Hum Reprod. 2014; 29(1): 83-89.
10. Bodenmann G. Dyadic coping and its significance for marital functioning. In: Revenson TA, Kayser K, Bodenmann G, editors. Couples coping with stress: emerging perspectives on dyadic coping. 2005: 33-49. American Psychological Association.

Available from: https://doi.org/10.1037/11031-002 (18 Jan 2022).
11. Chaves C, Canavarro MC, Moura-Ramos M. The role of dyadic coping on the marital and emotional adjustment of couples with infertility. Fam Process. 2019; 58(2): 509-523.
12. Sodani M, Momeni Javid M, Mehrabizadeh Honarmand M, Khojastehmeher R. The effectiveness of couple coping enhancement training on dyadic coping, conflict resolution style, ineffective dialogue and intimate safety in couples. Quarterly Journal of Women and Society. 2016; 7(25): 45-66.
13. Bodenmann G, Shantinath SD. The couples coping enhancement training (CCET): a new approach to prevention of marital distress based upon stress and coping. Fam Relat. 2004; 53(5): 477-84.
14. Kroemeke A, Kubicka E. Positive and negative adjustment in couples undergoing infertility treatment: the impact of support exchange. PLoS One. 2021; 13(6): e0200124.
15. Bodenmann G, Randall AK. Common factors in the enhancement of dyadic coping. Behav Ther. 2012; 43(1): 88-98.
16. Alves S, Fonseca A, Canavarro MC, Pereira M. Dyadic coping and dyadic adjustment in couples with women with high depressive symptoms during pregnancy. J Reprod Infant Psychol. 2018; 36(5): 504-518.
17. Taghipour A, Karimi FZ, Latifnejad Roudsari R, Mazlom SR. Coping strategies of women following the diagnosis of infertility in their spouses: a qualitative study. Evid Based Care J. 2020; 10(1): 15-24.
18. Bodenmann G, Pihet S, Kayser K. The relationship between dyadic coping and marital quality: a 2-year longitudinal study. J Fam Psychol. 2006; 20(3): 485-493.
19. Fallahchai R, Fallahi M, Chahartangi S, Bodenmann G. Psychometric properties and factorial validity of the dyadic coping inventory the Persian version. Curr Psychol. 2019; 38(2): 486-496.
20. Newton CR, Sherrard W, Glavac I. The fertility problem inventory: measuring perceived infertility-related stress. Fertil Steril. 1999; 72(1): 54-62.
21. Isanejad O, Alizade Z. The effectiveness of couples coping enhancement training (CCET) on marital adjustment and marital coping strategies. J Appl Psychol Res. 2020; 11(1): 67-85.
22. Cui C, Wang L, Wang X. Effects of self-esteem on the associations between infertility-related stress and psychological distress among infertile chinese women: a cross-sectional study. Psychol Res Behav Manag. 2021; 14:1245-1255.
23. Khalid A, Dawood S. Social support, self-efficacy, cognitive coping and psychological distress in infertile women. Arch Gynecol Obstet. 2020; 302(2): 423-430.
24. Kim M, Moon SH, Kim JE. Effects of psychological intervention for Korean infertile women under in vitro fertilization on infertility stress, depression, intimacy, sexual satisfaction and fatigue. Arch Psychiatr Nurs. 2020; 34(4): 211-217.
25. Li X, Ye L, Tian L, Huo Y, Zhou M. Infertility-Related stress and life satisfaction among chinese infertile women: a moderated mediation model of marital satisfaction and resilience. Sex Roles. 2020; 82(1): 44-52.
26. Dong YZ, Yang XX, Sun YP. Correlative analysis of social support with anxiety and depression in men undergoing in vitro fertilization embryo transfer for the first time. J Int Med Res. 2013; 41(4): 1258-1265.
27. Szatmari A. Impact of paramedical counseling on infertile male patients' coping strategies and care satisfaction. Orv Hetil. 2020; 159(31): 1263-1269.
28. Ordoni Avval Z, Rabiepoor S, Behroozilak T, Arefi M, Yas A. The effectiveness of counseling with a cognitive-behavioral approach on infertile women's stress. Maedica (Bucur). 2019; 14(4): 363-370.
29. Karaca A, Yavuzcan A, Batmaz S, Cangür Ş, Çalişkan A. The effect of cognitive behavioral group therapy on infertility stress, general health, and negative cognitions: a randomized controlled trial. J Ration-Emot Cogn-Behav Ther. 2019; 37(4): 375-394.
30. Starabadi M, Aminfakhraei A, Keramati K, Samavi A. Efficacy of acceptance and commitment therapy on stress and depression in infertile couples. Women Studies. 2020; 11(31): 1-18.
31. Lukse MP. The effect of group counseling on the frequency of grief reported by infertile couples. J Obstet Gynecol Neonatal Nurs. 1985; 14: S67-S70.
32. Hämmerli K, Znoj H, Barth J. The efficacy of psychological interventions for infertile patients: a meta-analysis examining mental health and pregnancy rate. Hum Reprod Update. 2009; 15(3): 279-295.
33. Chehreh R, Ozgoli G, Abolmaali K, Nasiri M, Mazaheri E. Comparison of the infertility-related stress among couples and its relationship with infertility factors. Int J Women's Health Reprod Sci. 2019; 7(3): 313-318.
34. Ying LY, Wu LH, Loke AY. Gender differences in experiences with and adjustments to infertility: a literature review. Int J Nurs Stud. 2015; 52(10): 1640-1652.
35. Omidian M, Rahimian Boogar I, Talepasand S, Najafi M, Kaveh M. The cultural tailoring and effectiveness of couples coping enhancement training on marital adjustment of wives. PCP. 2019; 7(1): 43-52.
36. Molgora S, Fenaroli V, Acquati C, De Donno A, Baldini MP, Saita E. Examining the role of dyadic coping on the marital adjustment of couples undergoing assisted reproductive technology (ART). Front Psychol. 2019; 10: 415.
37. Bodenmann G, Plancherel B, Beach SRH, Widmer K, Gabriel B, Meuwly N, et al. Effects of coping-oriented couples therapy on depression: a randomized clinical trial. J Consult Clin Psychol. 2008; 76(6): 944.
38. Martins MV, Peterson B, Almeida V, Mesquita-Guimarães J, Costa M. Dyadic dynamics of perceived social support in couples facing infertility. Hum Reprod. 2014; 29(1): 83-89.
39. Taghipour A, Karimi FZ, Roudsari RL. Exploring iranian women's perceptions and experiences of their spouses' behavior towards male factor infertility: a qualitative study. Curr Women's Health Rev. 2020; 16(1): 60-68.
40. Kiani Z, Simbar M, Hajian S, Zayeri F. Quality of life among infertile women living in a paradox of concerns and dealing strategies: a qualitative study. Nurs Open. 2021; 8(1): 251-261.

Original Article

# Is There any Role for Granulocyte Colony Stimulating Factor in Improvement of Implantation in Intrauterine Insemination? A Prospective Double-Blind Randomized Control Trial

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## Abstract

**Background:** Granulocyte colony stimulating factor (GCSF) has been introduced as an immunomodulatory agent by increasing implantation rate *in vitro* fertilization (IVF) patients but it has not been studied in intrauterine insemination (IUI) patients. The aim of this study is to answer the role of GCSF in implantation rate of IUI.

**Materials and Methods:** In this prospective double-blind randomized control trial, 320 eligible patients were enrolled, who were referred to the referral infertility clinic of Shiraz University of Medical Sciences from February 2018 till the end of 2019. They were divided into two groups randomly. After collecting the demographic data, all patients received clomiphene citrate from the 5th day of the menstruation cycle for 5 days. 50-150 units of recombinant purified follicle-stimulating factor (FSH) were started from the 8th day of the cycle. Follicle monitoring was done by transvaginal sonography till a mature follicle of 18 mm or more was developed. Human chorionic gonadotropin (HCG) injection was done in both groups with intrauterine administration of 300 µg GCSF in the case group and normal saline in the control group simultaneously. After 36 hours, IUI was performed. The clinical pregnancy, miscarriage, and ongoing pregnancy rates of both groups were calculated by SPSS software.

**Results:** The results showed improvement of clinical pregnancy rate [15.38% vs. 13.81% OR=1.17 (0.62-2.21)], miscarriage rate [3.84% vs. 5.26% OR=0.74 (0.25-2.20)] and ongoing pregnancy rate [11.53% vs. 8.55% OR=1.37 (0.65-2.92)] in the GCSF group compared to the control. However, the results revealed no statistically significance (P>0.05).

**Conclusion:** Although it was not statistically significant, 300 µg Intrauterine GCSF administration simultaneously with hCG injection in standard IUI procedure might increase the pregnancy outcomes. Further studies are warranted (registration number: IRCT201212079281N2).

***Keywords:*** Embryo Implantation, Granulocyte Colony-Stimulating Factor, Pregnancy Rates

**Citation:** Amooee S, Shomali Z, Namazi N, Jannati F. Is there any role for granulocyte colony stimulating factor in improvement of implantation in intrauterine insemination? A prospective double-blind randomized control trial. Int J Fertil Steril. 2022; 16(4): 281-285 doi: 0.22074/IJFS.2021.537125.1171.


## Introduction

Nowadays, unexplained subfertility is an issue of concern in infertility clinic visits among 30-50% of couples (1, 2). Expectant management controlled ovarian hyper-stimulation with intrauterine insemination (IUI) as a less invasive method, or the more aggressive technique of *in vitro* fertilization (IVF) are the accepted practices for managing unexplained subfertility (3-5). Although treatment strategies should be selected individually, some authors recommend stimulated IUI as the first method of therapy with a success rate of 12% per cycle that is followed by IVF after three cycles of failure (1, 2). In addition, some authors indicated that the success rate of IUI is defined to be more similar to IVF than previously recognized (6).

It is logical to manage unexplained subfertility patients stepwise and gradually start with inexpensive, less invasive, and low-risk treatments (2). As IUI is less invasive and more economic than IVF with considerable benefits, it is reasonable to improve the success rate of IUI in these patients. Normal semen analysis and patent uterine tubes of unexplained subfertility patients highlight the role of the uterus as the main target of therapy for IUI improvement of success rate by affecting the implantation rate (7).

Granulocyte colony stimulating factor (GCSF) is introduced as an effective cytokine in reproduction and fertility via overcoming immunologic factors by the final consequence of altering the implantation rate (8, 9). This cytokine is derived from the bone marrow and cells like the monocyte, macrophage, and fibroblasts; it triggers the proliferation of the neutrophils and promotes releasing them into the blood circulation (10). It plays a role in inflammatory prohibition, angiogenesis, and prevention

Received: 20/August/2021, Accepted: 18/December/2021
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Royan Institute
International Journal of Fertility and Sterility
Vol 16, No 4, October-December 2022, Pages: 281-285

of apoptosis (8, 11). Also, GCSF is responsible for advancing ovarian function, promoting oocyte maturation, regulating the endometrium by increasing receptivity, and improving embryo implantation (8, 12, 13). Although there are controversies, GCSF is introduced as a successful immunotherapy modality in IVF for advancing fertility in Recurrent Implantation Failure (RIF) patients by impacting the implantation process (8, 9, 11, 14-17). Also, GCSF is found in endometrial and fetal cells which may bold the possible role of this cytokine to improve pregnancy outcome (18). A noticeable point is the minimal harm of administration of GCSF for pregnancy outcome (19, 20).

To the best of our knowledge, there are limited data on IUI improvement by immunotherapy, especially on the effect of GCSF on IUI. Considering multiple aspects of IUI including low cost, less invasiveness, and patient-friendly points, and recognizing the uterus as the possible cause of IUI failure, we were encouraged to conduct this survey to evaluate the possible effects of intrauterine GCSF administration on the pregnancy success rate among patients with recurrent IUI failure to avoid the burden of IVF in unexplained subfertility.

## Materials and Methods

### The study protocol and setting

In this randomized control prospective study, we aimed to evaluate the effect of GCSF on the IUI success rate by measuring chemical and clinical pregnancy as primary outcome and miscarriage and ongoing pregnancy rates as secondary outcomes. It was approved by the Ethics Committee of Shiraz University of Medical Sciences following the Declaration of Helsinki Guideline (IR.SUMS.MED.REC.1395.60) and registered at the Iranian Registry of Clinical Trials (IRCT201212079281N2). To calculate the sample size based on a previous study (21), the success rate for the control and case groups was determined to be 19.6% and 44.6%, respectively. Considering the confidence interval of 95%, power of 80%, and type one and two errors of 0.05 and 0.20 respectively, the sample size was set to be 87 patients in each group (22). In previous studies on GCSF efficacy which were carried out on IVF protocol, the number of embryos was more than the patients due to the transfer of more than one embryo for most patients. Since this study was performed on the IUI protocol with an almost equal ratio of patients and embryos in each cycle, we increased the total studied samples to 320 eligible patients who were referred to the referral infertility clinic of Shiraz University of Medical Sciences from February 2018 till the end of 2019.

Patients were recruited after filling out the informed consent. Demographic data and basic fertility characters were checked. Randomization was done exactly performing IUI by a web-based software, considering each block size to be 4 (160 patients in each arm study). It should be mentioned that all laboratory tests of participants were done at the laboratory of our center, and the staff was blind to the study groups too. Also, all patients' endometrial thickness was examined by an expert sonographer using the Voluson E8 machine who was blind to allocations.

### Inclusion and exclusion criteria

The inclusion criteria were a mean age of 20-40 years, normal body mass index, and anti-Mullerian hormone level of 2-3.5 ng/ml, patent tubes in hysterosalpingography, and normal hormonal assay including follicle-stimulating factor (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH), and prolactin. The patients should have subfertility subtype of primary unexplained infertility for less than three years with normal endometrium thickness in women. The husband should have been examined by the urologist of our center in order to have a normal physical exam and normal laboratory studies including semen analysis with no medical diagnosis. It is emphasized that they should have a total motile count of more than 10 million in semen analysis. The exclusion criteria were the participants who had thin endometrium (less than 7 mm) on the day of human chorionic gonadotropin (HCG) injection, any chronic disease (like malignancy, chronic hypertension, Diabetes Mellitus, thyroid or kidney disease, anemia, polycystic ovarian disease), history of previous surgery on the uterus, ovulatory dysfunction, any contraindication for GCSF administration (patients with allergy to E. coli-derived proteins or previous history of severe side effects), severe male factor infertility, any stages of endometriosis, or unwillingness to continue the project.

### Treatment protocol and outcome

All patients had a basal evaluation of antral follicular count (AFC) on the second day of their cycle by transvaginal sonography. The enrolled patients received 100 mg clomiphene citrate (Iran Hormone Laboratory, Tehran, Iran) daily from the 5$^{th}$ day of the menstruation cycle for 5 days. In addition, starting from the 8th day of the cycle, 50-150 units of recombinant purified FSH (Gonal-F, Merck Serono, Switzerland) were prescribed individually. Then, on the 11$^{th}$ day of the cycle, transvaginal sonography was done by an assigned gynecologist who was blind to the group of patients by using the Voluson E8 machine. Based on the number and size of the dominant follicles, FSH dosage was adjusted for the next days till at least one mature follicle with a diameter of 18 mm or more was developing. At this time, 5000 units of hCG intramuscular injection (Choriomon, IBSA, Switzerland) was injected. Meanwhile, to make the study blind to the patients and remove the distributing factors, we inserted the IUI catheter (Prince medical, France) for all patients. Then, an

intrauterine injection of 300 µg of GCSF (1 cc, single-dose vial of Neupogen, Roche, Switzerland) was done for the case group, while 1 cc normal saline was injected in the control group in the same manner of the case group (23). Saline was in a bottle exactly like GCSF with the material the same in color and odor. There was an assigned staff in charge of preparing the syringe for injection of GCSF or saline after opening the sealed envelope of the patient group's allocation. The gynecologist who performed the procedure was blind to the group allocation and type of the substance in the syringe. 36 hours later, IUI was done by an expert gynecologist blinded to the group allocations by the standard local protocol method with swim-up technique of sperm preparation (24). After two weeks, the serum pregnancy test was done. Pregnancy was clinically established by transvaginal sonography at 6 weeks of gestational age in the patients with positive serum tests. The clinical pregnancy rate was calculated by dividing the number of patients with the presence of gestational sac in sonography divided into the total number of patients in each group. Also, miscarriage rate was defined as pregnancy loss before 12 weeks of gestational age. The ongoing pregnancy rate was calculated by subtracting the miscarriage rate from the total clinical pregnancy rate.

### Statistical analysis

Quantitative data were presented as mean ± SD while qualitative data were presented as number (n) and percentage. The comparison between two groups with quantitative data and normal distribution was done by using an independent Student t test while the Mann-Whitney U-test was used only with non-parametric data. Logistic regression analysis was used to assess the odds ratio of factors related to birth rates between two groups. Statistical analysis was carried out using SPSS version 21 (SPSS IBM, Armonk, NY, USA). P<0.05 was considered statistically significant.

## Results

As shown in Figure 1, 156 cases received GCSF (3 cases did not complete their follow up, one case had a technical problem in the administration of GCSF), and 152 control patients that not received GCSF (all omitted cases with not availability for follow up after IUI procedure) were enrolled at the end of the study. Six patients out of the GCSF group and 8 patients out of the control group had a miscarriage. In this study, all the ongoing pregnancies had live births. In the pregnancy course, one patient of each group (case at 27 weeks of gestation and control at 25 weeks of gestation) had alive premature birth that the neonates of both groups expired due to prematurity. Except for developing leukemia in one of the infants of the control group, no other specific event was notable in their follow-up. The demographic data of each group is presented in more detail in Table 1.

As demonstrated, both groups were not statistically different in age, endometrial thickness, number of follicles, parity, AFC, and body mass index (BMI).

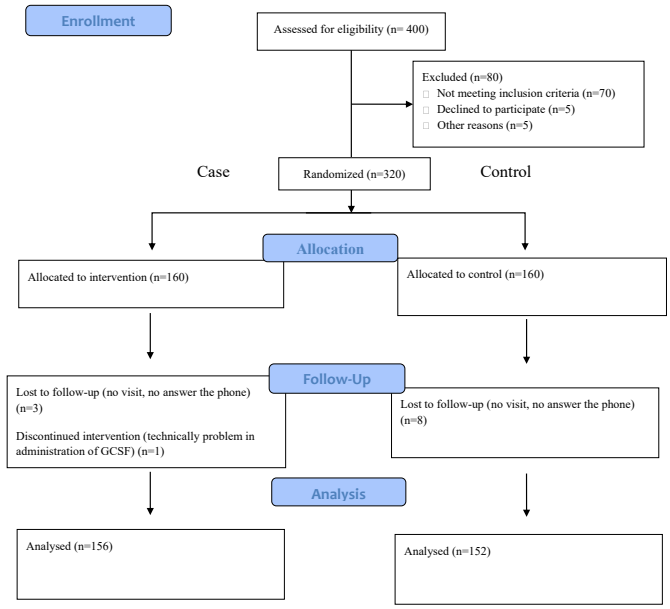


**Fig.1:** Flow chart of patients enrollment in the study that were randomly divided into groups of case and control.

**Table 1:** Demographic data of the case and control group

| Characteristics | Groups | | P value* |
|---|---|---|---|
| | GCSF (n=156) | Control (n=152) | |
| Age (Y) | 30.01 ± 5.18 | 29.05 ± 6.32 | 0.145 |
| ET (mm) | 7.47 ± 1.46 | 7.63 ± 1.49 | 0.38 |
| Follicle number (n) | 2.39 ± 0.82 | 2.33 ± 1.05 | 0.358 |
| Parity (n) | 0.2 ± 0.49 | 0.16 ± 0.44 | 0.546 |
| AFC on the second day of cycle (n) | 9.38 ± 3.31 | 9.42 ± 3.28 | 0.45 |
| BMI (kg/m$^2$) | 22.48 ± 2.43 | 22.5 ± 2.41 | 0.315 |

**Data are presented as mean ± SD. GCSF; Granulocyte colony stimulating factor, ET; Endometrial thickness, AFC; Antral follicular count, BMI; Body mass index, and *; Two-tailed t test.**

The pregnancy rate in the GCSF group was 24 out of 156 patients (15.38%) in comparison to 21 out of 152 patients (13.81%) calculated for the control groups. Although the data showed an improved pregnancy rate documented by sonography in the GCSF group, it was not significant (P=0.63). No specific side effects were seen among the case and control groups. Also, non-significant improvement in ongoing pregnancy and miscarriage is shown in the GCSF group (Table 2, P>0.05).

**Table 2:** IUI outcome in case and control group

| Characteristic | GCSF group (n=156) | Control group (n=152) | OR (95%CI) | P value |
|---|---|---|---|---|
| Clinical pregnancy rate | 24 (15.38) | 21 (13.81) | 1.17 (0.62-2.21) | 0.63 |
| Miscarriage rate | 6 (3.84) | 8 (5.26) | 0.74 (0.25-2.20) | 0.55 |
| Ongoing pregnancy rate | 18 (11.53) | 13 (8.55) | 1.37 (0.65-2.92) | 0.38 |

**Data are presented as n (%). IUI; Intrauterine insemination, GCSF; Granulocyte colony stimulating factor, OR; Odds ratio, and CI; Confidence interval.**

## Discussion

The results of this study showed no statistically significant improvement in fertility rate in patients who received GCSF on the day of hCG injection in the IUI cycle. To the best of our knowledge, we found no previous study on testing GCSF to improve the IUI success rate study. There are some articles in the literature focusing on GCSF in assisted reproductive techniques (ART) success among patients suffering from recurrent miscarriage (10, 25) or thin endometrium in ARTs (9, 26) although there are some non-specific side effects like nausea and vomiting, anorexia, and headache; moreover, chest pain, hypoxemia, and syncope are mentioned as its side effects (12).

There is a controversy on GCSF efficacy to treat RIF patients (20). Kamath et al. (27), in a recent systematic review, Kalem et al. (23) in a randomized control study on intrauterine administration of GCSF in normal endometrium patients (23), and Davari Tanha et al. (28), in a randomized double-blind placebo control trial presented GCSF as an ineffective treatment in RIF patients. They are all in line with the Practice Committee of the American Society for Reproductive Medicine which believes there is no effect of GCSF considering insufficient study on the issue (29). In contrast, the following mentioned studies indicated that GCSF was beneficial. Zhang et al. (15) revealed the positive effect of GCSF in either systematic or intrauterine root administration in RIF patients. Also, the potency of GCSF to increase fertility in RIF patients is shown in a systematic review as well as other immunotherapy methods (10). Zhao et al. (30), in a systematic review and meta-analysis presented this cytokine as a beneficial method of fertility improvement. These controversies occur due to national, ethical, and genetic variations as well as different sample sizes and study design studies, the dosage of administration, and root of injection (8, 31). In line with the Practice Committee of the American Society, Davari Tanha et al. (28), we found no significant improvement in the fertility rate although it was more in the groups that received GCSF. It may be attributed to the very short lag between the administration of GCSF and insemination (36 hours). More time might be needed to present the positive effects of GCSF. Also, we perfused GCSF once in the uterine cavity, with possible benefit in more times of administration of the cytokine.

The outstanding root of GCSF administration is uncertain. Zeyneloglu et al. (14) demonstrated the benefits of dual subcutaneous and intrauterine administration of GCSF in patients with recurrent implantation failure in the intracytoplasmic sperm injection process. Patients received GCSF subcutaneously for 15 days starting from the oocyte retrieval day. The intrauterine dose was injected on the day of ovulation induction. The result of the study revealed the effectiveness of combination therapy of GCSF as the best method of prescription. Kalem et al. (23) showed no effectiveness in intrauterine administration of GCSF daily on hCG. Recently, a systematic review emphasized the effectiveness of GCSF in both intrauterine and subcutaneous administration with more success for subcutaneous method (8). Cavalcante et al. (10) in a systematic review showed the subcutaneous root as the method of choice for recurrent miscarriage treatment purposes, while the intrauterine root was a suitable choice for RIF or thin endometrium. In a systematic review, the beneficial effect of GCSF was attributed to the subcutaneous root of administration (30). Incongruently, Xie et al. (32) presented the effectiveness of intrauterine administration of GCSF in patients suffering from thin endometrium. In the present study, although we presented a better outcome in patients who received intra-uterine GCSF, this improvement was not statistically significant in patients with normal endometrium thickness. Effects on the patients with thin endometrium were not studied in this survey, so the possible intrauterine positive effect of GCSF might have been ignored. The potential effects of systematic administration of GCSF on normal endometrium patients should be investigated in further studies.

The strength of our study is its large population with the study design of a double-blind randomized control trial. Sonographer, laboratory, and IUI performer were the same among all participants, leading to a reduction in bias. Also, to the best of our knowledge, there is limited data on the effect of GCSF administration on the IUI success rate. We focused on the possible effects of GCSF that could lead to altering the protocols of subfertility management. Finally, it is concluded that less expensive modalities with less invasive procedures should be used. Performing this study only on patients with normal endometrial thickness is the limitation of our study. It is recommended that further studies be conducted considering the thin endometrium group and those with normal endometrium. Also, considering different lags between GCSF prescription and insemination should be examined in future studies to evaluate the possible positive effects of the cytokine prescribed in systemic, intra-uterine, or both methods.

## Conclusion

Intrauterine 300 µg GCSF administration simultaneously with hCG injection in standard IUI procedure has increased the pregnancy outcome although it was not statistically significant. More studies are warranted that focus on the root and day of administration and studied population.

## Acknowledgements

This study is extracted from a thesis (registered no. 11161) submitted and financialy supported Shiraz University of Medical Sciences as a partial requirement for the degree of specialty in infertility fellowship. The authors wish to thank all staff and patients for their lovely cooperation. The authors would like to thank Shiraz University of Medical Sciences, Shiraz, Iran, and also the Center for Development of Clinical Research of Nemazee Hospital and Dr. Nasrin Shokrpour for editorial assistance. All authors declared no conflict of interest.

## Authors' Contributions

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

## References


1. Buckett W, Sierra S. The management of unexplained infertility: an evidence-based guideline from the Canadian Fertility and Andrology Society. Reprod Biomed Online. 2019; 39(4): 633-640.
2. Kandavel V, Cheong Y. Does intra-uterine insemination have a place in modern ART practice? Best Pract Res Clin Obstet Gynaecol. 2018; 53: 3-10.
3. Wang R, van Eekelen R, Mochtar MH, Mol F, van Wely M. Treatment strategies for unexplained infertility. Semin Reprod Med. 2020; 38(1): 48-54.
4. Ayeleke RO, Asseler JD, Cohlen BJ, Veltman-Verhulst SM. Intra-uterine insemination for unexplained subfertility. Cochrane Database Syst Rev. 2020; 3(3): CD001838.
5. Wang R, Danhof NA, Tjon-Kon-Fat RI, Eijkemans MJ, Bossuyt PM, Mochtar MH, et al. Interventions for unexplained infertility: a systematic review and network meta-analysis. Cochrane Database Syst Rev. 2019; 9(9): CD012692.
6. Bahadur G, Homburg R, Bosmans JE, Huirne JAF, Hinstridge P, Jayaprakasan K, et al. Observational retrospective study of UK national success, risks and costs for 319,105 IVF/ICSI and 30,669 IUI treatment cycles. BMJ Open. 2020; 10(3): e034566.
7. Wadhwa L, Mishra M. Therapeutic efficacy of endometrial scratching in repeated controlled ovarian stimulation (COS) failure cycles. J Hum Reprod Sci. 2018; 11(1): 59-71.
8. Jiang Y, Zhao Q, Zhang Y, Zhou L, Lin J, Chen Y, et al. Treatment of G-CSF in unexplained, repeated implantation failure: A systematic review and meta-analysis. J Gynecol Obstet Hum. 2020; 49(10):101866.
9. Bashiri A, Halper KI, Orvieto R. Recurrent implantation failure-update overview on etiology, diagnosis, treatment and future directions. Reprod Biol Endocrinol. 2018; 16(1): 121.
10. Cavalcante MB, Cavalcante C, Sarno M, Barini R. Intrauterine perfusion immunotherapies in recurrent implantation failures: Systematic review. Am J Reprod Immunol. 2020; 83(6): e13242.
11. Würfel W, Santjohanser C, Hirv K, Bühl M, Meri O, Laubert I, et al. High pregnancy rates with administration of granulocyte colony-stimulating factor in ART-patients with repetitive implantation failure and lacking killer-cell immunglobulin-like receptors. Hum Reprod. 2010; 25(8): 2151-2152.
12. Eftekhar M, Naghshineh E, Khani P. Role of granulocyte colony-stimulating factor in human reproduction. J Res Med Sci. 2018; 23: 7.
13. Dieamant F, Vagnini LD, Petersen CG, Mauri AL, Renzi A, Petersen B, et al. New therapeutic protocol for improvement of endometrial receptivity (PRIMER) for patients with recurrent implantation failure (RIF) - a pilot study. JBRA Assist Reprod. 2019; 23(3): 250-254.
14. Zeyneloglu HB, Tohma YA, Onalan G, Moran U. Granulocyte colony-stimulating factor for intracytoplasmic sperm injection patients with repeated implantation failure: which route is best? J Obstet Gynaecol. 2020; 40(4): 526-530.
15. Zhang L, Xu WH, Fu XH, Huang QX, Guo XY, Zhang L, et al. Therapeutic role of granulocyte colony-stimulating factor (G-CSF) for infertile women under in vitro fertilization and embryo transfer (IVF-ET) treatment: a meta-analysis. Arch Gynecol Obstet. 2018; 298(5): 861-871.
16. Kamath MS, Chittawar PB, Kirubakaran R, Mascarenhas M. Use of granulocyte-colony stimulating factor in assisted reproductive technology: a systematic review and meta-analysis. Eur J Obstet Gynecol Reprod Biol. 2017; 214: 16-24.
17. Rocha MNC, Florêncio RS, Alves RRF. The role played by granulocyte colony stimulating factor (G-CSF) on women submitted to in vitro fertilization associated with thin endometrium: systematic review. JBRA Assist Reprod. 2020; 24(3): 278-282.
18. Lédée N, Lombroso R, Lombardelli L, Selva J, Dubanchet S, Chaouat G, et al. Cytokines and chemokines in follicular fluids and potential of the corresponding embryo: the role of granulocyte colony-stimulating factor. Hum Reprod. 2008; 23(9): 2001-2009.
19. Cruz M, Alecsandru D, García-Velasco JA, Requena A. Use of granulocyte colony-stimulating factor in ART treatment does not increase the risk of adverse perinatal outcomes. Reprod Biomed Online. 2019; 39(6): 976-980.
20. Moustafa S, Young SL. Diagnostic and therapeutic options in recurrent implantation failure. F1000Res. 2020; 9: F1000 Faculty Rev-208.
21. Aleyasin A, Abediasl Z, Nazari A, Sheikh M. Granulocyte colony-stimulating factor in repeated IVF failure, a randomized trial. Reproduction. 2016; 151(6): 637-642.
22. Zhong B. How to calculate sample size in randomized controlled trial? J Thorac Dis. 2009; 1(1): 51-54.
23. Kalem Z, Namli Kalem M, Bakirarar B, Kent E, Makrigiannakis A, Gurgan T. Intrauterine G-CSF administration in recurrent implantation failure (RIF): an Rct. Sci Rep. 2020; 10(1): 5139.
24. Boomsma CM, Cohlen BJ, Farquhar C. Semen preparation techniques for intrauterine insemination. Cochrane Database Syst Rev. 2019; 10(10): CD004507.
25. Mehrafza M, Kabodmehri R, Nikpouri Z, Pourseify G, Raoufi A, Eftekhari A, et al. Comparing the Impact of autologous platelet-rich plasma and granulocyte colony stimulating factor on pregnancy outcome in patients with repeated implantation failure. J Reprod Infertil. 2019; 20(1): 35-41.
26. Ranisavljevic N, Raad J, Anahory T, Grynberg M, Sonigo C. Embryo transfer strategy and therapeutic options in infertile patients with thin endometrium: a systematic review. J Assist Reprod Genet. 2019; 36(11): 2217-2231.
27. Kamath MS, Kirubakaran R, Sunkara SK. Granulocyte-colony stimulating factor administration for subfertile women undergoing assisted reproduction. Cochrane Database Syst Rev. 2020; 1(1): CD013226.
28. Davari-Tanha F, Shahrokh Tehraninejad E, Ghazi M, Shahraki Z. The role of G-CSF in recurrent implantation failure: A randomized double blind placebo control trial. Int J Reprod Biomed. 2016; 14(12): 737-342.
29. Practice Committee of the American Society for Reproductive Medicine. Electronic address: ASRM@asrm.org; Practice Committee of the American Society for Reproductive Medicine. The role of immunotherapy in in vitro fertilization: a guideline. Fertil Steril. 2018; 110(3): 387-400.
30. Zhao J, Xu B, Xie S, Zhang Q, Li YP. Whether G-CSF administration has beneficial effect on the outcome after assisted reproductive technology? A systematic review and meta-analysis. Reprod Biol Endocrinol. 2016; 14(1): 62.
31. Zhang T, Chen X, Wang CC, Li TC, Kwak-Kim J. Intrauterine infusion of human chorionic gonadotropin before embryo transfer in IVF/ET cycle: The critical review. Am J Reprod Immunol. 2019; 81(2): e13077.
32. Xie Y, Zhang T, Tian Z, Zhang J, Wang W, Zhang H, et al. Efficacy of intrauterine perfusion of granulocyte colony-stimulating factor (g-CSF) for Infertile women with thin endometrium: a systematic review and meta-analysis. Am J Reprod Immunol. 2017; 78(2): e12701.

Original Article

# Endometrial Expression of Insulin Signaling Pathway Genes in Pregnancy Leading to Abortion under 20 Weeks in Infertile Women: A Case-Control Study

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## Abstract

**Background:** Impaired expression of genes which act on hormone signaling pathways is one of the factors affecting miscarriage. In this study, the expression levels of insulin receptor (*INSR*) and insulin receptor substrates-1 *(IRS-1)* genes in endometrial tissue of infertile women and fertile women with miscarriage in less than twenty weeks gestation for unknown reasons were evaluated.

**Materials and Methods:** In this case-control study, forty-two fertile women with children and 42 infertile women, who underwent *in vitro* fertilization (IVF), were selected. Both groups had abortions under twenty weeks gestation for unknown reasons. The endometrial tissue of all patients was prepared to evaluate the expression of *INSR* and *IRS-1* genes by quantitative real-time polymerase chain reaction (PCR) method after RNA extraction.

**Results:** There was a statistically significant relationship between the expressions of *INSR* and *IRS-1* genes in the endometrial tissue of the infertile women compared with the fertile women (P=0.002 and P=0.008, respectively). The expression level of genes was decreased in both groups by age and increasing body mass index (BMI). Comparison of genes expression levels in healthy and diabetic participants in each group showed a significant difference (P<0.0001), but no meaningful difference was indicated between diabetic infertile and fertile groups in terms of gene expression. *INSR* gene expression levels showed an increase in the fertile group in the second 10 weeks and a decrease in *IRS-1* gene expression. But in the infertile group, both genes showed a slight increase in expression.

**Conclusion:** It seems a decreased expression of insulin signaling pathway genes in the endometrial tissue of infertile women can be one of reasons for unspecified abortion. These genes may be strong molecular markers for infertility.

***Keywords:*** Abortion, Female Infertility, Insulin Receptor, *In vitro* Fertilization, Unexplained Symptom

**Citation:** Namvarsigaroudi N, Tahmasebi Fard Z. Endometrial expression of insulin signaling pathway genes in pregnancy leading to abortion under 20 weeks in infertile women: a case-control study. Int J Fertil Steril. 2022; 16(4): 286-291. doi: 10.22074/IJFS.2021.534736.1163.


## Introduction

Successful pregnancies in humans and non-human mammals rely on a unique set of events, such as embryo implantation, separation, mating, and parturition. Implantation is associated with molecular and physiological events regulated between the embryo and the receiving endometrium. In the implantation process in humans, fundamental events such as adhesion, adhesion / attachment, invasion, and immune regulation occur (1).

Spontaneous abortion is a significant issue in terms of social and economic effects. Today, most women face the possibility of reduced fertility and increased spontaneous abortion due to delayed pregnancy. Infertility has various causes, the most common of which are tubular and pelvic diseases, ovulation disorders, polycystic ovary syndrome (PCOS) and premature ovarian failure (2).

Insulin is a pivotal metabolic hormone for regulating energy homeostasis in the body. Insulin-dependent signaling also plays an important role in embryo reproduction and early growth (3). In humans, insulin and proinsulin levels (prohormones with less activity than insulin) are significantly associated with weight, height, head circumference, and skin thickness of infants at birth (4). Insulin sends messages through its heterotetrameric receptor. After binding of insulin to alpha extracellular subunits, deformation occurs in the second tyrosine kinase present in the two beta intracellular subunits, resulting in activation of tyrosine kinase to auto-phosphorylate tyrosine components in the Tyr-1158, Tyr-1162, and Tyr-1163 positions, followed by rapid phosphorylation of docking proteins such as insulin receptor substrates (IRS) and several other signaling proteins (5). In endometrial cancer, the insulin hormone, as a growth factor, can increase cell proliferation and inhibit the process of apoptosis through the PI3K/Akt and RAS/MAPK pathways (6, 7). Activation of insulin recep-

Received: 26/July/2021, Accepted: 13/December/2021
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Royan Institute
International Journal of Fertility and Sterility
Vol 16, No 4, October-December 2022, Pages: 286-291

tor *(INSR)*, insulin receptor substrates -1 *(IRS-1)* and *AKT* has also been linked to the invasive nature of endometrial cancer, and insulin has mitogenic and anti-apoptotic properties for these cells (6).

Human placental growth hormone is increased continuously during the first 20 weeks of gestation, and this hormone has a strong effect on insulin metabolism. Because of this, the insulin signaling pathway is necessary to regulate cell metabolism. In the present study, we hypothesized that energy balance was essential for embryo implantation and growth. Therefore, the disruption of the insulin signaling pathway due to decreased expression of *INSR* and *IR-1* genes in the endometrial tissue of infertile women is considered a factor affecting infertility and abortion in *in vitro* fertilization (IVF).

## Materials and Methods

### Sample collection

In this case-control study, two groups were selected from the clients referred to the infertility centers of Yas and Mirzakoochak Khan Hospitals in Tehran (2018-2019). Forty-two women with children, who had experienced at least one normal pregnancy, were selected as the fertile group. Forty-two women without children with a regular menstrual cycle that were married more than one year and also had an unknown reason for infertility were selected as the infertile group. The sample size was calculated based on the following assumption: type1 and 2 errors: 0.05 and 0.20, respectively; expected implantation rate in control group: 65%; expected frequency of abortion: 35%. The infertile group underwent the IVF method to get pregnant, but the fertile group had a normal pregnancy. Both groups had an abortion under twenty weeks for unknown reasons. The aborted fetus also had a normal karyotype. The selection criteria of the groups were as follows: regular menstrual cycles, normal ovarian function, and absence of abnormalities in the uterus and fallopian tubes, or signs of endometriosis on ultra-sonographic or laparoscopic examinations. In addition, the spouses of subjects had sufficient sperm volume; and analysis of semen was according to WHO criteria. Those who did not have this characteristic were excluded.

The subjects ranged in age from 24 to 36 years. Endometrial samples of individuals were collected using a Novak curette/ Pipelle catheter and transferred to a karyotype containing RNA to be stored in liquid nitrogen until RNA extraction.

### RNA extraction and cDNA synthesis

Approximately 150-200 mg of endometrial tissue samples were washed twice with phosphate buffered saline (PBS, Bioidea, IRAN). Then, the RNA of all samples was extracted with the help of a commercial kit instruction (Invitrogen, Carlsbad, CA, USA). After evaluating the quantity and quality of the extracted RNA according to the kit instructions (Takara Bio Inc., Japan) about 1 mg of the total RNA from each sample was added to random hexamer primers, RT enzyme, and enzyme buffer used for cDNA synthesis and placed in a thermos-cycler.

### Quantitative real-time polymerase chain reaction analysis

Using the ABI StepOne Plus™ system (Applied Biosystems, Germany), gene expression (*INSR* and *IRS-1*) was evaluated by quantitative real time polymerase chain reaction (qRT-PCR). Primers (F: 5′-TTCCGAGACCTCAGTTTCCC-3′ and R: 5′-AGATGACCAGCGCGTAGTTA-3′) were used to proliferate the *INSR* gene, primers (F: 5′-AGGTGGATGACTCTGTGGTG-3′ and R: 5′-GGGATTGTTGAGATGGTGCC-3′) were used for the *IRS-1* gene, and primers (F: 5′-CGTGCGTGACATTAAAGAGAA-3′ and R: 5′-GGGATTGTTGAGATGGTGCC-3′) were used for the *beta-actin* gene (internal control). The proliferation steps included 95°C for 5 minutes for initial DNA denaturation, then 35 cycles at 95°C for 30 seconds, 55°C and 60°C for 30 seconds, and 72°C elongation for 30 seconds. All tests were performed in pairs. Several proliferated products were sequenced. To analyze the sample proliferation, the threshold line was drawn based on the exponential phase of the products to be statistically analyzed using the $2^{-\Delta\Delta CT}$ method.

### Statistical analysis

Data were analyzed using Graph Pad software version 9. The normal distribution of data was first examined by the Kolmogorov - Smirnov test. Then the variables of age, BMI, duration of marriage and length of pregnancy were calculated based on an independent t test and were reported as mean ± SD. Other data such as diabetes, number of children and abortions were calculated based on Fisher's exact test between the two groups. The expression level of genes was reported as fold change according to the formula fold change=$2^{-\Delta\Delta Ct}$. The fertile and infertile groups were divided into two subgroups for age (30≥ and 30<), body mass index (BMI, 25≥ and 25<), diabetes (healthy and diabetic), length of pregnancy (10 ≥ and 10< week). Fold change of the *INSR* and *IRS-1* gene expression was compared between subgroups, using a two-sample t test. The differences in expression of INSR and IRS-1 genes in the two groups, the effect of age, BMI, diabetes, and length of pregnancy on gene expression were assessed by t-test. The missing data were excluded from the study. In all statistic testes, a P value of less than 0.05 was considered significant. Results were reported with 95% confidence intervals (CIs).

### Ethical considerations

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the Tehran Islamic Azad University of Medical Sciences (IR.IAU.TMU.REC.1397.007). After obtaining informed consent, the structured questionnaires were filled out by subjects.

## Results

There was no significant difference between the mean age of the two groups, duration of the marriage, number of abortions, smoking and diabetes. In terms of mean BMI, duration of pregnancy and the number of children the groups were statistically significant. Individual information is presented separately in Table 1.

### Evaluation of changes in *INSR* and *IRS-1* gene expression in endometrial tissue of infertile women compared to fertile women

In the fertile group, the expression of the *INSR* gene was 2.61 times higher (P=0.002, 95% CI: 0.639-2.622) and the *IRS-1* gene was 2.87 times higher (P=0.008, 95% CI: 0.177-1.137) than the infertile group. These differences were also statistically significant. The results are shown in Figure 1.

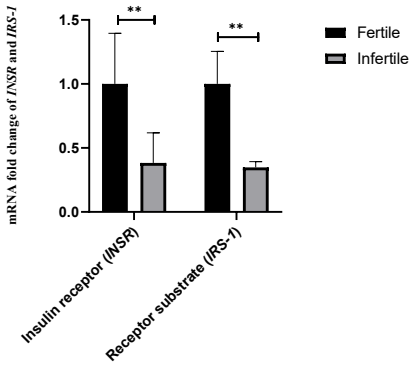


**Fig.1:** Quantitative real-time polymerase chain reaction (PCR) validation of transcriptome data for *INSR* and *IRS-1* genes. The mRNA fold change was used for comparative gene expression between fertile and infertile women. Independent samples student's t test was performed to compare *INSR* and *IRS-1* expression between fertile and infertile women. **; P<0.001.

### Evaluation of age parameters on the expression of *INSR* and *IRS-1* genes

In terms of age, each group was divided into two subgroups ≤30 and >30 years. Sixteen women in the fertile group and nineteen women in the infertile group were ≤30 years old; and 26 in the fertile group and 23 in the infertile group were >30 years old. In comparison with the fertile group, the expression of the *INSR* gene was 2.95 times (P=0.005, 95% CI: 0.397-4.010) higher and the *IRS-1* gene was 2.92 times (P<0.0001, 95% CI: 0.204-1.719) higher than that of the infertile group with the age of ≤30. The same comparison at age >30 showed that the expression of the *INSR* gene increased by 2.42 times (P=0.001, 95% CI: 0.147-2.459) and the expression of the *IRS-1* gene increased by 1.59 times (P=0.356, 95% CI: -0.131-0.333). Both groups did not differ in the expression of the *IRS-1* gene, except for the ≤30 age range. The results are shown separately in Figure 2.

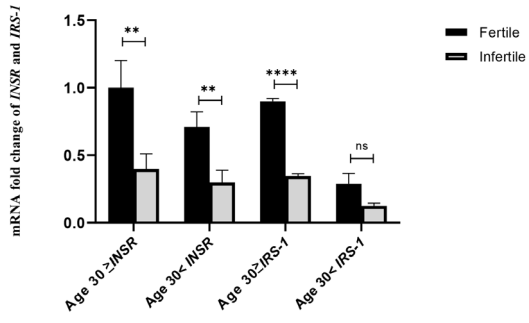


**Fig.2:** The effect of age parameter on the expression of *INSR* and *IRS-1* genes. The mRNA fold change was used for comparative gene expression between fertile and infertile women. Independent samples student's t test was performed to compare *INSR* and *IRS-1* expression between the two age groups. **; P<0.001, ****; P<0.00001, and ns; P>0.01.

**Table 1:** Baseline characteristics of the infertile and fertile women

| Variable | Fertile (n=42) | Infertile (n=42) | P value | 95% CI |
|---|---|---|---|---|
| Age (Y) | 34.1 ± 5.80 | 33.76 ± 5.37 | 0.770 | (-3.76-2.03) |
| BMI (kg/m$^2$) | 27.3 ± 2.80 | 24.09 ± 3.86 | <0.0001 | (-4.71--1.01) |
| Duration of marriage (Y) | 7.67 ± 3.74 | 6.50 ± 1.33 | 0.060 | (-2.38-0.05) |
| Chemical pregnancy or duration of pregnancy (days) | 86.1 ± 24.46 | 19.55 ± 9.93 | <0.0001 | (49.92-69.65) |
| Abortion | | | 0.234 | (0.47-0.76) |
| Non | 32 (76.19) | 36 (85.71) | | |
| 1 | 10 (23.81) | 5 (11.90) | | |
| 2 | 0 | 1 (2.39) | | |
| Diabetes | | | 0.131 | (0.15-0.42) |
| Positive | 4 (9.52) | 9 (21.43) | | |
| Negative | 38 (90.48) | 33 (78.57) | | |
| Child | | | <0.0001 | (0.01-0.07) |
| Non | 0 | 42 (100) | | |
| 1 | 17 (40.48) | 0 | | |
| 2 | 20 (47.62) | 0 | | |
| 3 | 5 (11.90) | 0 | | |

**Data are presented as mean ± SD or n (%). Age, BMI, duration of the marriage and length of pregnancy were calculated based on the independent t test. Fisher's exact test was used to compare the distribution of other variables (abortion, diabetes and number of children) between the two groups. BMI; Body mass index and CI; Confidence intervals.**

### Evaluation of the BMI parameter on the expression of *INSR* and *IRS-1* genes

Twenty-six fertile women and seven infertile ones had a BMI ≤25, and 16 fertile women and 35 infertile ones had a BMI >25. The expression of both genes was decreased by increasing BMI. Comparison of BMI ≤25 in the fertile women compared to the infertile women showed that the expression of the *INSR* gene was 10.07 times (P=0.002, 95% CI: 0.251-5.161) and *IRS-1* gene was 4.31 times (P<0.0001, 95% CI: 0.533-2.270) higher. Also, fertile and infertile persons at BMI >25 had 1.78 times (P=0.042, 95% CI: 0.214-2.026) more expression of the *INSR* gene and 2.19 times (P<0.0001, 95% CI: 0.069-0.812) more expression of the *IRS-1* gene. A comparison of the subgroups is shown in Figure 3.

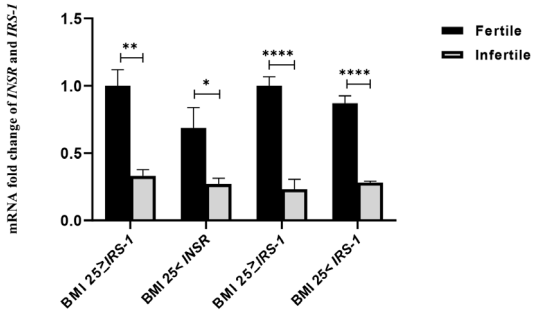


**Fig.3:** The effect of BMI parameter on the expression of *INSR* and *IRS-1* genes. The mRNA fold change was used for comparative gene expression between fertile and infertile women. Independent samples student's t test was performed to compare *INSR* and *IRS-1* expression between the BMI of the two groups of women. BMI; Body mass index, *; P<0.01, **; P<0.001, and ****; P<0.00001.

### Evaluation of diabetes on the expression of *INSR* and *IRS-1* genes

Nine fertile women and four infertile ones had diabetes (type II). Diabetes affected the expression of genes and caused a reduction in the expression of both genes in subjects with diabetes compared to healthy ones. This difference was statistically significant for the expression of the *INSR* gene (P=0.007) and *IRS-1* gene (P=0.029). Healthy fertile subjects had 23.82 times higher expression of the *INSR* gene than the fertile ones with diabetes (P<0.0001, 95% CI: 2.207-4.293) and 13.83 times higher *IRS-1* gene (P<0.0001, 95% CI: 0.679-1.813). Healthy infertile subjects showed 21.35 times more expression of the *INSR* gene (P<0.0001, 95% CI: 0.230-0.604) than the infertile ones with diabetes and 16.82 times more expression for the *IRS-1* gene (P<0.0001, 95% CI: 0.091-0.152). The comparison of the subgroups is shown in Figure 4.

### Evaluation of the duration of pregnancy on the expression of the *INSR* & *IRS-1* genes

The length of pregnancy was shorter in the infertile group than in the fertile group. This length was divided into two subgroups: ≥10 weeks and <10 weeks. In the fertile group, the expression of the *INSR* gene was 2.79 times (P<0.0001, 95% CI: 0.130-2.503) higher in the first ten weeks of pregnancy and 3.63 times (P<0.0001, 95% CI: 0.697-3.071) higher in the second ten weeks than the infertile group. In terms of *IRS-1* gene expression, the fertile group had 8.71 times (P<0.0001, 95% CI: 0.332-3.165) more expression in the first ten weeks and 1.48 times (P=0.653, 95% CI: -1.064-0.321) more in the second ten weeks. The results of gene expression in the first ten weeks and the second ten weeks are shown in Figure 5.

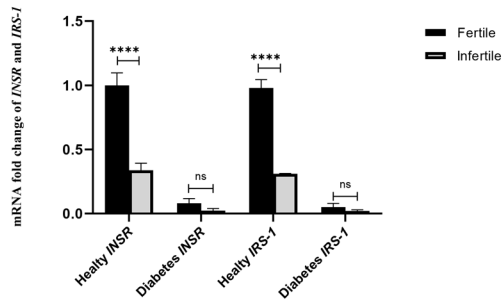


**Fig.4:** The effect of diabetes on the expression of *INSR* and *IRS-1* genes. The mRNA fold change was used for comparative gene expression between fertile and infertile women. Independent samples student's t test was performed to compare *INSR* and *IRS-1* expression between women who were healthy or with diabetic disease. ****; P<0.00001 and ns; P>0.01.

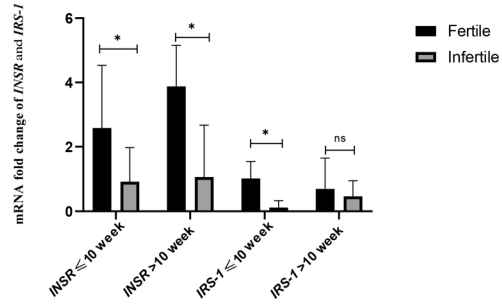


**Fig.5:** The effect of the length of pregnancy on the expression of *INSR* and *IRS-1* genes. The mRNA fold change was used for comparative gene expression between fertile and infertile women. Independent samples student's t test was performed to compare *INSR* and *IRS-1* expression and the length of pregnancy between groups. *; P<0.01 and ns; P>0.01.

## Discussion

Reproduction is controlled by the common function of several neuronal and hormonal signals (neuro-hormonal system). For central reproduction controlling, the deca-peptide gonadotrophin-releasing hormone (GnRH) is formed to activate the lower elements of the hypothalamus-pituitary-gonadal (HPG) axis, especially the secretion of the famous gonadotrophins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Also, environmental hormones affect GnRH activity. These gonadal hormones and various metabolic factors are essential for regulating energy homeostasis and fertility. Among these, insulin is a pivotal regulator of the HPG axis. Removing insulin receptors in animal models led to the development of severe metabolic disorders, hypogonadotropism, hypogonadism, and infertility (8). A study by Anjali and his colleagues demonstrated the effect of FSH on the expression of genes related to energy homeostasis. They showed that

FSH could increase the expression of the *IRS-2* gene and the functional deficiency of FSH reduced follicular growth and metabolism and led to infertility.

The pivotal role of the reproductive function of insulin activity in humans is determined by the expression of the insulin receptor in most tissues of the body, the hypothalamus, pituitary, uterus and ovaries (8). The binding of insulin to its INSR receptor causes induction of tyrosine phosphorylation in the insulin receptor substrate (IRS). Then the signal is transmitted through downstream enzymes such as *PI3K* and *AKT2*. Knockout mouse model of INSR causes hyperinsulinemia and hyperglycemia rapidly following diabetic ketoacidosis (9).

Human implantation is a complex and multifactorial process. Successful implantation requires some factors such as a healthy embryo, a receptive endometrium, the molecular coordination between them, and the protection of the host's immunity. Endometrial tissue has a transient functional state and allows blastocysts to be implanted and pregnancy to occur (10). Recent advances in the study of implantation processes have indicated that endometrial acceptance evaluation and pre-implantation genetic testing are necessary to overcome the possibility of implant failure (11, 12) and successful initiation of pregnancy. Early detection of endometrial abnormalities and the discovery of new strategies increase the chances of pregnancy, especially in infertile women.

In this study, a comparison between infertile women who had undergone IVF and fertile women was made. Both groups had an abortion less than twenty weeks for unknown reasons. The infertile group had lower expression of the *INSR* and *IRS-1* genes in uterine tissue compared to the fertile group. This difference of expression was statistically significant. The effect of some variables on gene expression was also evaluated.

Those in each group had less gene expression with aging (over 30 years). This reduction was more in the fertile group than in the infertile group. Comparing infertile with fertile women indicated a significant relationship between aging and the rate of decreased expression of insulin messaging genes. Also, Dunson et al. (13) examined the relationship between age and fertility. Their results demonstrated that women aged 19-26 were significantly more likely to become pregnant than women aged 27-29, and the infertility percent was estimated at 8% for women aged 19-26 and 13 to 14% for women aged 27 to 34, and 18 % for women aged 35 to 39.

The role of obesity is pivotal due to the increased production of hormones derived from adipose tissue, especially leptin (14). Leptin plays a role in energy balance and reproduction (8). Lack of leptin signaling in rats and humans causes obesity and infertility. Increased leptin in obese people reduces the activity of the hypothalamic-pituitary- gonadal (HPG) axis by creating a state of resistance (14). In the current study, the subgroups with BMI ≥25 and BMI <25 were also examined. Thirty infertile individuals and only sixteen fertile individuals had a BMI >25. Comparison of the two groups showed that the expression of both genes is decreased by increasing BMI. In obese fertile women, expression of both genes decreased significantly, but the infertile group showed a slight expression decrease in the *INSR* gene and an increased expression in the *IRS-1* gene.

Because insulin directly stimulates GnRH secretory activity (8), hyperglycemia occurs by decreased insulin secretion in diabetes. Also, diminished insulin secretion leads to infertility for reasons such as damage to the hypothalamic-pituitary-gonadal axis, increased DNA damage, oxidative stress, increased endoplasmic reticulum stress, mitochondrial function damage, and cell pathway modulation. Regulation of insulin levels directly affects *INSR* and *IGF1R* expression. Also, it leads to activation of signaling pathways associated with cell proliferation, differentiation, metabolism, and survival. In men with unexplained infertility, the lack of *INSR* and *IGR1R* in Sertoli cells causes reduction of testicular size by 75% and daily sperm production (15), insulin resistance also affects reproductive anomalies and their metabolism (16). In the present study, women with diabetes in both groups had a low-level expression of *INSR* and *IRS-1* genes compared to healthy subjects. But comparing infertile women with diabetes with fertile women with diabetes did not indicate a significant difference in terms of gene expression.

Concerning the length of pregnancy until termination, the two groups were divided into two subgroups of women less than 10 weeks pregnant and the women in the second 10 weeks of pregnancy. It aimed at evaluating the expression levels of *INSR* and *IRS-1* genes. The fertile women in the second 10 weeks of pregnancy showed that the expression levels of *INSR* and *IRS-1* genes increase and decrease, respectively. Infertile women in the second 10 weeks had a slight increase in *INSR* gene expression compared to the women in the first 10 weeks. They had a significant increase in *IRS-1* gene expression. It seems that decreasing or increasing one of the genes could disrupt the insulin signaling pathway.

## Conclusion

Hormones affect fertility and cause changes in gene expression for implantation and fetal growth through messaging pathways. Disorders in the signaling pathway of endometrial tissue can be one of the reasons for the lack of fetal growth and abortion. One of the most important hormones is insulin, which transmits the message inside the cell through the receptor and the receptor substrate. Genetic changes in infertile women lead to reduced expression of these proteins and disrupted hormone signaling. Other factors such as obesity, diabetes, old age and smoking also reduce the expression of these genes and aggravate the problem of infertility. Therefore, it is apparent that genetic disorders are one of the factors affecting infertility.

## Acknowledgments

The authors would like to thank the Medical Personnel at Yas and MirzaKuchak Khan Hospitals in Tehran and all the participants in this study. All research financial and material costs was provided by Nader Namvarsigaroudi. There is no conflict of interest in this study.

## Authors' Contributions

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

## References

1. Ochoa-Bernal MA, Fazleabas AT. Physiologic events of embryo implantation and decidualization in human and non-human primates. Int J Mol Sci. 2020; 21(6): 1973.
2. Wang HY, Qiao J, Sun XX, Wang ShY, Liang XY, Sun Y, et al. Epidemiological survey and risk factor analysis of recurrent spontaneous miscarriages in infertile women at large infertility centers. Chin Med J (Engl). 2017; 130(17): 2056-2062.
3. Laskowska D, Sjunnesson Y, Humblot P, Andersson G, Gustafsson H, Bage R. The functional role of insulin in fertility and embryonic development—What can we learn from the bovine model? Theriogenology. 2016; 86(1): 457-464.
4. Malaguarnera R, Sacco A, Voci C, Pandini G, Vigneri R, Belfiore A. Proinsulin binds with high affinity the insulin receptor isoform a and predominantly activates the mitogenic pathway. Endocrinology. 2012; 153(5): 2152-2163.
5. Draznin B. Molecular mechanisms of insulin resistance: serine phosphorylation of insulin receptor substrate-1 and increased expression of p85α. Diabetes. 2006; 55(8): 2392-2397.
6. Wang Y, Hua Sh, Tian W, Zhang L, Zhao J, Zhang H, et al. Mitogenic and anti-apoptotic effects of insulin in endometrial cancer are phosphatidylinositol 3-kinase/Akt dependent. Gynecol Oncol. 2012; 125(3): 734-741.
7. Tian W, Teng F, Gao J, Gao Ch, Liu G, Zhang Y, et al. Estrogen and insulin synergistically promote endometrial cancer progression via crosstalk between their receptor signaling pathways. Cancer Biol Med. 2019; 16(1): 555-570.
8. Codner E, Merino PM, Tena-Sempere M. Female reproduction and type 1 diabetes: from mechanisms to clinical findings. Hum Reprod. 2012; 18(5): 568-585.
9. Yang WM, Min KH, Lee W. Induction of miR-96 by dietary saturated fatty acids exacerbates hepatic insulin resistance through the suppression of INSR and IRS-1. PLoS One. 2016; 11(12): e0169039.
10. Neykova K, Tosto V, Giardina I, Tsibizova V, Vakrilov G. Endometrial receptivity and pregnancy outcome. J Matern Fetal Neonatal Med. 2020; 2: 1-15.
11. Cozzolino M, Diaz-Gimeno P, Pellicer A, Garrido N. Evaluation of the endometrial receptivity assay and the preimplantation genetic test for aneuploidy in overcoming recurrent implantation failure. J Assist Reprod Genet. 2020; 37: 2989-2997.
12. Barra F, Laganà AS , Scala C, Garzon S, Ghezzi F, Ferrero S. Pretreatment with dienogest in women with endometriosis undergoing IVF after a previous failed cycle. Reprod Biomed Online. 2020; 41(5): 859-868.
13. Dunson DB, Donna DB, Bernardo C. Increased infertility with age in men and women. Obstet Gynecol. 2004; 103(1): 51-56.
14. Borges BC, Garcia-Galiano D, Cruz-Machado SS, Han X, Gavrilina GB, Saunders TL, et al. Obesity-induced infertility in male mice is associated with disruption of Crisp4 expression and sperm fertilization capacity. Endocrinology. 2017; 158(9): 2930-2943.
15. Maresch CC, Stute DC, Alves MG, Oliveira PF, Kretser DM, Linn T. Diabetes-induced hyperglycemia impairs male reproductive function: a systematic review. Hum Reprod. 2018; 24(1): 86-105.
16. Mansour R, El-Faissal Y, Kamel A, Kamal O, Aboulserour G, Aboulghar M, et al. Increased insulin resistance in men with unexplained infertility. Reprod Biomed Online. 2017; 35(5): 571-575.

Original Article

# Association between Glucose Consumption and Oocyte Maturation Competence in Mice with Polycystic Ovarian Syndrome

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## Abstract

**Background:** This study evaluated association between glucose uptake by individually cultured oocyte and their maturation competence in mice with polycystic ovarian syndrome (PCOS).

**Materials and Methods:** In this experimental study, PCOS and non-PCOS cumulus-oocyte complexes (COCs), and cumulus-denuded oocytes (DOs) were cultured individually and categorized in four groups: i. PCOS DOs (n=83), ii. PCOS COCs (n=35), iii. Non-PCOS DOs (n=61) and iv. Non-PCOS COCs (n=62). After the culture period, 50 µl aliquots of the spent drops were used for glucose change analysis using high performance liquid chromatography. Polar NH2 column was used for the study of carbohydrates, acetonitrile with deionized water as the solvent phase and UV as detectors. Oocyte quality (growth differentiation factor 9: *GDF-9*), viability [bcl-2-like protein 4 (*BAX*) and B-cell lymphoma2 (*BCL2*)], in addition to fertilization and embryonic development rates were also evaluated in relation to glucose consumption rate of each oocyte.

**Results:** Maturation rate was significantly higher in non-PCOS COCs and DOs compared to PCOS COCs (IV: 70.9% vs. II: 45.71%) and DOs (III: 67.2% vs. 1: 53.01%), respectively. There was a significant negative correlation between high glucose intake (38.17 ppm) and *BCL2* gene expression (P=0.03) in PCOS COCs compared to non-PCOS COCs. There was a significant difference in the *GDF-9* gene expression from PCOS DOs (0.66 ± 0.02, P=0.003) and COCs (0.37 ± 0.02, P=0.0001) compared to non-PCOS DOs and COCs, respectively. A negative correlation was also observed between quality of PCOS-DOs and -COCs with glucose intake. Non-PCOS COCs significantly showed higher rate of successful IVF and development compared to PCOS COCs (P=0.01).

**Conclusion:** Based on the importance of metabolic analysis, the glucose consumption by DOs and COCs in culture medium can be a suitable criterion for their quality assessment. So that, glucose consumption may reflect oocyte maturation competence.

***Keywords:*** Glucose Intake, High Performance Liquid Chromatography, *In Vitro* Maturation, Oocyte Quality, Polycystic Ovarian Syndrome

**Citation:** **Kousheh F, Ghasemian F, Zahiri Z. Association between glucose consumption and oocyte maturation competence in mice with polycystic ovarian syndrome. Int J Fertil Steril. 2022; 16(4): 292-298. doi: 10.22074/IJFS.2021.532312.1142.**


## Introduction

*In vitro* maturation (IVM), a modified method of conventional *in vitro* fertilization (IVF), used smaller follicles after little or no exogenous gonadotropin stimulation (1, 2). During IVM, the immature oocytes at the stages of germinal vesicle (GV) or metaphase I (MI) were retrieved. The oocyte maturation and meiosis resumption were followed in the laboratory. Therefore, using gonadotrophin and an ovulation trigger were deleted or minimized during *in vivo* or *in vitro* maturation (1, 3). Additionally, a range of patients was treated in various manners [including follicle-stimulating hormone (FSH) resistance, oocyte donors, candidate for fertility preservation, the presence of severe effects of elevated estradiol, and patients with thrombophilia)] using IVM. Subsequently, there is an emerging interest to treat women with polycystic ovarian syndrome (PCOS) using IVM (1, 2). PCOS is commonly known as an endocrine disorder in the reproductive years of 4-12% of women. Anovulation and infertility are observed in the PCOS women (4). In addition, ovarian hyper-stimulation syndrome (OHSS) might be developed in women with PCOS undergoing IVF cycles to induce more antral follicles (1, 4).

Using animal models have been common, as a valuable resource, to elucidate potential mechanisms of PCOS pathology. The strategies have been introduced to develop animal models of PCOS, such as treatment with androgens, estrogens, progesterone receptor antagonists and genetic manipulations (5). The PCOS mouse models showed that exogenous androgen administration was

**Received: 16/June/2021, Accepted: 27/December/2021**
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**Royan Institute**
**International Journal of Fertility and Sterility**
**Vol 16, No 4, October-December 2022, Pages: 292-298**

sufficient to produce some symptoms of human PCOS, including hyper-androgenic and PCOS phenotypes [such as increased testosterone and luteinizing hormone (LH)], polycystic ovaries and acyclicity after treatment (6, 7).

Oocyte maturation has two stages: i. Nuclear maturation that is observed as resumption of the first meiosis and extrusion of the first polar body and ii. Cytoplasmic maturation. The other changes within oocyte was defined as cytoplasmic maturation, such as organelles development, accumulation of proteins and mRNA, cytoskeleton reorganization and changes in cellular metabolism. Insufficient cytoplasmic maturation leads to declined developmental potential of *in vitro* matured oocytes. The energy required for progression of all the dynamic processes during oocyte maturation is supplemented via energy metabolism from different substrates such as amino acids, lipids and carbohydrates. Increased glucose metabolism commonly occurs to produce mature oocyte at the metaphase II (MII) stage (6).

The successful fertilization, implantation and ongoing pregnancy influence quality of oocytes (7). So that, studies showed the association of fertilization failure with oocyte abnormalities. One of the most common methods to assay oocyte quality is observation of morphological oocyte characteristics at the light microscopy level (1). However, assessment of oocyte morphology is debatable for embryo selection and prediction of implantation potential. Pre-implantation genetic (PGD) diagnosis is another method for examination of embryo quality. Due to blastomere(s) and/or polar body biopsy as well as possible physical injuries, this is known as an invasive method that lead to a possible reduction of embryo quality (8, 9). Therefore, using a non-invasive oocyte evaluation such as metabolism of spent culture medium could introduce the best oocyte for subsequent fertilization processes.

As mentioned above, energy metabolism is necessary for oocyte maturation. So that, some studies indicated that glucose consumption was mediated gonadotropin-induced meiosis. In addition, the effect of glucose consumption on nuclear maturation of oocyte has been reported in many studies. For example, Xie et al. (6) showed that glucose consumption during IVM led to the release of metabolites from cumulus cells, their absorption by the oocyte and promotion of oocyte maturation. It has also been reported that glucose concentrations at certain level was important for normal mouse ovulation (10) and control of meiotic maturation in mouse cumulus oocyte complexes (11).

There are few studies on the effect of glucose consumption on oocyte maturation (10-13), while there is no data showing the effect of glucose consumption on IVM of oocytes with PCOS. Therefore, due to the importance of oocyte quality analysis and its role on the selection of the best embryo for implantation during assisted reproductive techniques, use of a non-invasive method to analyze oocyte quality is critical in IVF laboratory. Evaluation of maturation medium and changes of its metabolites is suggested as a suitable non-invasive method during *in vitro* oocyte maturation.

To the best of our knowledge, rate of glucose consumption by mouse PCOS oocyte as well as its association with *in vitro* oocyte maturation, viability and quality has not been evaluated. Therefore, the aim of this study was to answer the following questions: i. Is there an association between glucose consumption and IVM of mouse PCOS oocytes? ii. Does the rate of glucose consumption reflect quality of mouse PCOS oocyte and/or its apoptosis?

## Materials and Methods

### Study of animals

This experimental study was conducted in the University of Guilan (Rasht, Iran) between October 2020 and June 2021. Twenty adult Naval Medical Research Institute (NMRI) female mice (30-35 g, 7-8 weeks old) were used for the present study. Animals were housed in a central animal care room with controlled environment of 22 ± 3°C temperature, 45-55% humidity and 12 hours light/dark cycle. Each four mice were kept in a cage and fed with standard diet and water accessed ad libitum. All chemicals and reagents were purchased from Sigma Aldrich Company (Germany), unless otherwise specified. All investigations were confirmed with the ethical principles of research and they were approved by the Research Ethics Committee for Guilan University of Medical Sciences (IR.GUMS.REC.1399.255).

### Polycystic ovarian syndrome induction

All the experimental animals, except control groups (groups III and IV), were administered with estradiol valerate (Aburaihan Co., Iran) at a dose of 40 mg/kg body weight dissolved in 0.5% sesame oil by intramuscular injection once daily for 60 days (14). Vaginal epithelia smears were obtained daily and evaluated by light microscope using Giemsa stain to determine induction of PCOS. So that, the irregular estrous cycle and occurrence of persistent vaginal cornification phase were the symptoms of PCOS induction. With evidence of symptoms, ovaries were also cut through at longest longitudinal dimension and fixed in alcoholic Bouin's solution. After dehydration stage, the ovary was serially sectioned at 5 µm and stained with hematoxylin and eosin. The sections were used for histologic evaluation of PCOS ovaries.

In addition, to confirm the PCOS induction, the blood samples of PCOS mice were collected transcardially. Then, the separated serum was stored at – 20°C to estimate hormones. Levels of serum LH and FSH were evaluated using immunofluorometric techniques. The coefficient of variation for the total trial was 2.9 and 2.6%. Serum testosterone was measured directly through the Coat-A-Count RIA (CA) kit. The inter- and intra-assay coefficients of variation were 12% and 10%, respectively and they were considered with a sensitivity of 4 ng/dl (0.139 nmol/l).

### *In vitro* maturation of oocytes

After confirmation of PCOS induction, the PCOS ovaries were collected and placed in α-minimum essential

medium (α-MEM, Gibco, UK) supplemented with 5% fetal bovine serum (FBS, Gibco, UK). The ovaries were mechanically dissected and oocytes at the germinal vesicle (GV) stage and cumulus-oocyte complexes (COCs) were collected. In this way, only COCs with more than three layers of un-expanded cumulus cells and oocytes greater than 70 µm in diameter with a homogenous cytoplasm were selected. The selected COCs were denuded mechanically by pipetting and cumulus-denuded oocytes (DOs) were also prepared.

After three times washing, each PCOS DOs and COCs was cultured (one PCOS DOs/drop or one PCOS COCs/drop) in the α-MEM supplemented with 5% FBS, 0.23 mM sodium pyruvate, 75 mU/ml of follicle-stimulating hormone, 7.5 IU/ml human chorionic gonadotropin, 50 µg/ml penicillin and 50 µg/ml streptomycin (experimental group). Non-PCOS DOs and COCs was also cultured individually (one Non-PCOS oocyte or COCs/drop as control group). Therefore, groups were culture as following: i. PCOS DOs (n=83), ii. PCOS COCs (n=35), iii. Non-PCOS DOs (n=61) and iv. Non-PCOS COCs (n=62). Maturation rate of each DOs and COCs was examined 24 hours after culture in maturation medium at 37°C under 5% $CO_2$ in humidified air.

### *In vitro* fertilization and embryo culture

Sperms were collected from the caudal epididymis of male mice (n=8, 10-12 weeks old) and capacitated for 1 hour in Hams'F10 medium, at 37°C and 5% $CO_2$. PCOS and non-PCOS COCs as well as the matured DOs, *in vitro*, were inseminated with capacitated sperm in α-MEM supplemented with 10% of FBS for 4-5 hours, at 37°C and 5% $CO_2$. Subsequently, two pronucleus (2PN) zygotes were cultured in α-MEM medium with 10% FBS and incubated at 37°C and 5% $CO_2$. Only embryos with normal morphology from 2-cell, 8-cell to blastocysts were collected and studied.

### Measurement of glucose intake

High performance liquid chromatography (HPLC, Waters, USA) method was used to study glucose changes in the culture medium. One of the advantages of the HPLC method is that it detects the smallest changes in the amount of culture medium's glucose. At the end of each culture period (24 hours after DOs and COCs culture), 50 µl of the culture medium was taken from each dish to measure glucose level to measure glucose level. Therefore, the samples were included as the following: i. Pre-IVM culture medium, ii. Culture medium of matured PCOS DOs, iii. Culture medium of matured PCOS COCs, iv. Culture medium of matured non-PCOS Dos, and v. Culture medium of matured non-PCOS COCs. HPLC system in this study used UV detection made at 195 nm with column temperature of 50°C. The utilized column was $NH_2$ column (250 mm×4.6 mm). Ratio of the used acetonitrile and deionized water was 80 to 20%. A guard column was attached to the inlet of the column of prevent clogging (15).

### RNA isolation and quantitative reverse transcription polymerase chain reaction

Twenty-four hours after culture period, the matured DOs and COCs (experimental and control groups) were collected for RNA extraction. While the spent medium was also used to measure glucose intake using HPLC method at the same time. Extraction of total RNA was performed using RNeasy Mini Kit (Roche Molecular Bio Chemicals, Germany) and stored at -80˚C. Complementary DNA (cDNA) was synthesized by the cDNA kit (Thermo Scientific, EU) as directed by the manufacturer's instructions at 42˚C for 60 minutes, and stored at -20˚C.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to quantify mRNA transcript levels of *BAX*, *BCL2* and GDF9 genes. Primer pairs for amplifying these genes were designed using GenBank at NCBI. The primer sequences are shown in Table 1. In this study, housekeeping gene was Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Real time thermal cycler (Applied bio systems, USA) was used for analyzing gene expression. QuantiTect SYBR Green RT-PCR kit (Applied Bio systems, USA) was also employed for amplifying the targeted genes. Amplification of reference and target genes was performed in the same run, for each sample. The protocol of qRT-PCR was programmed as: the holding step at 95˚C for 5 minutes, cycling step at 95˚C for 15 seconds, 58˚C for 30 seconds and 72˚C for 15 seconds, which was followed by a melt curve step at 95˚C for 15 seconds, 60˚C for 1 minute, and 95˚C for 15 seconds. Determining relative quantitation for target genes was performed using ΔΔCt method. All experiments of qRT-PCR were done five times.

**Table 1:** Primer sequences used for quantitative reverse transcription polymerase chain reaction

| Genes | Primer pair sequence (5′-3′) | Annealing temperature (°C) | Size (bp) |
|---|---|---|---|
| *GDF-9* | F: CACCGTACTCATTCACCCT | 57.5 | 19 |
| | R: CACCTGGTCTTTTGTGCAT | 57.0 | 19 |
| *BAX* | F: CACTGGACTTCCTCCGTGA | 57.0 | 19 |
| | R: CTCCAGCCACAAAGATGGTCA | 57.2 | 21 |
| *BCL2* | F: GCGGATATACCTTCTTCCCT | 56.4 | 22 |
| | R: ATTCTGGTGTTTCCCCGTTG | 57.2 | 20 |
| *GAPDH* (endogenous) | F: CAAGGTCATCCATGACAACTTTG | 61.3 | 23 |
| | R: GTCCACCACCCTGTTGCTGTAG | 59.6 | 22 |

### Statistical analysis

All experiments were repeated five times and data were expressed as mean ± standard deviation (SD). The $\chi^2$, One-Way ANOVA and Tukey's post-hoc tests have been used to analyze differences among the groups and gene expression. Statistical analysis was performed using SPSS version 20 (IBM, USA). P<0.05 was considered statistically significant.

## Results

### PCOS ovaries evaluation

The irregular estrous cycles were confirmed in the PCOS mice and restricted to estrous stages upon estradiol valerate treatment. Histological examinations showed that number of pre-antral follicles was increased in the PCOS mice. In addition, the atretic and cystic follicles were observed in these mice (n=6 ovaries) and their ovaries contained fewer corpora luteal. Evaluation of steroid hormones showed that serum testosterone and luteinizing hormone levels were increased in the estradiol valerate-treated mice (P=0.04) at 60 days. Serum FSH level was not changed after treatment with estradiol valerate.

### *In vitro* maturation of PCOS oocytes and glucose intake

Overall rate of *in vitro* DOs and COCs matured in the different groups are shown in Table 2. The results indicated that 67.2% of non-PCOS DOs and 53.01% of PCOS DOs had the first polar body. In addition, 70.9% of non-PCOS COCs and 45.71% of PCOS COCs developed to MII stage. Maturation rate was significantly (P=0.001) higher in the group of non-PCOS COCs compared to PCOS COCs. In addition, significant difference was observed in the maturation rate of non-PCOS DOs compared to PCOS DOs (P=0.04). Simultaneously, proportion of GV and GVBD oocytes was higher in the group with PCOS COCs (Table 2).

The measured glucose levels in the MEM-α culture medium after IVM are shown in Table 2. Level of glucose in the MEM-α culture medium was 957.75 ppm. It should be mentioned that this level of glucose was detected in the culture medium before IVM (pre-IVM medium). Glucose measurement of medium culture after IVM indicated that level of glucose intake was significantly lower in the non-PCOS DOs and PCOS DOs compared to the PCOS COCs (P=0.001) and non-PCOS COCs (P=0.03). An increase was also observed in the rate of glucose intake in the PCOS COCs compared to non-PCOS COCs (P=0.001). There was no significant difference in the rate of glucose intake between non-PCOS DOs and POCs DOs groups (P=0.29). But non-PCOS DOs consumed more glucose than PCOS DOs. Maximum rate of the glucose intake for each oocyte was observed in PCOS COCs (38.17 ppm, Fig.1).

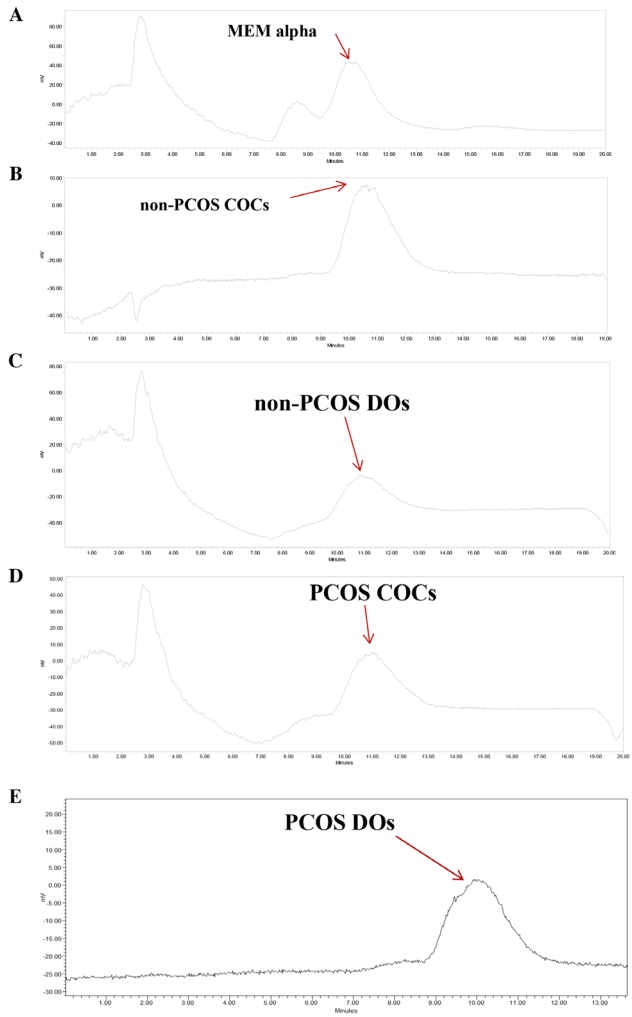


**Fig.1:** Distribution of relative amounts of glucose in the used culture medium from each group. **A.** MEM-α, **B.** Non-PCOS COCs, **C.** Non-PCOS DOs, **D.** PCOS COCs, and **E.** PCOS DOs. Maximum point of each curve (arrows) indicates the amount of glucose in each utilized culture medium. Obviously, amount of glucose in the used culture medium is inversely related to the amount of glucose consumed by the DOs/COCs. Rate of glucose consumption is higher in PCOS COCs (P=0.001). MEM-α; Modification of minimum essential medium-α, PCOS; Polycystic ovarian syndrome, COCs; Cumulus-oocyte complexes, and DOs; Cumulus-denuded oocytes.

**Table 2:** Association of glucose consumption with *in vitro* DOs/COCs maturation rate

| Groups | Number | GV (%) | GVBD (%) | M II (%) | Deg. oocytes (%) | Average of glucose intake/each oocyte (ppm) | No. of replicates |
|---|---|---|---|---|---|---|---|
| Normal DOs | 61 | 7 (11.47) | 8 (13.11) | 41 (67.2)[a] | 5 (8.19) | 25.05 ± 0.05 | 18 |
| Normal COCs | 62 | 6 (9.6) | 7 (11.29) | 44 (70.9)[**] | 5 (8.06) | 29.8 ± 0.05 | 16 |
| PCOS DOs | 83 | 10 (12.04) | 20 (24.09) | 44 (53.01) | 9 (10.84)[a] | 23.18 ± 0.06 | 25 |
| PCOS COCs | 35 | 5 (14.2) | 3 (8.57) | 16 (45.71) | 11 (31.42)[*] | 38.17 ± 0.07[**] | 16 |

**Data are presented as mean ± SD or n (%). DOs; Cumulus-denuded oocytes, COCs; Cumulus-oocyte complexes, PCOS; Polycystic ovarian syndrome, GV; Germinal vesicle, MII; Metaphase II, GVBD; Germinal vesicle breakdown, Deg. Oocytes; Degenerated oocytes, No; Number. There is significant difference in maturation rate of non-PCOS COCs compared to PCOS COCs. Higher degenerated oocytes were observed in PCOS COCs. *; P<0.05, **; P<0.001 vs. COCs, [a]; P<0.05 versus DOs by one-way ANOVA.**

### *In vitro* maturation- *in vitro* fertilization outcomes

To evaluate effects of glucose intake by PCOS and non-PCOS DOs/COCs on the development competence, their fertilization and development rate were analyzed among the groups (groups I-IV). In comparison with PCOS COCs, non-PCOS COCs significantly showed higher rate of successive IVF and development to 2-cells, 8-cells and blastocyst stages (P=0.01). As shown in Table 3, non-PCOS DOs had also significantly higher development rate compared to PCOS DOs (P=0.03).

### *BAX* and *BCL2* mRNA content and glucose intake status in PCOS oocytes

Expression level of two apoptosis marker genes (*BAX* and *BCL2*) are observed in Figure 2A-D. Our results showed equal expression levels of *BAX* gene in non-PCOS and PCOS groups. According to the data obtained from qRT-PCR assay and statistical analysis, it can be concluded that there is no significant difference in the rate of *BAX* expression gene between non-PCOS and PCOS groups (P=0.21). Therefore, statistical analysis revealed no significant difference at the expression level of *BAX* gene and glucose intake between non-PCOS and PCOS groups (Fig.2A, C).

Level of *BCL2* gene expression in the different groups of non-PCOS and PCOS DOs/COCs was also measured. A significant negative correlation (CR=-0.8) of glucose intake (38.17 ppm) and *BCL2* gene expression (0.605, P=0.0005) in PCOS COCs was detected (Fig.2D). Minimal expression level of this gene was observed for *BCL2* in PCOS COCs compared to non-PCOS COCs (P=0.0005). There is not significant difference in the expression level of *BCL2* gene between PCOS and non-PCOS DOs (P=0.058, Fig.2C). It can be calculated that this minimal expression level of *BCL2* gene can be correlated to more presence of atretic oocytes in the PCOS COCs than the other groups.

### *GDF-9* mRNA content and glucose intake status in PCOS oocytes

In this study, expression of gene related to oocyte quality (*GDF-9*) was investigated by qRT-PCR in non-PCOS and PCOS groups. The results showed a decline in *GDF-9* gene expression level of PCOS COCs and DOs compared to non-PCOS COCs and DOs, respectively. According to the data obtained and statistical analysis, it can be concluded that there was a negative correlation in the quality of DOs and COCs and glucose intake among the non-PCOS and PCOS groups. In this way, the non-PCOS DOs and COCs of control group had higher level of *GDF-9* expression than PCOS groups. Mean expression levels of *GDF-9* gene in non-PCOS DOs and COCs groups were 1.00 ± 0.08 and 1.24 ± 0.02, respectively. Whereas, the mean values of *GDF-9* gene expression in the PCOS DOs and COCs groups were 0.66 ± 0.02 and 0.37 ± 0.02, respectively. Statistical analysis revealed a significant difference at PCOS DOs (P=0.0002) and COCs (P=0.0001) groups compared to non-PCOS DOs and COCs, respectively (Fig.3).

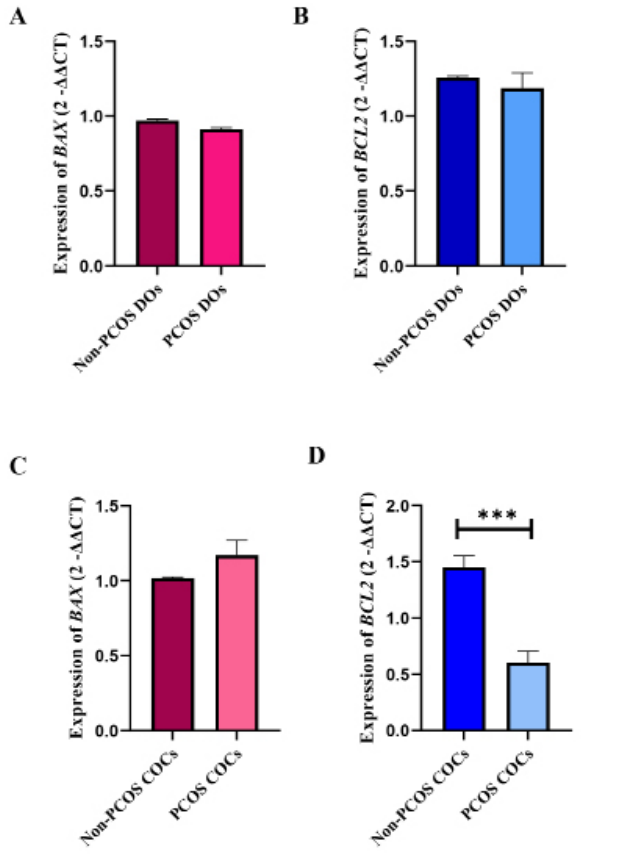


**Fig.2:** Relative expression of *BAX* and *BCL2* genes in the PCOS and non-PCOS groups. **A, B.** Non-PCOS and PCOS DOs and **C, D.** Non-PCOS and PCOS COCs. Statistical analysis shows significant difference of *BCL2* gene expression in PCOS COCs compared to non-PCOS COCs. PCOS; Polycystic ovarian syndrome, COCs; Cumulus-oocyte complexes, Dos; cumulus-denuded oocytes, and ***; P=0.0005.

**Table 3:** *In vitro* fertilization and embryo development outcomes

| IVF outcomes | Non-PCOS DOs | Non-PCOS COCs | PCOS DOs | PCOS COCs |
|---|---|---|---|---|
| No. of MII oocytes | 41 | 32 | 49 | 24 |
| Fertilization rate (%) | 58.23 ± 1.33[a] | 67.48 ±3.2** | 51.11 ± 2.01 | 43.62 ± 2.4 |
| 2-cell rate (%) | 43.13 ± 2.31[b] | 54.349 ± 2.2** | 30.52 ± 1.41 | 29.13 ± 2.26 |
| 8-cell rate (%) | 34.2 ± 2.24[a] | 41.25 ± 1.37* | 22.94 ± 2.12 | 21.95 ± 3.05 |
| Blastocyst rate (%) | 30.91 ± 2.12[a] | 38.29 ± 2.07** | 20.97 ± 2.54 | 18.87 ± 2.18 |

**Data are expressed as mean ± SD and percentage. IVF; *In vitro* fertilization, PCOS; Polycystic ovarian syndrome, COCs; Cumulus-oocyte complexes, Dos; cumulus-denuded oocytes, MII; Metaphase II, No; Number, *; P<0.05, **; P<0.01 vs. PCOS COCs, [a]; P<0.05, and [b]; P<0.01 vs. PCOS DOs by one-way ANOVA. There is a significant difference in fertilization and embryo development rate of oocytes in the non-PCOS COCs compared to the PCOS COCs as well as the non-PCOS DOs compared to the PCOS DOs.**

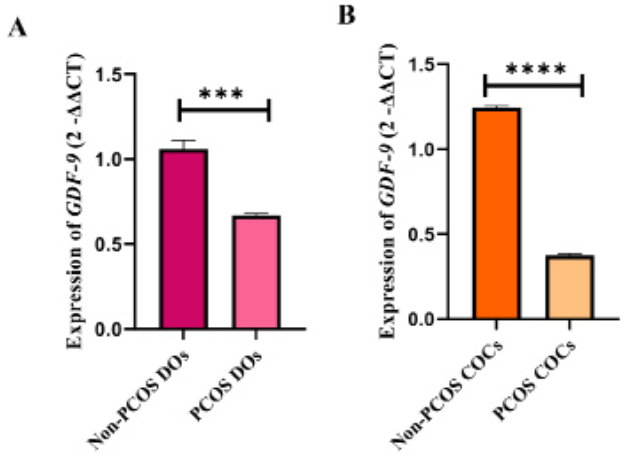


**Fig.3:** Relative expression level of *GDF-9* gene in the non-PCOS and PCOS groups. The level of *GDF-9* gene expression was significantly decreased in the **A.** PCOS DOs and **B.** COCs compared to non-PCOS DOs and COCs, respectively. PCOS; Polycystic ovarian syndrome, COCs; Cumulus-oocyte complexes, Dos; Cumulus-denuded oocytes, ***; P=0.0002, and ****; P=0.0001.

## Discussion

In the present study, relative abundance of genes potentially involved in oocyte quality, apoptosis markers and maturation competence of PCOS DOs and COCs was analyzed. Then, association of oocyte parameters with glucose intake from IVM culture medium was studied.

During the first part of the present study, a lower maturation competence and higher degeneration rate were observed in the PCOS COCs. In addition, the PCOS COCs showed decreased expression of anti-apoptotic marker (*BCL2* gene). In the second part, analyzing glucose consumption revealed that each PCOS COCs had a significant increase in glucose intake. Therefore, the results suggest that glucose consumption rate of each oocyte could introduce a non-invasive method to predict oocyte maturation competence and its quality. Attempts to identify potential biomarkers, to determine oocyte quality using metabolic pathways, have been reported in the literature. Although, they differ from this study in various aspects, such as evaluation of PCOS oocytes and using a non-invasive method to evaluate each oocyte through amount of the consumed glucose.

Given that the crosstalk between oocytes and granulosa cells is necessary for their survival and showing quality of oocyte growth, therefore, analysis of the used culture media for DOs and COCs IVM provides valuable information about the usage of different metabolites by oocytes (16, 17). Metabolism of oocytes and embryos has mainly been studied in antral follicles and mature oocytes. So that, metabolism of COCs plays an important role in oocyte quality (17). However, little information is known about metabolism of immature oocyte, especially oocyte with PCOS. Of note, to the best of our knowledge, this is the first study in which the rate of glucose intake by PCOS oocyte was studied during IVM.

In a study, McLennan et al. (18) reported that glucose intake played an important role in determination of the most suitable oocyte during IVM. It was also indicated that changes in the glucose concentration from culture medium affected cytoplasmic maturation of oocytes. Furthermore, it was well revealed that glucose was necessary for oocyte maturation and COCs expansion (19). In the other studies, it was reported that maturation process of bovine and porcine oocytes needed metabolite, such as glucose and fatty acids (20). It was documented that maturation of mouse COCs to the MII stage did not happen due to the lack of energy supply (6). This is in agreement with our study, whereby glucose intake was detected by both COCs and DOs. In addition, difference of glucose intake between normal and PCOS COCs or DOs was observed. Role of glucose consumption on the aging prevention of DOs and COCs has also been reported during IVM (21). Therefore, in the present study, it was shown that glucose consumption among PCOS oocytes was significantly increased, in comparison with the normal types. It seems that presence of cumulus cells plays an important role in glucose intake. In addition, glucose intake in the PCOS COCs was increased due to the increase in the thickness of theca layers, oocyte volume, glycolysis process and glucosamine synthesis by cumulus cells to proliferate. On the other hand, there was not significant difference in the glucose intake among PCOS DOs and non-PCOS COCs or DOs. Therefore, it was inferred that abnormal glucose uptake and maturation of PCOS oocytes was occured by cumulus cells. So that, it has been reported that bi-directional communication between the cumulus cells and oocyte facilitated glucose transport into the oocyte. Glucose transport has been demonstrated as a gradient through cumulus cell-corona radiate-oocyte by gap junction during bovine COC culture. It has been found that the cumulus cells metabolized the glucose and provided the metabolites for oocyte (22).

Due to the static IVM conditions, presence of a supra-physiological concentration of glucose in the culture medium led to improved nuclear maturation and developmental competence of oocytes (22). However, to the best of our knowledge, there is currently limited data on the influences of PCOS on the oocyte developmental competence and its association with glucose consumption. The results of present study showed different behavior of cumulus cells in the mouse PCOS COCs during IVM. The glucose consumption by PCOS cumulus cells was increased in comparison with intact COCs. However, the glucose intake by PCOS DOs was lower than non-PCOS DOs. Therefore, another possible explanation for the differences of glucose intake was related to oocyte quality (low expression of *GDF-9*). The lower capacity of PCOS DOs for intake and using glucose led to the accumulation of absorbed glucose in the follicular fluid and cumulus cells of PCOS COCs. Further evidences of metabolic cooperation between oocyte and cumulus cell should be provided to established the oocyte improve glucose metabolism in cumulus cells via influencing their transcriptome (17). So that, oocyte-secreted growth factors regulated metabolism of cumulus cells. Therefore,

simultaneous evaluation of the other metabolites and oocyte-secreted factors can also help understand the exact metabolism of oocytes and/or follicles which this is one of the most important limitations in this study.

## Conclusion

It is obvious that PCOS oocytes have poor maturation capacity than normal oocytes, due to poor folliculogenesis and the incidence of follicular apoptosis. So that, developmental oocyte competence may influence culture condition and metabolites consumption, especially glucose consumption. Therefore, glucose consumed by the COCs and DOs can be utilized for evaluation of oocyte quality and developmental capacity during IVM and pre-implantation processes and effects on the success of fertilization and subsequent embryo development.

## Acknowledgements

We thank Ms. Mirzanezhad for her skillful technical assistance (Genetic laboratory, University of Guilan, Rasht, Iran). This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors have no conflict of interest to disclose.

## Authors' Contributions

F.K., F.Gh.; Contributed to conception and design. F.K., Z.Z.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. F.Gh., Z.Z.; Were responsible for overall supervision. F.Gh.; Drafted the manuscript, which was revised by Z.Z. All authors read and approved the final manuscript.

## References

1. Shamsi M, Nejati V, Najafi G, Pour SK. Protective effects of licorice extract on ovarian morphology, oocyte maturation, and embryo development in PCOS-induced mice: an experimental study. Int J Reprod Biomed. 2020; 18(10): 865-876.
2. Hatırnaz Ş, Ata B, Hatırnaz ES, Dahan MH, Tannus S, Tan J, et al. Oocyte in vitro maturation: a sytematic review. Turk J Obstet Gynecol. 2018; 15(2): 112-125.
3. Julania S, Walls ML, Hart R. The place of in vitro maturation in PCO/PCOS. Int J Endocrinal. 2018; 5750298.
4. Ryan GE, Malik S, Mellon PL. Antiandrogen treatment ameliorates reproductive and metabolic phenotypes in the letrozole-induced mouse model of PCOS. Endocrinology. 2018; 159(4): 1734-1747.
5. Moore AM, Prescott M, Campbell RE. Estradiol negative and positive feedback in a prenatal androgen-induced mouse model of polycystic ovarian syndrome. Endocrinology. 2013; 154(2): 796-806.
6. Xie HL, Wang YB, Jiao GZ, Kong DL, Li Q, Li H, et al. Effects of glucose metabolism during in vitro maturation on cytoplasmic maturation of mouse oocytes. Sci Rep. 2016; 6: 20764.
7. Kauffman AS, Thackray VG, Ryan GE, Tolson KP, Glidewell-Kenney CA, Semaan SJ, et al. A novel letrozole model recapitulates both the reproductive and metabolic phenotypes of polycystic ovary syndrome in female mice. Biol Reprod. 2015; 93(3): 69.
8. Zanetti BF, Braga DPAF, Setti AS, Iaconelli A, Borges E. Effect of GnRH analogues for pituitary suppression on oocyte morphology in repeated ovarian stimulation cycles. JBRA Assist Reprod. 2020; 24(1): 24-29.
9. Zhang Y, Fang Z, Lu H, Li Y, Baloch Z, Liu Y, et al. A blastocyst biopsy approach for preimplantation genetic diagnosis technique that affects the expression of SNAP-α in mice. Reprod Biol. 2020; 20(3): 417-423.
10. Yan J, Zhou B, Yang J, Tai P, Chen X, Zhang H, et al. Glucose can reverse the effects of acute fasting on mouse ovulation and oocyte maturation. Reprod Fertil Dev. 2008; 20(6): 703-712.
11. Downs SM, Humpherson PG, Martin KL, Leese HJ. Glucose utilization during gonadotropin-induced meiotic maturation in cumulus cell-enclosed mouse oocytes. Mol Reprod Dev. 1996; 44(1): 121-131.
12. Liu W, Liu J, Du H, Ling J, Sun X, Chen D. Non-invasive pre-implantation aneuploidy screening and diagnosis of beta thalassemia IVSII654 mutation using spent embryo culture medium. Ann Med. 2017; 49(4): 319-328.
13. Frank LA, Sutton-McDowall ML, Brown HM, Russell DL, Gilchrist RB, Thompson JG. Hyperglycaemic conditions perturb mouse oocyte in vitro developmental competence via beta-O-linked glycosylation of heat shock protein 90. Hum Reprod. 2014; 29(6): 1292-1303.
14. Amini L, Tehranian N, Movahedin M, Ramezani Tehrani F, Soltanghoraee H. Polycystic ovary morphology (PCOM) in estradiol valerate treated mouse model. Int J Women's Health Reprod Sci. 2016; 4(1): 13-17.
15. Stoll T, Pugeaud P, von Stockar U, Marison IW. A simple HPLC technique for accurate monitoring of mammalian cell metabolism. Cytotechnology. 1994; 14(2): 123-128.
16. Liang B, Gao Y, Xu J, Song Y, Xuan L, Shi T, et al. Raman profiling of embryo culture medium to identify aneuploid and euploid embryos. Fertil Steril. 2019; 111(4): 753-762.
17. Richani D, Dunning KR, Thompson JG, Gilchrist RB. Metabolic co-dependence of the oocyte and cumulus cells: essential role in determining oocyte developmental competence. Hum Reprod Update. 2021; 27(1): 27-47.
18. McLennan HJ, Sutton-McDowall ML, Heng S, Abell AD, Thompson JG. Time-lapse confocal imaging-induced calcium ion discharge from the cumulus-oocyte complex at the time of cattle oocyte activation. Reprod Fertil Dev. 2020; 32(14): 1223-1238.
19. Walter J, Huwiler F, Fortes C, Grossmann J, Roschitzki B, Hu J, et al. Analysis of the equine “cumulome” reveals major metabolic aberrations after maturation in vitro. Genom. 2019; 20: 588.
20. Warzych E, Lipinska P. Energy metabolism of follicular environment during oocyte growth and maturation. J Reprod Dev. 2020; 66(1): 1-7.
21. Jiang X, Pang Y, Zhao S, Hao H, Zhao X, Du W, et al. Thioredoxin-interacting protein regulates glucose metabolism and improves the intracellular redox state in bovine oocytes during in vitro maturation. Am J Physiol Endocrinol Metab. 2020; 318(3): 405-416.
22. Gutnisky C, Breininger E, Dalvit GC, Cetica PD. Regulation of key enzymes of glucose metabolism in bovine COCs. Anim Reprod. 2017; 14: 406-412.

Original Article

# The Impact of Chrysin on The Folliculogenesis and Ovarian Apoptosis in Ischemia-Reperfusion Injury in The Rat Model

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## Abstract

**Background:** The ovarian Ischemia/reperfusion is one of the gynecological emergency concerns that may lead to the ovary damage and folliculogenesis. The present research aimed to evaluate the impact of the Chrysin (CH) on the ischemia-reperfusion (I/R) injury in the rat model.

**Materials and Methods:** In this experimental research, 48 adult female rats, 8 weeks age and 180-200 g weight, have been categorized into 6 equal groups (n=8) including one sham and 5 ovarian torsion groups (OT+CH groups) that received different treatments. Each group has been treated 30 min before detorsion with gavage of CH or normal saline for 1 week and pregnant mare serum gonadotropin (PMSG) has been injected on the day 5 for initiating folliculogenesis. Finally, bio-chemical, molecular, histopathological, apoptotic and hormonal evaluations were performed.

**Results:** The anti-oxidant enzyme, superoxide dismutase and glutathione peroxidase, ameliorated in the ovarian tissues of the OT+CH groups in comparison with the OT group ($P<0.001$). Moreover, the level of serum Luteinizing hormone considerably declined and estradiol level ($P<0.001$), partly enhanced in the rats treated with CH in comparison with the ones in the OT group ($P<0.05$). In addition, histopathological scores of the OT+CH groups ameliorated in comparison with the OT group scores ($P<0.05$). Furthermore, the expression *Caspase-3* and *Bax* genes were significantly increased while the expression of *Bcl-2* was notably decreased in the OT group in comparison with the sham group ($P<0.05$).

**Conclusion:** Here, it seems that CH is possibly beneficial for the protection of ovaries against reperfusion injury and ischemia.

***Keywords:*** Bax/Bcl-2, Chrysin, Ischemia, Ovary, Reperfusion Injury

**Citation:** Mohammadi Z, Hosseinianvari SM, Ghazalian N, Fani M, Mahmudian AS, Brazvan B, Shokoohi M, Abtahi-Eivary SH, Moghimian M. The impact of chrysin on the folliculogenesis and ovarian apoptosis in ischemia-reperfusion injury in the rat model. Int J Fertil Steril. 2022; 16(4): 299-305, doi: 10.22074/IJFS.2021.540364.1200.


## Introduction

Adnexal torsion refers to higher resistance or termination of blood circulation due to the ovaries' rotation around a suspensory ligament (1, 2). With regard to lower venous pressure, venous return ends as a result of torsion whereas the arterial blood flow continues regularly and edema expands in the ovarian tissues. Because of edema, supplying blood to ovaries discontinues that result in high ovarian pressure and ischemic damages (3). Then, prolonging this period leads to the necrosis as well as irreversible damages in the ovarian tissues. Notably, ovarian torsion has been considered the commonest gynecological emergency that accounts for 2.7% prevalence (4).

Nonetheless, detorsion of the twisted ovaries results in the other risk called ischemia/reperfusion (I/R) injury that has an association with the tissues neutrophil infiltration and reperfusion. The production of reactive oxygen species (ROS) enhances in the ovarian tissues due to the reperfusion procedure (5), leading to cellular damage via peroxidation of the poly-unsaturated fatty acids (6, 7).

Multiple anti-oxidants prevent oxidative injuries and inflammations in the ovarian tissue (8). In this regard, Karaçor et al. (9) found that providing Iloprost has a higher impact on the reduction of the ischemia-reperfusion (I/R) injuries in the ovarian tissues. Some studies have shown the helpful impact of administering

**Received: 05/October/2021, Accepted: 07/December/2021**
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**Royan Institute**
**International Journal of Fertility and Sterility**
**Vol 16, No 4, October-December 2022, Pages: 299-305**

Montelukast or Telmisartan that prevent the ovarian I/R injury (10, 11).

To sum up, any acceptable treatment has been not presented for curing the ovarian failure induced by I/R injury; therefore, we tried the usage of Chrysin (CH) for curing the ovarian failure in the rats.

It is well known that CH has been proposed as one of the natural flavonoids in the propolis, honey as well as several other plant extracts. Many investigations confirmed anti-inflammatory, anti-diabetogenic and anti-oxidant effects of CH (12-14). Researchers confirmed the advantages of the anti-oxidants for I/R injury in the brain, heart and ovary. As far as we know, there are no reports about the CH impact of the I/R injury in the course of folliculogenesis. Therefore, we aimed at the evaluation of the impacts of CH on experimental I/R ovaries injuries in the rat model.

## Materials and Methods

### Ethical considerations

The Ethical Committee of the Gonabad University of Medical Sciences (Khorasan Razavi, Iran) verified this experimental research (IR.GMU.REC.1398.134).

### Experimental design

Totally, 48 adult female Wistar rats with a weight of 230 ± 10 g and 8 weeks age have been stored at a constant temperature of 25 ± 2°C at 30 to 70% humidity with 12-h light/12-h dark cycle in the animal room with free access to food and water.

Then, they were classified into 6 equal groups (n=8):

1. Sham group: A longitudinal cutting (2.5 cm) was considered in the mid-line of lower abdomen and 5/0 nylon (SUPA medical divice, Iran) sutures were used to close the incision.
2. Torsion/detorsion group (OT): The left ovaries were chosen to induce torsion for 3 hours. After that, 30 min prior to the detorsion operation, the normal saline (Samen pharmacutical Co, Iran) was administered intra-peritoneally.
3. Torsion/detorsion/Chrysin group (OT+CH30): As well as OT group, we induced the ovarian torsion, but the 30 mg/kg CH 30 minutes prior to the detorsion operation was administered.
4. Torsion/detorsion/Chrysin group (OT+CH50): Like OT group, we induced the ovarian torsion, but the 50 mg/kg CH 30 minutes prior to the detorsion operation was administered.
5. CH groups (CH30): This group did not receive any operation; however, each rat received 30 mg/kg CH (Cat No: 480-40-0, Sigma Aldrich, Germany).
6. CH groups (CH50): This group did not receive any operation; however, each rat received 50 mg/kg CH.

Then, each group has been treated. The treatment was started 30 minutes before detorsion and continued for once daily for 1 week, with gavage CH or normal saline and PMSG has been injected on day 5 for initiating folliculogenesis. At the end, the animals have been sacrificed and the ovary and blood samples have been gathered.

### Surgical procedure

Following the acclimatization period, we used Xylazine (10 mg/kg) and Ketamine (50 mg/kg) (Sigma Aldrich, Germany) to anesthetize the rats. After that, we made a longitudinal cutting (2.5 cm) in the central area of the lower abdomen and a small peritoneal cutting has been made, which revealed the left uterine horns and adnexa. Then, the left ovary has been rotated 720° in a clockwise direction around its axis and fixed to the abdominal wall with 6/0 nylon for avoiding its detorsion. Next, 5/0 nylon sutures have been used to close the incision, which kept torsion for 3 hours. Moreover, 30 minutes prior to the opening of the ovarian torsion, CH extract has been injected intraperitoneally. With the completion of ischemic period that lasted 3 hours, the ovaries' twisting opened and a week has been allowed to the ovaries' reperfusion. By ending this operation, we injected Buprenorphine (0.02 mg/kg, Exir, Iran) as the analgesics and at the end of a week, the animals of each group have been were anesthetized by Xylazine and Ketamine. Afterwards, the blood specimens have been drawn out from the heart in order to assess the metabolic changes. In addition, the blood samples have been centrifuged at 4000 rpm for 5 minutes and serum has been separated. Next, all the serum samples have been added into 3 micro-tubes (500 µL, Shimi Tajhiz, Iran) and transported to freezer at a temperature of -70°C till the experiment time. Finally, we removed the ovarian tissues for evaluating the genes expression and histological changes (15).

### Tissue fixation, samples preparation, and histopathological evaluation

Following the ovariectomy process, we fixed the ovaries and put the samples in 10% formalin (Cat No: 1.1150, Notron, Iran) for 72 hours. After dehydration, we put them in paraffin (Cat No; CellWax, UK) and procured histological slides with 5 µm cutting by microtome. Then, hematoxylin-eosin (H&E) has been used to stain the samples (16). Moreover, for histo-metrical and histological investigations, the tissue sections of all ovaries have been examined from the cortex to medulla in a spiral clockwise direction. In the next step, we counted the number of pre-antral, antral and graafian follicles, as well as corpora lutea in each slide, and compared them with various groups for the respective analyses (10). The sections were observed by a microscope (BX63 , Olympus, Tokyo, Japan) and magnification x400.

### Apoptotic cell detection

TUNEL staining, the ovarian tissues were monitored to assay apoptotic cells. According to this method, the samples have been fixed in formalin 10% for 1 week and dehydrated by ascending degrees of alcohol (Razi, Iran). Then, Xylene has been used to clear the samples

and finally we blocked them in paraffin. In the next step, paraffin blocks have been incised into sections of 5 µm thickness and the slides have been transferred to Poly-L-Lysine slides (Sigma Aldrich, Germany). Afterwards, we deparaffinized the tissue sections and act with regard to the common histological procedures. It should be mentioned that we put the samples in 3% hydrogen peroxide solution (Chemicaliran, Iran) in ethanol (Razi , Iran) for 15 minutes for blocking the tissue internal peroxidase. The samples were washed, then proteinase K (Boehringer Mannheim, Germany) was used and the samples were incubated at the room temperature for 20 minutes. Then samples were rewashed and incubated with the reaction solution of the TUNEL staining kit (C10619, TermoFisher, Germany). Following, slides were incubated with a diaminobenzidine (DAB) solution (34002, TermoFisher, Germany) at room temperature for 15 minutes and then hematoxylin (TermoFisher, Germany) phosphate buffered saline (PBS) (6) staining was performed for all slides. At the end, those cells with brown nucleus have been assessed as the TUNEL-positive cells (17).

## RNA extraction

The Favor Prep Blood/Cultured Cell Total RNA Mini Kit (FABRK000, Favorgen, Taiwan) has been used to isolate the total RNA. While preparing the RNA extraction, we added 5-10 mg of the rat ovaries into 800 µl of lysis buffer (FABRK000, Favorgen, Taiwan). Upon the homogenization, we transported the solution and added 200 µl of Chloroform (CX1055-6, EMD Millipore, Germany). Then, centrifuge has been performed at 4ºC, 12000 rpm, and 10 minutes. Following the centrifugation, the three-phase process has been formed and we transferred the upper phase to a new 1.5 ml micro-tube and added 200 µl ethanol (70%) (Razi, Iran), mixed completely by vortexing for 30 seconds and transported cautiously to an RNA binding pure link spin column (FABRK000, Favorgen, Taiwan). In the next step, we washed the buffer to remove the impurities and eluted the extracted total RNA in 50 µl of RNase-free water stored at -80ºC. Afterwards, Nanodrop Epoch 2 microplate spectro-photometer (model No. UV-1100, Biotech, USA) has been applied for quantitative evaluation of 260/280 and 260/230 ratio of absorbance values so that a 260/280 ratio of 2.0 and a 260/230 ratio in ranges between 2.0 and 2.2 have been chosen as pure for RNA. Finally, %1.5 agarose gel electrophoresis was utilized for assessing the samples' integrity.

## cDNA synthesis

According to the research design, kit of cDNA synthesis (YT4500, Yekta Tajhiz Azma®, Iran) was used to convert the total RNA (> 500 ng) to cDNA. Then, 500 ng of total RNA was employed for the first-strand cDNA synthesis in a total volume of 20 µl based on the manufacturer's manual. Upon the centrifugation, we incubated the tubes at 70°C for 5 minutes. In addition, for oligo (dT), the tubes were incubated at 42°C for 60 minutes and the reaction was ended by heating at 70°C for 5 minutes. It is notable that for every reaction set, one RNA sample was procured without RevertAidTM M-MuLV reverse transcriptase (YT4500, Yekta Tajhiz Azma®, Iran) (RT reaction) for providing a negative control in the consequent PCRs. Finally, the RT reaction product was maintained at -20°C for less than a week. However, in order to enjoy a longer storage, the samples were transferred to -70°C.

## Real-time polymerase chain reaction

The real- time polymerase chain reaction (PCR) was performed in a total volume of 20 µl consisting of Primer (0.4 µM), BioFact™ 2X Real-Time PCR Smart mix Syber green (BioFact, Korea), cDNA (20 ng/µl) and nuclease-free water (model No. 7498 ABI, USA). These primers were designed by the Perlprimer 1.1.20 software (Table 1). All reactions were done in triplicate. Also, *β-actin* was chosen as an endogenous housekeeping gene. Therefore, 45 thermal cycles were run as follows: 5 minutes at 95ºC, 45 cycles, 95ºC for 15 seconds, and 61ºC for 1 minutes. Then, Delta CT values were computed using the *β-actin* CT values via $2^{-\Delta\Delta CT}$ method where ΔCt refers to the difference(s) between CT-value of the target genes and CT-value of *β-actin* (1).

**Table 1:** Primers for quantitative real-time reverse transcription-polymerase chain reaction

| Gene | Oligomer sequence (5'-3') | Amplicon size (bp) |
|---|---|---|
| *β-actin* | F: GTCGTGCTTGCCATTCAG<br>R: GGTATCTTCTTTCCATTCTTCAGTAG | 309 |
| *Bax* | F: TTTGCTACAGGGTTTCATCCAG<br>R: GTTGTCCAGTTCATCGCC | 145 |
| *Bcl2* | F: TGTGGATGACTGACTACCTGAACC<br>R: CAGCCAGGAGAAATCAAACAGAGG | 122 |
| *Caspase3* | F: GTGGAACTGACGATGATATGGC<br>R: CGCAAAGTGACTGGATGAACC | 135 |

## Evaluation of oxidative stress markers

The serum level of oxidative stress markers was measured according to the procedures described in details in our previous study (5).

## Measurement of luteinizing hormone and estrogen level

The serum level of the Estrogen hormones was determined by the Demeditec Diagnostics kit (E-FS-E117, Germany). And, the serum luteinizing hormone (LH) level was assayed by ELISA kit (CSB-E12654r. Cusabio: China) (18).

## Statistical analysis

SPSS version 20 (IBM, USA) was used to perform statistical analyses. The Kolmogorov-Smirnov test was employed to determine normal distribution of data. It is notable that all data have been written as the mean ± standard (mean ± SE) error. Also, one-way analysis of variance (ANOVA) as well as Tukey post-hoc test was run to comparing Oxidative Stress values and histopathological variables. Here, the statistical significance level was considered $P<0.05$.

## Results

### Histological assay

The number of follicles, antral, Graafian as well as pre-antral follicles were evaluated and its comparison between the OT group and the sham group showed a significant increase (P<0.001). We observed a significant reduction (P<0.001) in the corpus luteum in the OT group in comparison with the sham group. The number of Graafian and pre-antral follicles were decreased significantly in the OT group in comparison with the sham group (P<0.001, P=0.002). Comparing the number of preantral follicles, we observed a significant decline in the OT group and the OT+CH group (P<0.001) and a significant increase in the number of corpus luteum in the OT group (P<0.001, Table 2, Fig.1).

**Table 2:** The count of follicles in study groups

| Group | Preantral follicles | Antral follicles | Graafian follicles | Atretic bodies |
|---|---|---|---|---|
| Sham | 16.1± 0.22 | 4.3 ± 0.26 | 5.5 ± 0.18 | 2 ± 0 |
| OT | 4.6 ± 0.26*** | 2.5 ± 0.18*** | 2.7 ± 0.16*** | 5.6 ± 0.18*** |
| OT+CH30 | 6.5 ± 0.18### | 2.6 ± 0.18 | 3.3 ± 0.18 | 3.1 ± 0.12### |
| OT+CH50 | 7.7 ± 0.16### | 2.6 ± 0.18 | 3.3 ± 0.18 | 3.1 ± 0.12### |
| CH30 | 14.1 ± 0.12 | 4.3 ± 0.18 | 4.1 ± 0.22*** | 2 ± 0 |
| CH50 | 14.7 ± 0.16 | 4 ± 0.18 | 4.3 ± 0.18** | 2 ± 0 |

Data are presented as mean ± SD. OT; Ovarian torsion, CH; Chrycin, *; Significant difference with sham groups, #; Significant difference with OT group, **; P=0.002, ***; P<0.001, and ###; P<0.001.

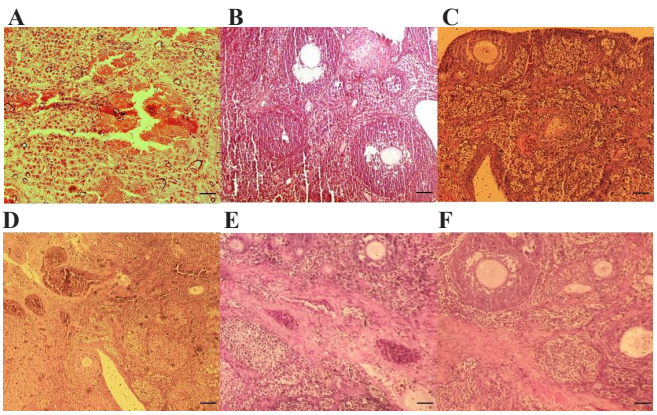


**Fig.1:** Histological findings. **A.** Sham group. **B.** Ovarian torsion/detorsion. **C.** Three hours' ovarian torsion and receiving 30 mg/kg of Chrysin. **D.** Three hours' ovarian torsion and receiving 50 mg/kg of Chrysin. **E.** Healthy rats receiving 30 mg/kg of Chrysin. **F.** Healthy rats receiving 50 mg/kg of Chrysin (scale bar: 20 µm).

### Apoptosis index

The count of TUNEL positive cells in the TD group in comparison with the sham group was higher significantly (Fig.2). Apoptosis index in the pre antral, antral and grafian follicles were enhanced in the OT group in compression with the sham group. Also, the apoptosis index of ovarian tissue cells and follicles of our treated groups with various doses of CH was significantly in comparison with the TD group.

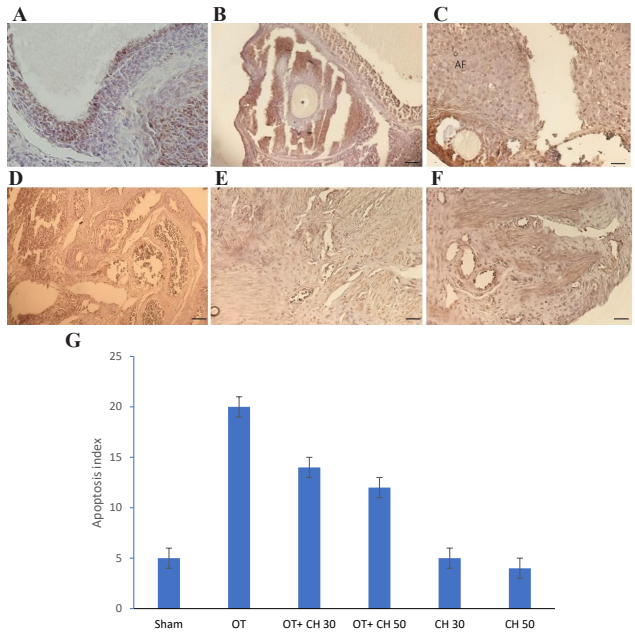


**Fig.2:** Apoptotic cells of ovarian tissue. **A.** Sham group. **B.** Ovarian torsion/detorsion. **C.** Three hours' ovarian torsion and receiving 30 mg/kg of Chrysin. **D.** Three hours' ovarian torsion and receiving 50 mg/kg of Chrysin. **E.** Healthy rats receiving 30 mg/kg of Chrysin. **F.** Healthy rats receiving 50 mg/kg of Chrysin. **G.** Apoptotic index of ovarian tissue in study groups.

### Expression of *Bax*, *Bcl-2*, and *Caspase-3*

Outputs obtained by the *Bax* gene expression indicated a significant reduction in the sham group in comparison with the OT+CH groups, OT+CH30 group and OT+CH50 group (P<0.001, Table 3). Moreover, comparing the OT group with OT+CH groups showed a significant reduction of Bax gene in OT+CH50 group (P<0.001); however, any differences were not seen in the OT+CH30 group.

**Table 3:** The Bax, *Bcl-2*, and *Caspase-3* genes experssion in study groups

| Group | *Bax* | *Bcl-2* | *Caspase-3* |
|---|---|---|---|
| Sham | 0.20 ± 0.02 | 1 ± 0.03 | 0.25 ± 0.03 |
| OT | 1 ± 0.04*** | 014 ± 0.01*** | 1 ± 0.02*** |
| OT+CH30 | 0.42 ± 0.06### | 0.45 ± 0.04 | 0.45 ± 0.06### |
| OT+CH50 | 0.35 ± 0.08### | 0.45 ± 0.07 | 0.40 ± 0.04### |
| CH30 | 018 ± 0.03*** | 0.92 ± 0.05 | 0.22 ± 0.02 |
| CH50 | 016 ± 0.01*** | 0.95 ± 0.04 | 0.18 ± 0.03 |

Data are presented as mean ± SD. The asterisk sign (*) represents a significant difference between the OT and sham groups and (#) indicates a significant difference between OT+CH0.5 and OT group. OT; Ovarian torsion, CH; Chrycin, ***; P<0.001, and ###; P<0.001.

We observed no significant difference expression of Bcl-2 in the control group in comparison with the OT group; while, CH30 and 50 groups showed a significant decline (P<0.001). Also, we considered a significant reduction of Bcl-2 expression in the OT+CH 50 group (P<0.001); although, the OT+CH30 group showed no changes.

The *Casp3* gene expression indicated a significant decline in the sham in comparison with the OT+CH groups, OT+CH 30 group and OT+CH 50 group (P<0.001). Also, we observed a significant reduction of *Casp3* expression in the OT+CH 50 group (P<0.001); although, the OT+CH 30 group showed no changes.

### Biochemical results

### Serum level of malondialdehyde

The malondialdehyde (MDA) level of serum significantly enhanced in the sham group in comparison with the OT group (P<0.001). Moreover, treatment with CH diminished the MDA level in the treated groups in comparison with the OT group (P<0.001, P=0.001, Fig.3A).

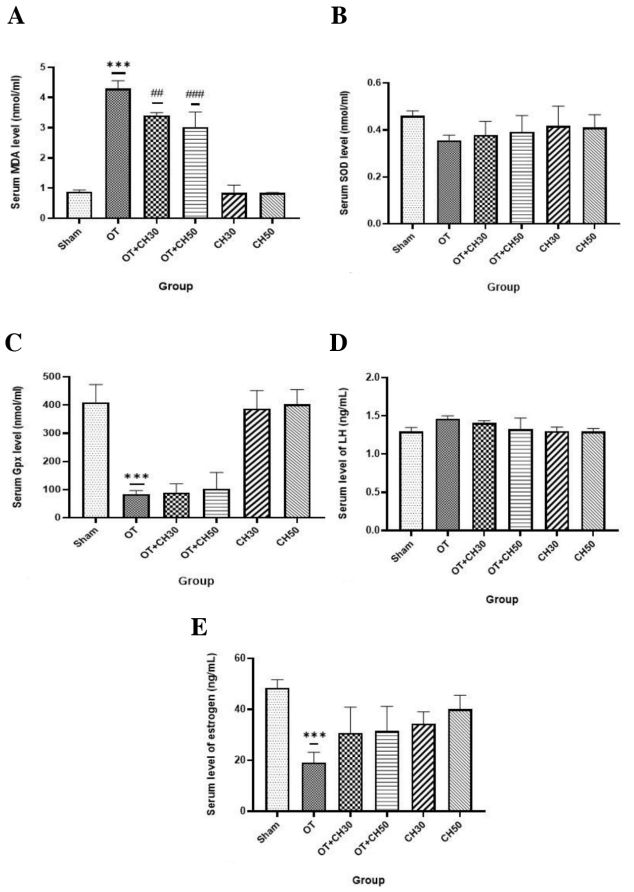


**Fig.3:** Biochemical and hormonal results. Comparison of the serum level of **A.** MDA, **B.** SOD, **C.** GPx, **D.** LH, and **E.** Estrogen in various groups. MDA; Malondialdehyde, SOD; Superoxide dismutase, GPx; Glutathione peroxidase, and LH; Luteinizing hormone.

### Serum level of superoxide dismutase

The level of superoxide dismutase (SOD) serum in the OT group indicated a decline in comparison with the sham group. In addition, treating with CH enhanced this level in treated groups, but not significant (Fig.3B).

### Serum level of glutathione peroxidase

According to our analysis, glutathione peroxidase (GPx) serum level remarkably diminished in the OT group in comparison with the sham group (P<0.001). Moreover, treatment with CH enhanced the GPx level in treated groups, but it was not significant (Fig.3C).

### Serum level of luteinizing hormone

The serum level of LH in the OT group demonstrated an enhancement in comparison with the sham group and treatment with CH declined the level of this hormone in treated groups but it was not significant (Fig.3D).

### Serum estrogen level

The level of serum estrogen declined in the OT groups in comparison with the sham group (P<0.001). Moreover, we observed no significant enhancement between the OT group and treatment groups (OT+CH) (Fig.3E).

## Discussion

In the present study, we have evaluated the CH effect on the ovary in a rat model of ovarian I/R injury. We found that I/R injury considerably diminishes the ovarian reserve while is related to the great oxidative stress. This amount of oxidative stress contributes to the hormonal changes, and enhances histological damages. On the contrary, oxidative stress variables, histopathological changes and ovarian reserve markers partly improved in the animals receiving CH following the I/R injury. An ovarian detorsion without oophorectomy may protect the ovarian functions; however, prophylactic measures would be necessary against I/R injury following the procedures. There is not enough information about the cellular damages following I/R injury. For this reason, knowledge of I/R injury mechanism can present a robust platform for new treatment options as well as prevention of injuries. Halestrap et al. (19) proposed the oxygen free radical generation as one of the essential mechanisms involving in the injuries of the post-ischemic tissues and cells.

The ovarian Ischemia/reperfusion led to the generation of the pro-inflammatory cytokines caused by the inflammatory cells could stimulate migration and adhesion of the circulating neutrophils to the endothelial cells as well as ROS production that raised the neutrophil infiltration and caused the ischemic injuries. Moreover, ROS and the respective poisonous products cause DNA damages and lipid peroxidation in the mitochondrial and cellular membranes, impair ion channels, and cause cells damages and even death. In addition, the cells' damages induced by the lengthy I/R injuries could cause autophagy, apoptosis, necroptosis and necrosis (20). It is notable that moderate I/R injury can result in the dysfunction of the cells through autophagy and actuate recovery systems to be survived. Severe damages can induce the cell death through necrotic or apoptotic pathways. However, in the human physiology, anti-oxidants and ROS preserve equilibrium. Furthermore, levels of antioxidant agents in the ischemic cells would be essential to eliminate the detrimental impact of ROS. The ROS production is enhanced as a result of the decreased concentration of anti-oxidative agents in the ischemic tissues (21). In this regard, numerous investigations emphasized the drug agents of the anti-oxidant and/or anti-inflammatory impacts in the ovarian I/R injuries preventing via animal models (22, 23). According to our data, this research is the first application of the CH in the I/R injury treatment thorough folliculogenesis period in the ovary. The mechanisms of anti-inflammatory and anti-oxidative impacts of CH are not fully known. Some studies showed

that the CH anti-oxidative features suppress the inducible nitric oxide synthase and cyclooxygenase-2 expression. This inhibits the pro-inflammatory nuclear factor kappa B (NF-κB) activities that proposes anti-inflammatory impacts of CH (24, 25).

Our outputs verified anti-oxidative effect as well as anti-inflammatory impacts of the CH via examining their protecting impacts against the I/R injury in the ovary of rats like the reduction of lipid peroxidation, improvement of histo-pathological scores and enhancement of anti-oxidant activities.

It is well known that MDA is an end product of the lipid peroxidation and the enhanced level of MDA reflects OS. On the contrary, greater activity of GPx and SOD indicated the tissues cure following the oxidative damages (26).

Melekoglu et al. (27) determined the effect of 50 mg/kg/day CH on the prevention of ovaries I/R injury. They demonstrated significant GPx and SOD activities increase and considerable reduction of MDA content in the CH treatment group following the I/R injuries.

Also, we found that CH influences beneficially the ovarian restoration following of I/R injuries and enhances the level of E2. Tsai et al. (28) addressed the evaluation of stem cells impact against damages to the rats' ovarian reserve. They reported a considerable increase of the serum LH and decrease of estradiol (E2) in the ovaries of I/R animals; however, treating the stem cells restored the impacts.

We observed that ovaries damages through an ovarian torsion and found that torsion-detorsion declines number of the follicles in each stage such as antral, graafian and preantral whereas numbers of the atretic bodies enhance. Put differently, CH therapy resulted in better histological signs. Also, other investigations confirmed the reduced number of follicles in each of these stages by ovarian torsion-detorsion. Therefore, they used the anti-oxidants to cure the disease ameliorated the condition (15, 29).

Nikoletopoulou et al. (30) introduced apoptosis as a one of the prominent mechanisms of the modulated mortality, which happens due to the cells damages or external stresses as well as in the course of morphogenesis and normal development. Multiple pathways mediate apoptosis. It seems two main, non-excluding, caspase-dependent pathways of apoptosis occur (31). Therefore, in the first pathway, extrinsic or receptor-mediated pathway, an external stimulus or signal is translated into an internal death signal. T-cell inactivation of the immune system is one of the examples where Fas receptor ligation starts a proteolytic cascade that results in cell death. However, in the second pathway, the upstream effector proteins such as Bax activates intrinsic pathway that is caspase machinery. Anyway, it is largely hypothesized that Bax and Bcl-2 apply antagonistic impacts (32).

The cells survival and death signals are related to apoptosis in the cells that are induced and integrated by the proteins of the Bcl-2 family as the anti-apoptotic proteins. On the contrary, higher expression of the Bax, a proapoptotic protein, mediates the great apoptosis. Sun et al. (33) determined the impacts of dexmedetomidine on the intestine I/R. They found that the level of Bax and caspase-3 considerable increases in the ischemia group and Bcl-2 decline in tissues with the completion of treatment but reverse outputs observed in the treatment group.

Ayan et al. (34) observed the protecting impact of Thymoquinone against the testicular torsion and demonstrated an apoptosis increase due to ischemia. Moreover, as an anti-oxidant, Thymoquinone remarkably decreased the damages.

In the present study, we detected that, the Bax expression level as well as a labeling index, caspase-3, that declines considerably in the ovary tissue of IR group, and the Bcl-2 expression diminishes to some extent. Some studies reported, that progesterone suppresses apoptosis and the uterine glandular cells exhibit maximum apoptotic index at the estrus and lower apoptotic index at diestrus and metestrus (35, 36). Such a change could result from the estrous cycle (metestrus) of rats or ischemia duration. Therefore, it is necessary to do additional investigations in this regard.

## Conclusion

Based on the findings, as one of the hydro-alcoholic sources, CH can modulate sexual hormone level, partly, protect ovarian tissues against the oxidative stress (OS) and tissue injuries, and diminish apoptosis induced by the ovarian torsion/detorsion.

## Acknowledgments

We appreciate the Deputy of Research and Technology Student Research Committee of Gonabad University of Medical Sciences, Khorasan Razavi, Iran and Clinical Research Development Unit of The Tabriz Valiasr Hospital, Azarbaijan Sharghi, Iran for their supports. A special grant has been given to the present manuscript from Deputy of Research and Technology and Student Research Committee of Gonabad University of Medical Sciences Khorasan Razavi, Iran. It is declared that there are not any conflicts of interest.

## Authors' Contribution

Z.M., M.M., A.S.M.; Contributed to study conception and design. M.Sh., Z.M., S.M.H.-A., N.G., M.F., B.B.; Contributed to all experimental work, data analysis, and data interpretation. M.M., S.-H.A.-E.; Supervisor and biochemical analyses. M.Sh.; Drafted the manuscript. M.Sh., M.M., A.S.M.; Manuscript reviser. All authors read and approved the final manuscript.

## References

1. Elmimehr R, Motamed-Sanaye A, Brazvan B, Abtahi-Eivary SH, Moghimian M, Fani M. Effects of hypothermia and pentoxifylline on the adnexal torsion/detorsion injuries in a rat testis model. Andrologia. 2021; 53(8): e14143.

2. McWilliams GD, Hill MJ, Dietrich III CS. Gynecologic emergencies. Surg Clin North Am. 2008; 88(2): 265-283.
3. Somuncu S, Cakmak M, Dikmen G, Akman H, Kaya M. Ischemia-reperfusion injury of rabbit ovary and protective effect of trapidil: an experimental study. Pediatr Surg Int. 2008; 24(3): 315-318.
4. Sasaki KJ, Miller CE. Adnexal torsion: review of the literature. J Minim Invasive Gynecol. 2014; 21(2): 196-202.
5. Khaje Roshanaee M, Abtahi-Eivary SH, Shokoohi M, Fani M, Mahmoudian A, Moghimian M. Protective effect of minocycline on Bax and Bcl-2 gene expression, histological damages and oxidative stress induced by ovarian torsion in adult rats. Int J Fertil Steril. 2022; 16(1): 30-35.
6. Akdemir A, Erbaş O, Ergenoğlu M, Yeniel AÖ, Oltulu F, Yavaşoğlu A, et al. Montelukast prevents ischaemia/reperfusion-induced ovarian damage in rats. Eur J Obstet Gynecol Reprod Biol. 2014; 173: 71-76.
7. Li C, Jackson RM. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. Am J Physiol Cell Physiol. 2002; 282(2): C227-C241.
8. Prieto-Moure B, Lloris-Carsí JM, Barrios-Pitarque C, Toledo-Pereyra LH, Lajara-Romance JM, Berda-Antolí M, et al. Pharmacology of ischemia–reperfusion. Translational research considerations. J Invest Surg. 2016; 29(4): 234-249.
9. Karaçor T, Dogan Z, Elibol E, Bulbul M, Nacar M. Effects of iloprost on experimental ischemia and reperfusion injury in rat ovary. Biotech Histochem. 2020; 95(5): 373-380.
10. Kumtepe Y, Odabasoglu F, Karaca M, Polat B, Halici MB, Keles ON, et al. Protective effects of telmisartan on ischemia/reperfusion injury of rat ovary: biochemical and histopathologic evaluation. Fertil Steril. 2010; 93(4): 1299-1307.
11. Oral A, Odabasoglu F, Halici Z, Keles ON, Unal B, Coskun AK, et al. Protective effects of montelukast on ischemia-reperfusion injury in rat ovaries subjected to torsion and detorsion: biochemical and histopathologic evaluation. Fertil Steril. 2011; 95(4): 1360-1366.
12. El Khashab IH, Abdelsalam RM, Elbrairy AI, Attia AS. Chrysin attenuates global cerebral ischemic reperfusion injury via suppression of oxidative stress, inflammation and apoptosis. Biomed Pharmacother. 2019; 112: 108619.
13. Goes AT, Jesse CR, Antunes MS, Ladd FVL, Ladd AAL, Luchese C, et al. Protective role of chrysin on 6-hydroxydopamine-induced neurodegeneration a mouse model of Parkinson's disease: Involvement of neuroinflammation and neurotrophins. Chem Biol Interact. 2018; 279: 111-120.
14. Khan R, Khan AQ, Qamar W, Lateef A, Tahir M, Rehman MU, et al. Chrysin protects against cisplatin-induced colon. toxicity via amelioration of oxidative stress and apoptosis: probable role of p38MAPK and p53. Toxicol Appl Pharmacol. 2012; 258(3): 315-329.
15. Shokri F, Shokoohi M, Abadi ARR, Kalarestaghi H. The ameliorative effect of Galega officinalis extract on histological damages, oxidative stress induced by torsion-detorsion in adult rats' ovarian. Int J Women's Health Reprod Sci. 2019; 7(1): 119-123.
16. Shokoohi M, Soltani M, Abtahi-Eivary S-H, Niazi V, Poor MJR, Ravaei H, et al. Effect of hydro–alcoholic extract of Olea europaea on apoptosis–related genes and oxidative stress in a rat model of torsion/detorsion–induced ovarian damage. Asian Pac J Reprod. 2019; 8(4): 148-156.
17. Bagheri-abassi F, Alavi H, Mohammadipour A, Motejaded F, Ebrahimzadeh-bideskan A. The effect of silver nanoparticles on apoptosis and dark neuron production in rat hippocampus. Iran J Basic Med Sci. 2015; 18(7): 644-648.
18. Abtahi-Eivari SH, Moghimian M, Soltani M, Shoorei H, Asghari R, Hajizadeh H, et al. The effect of Galega officinalis on hormonal and metabolic profile in a rat model of polycystic ovary syndrome. Int J Women's Health Reprod Sci. 2018; 6(3): 276-282.
19. Halestrap AP, Clarke SJ, Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion—a target for cardioprotection. Cardiovasc Res. 2004; 61(3): 372-385.
20. Zhu J, Yao K, Wang Q, Guo J, Shi H, Ma L, et al. Ischemic postconditioning-regulated miR-499 protects the rat heart against ischemia/reperfusion injury by inhibiting apoptosis through PDCD4. Cell Physiol Biochem. 2016; 39(6): 2364-2380.
21. Kalogeris T, Bao Y, Korthuis RJ. Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning. Redox Biol. 2014; 2: 702-714.
22. Bakan V, Çıralık H, Tolun Fİ, Atlı Y, Mil A, Öztürk Ş. Protective effect of erythropoietin on torsion/detorsion injury in rat model. J Pediatr Surg. 2009; 44(10): 1988-1994.
23. Kurt RK, Dogan AC, Dogan M, Albayrak A, Kurt SN, Eren F, et al. Protective effect of colchicine on ovarian ischemia–reperfusion injury: an experimental study. Reprod Sci. 2015; 22(5): 545-550.
24. Ha SK, Moon E, Kim SY. Chrysin suppresses LPS-stimulated proinflammatory responses by blocking NF-κB and JNK activations in microglia cells. Neurosci Lett. 2010; 485(3): 143-147.
25. Zhao S, Liang M, Wang Y, Hu J, Zhong Y, Li J, et al. Chrysin suppresses vascular endothelial inflammation via inhibiting the NF-κB signaling pathway. J Cardiovasc Pharmacol Ther. 2019; 24(3): 278-287.
26. Carden DL, Granger DN. Pathophysiology of ischaemia–reperfusion injury. J Pathol. 2000; 190(3): 255-266.
27. Melekoglu R, Ciftci O, Eraslan S, Alan S, Basak N. The protective effects of glycyrrhetinic acid and chrysin against ischemia-reperfusion injury in rat ovaries. Biomed Res Int. 2018; 2018: 5421308.
28. Tsai SC, Chen CP, Su TH, Kau MM, Lu CC. Involvement of ERK phosphorylation in the prevention of ischemia-induced ovarian follicular depletion by stem cells. Chin J Physiol. 2010; 53(3): 167-177.
29. Soltani M, Moghimian M, Abtahi H, Shokoohi M. The protective effect of Matricaria chamomilla extract on histological damage and oxidative stress induced by Torsion/Detorsion in adult rat ovary. Int J Women's Health Reprod Sci. 2017; 5(3): 187-192.
30. Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N. Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta. 2013; 1833(12): 3448-3459.
31. Jan R. Understanding apoptosis and apoptotic pathways targeted cancer therapeutics. Adv Pharm Bull. 2019; 9(2): 205-218.
32. Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol. 1997; 139(5): 1281-1292.
33. Sun Y, Gao Q, Wu N, Li SD, Yao JX, Fan WJ. Protective effects of dexmedetomidine on intestinal ischemia-reperfusion injury. Exp Ther Med. 2015; 10(2): 647-652.
34. Ayan M, Tas U, Sogut E, Caylı S, Kaya H, Esen M, et al. Protective effect of thymoquinone against testicular torsion induced oxidative injury. Andrologia. 2016; 48(2): 143-151.
35. Dharma SJ, Kholkute SD, Nandedkar TD. Apoptosis in endometrium of mouse during estrous cycle. Indian J Exp Biol. 2001; 39(3): 218-222.
36. Annie L, Gurusubramanian G, Roy VK. Estrogen and progesterone dependent expression of visfatin/NAMPT regulates proliferation and apoptosis in mice uterus during estrous cycle. J Steroid Biochem Mol Biol. 2019; 185: 225-236.

Original Article

# *USP7* and *SET9* Expression in The Oligospermic Human Semen: A Case-Control Study

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## Abstract

**Background:** Oligospermia is defined as a less than 15 million per milliliter sperm in each ejaculation of semen. Proper and complete spermatogenesis requires the expression of a large number of genes. As a result, stopping the expression of any of them may lead to disrupt the process of spermatogenesis. In order to understand the disorders of spermatogenesis, it is necessary to study expression of effective genes in the spermatogenesis process. Therefore, in the present study, *USP7* and *SET9* (*SETD7*) gene expression was examined in the healthy and oligospermic men.

**Materials and Methods:** In this case-control study, semen samples of individuals with normal sperm and oligospermia were collected from men who referred to the Roya Clinic (Qom, Iran) according to World Health Organization (WHO) parameters after obtaining consent. Then the expression of *USP7* and *SET9* genes in two groups was analyzed using quantitative polymerase chain reaction (qPCR).

**Results:** There was no difference forage between the healthy and oligospermic individuals (P=0.889). The data showed that, *USP7* gene expression in the patients was 3.99 times higher than the control group (P<0.001). The expression of *SET9* gene in the patient was 1.28 times less than the control group, which was not significant (P=0.231). The results indicated that *USP7* gene expression was increased in the 84% of oligospermic individuals.

**Conclusion:** The *USP7* gene can be considered as one of the molecular markers in the development of oligospermia.

***Keywords:*** Apoptosis, Male Infertility, Oligospermia, Ubiquitination

**Citation:** Farahani M, Yaghobi Z, Ramezani M, Piravar Z. USP7 and SET9 expression in the oligospermic human semen: a case-control study. Int J Fertil Steril. 2022; 16(4): 306-309. doi: 10.22074/IJFS.2021.537310.1174.



## Introduction

Infertility refers to a couple's inability to conceive after at least one year of intercourse without the use of contraceptives. According to the World Health Organization (WHO), today, almost 80 million couples suffer from infertility worldwide (1). The prevalence of infertility among Iranian couples ranges from 10.3 to 24.9% of which 50% is related to male causes (2).

Male-induced infertility is a complex disorder that affects a large portion of the male population, however many of its causes are unknown. Studies have shown that different genes affect the process of spermatogenesis and incomplete spermatogenesis is one of the important factors in the male infertility.

Oligospermia is not an uncommon disorder and is seen in about 5% of infertile couples. Also, 10-20% of infertile men are diagnosed with abnormal semen analysis. In recent years, our understanding of the genetic etiology of oligospermia has been advanced. Genetic factors cause more than 20% of oligospermia (3). If a gene is expressed at a certain stage of spermatogenesis, it is possible to predict how spermatogenesis will progress through molecular methods and its adaptation to histopathological findings. So far, the role of different genes in this category has been investigated and it has also been observed that many autosomal genes may be involved in male infertility. The two genes, *USP7* and *SET9*, can play a role in the spermatogenesis process (4, 5). The *USP7* gene is located at chromosomal position 16p13.2 and has 35 exons and seven RNA transcripts are made from it and the *SET9* gene is situated on chromosome 4q31.1 and has 10 exons and five RNA transcripts are made from it.

The *SET9* and *USP7* genes interfere in the FOXO (forkhead box O) signaling pathway. FOXO is one of the pathways involved in the process of spermatogenesis through involvement in ubiquitination. Also, FOXO as a critical factor influences the PI3K/AKT pathway in the spermatogonial cells (6).

The *SET9* has numerous activities such as chromatin

Received:24/August/2021, Accepted:25/December/2021
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Royan Institute
International Journal of Fertility and Sterility
Vol 16, No 4, October-December 2022, Pages: 306-309

binding, cellular response to DNA degradation, histone lysine N-methyltransferase activity, binding to P53, protein binding and lysine protein N-methyltransferase. Also, this gene is effective in the epigenetic process. One of the causes of oligospermia is epigenetic disorders which their importance has recently been realized (7).

The USP7 or ubiquitin specific protease 7 is a highly common deubiquitinating enzyme (DUB) involved in the cellular process such as apoptosis and DNA damage (8). USP7 has methyltransferase property and inhibits FOXO by methylation. Previous studies have documented the specific role of two factors, FoxO1 and FoxO3a in the fertility of mice (9). Since Foxo is a vital factor in the PI3K/AKT pathway in the spermatogonial stem cells, regulatory factors of this pathway like USP7 can be considered as a candidate for oligospermia in the diagnosis and treatment process. Therefore, in this research, the expression of *SET9* and *USP7* genes in the oligospermic individuals was studied and compared with the fertile men.

## Materials and Methods

### Sampling

This case-control study was approved by the Research Ethics Committee of Islamic Azad Tehran Medical Science University, Tehran, Iran, (IR.IAU.PS.REC.1398.325). Also, written consent was just obtained from all volunteer participants.

Fifty infertile men with oligospermia who referred to the Roya Fertility Clinic (Qom, Iran), between October and December 2020, were invited to this case-control study.

Also, 50 fertile men with at least one child and no family history of infertility were considered as a control group.

Exclusion criteria in the case group were varicocele, high agglutination in the semen sample and unhealthy karyotype and/or Y chromosome microdeletion. Also, all participants were asked for habitats concerning alcohol consumption, smoking, taking any herbal and chemical medication or special treatment such as radiotherapy and chemotherapy.

Semen samples were collected from the participants after 3-4 days of sexual abstinence. The samples were incubated for 20 minutes at 37°C for liquefaction and evaluated following the WHO criteria (1).

### RNA extraction and cDNA synthesis

Semen samples were washed with phosphate-buffered saline (P5-119, Sigma, Germany) and then the total RNA content was extracted using a GeneAll Biotechnology kit (404-304, GeneAll, South Korea). Quantity of the extracted RNA was determined using the NanoDrop spectrophotometer (NanoDrop Thermo Scientific, USA). RNA purity and concentration was indicated by measuring the absorbance ratio (260/280 and 260/230), after adjusting pH of the solution. Quality of RNA was monitored by electrophoresis on 1% agarose (116801, Merk, Germany) gel followed by ethidium bromide (111615, Merk, Germany) staining. The gel was visualized under the gel doc system at 260 nm UV wavelength (Fig.1). cDNA was synthesized by HyperScript TM RT premix with the Random Hexamer kit (501-025, GeneAll, South Korea) according to the manufacturer's protocol. A total amount of 1 µg of RNA was used for cDNA synthesis. In order to ensure cDNA synthesis, the reverse transcription polymerase chain reaction (RT-PCR) product was used as a template for amplification of *GAPDH*, *SET9* and *USP7* gene. Then the PCR product was run on 2% agarose gel (Fig.2).

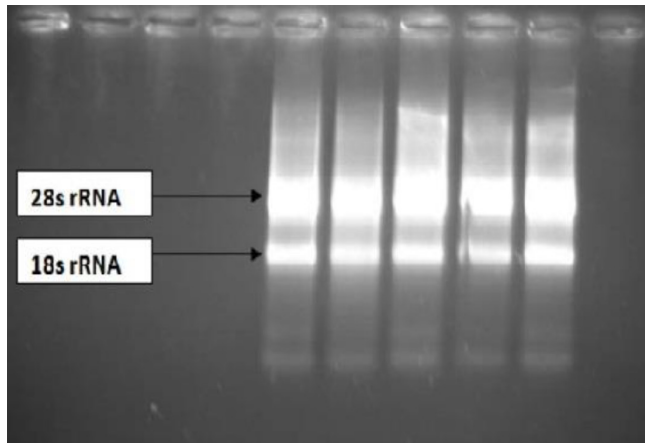


**Fig.1:** RNA gel electrophoresis.

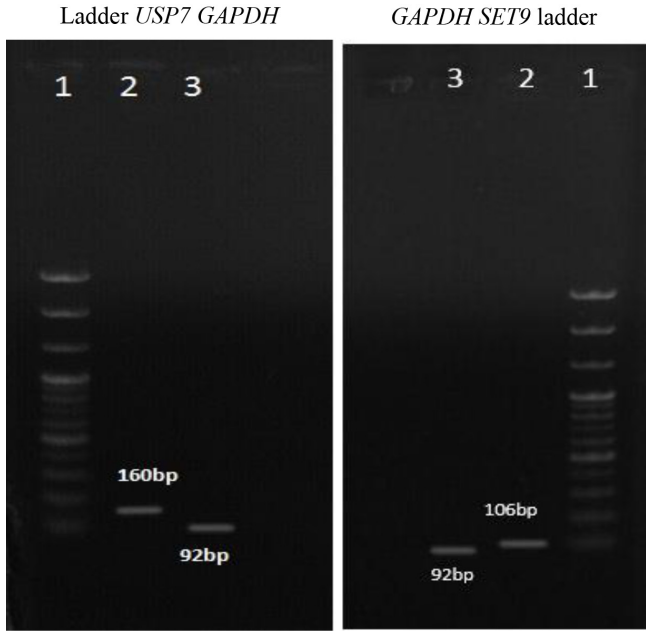


**Fig.2:** cDNA gel electrophoresis.

### Real-time polymerase chain reaction

FASTA sequences of *USP7* and *SET9* genes and housekeeping gene, *GAPDH*, were obtained from NCBI website, and then primers were designed and blasted using Oligo 5 software (Table 1). The efficiency of all primer pairs was calculated using the standard curve method.

**Table 1:** Sequences of the primers

| Genes | Primer sequence (5'-3') | Accession | Product length (bp) | Annealing temperature (◦C) |
|---|---|---|---|---|
| *Usp7* | F: GGGAGGAGGAGGAGGAGG<br>R: GCTTTCTGCTGCTGCTGC | NM_003470.3 | 160 | 60 |
| *SET9* | F: TACGGGCGGTCCAAGTGTC<br>R: GGCCCGTCAGCGTTTCTCT | NM_030648.3 | 106 | 61 |
| *GAPDH* | F: TGGCTACAGCAACAGGGTG<br>R: CTCTTGTGCTCTTGCTGGG | NM_001289746.2 | 92 | 58 |

Each PCR contained 10 µl of real Q-plus 2x Master mix SYBR Green High ROX (A325402, , Ampilicon, Denmark), 0.6 µl of forward primer and 0.6 µl of reverse primer (5 µm concentration), 2 µl of cDNA and deionized water in a final volume of 20 µl. Amplification was carried out on a STEP ONETM Real Time PCR (4376375, Applied Biosystems, USA) with the following program: 95°C for 10 minutes and 1 cycle for primary denaturation, 40 cycles of secondary denaturation 93°C for 20 seconds, Annealing 58°C for *USP7* and 61°C for *SET 9* for 35 seconds, and extension 72°C for 20 seconds. All runs were followed by a melting curve analysis at 55-59°C for 10 seconds. Non-template controls (deionized water) and Non-RT controls (without RT enzyme) were used to assess genomic DNA contamination.

After the end of the reaction, melting curve analysis was performed in order to confirm the specificity of the reaction product and the absence of non-specific products such as primer dimer. Finally, the expression of genes was analyzed using the $2^{-\Delta\Delta Ct}$ method. The *GAPDH* was used as reference gene in each sample to standardize the results. All products were analyzed at least in three technical replicates.

### Statistical analysis

The independent t test was used for the mean comparison in the control and oligospermic groups using SPSS software version 22 (BMI SPSS statistics version 22, USA).

The raw data were extracted as Ct from the Real time PCR device and analyzed using REST 2009 software by independent t test. P<0.05 was considered as significant.

## Results

The mean age of control and oligospermic individuals was 31.12 ± 2.14 years. and 31 ± 1.69 years, respectively. The age difference was not significant between our groups (P=0.889).

The results of the absorbance ratio confirmed purity of RNA. A ratio ≥1.8 and ≤2.2 was interpreted as a pure RNA sample. All qPCR experiments were conducted with three replications.

Table 2 shows the frequencies and values of USP7 and SET9 genes expression in all three modes (increase, decrease and non-significant) in the oligospermic group (n=50).

**Table 2:** Frequency of genes expression

| Genes | Total cases | Frequency of up expression cases (value) | Frequency of down expression cases (value) | Frequency of non-significant cases (value) |
|---|---|---|---|---|
| *USP7* | 50 | 84% (5.93) | - | 16% (1.41) |
| *SET9* | 50 | 20% (3.28) | 38% (3.125) | 42% (1.02) |

Figure 3 demonstrates the mean expression levels of *USP7* and *SET9* genes in the oligospermic and control groups. The *USP7* gene expression in the oligospermic group increased 3.99 times in comparison with the control group, which was significant (P<0.001). While, the expression of *SET9* gene in the oligospermic group was 0.783 ± 0.06 which decreased 1.28 times in comparison with the control group and was not significant (P=0.231).

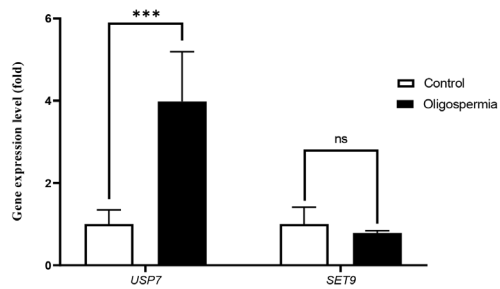


**Fig.3:** Expression levels of *USP7* and *SET9* genes. ***; P<0.001 and ns; No significant. All experiments were performed in triplicate.

## Discussion

Our results found that the *SET9* gene expression was reduced in the oligospermic group in comparison with the control group, but this decrease was not significant. This gene is involved in the histone methylation process which is a type of epigenetic process. Epigenetic factors can play an important role in the reproductive process by affecting the developmental stages of spermatozoa. Protamine-DNA interaction leads to spiraling of sperm DNA into cyclic subunits that each subunit consists of 50,000 bp (10). Each histone protein contains a central nucleus surrounded by DNA (nucleosome) and N-terminal tails protruding outwards and subjected to post-translational changes such as methylation, acetylation, phosphorylation and ubiquitination. The combined effect of these post-translational modifications are a key to DNA regulation, such as replication, repair, and activation or inhibition of DNA (11). Given that the *SET9* gene (*SETD7*) can influence the sperm lysine methylation process. We observed lysine methylation reduction in the oligospermic group that also was associated with sperm count decrease.

In this study, it was found that *USP7* gene expression was significantly increased in our oligospermic participants in comparison with the healthy fertile participants. This gene is involved in the deubiquitination process. USP7, which is primarily located in the nucleus, regulates the stability of several proteins involved in the various cellular processes, including DNA damage response, transcription regulation, epigenetic control of gene expression, and apoptosis (12). USP7 has been documented that regulates the cell level of p53 tumor inhibitor protein in most cancers due to its role in the ubiquitination process (13). Ubiquitin (Ub) is a labeling cellular proteolytic peptide that plays a controlling role in the complex functions of human and animal cells. Protein labeling for degradation is its main function through the proteasomal system. It also controls the stability, function, and location of intracellular proteins (14, 15).

P53 and E3Ub ligase MDM2 are USP7 targets. There are reports of switch-like manner function of USP7 in the these factors regulation. Under normal condition, USP7 associates with MDM2 to protect the E3Ub ligase from auto-ubiquitination. Therefore, MDM2 ubiquinates P53 and allows it for proteasomal degradation. However, under stress signals or patient circumstances, such as oligospermia that leads to DNA damage the expression of USP7 increases and it preferentially binds p53, stabilizes it through deubiquitination process and causes induction of the apoptosis pathway (16). This can be a good reason for reduction of sperm cells in the oligospermic men.

After the spermatozoa leave the testicle, they are stored in the epididymis, where they reach their final maturity (17). These stages of maturation and stabilization, protects sperm from oxidative damage during storage and after releasing into the female reproductive tract. The Ub enzymes exist in human semen plasma as well as in damaged spermatozoa that are secreted by epididymal epithelial cells during the epididymal passage (18). Damage to the DNA structure or other structures in the testicle may trigger apoptosis pathways, and ubiquitinated these sperms. Also, improper twisting of sperm surface antigens or the loss of their structure may cause ubiquitination of damaged sperm (19).

## Conclusion

Overall, our data showed that the RNA expression of *SET9* did not change significantly in the oligospermia. On the other hand, a significant increase in the *USP7* gene expression, RNA level, was observed and this increase was not related to the age. The *USP7* by involving in deubiquitination may cause a defect in the spermatogenesis process due to trigger apoptosis pathway. Therefore, the number of sperm is reduced as we observed in our oligospermic patients. Our results illustrated important effect of this gene that directed to further study on the oligospermia issue. The *USP7* can be used as a reference gene in the prognosis, detection and screening of oligospermia.

## Acknowledgments

The study group thanks all the volunteer participants in this study. The authors would like to thank all the lab assistants of the Islamic Azad University, Central Tehran Branch. There is no financial support and conflict of interest in this study.

## Authors' Contributions

M.R.; Participated as supervisor, study design, and data interpretation. Z.P.; Participated as advisor, conducted molecular experiments and PCR analysis. M.F., Z.Y.; Participated in doing experiments and statistical analysis. M.R., Z.P.; Drafted and revised the manuscript. All authors read and approved the final manuscript.

## References

1. Patel AS, Leong JY, Ramasamy R. Prediction of male infertility by the World Health Organization laboratory manual for assessment of semen analysis: a systematic review. Arab J Urol. 2018; 16(1): 96-102.
2. Morshed-Behbahani B, Lamyian M, Joulaei H, Rashidi BH, Montazeri A. Infertility policy analysis: a comparative study of selected lower middle-middle-and high-income countries. Global Health. 2020; 16(1): 1-9.
3. Gumus E, Kati B, Pelit ES, Ordek E, Ciftci H. A different look at genetic factors in individuals with non-obstructive azoospermia or oligospermia in our research study: to whom, which threshold, when, in what way? Rev Int Androl. 2021; 19(1): 41-48.
4. Luo M, Zhou J, Leu NA, Abreu CM, Wang J, Anguera MC, et al. Polycomb protein SCML2 associates with USP7 and counteracts histone H2A ubiquitination in the XY chromatin during male meiosis. PLoS Genet. 2015; 11(1): e1004954.
5. Shen Y, Tu W, Liu Y, Yang X, Dong Q, Yang B, et al. TSPY1 suppresses USP7-mediated p53 function and promotes spermatogonial proliferation. Cell Death Dis. 2018; 9(5): 1-4.
6. Sinha D, Kalimutho M, Bowles J, Chan AL, Merriner DJ, Bain AL, et al. Cep55 overexpression causes male-specific sterility in mice by suppressing Foxo1 nuclear retention through sustained activation of PI3K/Akt signaling. FASEB J. 2018; 32(9): 4984-4999.
7. Gunes S, Esteves SC. Role of genetics and epigenetics in male infertility. Andrologia. 2021; 53(1): e13586.
8. Wang Z, Kang W, You Y, Pang J, Ren H, Suo Z, et al. USP7: novel drug target in cancer therapy. Front Pharmacol. 2019; 10: 427.
9. Huang P, Zhou Z, Shi F, Shao G, Wang R, Wang J, et al. Effects of the IGF-1/PTEN/Akt/FoxO signaling pathway on male reproduction in rats subjected to water immersion and restraint stress. Mol Med Rep. 2016; 14(6): 5116-5124.
10. Samanta L, Swain N, Ayaz A, Venugopal V, Agarwal A. Post-translational modifications in sperm proteome: the chemistry of proteome diversifications in the pathophysiology of male factor infertility. Biochim Biophys Acta. 2016; 1860(7): 1450-1465.
11. Pozhidaeva A, Bezsonova I. USP7: Structure, substrate specificity, and inhibition. DNA Repair (Amst). 2019; 76: 30-39.
12. Crowe SO, Rana AS, Deol KK, Ge Y, Strieter ER. Ubiquitin chain enrichment middle-down mass spectrometry enables characterization of branched ubiquitin chains in cellulo. Anal Chem. 2017; 89(8): 4428-4434.
13. Jesenberger V, Jentsch S. Deadly encounter: ubiquitin meets apoptosis. Nat Rev Mol Cell Biol. 2002; 3(2): 112-121.
14. Wojcik C, Benchaib M, Lornage J, Czyba JC, Guerin JF. Proteasomes in human spermatozoa. Int J Androl. 2000; 23(3): 169-177.
15. Kim RQ, Sixma TK. Regulation of USP7: a high incidence of E3 complexes. J Mol Biol. 2017; 429(22): 3395-3408.
16. Ozkocer SE, Konac E. The current perspective on genetic and epigenetic factors in sperm maturation in the epididymis. Andrologia. 2021; 53(3): e13989.
17. Cannarella R, Condorelli RA, Mongioì LM, La Vignera S, Calogero AE. Molecular biology of spermatogenesis: novel targets of apparently idiopathic male infertility. Inter J Mol Sci. 2020; 21(5): 1728.
18. Brohi RD, Huo LJ. Posttranslational modifications in spermatozoa and effects on male fertility and sperm viability. Omics. 2017; 21(5): 245-256.
19. Li X, Yao Z, Yang D, Jiang X, Sun J, Tian L, et al. Cyanidin-3-O-glucoside restores spermatogenic dysfunction in cadmium-exposed pubertal mice via histone ubiquitination and mitigating oxidative damage. J Hazard Mater. 2020; 387: 121706.

Original Article

# The Relationship between Plant-Based Diet Index and Semen Parameters of Men with Infertility: A Cross-Sectional Study

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## Abstract

**Background:** Infertility is a major clinical problem that affects people psychologically and medically. For the past 40 years, studies have linked nearly 50% of childlessness to male infertility. It is worth noting that unlike other factors contributing to infertility, diet is a tunable factor and can be applied in counseling infertile men. The goal of this study was to determine the relationship between plant diet index (PDI) and semen parameters in Iranian infertile men.

**Materials and Methods:** In this cross-sectional study, dietary intake was determined by a valid 168-item questionnaire (FFQ). In this study, four dependent semen parameters, including total sperm motility (TSM), sperm concentration (SC), normal sperm morphology (NSM), and semen volume (SV) were measured.

**Results:** Results of this study stated that greater adherence to the healthful plant-based diet index (hPDI), can significantly increase sperm density and motility in men, as well as greater adherence to the PDI dietary pattern is related to a lower risk of sperm volume deficiency, and ultimately more adherence to the unhealthful plant-based diet index (uPDI), can reduce the risk of sperm motility.

**Conclusion:** In this study, for the first time, the relationship between PDI, hPDI, uPDI and male infertility was evaluated. Altogether, this study demonstrated that nutrition has an impact on semen quality and fertility of men.

***Keywords:*** Healthy Diet, Infertility, Plant-Based Diet, Sperm, Unhealthy

**Citation:** Nouri M, Abdollahi N, Leilami K, Shirani M. The relationship between plant-based diet index and semen parameters of men with infertility: a cross-sectional study. Int J Fertil Steril. 2022; 16(4): 310-319. doi: 10.22074/IJFS.2021.538675.1184.


## Introduction

The psychological impact of infertility has been debated for years as one major clinical problem. According to the World Health Organization (WHO), infertility is a disease determined by failure to become pregnant after 12 months of unprotected and ordered intercourse (1). Nearly 15% of couples worldwide face this problem and male infertility is responsible for not less than 50% of the occasions (2). While for many years women were considered the main issue causing infertility for couples, many recent studies have related nearly 50% of childlessness to male reproduction system problems (3, 4). So, considering the reproductive science progression in the female reproduction system, male infertility needs to be studied very well as it could result in many problems in pregnancy and embryo development (5).

Many physiological, environmental, and genetic factors could be respected in the pathogenesis of male infertility and sperm dysfunction (6). Disorders such as industrial chemicals exposures, alcohol consumption and smoking, infections, varicocele, stress, depression, nutritional deficiencies, and genetic disorders have been identified as factors that have negative impacts on semen quality (7). According to recent studies, lifestyle and nutritional factors play a key role in the functioning of the reproductive system (8, 9). Moreover, new researches showed that nutritional factors such as folate, omega-3 fats, saturated fats, soy, soy isoflavones, zinc, and antioxidants could affect semen quality (10). Spanish researchers introduced a positive relationship between sperm quality and consumption of folate-rich food sources, such as fruits and vegetables (11). Vujkovic et al. (12) reported

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Royan Institute
International Journal of Fertility and Sterility
Vol 16, No 4, October-December 2022, Pages: 310-319

a better quality of sperm DNA for men with a "health-conscious" diet (high intake of fruits, vegetables, fish, and whole grains). Another study by Jurewicz et al. (13) of dietary patterns also showed a diet rich in vegetables, fruits, fish, poultry, and whole grains could correlate positively with the percentage of motile sperm. Diet is a complex variable, and traditional analyzes in nutritional epidemiology often examine the association between a disease and a nutrient, or a small number of them. People eat a variety of nutritious foods daily that contain complex nutrient compositions. Therefore, dietary pattern analysis is a method that can determine the relationship between diet and disease (14).

Today, researchers have developed new indices to assess the quality of a diet that measures adherence to a predominantly plant-based diet (15). Plant-based diet index (PDI), healthful plant-based diet index (hPDI), and unhealthful plant-based diet index (uPDI) evaluated the consumption of animal foods and plant foods according to the health of plant foods (16). Considering many studies in the field of male infertility, the relationship between PDI and male infertility has not yet been studied. Therefore, more research is needed. It is worth noting that unlike other factors contributing to infertility, diet is a tunable factor and can be applied in counseling infertile men. The goal of this study was to determine the relationship between PDI and sperm quality in Iranian infertile men.

## Materials and Methods

This cross-sectional study was performed in a major infertility clinic in Isfahan province in 2018. 270 infertile adult men aged 18-55 years who met the inclusion criteria were selected. Before entering the study, participants signed an informed consent form. Subjects with the following criteria were not included in the study: (history of testicular atrophy, urinary tract infection, azoospermia, testicular torsion, genital surgery, and other genital diseases, endocrine, anatomical disorders), metabolic diseases (cardiovascular disease, cancer, diabetes, kidney disease or osteoporosis), psychiatric and physiological disorders such as depression, alcohol and drug abuse, supplement use, previous hormone therapy, anticoagulants, anti-androgens, androgens, cytotoxic drugs or immunosuppressants (17). Ten participants were excluded from the study due to calorie consumption of more than 4200 or less than 800 kcal per day or lack of basic information. Finally, 260 data were used for the final analysis Ethics Committee of Isfahan University of Medical Sciences (IUMS), Isfahan (IR.MUI.RESEARCH.REC.1397.232).

### Assessment of semen parameters

Semen samples were taken from the participant after 3 days of abstinence and collected in sterile containers and half an hour before analysis was liquefied at 37°C. The 5th edition of the World Health Organization (WHO) laboratory manual was used to assess semen. Accordingly, sperm motility was expressed as A to D. A+B is defined as total progressive motility, C is defined as non-progressive motility, A+B+C is defined as total motility, and D is defined as immotile sperm. In this study, four dependent semen parameters, including total sperm motility (TSM), sperm concentration (SC), normal sperm morphology (NSM), and sperm volume were measured for evaluation (18).

### Assessment of dietary intakes

Dietary intake was determined by a valid 168-item questionnaire (FFQ). FFQ validation is confirmed in Iran (19). This questionnaire contains common dietary guidelines in the country and can be used for adults. Participants specified their average frequency of consumption over the past 12 months (number of daily, weekly, monthly and annual) in this form. Specific groups were categorized as follows: 6 times or more a day, 3–5 times a day, 2-3 times a day, every day (once daily), 5-6 times /week, 2-4 times /week, once a week, 1-3 times a month and less than once a month. Finally, the frequency category chosen for each food item was converted into a daily intake for evaluation. Data extracted from the FFQ questionnaire were calculated by modified Nutritionist IV software for Iranian food.

### Plant Diet Index

To create three versions of plant based diets PDI, hPDI, and uPDI, we use the Satije et al. method (20). All foods were grouped into 18 food groups in three main classes. According to food groups, hPDI included whole grains, fruits, vegetables, nuts, legumes, vegetable oils, and tea/coffee, uPDI included fruit juices, sugar-sweetened beverages, refined grains, potatoes, and sweets/desserts, and animal food included animal fat, dairy, egg, fish/seafood, meat, and miscellaneous animal-based foods. In each group, food items were altered to deciles and received 1-10 score according to the lowest and highest intake in each group. In PDI and hPDI index, the highest intake gets a 10 score and the lowest intake get a 1 score. In uPDI, scored 1 for highest intake and 10 for lowest intake of animal food intake by participants. Scores were summed up to get a score ranging from 18 to 180 for each PDI, hPDI, and uPDI index. A higher total score for every index showed higher conformity to that pattern.

### Assessment of other variables

All participants were interviewed face-to-face and the height (in centimeters) and weight (in kilograms) of the subjects were measured by standard methods for calculating body mass index (BMI) in kilograms per square meter.

### Statistical analysis

Participants were classified based on tertiles of PDI, hPDI, and uPDI. To compare continuous variables across tertiles of PDI, hPDI and uPDI, we use One-way analysis of

variance (ANOVA) and chi-squared test used to compare the categorical variables across the tertiles of each pattern score. To determine the relation between plant-based diet scores and odds of sperm parameters, multivariable logistic regression was used in different models. This relation was observed in both crude and adjusted models. In the first model, we adjusted age and energy intake. In the second model, additional controls for BMI, physical activity, marriage time, educational status, smoking, and alcohol history were done. Statistical analyses were conducted using SPSS for Windows software (version 20.0), SPSS Inc., and Chicago IL. P<0.05 was considered statistically significant.

## Results

Baseline characteristics of participants according to tertiles of PDI, hPDI, and uPDI are shown in Table 1. The mean age, body mass index, waist circumference, and physical activity of infertile men were 31.24 years, 26.94 kg/m$^2$, 94.51 cm, and 29.27 Met/day, respectively. BMI and waist circumference were higher in the last tertile of hPDI and waist circumference was higher in the first tertile of uPDI scores. Furthermore, there was a significant change between tertiles of uPDI with alcohol history and supplement use.

The energy-adjusted dietary nutrients and food items intakes of participants through tertiles of PDI, hPDI, and uPDI are shown in Table 2. Participants in the last tertile of PDI had a higher intake of fiber, vitamin E, B9, C, whole grains, fruits, legumes, vegetable oils, tea/coffee, and sugar sweetened beverages, but lower intake of carbohydrate, protein, SFA, cholesterol, B12, calcium, dairy, eggs and fish/seafood compared to lowest tertile.

Moreover, in the highest tertile of hPDI, participants consumed higher amounts of energy, carbohydrate, protein, fat, fiber, vitamin E, B9, magnesium, iron, whole grains, fruits, vegetables, legumes, vegetable oil, and tea/coffee, but lower intake of SFA, cholesterol, B12, refined grains, potatoes, sugar sweetened beverage, sweets desserts, animal fat, dairy, eggs, fish/seafood, and meats compared to the first tertile.

Furthermore, higher intake of refined grains and sugar sweetened beverages, but a lower intake of protein, cholesterol, vitamin A, B6, B12, and C, as well as fruits, vegetables, nuts, legumes, vegetable oil, animal fat, dairy, eggs, fish/seafood, and meats, as well as fruits, vegetables, nuts, legumes, vegetable oil, animal fat, dairy, eggs, fish/seafood and meats were observed in the highest uPDI tertile in comparison with those the lowest tertile.

The mean and standard deviation (SD) of sperm parameters in crude and adjusted models across tertile of PDI, hPDI, and uPDI are shown in Table 3. For the hPDI score, participants in the highest tertile had a higher mean of sperm density compared with those in the lowest tertile in the crude model. Also, after adjustment for potential covariates, the difference was significant. In addition, the mean of sperm motility in the third tertile of uPDI was higher than those in the first tertile in crude and adjusted models.

**Table 1:** Basic characteristics of participants across the tertiles of PDI, hPDI, and uPDI scores

| Variable | PDI | | | | hPDI | | | | uPDI | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T1 (n=96) | T2 (n=76) | T3 (n=82) | P | T1 (n=85) | T2 (n=89) | T3 (n=80) | P | T1 (n=90) | T2 (n=83) | T3 (n=81) | P |
| Age (Y) | 30.94 ± 3.73 | 31.94 ± 4.88 | 31.12 ± 4.40 | 0.70 | 30.75 ± 3.69 | 31.26 ± 4.85 | 31.48 ± 4.29 | 0.53 | 30.98 ± 4.15 | 31.40 ± 4.05 | 31.12 ± 4.75 | 0.81 |
| Body mass index (kg/m$^2$) | 26.68 ± 27.43 | 27.43 ± 4.35 | 26.82 ± 3.88 | 0.46 | 26.67 ± 4.02 | 26.38 ± 3.76 | 27.88 ± 4.47 | 0.04* | 27.49 ± 4.08 | 27.23 ± 4.28 | 26.07 ± 3.89 | 0.06 |
| Waist (cm) | 94.47 ± 10.11 | 95.01 ± 10.23 | 94.65 ± 10.88 | 0.94 | 94.66 ± 9.63 | 92.99 ± 9.52 | 96.61 ± 11.72 | 0.07 | 95.29 ± 10.92 | 95.11 ± 10.33 | 93.59 ± 9.78 | 0.51 |
| Marriage time (Y) | 5.66 ± 3.17 | 5.38 ± 2.71 | 5.43 ± 3.20 | 0.80 | 5.37 ± 2.40 | 5.33 ± 3.02 | 5.84 ± 3.62 | 0.49 | 5.86 ± 3.23 | 5.71 ± 2.96 | 4.89 ± 2.84 | 0.08 |
| Physical activity (Met/day) | 29.51 ± 2.15 | 29.11 ± 2.20 | 29.08 ± 1.99 | 0.45 | 29.35 ± 2.10 | 29.30 ± 1.97 | 29.09 ± 2.34 | 0.78 | 29.20 ± 2.14 | 29.20 ± 2.25 | 29.44 ± 1.96 | 0.79 |
| Smoking history | | | | 0.85 | | | | 0.08 | | | | 0.95 |
| Yes | 35 (36.45) | 30 (39.47) | 29 (35.36) | | 37 (43.53) | 35 (39.33) | 22 (27.50) | | 33 (36.67) | 30 (36.15) | 31 (38.30) | |
| No | 61 (63.55) | 46 (60.53) | 53 (64.64) | | 48 (56.47) | 54 (60.67) | 58 (62.50) | | 57 (63.33) | 53 (63.85) | 50 (61.70) | |
| Alcohol history | | | | 0.22 | 19 (22.35) | | | 0.46 | | | | 0.01* |
| Yes | 17 (17.70) | 21 (27.63) | 15 (18.30) | | 66 (77.65) | 21 (23.60) | 13 (16.25) | | 28 (31.11) | 12 (14.46) | 13 (16.05) | |
| No | 79 (82.30) | 55 (72.37) | 67 (81.70) | | | 68 (76.40) | 67 (83.75) | | 62 (68.89) | 71 (85.54) | 68 (83.95) | |
| Supplement use | | | | 0.94 | 32 (37.65) | | | 0.24 | | | | 0.03* |
| Yes | 30 (31.25) | 25 (32.89) | 25 (30.49) | | 53 (62.35) | 23 (25.85) | 25 (31.25) | | 37 (41.11) | 19 (22.90) | 24 (29.63) | |
| No | 66 (68.75) | 51 (67.11) | 57 (69.51) | | | 66 (74.15) | 55 (68.75) | | 53 (58.89) | 64 (77.10) | 57 (70.37) | |
| Education status | | | | 0.29 | | | | 0.14 | | | | 0.63 |
| Less than high school | 16 (16.66) | 17 (22.37) | 23 (28.05) | | 14 (16.47) | 17 (19.10) | 25 (31.25) | | 18 (20.00) | 18 (21.70) | 20 (24.70) | |
| High school diploma | 28 (29.17) | 23 (30.26) | 27 (32.93) | | 25 (29.41) | 30 (33.70) | 23 (28.75) | | 33 (36.66) | 23 (27.70) | 22 (27.16) | |
| Bachelor degree or higher | 52 (54.17) | 36 (47.37) | 32 (39.02) | | 46 (54.12) | 42 (47.20) | 32 (40.00) | | 39 (43.34) | 42 (50.60) | 39 (48.14) | |

**Values are mean (SD) for continuous and percentage for categorical variables. Using one-way ANOVA for continuous and Chi-square test for categorical variables. PDI; Plant-base diet index, hPDI; Healthful plant-base diet index, uPDI; Unhealthful plant-base diet index, T1; First tertile, T2; Second tertile, T3; Third tertile, P; P value, and *; P<0.05 was considered as significant (more explanation are reported in result section).**

**Table 2:** Dietary intakes of participants across the tertiles of PDI, hPDI, and uPDI scores

| Variable | PDI | | | | hPDI | | | | uPDI | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T1 (n=96) | T2 (n=76) | T3 (n=82) | P value | T1 (n=85) | T2 (n=89) | T3 (n=80) | P value | T1 (n=90) | T2 (n=83) | T3 (n=81) | P value |
| Nutrient items | | | | | | | | | | | | |
| Energy (kcal/d) | 2640.21 ± 751.37 | 2465.46 ± 632.88 | 2427.66 ± 661.09 | 0.08 | 2413.03 ± 635.32 | 2410.82 ± 611.83 | 2752.91 ± 780.83 | 0.001* | 2523.91 ± 552.97 | 2523.86 ± 840.85 | 2509.51 ± 672.53 | 0.98 |
| Carbohydrate (g/d) | 364.83 ± 113.98 | 360.26 ± 96.95 | 357.55 ± 103.55 | 0.003* | 331.48 ± 87.00 | 345.99 ± 92.27 | 409.41 ± 120.31 | <0.001* | 354.53 ± 88.74 | 364.30 ± 121.50 | 365.14 ± 105.63 | 0.19 |
| Protein (g/d) | 105.00 ± 27.24 | 92.02 ± 24.38 | 84.44 ± 25.16 | <0.001* | 95.38 ± 24.41 | 89.79 ± 25.37 | 98.74 ± 30.92 | 0.004* | 98.39 ± 24.23 | 93.77 ± 31.30 | 90.86 ± 25.16 | 0.009* |
| Fat (g/d) | 95.44 ± 45.20 | 81.98 ± 31.06 | 81.95 ± 28.41 | 0.15 | 86.49 ± 35.15 | 82.63 ± 29.04 | 92.58 ± 45.06 | 0.01* | 88.43 ± 27.52 | 88.70 ± 47.20 | 83.84 ± 33.79 | 0.31 |
| Dietary fiber (g/d) | 38.79 ± 16.61 | 41.22 ± 14.64 | 42.26 ± 18.03 | 0.009* | 32.54 ± 11.05 | 40.61 ± 13.59 | 49.27 ± 19.86 | <0.001* | 41.95 ± 16.40 | 39.48 ± 14.57 | 40.37 ± 18.57 | 0.51 |
| SFA (g/d) | 31.98 ± 13.18 | 25.77 ± 9.80 | 24.49 ± 8.89 | <0.001* | 28.92 ± 11.63 | 26.30 ± 9.26 | 27.97 ± 13.21 | <0.001* | 28.36 ± 9.31 | 27.97 ± 14.00 | 26.70 ± 10.64 | 0.37 |
| MUFA (g/d) | 30.11 ± 15.16 | 26.99 ± 11.68 | 27.85 ± 10.60 | 0.55 | 27.51 ± 11.78 | 27.38 ± 10.94 | 30.62 ± 15.46 | 0.28 | 29.35 ± 9.92 | 28.58 ± 16.30 | 27.30 ± 11.68 | 0.33 |
| PUFA (g/d) | 17.87 ± 10.13 | 15.79 ± 6.89 | 16.99 ± 7.02 | 0.28 | 15.50 ± 7.56 | 16.98 ± 7.72 | 18.50 ± 9.45 | 0.20 | 17.69 ± 5.88 | 17.23 ± 10.69 | 15.88 ± 7.80 | 0.17 |
| Cholesterol (mg/d) | 390.35 ± 246.70 | 303.54 ± 204.51 | 238.87 ± 102.62 | <0.001* | 369.87 ± 198.90 | 293.31 ± 231.29 | 282.33 ± 173.86 | <0.001* | 345.37 ± 210.16 | 317.91 ± 228.66 | 279.75 ± 173.18 | 0.07 |
| Vitamin A (RAE/d) | 844.51 ± 428.17 | 818.43 ± 439.16 | 752.68 ± 348.31 | 0.62 | 780.59 ± 382.89 | 745.43 ± 357.69 | 903.76 ± 468.98 | 0.51 | 919.07 ± 447.39 | 802.77 ± 398.12 | 687.01 ± 335.16 | <0.001* |
| Vitamin E (mg/d) | 12.80 ± 5.99 | 13.46 ± 7.87 | 14.51 ± 6.17 | 0.001* | 11.57 ± 5.44 | 14.01 ± 7.62 | 15.14 ± 6.28 | 0.009* | 13.83 ± 5.36 | 13.86 ± 8.43 | 12.92 ± 5.95 | 0.51 |
| Vitamin B6 (mg/d) | 2.27 ± 0.57 | 2.22 ± 0.61 | 2.06 ± 0.60 | 0.05 | 2.05 ± 0.54 | 2.12 ± 0.58 | 2.40 ± 0.62 | 0.10 | 2.29 ± 0.59 | 2.14 ± 0.57 | 2.12 ± 0.62 | 0.004* |
| Vitamin B9 (µg/d) | 552.80 ± 141.71 | 555.77 ± 139.59 | 560.61 ± 161.81 | 0.02* | 502.36 ± 114.18 | 551.46 ± 121.72 | 618.71 ± 179.05 | <0.001* | 559.11 ± 134.36 | 544.48 ± 149.54 | 656.01 ± 159.44 | 0.37 |
| Vitamin B12 (µg/d) | 7.22 ± 3.68 | 5.96 ± 3.08 | 5.00 ± 2.68 | <0.001* | 7.16 ± 3.82 | 5.64 ± 2.98 | 5.56±2.89 | <0.001* | 6.90 ± 3.49 | 5.98 ± 3.41 | 5.41 ± 2.89 | 0.004* |
| Vitamin C (mg/d) | 233.35 ± 121.22 | 264.20 ± 142.11 | 233.87 ± 117.18 | 0.02* | 219.51 ± 113.90 | 226.49 ± 118.10 | 285.53 ± 139.62 | 0.79 | 269.05 ± 125.80 | 247.20 ± 128.30 | 208.95 ± 120.31 | 0.002* |
| Calcium (mg/d) | 1362.73 ± 435.79 | 1183.35 ± 405.30 | 1076.67 ± 389.21 | <0.001* | 1178.37 ± 422.94 | 1176.70 ± 385.98 | 1301.70 ± 469.14 | 0.89 | 1262.96 ± 423.62 | 1213.67 ± 436.52 | 1168.42±436.52 | 0.18 |
| Magnesium (mg/d) | 477.22 ± 156.36 | 438.89 ± 114.41 | 436.31 ± 148.21 | 0.77 | 405.38 ± 102.31 | 439.90±133.43 | 516.72 ± 166.84 | <0.001* | 458.62 ± 118.24 | 455.64 ± 164.39 | 442.63 ± 146.01 | 0.54 |
| Selenium (mg/d) | 124.95 ± 51.89 | 109.91 ± 36.21 | 110.70 ± 43.85 | 0.31 | 104.26 ± 27.39 | 115.66±45.36 | 128.37 ± 56.77 | 0.06 | 111.53 ± 31.04 | 115.56 ± 52.62 | 120.95 ± 50.75 | 0.18 |
| Zinc (mg/d) | 15.88 ± 4.67 | 13.85 ± 4.37 | 12.76 ± 4.08 | <0.001* | 14.17 ± 4.17 | 13.77 ± 4.56 | 14.92 ± 4.97 | 0.10 | 14.71 ± 4.08 | 14.14 ± 5.21 | 13.90 ± 4.42 | 0.22 |
| Iron (mg/d) | 18.07 ± 5.89 | 17.62 ± 4.82 | 17.79 ± 5.56 | 0.05 | 16.01 ± 3.83 | 17.52 ± 4.95 | 20.17 ± 6.60 | <0.001* | 17.90 ± 4.46 | 17.72 ± 6.32 | 17.92 ± 5.60 | 0.84 |
| Food items | | | | | | | | | | | | |
| Whole grains (g/d) | 43.47 ± 64.81 | 65.20 ± 65.23 | 74.40 ± 93.48 | 0.01* | 34.50 ± 41.11 | 54.32 ± 59.54 | 93.28 ± 104.89 | <0.001* | 71.08 ± 85.03 | 54.80 ± 66.84 | 52.88 ± 74.50 | 0.22 |
| Fruits (g/d) | 408.90 ± 175.35 | 490.74 ± 177.37 | 599.12 ± 354.15 | <0.001* | 424.43 ± 158.97 | 471.87 ± 171.17 | 595.07 ± 374.31 | <0.001* | 582.62 ± 338.87 | 477.76 ± 191.76 | 414.47 ± 179.17 | <0.001* |
| Vegetables (g/d) | 327.70 ± 198.10 | 367.37 ± 169.88 | 373.79±165.95 | 0.17 | 311.90 ± 179.31 | 362.13 ± 175.42 | 391.12 ± 180.01 | 0.01* | 414.36 ± 167.57 | 334.12 ± 182.14 | 308.72 ± 176.40 | <0.001* |

**Table 2:** Continued

| Variable | PDI | | | | hPDI | | | | uPDI | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T1 (n=96) | T2 (n=76) | T3 (n=82) | P value | T1 (n=85) | T2 (n=89) | T3 (n=80) | P value | T1 (n=90) | T2 (n=83) | T3 (n=81) | P value |
| Nuts (g/d) | 18.71 ± 22.75 | 15.95 ± 12.17 | 17.65 ± 13.00 | 0.57 | 17.57 ± 14.96 | 18.21 ± 20.43 | 16.77 ± 15.38 | 0.85 | 23.46 ± 18.68 | 15.96 ± 16.86 | 12.58 ± 13.50 | <0.001* |
| Legumes (g/d) | 30.17 ± 23.82 | 31.15 ± 17.57 | 38.14 ± 17.51 | 0.02* | 28.46 ± 16.49 | 37.53 ± 24.50 | 32.89 ± 18.09 | 0.01* | 40.66 ± 21.15 | 32.63 ± 19.54 | 24.98 ± 17.13 | <0.001* |
| Vegetable oil (g/d) | 9.86 ± 7.32 | 14.11 ± 9.42 | 21.01 ± 11.47 | <0.001* | 12.35 ± 8.91 | 14.85 ± 9.78 | 17.13 ± 12.28 | 0.01* | 18.03 ± 11.95 | 13.45 ± 9.14 | 12.38 ± 9.22 | 0.001* |
| Tea and coffee (g/d) | 274.75 ± 247.36 | 361.96 ± 256.35 | 462.37 ± 257.30 | <0.001* | 286.73 ± 216.92 | 375.55 ± 237.01 | 425.04 ± 317.20 | 0.002 | 384.94 ± 242.90 | 374.56 ± 270.01 | 321.81 ± 279.28 | 0.25 |
| Fruit juice (g/d) | 28.81 ± 42.38 | 48.55 ± 81.39 | 47.57 ± 62.35 | 0.05 | 53.93 ± 49.01 | 32.35 ± 48.00 | 36.16 ± 85.48 | 0.05 | 36.82 ± 62.48 | 38.96 ± 55.76 | 47.01 ± 70.47 | 0.54 |
| Refined grains (g/d) | 278.82 ± 143.28 | 292.02 ± 131.91 | 325.65 ± 124.46 | 0.06 | 295.28 ± 115.53 | 323.18 ± 132.79 | 272.53 ± 152.15 | 0.04 | 262.57 ± 122.06 | 292.23 ± 113.01 | 342.93 ± 156.46 | <0.001* |
| Potatoes (g/d) | 25.48 ± 29.19 | 25.82 ± 22.77 | 29.20 ± 17.14 | 0.53 | 36.97 ± 29.17 | 24.06 ± 18.70 | 18.99 ± 18.75 | <0.001* | 27.72 ± 23.53 | 24.88 ± 25.03 | 27.70 ± 23.26 | 0.67 |
| Sugar sweetened beverage (g/d) | 34.94 ± 46.91 | 51.12 ± 60.96 | 77.79 ± 84.47 | <0.001* | 63.91 ± 70.90 | 60.58 ± 72.13 | 34.92 ± 53.52 | 0.008* | 37.09 ± 45.25 | 45.75 ± 45.31 | 80.04 ± 94.26 | <0.001* |
| Sweets desserts (g/d) | 41.38 ± 43.31 | 35.68 ± 25.13 | 47.93 ± 24.66 | 0.06 | 56.92 ± 36.58 | 41.34 ± 31.02 | 26.21 ± 23.81 | <0.001* | 40.59 ± 34.34 | 40.13 ± 30.47 | 44.82 ± 35.10 | 0.61 |
| Animal fat (g/d) | 8.37 ± 12.93 | 5.93 ± 10.17 | 4.96 ± 5.87 | 0.06 | 8.26 ± 8.19 | 7.19 ± 11.71 | 3.98 ± 10.35 | 0.01* | 9.04 ± 13.15 | 5.65 ± 7.64 | 4.67 ± 8.59 | 0.01* |
| Dairy (g/d) | 527.79 ± 243.42 | 463.57 ± 225.46 | 386.20 ± 170.12 | <0.001* | 535.87 ± 231.23 | 442.53 ± 199.84 | 407.92 ± 223.18 | <0.001* | 505.34 ± 203.11 | 485.93 ± 216.57 | 392.04 ± 238.28 | 0.002* |
| Eggs (g/d) | 23.24 ± 20.88 | 18.67 ± 14.09 | 15.72 ± 11.24 | 0.008* | 24.40 ± 19.82 | 17.39 ± 12.69 | 16.47 ± 15.40 | 0.003* | 23.14 ± 17.39 | 20.04 ± 12.87 | 14.74 ± 17.86 | 0.004* |
| Fish and seafood (g/d) | 27.83 ± 23.27 | 21.08 ± 22.70 | 14.29 ± 10.75 | <0.001* | 30.69 ± 22.67 | 17.85 ± 16.33 | 15.61 ± 19.41 | <0.001* | 26.59 ± 23.29 | 19.12 ± 17.57 | 18.10 ± 19.43 | 0.01* |
| Meats (g/d) | 70.71 ± 39.39 | 68.72 ± 50.87 | 57.04 ± 34.86 | 0.07 | 82.13 ± 36.32 | 62.50 ± 41.23 | 51.80 ± 43.51 | <0.001* | 75.01 ± 45.88 | 58.91 ± 35.79 | 62.32 ± 42.48 | 0.02* |
| Miscellaneous animal based foods (g/d) | 25.25 ± 26.26 | 24.19 ± 32.35 | 18.18 ± 11.66 | 0.13 | 24.76 ± 18.52 | 25.45 ± 26.42 | 17.29 ± 28.44 | 0.05 | 24.15 ± 22.79 | 25.25 ± 33.61 | 18.32 ± 14.29 | 0.16 |

**Values are mean ± SE. All values are adjusted for energy intake using ANCOVA. SFA; Saturated fatty acid, PUFA; Polyunsaturated fatty acid, MUFA; Monounsaturated fatty acid, T1; First tertile, T2; Second tertile, T3; Third tertile, and *; P<0.05 was considered as significant (more explanation are reported in result section).**

**Table 3:** Mean sperm parameters across tertiles of PDI, hPDI, and uPDI scores

| Variable | PDI | | | | hPDI | | | | uPDI | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T1 (n=96) | T2 (n=76) | T3 (n=82) | P value | T1 (n=85) | T2 (n=89) | T3 (n=80) | P value | T1 (n=90) | T2 (n=83) | T3 (n=81) | P value |
| Volume (ml) | | | | | | | | | | | | |
| Model I[a] | 4.19 ± 2.23[d] | 3.97 ± 1.92 | 4.31 ± 2.04 | 0.57 | 4.17 ± 2.18 | 3.82 ± 1.65 | 4.54 ± 2.33 | 0.07 | 4.21 ± 2.05 | 4.13 ± 1.80 | 4.14 ± 2.37 | 0.96 |
| Model II[b] | 4.19 ± 2.23 | 3.97 ± 1.92 | 4.31 ± 2.04 | 0.56 | 4.17 ± 2.18 | 3.82 ± 1.65 | 4.54 ± 2.33 | 0.14 | 4.21 ± 2.05 | 4.13 ± 1.80 | 4.14 ± 2.37 | 0.96 |
| Model III[c] | 4.19 ± 2.23 | 3.97 ± 1.92 | 4.31 ± 2.04 | 0.59 | 4.17 ± 2.18 | 3.82 ± 1.65 | 4.54 ± 2.33 | 0.13 | 4.21 ± 2.05 | 4.13 ± 1.80 | 4.14 ± 2.37 | 0.93 |
| Density ($\times 10^6$/ml) | | | | | | | | | | | | |
| Model I[a] | 10.83 ± 10.75 | 15.54 ± 17.51 | 13.32 ± 18.55 | 0.14 | 9.35 ± 6.93 | 15.58 ± 19.22 | 14.15 ± 17.59 | 0.02* | 11.96 ± 13.18 | 13.36 ± 15.24 | 13.93 ± 18.75 | 0.70 |
| Model II[b] | 10.83 ± 10.75 | 15.54 ± 17.51 | 13.32 ± 18.55 | 0.11 | 9.35 ± 6.93 | 15.58 ± 19.22 | 14.15 ± 17.59 | 0.02* | 11.96 ± 13.18 | 13.36 ± 15.24 | 13.93 ± 18.75 | 0.69 |
| Model III[c] | 10.83 ± 10.75 | 15.54 ± 17.51 | 13.32 ± 18.55 | 0.07 | 9.35 ± 6.93 | 15.58 ± 19.22 | 14.15 ± 17.59 | 0.03* | 11.96 ± 13.18 | 13.36 ± 15.24 | 13.93 ± 18.75 | 0.93 |
| Total motility (%) | | | | | | | | | | | | |
| Model I[a] | 28.38 ± 17.99 | 31.68 ± 18.91 | 28.51 ± 17.52 | 0.43 | 26.60 ± 16.22 | 31.74 ± 19.72 | 29.81 ± 17.97 | 0.16 | 25.65 ± 16.74 | 28.09 ± 19.33 | 34.94 ± 17.14 | 0.002* |
| Model II[b] | 28.38 ± 17.99 | 31.68 ± 18.91 | 28.51 ± 17.52 | 0.43 | 26.60 ± 16.22 | 31.74 ± 19.72 | 29.81 ± 17.97 | 0.19 | 25.65 ± 16.74 | 28.09 ± 19.33 | 34.94 ± 17.14 | 0.002* |
| Model III[c] | 28.38 ± 17.99 | 31.68 ± 18.91 | 28.51 ± 17.52 | 0.36 | 26.60 ± 16.22 | 31.74 ± 19.72 | 29.81 ± 17.97 | 0.19 | 25.65 ± 16.74 | 28.09 ± 19.33 | 34.94 ± 17.14 | 0.009* |
| Normal morphology (%) | | | | | | | | | | | | |
| Model I[a] | 3.47 ± 8.29 | 4.67 ± 11.54 | 4.14 ± 10.65 | 0.74 | 3.85 ± 9.98 | 4.24 ± 9.92 | 4.03 ± 10.52 | 0.96 | 4.27 ± 10.64 | 3.34 ± 7.40 | 4.51 ± 11.80 | 0.73 |
| Model II[b] | 3.47 ± 8.29 | 4.67 ± 11.54 | 4.14 ± 10.65 | 0.87 | 3.85 ± 9.98 | 4.24 ± 9.92 | 4.03 ± 10.52 | 0.98 | 4.27 ± 10.64 | 3.34 ± 7.40 | 4.51 ± 11.80 | 0.67 |
| Model III[c] | 3.47 ± 8.29 | 4.67 ± 11.54 | 4.14 ± 10.65 | 0.91 | 3.85 ± 9.98 | 4.24 ± 9.92 | 4.03 ± 10.52 | 0.96 | 4.27 ± 10.64 | 3.34 ± 7.40 | 4.51 ± 11.80 | 0.67 |

**Data are presented as mean ± SE. [a]; Crude, [b]; Adjusted for age and energy intake, [c]; Additionally adjusted for BMI, physical activity, marriage time, educational status, smoking and alcohol history, [d]; These values are mean (SE), T1; First tertile, T2; Second tertile, T3; Third tertile, and *; P<0.05 was considered as significant (more explanation are reported in result section).**

**Table 4:** Crude and multivariable-adjusted odds ratios and 95% CIs for sperm parameters rs across tertiles of PDI, hPDI, and uPDI scores

| Variable | PDI | | | | hPDI | | | | uPDI | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T1 (n=96) | T2 (n=76) | T3 (n=82) | P value | T1 (n=85) | T2 (n=89) | T3 (n=80) | P value | T1 (n=90) | T2 (n=83) | T3 (n=81) | P-value |
| Volume (ml) | | | | | | | | | | | | |
| Model Ia | 1.00 | 1.11 (0.59, 2.08) [d] | 0.53 (0.27, 1.05) | 0.08 | 1.00 | 1.48 (0.78, 2.79) | 0.80 (0.40, 1.59) | 0.56 | 1.00 | 0.85 (0.44, 1.63) | 0.99 (0.52, 1.89) | 0.97 |
| Model IIb | 1.00 | 1.05 (0.55, 1.99) | 0.49 (0.25, 0.98) | 0.05 | 1.00 | 1.48 (0.78, 2.80) | 0.86 (0.42, 1.74) | 0.75 | 1.00 | 0.84 (0.44, 1.62) | 0.98 (0.51, 1.88) | 0.95 |
| Model IIIc | 1.00 | 0.92 (0.47, 1.78) | 0.43 (0.21, 0.87) | 0.02* | 1.00 | 1.47 (0.77, 2.81) | 0.84 (0.40, 1.75) | 0.72 | 1.00 | 0.85 (0.43, 1.68) | 0.99 (0.50, 1.96) | 0.90 |
| Density (×106/ml) | | | | | | | | | | | | |
| Model Ia | 1.00 | 0.60 (0.31, 1.18) | 0.97 (0.49, 1.94) | 0.89 | 1.00 | 0.46 (0.23, 0.93) | 0.70 (0.33, 1.45) | 0.34 | 1.00 | 0.89 9 0.45, 1.75) | 0.86 (0.44, 1.70) | 0.67 |
| Model IIb | 1.00 | 0.59 (0.30, 1.17) | 0.98 (0.49, 1.97) | 0.91 | 1.00 | 0.45 (0.22, 0.91) | 0.66 (0.31, 1.40) | 0.26 | 1.00 | 0.88 (0.44, 1.73) | 0.86 (0.43, 1.70) | 0.66 |
| Model IIIc | 1.00 | 0.52 (0.26, 1.07) | 1.02 (0.50, 2.11) | 0.97 | 1.00 | 0.44 (0.21, 0.89) | 0.71 (0.33, 1.56) | 0.36 | 1.00 | 0.97 (0.48, 1.96) | 0.92 (0.45, 1.89) | 0.84 |
| Total motility (%) | | | | | | | | | | | | |
| Model Ia | 1.00 | 0.72 (0.37, 1.41) | 1.18 (0.59, 2.38) | 0.68 | 1.00 | 0.69 (0.35, 1.38) | 0.80 (0.39, 1.65) | 0.55 | 1.00 | 0.45 (0.21, 0.95) | 0.34 (0.16, 0.72) | 0.005* |
| Model IIb | 1.00 | 0.69 (0.35, 1.37) | 1.13 (0.56, 2.30) | 0.77 | 1.00 | 0.69 (0.35, 1.39) | 0.87 (0.42, 1.81) | 0.70 | 1.00 | 0.45 (0.21, 0.95) | 0.34 (0.16, 0.72) | 0.005* |
| Model IIIc | 1.00 | 0.66 (0.32, 1.33) | 1.17 (0.56, 2.42) | 0.75 | 1.00 | 0.68 (0.34, 1.38) | 0.88 (0.41, 1.88) | 0.66 | 1.00 | 0.49 (0.23, 1.06) | 0.39 (0.18, 0.85) | 0.01* |
| Normal morphology (%) | | | | | | | | | | | | |
| Model Ia | 1.00 | 0.65 (0.21, 2.04) | 0.84 (0.26, 2.72) | 0.76 | 1.00 | 1.05 (0.29, 3.76) | 0.49 (0.15, 1.54) | 0.20 | 1.00 | 1.52 (0.47, 4.85) | 1.22 (0.40, 3.67) | 0.70 |
| Model IIb | 1.00 | 0.73 (0.23, 2.30) | 0.93 (0.28, 3.06) | 0.90 | 1.00 | 1.10 (0.30, 4.01) | 0.43 (0.13, 1.39) | 0.14 | 1.00 | 1.64 (0.50, 5.31) | 1.28 (0.42, 3.91) | 0.63 |
| Model IIIc | 1.00 | 0.71 (0.21, 2.30) | 0.86 (0.25, 2.96) | 0.82 | 1.00 | 1.01 (0.27, 3.78) | 0.35 (0.10, 1.21) | 0.09 | 1.00 | 2.08 (0.61, 7.09) | 1.72 (0.51, 5.73) | 0.42 |

[a]; Crude, [b]; Adjusted for age and energy intake, [c]; Additionally adjusted for BMI, physical activity, marriage time, educational status, smoking and alcohol history, [d]; These values are odd ratio (95% CIs), e ; Obtained from logistic regression, T1; First tertile, T2; Second tertile, T3; Third tertile, BMI; Body mass index, CI; Confidence interval, and * ; P<0.05 was considered as significant (more explanation are reported in result section).

Multivariable-adjusted odds ratio (OR) and 95% confidence intervals (CIs) for sperm parameters across tertiles of PDI, hPDI, and uPDI are indicated in Table 4. Although there was no significant association between volume and PDI in the crude model (OR=0.53, 95% CI: 0.27, 1.05, P=0.08), which became significant in the fully-adjusted model and participants in the highest PDI tertile had a lower risk of volume deficiency (OR=0.43, 95% CI: 0.21, 0.87, P=0.02). In the crude model, there was a significant association between total motility and uPDI, and participants in the highest uPDI tertile had a lower risk of sperm motility (OR=0.34, 95% CI: 0.16, 0.72, P=0.005). After adjustment for potential confounders including age, energy intake, BMI, physical activity, marriage time, educational status, smoking, and alcohol history, the association was significant and participants in the highest uPDI tertile had a lower risk (OR=0.34, 95% CI: 0.16, 0.72, P=0.005 and OR=0.39, 95% CI: 0.18, 0.85, P=0.01).

## Discussion

In this study, for the first time, the relationship between PDI, hPDI, uPDI and male infertility was studied and the results of this investigation revealed that greater adherence to the hPDI dietary pattern could significantly increase sperm concentration and motility in men. Greater adherence to the PDI dietary pattern also could associate with a lower risk of sperm volume deficiency, and ultimately more adherence to the uPDI dietary pattern could reduce the risk of sperm motility.

We create three different plant food patterns to be able that compare them more easily and even distinguished between healthy and unhealthy plant food according to their effect on various diseases such as type 2 diabetes mellitus, cancers, cardiovascular disease, and also some hazardous conditions (hypertension, hyperlipidemia, obesity, and inflammation). Previous studies have applied this type and category of dietary patterns (20, 21), but the association of it with infertility in men, has not been evaluated, yet.

Participants who have a higher hPDI score and consumption more amount of energy, carbohydrate, protein, fat, fiber, vitamin E, B9, magnesium, iron, whole grains, fruits, vegetables, legumes, vegetable oil, and tea/coffee, have a higher mean of sperm density and motility. These findings can partly confirm the results of one study that reported diet rich in vegetables, fruits, whole grains, fish and chicken can be a suitable way to improve semen quality (12). Joanna Jurewicz et al. (13) also conducted one important study to evaluate the association between dietary patterns and male infertility. The results indicated that men who consumed more fruits, cruciferous, vegetables, tomatoes, leafy green vegetables, whole grains, legumes, fish, and chicken had higher sperm concentration and testosterone levels. Besides, in a specific evaluation of the effect of extra virgin olive oil (vegetable oil) consumption on male fertility was found that extra virgin olive oil, due to changes in plasma lipid profile, affects the activity of several peptidases in the testes. In addition, with changes in angiotensinase activity in the testis, it is able to modulate the renin-angiotensin system and its functions in male fertility (22).

The suggested mechanism that a healthy diet is correlated with better semen quality maybe is related to a high amount of fiber sources such as fruits, vegetables, and whole-grain can bind to estrogen and reduce its level in the blood (23, 24). Also, a healthy dietary pattern is associated with more consumption of antioxidants, and several studies showed that more intake of an antioxidant such as carotenoids, vitamin E, and vitamin C can affect semen quality and especially enhance sperm motility (25, 26). Because one of the main reasons for male infertility is direct damage to the DNA of sperm cells and peroxidation of their membranes by reactive oxygen species (ROS) (22).

Even a review article that is about the effect of antioxidants and phytocompounds on seminal oxidative stress concludes that plant foods not only can reduce oxidative stress, but also can improve male reproductive functions (3). Recent studies conducted in the Iranian male population have also shown that there is a positive relationship between healthy dietary patterns and improvement of sperm indices, even following a healthy and traditional dietary pattern has been introduced as a protective factor against male infertility and western and fat-based dietary pattern as a risk factor (27, 28).

Our study also shows that men who follow most of the PDI dietary patterns with lower intake of carbohydrates, protein, SFA, cholesterol, B12, calcium, dairy, eggs, and fish, had a lower risk of volume deficiency. In the same way, Attaman et al in their study about dietary fat and semen quality have reported that high consumption of saturated fat can diminish sperm concentration (29).

In both PDI and hPDI patterns, men in the highest tertile with better semen quality had a lower intake of vitamin B12 and it is in contrast with many previous investigations that said vitamin B12 and folate are important for DNA methylation and improve sperm motility and concentration (30, 31). That may be this role can be more associated with folate compare to vitamin B12. Also in Vujkovic et al. (12) study, there is a positive association between traditional Dutch dietary patterns and seminal vitamin B12 concentration, which maybe is related to the high consumption of meat in this dietary Patten.

Also, the results of this study demonstrate that participants in the highest tertile of uPDI that have a higher intake of refined grains and sugar sweetened beverages were at lower risk of abnormal sperm motility. That could be because a high intake of simple sugar causes insulin resistance and oxidative stress that can affect sperm motility (32, 33). However, excessive fats and carbohydrates have always been the cause of obesity, but today it has been found that more complex relationships of macronutrients or even micronutrient deficiency in unhealthy dietary patterns can be involved in this case and consequently its relationship with infertility in men (34).

Some of the straight points of the current study are that

this topic is new and we had innovation for choose of it, also an appropriate sample population of infertile men was available who were accurately evaluated for inclusion and exclusion criteria and also were relatively homogenous in age, ethnicity and anthropometric indexes, that reduces the chance of finding results be related to peripheral and uncontrolled factors. Additionally, to minimize errors in the results of this study after evaluating the cured model in another model adjusted in terms of BMI, physical activity, age, energy intake, BMI, marriage time, educational status, smoking, and alcohol history conducted that cause results become more valid and reliable.

In the present study for evaluating male infertility, semen samples were applied in a standard situation and taking into account all of the WHO criteria, but we have access only to one sample of each man, similar to other epidemiological studies, whereas it can be better to have several semen samples collected over 1-2 weeks (35). In this investigation, we use the dietary pattern method which is considered a complex of food consumption and different connections between food compounds, since dietary intake is a multidimensional variable and people do not consume food individually, using this dietary pattern method has more potential to be associated with health outcomes and provide a basis for dietary recommendations (36, 37).

In this study, 168-item FFQ was used for collecting nutritional data. This tool has adequate validity and reproducibility (19), However, there is likely to have some measurement error, which can usually cause errors in dietary classifications and reduce associations of interest in all observational studies (38, 39). But so far, it is the only tool available and suitable tool. There were not numerous limitations in this study, just because it was observational cross-sectional we cannot prove causality between diet and semen quality parameters.

## Conclusion

In this study, for the first time, the relationship between plant PDI, hPDI, uPDI and male infertility were evaluated and demonstrated very important results, including that greater conformity to the hPDI dietary pattern could significantly increase sperm density and motility in men, as well as greater adherence to the PDI dietary pattern is associated with a lower risk of sperm volume deficiency, and ultimately more adherence to the uPDI dietary pattern, can reduce the risk of low sperm motility. Altogether, this cross-sectional study demonstrated that nutrition has an impact on semen quality and fertility of men.

## Acknowledgments

There is no financial support and conflicts of interest in this study.

## Authors' Contributions

M.Sh.; Participated in study design, data collection and evaluation. M.N.; Participated in data collection and evaluation. N.A.; Participated in statistical analysis of data and interpretation of data. K.L.; Participated in statistical analysis of data and editing the manuscript. All authors read and approved the final manuscript.

## References

1. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. Reprod Biol Endocrinol. 2015; 13: 37.
2. Alkhedaide A, Alshehri ZS, Sabry A, Abdel-Ghaffar T, Soliman MM, Attia H. Protective effect of grape seed extract against cadmium-induced testicular dysfunction. Mol Med Rep. 2016;13(4): 3101-3109
3. Brake A, Krause W. Decreasing quality of semen. BMJ. 1992; 305(6867): 1498.
4. Sikka S, Hellstrom W, Naz R. Pentoxifylline: role in management of male infertility/mechanisms of action. Molecu Androl. 1993; 5: 220-231.
5. Adewoyin M, Ibrahim M, Roszaman R, Isa MLM, Alewi NAM, Rafa AAA, et al. Male infertility: the effect of natural antioxidants and phytocompounds on seminal oxidative stress. Diseases. 2017; 5(1): 9.
6. Kamischke A, Nieschlag E. Analysis of medical treatment of male infertility. Hum Reprod. 1999; 14 Suppl 1: 1-23.
7. Sengupta P, Dutta S, Krajewska-Kulak E. The disappearing sperms: analysis of reports published between 1980 and 2015. Am J Mens Health. 2017; 11(4): 1279-1304.
8. Merzenich H, Zeeb H, Blettner M. Decreasing sperm quality: a global problem? BMC Public Health. 2010; 10(1): 1-5.
9. Shirani M, Saneei P, Nouri M, Maracy M, Abbasi H, Askari G. Associations of major dietary patterns and dietary diversity score with semen parameters: a cross-sectional study in iranian infertile men. Int J Fertil Steril. 2020; 14(3): 185-192.
10. Braga DPdAF, Halpern G, Rita de Cássia SF, Setti AS, Iaconelli Jr A, Borges Jr E. Food intake and social habits in male patients and its relationship to intracytoplasmic sperm injection outcomes. Fertil Steril. 2012; 97(1): 53-59.
11. Giahi L, Mohammadmoradi S, Javidan A, Sadeghi MR. Nutritional modifications in male infertility: a systematic review covering 2 decades. Nutr Rev. 2016; 74(2): 118-130.
12. Vujkovic M, de Vries JH, Dohle GR, Bonsel GJ, Lindemans J, Macklon NS, et al. Associations between dietary patterns and semen quality in men undergoing IVF/ICSI treatment. Hum Reprod. 2009; 24(6): 1304-1312.
13. Jurewicz J, Radwan M, Sobala W, Radwan P, Bochenek M, Hanke W. Dietary patterns and their relationship with semen quality. Am J Mens Health. 2018; 12(3): 575-583.
14. Gaskins AJ, Colaci DS, Mendiola J, Swan SH, Chavarro JE. Dietary patterns and semen quality in young men. Hum Reprod. 2012; 27(10): 2899-2907.
15. Hu FB, Rimm E, Smith-Warner SA, Feskanich D, Stampfer MJ, Ascherio A, et al. Reproducibility and validity of dietary patterns assessed with a food-frequency questionnaire. Am J Clin Nutr. 1999; 69(2): 243-249.
16. Huijbregts P, Feskens E, Räsänen L, Fidanza F, Nissinen A, Menotti A, et al. Dietary pattern and 20 year mortality in elderly men in Finland, Italy, and The Netherlands: longitudinal cohort study. BMJ. 1997; 315(7099): 13-17.
17. Satija A, Hu FB. Plant-based diets and cardiovascular health. Trends Cardiovasc Med. 2018; 28(7): 437-441.
18. Lee K, Kim H, Rebholz CM, Kim J. Association between different types of plant-based diets and risk of dyslipidemia: a prospective cohort study. Nutrients. 2021; 13(1): 220.
19. Biswas TK, Pandit S, Mondal S, Biswas SK, Jana U, Ghosh T, et al. Clinical evaluation of spermatogenic activity of processed Shilajit in oligospermia. Andrologia. 2010; 42(1): 48-56.
20. Künzle R, Mueller MD, Hänggi W, Birkhäuser MH, Drescher H, Bersinger NA. Semen quality of male smokers and nonsmokers in infertile couples. Fertil Steril. 2003; 79(2): 287-291.
21. Jensen TK, Swan S, Jørgensen N, Toppari J, Redmon B, Punab M, et al. Alcohol and male reproductive health: a cross-sectional study of 8344 healthy men from Europe and the USA. Hum Reprod. 2014; 29(8): 1801-1809.
22. Mirmiran P, Esfahani FH, Mehrabi Y, Hedayati M, Azizi F. Reliability and relative validity of an FFQ for nutrients in the Tehran lipid and glucose study. Public Health Nutr. 2010; 13(5): 654-662.

23. Satija A, Bhupathiraju SN, Rimm EB, Spiegelman D, Chiuve SE, Borgi L, et al. Plant-based dietary patterns and incidence of type 2 diabetes in US men and women: results from three prospective cohort studies. PLoS Med. 2016; 13(6): e1002039.
24. Zamani B, Milajerdi A, Tehrani H, Bellissimo N, Brett NR, Azadbakht L. Association of a plant-based dietary pattern in relation to gestational diabetes mellitus. Nutr Diet. 2019; 76(5): 589-596.
25. Chiu YH, Afeiche MC, Gaskins AJ, Williams PL, Petrozza JC, Tanrikut C, et al. Fruit and vegetable intake and their pesticide residues in relation to semen quality among men from a fertility clinic. Hum Reprod. 2015; 30(6): 1342-1351.
26. Eslamian G, Amirjannati N, Rashidkhani B, Sadeghi MR, Hekmatdoost A. Intake of food groups and idiopathic asthenozoospermia: a case–control study. Hum Reprod. 2012; 27(11): 3328-3336.
27. World Health Organization. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. Geneva: World Health Organization: 2000; 894: i-xii, 1-253.
28. Mendiola J, Torres-Cantero AM, Vioque J, Moreno-Grau JM, Ten J, Roca M, et al. A low intake of antioxidant nutrients is associated with poor semen quality in patients attending fertility clinics. Fertil Steril. 2010; 93(4): 1128-1133.
29. Eskenazi B, Kidd S, Marks A, Sloter E, Block G, Wyrobek A. Antioxidant intake is associated with semen quality in healthy men. Hum Reprod. 2005; 20(4): 1006-1012.
30. Nouri M, Tavasoli M, Nozari A, Askari G. The association of food pattern and sperm quality parameters and biochemical markers in infertile men: a review study. HSR. 2020; 16(3): 206-211.
31. Haeri F, Pourmasoumi M, Ghiasvand R, Feizi A, Salehi-Abargouei A, Marvasti LD, et al. The relationship between major dietary patterns and fertility status in iranian men: a case-control study. Sci Rep. 2021; 11(1): 18861.
32. Attaman JA, Toth TL, Furtado J, Campos H, Hauser R, Chavarro JE. Dietary fat and semen quality among men attending a fertility clinic. Hum Reprod. 2012; 27(5): 1466-1474.
33. Eslamian G, Amirjannati N, Rashidkhani B, Sadeghi MR, Baghestani AR, Hekmatdoost A. Adherence to the western pattern is potentially an unfavorable indicator of asthenozoospermia risk: a case-control study. J Am Coll Nutr. 2016; 35(1): 50-58.
34. Dattilo M, Cornet D, Amar E, Cohen M, Menezo Y. The importance of the one carbon cycle nutritional support in human male fertility: a preliminary clinical report. Reprod Biol Endocrinol. 2014; 12(1): 1-9.
35. Stanhope KL, Schwarz JM, Keim NL, Griffen SC, Bremer AA, Graham JL, et al. Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. J Clin Invest. 2009; 119(5): 1322-1334.
36. Benedetti S, Tagliamonte MC, Catalani S, Primiterra M, Canestrari F, De Stefani S, et al. Differences in blood and semen oxidative status in fertile and infertile men, and their relationship with sperm quality. Reprod Biomed Online. 2012; 25(3): 300-306.
37. Pini T, Raubenheimer D, Simpson SJ, Crean AJ. Obesity and male reproduction; placing the western diet in context. Front Endocrinol (Lausanne). 2021; 12: 622292.
38. Stokes-Riner A, Thurston SW, Brazil C, Guzick D, Liu F, Overstreet JW, et al. One semen sample or 2? Insights from a study of fertile men. J Androl. 2007; 28(5): 638-643.
39. Centritto F, Iacoviello L, di Giuseppe R, De Curtis A, Costanzo S, Zito F, et al. Dietary patterns, cardiovascular risk factors and C-reactive protein in a healthy Italian population. Nutr Metab Cardiovasc Dis. 2009; 19(10): 697-706.

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

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

**A. Original articles**
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, Author's 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, Author's 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, Author's Contributions, and References **(Up to 70)**.

**D. Short communications**
Short communications are 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, Author's Contributions, and References **(Up to 30)**.

**E. Case reports**
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, Conclusion, Acknowledgements, Author's Contributions, and References **(Up to 30)**.

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

**G. Imaging in reproductive medicine**
Imaging in reproductive medicine should focus around 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 editors**
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 to 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

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It is essential for authors to include a statement of responsibility in the manuscript that specifies all the authors' contributions. This participation must include: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, and Funding acquisition. Authors who do not meet the above criteria should be acknowledged in the Acknowledgments section.

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Each manuscript 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). **Also, no manuscript would be accepted in case it has been pre-printed or submitted to other websites.** I hereby assign the copyright of the enclosed manuscript to Int J Fertil Steril".

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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 other 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.**

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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.
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**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 the mobile number and full postal address of the corresponding author).

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**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 after the abstract:

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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.

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It should include 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.

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

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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 the manuscript while their location should be mentioned in the main text.

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**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".

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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.
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**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.

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Name of author. Thesis title. Degree. City name. University. Publication date (year).
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**Example:** Jahanshahi A, Mirnajafi-Zadeh J, Javan M, Mohammad-Zadeh M, Rohani M. Effect of low-frequency stimulation on adenosineA1 and A2A receptors gene expression in the dentate gyrus of perforant path kindled rats. Cell J. 2008; 10 (2): 87-92. Available from: http://www.celljournal.org. (20 Oct 2008).

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

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

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3. Tables should be in a separate page. Figures must be sent in color and also in GIF or JPEG format with 300 dpi resolutions.
4. Cover Letter should be uploaded with the signature of all authors.
5. An ethical committee letter should be inserted at the end of the cover letter.

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