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Contents

Systematic Review

The Effect of Lifestyle Intervention on Pregnancy and Birth Outcomes on Obese Infertile Women: A Systematic Review and Meta-Analysis

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Abstract

Obesity has been associated with negative effects on natural fertility and poor prognosis when assisted reproductive technologies (ART) are performed. Patients attending for fertility treatments are often advised to optimize their weights to improve the outcomes. There is lack of enough information on how weight-loss would be effective for improving fertility in women who are overweight or obese. We conducted a systematic review to evaluate whether weight-loss achieved by lifestyle program improves natural or assisted reproduction in obese infertile women. We searched CENTRAL, MEDLINE, and EMBASE up to March 2018. Two reviews were selected as randomised trials assessing a lifestyle intervention in women with obesity before receiving treatments for infertility and appraised their risk of bias. We extracted data on pregnancy, birth, and miscarriage rates as the primary outcomes and pooled effect estimates using a random effects model. The primary outcome was the live birth rate. We reported summary measures as the relative risk (RR), 95% confidence interval (CI), and percentage of heterogeneity (I^2). We included eight randomised trials with 1175 women. Lifestyle programmes, improved pregnancy rates (RR: 1.43, CI: 95% 1.02 to 2.01; I^2=60%; 8 RCTs; N=1098) but had no impact on live births (RR: 1.39, CI: 95% 0.90 to 2.14; I^2=64%; 7RCTs; N=1034). Our findings suggest that women participating in lifestyle interventions had an increased risk of miscarriage (RR: 1.50, CI: 95% 1.04 to 2.16; I^2=0; 6RCTs; N=543). We rated the quality of evidence for these outcomes as the moderate-to-low. Lifestyle interventions slightly increased the pregnancy rate, while it would be uncertain whether it can improve the live birth. Lifestyle interventions can increase the risk of miscarriage. More research is needed to further explore lifestyle interventions on reproductive outcomes in obese infertile women.

Keywords: Diet, Infertility, Live Birth Rate, Obesity, Physical Exercise

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Introduction

The prevalence of overweight and obesity among women have increased more than three times in the last years, creating a global pandemic affecting both industrialized and developing countries (1, 2). Obesity has been associated with negative effects on both general and reproductive health. Natural fertility is compromised in both, men and women (3). In the last, polycystic ovarian disease (which is typically associated with central obesity, insulin resistance, and hyperinsulinism) and alterations affecting obesity-related hormones (e.g., leptin, adipokines, ghrelin, and endorphins) can affect oocyte quality, fertilization, embryo development, and implantation, as well as reducing the fertility rate in women with a normal menstrual cycle (4-7). The extent of impact of obesity on *in vitro* fertilization (IVF) outcomes is unknown due to the heterogeneity of studies conducted in this area, the retrospective nature of most investigations, and lack of standardized criteria (8-10). Obesity has been associated with an increase in gonadotropin need, more days of treatment, higher cancellation rates of cycles due to the inadequate response, decreased numbers of total and mature eggs, reduced rates of fertilization, and consequently fewer high-quality embryos. Obesity has also been associated with endometrial abnormalities and lower implantation rates (11-14).

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Weight-loss has been appreciated as one of the most effective means of increasing the probability of fertility in infertile overweight or obese women (15, 16). Few studies have analyzed the actual effects of a lifestyle intervention, including diet and exercise on obese women wishing to become pregnant. Additionally, the findings of these studies have been inconsistent, probably owing to methodological shortcomings (17). A prior systematic review, including randomized and non-randomized controlled trials and studies using weight reduction drugs showed an increase in the feasibility of becoming pregnant, with no significant adverse effect on live birth rates (18).

In this systematic review, we aimed to evaluate whether weight-loss achieved by a lifestyle intervention improved the pregnancy outcomes in obese infertile women, with a specific focus on the live birth rate.

Materials and Methods

We conducted this systematic review according to the methodological guidance of Cochrane (19). We reported the findings from the review according the PRISMA statement (20).

Search strategies

We searched MEDLINE (via PubMed), EMBASE (via Ovid), and CENTRAL (via The Cochrane Library) from the databases inception up to March 2018. We designed a search strategy combining text words and controlled vocabulary adapted to the requirements of each database. We included the complete search strings in the Materials S1 (See Supplementary Online Information at www.ijfs.ir). Additionally, we searched the reference list of all eligible studies and contacted authors of the included trials to request additional information.

Study selection

We included randomised controlled trials assessing a lifestyle intervention in obese women before receiving treatments for infertility. The lifestyle interventions that we considered in this study consisted of any type of structured physical exercise and/or any low calorie intake diet refered by the primary included studies. Eligible trials included women with a body mass index of 29 or higher who were candidates for IVF. The selected trials assessed the structured health promotion programmes consisting of dietary intake reduction alone or combined with physical activity compared with an inactive control group (e. g. women on a waiting list) or women receiving weight loss advice. Three authors independently evaluated whether the references retrieved from the searches met the inclusion criteria and resolved disagreements by discussion or through adjudication by an additional author. We obtained full copies of eligible references for a final decision with respective to their inclusion and reported the reason that led to exclusion of studies.

Outcomes

We set the following primary outcomes: live birth (including spontaneous live birth, IVF live birth and cumulative live birth per initial cycle), cumulative pregnancy rate and miscarriage (pregnancy ending within the first 20 weeks of gestation). Secondary outcomes were pregnancy (including multiple pregnancies), ongoing pregnancy, and implantation rates.

Data extraction and risk of bias assessment

Two authors extracted independently the relevant data from chosen trials using a predefined extraction form and an additional author revised the process for accuracy. We registered the characteristics of included studies in descriptive tables. We contacted authors from included studies to request missing data in published papers.

We assessed independently the risk of bias from included trials using the Cochrane tool for that purpose (21). We assessed the trial randomisation sequence generation and its concealment, the concealment of the intervention to participants, researchers, and outcomes assessors, attrition, and incomplete outcome data and selective outcome reporting.

Data analysis and findings description

We analysed the effect measures for dichotomous variables using risk ratios (RR) and mean differences (MD) for continuous variables calculating their 95% confidence intervals (CI). We considered statistic significant difference between compared groups when 95% CI was not included. The unit of the analysis of interest was the participants in included trials and we used the available-case analysis approach to calculate the effect estimates.

When appropriate, we calculated pooled effect estimates for each outcome using a fixed-effect model or a random effect model when there was statistical heterogeneity (22). We assessed heterogeneity comparing characteristics from included studies and through the I square statistics (23) considering a substantial statistical heterogeneity for values greater than 50% and considerable heterogeneity for values greater than 75% scenario in which we did not perform the pool effect estimates. We performed sub-group analyses according to the lifestyle programme assessed in the included trials (diet alone or combined with physical activity). We planned sensitivity analyses excluding trials with the highest risk of bias or those that were a suspected source of heterogeneity. As any pooled analyses included more than 10 trials, we were not able to conduct formal tests to assess the impact of publication bias (24). We used the statistical package in the open access software Review Manager (v 5.3.5) to conduct all of the analyses (25). We assessed the quality of evidence to judge the confidence in the effect estimates obtained from each primary outcome. We

rated the quality of evidence as high, moderate, low or very low according to the impact of each outcome on the risk of bias, indirectness, and effect estimates inconsistency, and imprecision (26). We summarized the effect estimates for primary outcomes and their quality of evidence in a summary of the Table of findings (27).

Results

Study selection and characteristics

Our search strategy yielded 726 records of which 48 were potentially eligible to be included. The flowchart (Fig.1) describes the complete eligibility process, and we describe the reasons for excluding 40 studies and the main characteristics of eight included trials (28-34) in the Materials S2 (See Supplementary Online Information at www.ijfs.ir) and the Table 1, respectively. Table 2 shows the summary of findings of the review with a judgement on their quality of evidence.

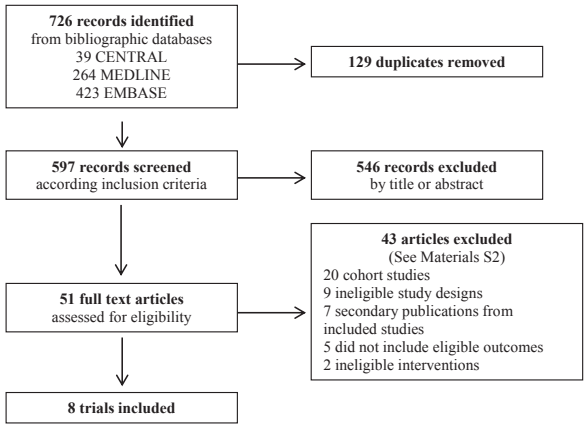

Fig.1: Flowchart for study eligibility.

In total, we included 1175 infertile women. The mean age ranged from 29 to 34 years old, and the body mass index (BMI) from 24 to 38. The included trials compared lifestyle-structured programmes with the usual care. The assessed programmes consisted of dietary intake reduction (28-30) or combined with physical activity interventions (6, 30-33). Women in control groups immediately received infertility treatment with no history of interventions or were included in a waiting list for IVF (28-30, 32) or received standard advice for weight-loss (16, 31, 34). All lifestyle interventions significantly reduced the weight of infertile women compared with control group in the Materials S3 (See Supplementary Online Information at www.ijfs.ir). The mean weight loos values ranged between 3 and 10 kg at the end of the intervention. We did not pooled the results of studies reporting weight-loss due to the presence of high heterogeneity (95%).

Risk of bias

Most trials implemented random sequences generated adequately using lists of computer generated numbers (16, 29-34) and had proper allocation concealment, using opaque envelopes in most of the cases (16, 31-34). With the exception of one trial (30), the rest was open or did not provide details on blinding of researchers or participants, but four implemented a blinded outcome assessment (29-32). We considered three trials having high risk of bias because the data available for the analysis were partially complete (16, 28, 32). Finally, two trials had high risk of selective reporting bias because some outcomes included in their protocols did not coincide with those reported in the published reports of their findings (Fig.2) (28, 34).

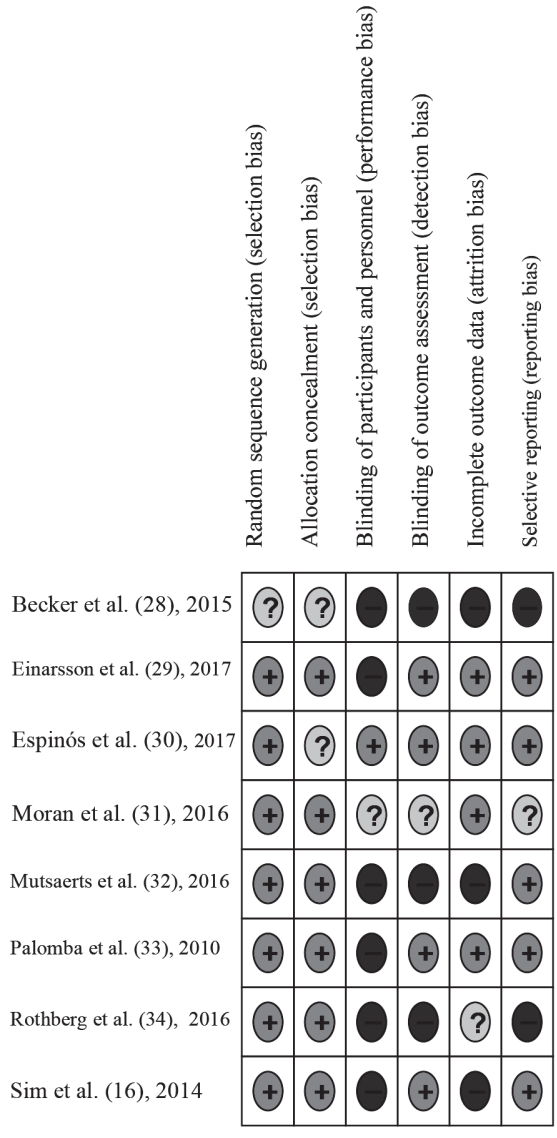

Fig.2: Risk of bias.

Table 1: Characteristics of included studies

Study ID, Setting, country	Women	Age (Y) Mean years (SD) Experimental/ control group	BMI at baseline Mean (SD) Experimental/ control group	Experimental intervention	Control intervention	Outcomes	Follow-up (months)	Funding
Becker et al. (28), 2015 Obstetrics and Gynaecology Service of the Hospital de Clinicas de Porto Alegre, Brazil	35	31.36 (SE 0.89)/ 31.25 (SE 0.78)	28.67 (SE 0.60)/ 28.82 (SE 0.98)	Hypocaloric diet with a low glycemic index and low glycemic load	Maintenance of the body weights and usual diets	Live birth (spontaneous) ndesirable effects (miscarriage) Pregnancy rate (clinical) BMI change Weight change	12	Not reported
Einarsson et al. (29), 2017 Infertility clinics Sweden, Denmark and Iceland	317	31.5 (4.3)/ 31.7 (4.1)	33.1 (1.3)/ 33.0 (1.5)	A low calorie liquid formula diet of 880 kcal/ day	IVF with no previous interventions	Live birth (spontaneous IVF) Undesirable effects (miscarriage, ectopic pregnancy) Pregnancy rate (clinical, multiple) BMI change Weight change	12	Sahlgrenska University Hospital (ALFGBG-70 940), Merck AB, Solna, Sweden (an affiliate of Merck KGaA, Darmstadt, Germany), Impolin AB, Hjalmar Svensson Foundation and Jane and Dan Olsson Foundation
Espinós et al. (30), 2017 Fertility Unit of Hospital de la Santa Creu i Sant Pau-Fundacio Puigvert, Barcelona Spain	41	32.0 (3.2)/ 32.9 (3.9)	34.6 (3.0)/ 34.0 (4.1)	Diet and exercise	IVF/ICSI with no previous interventions	Live birth (IVF, cumulative) Undesirable effects (miscarriage) Pregnancy rate (clinical, multiple) Weight change Implantation rate Fertilization rate	12	Grant from the Instituto de Salud Carlos III (PI11/02816)
Moran et al. (31), 2016 Repromed, Adelaide Australia	46	33.8 (3.5)/ 32.5 (3.3)	34.0 (4.5)/ 33.9 (4.4)	A nutritionally adequate reduced energy diet and exercise intervention andcontact with investigators	A standard advice on appropriate diet and lifestyle factors influencing fertility provided face-to face at one session with no active follow-up	Live birth Undesirable effects (miscarriage) Pregnancy rate BMI change Weight change	Not reported	NHMRC Program Grant to RJN, a Brailsford Robertson Grant and The University of Adelaide in Adelaide, Australia, and sponsored with a product (Optifast VLCD) by Novartis USA
Mutsaerts et al. (32), 2016 University medical centres and general hospitals Netherlands	577	29.7 (4.5)/ 29.8 (4.6)	27.7 (range 24.4-31.0)/	Motivational counselling: outpatient visits, telephone consultations, assistance of an online diet diary, advise to engage in moderate intensity physical activity	Prompt infertility treatment with no previous interventions	Live birth Undesirable effects (miscarriage) Pregnancy rate (clinical, multiple) BMI change Weight change	24	Grant (50-50110-96-518) from the Netherlands Organization for Health Research and Development
Palomba et al. (33), 2010* Setting Units of Reproductive Medicine and Surgery Italy	96	28.43 (8.31)/ 26.50 (4.26)	31.05 (2.98)/ 32.3 (3.73)	Structured exercise training plus hypocaloric diet for 6 weeks, with one cycle of CC after the first 2 weeks	2 weeks of observation followed by one cycle of CC therapy	BMI change Weight change Ovulation rate Reproductive outcomes Changes in anthropometric and hormonal and metabolic parameters Compliance with the interventions	Not reported	Not reported

Table 1: Continued

Study ID, Setting, country	Women	Age (Y) Mean years (SD) Experimental/ control group	BMI at baseline Mean (SD) Experimental/ control group	Experimental intervention	Control intervention	Outcomes	Follow-up (months)	Funding
Rothberg et al. (34), 2016 University of Michigan (UM) Health System, Ann Arbor, Michigan USA	14	33 (5.0)/ 30 (4.0)	41 (4)/ 41 (4)	Intensive weight loss interventions consisted of 12 weeks of very-low-energy diet (800 kcal/day) plus 4 weeks of a low-calorie conventional food-based diet	Standard-of-care nutritional counselling consisted of 16 weeks of conventional food-based diet	Live birth Pregnancy rate BMI change Weight change	12	Grant from the Michigan Institute for Clinical Research (grant U040012 PI to A.R.); the core services of the Michigan Nutrition Obesity Research Centre (grant DK089503); and the Michigan Centre for Diabetes Research (grant P30DK020572)
Sim et al. (16), 2014, Royal Prince Alfred Hospital (RPAH) Fertility Unit, Sydney, Australia	49	32,9 (3.3)/ 32,8 (3.1)	35.1 (3.8)/ 38.0 (5.2)	A very-low-energy diet for the initial 6 weeks followed by a hypocaloric diet, combined with a weekly group multidisciplinary programme	Recommendations for weight loss and the same printed material as the intervention.	Live birth Undesirable effects (miscarriage) Pregnancy rate (clinical, assisted, natural) BMI change Weight change	12	National Health and Medical Research Council of Australia and from the Sydney University Nutrition Research Foundation to KAS. Prima Health Solutions provided the VLED (KicStart)

SD; Standard deviation, SE; Standard error, CC; Clomiphene citrate, BMI; Body mass index, IVF; *In vitro* fertilization, ICSI; Intracytoplasmic sperm injection, *; Palomba et al. study had 3 groups, but we include only group B and C described in the Table. The group A received structured exercise training plus hypocaloric diet for 6 weeks without CC.

Table 2: Summary of review findings

Outcomes	Anticipated absolute effects (95% CI)		Relative effect (95% CI)	Number of participants	Quality of the evidence
	Risk with usual care	Risk with lifestyle interventions (*)			
Live births IVF live births	242 per 1.000	346 per 1.000 (181 to 655)	RR 1.43 (0.75 to 2.71)	433 (4 RCTs)	⊕⊕⊖⊖ Low[1, 2]
Live births All live births	405 per 1.000	563 per 1.000 (365 to 867)	RR 1.39 (0.90 to 2.14)	1034 (7 RCTs)	⊕⊕⊖⊖ Low[2, 3]
All pregnancies	502 per 1.000	718 per 1.000 (507 to 1.000)	RR 1.43 (1.01 to 2.02)	1034 (7 RCTs)	⊕⊕⊕⊖ Moderate[3]
Miscarriage	142 per 1.000	213 per 1.000 (148 to 307)	RR 1.50 (1.04 to 2.16)	543 (6 RCTs)	⊕⊕⊕⊖ Moderate[4]

***; The risk in the intervention group [and its 95% confidence interval (CI)] is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI), [1]; Two studies had high risk of performance bias (open trials), and additional one high risk of attrition bias, [2]; The confidence interval of effect estimate includes both an effect for the intervention and the control condition, [3]; Five studies had high risk of performance bias or detection bias (open trials), and two reported selectively their outcomes, and [4]; Four studies had high risk of performance bias or detection bias (open trials), three had high risk of attrition bias and one reported selectively its outcomes. Grade working group grades of evidence: High quality; Further research is very unlikely to change our confidence in the estimate of effect. Moderate quality; Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate. Low quality; Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate. Very low quality; We are very uncertain about the estimate.**

Effect of lifestyle interventions in primary outcomes

Seven studies reported the live birth with a total number of 1034 patients (28-34), and showed that lifestyle interventions had no effect on live birth rates (RR: 1.39, CI: 95% 0.90 to 2.14; I^2=65%; Fig.3).

We rated this outcome as low-quality due to limitations in study designs and imprecision in the effect estimate. On the other hand, the intervention led to higher pregnancy rates according the pooled results of seven trials including 1098 women (RR: 1.43, CI: 95% 1.02 to 2.01; I^2=60%; Fig.4) (16, 28-31, 33). Twenty-one more women out 100 participating in a lifestyle intervention became pregnant in comparison to women receiving usual care (CI: 95% 0.5 to 38 more).

A subgroup analysis of studies assessing interventions based on dietary restriction (28, 29) or in combination with physical activity (16, 30-32, 34) did not show changes any the effect estimates magnitude or direction (in the Materials S4, See Supplementary Online Information at www.ijfs.ir).

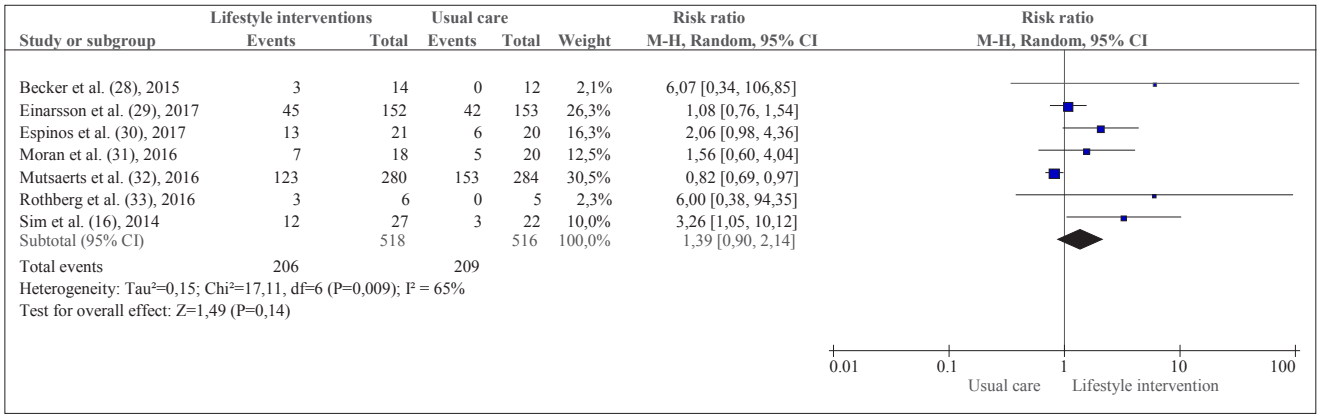

Fig.3: Live Birth-pooled analysis.

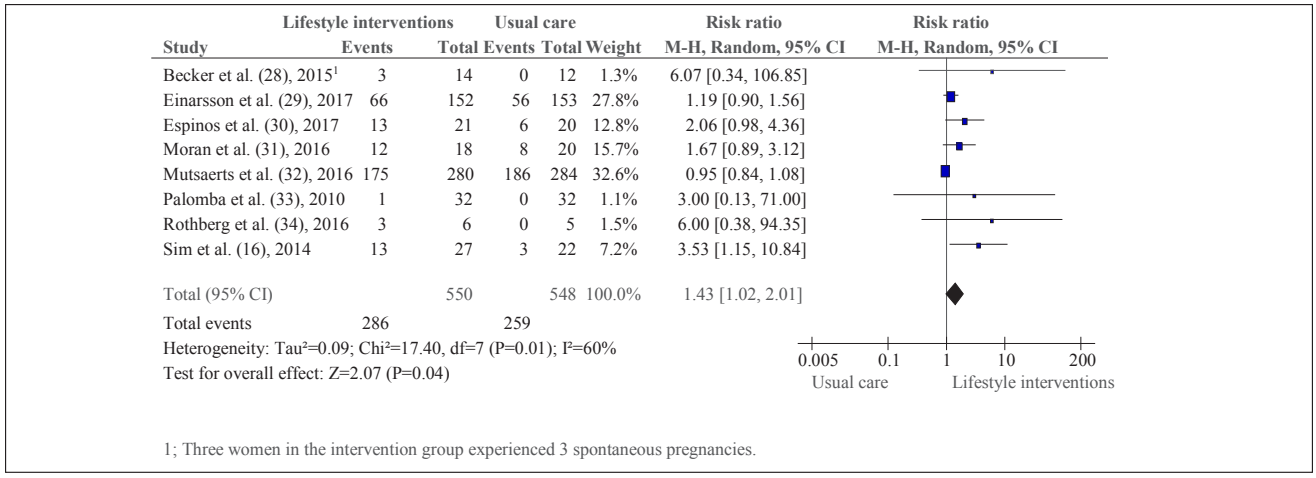

Fig.4: Pregnancy rate-pooled analysis.

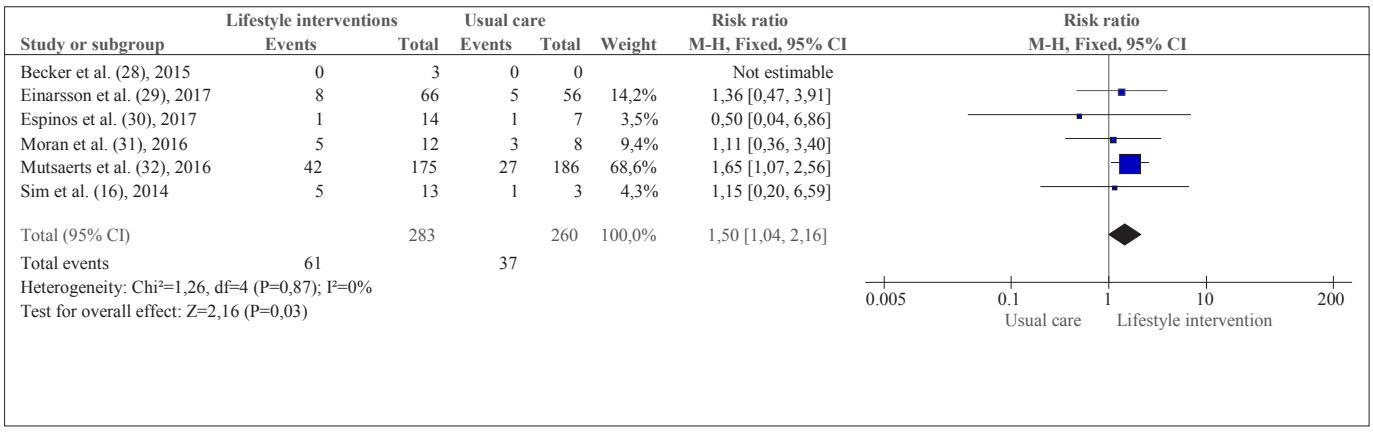

Fig.5: Miscarriage-pooled analysis.

Notably, the results from six studies with a total number of 543 participants (16, 28-32) showed a statistically significant increase in the risk of miscarriage in women allocated to lifestyle interventions (RR: 1.50, CI: 95% 1.04 to 2.16; I^2=0; Fig.5), resulting in seven women more out of 100 allocated to lifestyle interventions having a miscarriage in comparison to women receiving usual care (CI: 95% 0.6 to 9 more). We rated pregnancy rates and miscarriage as moderate quality due to limitations in studies design. This increase in the risk of miscarriage disappeared in a subgroup analysis of interventions that were exclusively based on a dietary restriction according

the pooled results from two trials (125 participants; RR: 1.36; 95% CI: 0.47 to 3.91; I^2=0) (Materials S4, See Supplementary Online Information at www.ijfs.ir).

After exploring possible sources of heterogeneity, we performed a sensitivity analysis excluding from the pooled analyses one trial that could have an impact on the consistency of effect estimates (32). The results of these analyses resulted in a statistically significant increase in live birth rates that favoured the intervention (6 trials, 470 participants; RR: 1.69; 95% CI: 1.05 to 2.70; I^2=34%), while the impact on miscarriage switched to a non-significant difference (5 trials, 182 participants; RR: 1.16; 95% CI: 0.59 to 2.30; I^2=0%) (Materials S5) (See Supplementary Online Information at www.ijfs.ir).

Cumulative pregnancy rate was not reported in the included studies.

Effect of lifestyle interventions in secondary outcomes

The participation in a lifestyle intervention did not show differences, compared to the usual care, in the rate of ongoing pregnancies (32) (317 participants; RR: 0.91, CI: 95% 0.79 to 1.05) or implantation rates (30) (65 participants; RR: 1.32, CI: 95% 0.72 to 1.69). We rated these outcomes as low due to the imprecision in effect estimates.

Discussion

We included eight trials, providing a total of 1175 infertile obese women randomised to receive a type of diet and/or exercise structured program versus usual care before undergoing an assisted reproduction program. In all included studies, experimental interventions significantly lowered the women's weight; however, there were some variations in the measure effects between the studies. The main findings of our systematic review suggests that lifestyle interventions may have little or no impact on the live birth rates of obese infertile women who wish pregnancy.

On the other hand, our results showed an increase in the risk of miscarriage rate in seven more pregnant women out of 100 receiving the intervention instead of the usual care. The sub-group analyses according to the components of the intervention of interest (dietary restriction alone or in combination with physical activity) did not have major impact on our findings. No studies reported the cumulative pregnancy rate.

Our review surveyed rigorous methodological standards, and we set the methods used in our review in a protocol prospectively registered. Most of the review steps were conducted independently by pairs of reviews to ensure the accuracy of judgements and data. We made an effort to identify all the relevant trials eligible for our inclusion criteria and asked missing data in published reports to the authors to avoid selective reporting bias. The review has also some limitations, and we obtained few missing data from trials and the data extracted from trial reports. This fact did not allow us to undertake reliable analysis to explore the effects of the intervention in terms of different characteristics of women participating in other studies, the interventions assessed or the control conditions. Also, we limited inclusion to randomised trials that allowed us to obtain reliable effect estimates but omitted the results from a body of controlled observational studies (see excluded studies at Materials S2, See Supplementary Online Information at www.ijfs.ir) that could bring light to the findings of our review. We also found some high heterogeneity related with the different types of interventions for reducing weight and the discrepancies in women's characteristics, such as age and the baseline values of women's weight between studies. We rated the quality of evidence for primary outcomes as moderate-to-low due to the limitations in the included studies design and the imprecision in effect estimates.

The increase in the miscarriage rate is an unexpected finding since obesity has been related to a lower oocyte quality and endometrial receptivity increasing the risk of pregnancy loss. However, the study by Mutsaerts et al. (32) in comparison with the other studies introduced clinical heterogeneity because women had lower BMI and the control group received a higher number of infertility treatments; furthermore, the assessed intervention lasted for a longer period and the study presented attrition bias (22% of losses). For these reasons, we excluded Mutsaerts et al. (32) study in the sensitivity analysis. In consequence, results changed to lifestyle interventions increased of live birth and there was not difference in the risk of miscarriage compared with the control group. These results are more consistent with recent data that show an association of weight gain ≥5% with a higher risk of pregnancy loss compared with maintaining a constant weight. The weight loss ≥5% did not associate with the increased risk of pregnancy loss (35). Other systematic reviews have reported the effect of diet and/or exercise on obese fertile women. One review (36) assessed the effect of low carbohydrate diet on fertility hormones and pregnancy in overweight and obese women with a methodology that differed from our review and with inconclusive results regarding the impact of intervention on the pregnancy rate. Another review also focused on assessed weight-loss interventions in overweight and obese women with broader inclusion criteria (the review included non-randomized studies and also assessed weight reduction drugs) (18). Pooled analysis from randomized trials showed similar results for the pregnancy rate and live birth, but did not show any increase in the rate of miscarriage, as shown by our findings.

Lifestyle intervention programmes targeted to people with overweight or obesity usually result in poor compliance rates and gender have been identified as one of the critical predictors for adherence, which is lower in women (37). On the other hand, a great majority of obese women facing an infertility treatment with interest

in a supervised medical weight-loss programme would not be willing to delay the fertility treatment more than three months to attempt weight-loss (38). These considerations are relevant in the light of the review findings when making a decision to initiate a programme such those described but facing low expectations from it in terms of the fertility treatment success. In that context, an individualized and shared decision should be made exploring patient motivation and other compliance predictors, such as age, baseline BMI, and mood (37).

Conclusion

Lifestyle interventions in obese infertile women based on dietary restrictions and physical activity probably lead to a slightly increase in the pregnancy rate compared with the usual care and make little difference in the improvement of live birth. Furthermore, our findings suggested a link between these interventions and a slightly increase of the risk of miscarriage. More research is needed in obese women undergoing infertility programs to further confirm or refute our findings.

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Authors' Contributions

J.J.E.; Conceived the study. I.S.; Designed and conducted the search. C.V., L.Z., M.J.M.-Z.; Screened search results for eligibility and extracted data from relevant studies. I.S., C.V., L.Z., M.J.M.-Z.; Assessed the risk of bias. M.J.M.-Z., J.J.E., I.S., C.V.; Drafted the manuscript and the rest of authors contributed to preparation of the manuscript. J.J.E., A.P.; Designed and conducted one of the included trials. All authors read and approved the final manuscript.

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Original Article

Effect of Mindfulness-Based Group Counseling on Depression in Infertile Women: Randomized Clinical Trial Study

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Abstract

Background: Assisted reproductive technology (ARTs) such as *in vitro* fertilization (IVF) can lead to depressive symptoms in infertile women due to their low success and high costs. Mindfulness-based group counseling can decrease depressive symptoms by increasing mental concentration. The aim of the present study was to evaluate the effect of mindfulness-based group counseling on depression in infertile women undergoing IVF.

Materials and Methods: The present clinical trial included 90 infertile women undergoing IVF treatment in an infertility center in 2016. Women were divided into two groups, intervention and control. Both groups completed a demographic questionnaire and the Beck depression inventory (BDI). Eight 90-minute sessions (two each week) of mindfulness-based group counseling were held with the intervention group, while the control group received treatment as normal. Following the intervention, the BDI was again completed by both groups. The data were analyzed and independent t tests and, paired t tests conducted at a significance level of $P<0.05$.

Results: No statistically significant demographic differences were observed between the two groups. Women in the control group had a somewhat lower depressive symptom score than the intervention group before the intervention. However, compared with before, the depressive symptom score among women in the intervention group decreased significantly (48%) ($P<0.001$) after the intervention. In contrast, the depressive symptom score in control women was higher after the intervention than before.

Conclusion: According to the findings of the present research, mindfulness-based group counseling is able to reduce depressive symptoms in infertile women under IVF treatment. Therefore, group counseling sessions are suggested for all depressed women undergoing infertility treatment (Registration number: IRCT2015082013405N14).

Keywords: Counseling, Depression, Female, Infertility, Mindfulness

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Introduction

Primary infertility is defined as an inability to conceive after 1 year of unprotected sex (without using contraceptives), and can be related to the male or female partner or both (1). Worldwide, more than 80 million people are infertile (2).

The WHO states that inability to bear a child, either due to the inability to become pregnant or the inability to carry a pregnancy to a live birth following either a previous pregnancy or a previous ability to carry a pregnancy to a live birth. In 2010, among women 20-44 Y of age who were exposed to the risk of pregnancy, 1.9% (95% uncertainty interval 1.7%, 2.2%) were unable to attain a live birth (primary infertility). Out of women who had had at least one live birth and were exposed to the risk of pregnancy, 10.5% (9.5%, 11.7%) were unable to have another child (secondary infertility) (3).

Prevalence varies between countries with a global average of 12 to 15%. Infertility can be divided into two groups; primary (no conception occurring over the past year) and secondary infertility (conception without giving birth to a living child). In Iran the prevalence of primary infertility based on the WHO's clinical, epidemiological and demographic definitions. is 20.2, 12.8 and 9.2%, respectively (2, 3). At a global level, the primary infertility rate is 0.6 to 3.4%, and the secondary infertility rate is 8.7

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to 32.6%. In Iran, the mean primary and secondary infertility rates are 10.6% and 2.7%, respectively (4).

In response to the infertility rate, rapid progress in reproductive medicine has contributed to new technologies associated with the care and treatment of infertile couples across the world (4). Assisted reproductive technology (ART), including a wide range of treatments and approaches, is a common and successful treatment in many countries (5). One of the techniques is *in vitro* fertilization (IVF), a complex series of procedures commencing with extreme and controlled ovarian stimulation by exogenous gonadotropin, including techniques wherein fertilization is undertaken using intra-cytoplasmic injection of sperm, gamete transference to the fallopian tube, transfer of zygote into the fallopian tube, and the transfer of the peritoneal tube by laparoscopy (6). Epidemiological findings have documented high levels of depression in different countries. In 1990, the prevalence of depression was 472 million worldwide with, around 5 million in Iran, showing the high prevalence and importance of depression disorder on both global and national scales (7). Depression can increase during periods of infertility, and it is estimated that approximately 86% of infertile couple experience depression (8). One study showed that although the events and conditions that reveal depression, anxiety and stress differ from person to person, depression in infertile women is twice that in fertile women (9).

Although most people who seek infertility treatment seem to be emotionally stable, infertility is known to be a life-long crisis. Most infertile people have to deal with depression, feelings of loss and guilt, detachment, meaninglessness, and sexual and marriage problems. In addition, physical, psychological and economic problems associated with ART influence the psychological stability of couples (10). Psychological treatments administered along side infertility treatment programs, make infertile women more resistant to stress, increase the effectiveness of infertility treatments, and encourage infertile patients to follow the treatment by enhancing their mental health (11).

Studies conducted in infertile women have indicated the positive effect of counseling and psychological interventions on improving life quality (12). Mindfulness-based interventions are a common type of cognitive-behavioral therapy. Mindfulness is a form of meditation rooted in the eastern religious rituals, especially those related to Buddhism (13). Mindfulness is one of which is high awareness, focusing on the reality of the present, accepting and acknowledging it, regardless of the thoughts about the situation or emotional reactions to the situation (14). In essence, mindfulness consists of an informed and nonjudgmental sense of what is happening now (15). Pots et al. (16) document the important role of the learned skills of attention control in mindfulness meditation in preventing depression relapse. Based on their information processing theory, those who have experienced major periods of depression are susceptible to relapse when faced with a dysphoric state, because these states can activate the depressed thinking patterns of the period of depression. In this study, Mindfulness-Based Cognitive Therapy (MBCT) was employed as it includes meditation techniques for mindfulness and meditation along with daily activities for depression (17).

Given the problems of infertile women, such as depression, the prevalence of infertility and the few studies conducted in Iran, especially on the impact of group counseling on infertility and the lack of comprehensive therapeutic methods in the field of counseling, the present study aimed to evaluate the effect of mindfulness-based group counseling on depression in infertile women under IVF treatment.

Materials and Methods

Instruments

Demographic questionnaire

Demographic characteristics were assessed using a questionnaire designed by the researchers. It included questions about the personal characteristics of infertile women and their partners (10 questions), expenditures and the existence of health insurance coverage (2 questions), duration of marriage, duration of infertility, number of infertility years, frequency of IVF use and questions regarding psychiatric history (5 questions). Personal information included: first and last name, place of residence, age, employment, and education of the women and their partners, and monthly family income. Infertility was either primary (no pregnancy) or secondary (only pregnant once). Questions related to psychiatric histories included history of admission to psychiatric hospitals, history of mental illness, and use of psychiatric drugs and narcotics.

Beck depression inventory

The second Beck depression inventory (BDI-II) is a depression inventory and a self-report index for measuring depression symptoms in different clinical and nonclinical populations. Published in 1996 the second edition of BDI-II inventory was developed in response to the American Psychiatric Association's publication of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), which changed many of the diagnostic criteria for Major Depressive Disorder (American Psychiatric Association, 1994). This inventory is a 21-item self-reported measure of depression with 15 questions related to psychological symptoms and 6 questions related to physical symptoms. Time frame for BDI-II is consistent with the 1-2 weeks time frame for major depressive disorders in DSM-IV. All the questions assess the severity of the disorder based on a Likert scale (0-3). The total score of a participant is obtained by aggregating the scores of all questions of 0 to 63. Based on Beck's suggested scoring, a score of 0-9 indicates the absence of depression, 10-18 indicates mild to moderate depression, 19-29 moderate to severe depression, and

30-63 severe depression. Since the results of many studies of the BDI-II have shown its validity and reliability in different countries, the same questionnaire was used in the present research. Rajabi and Karjo (18) (according to Karmoudi study) obtained a Cronbach's alpha coefficient of 0.91 for a student sample and reliability coefficients of 0.90, 0.87, and 0.44 for the whole questionnaire, the cognitive-emotional factor, and the physical factor, respectively. In the study of Khormaei et al. (19) (according to Dobson and Mohamadkhani study), the reliability coefficient measured as Cronbach's alpha was 0.91 and Goodarzi reported a Cronbach's alpha of 0.84 for internal consistency. In this study, Cronbach's alpha coefficient for the reliability of the BDI-II was 0.78.

Procedures

The present clinical trial (IRCT2015082013405N14) which included a pre-test, post-test, and control group was conducted in women with diagnosed primary infertility who were in the early stages of IVF. Inclusion criteria were age 25-40 years, high school education or more, residency in Hamedan, no psychiatric hospital admissions, no addiction, no neurological or other progressive diseases, and no psychiatric drug use. Level of depression [mild mood disturbances, moderate depression, and severe depression (up to 63)] were determined using cut-off points of the BDI-II. Exclusion criteria were absence from more than two counseling sessions in the test group, natural pregnancy and no use of ART during treatment, and incidence of physical or psychological illness during the study. Women meeting the inclusion criteria and who agreed to participate in the study were selected prior to IVF treatment. Based on the eligibility criteria, a convenience sampling approach was used to select the participants. Among the 120 women who met the inclusion criteria, 90 women were enrolled in the present study.

According to Khormaei et al. Study, if the first type error is 5% and the study power is 90%, the mean score of the first group is 12 and the second group is 10, with a standard deviation of 3 need 41 persons in both groups (82 persons in total). On the other hand, the sample size is increased to 45 persons in each group in order to counter the probable loss of 10% (19).

It should be noted that applying the above equation is equivalent to using the following formula:

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

After enrollment, the women were divided into intervention and control groups by block randomization, and group counseling was delivered to the intervention group. We constructed 10 blocks of 4 and one block of 5 (45 women), and randomly assigned the participants to the two study groups by assigning the next block of participants according to the specified sequence (Fig.1).

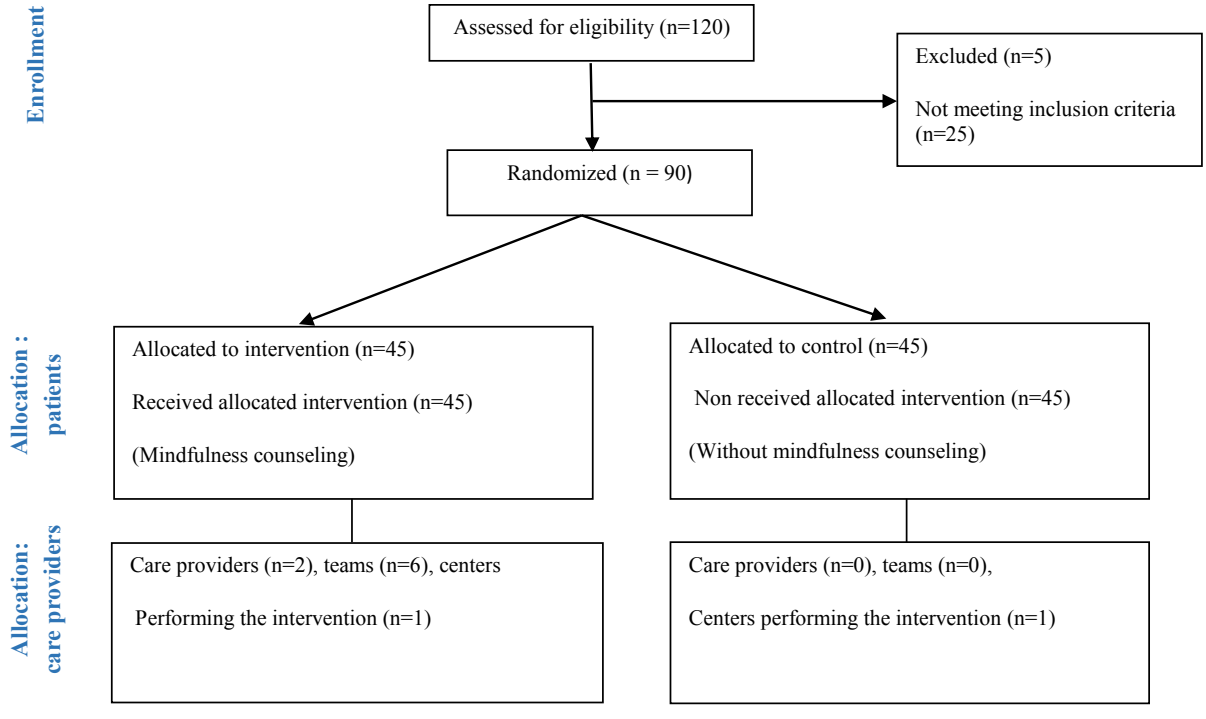

Fig.1: Modified CONSORT flow diagram for individual randomized controlled trials of nonpharmacologic treatments.

Before starting the study, the aim of the study was explained and verbal and written informed consent was obtained from the women. First, the 45-member intervention group was divided into three 8-member groups and three 7-member groups to increase the efficiency of group counseling sessions. After that, eight 90-minute group counseling sessions were held twice a week (the IVF process can last for 4 to 6 weeks) using mindfulness training packages. Counseling axes included auto-guidance, confrontation with obstacles, breathing with mindfulness, staying in the moment, the untruthfulness of thoughts, and how to take optimal care of oneself (Table 1). These counseling sessions were organized by a researcher trained by a senior researcher with a Ph.D. in clinical psychology, overseen by the professor of psychology in the research team. At the end of each session, an educational note and a CD related to that session were given to participants. During the counseling sessions, participants were divided into small class groups to interact with group members, and state and explain their problems. We used R software (version 3.5.2), a free and open source software for the statistical analysis.

The participants in the intervention group were asked to practice conscious yoga exercises at home and present the principles of counseling, goals, and exercises of the previous session at the beginning of each session. Moreover, in order to resolve possible ambiguities, women in the intervention group were asked to do all exercises in class with the researcher. This resulted in more repetition and training, and helped the creation of a new mindset. During the counseling sessions, we tried to fully explain the meaning and concept of mindfulness through daily routine examples, stories, and conscious yoga exercises. This method was also employed in the infertility center while the women were undergoing their IVF treatment. The control group received routie programs of infertility center and did not recive any intervention. Due to ethical considerations at the end of the study the educational pamphlets and the CD were administered to the control group. Pre-test assessments were conducted on the 90 randomised participants prior to commencing IVF treatments, meaning all members of both groups completed the demographic information and Beck depression inventories. After the intervention the post-test was performed using the BDI-II 3-7 days before the embryo transfer stage. At this point depression is at its minimum and the effect of the intervention on intervention group can be determined better. The counseling sessions are shown in Table 1.

Table 1: Mindfulness training taken from Crane R. Mindfulness-based cognitive therapy (20)

Session	Goals	Practical exercise per session	Each session's program	Homework
1	Automated guidance	Eating a raisin with mindfulness meditation on body checking	Making a group, presenting the moral code of the method and group boundary, introducing participants, providing explanations about infertility and the resulting depression and the necessity of mindfulness training, explaining the automated guidance system	1. Concentration on body checking for 45 min 2. Attention to daily routines such as daily showers 3. Eating a meal once a week with mindfulness
2	Facing obstacles	Body checking meditation, 10-minute breathing with meditation and mindfulness	Thinking about practices and the exact feeling of each	1. Reviewing the previous session 2. 4-minute body checking meditation 3. 10-minute breathing with mindfulness 4. Focusing on continuous activity to experience a pleasant day or event
3	Breathing with mindfulness	Conscious breathing and stretching practice, breathing and stretching with mindfulness	3-minute breathing, Identifying and recording pleasant experiences or unpleasant ones to be studied in the fourth session	1. Reviewing the previous session 2. Continuity and breathing exercises on days 1, 3 and 5 of a week 3. Practicing movements consciously on days 2, 4 and 6 in a week 4. Daily recording of pleasant experiences 5. Three minutes of breathing over three periods of time
4	Staying in the moment	5-Minute seeing or listening with mindfulness 3-minute breathing space, and walking with mindfulness	Discovering unpleasant experiences, detecting and defining depression problems or alternate group focus.	1. Revewing the previous session 2. Creating relaxation and meditation 3. 3-minute normal breathing (3 times a day) 4. 3-minute patterning breathing (as a meditative strategy while experiencing unpleasant feelings)
5	Acceptance and authorization of presence	Awareness of breathing and body, emphasizing the perception of how to react to thoughts, feelings and body sensations. 3-minute breathing	Reading Guest House poems by Rumi's works and identifying them in the group, practicing the discovery of reactions to normal patterns and the application of the potential talents of mindfulness skills to facilitate the response to the present-day experiences.	1. Reviewing previous session's assignments. 2. Thinking in sitting position. 3. 3-minute normal breathing (three times a day) 4. Four minutes of patterned breathing (as a meditative strategy in the experience of unpleasant feelings) 5. Reopening (body doors) and entering the outside realm of the body (in the body)

Table 1: Continued

Session	Goals	Practical exercise per session	Each session's program	Homework
6	Thoughts do not have a real origin	Meditation sessions, awareness of breathing and body, highlighting the patient's problems during exercise and detecting their effects on the body and mind.	Training in changing behaviors, thoughts, and attitudes, start the development of personal rehabilitation and activity plans, and preparing the participants for the end of the course	1. Reviewing the previous session's assignments 2. 40 minutes of daily practice, with different combinations of the three main exercises 3. Exploring the use of short-term exercises 4. 3-minute normal breathing (three times a day) 5. 3-minute patterned breathing (as a meditative strategy when experiencing unpleasant feelings) 6. Reflection and work on the plan to prevent personal recurrence
7	How can we look after ourselves?	-Meditation sessions - Awareness of breathing, organs, sounds, thoughts, and emotions. -3 minutes of breathing - Highlighting a problem during exercise and detecting its effect on the body and mind.	Discovering the relationship between activity and mood, a general list of daily activities and considerations (emotional drainage) that empowers the body, exploring ways to increase activity (useful), recognizing relapses and activities that cause recurrence.	1. Reviewing previous session's assignments 2. The breathing space in accordance with the routine as a coping strategy 3. Discovering a wa to do dexterous work after practice 4. Developing an early warning system for recurrence detection 5. Developing a practical plan that can be used in depressed moods
8	How to use these factors in future decision making	End course body checking meditation	Reviewing early warning system and practical plans (to use in high-risk relapsing time), reviewing all previous sessions, discussing the way of preserving motor power, developed in formal and informal exercises. End of course and acknowledgments.	1. Reviewing previous session's assignments 2. Making questions to answer the personal reflections during the day 3. Doing homework(Mindfulness exercise with booklet study) and practice at home alone (21).

Data analysis

The Kolmogorov-Smirnov test was used to confirm the normal distribution of all the variables. Data were analyzed includes independent t test and using IBM SPSS V.21, (http://www.meta-analysis.com), to provide descriptive statistics, such as mean and standard deviation, for the quantitative data. Independent tests and Chi-square tests were employed to compare the variables before and after the intervention; paired t tests were employed to compare variations between the groups. The significance level was assumed to be $P<0.050$.

Ethical considerations

This study code IR.UMSHA.REC.1395.336 was approved by the Ethics Committee and Research Council of Hamedan University of Medical Sciences. For ethical considerations, at the end of the study, educational notes and CDs were given to the control group.

Results

In the present study, 90 women meeting the inclusion criteria were divided into two groups of intervention (45 women) and control (45 women); and the effect of mindfulness-based group counseling on depression in infertile women undergoing IVF treatment was evaluated. The mean age of the infertile women in the intervention and control groups was 30.28 ± 5.39 and 29.64 ± 4.71 years, respectively and the mean age of their partners was 34.82 ± 4.97 and 34.37 ± 5.39 years, respectively. Mean marriage duration in the intervention and control group was 8.28 and 8.16 years, and the mean infertile period was 5.26 and 4.39 years, respectively. The majority of infertile women in the intervention (84.4%) and control (71.1%) group were unemployed and most of their partners were employed, 97.8% in the intervention group and 95.6% in the control group. The majority of infertile women in the intervention (57.8%) and control group (57.8%) had a high school diploma. Others had a license and master's degree; intervention group (40.0-2.2%) and control group (35.6-6.7%) (P=0.08). Most of their partners, 66.7% in the intervention group and 44.4% in the control group, had high school diploma. Others had a license and master's degree; intervention group (24.4-8.9%) and control group (33.3-22.2) (P=0.63). Most of the patients in the intervention (86.7%) and control group (62.2%) had health insurance, although most of the treatment costs in both groups were not paid by their health insurance [intervention group (73.3%) and control group (84.4%)]. The frequency of IVF was divided into five categories (0-1, 2, 3, 4 or 5 times): the majority of women in the intervention group had used 0 and 1 times (37.8%) and the majority of subjects in the control group had used 0 times (35.6%) of the IVF treatment. The mean number of previous IVF treatments in the intervention and control groups was 1.11 and 1.24 respectively (Table 2). Mean depressive symptoms scores in the intervention and control group before and after the intervention (the intervention in mindfulness counseling in the intervention group) were 20.77, 10.82, and 17.95, 21.33, respectively (Table 3). Before the intervention the mean depression score was lower in the control group than in the intervention group (P=0.046). As seen in Table 3, there is a significant relationship between before and after intervention in the

Table 2: Comparison of the mean and standard deviation of certain demographic characteristics (age of men and women, male income, duration of marriage and duration of infertility) in the two groups

Group	Intervention group	Control group	P value
Infertile women's age (Y)	30.28 ± 4.41	29.64 ± 4.71	0.500
Partners' age (Y)	34.82 ± 4.97	34.37 ± 5.39	0.680
Partner's income (Toman)	10681707 ± 2215337.3	1617777 ± 669222.3	0.850
Marriage duration (Y)	8.28 ± 3.45	8.16 ± 4.12	0.870
Infertility duration (Y)	5.26 ± 3.20	4.93 ± 3.38	0.620

Data are presented as mean ± SD.

intervention group (P<0.001), meaning after intervention, the mean depression was significantly reduced. After the intervention the mean depression score in the intervention group was reduced by 48% (P<0.001). In contrast, the mean depression score in the control group had increased by 19% (P<0.001), so that the depression score among women in the intervention group after the intervention was less than half that in the control group (P<0.001). The heterogeity and bias in base line data were solved by using ANCOVA Test.

Table 3: Comparison of average depression scores in infertile women before and after intervention in the experimental and control groups

Group	Depression (Before)	Depression (After)	P value for test of difference
Experimental	20.77 ± 6.35	10.82 ± 7.16	<0.001
Control	17.95 ± 6.85	21.33 ± 6.48	<0.001
P value for test of difference	0.0460	<0.001	

Data are presented as mean ± SD.

Discussion

The aim of the present study was to evaluate the effect of mindfulness-based group counseling on depression in infertile women undergoing IVF treatment. Our results showed that mindfulness-based group counseling reduced depression scores in infertile women. This findings is in line with Hoveyda et al. (21) who measured the effect of stress reduction-based mindfulness and conscious yoga on anxiety, depression, and stress in infertile women, and observed a significant reduction in depression from before to after the intervention in the intervention group. In the present study, 8 X 90-minute sessions of cognitive therapy-based mindfulness counseling were held, while in the mentioned study, there were 8 X 120-minute sessions. However, the content of mindfulness sessions was the same in both studies.

Galhardo et al. (22) studied the effectiveness of mindfulness programs in infertility, and showed a reduction in depression symptoms after the intervention in the intervention group in line with the current study. In addition, their study, like ours, showed an increase in depression scores in the control group after the intervention. Hoveyda et al. (21), in contrast, found no significant difference in depression scores before and after the intervention in the control group. In the present study, there was significant difference between the intervention and control groups regarding the symptoms of depression prior to the intervention. Mentioned study demonstrated that there was no significant difference concerning depression between the intervention and control groups before and after the intervention, which is against of the current results. The study of Galhardo et al. (22) consisted of 55 infertile women in the intervention group and 37 in the control group. The content of this study is similar to that of the present study, including body checking meditation, 3-minute body space, thought and sound meditation, and staying in the present. However, in the study, 10 X 120-minute counseling sessions were held.

Panahi and Faramarzi (17) found a significant improvement in depression symptoms in premenstrual women of the intervention group using mindfulness-based cognitive treatment, compared with the control. Also in findings similar to ours, they found mindfulness-based cognitive treatment to produce a significant improvement in depression symptoms in premenstrual women, P=0.007 compared to control women. Strege et al. (23) showed that depression scores of pregnant women in the intervention group (Positive Affect and Social Anxiety Symptoms) were considerably less than in the control group. We conclude that a counseling approach can play a major role in the reduction of mental disorders such as depression (24) and suggest that it should be included as routine during IVF treatment in infertile women.

One of the limitations of this study was the inadequate completion of the questionnaires (due to their anxiety) by the study sample. In an attempt minimize the error rate in this case, the investigators talked to the participants in the study to resolve this problem and inspire confidence that information would remain confidential. Finally, it was explained to infertile women that reducing anxiety may have the effect of speeding up their pregnancy. Also, due to the length of the counseling sessions (8 sessions), some of the women in the study were not able to attend all the scheduled sessions. To minimize this problem, meeting times were adjusted based on the participants' suggested time.

Conclusion

The findings of the present study point to the effectiveness of mindfulness-based cognitive group therapy on depression in infertile women undergoing IVF treatment.

Mindfulness counseling reduced depression in the intervention group. In the control group, where no intervention was performed, the depression score increased. As mindfulness-based cognitive group therapy results in a significant decrease in depression symptoms in infertile women under IVF treatment, it is suggested that it should be available to all depressed women undergoing IVF treatment.

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Authors' Contributions

F.K., S.Z.M.; Were the project leaders and responsible for the study conception and design. F.Sh.; Was involved in the acquisition of data. Y.M.; Contributed significantly to the analysis. M.Y.; Was in charge of interpreting the data, drafting and critically revising the manuscript. All the authors provided their final approval for the completed manuscript.

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Original Article

The Effect of Lycopene Supplementation on Mood Status and Quality of Life in Infertile Men: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial

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Abstract

Background: Infertility is a major worldwide problem which is caused by several factors such as environmental, physiological, and genetic conditions. Lycopene is considered to be one of the most important antioxidants that can contribute to reducing or preventing the psychological damage that leads to infertility. Thus, the aim of this study was to evaluate the effect of lycopene supplementation on depression, anxiety and stress scales and quality of life in infertile men.

Materials and Methods: In this randomized clinical trial, 44 infertile men with oligozoospermia were randomly divided into the following two groups: the experimental group was supplemented with 25 mg lycopene, once per day for 12 weeks, and the control group received a placebo, for 12 weeks. Anthropometric and dietary data, physical activity, mood status, including depression, anxiety, stress, and quality of life scores were recorded pre- and post-intervention. Depression, anxiety and stress were assessed using a 21-item questionnaire (DASS-21) and quality of life was examined using the WHO 26-qustion questionnaire (WHOQOL).

Results: The baseline age and body mass index (BMI) were not significantly different between the two groups (age: 31.89 ± 2.51 and 32.15 ± 2.16 years old for intervention and placebo, respectively; P=0.732 and BMI: 27.20 ± 1.68 and 26.53 ± 1.53; for intervention and placebo, respectively; P=0.206). There were no significant differences in depression, anxiety and stress values between the two groups; however, depression score significantly decreased in both groups compared to the baseline levels (P=0.028 and P=0.031). No significant differences were observed in four domains of quality of life, except for psychological domain that was improved in the lycopene group compared to the baseline values (P=0.049).

Conclusion: Short term supplementation of lycopene had no effect on mood status and quality of life, except for psychological status in infertile men (Registration number: IRCT20171105037249N1).

Keywords: Anxiety, Depression, Lycopene, Quality of Life, Stress

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Introduction

Infertility is a disease of the reproductive system defined by the inability to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (1). The World Health Organization (WHO), as well as epidemiological studies estimated that the prevalence of infertility in the world is about 15%, and in developing countries, in one out of every four couples, infertility is detected (2, 3). Various psychological and environmental factors such as age, environmental and occupational pollution, ionizing radiation, heavy metals, toxic chemicals, inadequate nutrient intakes, change in lifestyle, exposure to toxin, oxidative stress, depression and anxiety contribute to spread of this disorder (4, 5). For decades, stress-related illnesses like depression and anxiety, have risen (6); for instance, Maroufizadeh et al. (7) reported that prevalence of depression and anxiety scale was 33.0 and 49.6% in infertile participants, respectively. Furthermore, various studies showed that the quality of life in infertile people is significantly lowered (8); Valsangkar et al. (9),

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in a study on 106 women who referred to the infertility center, showed that women with infertility had compromised quality of life.

The relationship between mental status and infertility is complex as infertility is a risk factor for mental illness and, psychological distress, can also be a risk factor for infertility (10). For various reasons, infertility increases the stressful conditions that cause mental harm (11). Several studies showed that oxidative stress or reduction of antioxidant defense, as well as the reduction of antioxidant enzymes, can contribute to mood symptoms (6). Moreover, changes in neurological signals, as well as inhibition of neurogenesis, which reduce the secretion of hormones and affect the synthesis of testosterone, can contribute to male infertility (12).

Dietary antioxidants, mainly vitamins E and C and β-carotene, have an important role in preventing reactive oxygen species (ROS) production lipid peroxidation (LPO) and DNA damage (13, 14). Recent studies showed antioxidants have protective effects on depressive symptoms (15, 16). Lycopene as a fat-soluble aromatic carotenoid and one of the most important antioxidants to protect against free radicals, protects against infertility in men (6). Since lycopene can alter the levels of antioxidant enzymes by altering the levels of ROS, it can contribute to reducing or preventing the psychological damage that affects infertility (6, 17).

Previous studies showed that deficiencies in antioxidants levels are major causes of oxidative stress and affect the mood status; also, they found a relationship between the level of ROS, mood status, quality of life and fertility, suggesting that various factors can negatively affect spermatogenesis through increasing the levels of ROS, and alteration of the redox balance, which favors oxidants over antioxidants (18-20). Little work has been done to explore the role of antioxidants in ameliorating mood status and quality of life in infertile people. Thus, we sought to evaluate the effect of lycopene supplementation on mood (i.e. depression, anxiety, and stress) and quality of life in infertile men.

Materials and Methods

Subjects

This double-blind clinical trial was conducted in winter and spring of 2018 at Isfahan Fertility and Infertility Center. Initially, individuals who had a history of primary and secondary infertility, for at least 5 years, were selected. After a thorough examination, 44 infertile men met the inclusion criteria. The inclusion criteria included infertile men with a sperm count less than 20 million per milliliter, normal sperm <65%, volume <3.0 ml, and average motility <60%, aged between 25 and 45 years, and not receiving any other treatments. All patients were required to stop all prior medical treatments for a period of ≥12 weeks and to sign written consent form to enter the study. The exclusion criteria included having a history of all related disorders including testicular atrophy, urinary tract infection, testicular torsion, asthenospermia, azoospermia, genital trauma, inguinal and genital surgery or other genital diseases such as current genital inflammation and cryptorchidism, anatomical disorders for example meatal stenosis, or endocrinopathy, use of androgens or antiandrogens, previous hormonal therapy, or use of cytotoxic drugs, anticoagulants, immunosuppressants or any antioxidant supplements. Patients with physiological and psychiatric disorders that may affect sperm and sexual performance, drug abuse and body mass index (BMI) ≥30 kg/m2, were also excluded (21, 22). The study protocol was approved by the Medical Ethics Committee at the Isfahan University of Medical Sciences (IR.MUI.REC.1396.3.325) and registered under the code of IRCT20171105037249N1 in the clinical trials registry of Iran.

Study design

At the beginning of the study, subjects were randomly assigned to the intervention group [that was supplemented with 25 mg lycopene (produced by 21st Century Company, USA) once per day for 12 weeks], or the control group [that received placebo (starch) for 12 weeks] and patients were advised to take the palacebo pills at lunch or dinner meal. We used the standard formula suggested for clinical trials by considering the study with type I error of 5% (α=0.05) and type II error of 20% (β=0.20) to calculate the sample size. For randomization group, the intervention 22 patients were assigned to code A, and for the placebo group 22 were assigned to code B, through the method of convenience sampling. Sample size was calculated based on sperm concentration (23). All patients and the clinician that prescribed the supplements were blind to the treatment. In order to guarantee blindness, lycopene and placebo were prepared in similar appearance.

Lifestyle information, medical history, demographic data, alcohol and tobacco use, and supplement intake were recorded for all participants. Body weight was measured (in minimal clothing), and body fat was determined by bioelectrical impedance analysis (BIA) method using the Omron BF-511 set. To measure height, a fixed non-stretchable tape was used in standing position. Then, BMI was calculated in kg/m2. The Physical activity level was assessed using the short form International Physical Activity Questionnaire (IPAQ) (24). After initial screening, dietary intakes of all patients were collected using a 3-day dietary record at the beginning and end of the study and we calculated nutrient composition of the portion size, and subsequently, energy, carbohydrate, protein, fat and lycopene intakes were obtained from food composition Tables provided by the United States Department of Agriculture sources.

Assessment of depression, anxiety and stress

Depression, anxiety and stress were assessed using a 21-item Questionnaire (DASS-21) for all participants pre- and post-intervention. The Cronbach's alpha coefficient was obtained to show the reliability of the questionnaire (0.84). Similar internal consistency coefficients were reported previously (25). Each item uses a four-point response scale ranging from 0 (did not apply

to me at all) to 3 (applied to me very much or most of the time). For each 3 subscales, 7 questions are considered. Then, based on the score given for each question, the score of each parameter of depression, anxiety and stress was calculated.

Assessment of quality of life

A 26-questions form of World Health Organization Quality of Life Questionnaire (WHOQOL) was applied. The Cronbach's alpha for all sample, non-clinical and clinical was 0.82, 0.84 and 0.82, respectively (26). It should be noted that questions 1 and 2 are used to measure the overall QoL, and 24 items encompass dimensions including social, psychological, environmental and physical issues. Environmental health was measured by 8 items, physical 7 items, psychological 6 items and social 3 items. There is no overall score for the WHOQOL and each domain is calculated by summation of their specific items. Individual's perception of quality of life is measured by summing the total scores for each particular domain. All domain scores are scaled in a positive direction (higher score indicates higher QOL).

Statistical analysis

All data are reported as mean ± standard deviation or frequency (%). The Kolmogorov-Smirnov test was used to evaluate the distribution of data. An independent samples t test was used to analyze the initial variables, dietary intake, mood status and quality of life between the two groups considering normal distribution of variables. A paired t test samples was used to compare the intragroup variables pre- and post-intervention. To control the confounding variables (energy and carbohydrate), a MANCOVA test was applied to determine the differences between the groups post-intervention. Statistical analysis was performed using SPSS software version 16 (SPSS Inc., Chicago, IL, USA). A $P<0.05$ was considered significant.

Results

In total, 44 patients were recruited for this clinical trial and divided into two groups of 22 individuals; finally, 38 subjects completed the study: 19 in the lycopene group and 19 in the placebo group. In each group 3 participants refused to take supplements or participate in the final test, and thus, were removed from the study (Fig.1). Table 1 shows the basic characteristics and dietary intake of the patients. There were no significant differences regarding the baseline characteristics between the two groups, except for energy and carbohydrate intakes ($P<0.05$).

There were no significant differences in depression, anxiety and stress values between the two groups before and after adjustment of confounders using MANCOVA test (Table 2). Depression score decreased in both groups compared to the baseline values as assessed by pair t test ($P=0.028$ and $P=0.031$).

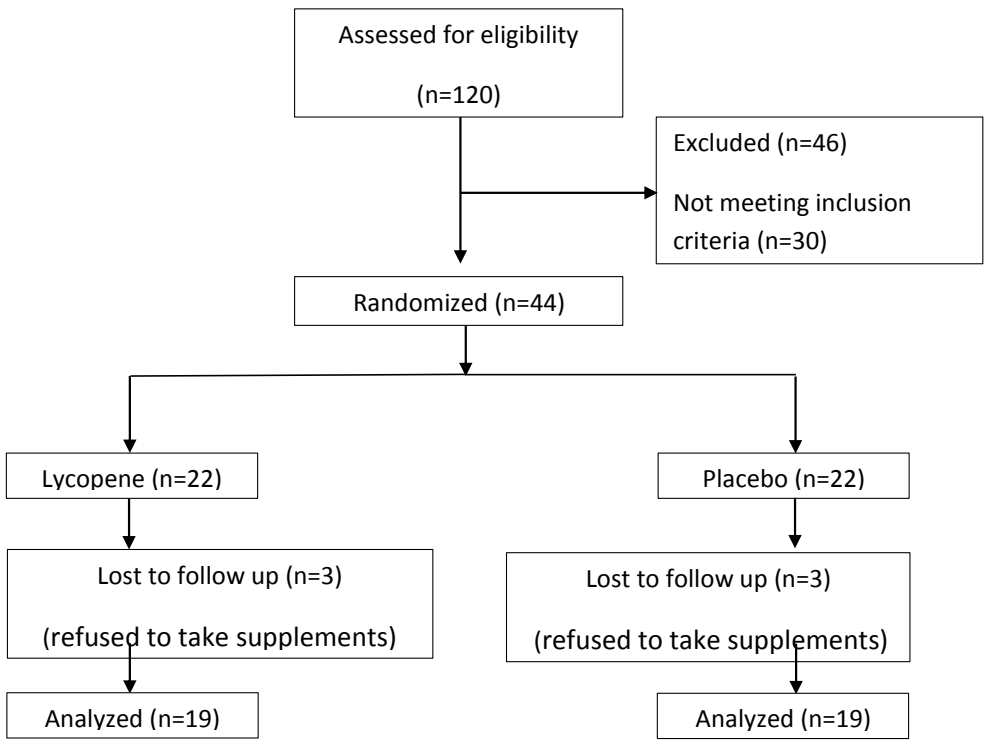

Fig.1: Flowchart of patient recruitment for the double-blind, placebo-controlled, randomized trial of lycopene supplementation in infertile men.

Table 1: Anthropometric and demographic characteristics and dietary intake of participants at baseline and end

Characteristic	Lycopene n=19	Placebo n=19	P value
Age (Y)	31.89 ± 2.51	32.15 ± 2.16	0.732
Smoking history			0.740
Yes	7 (36.84)	8 (42.1)	
No	12 (63.16)	11 (57.9)	
Drinking alcohol history			0.721
Yes	6 (31.57)	14 (73.69)	
No	13 (68.43)		
Education			0.896
≤12	3 (15.79)	4 (21.06)	
High school diploma	7 (36.84)	6 (31.57)	
Bachelor degree or higher	9 (47.37)	9 (47.37)	
Height (cm)	177.57 ± 4.79	178.78 ± 3.45	0.378
Weight (kg)	85.78 ± 6.10	84.78 ± 4.93	0.582
Body mass index (kg/m^2)	27.20 ± 1.68	26.53 ± 1.53	0.206
Body fat (kg)	28.65 ± 3.37	27.98 ± 3.69	0.564
Physical activity (MET-h/week)	30.83 ± 1.95	31.0 ± 1.71	0.707
Energy intake (kilocalories/day)			
Before	2251.39 ± 230.54	2115.53 ± 175.082	0.048
After	2326.70 ± 200.01	2113.63 ± 199.87	0.002
Carbohydrate intake (g/d)			
Before	316.56 ± 34.57	294.43 ± 28.0730	0.037
After	330.41 ± 24.83	301.02 ± 27.96	0.002
Protein intake (g/d)			
Before	88.04 ± 12.29	88.38 ± 8.52	0.922
After	90.15 ± 12.65	86.11 ± 9.64	0.27
Fat intake (g/d)	78.07 ± 16.35	72.61 ± 10.487	0.229
Before	79.59 ± 16.41	71.02 ± 11.11	0.068
After			
Lycopene intake (µg/d)	4306.46 ± 133	4664.39 ± 935.43	0.345
Before	4895.57 ± 1362.35	4839.47 ± 961.29	0.885
After			

Data are presented as n (%) or mean ± SD. Analysis done using independent-sample t test.

Table 2: Depression, anxiety and stress score of participants at baseline and end

Variable	Lycopene n=19	Placebo n=19	P value[a]	P value[b]
Depression				
Baseline	14.10 ± 2.94	13.78 ± 3.11	0.750	
End	12.73 ± 2.02	12.31 ± 2.13	0.537	0.424
P value[c]	0.028	0.031		
Anxiety				
Baseline	11.26 ± 2.23	11.47 ± 3.04	0.809	
End	10.31 ± 2.13	10.84 ± 2.43	0.483	0.510
P value[c]	0.132	0.380		
Stress				
Baseline	15.05 ± 2.34	14.52 ± 2.73	0.528	
End	14.52 ± 2.09	14.21 ± 1.98	0.636	0.700
P value[c]	0.331	0.546		

Data are reported as mean ± SD. [a]; Analysis done using Independent-sample t test, [b]; Multivariate analysis of covariance done following adjustment (for energy and carbohydrate), and [c]; Analysis done using paired-sample t test.

The effect of lycopene supplementation on four domains of quality of life (physical, psychological, social, and environmental) is presented in Table 3. There were no significant differences in all domains between the two groups before and after adjustment of confounders using MANCOVA test. Aside from the psychological domain in the lycopene group (P=0.049), no significant changes were observed in other quality of life domains as assessed by pair t test.

Table 3: Quality of life score of participants at baseline and end

Variable	Lycopene n=19	Placebo n=19	P value[a]	P value[b]
Physical health (%)				
Baseline	67.73 ± 11.21	70.31 ± 17.01	0.585	
End	71.89 ± 10.20	72.89 ± 15.34	0.814	0.743
P value[c]	0.111	0.238		
Psychological health (%)				
Baseline	66.36 ± 13.75	69.00 ± 19.39	0.632	
End	69.52 ± 10.99	71.57 ± 15.47	0.640	0.998
P value[c]	0.049	0.233		
Social relation health (%)				
Baseline	72.05 ± 17.57	71.31 ± 22.28	0.911	
End	71.89 ± 12.16	72.89 ± 17.50	0.839	0.680
P value[c]	0.936	0.480		
Environmental health (%)				
Baseline	67.36 ± 13.38	65.57 ± 21.45	0.760	
End	67.42 ± 11.25	65.52 ± 15.95	0.675	0.578
P value[c]	0.977	0.980		

Data are reported as mean ± SD. [a]; Analysis done using Independent-sample t test, [b]; Multivariate analysis of covariance done following adjustment (for energy and carbohydrate), and [c]; Analysis done using paired-sample t test.

Discussion

This study was a randomized clinical trial designed to evaluate the effect of lycopene supplementations on depression, anxiety, stress, and quality of life. To the best

of our knowledge this is the first study that assessed the effect of lycopene on mood state and quality of life scale. No significant differences were observed between the groups in terms of depression, anxiety and stress scores, or quality of life, after lycopene supplementation. Energy and carbohydrate intakes were different.

Our findings were in line with those reported by Tsuboi et al. (27) who assessed the correlations between serum lycopene and depressive score in 66 healthy female volunteers aged 38-70 years in 2000, and found no significant correlation between lycopene and depressive score. However, the results of other studies are equivocal. By conducting a cross-sectional study on 986 elderly Japanese individuals, Niu et al. found that a tomato-rich diet is independently related to lower prevalence of depressive symptoms. However, they were ambivalent whether the protective effect of lycopene was directly caused by affecting the brain cells or by preventing depression-inducing diseases (6).

The antidepressant properties of lycopene were also observed in animal studies; for instance, Zhang et al. administered 6 mg/kg body weight per day lycopene for seven days to mice, and observed attenuated depression-like behaviors (28). Moreover, Jain et al. (29) investigated the synergistic effect of a combination of lycopene, quercetin, and poloxamer 188 in a 3-nitropropionic acid-induced Huntington's disease model, indicating that the combination of lycopene and quercetin is an effective nutritional component to alleviate and/or prevent the complications of Huntington's disease, such as anxiety and depression.

Depression, anxiety, and stress are among the most prevalent mood disorders in the world (30). Since they can adversely affect the quality of life and are associated with infertility (31), the biological processes involved in the etiology of psychiatric disorders were studied. Oxidative stress is defined as an imbalance between cellular production of ROS and the counteracting antioxidant mechanisms (32). Since the brain consumes a high amount of oxygen and has a lipid-rich environment, it is highly vulnerable to oxidative stress (31). Also, due to the effects of smoking on oxidative status, and sperm quality, concentration, motility, and morphology, in this study, we recorded the history of smoking.

Besides, new studies point out that psychiatric disorders are resulted from alterations, not only in brain function, but also in neuronal plasticity (33). Increased free radicals could trigger such alterations, leading to cell death and atrophy of neuronal and glial cell population in the brain (34). Hence, powerful antioxidants, such as lycopene, are speculated to be protective agents against oxidative stress-induced neuronal damage since they are able to remove ROS. Lycopene could conceivably protect against this damage, resulting in the remission and functional recovery of depression or anxiety symptoms (35).

Another putative explanation for the potential protective effect of lycopene is based on its protective role against atherosclerotic cardiovascular diseases and cancer (36, 37). Since these chronic illnesses are also related to the occurrence of depressive symptoms.

The non-significant nature of our findings might be due to the relatively short duration and/or low dosage of administered lycopene, which might have been not high enough to exert stronger acute effects, highlighting the need for further work to investigate the impact of both duration and dosage. Nevertheless, contrary to contemporary theories, lycopene did not show any clinical effects on psychiatric disorders. It is noteworthy that within-group analysis showed that depression scores decreased in both groups compared to the baseline values. This could simply be due to the fact that the patients merely felt they are getting better while no clinical response to lycopene was evident. However, due to some constraints, we were unable to measure the seminal levels of lycopene, the receptors, and enzymes such as super oxidase dismutase (SOD) and catalase (CAT).

The authors of the present study strongly suggest that further work using varying doses, done in larger sample sizes, including both genders, and for longer periods should be conducted to evaluate the effects of potent antioxidants on different psychological aspects of infertile individuals.

Conclusion

12-week lycopene supplementation, did not have any significant effects on psychiatric disorders and quality of life, urgently highlighting the need for further evidence of the efficacy of lycopene, for improving mood status and quality of life in infertile men.

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Authors' Contributions

M.N.; Contributed to data collection and writing the first draft. M.H.N.-E.; Contributed to patient management and providing the lab facility. M.J.T.; Contributed to all experimental work, data and statistical analysis, and interpretation of data R.A.; Contributed to the research concept, supervised the work and revised the manuscript. All authors read and approved the final manuscript.

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Original Article

Effect of Single-Dose Methotrexate Treatment on Ovarian Reserve in Women with Ectopic Pregnancy Undergoing Infertility Treatment: A Single-Center Experience

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Abstract

Background: The aim of this study was evaluation of the impact of single-dose methotrexate (MTX) treatment on ovarian reserve in women with ectopic pregnancy (EP) undergoing infertility treatment in Iranian population.

Materials and Methods: This prospective cohort study was done between March 2015 and March 2017 in Tehran General Women Hospital, Tehran, Iran. We enrolled 20 patients with EP who conceived during infertility treatment and received a single-dose MTX (50 mg/m^2) intramuscularly. Serum anti-Mullerian hormone (AMH), 17 beta-estradiol (E2), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and antral follicle count (AFC) on transvaginal ultrasonography, were evaluated before and 8 weeks after administration of MTX.

Results: AMH did not significantly vary after the administration of MTX, compared to before treatment value (P=0.36). FSH, E2 and AFC changes were not statistically significant, while increment of LH was significant (P=0.02).

Conclusion: Results indicated that single-dose MTX treatment did not reduce ovarian reserve in women with EP. Further randomized controlled clinical trial studies with larger sample sizes, by using multiple dosages of MTX, and with long-term follow up are suggested to be done.

Keywords: Anti-Mullerian Hormone, Assisted Reproductive Techniques, Ectopic Pregnancy, Methotrexate, Ovarian Reserve

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Introduction

Ectopic pregnancy (EP) occurs when a fertilized egg implants somewhere other than the main cavity of the uterus. EP cannot continue as a normal pregnancy. EP comprises about 1.6% of all pregnancies and it is a potential leading cause of pregnancy-related mortality in the first trimester of pregnancy (1).

Laparoscopy is the gold standard for managing EP (2). Conservative management can be medical or expectant, however, watchful selection and counseling are important, as non-surgical approach may expose the women to the risk of tubal rupture. The most commonly used drug for medical management is methotrexate (MTX). It is an anti-metabolite drug that acts on actively growing cells (3). This mechanism effectively treats EP but it is supposed that MTX has an impact on fertility by targeting the actively dividing granulosa cells (GC) within the ovary. This, in turn, may decrease ovarian reserve and further responsiveness (4).

Anti-Mullerian hormone (AMH) is an endocrine marker considered for assessing the ovarian reserve and it is not affected by gonadotropins (5). Also, luteinizing hormone (LH) and follicle stimulation hormone (FSH) were assessed during the course of the treatment and monitoring. AMH values can be measured at any point during the menstrual cycle without the need for a sonogram, which makes it a unique and specific test in evaluation of ovarian reserve (6). Assisted reproductive techniques (ART) may be an opportunity to study the effect of MTX on ovarian reserve. The novelty of our study was related to the time interval between measurements and assessment of the hormonal levels after administration of only one dose of MTX. The present study was conducted to assess the effect of single-dose MTX on ovarian reserve by measuring AMH in women with EP undergoing infertility treatment in Iranian population.

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Materials and Methods

This prospective cohort study was conducted from 2015 to 2017 in Tehran General Women Hospital, an educational hospital affiliated with Tehran University of Medical Sciences (TUMS), Tehran, Iran. The study was approved by the Ethical Board Committee of Tehran University of Medical Sciences by number 90-0339-14127. Patients were thoroughly informed of the experiment and fully consented to taking part in the study.

Sample selection

Patients who referred to our hospital with suspicious EP, were assessed for eligibility. AFC, AMH, (FSH and LH on the 3rd day of the cycle) and 17 beta estradiol (E2) in mid cycle were assessed before recruiting in the ART cycle. For baseline assessment, beta-human chorionic gonadotropin (β-hCG) concentration was measured and trans-vaginal ultrasound was performed to evaluate the pregnancy sac in spontaneous course and at baseline before any intervention. The diagnosis of EP was made based on an increasing serum β-hCG concentration (>2000 mIU/ml) and no intrauterine sac visualized by trans-vaginal ultrasound after 6 weeks of gestational age. The unnecessary tests are not mentioned. All tests were done in a single lab in the reference hospital.

Eligible subjects were adult women with at least 18 years of age with history of infertility that had previously received *in vitro* fertilization (IVF) and received single-dose MTX (50 mg/m^2) intramuscularly for mangling EP.

Eligibility for MTX administration included: stable homodynamic status, the size of ectopic mass below 4 cm on ultrasound examination, unruptured EP and no contraindication either relative or absolute for MTX use. Serum concentration of β-hCG >5000 mIU/ml and fetal heart activity were relative contraindications for non-surgical management of EP. Absolute contraindications were chronic liver disease, pre-existing blood dyscrasias, pulmonary disease, peptic ulcer disease and immunodeficiency. In addition, the participants who had sensitivity to MTX, or were breastfeeding, were not included for MTX therapy (7). Patients were thoroughly informed about the experiment and fully consented to taking part in the study.

Intervention and outcome

Plasma levels of 17 beta-estradiol (in mid cycle), LH and FSH (on the 3rd day of the cycle) as well as AMH were measured at baseline (definitely before MTX administration) and 8 weeks after treatment with MTX. Selection of the time point (i.e. after eight weeks) was according to our pilot study that showed the highest alteration at this time. Antral follicle count (AFC) was estimated by trans-vaginal ultrasound before and after the study. These markers had been checked in the same laboratory before pregnancy.

Statistical analysis

Statistical analysis was done by SPSS version 19.0 software (IBM SPSS Statistics, USA). Mean ± SD and numbers (%) were calculated for continuous and categorical variables, respectively. T test for paired observation was used (after running Kolmogorov-Smirnov test reassurance for parametric distribution) to evaluate any differences in AMH, LH, FSH, AFC and E2 values between the pre-pregnancy values and those obtained 8 weeks after MTX administration. A $P \leq 0.05$ was considered statistically significant.

Sample size was calculated by Cochran's formula. Given the probability of 1.6% of EP (in normal population) of which 35% are eligible for receiving medical treatment, the estimated sample size was 20. The sample size was determined by a pilot study done on ten subjects before initiation of the main study.

Results

Twenty patients were recruited and all of them were followed until the end of the study. None of our cases needed extra dose of MTX nor needed an emergent surgery due to ruptured EP. No serious adverse effect was reported during the study. None of our cases had persistent EP. In other word, all patients were cured both clinically and according to laboratory tests.

The mean (± SD) age of patients was 30.9 ± 5.37 years (range: 21 to 43 years old). Table 1 illustrates the age and obstetrics background of the participants. Two patients had a history of EP. None of the participants had heterotopic pregnancy.

Table 1: Age and obstetrics background of the participants

Variables	n=20
Age (Y)	30.9 ± 5.37
Gravidity	
1	4 (20)
2	7 (35)
3	6 (30)
4	3 (15)
Parity	
0	10 (50)
1	6 (30)
2	4 (20)
Abortion status	
0	10 (50)
1	8 (40)
2	2 (10)
BMI (Kg/m^2)	27.1 ± 4.7

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

The mean (± SD) of AMH levels at baseline and after 8 weeks were 9.5 ng/ml (± 4.23) and 9.15 ng/ml (± 4.24), respectively which were not statistically significantly different (P=0.36). For FSH, E2 and AFC, there was a non-statistically significant difference between baseline value and the value obtained 8 weeks after MTX administration. However, the mean (± SD) LH values were 6.63 IU/l (± 3.03) and 8.1 IU/l (± 2.63) at baseline and 8 weeks after MTX administration, respectively (P=0.02). Table 2 compares lab data and AFC, before and after EP treating by MTX.

Table 2: Comparison lab data and AFC, before and after EP treated by MTX

Variables	Pre-MTX	Post-MTX	P value
FSH (mIU/ml)	6.6 ± 3.1	8.2 ± 4.9	0.1
LH (IU/L)	6.6 ± 3.0	8.1 ± 2.6	0.02
AMH (ng/ml)	9.5 ± 4.2	9.1 ± 4.2	0.36
AFC	9.1 ± 2.0	8.6 ± 2	0.49
E2 (pg/ml)	21.9 ± 20.1	16.3 ± 13.5	0.15

Data are presented as mean ± SD.
AFC; Antral follicle count, EP; Ectopic pregnancy, MTX; Methotrexate, FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, AMH; Anti-Mullerian Hormone, and E2; 17-beta estradiol.

Discussion

Our study demonstrated that medical management of EP using MTX does not have adverse effects on ovarian reserve in infertile women undergoing various types of ART. EP management by using MTX is safe and effective in carefully selected patients. It is beneficial in cases with no tendency for surgery (7) and helps to decline the rate of surgery. Indeed, it is safe for a later pregnancy. The fetal exposure to MTX from maternal organs is considered to be low and the outcomes of pregnancies shortly after MTX therapy, are almost favorable (8).

Another important issue in management of patients is the maintenance of women's fertility. Infertile women under infertility treatment, may already have compromised ovarian reserve (4). Furthermore, the data known about the effects of MTX on ovarian reserve and effectiveness of subsequent ART, is minimal.

Evaluation of ovarian reserve is important in assessment and treatment of infertility. Ovarian reserve will decline by age. The most commonly used approach to assess ovarian reserve, is the measurement of FSH and LH. However, AMH and inhibin-B are other biomarkers of ovarian reserve that are most popular since they provide direct determination of ovarian status (9). In this study, LH had significant difference despite narrow difference of values.

Ovarian reserve, ovarian responsiveness, or subsequent IVF outcomes was discussed in other studies and most of such reports did not reveal a significant difference in IVF cycle parameters or outcomes. Orvieto et al. (10) demonstrated no difference in FSH, ovarian stimulation characteristics, or oocytes retrieved in IVF patients before and after receiving MTX treatment for EP. Also, in another study on women undergone IVF/intra-cytoplasmic sperm injection (ICSI), no difference in AMH, stimulation parameters, oocytes retrieved, or number of embryos was found between before and after MTX administration (11).

Pregnancy rate after MTX administration was 36.4%, that is similar to normal rate showing no modification of the characteristics of the endometrial or follicles during IVF after MTX treatment for EP (12). In another study on patients who underwent an IVF cycle that resulted in an EP and patients treated with MTX, no adverse effect on ovarian reserve or ovarian responsiveness was found (4). Similarly, in our study, AMH, AFC and FSH as a main biomarker for ovarian function, did not change after MTX administration. Ohannessian et al. (13) in a meta-analysis, similarly demonstrated that comparisons between before and after treatment with MTX showed no statistically significant differences in the basal plasma FSH level, total gonadotrophin dose used for stimulation, duration of stimulation, and E2 level on the day ovulation was triggered.

While the evidence from human studies is limited, results of studies that assessed the effect of MTX on AMH as an ovarian reserve marker, are controversial. Dosages of MTX, sample size and follow up period length have been suggested as factors altering the results.

Our study had some limitations that should be mentioned. It was a retrospective study with small sample size. Considering our results which were similar to those of other studies, it was not possible to definitely prove that MTX has no adverse effect on ovarian reserve and responsiveness in Iranian population.

The strength of our study was evaluation of AMH during evaluation of ovarian reserve that was absent in a similar study by Boots et al. (4). Nowadays, AMH is routinely measured in multiple occasions especially for infertility evaluation. Measurement of AMH is considered a highly effective approach of assessing ovarian reserve because of its independence of the menstrual cycle as well as the higher inter-cycle and intra-cycle reproducibility (8).

Conclusion

Our results showed that single-dose MTX treatment in EP did not decrease the ovarian reserve in infertile women.

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Authors' Contributions

M.S.; Study conception and design, analysis and interpretation of data, and critical revision. P.P.; Study conception and design. N.H.; Acquisition of data and patient collection, helping on drafting. Z.S.; Drafting of the manuscript and patient collection, helping on data analysis. M.Ghaz.; Acquisition of data and data analysis. F.D.T.; Study conception and design. B.G.Y; Article revision, and statistical analysis. M.Ghae.; Drafting and editing the manuscript, analysis and interpretation of data, and critical revision of the final manuscript. All authors read and approved the final manuscript.

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Original Article

Relationship between Serum Levels of Anti-Mullerian Hormone, Adiponectin and Oxidative Stress Markers in Patients with Polycystic Ovary Syndrome

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Abstract

Background: Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age. Anti-Mullerian hormone (AMH) is a valid indicator of ovarian function and is used for PCOS diagnosis. Some studies have shown that adipokines affect the synthesis of AMH, and therefore they are somehow related in function. The aim of the present study was to determine the relationship between serum levels of AMH, adiponectin and oxidative stress markers in PCOS patients.

Materials and Methods: In this cross-sectional study, PCOS patients and healthy women (80 cases in total) were investigated. Serum levels of AMH, adiponectin, gonadotropins, androgens, total antioxidant capacity (TAC), nitric oxide (NO) and insulin resistance (IR) were measured by standard methods. An independent t test was used to compare the two groups and Pearson correlation coefficient was used to determine the relationship between variables.

Results: There was a significant difference between the means of AMH (5.16 ± 5.3 vs. 2.44 ± 2.5 ng/mL) (P=0.007) and adiponectin (24.55 ± 9.41 vs. 30.57 ± 14.2 µg/L) (P=0.029) among the PCOS and control groups, respectively. The correlation between AMH and adiponectin in the control group was statistically significant and negative (P=0.028, r=-0.35), while in the PCOS group it was not significant (P=0.11, r=-0.25).

Conclusion: Various biochemical and hormonal factors differ between PCOS and healthy women. Different factors can influence AMH and adiponectin levels independently of PCOS in women of reproductive age.

Keywords: Adiponectin, Anti-Mullerian Hormone, Polycystic Ovary Syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is a metabolic disorder and one of the most common endocrine disorders in women of reproductive age, with an incidence of 4-18% (1). This syndrome is the main cause of anovulation in infertile women. Although PCOS was initially recognized by increasing androgen secretion from adrenal glands and ovaries, hirsutism, irregular menstruation, large ovaries, increased number of primary and pre-anteral ovarian follicles, and disturbances in the dominant follicle selection, today it is introduced as a disorder with multiple causes and metabolic consequences (2). However, the pathogenesis of PCOS is complex and not completely understood. Previous studies have shown that androgens and insulin play key roles in the development of this disease (3, 4). PCOS patients have higher serum levels of testosterone and insulin, triglycerides, cholesterol, and lower serum levels of sex hormone-binding globulin (SHBG) and follicle stimulating hormones (FSH) compared to healthy women (4, 5). Many studies have found effective oxidative stress in the pathogenesis of anovulation, insulin resistance (IR), and hyperandrogenism in PCOS patients (6). Also, signs of high serum levels of oxidative stress, such as malondialdehyde (MDA) and reduction of total antioxidant capacity (TAC) have been observed in PCOS patients (7).

Anti-Mullerian hormone (AMH) is a glycoprotein from the family of transforming growth factor-beta (TGF-β), secreted by granulosa cells of the antral follicles (4-6 mm). AMH secretion gradually decreases during follicular growth and cannot be distinguished in follicles larger than 8 mm. Currently the serum level of AMH, as a valid indicator of ovarian function, is determined in women's fertility screening and PCOS diagnosis, allowing for targeted treatment of infertility (8). The concentration of AMH is related to the number of small follicles and ovarian reserve (6, 9). The number

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of the small follicles is relatively constant during the menstrual cycle and it seems that AMH concentration has insignificant fluctuation during this time. As age increases, AMH decreases gradually, indicating a decrease in the number of ovarian follicles and reaching the menopausal stage (9).

AMH has an inhibitory effect on the growth of primordial follicles, thus preventing them from finishing early in the life of a woman (10, 11). In PCOS women, the number of small follicles (2-5 mm) is 2 to 3 times that of healthy women, which leads to an increase in the concentration of AMH in these individuals (12, 9) and it seems that AMH concentration is effective in the pathogenesis of PCOS and anovulation. The increased AMH reduces the sensitivity of the antral follicles to the follicle-stimulating hormone (FSH) and subsequently prevents both the selection of the dominant follicle and the growth of follicles in the antral phase (13). Also AMH inhibits the aromatase enzyme, leading to a decrease in the production of follicular estradiol, which in turn may be accompanied by a defect in the selection of the dominant follicle (14).

Nutritional status and obesity may affect the synthesis of AMH, as some studies have reported a decrease in AMH levels in obese women, indicating a negative correlation between AMH and BMI, while others have not mentioned a correlation between nutritional factors, body mass index (BMI) and AMH (14, 15). The prevalence of obesity is more than 50% in patients with PCOS, leading to IR and increased insulin levels in these patients. Obesity may contribute to the clinical complications of PCOS, and hyperinsulinemia can be associated with the termination of ovarian follicle growth (16).

In addition to energy storage, adipose tissue can synthesize and secrete important metabolic proteins, including adipokines that regulate multiple biological actions (17). An adipokine, which accounts for about 0.01% of plasma proteins, is adiponectin (18). This protein has two receptors (ADIPO R1 and ADIPO R2) and pivotal roles in lipid metabolism, such as increasing insulin sensitivity and employing anti-inflammatory effects (19). Several studies have shown that there is correlation between adiponectin deficiencies in adipose tissue and the reduction of ovarian reserve in obese PCOS and non-PCOS women (9, 20). Some studies have reported adiponectin reduction in PCOS patients, which may be due to obesity and IR (21). Also, it has been suggested that leptin and not adiponectin may affect the synthesis of AMH in women. It seems that there is a negative correlation between insulin and AMH levels, while there is a positive correlation between AMH and adiponectin (22).

Undoubtedly, the recognition of the factors involved in the pathogenesis of PCOS and how they interferes with the syndrome can lead to a better understanding of PCOS and therefore provides access to appropriate methods for its diagnosis and treatment. Regarding the importance of AMH, the prevalence of obesity and related dysfunction of adiponectin in PCOS, the aim of present study was to determine the correlation between AMH, adiponectin and oxidative stress markers in PCOS patients.

Materials and Methods

Study subjects

In this cross-sectional study, 40 PCOS patients and 40 healthy women aged 18-40 years were randomly divided and evaluated in two groups. The sample size was accepted by an academic static consult in related committee. PCOS and healthy subjects were selected by our gynecologist from her private clinic. The diagnosis of PCOS was done based on Rotterdam Criteria (23). Exclusion criteria were: subjects with diabetes, or underlying systemic disease, galactorrhea, any endocrine disease associated with thyroid stimulating hormone (TSH), prolactin or 17α-hydroxy progesterone levels, usage of drugs that affect the function of the hypothalamus-pituitary-ovarian axis or insulin-sensitizing drugs such as metformin during last three months and using contraceptives during last 4 weeks. Also, women with addiction to cigarette, narcotics or alcohol, as well as women who were involved in regular exercise activities during the study period were excluded. This study was approved by the Ethics Committee of Kermanshah University of Medical Sciences (KUMS.REC.1395.626) and the patients signed informed consent.

Sample collection

Blood samples were collected in similar conditions for each participant on the 3rd and 5th days of their menstrual cycle and after 8 hours of fasting. AMH Enzyme-linked immunosorbent assay (ELISA, Beckman Coulter, USA) was performed according to manufacturer's instructions. Adiponectin, gonadotropins and androgen were detected by chemiluminescence technique (Immulite 2000, Siemens, Germany). To evaluate IR, the HOMA-IR index (Homeostasis Model Assessment for IR) was used as follows: fasting blood glucose (mmol/L) concentration x fasting insulin (μIU/mL) divided by constant 22.5; an index > 2 indicated IR (21).

Ferric reducing antioxidant power assay

The TAC of the sera was assessed by Ferric reducing antioxidant power assay (FRAP) method. Briefly, serum (150 μl) was mixed with 1.5 ml of fresh FRAP reagent (10 mM 2, 4, 6- Tripyridyl-s-Triazine, 20 mM $Fecl_3$, $6H_2O$ solution and 300 mM acetate buffer pH=3.6), and incubated at 37˚C for 10 minutes. Then the absorbance was measured at 593 nm using a spectrophotometer (Pharmacia, Novaspec II, Biochrom, England) and was compared to a standard curve constructed with known concentrations of FeSO4 $7H_2O$. Results were expressed in μM (24).

Nitric oxide assay

Griess method was used for determination of the serum levels of NO, which includes the conversion of nitrate to nitrite. Griess reagent facilitates the conversion of nitrite to a deep pink azo substance (25). Briefly, equal volumes of serum samples and Griess reagent were mixed and incubated at room temperature for 30-45 minutes. Next, the absorbance rate was determined at 540 and 630 nm using ELISA reader (STAT Fax 100, USA).

Statistical analysis

All data were analyzed by SPSS software version 18.0 (Inc., Chicago, IL, USA) and presented as mean ± SE. Kolmogorov-Smirnov test was used to check the normality of the data. To compare the two groups, independent t test was used and Pearson correlation coefficient was used to determine the relationship between variables. The significance level was considered at P≥0.05.

Results

In this study 80 women with a mean age of 31.36 ± 6.19 years were evaluated in two PCOS and control groups. The subjects were similar in age in both groups. Although the mean of BMI was higher in PCOS patients than in the control group, this difference was not statistically significant (Table 1).

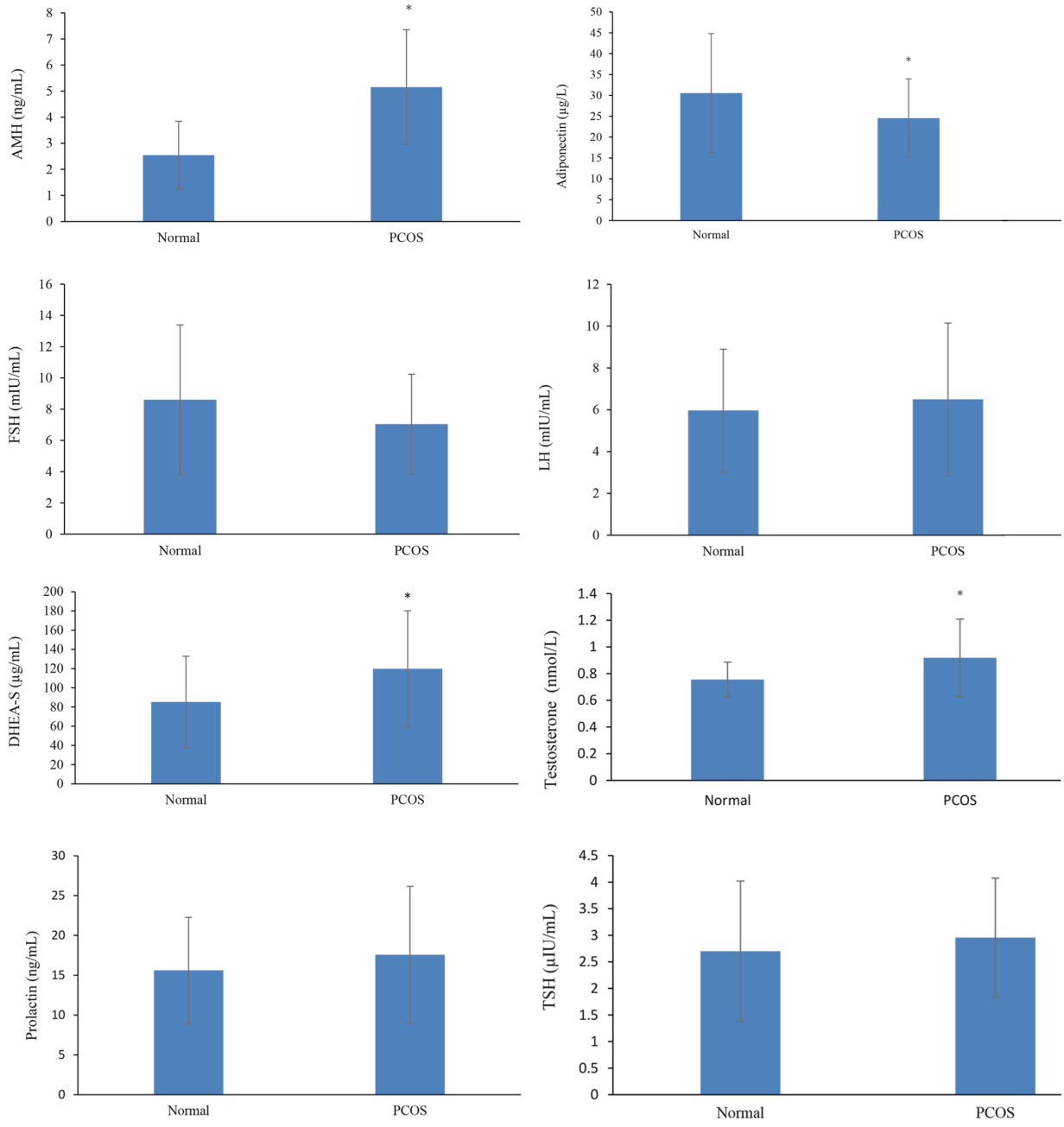

Fig.1: Comparison of the mean levels of AMH, Adiponectin and other hormones in control and PCOS groups.
AMH; Anti-mullerian hormone, DHEA-S; Dehydroepiandrosterone sulfate, TSH; Thyroid stimulating hormone, FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, and PCOS; Polycystic ovary syndrome. *; Significant difference between groups (P<0.05).

Table 1: Comparison of the mean levels of AMH, Adiponectin and other factors between control and PCOS groups

Variables	Control	PCOS	P value*
Age (Y)	32.02 ± 6.24	30.70 ± 6.14	0.341
BMI (Kg/m²)	25.33 ± 3.15	26.66 ± 4.24	0.117
AMH (ng/mL)	2.54 ± 2.44	5.16 ± 5.30	0.007
Adiponectin (µg/L)	30.57 ± 14.23	24.55 ± 9.41	0.029
DHEA-S (µg/mL)	85.25 ± 47.58	119.78±60.31	0.006
Testosterone (nmol/L)	0.76 ± 0.13	0.92 ± 0.29	0.002
Prolactin (ng/mL)	15.62 ± 6.66	17.57 ± 8.59	0.259
TSH (µIU/mL)	2.70 ± 1.32	2.96 ± 1.12	0.354
FSH (mIU/mL)	8.60 ± 4.79	7.04 ± 3.19	0.090
LH (mIU/mL)	5.97 ± 2.93	6.50 ± 3.65	0.476
FBG (mg/mL)	86.96 ± 9.52	85.63 ± 7.53	0.488
Insulin (µIU/mL)	5.68 ± 3.48	8.66 ± 3.98	0.001
IR-HOMA	1.25 ± 0.87	1.86 ± 0.90	0.003
TAC (µmol)	260.02 ± 212.71	231.26 ± 178.51	0.517
NO (µmol)	26.01 ± 12.41	31.11 ± 14.54	0.213

Data are presented as mean ± SD.
AMH; Anti-mullerian hormone, PCOS; Polycystic ovary syndrome, BMI; Body mass index, DHEA-S; Dehydroepiandrosterone sulfate, TSH; Thyroid stimulating hormone, FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, FBG; Fasting blood glucose, IR-HOMA; Insulin resistance- homeostatic model assessment, TAC; Total antioxidant capacity, NO; Nitric oxide, and *; Independent Sample t test.

Biochemical analyzes

AMH level in PCOS group was significantly higher than in the normal group (5.16 ± 5.30 vs. 2.44 ± 2.49) (P=0.007). Also, there was a significant difference in the adiponectin level between the two groups (P=0.029), as it was lower in the PCOS group compared to the control group (24.55 ± 9.41 vs. 30.57 ± 14.23) (Table 1, Fig.1). There was no statistically significant difference in the mean of FSH and luteinizing hormone (LH) levels between the two groups, while the mean of both androgens in the PCOS group was significantly higher than in the control group (P=0.006 and P=0.002, respectively). Also, the mean of prolactin and TSH levels was higher in the PCOS group, but this difference was not significant (Table 1, Fig.1).

The mean of fasting blood glucose (FBG) was not significantly different between the two groups, but the mean of insulin in the PCOS group was significantly higher than in the control group (P=0.001). Also, the mean of insulin resistance-homeostatic model assessment (IR-HOMA) was significantly different between the two groups (P=0.003), It was higher in the PCOS group than control group (Table 1, Fig.2). Anti-oxidants and oxidative stress (OS) levels were evaluated in this study with two variables: TAC and serum NO. The mean of TAC was lower in the PCOS group and the mean of NO was higher than that of healthy subjects, but the difference was not statistically significant (Table 1, Fig.2).

Correlation of variables in the PCOS patients

In the PCOS group, there was a significant negative correlation between age and AMH (P=0.002, r=-0.46), age and dehydroepiandrosterone sulfate (DHEA-S, P=0.045, r=-0.32), body mass index (BMI) and FSH (P=0.03, r=-0.34), and adiponectin and testosterone (P=0.02, r=-0.36). Also, There was a significant positive correlation between BMI and insulin (P=0.04, r=0.32) and IR (P=0.04, r=0.32), AMH and LH (P=0.10, r=0.4), DHEA-S and testosterone (P=0.003, r=0.45), DHEA-S and TAC (P=0.005, r=0.43), prolactin and nitric oxide (NO, P=0.04, r=0.42), and TSH and TAC (P=0.005, r=0.43). FBG (P=0.000, r=0.59) and insulin (P=0.000, r=0.99) also had a significant positive correlation with the IR index (IR-HOMA).

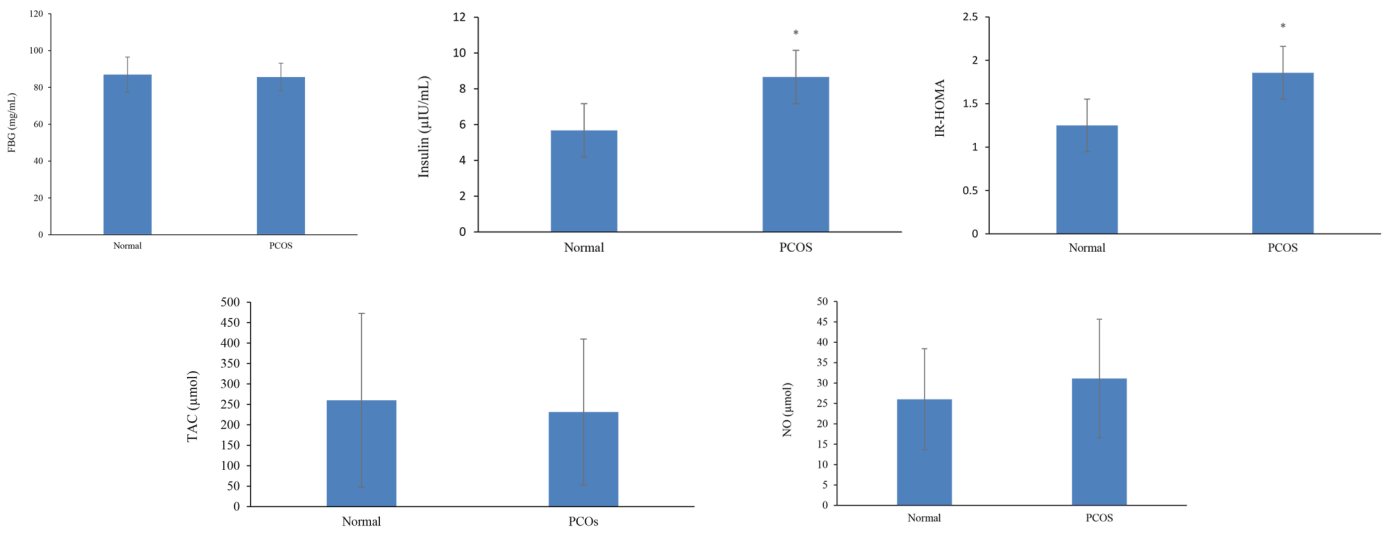

Fig.2: Comparison of insulin resistance index, TAC and NO in in control and PCOS groups.
FBG; Fasting blood glucose, IR-HOMA; Insulin resistance-homeostatic model assessment, TAC; Total antioxidant capacity, NO; Nitric oxide, and PCOS; Polycystic ovary syndrome.*; Significant difference between groups (P<0.05.)

Correlation between variables in the control subjects

In control subjects, there was a significant negative correlation between Age and AMH (P=0.000, r=-0.76), Age and testosterone (P=0.01, r=-0.39), AMH and adiponectin (P=0.03, r=-0.35), and AMH and FSH (P=0.005, r=-0.43). There was a significant positive correlation between age and FSH (P=0.037, r=0.33). AMH and testosterone (P=0.01, r=0.39), prolactin and TAC (P=0.002, r=0.48), FBG and insulin (P=0.004, r=0.45), FBG and IR-HOMA (P=0.000, r=0.61), insulin and IR-HOMA (P=0.000, r=0.98), and IR-HOMA and NO (P=0.45, r =0.44). In the control subjects, increasing in BMI leads to decreasing in adiponectin (P=0.001, r=-0.5) and DHEA-S (P=0.04, r=-0.34).

Discussion

Several factors were studied in this study, but the most important results were the significant differences between AMH, adiponectin, androgens and IR between the two groups of PCOS patients and healthy controls. We observed significant correlations between these variables in the two groups independently. PCOS group showed biochemical features associated with PCOS, such as higher levels of androgens, insulin and IR. Also, there was a higher AMH and lower adiponectin level in PCOS patients. The most important correlation found in the PCOS group was a significant positive correlation between AMH and each of the factors LH, DHEA-S, TAC, prolactin, NO, BMI, insulin and IR. In addition, there was a significant negative correlation between AMH and DHEA-S, BMI and FSH, adiponectin and testosterone in the PCOS group. However, in the control group, there was a significant positive correlation between age and FSH, AMH and testosterone, prolactin and TAC, FBG and insulin, and IR and NO. On the other hand, there was a significant negative correlation between age and AMH, age and testosterone, BMI and adiponectin, BMI and DHEA-S, AMH and adiponectin, and AMH and FSH in this group.

The results of our study, similar to Olszanecka-Glinianowicz et al. (21), showed that PCOS as the most common endocrinopathology of women of reproductive age is accompanied with multiple metabolic changes, including increased androgen and insulin levels, and the emergence of IR. Many studies have shown that at least half of the people with PCOS are obese and that obesity plays a major role in the advent of IR in these individuals (15, 18). In our study, the mean of BMI of PCOS patients was higher than the control group but it was not significant. This finding is in contrast with the study of Woo et al. (26).

Adiponectin plays an important role in anti-inflammatory processes, insulin sensitivity and obesity. The results of some studies (16, 17), consistent with our study, show that adiponectin levels in PCOS patients are lower than in the healthy subjects, while in the study of Emadi et al. (27), there was not a significant difference in the level of adiponectin when comparing the two groups. Considering the prevalence of obesity in PCOS patients and the higher BMI in these subjects in our study, the lower mean of adiponectin and the increased mean of IR in this group was predictable.

In recent years, AMH has been used as a key factor for evaluating ovarian function and an indicator for determining the number of ovarian follicles and reverse. Due to the increase in the number of small follicles in the ovaries of PCOS patients, the increase of this hormone is not unexpected. In our study, after adjustment for age, AMH was significantly higher in the PCOS group, which was similar to the results of Woo et al. (26). Also, an increase in androgens and the number of follicles in PCOS group can lead to an increase the production of AMH, which may play a vital role in decreasing the sensitivity of growing follicles to FSH hormone. In the present study, similar to the findings of Mahdi et al. (28), the rate of androgens and AMH in the PCOS group is higher than in the control group, which may be due to an impairment in the production of AMH and androgens in these individuals. While in the control group with normal levels of androgens and AMH, there was a significant positive correlation between AMH and testosterone, which was similar to that of Woo et al. (26).

In some studies, the mean of FSH in patients with PCOS was higher than in the control group (26, 28), while in the present study, the mean of FSH was lower in the PCOS group. Nonetheless, similar to Hamza et al. (6) the difference that we observed was not significant. It can be suggested that increasing the number of small follicles and the AMH secreted from them, which lowers the sensitivity of the follicles to FSH, can affect the level of FSH and decrease its effect on PCOS patients. In a number of studies, levels of LH have increased dramatically in the follicular phase in PCOS patients. In our study, similar to Mahdi et al. (28), the mean of LH was higher in the PCOS group. Also, Hamza et al. (6) did not show any significant difference in the LH between the two groups.

There was a significant positive correlation between AMH and LH in the PCOS group and a significant negative correlation between AMH and FSH in the control group in the present study. In both groups the mean of AMH decreased with aging, which was similar to other previous studies (26-28). This decrease was due to a decrease in the number of follicles and ovarian reserves in women with an approach to menopause. It is also thought that with increasing age, the gonadotropins content in women should be increased (29). In our study, only in the control group age had a significant positive correlation with FSH. In the study by Swellam et al. (30), there was a significant correlation between age and decreasing of androgens in both groups, but in our study we did not find such correlation.

Although some studies have reported a negative correlation between AMH and BMI (31, 15), in our study,

this correlation was not seen in either of the groups. Interestingly, the study by Nardo et al. (32) showed that AMH increased with increased activities of the subjects, and did not correlate with BMI. In our study we show that the BMI of PCOS individuals has a positive correlation with insulin level resistance, and a negative correlation with FSH. The results of various studies (17, 27) have shown that with increasing BMI, the levels of adiponectin in women decrease. However, in our study this was only observed in the control group.

In the present study, the correlation between AMH and adiponectin was negative in both groups, but it was significant only in the control group. In the study of Woo et al. (26), the correlation between AMH and adiponectin in the control group was direct and significant, which is the opposite of our findings; and in their PCOS group, there was no significant correlation between the two factors. In some of the previous studies (3, 6), in the PCOS group AMH has only a significant positive correlation with testosterone, which is different from our study results. The positive correlation between AMH and testosterone can be biologically normal for all women of reproductive age. These findings confirm that ovarian hyperandrogenesis has stopped the growth of follicles and in turn has increased AMH production. In our study, the absence of this association in the PCOS group may be due to a significant increase of the androgens and AMH in the PCOS patients, which can disturb the study of correlations.

Regarding prolactin, there is a hypothesis that polycystic ovaries affect the activity of dopamine in the hypothalamus and cause hyperprolactinemia in these patients (32). In our study, the level of prolactin in PCOS patients was higher than in healthy controls, and it was significantly correlated with an increase in NO levels, while in the control group there was a significant positive correlation between prolactin and TAC. In the present study the rate of IR in PCOS patients was higher than in the control group, similar to another previous study (17). Also, the correlation between IR and NO was found to be significant in the control group, which can be due to the effect of IR, which may also increase the level of oxidative stress in reducing ovulation (7).

The correlations between variables in the present study and the significant differences between the two groups can indicate the role of these factors in the pathogenesis of PCOS, which is a multifactorial disorder. More in depth research is needed for a better understanding of the molecular mechanism, cellular changes and gene expression that initiate PCOS pathogenesis.

Conclusion

Adiponectin changes can lead to impaired ovarian function and ovarian hormones in the reproductive age and its deficiency in PCOS patients may be associated with IR and increased insulin levels. Insulin is one of the effective factors in increasing the number of antral follicles and ultimately increasing ovarian volume. In women suffering from PCOS hyperinsulinemia may increase AMH levels. So it can be concluded that the role of adiponectin in increasing insulin sensitivity plays a key role in controlling the synthesis of AMH in women of reproductive age.

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Authors' Contributions

M.K.; Data collection and biochemical assay. M.R.Kh.; Writing the manuscript and OS assay. F.Ch.; Patient selection and data collection. M.Kh.; Study design, data analysis and revised manuscript. All authors read and approved the final manuscript.

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Original Article

Cumulative Live-Birth Rates by Maternal Age after One or Multiple *In Vitro* Fertilization Cycles: An Institutional Experience

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Abstract

Background: The aim of this retrospective study is to investigate the cumulative live birth rate (CLBR) following one or more completed *in vitro* fertilization (IVF) cycles (up to 6 cycles) stratified by maternal age and type of infertility.

Materials and Methods: In this retrospective study, five hundred forty-seven women who received 736 fresh ovarian stimulation/embryo transfer cycles between January 2016 and December 2016 were included in the study at a tertiary care center located in Lebanon.

Results: In all women, the live birth rate for the first cycle was 33.0% [95% confidence interval (CI): 27.8-38.2]. The CLBR showed an increase with each successive fresh cycle to reach 56.9% (95% CI: 51.2-62.4) after 3 cycles and 67.9% (95% CI: of 62.5-73.0) after 6 cycles. The CLBR following 6 cycles reached 69.9% (95% CI: 63.8-75.6) in women younger than 35 years. In women older than 40 years, however, the live birth rate for the first cycle was significantly low at 3.1% (95% CI: 0.3-9.5) with a plateau in success rates after 4 cycles reaching 21.9% (95% CI: 9.2-40.0). Couples with different types of infertility had CLBRs ranging from 65% to 72%, with the exception of women with low ovarian reserve, where CLBRs reached 29.4% (95% CI: 10.3-56.0).

Conclusion: The CLBR at a referral center in a Middle Eastern country reached 67.9 % after 6 cycles, with variations by age and type of infertility treatment. These findings are encouraging for patients insisting to extend their treatment beyond 4 to 5 cycles.

Keywords: Assisted Reproductive Techniques, Live Birth Pregnancy Rate, Maternal Age, Multiple Pregnancy

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Introduction

The prevalence of infertility is around 9% worldwide (1), while it is 10-15% in the Middle East (ME) for many reasons, including a high incidence of postpartum infections, iatrogenic tubal and pelvic infertility and women delaying childbearing (2, 3). The number of women treated with *in vitro* fertilization (IVF) in the ME has increased from 8305 cycles in 2005 to 11876 cycles in 2008 (4). The live birth rate per cycle is the ultimate success, and therefore it has been used in multiple studies (5-7). The outcome as livebirth per fresh IVF cycle is more evocative for patients coming for counseling, than the outcome as a positive pregnancy test per cycle. However, the best way is to counsel patients about the cumulative chances of success after a defined number of IVF cycles (8). Some centers who have adopted the single embryo transfer policy have reported cumulative live birth rates (CLBRs) as a fresh embryo transfer cycle followed by cryo-warmed cycles, all resulting from one episode of ovarian stimulation (8-12). On the other hand, others have included only fresh cycles for CLBR assessment (6, 13-15). Although it has been previously reported that the live birth rates decrease after the 4th cycle (13, 16), there is no medical reason behind limiting the number of cycles. Many patients are likely to discontinue their infertility treatments because of the psychological burden of the process and the cost of repetitive failed IVF cycles (17). On the other hand, the decision of the couple to proceed with further fresh cycles is bounded by cultural factors where the continuation of marriage is dependent on having children and many couples are reluctant to seek egg or sperm donation cycles for ethical and religious reasons.

To the best of our knowledge, CLBR after IVF/intra-cytoplasmic sperm injection (ICSI) cycles has never been reported at a national level in Lebanon, nor in the ME. It is important to determine these rates and how they change with repeated cycles, according to maternal age and type of infertility. It is essential to define an IVF cycle for these patients as the initiation of ovarian stimulation with subsequent fresh embryo transfer.

We aim to determine whether the CLBR increases over multiple successive IVF cycles, providing patients with a better estimation of their chances of a live birth.

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Materials and Methods

Ethical approval

The Ethical approval for this study was obtained from the Institutional Review Board at the AUBMC (BIO – 2017 - 0331).

Study population

This retrospective cohort study was performed on all patients scheduled to have fresh IVF/ICSI cycles at the AUBMC between January 2016 and December 2016. One IVF cycle is defined as a fresh embryo transfer attempt resulting from one episode of ovarian stimulation. All embryo transfers involving the transfer of one or more embryos were included in the study to reproduce the daily practice of assisted reproductive technologies in our region.

Cycles that were excluded are those which were cancelled before the oocyte retrieval or before the embryo transfer, patients who had their IVF cycles after December 2016 and cycles with frozen embryos/frozen oocytes. Cancellation rate was 5%.

Baseline characteristics

Baseline characteristics included different age categories (≤35, 36-39 and ≥40 years) and different types of infertility (male factor, unexplained infertility, ovulatory disorders, endometriosis, low ovarian reserve, tubal infertility and combined factors). Data collected included levels of anti-müllerian hormone and/or day 3 follicle stimulating hormone (FSH) and estradiol.

Fresh embryo transfer

Patients underwent controlled ovarian stimulation and oocyte retrieval after 10-12 days of stimulation. All cycles included were ICSI cycles. Fresh embryo transfer took place two, three or five days after the oocyte retrieval. All cycles with pre-implantation genetic testing (PGT) or frozen embryo transfer were excluded.

Outcomes

Live birth and CLBRs per cycle were the main outcome measures, stratified by maternal age and type of infertility in up to six IVF cycles. Live birth was defined as a newborn delivered after 24 weeks of gestation. Once a woman succeeded in achieving her first live born baby from IVF, she does not contribute further to the cumulative rates calculation. All women without a live birth in a previous cycle were eligible for a subsequent cycle. The CLBR at one cycle expressed the likelihood of a live birth at that cycle and from all preceding cycles.

Statistical analysis

For all patients included, descriptive statistics of demographics and treatment characteristics were analyzed. A summary of the statistics was prepared as percentages for categorical variables and is compared using the chi-square test. The mean ± standard deviation (SD) was used for continuous variables and was compared using Student's t test or one-way analysis of variance (ANOVA).

The primary outcome of this study was the CLBR. Patients were not re-enrolled after having a first live birth in a previous IVF cycles.

The live birth rate per fresh IVF treatment was calculated at different number of cycles, through dividing the number of women in each cycle who had their first live birth by the total number of IVF cycles. Conservative CLBR was also calculated by dividing the total number of women who had their first live birth up to the corresponding cycle by the total number of women who ever attempted IVF (18). The binomial distribution was used to calculate the 95% confidence intervals. A log-rank test compared the live birth rate and CLBR within each cycle and across all cycles.

Statistical analysis and computations were performed using Statistical Package for Social Sciences (SPSS IBM version 24 software, AUBMC, Lebanon), and a value of $P<0.05$ was considered to be statistically significant.

Results

In this cohort study a total of 706 women underwent fresh IVF cycles at the AUBMC from January 2016 to December 2016. After exclusions, 547 women with 736 fresh ovarian stimulation cycles were included in the analysis (Fig.1), with a yield of 10.4 ± 7.8 oocytes retrieved per cycle.

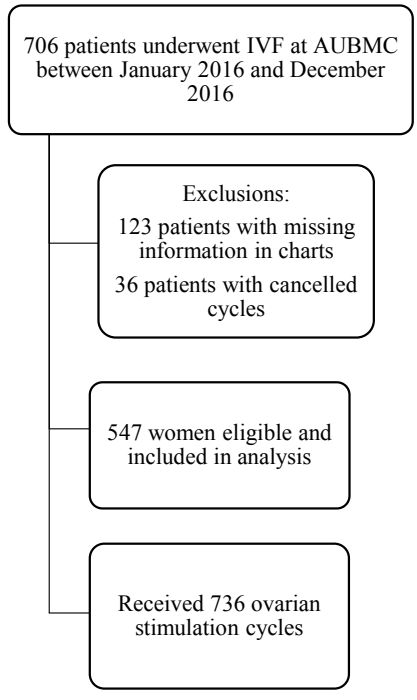

Fig.1: Flow chart of eligible cycles.
AUBMC; American University of Beirut Medical Center.

Tables 1 and 2 summarize the baseline characteristics of the cohort. Sixty-five percent of the patients undergoing IVF cycles were younger than 35 years of age. The mean duration of infertility was 4.2 years with male infertility being the most frequent diagnosis (42.5%).

Table 1: Characteristics of the 736 fresh IVF Cycles at the American University of Beirut Medical Center in 2016

Variables	For all cycles number (%)
Nationality	
Lebanese	669 (91.1)
Syrian	32 (4.4)
Iraqi	20 (2.7)
Others	13 (1.7)
Age (Y)	
≤35	479 (65.1)
36-39	137 (18.6)
≥40	120 (16.3)
Medical history	
Healthy	629 (86.0)
Thyroid disorder	47 (6.4)
Smoking status	
Non-smoker	587 (81.3)
Menstrual regularity	
Regular	652 (88.6)
Type of infertility	
Primary	425 (58.2)
Secondary	308 (41.8)
Cause of infertility	
Male factor	311 (42.5)
Unexplained infertility	136 (18.6)
Combined factors	97 (13.3)
Ovulatory disorder	55 (7.5)
Endometriosis	51 (7.0)
Low ovarian reserve	48 (6.6)
Tubal factor	33 (4.5)
Total number of cycles	
1	318 (43.2)
2	172 (23.4)
3	102 (13.9)
4	64 (8.7)
5	39 (5.3)
6	41 (5.6)
COS	
Antagonist protocol	631 (85.7)
Long protocol	85 (11.5)
Mild stimulation	19 (2.7)
Trigger	
hCG trigger	574 (78.0)
GnRHa trigger	159 (22.0)
Day of embryo transfer	
Day 2	139 (19.0)
Day 3	454 (62.0)
Day 5	139 (19.0)

Spring (March to May), Summer (June to August), Fall (September to November), Winter (December to February). IVF; *In vitro* fertilization, COS; Controlled ovarian stimulation, hCG; Human chorionic gonadotropin, and GnRHa; Gonadotropin releasing hormone agonist.

Table 2: Characteristics of the 736 fresh IVF Cycles at the American University of Beirut Medical Center in 2016

Variables	For all cycles
BMI (Kg/m^2)	25.5 ± 4.7
Duration of infertility (Y)	4.2 ± 3
Day 3 FSH (mIU/mL)	7.1 ± 2.6
Day 3 Estradiol (ng/mL)	63.5 ± 62.0
AMH (ng/mL)	2.1 ± 2.3
Number of oocytes retrieved	10.4 ± 7.8
Number of mature oocytes	7.4 ± 5.2
Number of 2PN zygotes on day 1	5.5 ± 3.9
Number of embryos transferred	2.7 ± 0.9

Data are presented as mean ± SD.
IVF; *In vitro* fertilization, BMI; Body mass index, FSH; Follicle-stimulating hormone, AMH; Anti-mullerian hormone, and PN; Pronuclear.

Cycles were stimulated with various protocols, with the antagonist protocol being the most commonly used (85.7%). Final oocyte maturation was mainly triggered by human chorionic gonadotropin (hCG) (78% of cycles), while the remaining cycles were triggered by gonadotropin releasing hormone agonist (GnRH) agonist. Transvaginal oocyte collection was performed 35-36 hours after the trigger. The luteal phase was supported by vaginal (micronized progesterone suppositories), intra-muscular and/or oral progesterone (Dydrogesterone).

The average number of embryos transferred per patient was 2.7, and 81% of the embryo transfers were performed on day 2 or day 3 with a fresh cleavage-stage embryo. This resulted in 216 live births (29.3%), where 61.6% were singletons and 38.4% were multiple gestations (Table S1, See Supplementary Online Information at www.ijfs.ir).

Cumulative live birth rates

The overall CLBR for all treatment cycles and all age groups is shown in Figure S1 (See Supplementary Online Information at www.ijfs.ir). The conservative CLBRs across all cycles up to cycle number 6 were calculated (Table 3). Overall, the live birth rate resulting from the first fresh IVF cycle is 33.0% (95% CI: 27.8-38.2). This value remained above 20% up to the sixth cycle. The conservative CLBR showed an increase with each successive fresh cycle to reach 56.9% (95% CI: 51.3-62.4) after 3 cycles and 67.9% (95% CI: 62.5-73.0) after 6 cycles.

Conservative CLBR stratified for the different age groups are presented in Figure S2 (See Supplementary Online Information at www.ijfs.ir) and in Table 4. The live birth rates fluctuated with an overall decrease with progressive cycles and in patients younger than 35 years were 37.4%, 34.2%, 30.6%, 34.5%, and 33.3% at cycles 1 through 5, respectively. Following 6 cycles, CLBRs reached 69.9% (95% CI: 63.8-75.6) in patients younger than 35 years and 83.7% (95% CI: 69.3-93.2) in patients between 36 and 39 years old. The CLBR decreased after the age of 40, as a plateau in success rates was reached after the 4th cycle with 21.9% (95% CI: 9.3-40.0). The log-rank test revealed significantly different age-specific rates ($P<0.05$).

Table 3: Live birth rates within initiated treatment cycle and conservative cumulative live birth rates across all cycles

Cycle number	Number of cycles	Number of live births	Live birth rate with-in each cycle, % (95% CI)	Cumulative live birth rates across all cycles, % (95% CI)
1	318	105	33.0 (27.8-38.2)	33.0 (27.8-38.2)
2	172	49	28.5 (21.7-35.3)	48.4 (42.8-54.1)
3	102	27	26.5 (17.8-35.2)	56.9 (51.3-62.4)
4	64	16	25 (14.1-35.9)	61.9 (56.4-67.3)
5	39	8	20.5 (7.2-33.8)	64.5 (58.9-69.7)
6	41	11	26.8 (12.7-41.0)	67.9 (62.5-73.0)

CI; Confidence interval.

Table 4: CLBRs across all age groups

Cycle number	Number of cycles	Number of live births	Live birth rate within each cycle, % (95% CI)	Cumulative live birth rates across all cy-cles, % (95% CI)
1. Women aged ≤35 years' old				
1	243	91	37.4 (31.3-43.6)	37.4 (31.3-43.6)
2	117	40	34.2 (25.5-42.9)	53.9 (47.4-60.3)
3	62	19	30.6 (18.8-42.4)	61.7 (55.3-67.9)
4	29	10	34.5 (16.1-52.9)	65.8 (59.5-71.8)
5	18	6	33.3 (9.2-57.5)	68.3 (62.1-74.1)
6	10	4	40.0 (3.1-76.9)	69.9 (63.8-75.6)
2. Women aged 36-39 years' old				
1	43	13	30.2 (15.9-44.5)	30.2 (15.9-44.5)
2	32	9	28.1 (11.7-44.6)	51.2 (35.5-66.7)
3	22	4	18.2 (0.7-35.7)	60.5 (44.4-75.0)
4	20	4	20.0 (0.8-39.2)	69.8 (53.9-82.8)
5	11	2	18.2 (0.9-45.4)	74.4 (58.8-86.5)
6	9	4	44.4 (3.9-85.00)	83.7 (69.3-93.2)
3. Women aged ≥ 40 years' old				
1	32	1	3.1 (0.3-9.5)	3.1 (0.3-9.5)
2	23	0	0	3.1 (0.3-9.5)
3	18	4	22.2 (0.9-43.5)	15.6 (5.3-32.8)
4	15	2	13.3 (-6.1-32.8)	21.9 (9.3-40.00)
5	10	0	0	21.9 (9.3-40.00)
6	22	3	13.6 (-1.9-29.2)	31.2 (16.1-50.0)

CLBRs; Cumulative live birth rate and CI; Confidence interval.

Conservative CLBR categorized by the different types of infertility are presented in Figure S3 (See Supplementary Online Information at www.ijfs.ir). With the exception of women with low ovarian reserves, couples with different types of infertility have a similar live birth rate at the first cycle when compared to all other cycles. The CLBR after 6 cycles for couples with low ovarian reserves is the lowest with 29.4% (95% CI: 10.3-56.0).

Discussion

This 1-year cohort showed significant CLBRs based on fresh IVF cycles, even in women older than 40 years of age. These numbers can help physicians counsel patients about the chances of successful live births in terms of age and type of infertility with repeated cycles. Because of the health system differences between the ME and Western countries (financial constraints, lack of insurance coverage, ethical and religious reasons), we assessed the CLBRs in fresh IVF cycles only. We chose 6 cycles, because of the significant reduction in success in CLBRs after 4 to 6 cycles noted in the literature (6, 13). Moreover, the number of patients receiving more than 6 cycles is low. In this study, the CLBRs following 1 to 6 successive IVF cycles in a referral tertiary center in the ME was calculated. The conservative estimates of the CLBR increased by more than 50% from cycle number 1 (33.0%, 95% CI: 27.8-38.2) to cycle number 6 (67.9%, 95% CI: 62.5-73.0) across all cycles, whilst it increased by 53.5% in patients who were ≤35 years old, by 36% in patients between 36 and 39 years of age and by only 10% in patients ≥40.

It is believed that the success rate within a cycle decreases with an increase in the number of cycles (5), however, the cumulative rates in our cohort increased up to the sixth cycle. The cumulative rates also increased up to the fourth cycle in women aged ≥40 years old (21.9%, 95% CI: 9.3-40.00). Occasional live births were achieved in patients older than 40 with a probability of 3.1% per started cycle in our cohort compared to 0.46% in a single-center Japanese cohort study (19). These findings are in line with a study published by Smith et al. (20) who categorized women older than 40 years of age into 2 groups and showed that women aged 40 to 42 still have acceptable chances up to the ninth cycle, while women older than 42 show an increase up to the fifth cycle only. The same authors also showed that patients with a low yield of oocytes retrieved in previous cycles still benefit from continuing successive cycles if they are younger than 40 years. On the contrary, we showed that when including all reproductive ages in the study, patients with low ovarian reserve and low number of oocytes retrieved have the lowest cumulative rates, plateauing after the second cycle with a 29.41% chance of success. Moreover, our rates were similar to those reported in previous studies, as the CLBRs decreased in older ages (21).

When the cause of infertility was taken into account, the differences noted in CLBRs were insignificant among patients with male factor, unexplained, tubal and combined infertility. In addition, couples with a male factor had the highest CLBRs as it is also outlined in the biggest US study by Luke et al. (22). Furthermore, it is worth mentioning that in patients with anovulation the CLBRs reach plateaus after the third cycles at 65.5%. These results may be explained by the distorted steroidogenesis of the theca cells and metabolic imbalance found in patients with polycystic ovary syndrome (PCOS). The quality of the oocytes has previously been showed to be poorer in patients suffering from PCOS and the finest dosage of ovarian hormonal stimulation in patients undergoing IVF is still debatable (23). Thus, multiple new therapies are implemented in order to improve pregnancy outcomes in this subcategory of patients. Among them, myo-inositol has a pivotal role in cellular signaling, as it has been shown to improve glucose uptake and FSH signaling affecting positively the oocyte quality (24). Nonetheless, data is not strong enough to support this improvement in pregnancy outcomes and additional clinical trials are needed in this regard (24-26).

Only patients with low ovarian reserve had their CLBR plateauing after the second cycle with only 29.4%, which is significantly different from the rest of our study cases mentioned here. With an improvement in cumulative rates of only 7% after 2 cycles and subsequent stabilizing after 6 consecutive cycles, it may be concluded that assisted reproductive technologies in patients with low ovarian reserves may be futile and especially after 3 cycles. Nevertheless, the number of events in this particular group was too small to draw definite conclusions. These findings contradict previous reports that showed no substantial differences in the CLBRs among women with various causes of infertility (27-30).

These results show that for patients willing to continue their treatment, the CLBRs after 6 cycles would be 69.9% (95% CI: 63.8-75.6) at the age of 35 years or younger, which is close to the live birth rate of 75% in a woman trying to conceive naturally. However, the CLBR at the age of 40 years for our subjects is 31.2% (95% CI: 16.1-50.0), which is slightly lower than the 44% of natural conception (31, 32). Considering the age-related reduction in success rates in IVF treatments, our results are reassuring that a CLBR up to 83.7% in women aged 36 to 39 years (95% CI: 69.3-93.2) is achievable, encouraging women younger than 40 years to repeat their IVF treatment cycles when the cost is not a barrier to the treatment. Our findings are in line with a previous report showing that patients older than 40 years are less likely to conceive with repeated cycles compared to the younger ones (27), thus patients older than 40 years of age should be adequately counseled that IVF at this point does not improve the age-related decrease in fertility.

In a retrospective study on 4810 transfers, the possible beneficial effects of transvaginal ultrasound-guided ET was assessed and it was shown that the number of pregnancies per ET significantly increased when performed under transvaginal ultrasound compared to trans-abdominal (38% vs. 30%, P<0.001). Transvaginal ultrasound may simplify difficult transfers via a better monitoring of the trans-cervical area improving the overall technique (33).

The multiple pregnancy rate was 38.4 %, with 83.1% twins, and 15.7% triplets, reflecting the continuing practice of transferring more than 2 embryos in the ME. The mean number of embryos transferred in this study was 2.7 (± 0.9). These rates are high when compared to averages reported in the American and European registries, with only 25.1% risk of multiple births (29). The percentage of multiples is slightly lower than the ones observed in Argentina (43.1%), Brazil (55.9%) and Taiwan (40.5%) (34). This indicates the utmost priority for establishing new policies and regulations regarding the number of embryos transferred per cycle to lower the increased risk of perinatal and maternal morbidity and mortality associated with multiple pregnancies (35). With improvements in cryopreservation methods, consecutive fresh and frozen single-embryo transfer cycles should be encouraged, thus taking into account frozen cycles when estimating CLBRs.

This is the first study in the ME to report CLBRs per cycle following fresh IVF treatment over a one-year period. We classified our patients according to age and the type of infertility when to our knowledge other studies have failed to do so. In addition, we included all patients presenting for their first cycle and undergoing fresh cycles, thus increasing the generalizability of our results. CLBRs were calculated on the basis of conservative estimates reflecting that women who do not achieve a live birth at their first attempt, will have their chances increased after

successive attempts. In our study, we used live birth rates as a primary outcome while other studies reported pregnancy rates only (14, 15).

Because of the retrospective aspect of the study, confounders were not reliably controlled, and significant biases affected the outcome. Our study has several other drawbacks. For instance, the cycles that were cancelled before oocyte retrieval were not recorded. This might have led to a minor overestimation of the CLBRs, as patients with severely poor prognosis did not account for the number of cycles and were excluded. However, only 36 patients were deemed ineligible, concluding that our findings are very close to the actual rates and the methodological bias had a relatively small influence on the final results. Patients who usually discontinue treatment are patients with very poor prognosis and are older than 40 years. In our cohort, only 16.3% of the cases were older than 40 years and most women had a high oocyte yield (10.4 ± 7.8). Because of these two important factors, we expect a very small difference between the rates that we calculated and the actual rates. On the other hand, some patients had undergone previous IVF cycles in other centers, adding some bias to the results since different laboratories and techniques may have been used. Furthermore, there was extensive heterogeneity in the different controlled ovarian stimulation protocols used limiting the generalizability of the results.

Our observed results postulate the chances of obtaining a live birth after one or multiple consecutive cycles, basing our decisions on some realistic expectations of CLBRs. In addition, it provides hope for older patients whose CLBRs are not affected by their age up till the age of 40. This reveals the advancements in reproductive technologies with the growth of ICSI (35).

In a region that is highly influenced and controlled by religious beliefs, different barriers exist for using assisted reproductive technologies, preventing the performance of oocyte and sperm donation. Therefore, with these unanticipated findings, couples have no other options except to extend their treatment cycles beyond 4 cycles.

Conclusion

This study provides an approach for estimating the effectiveness of IVF over 6 successive cycles. We showed an increase in the CLBRs over multiple cycles reaching a 67.9% chance of conception after 6 cycles, with variations by age and type of diagnosis. These findings are reassuring for patients insisting to continue with their treatments given the meaningful cumulative chances of success. Thus, barriers to continuation of treatment should be reduced with improvement in couples' counseling. Moreover, our results show that IVF treatments approach the natural fertility rates in patients younger than the age of 40.

However, the multiple pregnancy rate is still high in this part of the world due to the lack of regulations and policies. The practice is surrounded by an inequity in accessibility to this expensive form of health resource with fluctuation in the proportion of treatment cycles where few patients have the privilege of starting another IVF treatment in the case of a previous failed one.

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Authors' Contributions

D.K., G.G.; Participated in the study desing, data collection and drafting the manuscript. D.K., J.A., M.A., A.A.M., A.H., L.E.T., F.K.; Participated in patient recruitment, data entry, statistical analysis, and revision of the final manuscript. D.K.; Was responsible for data analysis and interpretation. A.K., A.N., G.G.; Participated in finalization of the manuscript and approval of the final draft. All authors read and approved the final manuscript.

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Original Article

Different Polymorphisms of *Vascular Endothelial Growth Factor* Gene in Patients with Pre-Eclampsia among The Iranian Women Population

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Abstract

Background: Pre-eclampsia (PE) is a pregnancy complication and one of the leading causes of maternal and neonatal morbidity and mortality in the world. PE is characterized by high blood pressure and signs of damage to the other organs, most often the liver and kidneys. Given the importance of mutation in the vascular endothelial growth factor (*VEGF*) gene and its correlation with the incidence of PE, the relationship of *VEGF* encoding gene polymorphisms rs922583280, rs3025040 and rs10434 with the incidence of PE in the population of Iranian women was studied, in this research.

Materials and Methods: In this case-control study, 100 pregnant women with PE diagnosis and 50 healthy pregnant women were evaluated using Sanger sequencing method to determine genotypes rs922583280, rs3025040 and rs10434.

Results: There was no significant difference in the allele frequency of rs922583280 and rs3025040 polymorphisms between case and control groups (P>0.05), while frequency of the recessive allele (G) for rs10434 polymorphism was significantly higher in the case group compared to the control group (P=0.014, case=24%, control=12%). Frequency of the allele A in the control group was higher than the patient group (case=76%, control=88%). Frequency of AG genotype in the patient group was also higher than the control group. In addition, frequency of AA genotype in the control group was higher than the patient group (case=57%, control=78).

Conclusion: The results of this study demonstrated a significant difference between patient and control groups for the *VEGF* coding gene polymorphism rs10434 and it can affect the incidence of PE among Iranian women.

Keywords: Iranian Women, Pre-Eclampsia, Single Nucleotide Polymorphism, Vascular Endothelial Growth Factor

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Introduction

Pre-eclampsia (PE) is one of the most common types of abnormality in pregnancy associating with increased blood pressure in the second half of pregnancy and proteinuria (protein excretion in the urine) and is considered as one of the three main causes of maternal and fetal mortality and related complications (1). This complication is a systemic disorder and it can cause different complications in the mother such as kidney and liver dysfunction, cerebral edema associated with seizure and affliction to hemolysis, elevated liver enzymes, and a low platelet count (HELLP) syndrome. It can also increase risk of abnormality in the fetus, such as fetal growth restriction, which is considered as one of the most important causes of neonatal mortality (2, 3).

Expulsion of the fetus and placenta from the mother's body eliminates symptoms of the disease, but complications of the disease can be problematic for the child and mother until the end of life (4).

There are two types of PE: mild and severe. Mild form of PE is diagnosed when pregnancy is greater than 20 weeks, blood pressure is greater than 140 systolic or 90 diastolic, 0.3 g of protein is collected in a 24-hours urine sample or persistent 1+ protein measurement on urine dipstick. There is no other sign of problem in the mother or baby (5). Severe type of PE is characterized by a diastolic blood pressure of 110 mm Hg or more, 2+ or higher proteinuria, high creatinine, increased liver enzymes and headache, oliguria, pulmonary edema, upper abdominal pain, visual impairment and thrombocytopenia (6).

Many studies have pointed the importance of vascular endothelial growth factor (*VEGF*) gene in the pathogenesis of PE (6-8). *VEGF* gene (that produces an angiogenic protein) is located on chromosome 6 and it contains 4 ex-

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ons, playing essential role in normal function of the endothelial cells (9). Findings constantly reported reduced free and accessible amount of biological VEGF in preeclamptic women. Production of direct VEGF inhibitor in response to ischemic placenta, as a characteristic of the disease, is a mechanism which often leads to reduced level of free VEGF (10). All members of VEGA family stimulate cellular response by binding to its tyrosine kinase receptors on the cell surface (11). Single nucleotide polymorphisms (SNPs) are intended as a major genetic source of phenotypic changes within a species. They are considered as important markers that are used in diagnosis of disease (12, 13).

Today, a large number of women are suffering from PE during pregnancy. Unfortunately, the main causes of this disease have not yet been known. It seems that the mutation in the *VEGF* gene is one of the main causes of this disease (6). The aim of this study was to examine the polymorphisms of *VEGF* gene in women patients. Regarding the importance of this issue, we attempted to obtain enough statistical information to consider SNPs for identifying individuals predisposed to the disease through determining possible mutations associated with PE in the affected individuals.

Materials and Methods

This study was approves by Ethics committee of Islamic Azad University- Science and research branch (Tehran, Iran, approval number: IR.I-AU.SRB.REC.1397.111).

This is a case-control study in which three SNPs of *VEGF* gene including rs922583280, rs3025040 and rs10434 were examined in 100 pregnant women diagnosed with PE and 50 healthy pregnant women referred to the hospitals in Tehran, between 2017 and 2018 (inclusion criteria consists of pregnant women with no history of hypertension and with average of 110 mm hg systolic and 70 diastolic blood pressures).

Exclusion criteria included history of any cardiovascular disease, metabolic disease, hypertension before pregnancy, smoking of cigarette, chronic hypertension and kidney disease before or during this research (14), since these criteria might cause disorders in our studies due to the interference of gene function. Sanger sequencing method was used to determine genotypes. After completing the consent form by the participants, 5 ml volume of venous blood was taken from qualified individuals in the studied groups and it was divided into two tubes; clotting tube for serum separation and Ethylenediaminetetraacetic acid (EDTA) anticoagulant tube for DNA extraction. Blood samples were stored at -20°C. All samples were evaluated using similar methods and conditions. DNA was extracted from all samples using a salting out method. DNA purity and quantity were determined using a Nanodrop 2000 spectrometer (Termo-nanodrop 2000c-USA). Primers were designed using Primer blast software (http:// www.ncbi.nlm.nih.gov/tools/primer-blast). The primers and size of the amplified sequence are mentioned in Table 1.

Table 1: The primers and size of the amplified sequences

Gene	Primer sequences (5´-3´)	PCR product size
VEGF	F: TGGTGAAGTTCATGGATGTCTATC R: ACACAGGATGGCTTGAAGATG	115
	F: GTGCTAATGTTATTGGTGTCTTC R: CAATGTGTCTCTTCTCTTCGC	508

PCR; Polymerase chain reaction.

Polymerase chain reaction (PCR) reaction was performed in 25 µl volume, containing 100-300 ng of extracted DNA, 1X PCR buffer (included 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$), 2 mM $MgCl_2$, 200 µM dNTP mix and double distilled water, 1 U of Taq DNA polymerase (super Taq DNA polymerase, Gen Fanavaran Co., Iran) and 0.4 µM of each oligoneucleotide primer in Thermocycler (Epperndorf-Nexus, Germany). PCR program was performed as follow: enzyme activation at 95ºC for 5 minutes, denaturation at 95ºC for 30 seconds, annealing at 58ºC for 30 seconds, elongation at 72ºC for 30 seconds for 30 cycles and a final extension at 72ºC for 5 minutes. PCR products were loaded on 1% agarose gel followed by ethidium bromide staining to confirm specificity and quality of the amplified fragments (PCR kit, Gen Fanavaran Co. DATA sheet).

To determine genotype of the PCR products, the samples were sequenced. They were next analyzed by FinchTV software and the accuracy of work was ultimately confirmed.

SPSS software (BMI SPSS statistics version 22, USA) was used for data analysis and only 5% was considered as acceptable rate of the type 1 error. The SHEsis software was used to examine the Hardy-Weinberg equilibrium and to evaluate the extent of linkage disequilibrium (LD), D′ and r^2 between pairs of polymorphisms. Given the status of data distribution, independent samples t test, Mann-whitney U and one-way ANOVA or Kruskal-Wallis were also used. Odds ratios (OR) with 95% confidence intervals were calculated to determine the odds of developing PE when the individual has gene variants of interest. Comparison of genotype frequencies, association with the disease using the best inheritance model, LD statistics and haplotype analysis, including haplotype frequency estimation, as well as the analysis of association between haplotypes and PE were performed using SNP Stats software. $P<0.05$ was considered statistically significant.

Results

The demographic and clinical characteristics of the studied subjects are presented in Table 2. The results of this study showed that there is a significant difference between the groups of patient (case) and control in terms of pregnancy weight gain and blood pressure; so that the weight in the patient group was significantly higher than the control group ($P<0.001$, OR=2.556).

According to this study, it was also found that there is significant difference between systolic (P<0.001) and diastolic blood pressures (P<0.001) of these groups. So that blood pressure in the patient group was higher than the control group (Table 2), but there was no significant difference in BMI (P=0.131, OR=0.575) and age (P=0.217, OR=0.364) between the case and control groups. PCR fragments of this gene were detected after electrophoresis on 1% agarose gel. Sizes of fragments are 520 bp and 256 bp (Fig.1).

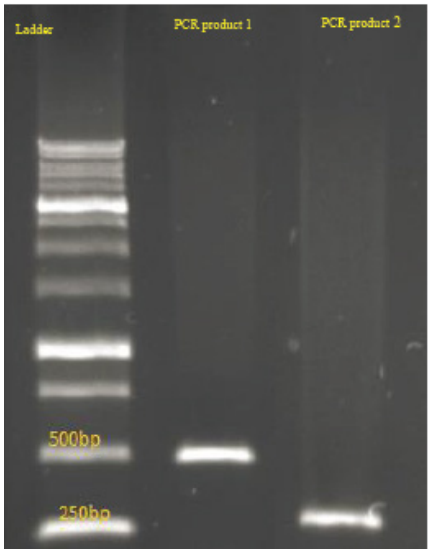

Fig.1: Polymerase chain reaction (PCR) amplification. Fragments of the gene were detected after electrophoresis on 1% agarose gel. Sizes of fragments are 520 bp and 256 bp.

We determined which allele combination from the two SNPs associated with preeclampsia (Table 3).

Three allele combinations including C-C-A and C-C-G haplotypes, with 7-23% frequency, were associated with preeclampsia (Figs. S1-3, See Supplementary Online Information at www.ijfs.ir).

In addition, no significant difference was determined in the frequency of rs922583280 and rs3025040 polymorphisms between the patient and control groups. The frequency of recessive allele G, in the rs10434 polymorphism was significantly higher in the patient group rather than that the control group (case=24, control=12 OR, R=2.316: from 1.167 to 4.594, P=0.014), while the frequency of allele A in the control group was higher than the patient group (case=76, control=88, OR=2.316: from 1.167 to 4.594, P=0.014). The frequency of AG genotypes in the patient group was also higher than control group (OR=2.452: from 1.099 to 5.467, P=0.026), while frequency of AA genotype in the control group was higher than the case group (OR=2.675: from 1.229 to 5.820, P=0.012, Table 4).

The interaction between any possible pair of SNPs was visualized by SHEsis program. Analysis revealed linkage disequilibrium (LD) between rs922583280 and rs3025040 (D´=1.000 and r^2=0.001), while weak LD was determined between rs922583280 and rs10434 as well as rs3025040 and rs10434 (D´=0.062 and r^2=0.001; D´=0.299 and r^2=0.001, respectively, Table 3).

Minor allele frequency for VEGF SNPs were rs10434: A>G (A=0.3476/1741; 1000Genomes), rs3025040: C>T (T=0.1512/757; 1000Genomes), rs922583280 C>T (minor allele frequency is not specified.

Table 2: Anthropometry and blood pressure data in the patient (case) and control groups

Characteristics	Case group	Control group	P value
Age (Y)[a]	25.8 (7.16)	24.7 (6.22)	0.217
Gestational age (weeks)[a]	32.9 (4.02)	33.1 (4.71)	0.797
Gestational weight gain (kg)[b]	12.7 (8.50-16.50)	10.0 (6.75-13.55)	0.003*
Systolic blood pressure (mmHg)[b]	170 (160.0-180.0)	110 (100.0-120.0)	<0.001*
Diastolic blood pressure (mmHg)[b]	110 (100.0-120.0)	70 (70.0-80.0)	<0.001*
Body mass index (kg/m^2)[b]	24.05 (21.63-28.13)	23.35 (20.53-26.90)	0.131

[a]; Characteristics are presented as mean (standard deviation), [b]; Characteristics are presented as median (ranges), and *; P<0.05: statistic significant.

Table 3: Haplotype frequencies of *VEGF* rs922583280, rs3025040 and rs10434 polymorphisms in case and control groups

Haplotypes	Cases (%) n=100	Controls (%) n=50	P value[a]	OR (95% CI)
VEGF rs922583280, rs3025040 and rs10434				
C - C - A	0.070	0.847	0.002	0.365 (0.187-0.712)
C - C - G	0.235	0.103	0.006	2.648 (1.283-5.467)
C - T - A	0.050	0.023	0.274	2.214 (0.514-9.543)

VEGF; Vascular endothelial growth factor, OR; Odds ratio, CI; Confidence interval, and [a]; Evaluated by Pearson's Chi-squared test.

Table 4: Haplotype frequencies of *VEGF* rs922583280, rs3025040 and rs10434 polymorphisms in case and control groups

Gene	Case group (%)	Control group (%)	P value[a]	OR (95% CI)
Rs922583280				
CC	97.00	98.00	0.593	0.660 (0.067-6.507)
CT	3.00	2.00	0.593	0.660 (0.067-6.507)
TT	0.00	0.00	ND	ND
Frequency of C allele	98.50	99.00	0.722	1.508 (0.155-14.681)
Frequency of T allele	1.50	1.00	0.722	1.508 (0.155-14.681)
Rs3025040				
CC	91.00	92.00	0.552	1.137 (0.332-3.891)
CT	8.00	8.00	1.00	1.00 (0.286-3.495)
TT	1.00	0.00	0.478	0.990 (0.971-1.010)
Frequency of C allele	95.00	96.00	0.102	0.474 (0.190-1.179)
Frequency of T allele	5.00	4.00	0.302	0.605 (0.231-1.585)
Rs10434				
AA	57.00	78.00	0.012*	2.675 (1.229-5.820)
AG	38.00	20.00	0.026*	2.452 (1.099-5.467)
GG	5.00	2.00	0.377	2.579 (0.29312.690)
Frequency of A allele	76.00	88.00	0.014*	2.316 (1.167-4.594)
Frequency of G allele	24.00	12.00	0.014*	2.316 (1.167-4.594)

OR; Odds ratio, CI; Confidence interval, [a]; P<0.05: Statistically significant, and ND; Not defined.

Table 5: Minor allele frequency and Hardy-Weinberg tests for the study of population

SNP	MAF	HWE P
rs10434	0.3476	0.676
rs3025040	0.1512	0.114
rs922583280	-	0.878

SNP; Single nucleotide polymorphism, MAF; Minor allele frequency, and HWE P; Hardy-Weinberg equilibrium P value.

Discussion

Untreated PE causes serious and fatal complications for mother and baby (1). Given the importance of mutations in the *VEGF* gene, their correlation with the incidence of PE and early delivery, and the risk of afflicting to eclampsia and mortality caused by it for mother and fetus, we attempted to identify possible mutations and early genetic detections.

In this study which was carried out on Iranian pregnant women with PE, a total number of 150 pregnant women, including 100 pregnant women diagnosed with PE and 50 healthy pregnant women, were examined. Mean age in the patients group was 25.8 ± 7.16 and in the control group was 24.7 ± 6.22. This difference was not significant. Analysis represented that frequency and distribution of rs10434 polymorphism allele and genotype in both control and case groups showed a significant difference, so that the frequency of recessive allele G in the patient group was significantly higher than the control group.

It was also found that frequency of the allele A in the control group was higher than that of the patient group. In the genotypic frequency study, the results showed that frequency of AG genotype in the patient group was higher than the control group and frequency of AA genotype in the control group was higher than that of the patient group. In the case of rs922583280 and rs3025040, there was no significant difference in the allele and genotype frequency between these groups. The results of our data were similar to the studies conducted on PE patients in other populations, as it was found in the meta-analysis study conducted on four *VEGF* gene polymorphisms by Song et al. (15). In this study, it was demonstrated that rs2010963 polymorphism was associated with the incidence of PE in Asian and European populations. Moreover, rs3025039 polymorphism was associated with this disease in the Asian population, while rs1570360 and rs699947 polymorphisms had no correlation with the incidence of the disease (16). Similarly, in a study done by Salimi et al. (17) on Iranian population, it was found that rs2010963 polymorphism in the *VEGF* gene was associated with the incidence of PE. In similar studies conducted by Hansen et al. (18) and Chedraui et al. (16), it was found that there was no significant association between the polymorphisms of *VEGF* gene and the incidence of disease. A notable point in the present study and the studies conducted on different populations is that polymorphisms in the untranslated regions (UTRs) of *VEGF* gene exons are mainly associated with the incidence of disease. Because these regions play an important and vital role in the trimming process, it can be concluded that mutation in these regions can affect function of VEGF and lead to disorders encountered PE. However, this was not observed in some cases, such as rs3025040 polymorphism, which is

in the UTR region. The reason of controversial findings in different studies can be due to differences in populations and breeds of these studies, considering the fact that gene polymorphisms are affected by this important factor (19).

In general, it can be concluded that *VEGF* gene polymorphisms are associated with the incidence of PE in Iranian women. So that, it is concluded in this study that allele G in the polymorphism rs10434 as well as genotype AG in the same polymorphism may lead to the increased incidence of PE in this population, while no relationship of rs3025040 and rs922583280 alleles and genotypes with the incidence of PE was determined. Moreover, there was no significant relationship between anthropometric factors and genotype of all three polymorphisms (rs922583280, rs3025040 and rs10434) in both patient group with PE and control group.

Conclusion

Investigations showed that frequency and distribution of rs10434 polymorphism alleles and genotypes had significant difference between control and case groups, so that the frequency of recessive allele G in the patient group was significantly higher than the control group. In the genotypic frequency study, the results showed that frequency of AG genotype in the patient group was higher than the control. In the case of the frequency of alleles and genotypes for two polymorphisms rs922583280 and rs3025040, there was no significant difference between patient and control groups. Some of the limitations which can be mentioned here include small size population of the study and lack of the concomitant VEGF level in plasma. Further studies consisting of the larger and classified cohort are needed to validate our initial findings and to determine association of the other clinical variables and SNPs with the subtypes of PE. Moreover, several clinical parameters, including plasma VEGF, PlGF and sFlt-1 levels and polymorphisms of the other VEGF family (PlGF) members should be put into the prospective account.

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Authors' Contributions

Z.P.; Participated as supervisor, study design, sample collection and evaluation. R.B.; Participated as advisor, conducted molecular experiments and PCR analysis. R.N.; Participated in data collection and statistical analysis. All authors read and approved the final manuscript.

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Original Article

Presence of The NLRP3 Inflammasome Components in Semen of Varicocele Patients

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Abstract

Background: Varicocele is a common cause of male infertility with multifactorial etiology. Inflammation is a characteristic pathological event that occurs in the testis tissue following the varicocele. The aim of this study was to investigate expression of nod-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome components and cytokines in semen of varicocele and control subjects.

Materials and Methods: In this case-control study, seminal plasma was collected from 32 varicocele patients (with grades 2 and 3) and 20 fertile men as control group. Semen analysis was performed in all subjects. Concentrations of interleukin-1b (IL-1b), IL-18 and caspase-1 in seminal plasma were measured by enzyme-linked immunosorbent assay (ELISA). Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain, in addition to NALP3 were identified in seminal plasma by Western blot. Statistical significance between the mean values was determined by student's t test.

Results: According to our data, the level of IL-1b was significantly (P=0.03) increased in the seminal plasma of varicocele patients, compared to the control subjects. We analyzed amount of IL-18 in the both groups. The level of this interleukin was markedly (P=0.002) decreased in varicocele patients. No change was observed in the level of caspase-1 in both groups. Western blot analysis revealed that apoptosis associated speck-like protein (ASC, P=0.0002) and NLRP3 (P=0.005) were significantly elevated in the semen of varicocele patients.

Conclusion: This study provides the first evidence of activation of NLRP3 components in semen of men with varicocele.

Keywords: Inflammasome, Semen, Varicocele

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Introduction

Varicocele is one of the most common causes of male infertility. Approximately, 15% of healthy men and 40% of infertile men suffer from varicocele (1). This gonadal disease is defined as a pathological dilation of testicular venous plexus (pampiniform plexus) and it is associated with pathological problems in the testicular tissue. Typically, it occurs on the left side (2). Varicocele could interfere with normal spermatogenesis which leads to production of abnormal spermatozoa (3, 4).

In varicocele disease, heat stress induces undesirable adverse effects on testis tissue, such as spermatogenesis impairment, increase in production of reactive oxygen species (ROS) and apoptosis (5). On the other hand, the stasis of venous blood in the dilated pampiniform plexus impairs arterial blood flow and restricts oxygen supply necessary for testis tissue which can lead to the testicular hypoxia (6). It is believed that hypoxia signaling pathway is responsible for pathogenesis of varicocele (7). However, there are studies suggesting that varicocele stimulates pro-inflammatory and inflammatory cytokines release, such as interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α) (8-11).

Inflammation is an immune response to pathological events, such as bacterial/viral infection and tissue damage to protect other cells from injury (12). Inflammation can be triggered by necrosis or pyroptosis. The latter is involved in receptor-mediated sensing of pathogens, cell fragments,

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ATP as well as the activation of intracellular multiprotein complex named inflammasome. Inflammasomes exist in different subtypes and represent a complex of proteins assembly. They activate caspase-1 which, in turn, promotes maturation of pro IL-1 and IL-18 into their active forms. Among the different types of inflammasome (NLRP1, NLRP2, NLRP3 and AIM), the role and regulation of Nod-like receptor family, pyrin domain containing 3 (NALP3) inflammasome is well studied. The structure of NLRP3 consists of three components: a central nucleotide-binding and oligomerization (NACHT) domain, a ligand-sensing leucine-rich repeat domain (LRRs) and a pyrin (PYD) domain. NLRP3 activation leads to the oligomerization of apoptosis associated speck-like protein (ASC) which contains a caspase activation and recruitment domain (CARD). Apoptosis associated speck-like protein (ASC) interacts with the CARD of pro-caspase-1 and converts it to the active form. Activated caspase-1 then proceeds to generate active form of IL-1b and IL-18 from the immature forms (13).

In testis of rodents and primates, Sertoli cell is responsible for NLRP3 expression and it is believed that alteration in NLRP3 expression might impair fertility (14). Increased level of *NLRP3* mRNA in rat testis tissue was induced seven days after spinal cord injury (SCI) (15). In addition, overexpression of the NLRP3 components (ASC, caspase-1, IL-1β and IL-18) were identified in the seminal plasma of patients with SCI, while the previous studies showed that inflammasomes are responsible for abnormal semen quality in these patients (16). Recently we showed NLRP3 complex expressed in testis tissue of varicocelized rats and resveratrol as an antioxidant could decrease its expression (17). Due to this finding and presence of some pro-inflammatory cytokines during the course of varicocele (10, 11), we hypothesized that NLRP3 inflammasome components might be present in semen of varicocele patients and inflamatory events are involved in the pathogenesis of varicocele in addition to the hypoxia pathway. Therefore, the aim of this study was to investigate presence of NLRP3 complex in seminal plasma of varicocele patients.

Materials and Methods

Study design

This study was performed from December 2017 to September 2018. Sample size was calculated by the following formula: $n \geq \frac{\left(z_{\alpha/2} + z_{\beta}\right)^2 \sigma^2}{\varepsilon^2}$

where type one (α) and type two errors (β) were 0.05 and 0.20 (power ¼; 85%), respectively according to previous studies (7, 18). Based on this, we needed at least 20 subjects in each group. Semen samples were collected from 32 men with varicocele and 20 age-matched control subjects attending the Iranian Academic Centre for Education, Culture and Research (ACECR). All control subjects had no history of infertility with normal sperm analysis who volunteered to take part in this research. The mean ± standard error of the mean (SEM) age of varicocele patients and control subjects were 27 ± 2.1 years and 26 ± 1.8 years, respectively. The patients had palpable varicocele (at grades 2 and 3) with a clear history of infertility (for 2-3 years). Infertility is defined as inability to have children after at least one year of unprotected intercourse (19). The study was approved by the Ethics Committee of Arak University of Medical Sciences (code: 93-175-10; Arak, Iran) and all patients signed the informed consent for this study.

Seminal collection

Semen samples were collected from varicocele and control subjects by masturbation after at least 48 hours of sexual abstinence. After liquefaction for 30 minutes, semen parameters including volume, pH, concentration, morphology and motility were analyzed according to the World Health Organization criteria (20). Analysis of sperm concentration was performed with a Neubar chamber on two separate preparations of the semen sample (dilution 1:20 in Ringer's solution). A standard volume of semen (approximately 10 µl) was placed onto a glass slide and covered by the cover slide, then 200 spermatozoa were assessed under a light microscope (×400 magnification) for the percentage of sperm motility. To evaluate sperm morphology Papanicolaou staining was used and one hundred sperm from different fields were counted to determine the morphological abnormalities (21).

Seminal plasma preparation

Semen samples were centrifuged at 1000 xg for 15 minutes at room temperature. The supernatant was then collected and stored at -70°C for further analysis (16).

ELISA analysis

Concentrations of the mature IL-1β, IL-18 and caspase-1 (all from Abcam, USA) were measured by an enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol. Seminal plasma samples were thawed at room temperature and placed in plates pre-coated with a specific monoclonal antibody for each of IL-1b, IL-18 or caspase-1. Each sample was duplicately assayed.

Western blot

Seminal plasma samples were thawed at room temperature. One microliter of seminal plasma from each subject was mixed with loading buffer (containing a final concentration of 50 mmol/l Tris-HCl pH=7.0, 2% sodium dodecyl sulfate, 10% glycerol, 5% b-mercaptoethanol and 0.002% bromophenol blue), and heated at 95°C for 10 minutes. Protein concentrations were determined using the BCA™ Protein Assay Kit (Pierce, Germany) according to the manufacturer's protocol. The same amount of protein samples was loaded, separated on 8-12% (v/v) discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and

transferred onto a polyvinylidenefluoride (PVDF) membrane (Roche, Germany). After blocking with 5% skimmed milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 hour at room temperature, PVDF membranes were incubated with anti-ASC antisera (Santa Cruz, USA, diluted 1:1000) or anti-NLRP3 antisera (Bioss, USA, diluted 1:1000) overnight at 4oC. After washing with TBS-T, membranes were incubated with a peroxidase-conjugated goat anti-rabbit (BioRad, USA, diluted 1:500) secondary antibody for 2 hours at room temperature. Visualization was performed using the enhanced chemiluminescence method (ECL plus, Pierce Scientific, USA) according to the manufacturer's protocol. For densitometric quantification, intensity of the specific bands was normalized to β-actin (Bioss, USA, diluted 1:1000) in the same blot using Image J software (free Java software provided by the National Institute of Health; Bethesda, USA) (22).

Statistical analysis

The results are expressed as means ± standard errors (SE). The Shaprio-wilktest was used to determine normal distribution. Independent sample t test was applied to check the matched factor between the case and control groups. Statistical significance between the mean values was determined by paired t test and $P \leq 0.05$ was considered statistically significant.

Results

In this study, we analyzed relationship of sperm parameters with NLRP3, ASC, IL-18 and caspase-1 in both control and varicocele groups. We could not find any correlation in our study.

Semen quality is lower in varicocele patients compared to the control subjects

The semen volume (5.1 ± 1.8 ml in control vs. 4.06 ± 1.5 in varicocele) and pH (7.8 ± 0.2 in control vs. 7.7 ± 0.1 in varicocele) were not significantly different between the control and varicocele subjects. The median sperm concentration in the varicocele group (44 million/ml) was significantly lower than the control group (97 million/ml, P=0.0001).The median sperm total motility was equal in the both groups (61% in control vs. 55% in varicocele) while the sperm progressive motility in varicocele patients showed a significant decrease (P=0.0003) compared to the control subjects. In addition, varicocele patients had more abnormal sperm (especially abnormal head) morphology compared to the control group (99% in varicocele vs. 80% in control).

Alteration in inflammatory cytokine levels in varicocele patients

The levels of IL-1β, IL-18 and caspase-1 were investigated in seminal plasma by ELISA. Our data showed that IL-1β was significantly increased (P=0.03) in seminal plasma of varicocele patients in comparison with control subjects [optic densitometry (OD)=1 ± 0.016 vs. 0.94 ± 0.021, respectively; Fig.1A]. In these patients, concentration of caspase-1 showed no obvious change (Fig.1B), while seminal plasma concentration of IL-18 revealed a small but significant (P=0.002) decline in varicocele versus controls (OD=0.71 ± 0.036 vs. 0.82 ± 0.032, Fig.1C).

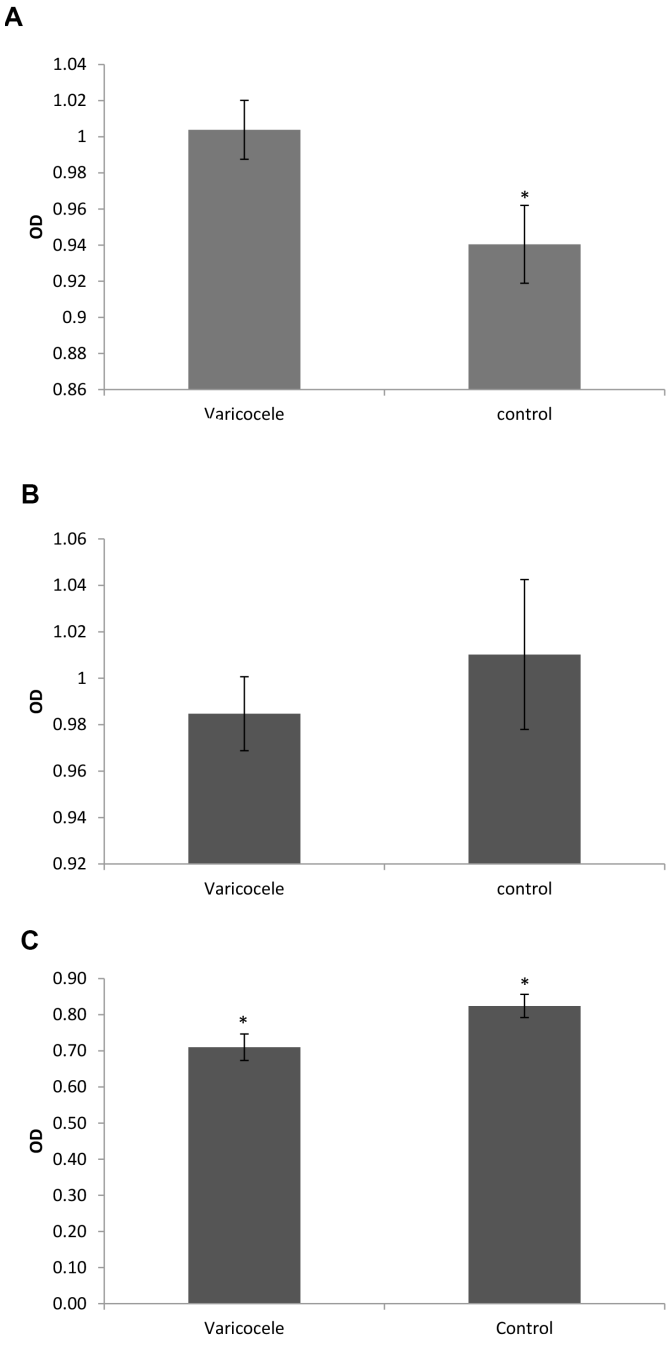

Fig.1: Measurement of the inflammatory cytokines in seminal plasma of varicocele patients using ELISA. **A.** Note the significant increase of IL-1ß protein levels in varicocele patients compared to the control subjects. **B.** Caspase-1 protein level did not reveal significant change between two groups. **C.** Note the significant decline of IL-18 protein levels in the varicocele group compared to the controls.
OD; Optic densitometry and *; P≤0.05 compared to the controls.

Inflammatory NLRP3 and ASC protein are elevated in seminal plasma of varicocele subjects

To investigate whether inflammasome components are expressed and changed in seminal plasma of varicocele patients, we quantified respectively ASC and NLRP3 protein levels by Western blot. NLRP3 (P=0.005) and ASC (P=0.0002) protein levels were significantly elevated in varicocele patients versus control subjects (relative intensity of ASC was 2.02 ± 0.09 vs. 0.32 ± 0.28 and relative intensity of NLRP3 was 1.5 ± 0.13 vs. 0.56 ± 0.1, respectively, Fig. 2 A-C).

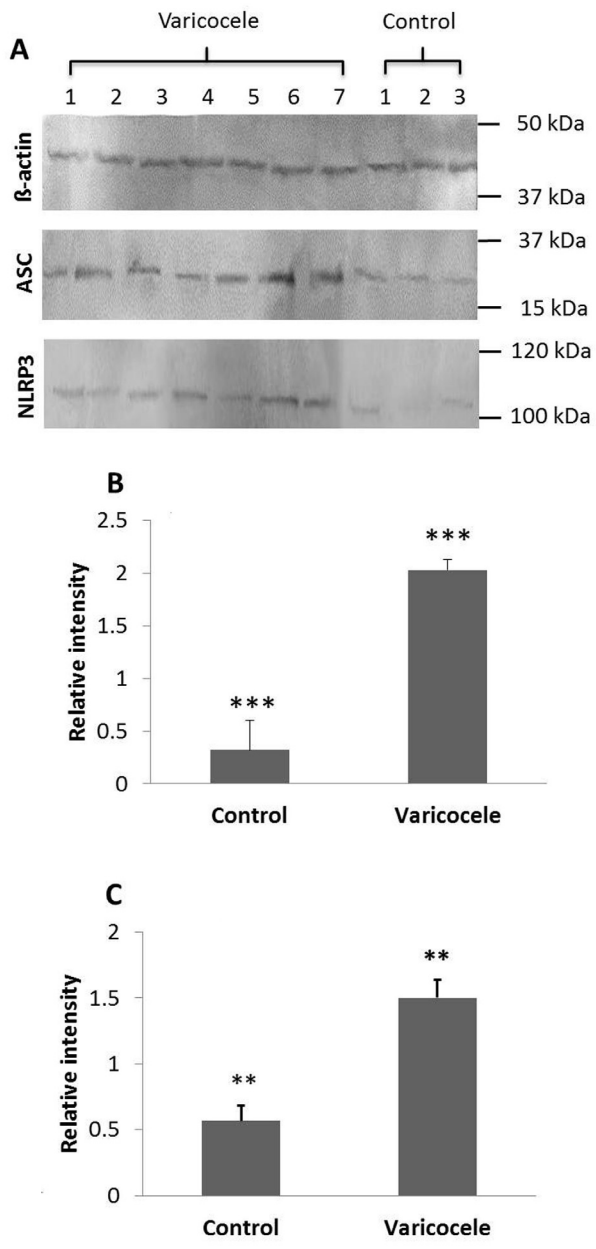

Fig.2: Analysis of inflammasome components in seminal plasma of varicocele patients by Western blotting and subsequent measurement of optical densities of immune-labelled bands. **A.** Respresentative Western blots with seven samples of varicocele patients and three control subjects. Note the increased intensities of NLRP3 and ASC in all varicocele patients. Quantification of the respective band intensities for **B.** ASC and **C.** NLRP3 given as relative intensities compared to ß-actin bands. **; P≤0.01 and ***; P≤0.001 compared to controls.

Discussion

In this study, quality of semen in all varicocele patients was lower than the control group. Varicocele patients had abnormal semen quality (23) which might be because of high ROS level (18), germ cell apoptosis and release of the inflammatory cytokines (5). Although previous studies suggesting the inflammatory cytokines are present in semen of men with varicocele, the inflammasome signalling mechanism in varicocele has not been previously tested. The current study indicates, for the first time, that inflammasome components ASC and NLRP3 are present in semen of varicocele patients.

In this study our results showed that ASC and NLRP3 levels in semen of varicocele subjects were significantly elevated compared to the control subjects. Additionally, concentration of IL-1β was higher in varicocele versus control subjects, whereas IL-18 was decreased in seminal plasma of varicocele patients and caspase-1 was not changed. In addition, we could not find any significant correlation between sperm parameters and NLRP3 inflammasome components.

Sahin et al. (24) acclaimed that amount of pro-inflammatory cytokines such as IL-1α and IL-1β were increased 11 and 13 weeks after the induction of varicocele in rats. They showed that during progression of the disease, IL-1α was expressed in round spermatids, spermatogonia, primary spermatocytes, Sertoli and Leydig cells, while IL-1β was found only in spermatogonia, Leydig and Sertoli cells. Concentration of the caspase-1 which is involved in IL-1β and IL-18 maturation (13), was equal in testis tissue of the both varicocele and normal subjects (25). During varicocele condition, an excessive release of nitric oxide (NO) into the seminal plasma occurs and this is believed to be a reason of low motility in sub-fertile patients with varicocele (26, 27). Kim et al. (28) revealed that NO production inhibits caspase-1 activity and subsequently inflammatory responses. However, NO accumulation could damage tissue itself. In the current study, absence of any obvious changes of caspase-1 protein level might therefore reflect neither the enzyme activity nor the influence of an excessive NO production in varicocele patients.

In this study, we expected that IL-18 level would be increased in varicocele subjects (29), while it was decreased. The difference between our results and the investigation performed by Zeinali et al. (29) could be related to different numbers and ages of the studied samples.

Western blot analysis showed high level of NLRP3 and ASC protein expressions in seminal plasma of varicocele patients. In our previuos work, we showed high levels of ASC, NLRP3 and caspase 1 expression in the testis tissue of varicocele-induced rats, three months after surgery (17).

Novelty is the strength of this study, as this is the first evidence for the existence and presence of the NLRP3 inflammasome in seminal plasma of varicocele patients.

Thus, it highlights the importance of anti-inflammasome therapies to improve the fertility rate in varicocele patients. However, a limitation is about the control subjects. It was better to choose fertile varicocele subjects as control group. The other weak point is that the immunohistochemistry was not done in this study to localize this complex.

Conclusion

Findings obtained from this study suggest that NLRP3 activation occurs in varicocele and it might be responsible for pathological procedure occurring in varicocele patients. Details of the NLRP3 inflammasome activation process has not been clarified yet. At present, it is not clear whether NLRP3 is causally related to the onset of varicocele or the result of pathological damages appearing in the course of this gonadal disease. Further study is underway to determine time course of activation of inflammasome in varicocele disease.

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Authors' Contributions

M.B.; Contributed to designing, conducting, and writing the manuscript. A.A.Gh., A.R.N.K; Contributed to sampling and sperm parameter analysis. C.B., A.Z.; Contributed to analysing the data and writing the manuscript. All authors read and approved the final manuscript.

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Original Article

Comparison of Sperm Telomere Length between Two Sperm Selection Procedures: Density Gradient Centrifugation and Zeta Potential

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Abstract

Background: Telomeres are particular sequences of DNA located at the end of the eukaryotic chromosomes that are essential for genome integrity. Telomere length in spermatozoa differs among males, as well as spermatozoa. Also, decreased telomere length in spermatozoa of infertile men is associated with the reduction of fertility potential and embryo quality. Density gradient centrifugation (DGC) and swim-up are useful techniques for separation of spermatozoa with longer telomeres. Also, the selection of sperm based on surface negative electric charge or "Zeta potential", can separate high percentage of spermatozoa with intact chromatin compared to DGC alone, and also the combination of DGC-Zeta can improve clinical outcomes of infertile men candidate for intracytoplasmic sperm injection (ICSI). Therefore, we compared sperm telomere length and DNA fragmentation between two sperm preparation procedures, namely DGC and zeta potential.

Materials and Methods: In this experimental study, we assessed sperm telomere length and DNA fragmentation by quantitative real-time polymerase chain reaction (PCR) and TUNEL assay methods, respectively. The spermatozoa were obtained from infertile men with normozoospermia between September 2017 and December 2017 and prepared either by DGC or zeta potential methods. Sperm telomere length was expressed as relative and absolute units.

Results: Compared with washed semen samples or control, no significant ($P>0.05$) difference was observed in the mean relative or absolute sperm telomere length when the two methods DGC or zeta potential were compared. However, the mean percentage of DNA fragmentation was significantly ($P<0.05$) lower in spermatozoa prepared by DGC or zeta potential methods than spermatozoa obtained from control samples.

Conclusion: This is the first study that compared the effect of DGC and zeta potential as the sperm preparation methods on sperm telomere length. It seems that both methods can select sperm population with high DNA integrity and the same sperm telomeres length.

Keywords: Density Gradient Centrifugation, DNA Fragmentation, Telomere

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Introduction

Lack of pregnancy following one year of unprotected sexual intercourse is termed" infertility", and its frequency is around 15%, that 40% of which is related to male infertility factors. Male infertility can be cured by intracytoplasmic sperm injection (ICSI), which almost bypasses all-natural selection barriers that sperm faces during natural fertility (1).

Quality of oocyte and sperm are two critical parameters determining ICSI outcomes. Quality of sperm is commonly defined based on the assessment of routine seminal indices, such as sperm concentration, motility, and morphology which reflect the efficiency of the male reproductive system (2, 3). During ICSI, despite the selection of motile or viable spermatozoa with normal morphology, the overall outcome remains limited. This dearth partly contributes to other functional aspects of spermatozoa, especially the genomic integrity of these cells, as this structure approximately participates in 50%

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of the genetic constitutions of the next generation (4, 5). In this regard, Avendaño et al. (6) demonstrated that the spermatozoa with normal morphology may have DNA fragmentation. Therefore, sperm preparation or processing in addition to the selection of spermatozoa based on sperm functional characteristic may have significant effects on ICSI outcomes. When it comes to sperm selection, researchers have taken different approaches to choose the most "fecund or physiological" spermatozoa. This is one of the hot topics in the field of andrology. For further explorations, please refer to reviews published by Henkel (7), Rappa et al. (8), and Sakkas (9).

One of the approaches for the separation of functional spermatozoa according to cellular and molecular characteristics is the selection of sperm cells based on surface negative electric charge or "zeta potential", which is induced by sialic acid added to sperm surface during maturation or the passage through the epididymis (10, 11). Selected sperm based on zeta potential has been shown to exhibit higher degrees of chromatin and DNA integrity compared to sperm selection based on the density gradient centrifugation (DGC) method and results in improved embryos quality (10, 12-14). In a randomized clinical study, it has been shown that the pregnancy rate was significantly higher when the combined methods of zeta potential and DGC procedures were applied in comparison with the DGC method alone in infertile men candidate for ICSI (15). Considering increased interest for clinical application of ICSI for severe male infertility, who are candidates for ICSI, there is urgent need to assess the molecular facets of sperm selection based on this technique compared to the DGC method.

Despite novel approaches for sperm selection/preparation, routine sperm processing has a historical background and lies in the way of assisted reproductive techniques (ARTs), especially intrauterine insemination (IUI). Previous studies indicate that several approaches have been taken to process spermatozoa for insemination, including swim-up, swim-down, DGC, albumin gradient, glass wool filtration, and Sephadex beads (7-9, 15). Among these techniques, the DGC method which separates spermatozoa based on their density (mass/volume) exposed to the gradient in the centrifugation field is currently the most popular common technique in andrology (16-18). DGC is almost used for all types of ARTs including, IUI, *in vitro* fertilization (IVF), and ICSI due to several advantages, such as the clean fraction of highly mature and motile spermatozoa, and also, it can be used for processing of semen samples. Also, the DGC method removes leukocytes or other cells and markedly reduces reactive oxygen species (ROS) (17). However, one of the disadvantages of this technique is sperm exposure to shear forces during centrifugation which is believed to induce ROS, and it can lead to a decrease in genomic integrity of spermatozoa. However, this shortcoming could be partially resolved by supplementation of processing media with antioxidants when the DGC method is applied (19, 20).

One of the critical aspects of sperm selection/preparation procedures and sperm process techniques such as DGC is the genomic integrity of sperm cells. Spermatozoa have very highly condensed nucleus protected against any chemical and physical insults during *in vivo* or *in vitro* studies (7, 21). One of the cellular facets affecting genomic integrity is the telomere length.

Telomeres are guanine-rich sequences that are more prone to undergo DNA break than non-telomeric DNA regions. They are considered important targets for free oxygen radicals. In this line, several studies showed significant negative correlations between sperm telomere length and sperm parameters, such as DNA fragmentation, protamine deficiency, and oxidative stress (22-25). Besides, there are significant associations between sperm telomere length and the percentage of sperm motility and viability (25). Therefore, short telomere length in spermatozoa denotes different functional defects at the cellular and molecular levels. Several lines of evidence demonstrate significant positive correlations between sperm telomere length and other factors, such as male age, fertilization, and embryo quality (22, 25-27). Indeed, it has been shown that children born with short telomere length present a high load of genetic damages (28).

Considering the fundamental roles of the DGC method in andrology or ARTs, as well as Zeta potential for sperm preparation as a novel approach to select the most fecund sperm, we aimed, for the first time, to evaluate and compare the sperm telomere length as a parameter of sperm quality between DGC and Zeta potential methods used for sperm preparation.

Materials and Methods

Ethical approval and subjects

In this experimental study, was approved by the Research Ethics Committee of the Royan Institute (IR.ACECR.ROYAN.REC.1397.89). Between September 2017 and December 2017, semen samples were obtained from 15 infertile men with normozoospermia who referred to the Andrology Unit of the Isfahan Fertility and Infertility Center for semen analysis. Total sperm count, sperm concentration, sperm motility, and morphology of spermatozoa were equal to or above the lower reference limit according to the criteria for the selection of normozoospermia established by World Health Organization (WHO) (29). Men with leukocytospermia, age >40 years or other infertility-related diseases, such as varicocele, Y-chromosome microdeletion, a history of cryptorchidism and orchitis, abnormal hormonal profile, and semen samples with sperm autoantibodies were excluded from the study. Written informed consent was obtained from all participants.

Sperm preparation

Semen samples were collected after 2-7 days of sexual abstinence and standard semen analysis was performed

according to WHO (29). Each semen sample was aliquoted into three parts. The first part was considered "control" or "washed sample" group that was rinsed with VitaSperm (Inoclon, Iran). The second and third parts of the semen sample were processed by DGC and zeta potential methods, respectively. Then, sperm telomere length and DNA fragmentation were assessed by quantitative real-time polymerase chain reaction (PCR) and TUNEL assay, respectively.

Sperm preparation by the density gradient centrifugation procedure

Semen samples were washed with sperm washing media (VitaSperm, Inoclon, Iran) supplemented with 10% human serum albumin. Then, the DGC procedure was performed with PureSperm (Nidacon International, Sweden). In this method, 1.5 ml of 45% PureSperm was layered over 1.5 ml of 90% PureSperm, and then, 1.5 ml washed samples were mounted on the 45% PureSpermand layer and centrifuged for 15 minutes (300 g). Subsequently, sperm pellet was regarded as processed spermatozoa and used for the assessment of sperm telomere length and DNA fragmentation (30).

Sperm preparation by the zeta potential procedure

The zeta potential method was carried out based on a study conducted by Chan et al. (31). Briefly, semen specimens were rinsed with the serum-free VitaSperm processing medium, and their concentration was adjusted to 5×10^6 spermatozoa/ml. Afterwards, 4 ml of adjusted sperm solutions were transferred to a 5-ml Falcon tube induced by gaining a positive surface charge using the rotation of the tube, two or three turns, inside a latex rubber tubing. One minute was specified for spermatozoa to adherence to the charged wall of the tube. Finally, the medium was collected to remove the non-adhering sperm cells.

Subsequently, the surface of the tube was washed thoroughly with VitaSperm plus 10% human serum albumin to detach adhering spermatozoa from the tube wall. Subsequently, the selected spermatozoa were centrifuged and used for further assessments.

Evaluation of sperm DNA fragmentation using the TUNEL assay

For each sample, washed semen that obtained spermatozoa after DGC and zeta potential methods were used for assessment of DNA fragmentation according to the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (32). For conducting this method, a commercial detection kit was employed purchased from Promega company (Apoptosis Detection System Fluorescein, Promega, and Mannheim, Germany), and all the procedures were performed according to the manufacturer's instructions. Lastly, the percentage of sperm DNA fragmentation for each group was evaluated under an Olympus fluorescent microscope (BX51, Japan). Spermatozoon without fragmented DNA or TUNEL-negative spermatozoa were red, whereas spermatozoa with fragmented DNA or the TUNEL-positive were bright green.

DNA extraction and telomere length measurement by quantitative real-time polymerase chain reaction

The extraction of DNA sperm and peripheral blood leukocytes were carried out by the QIAamp DNA Mini Kit (Qiagen, Italy) according to the manufacturer's recommendations. Real-time PCR was performed according to the study by Cawthon (33). The results were expressed as the "relative telomere length" ($2^{-\Delta\Delta ct}$) (33) and "absolute telomere length" according to a modified method introduced by O'Callaghan and Fenech (34).

Statistical analyses

Statistical analyses were performed by the Statistical Program for Social Sciences (SPSS Inc., Version 11.0, Chicago, IL, USA). Data are expressed as the means and standard error of the mean (means ± SEM), except for the age reported as the standard deviation of the means (means ± SD). One-way ANOVA was used, followed by LSD t tests to analyze the differences of parameters before and after semen preparation. Pearson's correlation coefficient was applied to calculate the association between different parameters. The $P<0.05$ was considered statistically significant.

For this study, the sample size was determined according to the sample size formula mentioned below:

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

In this formula, $\sigma_1=2.5$; $\sigma_2=3.8$; $\mu_1=6.51$; $\mu_2=9.73$, Z_1-$\beta=0.8$, and $\alpha=0.05$. Accordingly, the minimum number of cases in each group was 15.

Results

Sperm characteristics and DNA fragmentation

Table 1 shows the semen characteristics of 15 infertile men with normozoospermia that participated in this study. Sperm parameters, such as sperm concentration, motility, morphology, and semen volume, were higher than the defined threshold levels in accordance with the criteria established by the WHO (29). Sperm DNA fragmentation was assessed by the TUNEL assay, and the mean percentages of sperm DNA fragmentation were 4.97 ± 0.53, 3.10 ± 0.49, and 2.97 ± 0.47 in washed samples, DGC, and zeta potential groups, respectively. The analysis of the data revealed that the percentage of sperm DNA fragmentation was significantly lower in DGC and zeta potential-processed samples compared with the washed samples ($P<0.05$). Although, the percentage of sperm DNA fragmentation was lower in Zeta potential group

compared with the DGC processed samples, but the difference was not statistically significant (Fig.1).

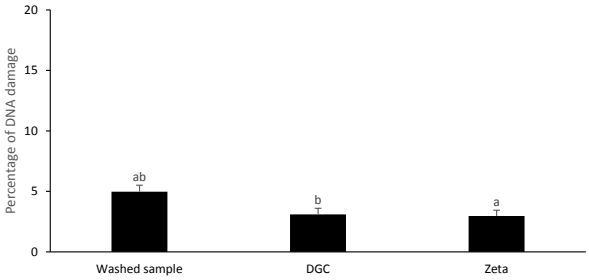


Fig.1: The comparison of the mean percentage of DNA fragmentation among washed samples, density gradient centrifugation (DGC), and zeta potential-processed samples. Common letter indicate significant differences between groups.

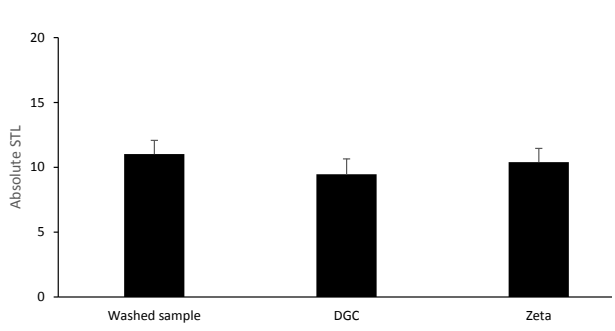
Table 1: Fresh semen characteristics of men with normozoospermia (n=15)

Parameters	Mean ± SE	Min	Max
Male age (Y)*	32 ± 5.02	25.00	45.00
Sperm concentration (10^6/ml)	91.40 ± 4.1	70.00	125.00
Sperm count (10^6/ejaculate)	339.34 ± 34.14	121.00	621.6
Sperm motility (%)	63.66 ± 1.5	55.00	70.00
Abnormal sperm morphology (%)	95.93 ± 0.43	92.00	97.00
Semen volume (ml)	3.78 ± 0.38	1.1	7.4

*; Mean ± SD.

Sperm telomere length measurement

The results of absolute and relative sperm telomere length among washed samples, DGC, and zeta potential-processed samples were compared (Fig.2). The mean absolute telomere length in the washed samples, DGC, and zeta potential-processed samples were 11.01 ± 1.06, 9.46 ± 1.18, and 10.39 ± 1.05, respectively. The differences among these groups were not statistically significant. Also, the mean relative telomere length in the washed samples, DGC, and zeta potential-processed samples were 1.02 ± 0.12, 0.85 ± 0.14, and 1.00 ± 0.1, respectively. The differences between the values of experimental groups were not statistically significant.



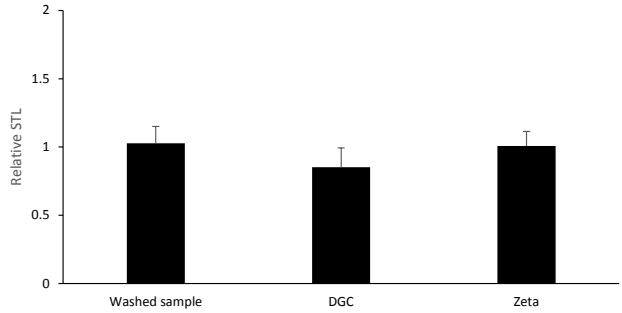


Fig.2: The comparison of sperm telomere length (STL) between experimental groups. **A.** Comparison of absolute and **B.** Relative of STL among washed semen samples, density gradient centrifugation (DGC), and zeta-processed samples (n=15).

Correlation between sperm telomere length and sperm parameters

The correlative analysis between absolute sperm telomere length and sperm parameters revealed a significant correlation between this parameter with sperm abnormal morphology (r=-0.561, P=0.03). The results of the correlation analysis of absolute and relative telomere length, sperm parameters, and sperm DNA fragmentation with the male age are presented in Table 2. The results indicated significant correlations of the male age with sperm abnormal morphology (r=-0.75, P=0.001), absolute (r=+0.64, P=0.009) and relative telomere length (r=+0.64, P=0.01).

Table 2: The correlation of male age with semen parameters, absolute, and relative sperm telomere length, as wel as sperm DNA fragmentation (n=15)

Parameters	r (P value)
Semen volume (ml)	0.17 (0.54)
Sperm concentration (×10^6/ml)	0.31 (0.26)
Total sperm count (×10^6)	0.29 (0.28)
Sperm motility (%)	-0.11 (0.69)
Abnormal sperm morphology (%)	-0.75 (0.001)
Sperm DNA fragmentation (%)	-0.008 (0.97)
Absolute sperm telomere length	0.64 (0.009)
Relative sperm telomere length	0.64 (0.01)

Discussion

Numerous studies in the field of andrology emphasize on sperm telomere length as a sperm marker which has the ability to distinguish fecund sperm from non-fecund ones (22, 25, 35). In this regard, many studies have assessed the relationship between sperm telomere length and different sperm functional characteristics, showing that sperm telomere length has positive correlations with sperm count, sperm progressive motility, vitality, individual age, paternal, and the maternal age of the male parents at the time of conception and negative correlation with sperm DNA fragmentation and ROS production (22-25). In this study, we also observed a significant negative

correlation between absolute telomere length and the percentage of abnormal sperm morphology. Thus, this result has further emphasized on sperm telomere length as a positive marker for sperm quality. Unlike previous studies (36), we observed negative correlations between sperm telomere length with male age, indicating that similar to many sperm functional characteristics, this parameter is inversely associated with the male age.

As mentioned above, sperm selection/preparation procedures play a pivotal role in the management of ARTs and have profound effects on ICSI outcomes (7, 8). Previous studies have shown that the selection of sperm based on the surface electrical charge reduces the degree of sperm DNA fragmentation (10, 12). Therefore, we assessed the efficiency of zeta potential as a sperm selection procedure compared with DGC and neat semen in this study. As expected, and in accordance with the literature (10, 31), both techniques significantly reduced the degree of DNA fragmentation in the selected populations. Comparison of sperm DNA fragmentation in spermatozoa prepared by DGC and zeta potential methods showed a lower level of DNA fragmentation in spermatozoa prepared by the zeta potential technique, but such a difference was not statistically significant. This observation is in line with the previous literature (12, 31, 37) but a reduction (not statistically significant) may be due to population selection. In other studies, zeta potential and DGC procedures were conducted on semen samples obtained from infertile men with severe male fertility (12, 31, 37), while in this study, individuals were normozoospermic men according to WHO criteria due to minimizing heterogeneous factors (29).

Comparison of absolute and relative sperm telomere length among the three groups demonstrated the lack of a significant difference among experimental groups. In contrary to our results, Yang et al. (27) have shown that sperm processing by DGC and swim-up methods, presents higher telomere length. Although it is difficult to explain the differences between the two studies, one of the major differences in that study is the much higher population compared to our study. It is also important to note that in a study performed by Lafuente and colleagues (38), they used the fluorescent in-situ hybridization (FISH) technique to detect telomeres length. They failed to observe any difference among neat, DGC, and swim-up a processed sample in normozoospermic individuals. They explain that the difference may be related to the methodology and sample size. However, another reason could be owing to the low oxidative stress levels, which account for shorter telomere length between experimental groups in different studies. In this study, due to the selection of normozoospermic individuals and the low mean of DNA fragmentation, it is not unexpected to observe any difference in telomere length between the groups.

In accordance with the literature, in this study, we detected a significant positive correlation between sperm telomere length and male age, indicating spermatozoa derived from old age men present higher telomeres length. It is also important to note that numerous factors, including oxidative stress, aging, psychological stress, obesity, infection, smoking, lifestyle, diet, etc., can affect telomere length (35, 38-40). Therefore, the contradiction observed in this study could be partially linked to these confounding factors and the low number of participants, considered one of the limitations of this study.

Conclusion

The results of this study show that both DGC and zeta potential procedures can select sperm population with higher DNA integrity, but no difference was observed between the sperm selected samples in terms of telomeres length.

Acknowledgements

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Authors' Contributions

M.H.N.-E.; Conception, design, data analysis, interpretation, manuscript writing, and final approval of the manuscript. M.T.; Conception, design, collection and/or assembly of data, data analysis, interpretation, manuscript writing and final approval of the manuscript. T.I.; Analysis of quantitative real-time PCR and molecular exams. R.G.-s.; Semen analysis, preparation of samples, and collected data. M.H.; Data collection and data analysis. L.A.; Preparation of samples and data collection. M.R.Z.; Semen analysis and data collection. All authors read and approved the final manuscript.

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Original Article

Micronutrients in Support to The Carbon Cycle Activate Antioxidant Defences and Reduce Sperm DNA Damage in Infertile Men Attending Assisted Reproductive Technology Programs: Clinical Trial Study

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Abstract

Background: Micronutrients in support to the carbon cycle were shown to reduce sperm DNA damage both in animal models and infertile men. Besides supporting DNA methylation, their positive effect may be mediated by an improved performance of the endogenous antioxidant system but this has not yet been proven in clinical settings. The present study aimed at evaluating the effects of micronutrient supplementation in infertile male partners of assisted reproductive technology (ART) resistant couples.

Materials and Methods: In this experimental clinical trial study, infertile male partners of couples resistant to at least one ART cycle, with a sperm fragmentation rate >20% (TUNEL), underwent a 4-month oral supplementation with micronutrients in support to the carbon cycle including folates, B vitamins, zinc and cysteines. Semen, sperm DNA fragmentation (TUNEL), nuclear maturation (CMA3 and blue aniline staining) and lipid peroxidation (BODIPY) were assessed before and after treatment. The couples were followed-up to record clinical outcomes.

Results: Forty-three patients completed the program but full data of pre- and post-treatment were available only for 25 patients. The treatment did not modify sperm concentration or motility but improved morphology. Nuclear maturation, DNA fragmentation and lipid peroxidation significantly improved after the treatment. Overall, 10 clinical pregnancies (23.3%) and 4 live births (9.3%) were recorded during the follow-up following expectant management (25 couples) or a new intracytoplasmic sperm injection (ICSI) cycle (18 couples).

Conclusion: The micronutrients appeared to induce both DNA methylation, resulting in improved sperm nuclear maturation, and antioxidant defences, resulting in less DNA fragmentation and lipid peroxidation. The clinical outcomes were aligned with a possible positive effect on reproductive function. Micronutrients could be regarded as an alternative to antioxidants in correcting oxidative damage in infertile men; however, to confirm such findings, further clinical investigations are warranted (Registration number: IRCT201510207223N6).

Keywords: Antioxidant, DNA Methylation, Male Infertility, Micronutrients, Sperm

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Introduction

Oxidative stress has been recognized as a main cause of male subfertility with sperm DNA damage affecting fertilization rates, embryo quality and pregnancy rates within assisted reproductive technology (ART) cycles (1). This has triggered efforts to improve the male fertility, and possibly the ART outcomes, by administering oral antioxidants. The latest available Cochrane review on male infertility (2) concluded that although the quality of evidence is weak, oral antioxidants significantly improve the chances of live birth for couples attending fertility clinics, which confirms the primary role of oxidative imbalance in male infertility. The evidence is so far weaker than expected, mostly due to the low quality of available studies. It is very difficult to establish the amount of antioxidants needed in the single subject and too strong supports may result in excess sperm nuclear decondensation and worsening of male reproductive potential (3), which has been defined as "reductive stress"(4). The available products often contain high doses of antioxidants and multiple exposures, including food fortification, may easily lead to excess of antioxidants with possible negative effects (5).

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Under physiologic conditions, the oxy-redox balance, besides leveraging on the assumption of reducing substances with diet, is largely based on the endogenous production of reducing power in the form of glutathione (GSH), a reducing tripeptide with an activated sulfhydryl group (-SH) able to donate reducing equivalents and reactivate most of the physiologic antioxidants (6). GSH de novo biosynthesis occurs within the one carbon cycle, responsible for DNA methylation and epigenetics, by transulfuration of its end-product, homocysteine, to cysteine. Cysteine is then complexed with glutamate and glycine to form GSH. The ability of the carbon cycle to support DNA methylation is well-known to be regulated by the availability of dietary micronutrients (7) and this may also be true for the induction of GSH synthesis and antioxidant defences. Indeed, the functions of DNA methylation and antioxidant power generation are cross regulated and may respond to the same micronutrients within a homeostatic unit named "methoxistasis"(8). Methyl donors (e.g. folates), vitamins B2, B3 and B12 and zinc are essential to activate the methylations, which in turn result in activation of GSH synthesis. Vitamin B6, zinc and extra cysteines directly feed GSH synthesis and thus, facilitate the methylations. The concept of micronutrients administration in support to DNA methylation and GSH synthesis, is depicted in Figure 1.

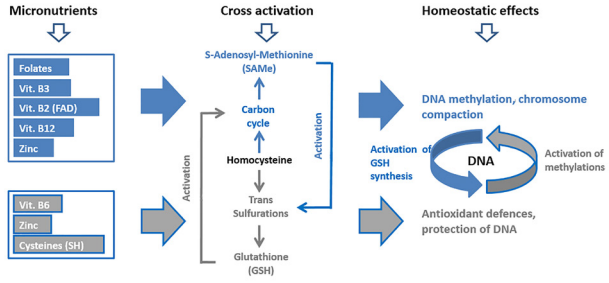

Fig.1: Concept model of micronutrients administration in support to DNA methylation (blue pathway) and antioxidant defences (grey pathway). Micronutrients: small amounts of folates, vitamins B2, B3 and B12 and zinc are needed daily to feed the carbon cycle and the methylations (SAMe). The same applies to vitamin B6, zinc and cysteine to feed glutathione (GSH) synthesis. Cross activation: SAMe acts on the enzyme CBS to increase GSH synthesis. Reducing power from GSH synthesis in turn activates the carbon cycle. Homeostatic effects: DNA methylation and antioxidant defences synergize in keeping a healthy DNA status.

The above substances administered to male partners of couples resistant to ART due to a male factor, resulted, differently from what seen with strong oral antioxidants (9), in a significant correction of both sperm DNA fragmentation and nuclear decondensation suggesting that a boost to the natural antioxidant defences may be effective and devoid of rebound effects (10). Many of the treated patients achieved a pregnancy, either spontaneously or following a new ART cycle, and the pregnancies were strongly correlated with the improvement of sperm nuclear condensation. This was expected due to the known ability of these micronutrients to support DNA methylation (7) and the ability of DNA methylation to trigger chromosome compaction (11). The nuclear compaction could also improve the resistance of DNA to oxidative attacks resulting in less DNA fragmentation. Thus, the induction of the endogenous antioxidant system has not been definitively proven. We tested the same micronutrients in a model of varicocele induction in rats (12) and found that the improvements of sperm DNA damage were paralleled by a sharp reduction of lipid peroxidation, which vouches for a strong induction of antioxidant activity. The present study intended to test the same combination of micronutrients in ART-resistant infertile men and confirm that its effect is linked to a true antioxidant induction as previously seen in the animal model.

Materials and Methods

Patients and study design

This experimental clinical trial study was approved by the Ethical Committee of Royan Institute (IR.ACECR.Royan.REC.1394.9) and carried out between April 2015 and April 2018 at the Isfahan Fertility and Infertility Center, Iran.

We included couples with male factor infertility, at least 1 failed ART cycle [either intra uterine insemination (IUI), *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI)] and a male partner with high rate of sperm DNA fragmentation (>20% as measured by TUNEL) with no female factor. Female partners were defined as normal based on regular ovarian cycles, normal hormonal profile and no findings found in the hysterosalpingogram. All subjects were informed about the details of the study and a consent form was signed by both partners.

The male partners of the enrolled couples were prescribed with a 4-month treatment with a nutritional supplement containing micronutrients in support to the carbon cycle: folic acid (800 µg), vitamins B2 (2.8 mg), B3 (32 mg), B6 (2.8 mg) and B12 (5 µg), zinc (25 mg) and N-acetyl cysteine (500 mg) per day. Sperm quality was assessed at baseline and the end of the treatment.

The couples were followed-up for 3 to 10 months from treatment termination and pregnancies, either spontaneous or following a new ICSI cycle, were recorded.

Sperm quality testing

The semen sample was collected by masturbation after 3-7 days of abstinence before and after the supplementation. Sperm parameters were assessed according to the WHO (2010) criteria and motility was assessed by CASA (CASA, Video Test, ltd: version Sperm 2.1© 1990-2004, Russia). DNA fragmentation was assessed by TUNEL (Apoptosis Detection System Fluorescein, Promega, Germany) as previously reported (13). Lipid peroxidation was assessed using BODYPI as previously shown (14). Chromatin condensation and maturation were assessed by CMA3 and aniline blue staining, respectively (15, 16).

Sperm preparation and intracytoplasmic sperm injection procedure

In case of couples undergoing a new ART cycle post-treatment, ovulation induction in the female partner was achieved by administration of recombinant follicle stimulating hormone (FSH, Sinal F, SinaGene, Iran) and human menopausal gonadotropin (hMG, Menogon, Ferring, Germany) after pituitary suppression by a gonadotropin release hormone (GnRH) antagonist (Cetrotide, Merk-Serono, USA). Follicular maturation was monitored by trans-vaginal ultrasounds and final ovulation was induced by 10000 IU human corionic gonadotropin (hCG, Choragon, Ferring, Germany). Oocytes were collected using ultrasound-guided trans vaginal aspiration and cumulus and coronal cells were removed to evaluate oocyte maturity. MII oocytes were selected for the following ICSI procedure.

Partner's sperm was prepared for ICSI by density gradient centrifugation (13). Morphologically normal and motile sperm were selected under 200-400X magnification and injected into the MII oocytes. Inseminated oocytes and embryos were cultured at 37°C in 6% CO_2, 6% O_2 under humidified conditions. ICSI and the following embryo culture, were performed in Vitrolife culture media (G-V series, Vitrolife, Sweden).

Fertilization, cleavage, implantation, clinical pregnancy and abortion rate were defined and assessed based on the terminology of the international committee for monitoring assisted reproductive technologies (ICMART) (17). Embryo quality was assessed on day 3 according to f Giorgetti et al. (18) and Terriou et al. (19). Embryos with 6-8 cells, equal blastomere size and less than 25% fragmentation, were rated as "top quality" and fresh transferred or vitrified for later use. The fertilization, cleavage and top quality embryo rates were compared to those achieved by the same couples in their previous ICSI cycle.

Statistical analysis

Statistical Package for the Social Sciences software (SPSS 18, Chicago, IL, USA) was used for data analysis. Data are expressed as mean ± error of the mean (SEM) and differences were considered significant at $P<0.05$. Comparison of sperm parameters, chromatin status, and lipid peroxidation, before and after treatment and clinical outcomes (fertilization, cleavage rate, top quality embryos) between current cycle and previous cycle was performed by Student's t test. For comparison of pregnancy rate, Chi-square was used.

Results

In total, 51 patients were enrolled and 8 of them quit for personal reasons. Data on semen analysis pre- and post-treatment were available for all 43 patients completing the study whereas some data concerning TUNEL (n=36), BODIPY, CMA3 and blue aniline (n=25) methods, were missing.

Effect of micronutrients on sperm quality

The supplementation with micronutrients had no effect on sperm concentration, motility and volume but significantly improved sperm morphology (n=43, P=0.001, Fig.2). The treatment was of benefit with respect to sperm nuclear maturation with a significant reduction of the rate of sperms stained by CMA3 (n=24, $P<0.05$) and aniline blue (n=24, $P<0.001$, Fig.3). Micronutrients also improved oxidative damage-induced sperm DNA fragmentation measured by TUNEL (n=36, P=0.001) and lipoperoxidation measured by BODIPY (n=24, $P<0.001$), that significantly improved after treatment (Fig.4).

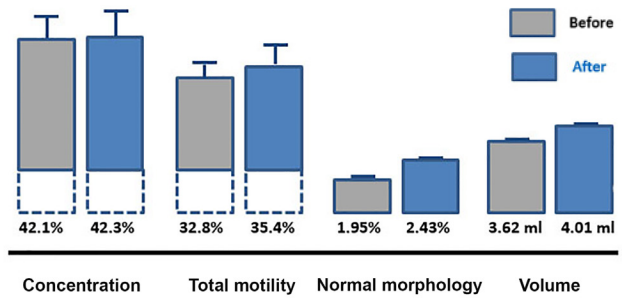

Fig.2: Sperm concentration (P=0.8), total motility (P=0.4), normal morphology (P=0.001), and volume (P=0.06) before and after a 4 month exposure to micronutrients, mean values ± SE.

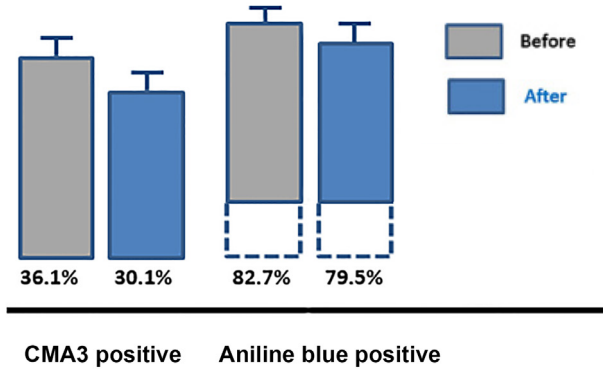

Fig.3: Sperm protamine deficiency ($P<0.05$) and nuclear maturation ($P<0.001$) before and after a 4 month exposure to micronutrients, mean values ± SE (n=24). CMA3 reports on sperm protamine deficiency, Aniline blue reports on sperm nuclear maturation.

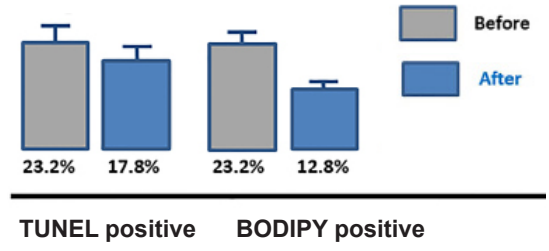

Fig.4: Sperm DNA fragmentation (P=0.001) and lipid peroxidation ($P<0.001$) before and after a 4 month exposure to micronutrients, mean values ± SE (n=24). TUNEL reports on sperm DNA fragmentation, BODIPY reports on lipid peroxidation.

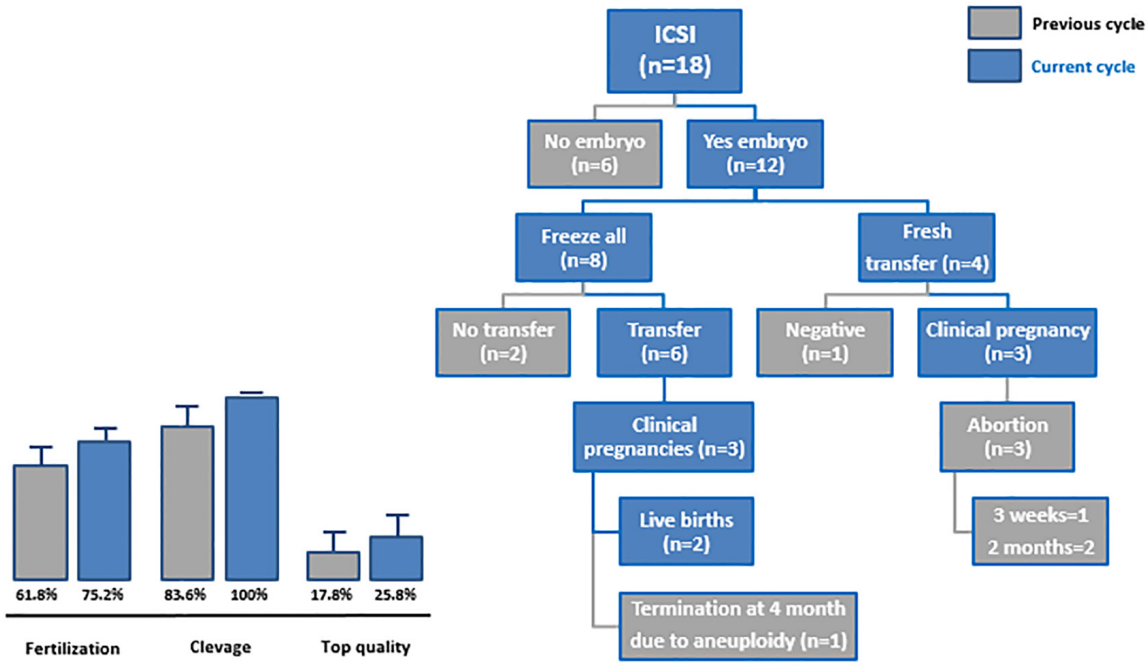

Fig.5: Outcomes from the ART cycles and couples disposition. Fertilization, cleavage (P<0.05) and top quality embryo rates were compared to those achieved by the same couples in their previous ICSI cycle. ART; Assisted reproductive technology and ICSI; Intracytoplasmic sperm injection

Clinical outcomes

Out of the 43 couples whose male partner completed the treatment, 25 couples did not undergo a new ART cycle and opted for an expectant management strategy. Four of these couples, achieved a spontaneous pregnancy during the study follow-up period resulting in 2 deliveries and 2 spontaneous miscarriages.

The remaining 18 couples underwent a new ICSI cycle but only 12 of them had embryos suitable for transfer or vitrification. Four of them underwent a fresh transfer cycle resulting in 3 clinical pregnancies and 3 abortions at either 3 weeks (n=1) or 2 months (n=2). Only 6 of 8 couples with vitrified embryos, underwent a thawed embryo transfer during the study period resulting in 3 clinical pregnancies with 2 deliveries and one early termination due to aneuploidy. The embryo cleavage rate was significantly higher (P<0.05) compared to the one in the previous cycle in the same couples whereas the improvement of fertilization rate and top quality embryo rate was not significant. The couples' disposition and outcomes after a new ICSI cycle are summarized in Figure 5. Overall, treatment of 43 male partners of ART-resistant couples with micronutrients in support to the carbon cycle, resulted in 10 clinical pregnancies (23.3%) and 4 live births (9.3%).

Discussion

This was a small size explorative study aimed at testing the hypothesis that a nutritional intervention using micronutrients in support to the carbon cycle, is of benefit to infertile men attending ART programs and that it is able to induce both improved DNA methylation and resumption of the endogenous antioxidant activity.

The administration of micronutrients did not modify sperm count or motility but improved sperm morphology, which can be assumed as a possible effect on the processes of DNA methylation that contribute to the epigenetic programming of the cell phenotype. The parallel improvement of sperm nuclear maturation shown by CMA3 and blue aniline staining, also points to a possible effect on DNA methylation. Indeed, DNA and histone methylation plays a pivotal role in sperm nuclear maturation and is also involved in the process of protamination (20). The induction of DNA methylation is explained by the ability of the administered folates to feed homocysteine re-methylation to methionine. Methionine is adenylated to generate the universal methyl donor S-adenosylmethionine (SAMe) acting as a substrate for DNA N-methyltransferases. We also supplemented vitamin B12 that is needed to pass the methyl group from folates to homocysteine, vitamins B2 and B3 as cofactors for methylentetrahydrofolate reductase (MTHFR) to activate folates, and zinc that is as well necessary to MTHFR and methionine synthase for homocysteine re-methylation.

Micronutrients supplementation achieved a significant reduction of sperm DNA fragmentation and lipid peroxidation. The improvement of DNA fragmentation was likely of clinical relevance because the average rate moved from 23.2 to 17.8% (i.e. it dropped below the critical threshold of 20% that is assumed as clinically relevant) (21). This may imply an improved performance of the endogenous antioxidant metabolism because the administered micronutrients did not include any

direct antioxidant substances. However, the origin of sperm DNA fragmentation is controversial with several processes showing the ability to contribute. According to Muratori et al. (22), oxidative aggression does not behave as the primary trigger, rather it is a process of apoptosis, likely triggered by an impairment of chromatin maturation in the testis and oxidative stress during the transit in the male genital tract. Therefore, the positive effect of micronutrients on DNA fragmentation may be already explained by the protection from an improved chromatin packaging resulting from the induction of methylations. However, an improvement of the antioxidant defences may also be involved.

Micronutrients also achieved a significant drop in sperm lipid peroxidation to half of the baseline value. Lipid peroxidation is a process where oxidants attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs), resulting in production of other radicals attacking other double bonds in a self-amplified process and is a clear-cut oxidative damage (23). The abundance of PUFAs in the sperm membrane is a reason for the particular sensitivity of sperms to lipid peroxidation if an oxidative imbalance occurs (24). GSH is produced as a reaction to oxidative stress by activation of the redox-sensitive transcription factor, nuclear factor erythroid-2-related factor 2 (Nrf2), that activates the enzyme γ-glutamyl cysteine ligase. This enzyme is further regulated by the availability of intracellular cysteines from both the diet and endogenous synthesis (25). Our micronutrients included soluble cysteine in the form of N-acetylcysteine and supported the endogenous cysteine synthesis by zinc and vitamin B6 acting as the necessary co-factor for cystathinonine β-synthase (CBS), the enzyme addressing homocysteine from the carbon cycle to cystathionine and then, cysteine synthesis. These micronutrients had therefore the ability to support antioxidant defences by facilitating GSH synthesis and the reduction of lipid peroxidation was likely a sign of antioxidant defences induction.

In addition, a synergy with other micronutrients is likely to apply. Abundance of SAMe from the carbon cycle can bind CBS resulting in an allosteric activation with a fivefold increase of enzyme activity (26). Thus, the administered micronutrients had the potential to induce a strong boost to intracellular cysteine availability by increasing both its nutritional intake and the endogenous synthesis for a better response of the GSH system to an increased oxidative load and this may account for the effect on lipid peroxidation. Worth to note, the synthesis of cysteine from homocysteine is the only source of the intracellular gasotransmitter H_2S (27). H2S in turn promotes the activation of vitamin B12 (28) and plays as a main inducer of the carbon cycle designing a cross activated homeostatic unit.

Our study was too small in size to provide meaningful clinical findings; however, the outcomes were aligned with a possible positive effect of the treatment on the patients reproductive performance. Four out of 25 (16%) couples opting for an expectant management strategy, achieved a clinical pregnancy during the follow-up period. Interestingly, the male partners of these couples also showed a good response to the micronutrients of the sperm damage indexes (data not shown), which endorses a possible link between the positive pregnancy outcome and the treatment. Out of 18 couples opting for a new ART cycle, only 12 had viable embryos and only 10 of them underwent either fresh (n=4) or thawed (n=6) embryo transfer, which resulted in 6 clinical pregnancies. Again, the male partners of the couples achieving a pregnancy were good responders for sperm parameters. Altogether, these clinical outcomes indicate good chances that the treatment can be of help to male reproductive function at least in good responders. Reasons for lack of response may be bad treatment compliance, a negative genetic background and occurrence of other pathogenic mechanisms beside oxidative aggression, but our data do not allow to further speculate on this.

In summary, the oral administration of a combination of micronutrients including folates, B vitamins, zinc and cysteines to male partners of ART-resistant couples, showed the ability to reduce sperm DNA damage and improve sperm nuclear maturation and the clinical outcomes reflected a positive effect. These outcomes were related to the ability of the micronutrients to activate the one carbon cycle resulting in both stronger methylation ability and activation of the endogenous antioxidant defences. The recorded antioxidant effect, which was strong and likely of clinical value, was achieved without perturbations of the cell homeostasis as happen with oral antioxidants (9).

Conclusion

The present study failed to address a series of relevant questions including the actual clinical gain that can be achieved by micronutrients, how to individuate potential good responders, dose and duration of the treatment and whether the add-on of oral antioxidants may further improve the effect or rather just derange the homeostatic regulations. All of these questions should be addressed by larger size clinical trials centred on the clinical outcomes. Meantime, micronutrients qualify as an alternative to antioxidants in correcting oxidative damage in infertile men.

Acknowledgements

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Authors' Contributions

M.H.N.-E., M.T.; Conception, design, data analysis, interpretation, manuscript writing and final approval of manuscript. M.D.; Conception, design, interpretation, and manuscript writing. F.B.; Semen analysis, samples

preparation, experimental tasks, data collection, and manuscript writing. All authors read and approved the final manuscript.

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Original Article

Temperature Decline in Embryological Culture Dishes outside Incubator

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Abstract

Background: In embryological culture dishes, there is a temperature decline when they are removed outside incubators. This study aimed at investigating the effects of this temperature decline within a certain time frame, the type of culture dish with or without the use of laminar air flow and whether it is possible to achieve a sufficient thermal control with the use of a heating stage.

Materials and Methods: In this experimental study, the temperatures of four different types of polystyrene dishes [50 mm intracytoplasmic sperm injection (ICSI), 35 mm, 60 mm, 90 mm], filled with culture medium and oil were recorded for a period of 10 minutes outside the incubator. Temperature was measured with an infrared thermographic camera. The reference temperature was 37°C. Four parameters were analyzed: the type of dishes, air flow, a heating stage at 37°C and 38.5°C.

Results: There was a time-dependant significant temperature decline outside the incubator in all types of dishes and under all experimental conditions. Under air flow temperature decline increased compared to the no air flow condition. The use of a heating stage at either 37°C or 38.5°C slightly improved the situation in most cases. After three minutes out of the incubator without a heating stage and air flow, the temperature was <34°C; with air flow and without a heating stage the temperature was <33°C. When a heating stage was used, the temperature was <36°C, except when using ICSI dishes. When ICSI dishes were on a heating stage they maintained a temperature close to 37°C with or without air flow. In all experimental conditions the highest decline was recorded with the 90 mm dishes.

Conclusion: Time is crucial for managing the temperature decline in culture dishes when out of the incubator. Under air laminar flow, the heat loss is greater, when with a heating stage at 37°C or better at 38.5°C this loss decreases but still exists. ICSI flat bottom dishes give the best results when heated stages are used. Flat bottom dishes maintain the temperature rather efficiently. Based on our findings, the use of flat bottom dishes should become a universal practice in *in vitro* fertilization (IVF).

Keywords: Culture Dish, Embryo Culture, Temperature

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Introduction

Temperature is a crucial factor for *in vitro* cell culture, particularly for in vitro culture of human gametes and embryos. The human core body temperature, 37°C, is considered optimal for in vitro culture of human gametes and embryos although several studies have shown that the temperature in ovaries and fallopian tubes is lower (1-5). However, attempts to culture human embryos in lower temperatures than 37°C have not given satisfactory results (6, 7). Moreover, experiments with oocytes of both domestic animals and humans have shown that exposure to low temperatures has an impact on spindle integrity, chromosomal organization and fertilization (8-11). Incubators effectively maintain a constant temperature of 37°C, although the temperature inside the incubators may be affected by the frequency of door openings and incubator type.

In the every-day clinical practice, gametes and embryos are routinely exposed to room temperature: during oocyte retrieval, preparation for *in vitro*

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fertilization (IVF), intracytoplasmic sperm injection (ICSI), assessment of embryo development and embryo transfers. In all of these cases, the temperature decreases despite the fact that embryologists emphasize on minimizing this decrease by reducing the time outside of incubators and by using heating stages. The magnitude of the temperature drop is an issue of concern but has not been studied in detail.

In the present study we have investigated the following questions regarding temperature decline in embryological culture dishes outside incubators: how much is the reduction of the temperature in a certain time frame and type of dish? Is it possible to achieve a sufficient thermal control with the use of heating stages? How much does the laminar air flow affect the temperature?

Materials and Methods

This was an experimental study, conducted in the Laboratory of Physiology, Faculty of Medicine, School of Health Sciences, Democritus University of Thrace, Alexandroupolis, Greece, from April to June 2018. In this study, no human gametes, embryos, body fluid samples or personal data were utilized.

Experimental design

Four different types of polystyrene dishes (Nunc IVF Petri Dish; Thermo Scientific, Roskilde, Denmark), were used. All dishes were prepared exactly as if for embryo culture in order to be as close as possible to the working conditions of an embryological laboratory:

- 50 mm ICSI dishes with 5 droplets of culture medium (4 µl each) and a line of 10 µl polyvinyl pyrrolidone (PVP), with 4 ml of oil (group 1).

- 35 mm dishes with 5 droplets of 50 µl culture medium, covered with 4 ml of light mineral oil (group 2).

- 60 mm dishes with 5 droplets of 50 µl culture medium, covered with 12 ml of light mineral oil (group 3).

- 90 mm dishes containing 14 ml of culture medium, without oil (group 4).

Universal IVF Medium (Origio-Sage, Måløv, Denmark) was used for culture medium. Oil for Tissue Culture (Origio-Sage, Måløv, Denmark) was used to overlay the medium droplets. PVP in ICSI dishes was purchased from Origio-Sage (Måløv, Denmark). All dishes were prepared in a vertical laminar flow cabinet Class II (Type A2, ECSO) and then incubated overnight in a Hera Cell 150 incubator (Thermo Electron Co., Germany) set at 37°C. In order to replicate the working conditions in an embryological laboratory, the experimental procedure involved removal of culture dishes from the incubator, abolition of the protective lids of the dishes and recording the temperature of the culture dishes under six experimental conditions: i. On a stereomicroscope without air flow, ii. On a stereomicroscope under vertical laminar air flow, iii. On a microscope with a heating stage set at 37°C without air flow, iv. On a microscope with a heating stage set at 38.5°C without air flow, v. On a microscope with a heating stage set at 37°C under vertical laminar air flow, vi. On a microscope with a heating stage set at 38.5°C under vertical laminar air flow.

For vertical laminar air flow, a class II cabinet (type A2, ECSO) was used with inflow 0.45 m/second and downflow 0.36 m/second. The heating stage used was a MATS-U505R (Tokai Hit Co, LTD, Japan); the dishes were always placed in the centre of the heating stage. The room temperature was monitored at 22°C. Each experiment was repeated 20 times. Overall, twenty four experimental groups were formed:

Group 1: no laminar air flow, no heating

Group 2: no laminar air flow, no heating

Group 3: no laminar air flow, no heating

Group 4: no laminar air flow, no heating

Group 1 air: laminar air flow, no heating

Group 2 air: laminar air flow, no heating

Group 3 air: laminar air flow, no heating

Group 4 air: laminar air flow, no heating

Group 1-37: no laminar air flow, heating at 37°C

Group 2-37: no laminar air flow, heating at 37°C

Group 3-37: no laminar air flow, heating at 37°C

Group 4-37: no laminar air flow, heating at 37°C

Group 1-38.5: no laminar air flow, heating at 38.5°C

Group 2-38.5: no laminar air flow, heating at 38.5°C

Group 3-38.5: no laminar air flow, heating at 38.5°C

Group 4-38.5: no laminar air flow, heating at 38.5°C

Group 1 air 37: laminar air flow, heating at 37°C

Group 2 air 37: laminar air flow, heating at 37°C

Group 3 air 37: laminar air flow, heating at 37°C

Group 4 air 37: laminar air flow, heating at 37°C

Group 1 air 38.5: laminar air flow, heating at 38.5°C

Group 2 air 38.5: laminar air flow, heating at 38.5°C

Group 3 air 38.5: laminar air flow, heating at 38.5°C

Group 4 air 38.5: laminar air flow, heating at 38.5°C

Temperature assessment

Temperature was measured with a high precision infrared thermographic camera (OPTRIS PI4500, Germany) having thermal sensitivity of 40 mK and optical resolution of 382X288 pixels. The thermographic camera

was calibrated by the manufacturer (OPTRIS GmbH, Germany). Additionally, its precision was verified against a resistance temperature detector (Digi-Sense Traceable Memory-Los Model 6442, Cole-Parmer, IL, USA) in the Laboratory of Mechatronics and Systems Automation (Democritus University of Thrace). The camera was connected to a computer allowing continuous recording of temperature. During recordings the camera was always set at the same fixed angle and distance (20 cm) from culture dishes. For statistical analysis we used the recorded temperatures 3, 5 and 10 minutes after removing the culture dishes out of the incubator.

Statistical analysis

Data was analyzed using the Statistical Package for Social Sciences (SPSS), version 19.0 (IBM, NY, USA). The normality of quantitative variables was tested by Kolmogorov-Smirnov test. All parameters were expressed as mean ± standard deviation (SD). Within groups, differences of quantitative variables were examined by one-way repeated measures analysis of variance (rmANOVA) and post h°C analysis was performed using Sidak's test. Amongst groups differences at each time point were assessed by one-way ANOVA and Tukey's test was used for multiple comparisons. The interaction group x time was established by a two-way ANOVA. All tests were two tailed and $P<0.05$ were considered statistically significant.

Results

The use of a thermographic camera proved to be very convenient as it measures temperature from a distance (20 cm) allowing for unobstructed manipulation of the culture dishes. The temperature of the culture dishes, as expected, declined significantly after 3, 5 and 10 minutes outside the incubator. The decline depended on the time, the experimental conditions and the type of dish. Working under air flow worsened the decline of temperature and, in general, larger dishes lost temperature faster than the smaller ones. The detailed results are presented in Tables 1-6.

Table 1: Temperatures recorded without a heating stage and air laminar flow

Group	Time			
	Onset	3 minutes	5 minutes	10 minutes
1	36.98 ± 0.01^{A}	31.30 ± 0.21	30.89 ± 0.18	30.45 ± 0.07^{B}
2	36.98 ± 0.01^{A}	32.58 ± 0.21	31.51 ± 0.16	30.47 ± 0.09^{B}
3	36.98 ± 0.01^{A}	33.57 ± 0.13	32.54 ± 0.18	31.16 ± 0.08
4	36.98 ± 0.01^{A}	28.56 ± 0.13	27.59 ± 0.19	26.78 ± 0.23

Data are presented as mean ± SD. In each type of culture dish, the differences among the recorded temperatures at different time points were statistically significant (Sidak's test). In pair-wise comparisons between groups (Tukey's test) all differences were statistically significant except the cases denoted with the same capital letters. Group 1; 50 mm ICSI dishes with 5 droplets of culture medium (4 µl each) and a line of 10 µl PVP, with 4 ml of oil, Group 2; 35 mm dishes with 5 droplets of 50 µl culture medium, covered with 4 ml of light mineral oil, Group 3; 60 mm dishes with 5 droplets of 50 µl culture medium, covered with 12 ml of light mineral oil, Group 4; 90 mm dishes containing 14 ml of culture medium, without oil.
ICSI; Intracytoplasmic sperm injection and PVP; Polyvinyl pyrrolidone.

Table 2: Temperatures recorded with laminar air flow and without a heating stage

Group	Time			
	Onset	3 minutes	5 minutes	10 minutes
1 air	36.98 ± 0.01^{A}	28.95 ± 0.11	28.33 ± 0.13^{B}	27.95 ± 0.05
2 air	36.97 ± 0.01^{A}	29.89 ± 0.27	28.18 ± 0.19^{B}	26.75 ± 0.07
3 air	36.98 ± 0.01^{A}	31.93 ± 0.13	30.46 ± 0.15	28.77 ± 0.07
4 air	36.98 ± 0.01^{A}	23.62 ± 0.13	23.05 ± 0.19	22.42 ± 0.08

Data are presented as mean ± SD. In each type of culture dish, the temperature differences recorded at different time points were statistically significant (Sidak's test). In pair-wise comparisons between groups (Tukey's test) all differences were statistically significant except the cases denoted with the same capital letters. Group 1 air; 50 mm ICSI dishes with 5 droplets of culture medium (4 µl each) and a line of 10 µl PVP, with 4 ml of oil, Group 2 air; 35 mm dishes with 5 droplets of 50 µl culture medium, covered with 4 ml of light mineral oil, Group 3 air; 60 mm dishes with 5 droplets of 50 µl culture medium, covered with 12 ml of light mineral oil; Group 4 air; 90 mm dishes containing 14 ml of culture medium, without oil.
ICSI; Intracytoplasmic sperm injection and PVP; Polyvinyl pyrrolidone.

Table 3: Temperatures recorded with a heating stage at 37°C, without air laminar flow

Group	Time			
	Onset	3 minutes	5 minutes	10 minutes
1-37	36.98 ± 0.01^{A}	35.92 ± 0.07	$35.62 \pm 0.17^{*}$	$35.38 \pm 0.20^{*}$
2-37	36.99 ± 0.01^{A}	35.12 ± 0.16	34.61 ± 0.24^{B}	33.53 ± 0.12
3-37	36.99 ± 0.01^{A}	$34.83 \pm 0.26^{*}$	$34.48 \pm 0.42^{B*}$	33.80 ± 0.05
4-37	36.98 ± 0.01^{A}	30.79 ± 0.15	29.48 ± 0.21^{B}	28.37 ± 0.10

Data are presented as mean ± SD. In each type of culture dish, the temperature differences recorded at different time points were statistically significant except the cases denoted with *(Sidak's test). In pair-wise comparisons between groups (Tukey's test) all differences were statistically significant except the cases denoted with the same capital letters. Group 1-37; 50 mm ICSI dishes with 5 droplets of culture medium (4 µl each) and a line of 10 µl PVP, with 4 ml of oil, Group 2-37; 35 mm dishes with 5 droplets of 50 µl culture medium, covered with 4 ml of light mineral oil, Group 3-37; 60 mm dishes with 5 droplets of 50 µl culture medium, covered with 12 ml of light mineral oil, Group 4-37; 90 mm dishes containing 14 ml of culture medium, without oil.
ICSI; Intracytoplasmic sperm injection and PVP; Polyvinyl pyrrolidone.

Table 4: Temperatures recorded with heating stage at 37°C and air laminar flow

Group	Time			
	Onset	3 minutes	5 minutes	10 minutes
1 air 37	36.98 ± 0.01^{A}	34.68 ± 0.08	34.48 ± 0.15	34.18 ± 0.10
2 air 37	36.98 ± 0.01^{A}	33.91 ± 0.07	33.23 ± 0.07	32.77 ± 0.07
3 air 37	36.99 ± 0.01^{A}	34.46 ± 0.08	33.79 ± 0.07	32.94 ± 0.02
4 air 37	36.98 ± 0.01^{A}	27.78 ± 0.20	26.42 ± 0.22	25.34 ± 0.20

Data are presented as mean ± SD. In each type of culture dish, the temperature differences recorded at different time points were statistically significant (Sidak's test). In pair-wise comparisons between groups (Tukey's test) all differences were statistically significant except the cases denoted with the same capital letters. Group 1 air 37; 50 mm ICSI dishes with 5 droplets of culture medium (4 µl each) and a line of 10 µl PVP, with 4 ml of oil, Group 2 air 37; 35 mm dishes with 5 droplets of 50 µl culture medium, covered with 4 ml of light mineral oil, Group 3 air 37; 60 mm dishes with 5 droplets of 50 µl culture medium, covered with 12 ml of light mineral oil, Group 4 air 37; 90 mm dishes containing 14 ml of culture medium, without oil.
ICSI; Intracytoplasmic sperm injection and PVP; Polyvinyl pyrrolidone.

Table 5: Temperatures recorded with the heating stage at 38.5°C, without laminar air flow

Group	Time			
	Onset	3 minutes	5 minutes	10 minutes
1-38.5	36.98 ± 0.01^{A}	$36.71 \pm 0.17^{*}$	$36.77 \pm 0.16^{*}$	$36.65 \pm 0.11^{*}$
2-38.5	36.99 ± 0.01^{A}	35.65 ± 0.24	35.31 ± 0.20	34.63 ± 0.12
3-38.5	36.98 ± 0.01^{A}	$36.20 \pm 0.21^{*}$	$36.19 \pm 0.21^{*}$	35.86 ± 0.07
4-38.5	36.98 ± 0.01^{A}	31.48 ± 0.29	30.36 ± 0.20	29.47 ± 0.07

Data are presented as mean ± SD. In each type of culture dish, the temperature differences recorded at different time points were statistically significant (Sidak's test) except the cases denoted with *. In pair-wise comparisons between groups (Tukey's test) all differences were statistically significant except the cases denoted with the same capital letters. Group 1-38.5; 50 mm ICSI dishes with 5 droplets of culture medium (4 µl each) and a line of 10 µl PVP, with 4ml of oil, Group 2-38.5; 35 mm dishes with 5 droplets of 50 µl culture medium, covered with 4 ml of light mineral oil, Group 3-38.5; 60 mm dishes with 5 droplets of 50 µl culture medium, covered with 12 ml of light mineral oil, Group 4-38.5; 90 mm dishes containing 14 ml of culture medium, without oil.
ICSI; Intracytoplasmic sperm injection and PVP; Polyvinyl pyrrolidone.

Table 6: Temperatures recorded with the heating stage at 38.5°C and air laminar flow

Group	Time			
	Onset	3 minutes	5 minutes	10 minutes
1 air 38.5	36.99 ± 0.01[A]	36.32 ± 0.11[*]	36.20 ± 0.12[*]	36.00 ± 0.04
2 air 38.5	36.99 ± 0.01[A]	35.65 ± 0.10	35.20 ± 0.11	34.73 ± 0.04
3 air 38.5	36.99 ± 0.01[A]	35.36 ± 0.15	34.85 ± 0.16	34.03 ± 0.22
4 air 38.5	36.99 ± 0.01[A]	28.75 ± 0.13	27.85 ± 0.16	27.56 ± 0.08

Data are presented as mean ± SD. In each type of culture dish, the temperature differences recorded at different time points were statistically significant (Sidak's test) except the cases denoted with *. In pair-wise comparisons between groups (Tukey's test) all differences were statistically significant except the cases denoted with the same capital letters. Group 1 air 38.5; 50 mm ICSI dishes with 5 droplets of culture medium (4 µl each) and a line of 10 µl PVP, with 4 ml of oil, Group 2 air 38.5; 35 mm dishes with 5 droplets of 50 µl culture medium, covered with 4 ml of light mineral oil, Group 3 air 38.5; 60 mm dishes with 5 droplets of 50 µl culture medium, covered with 12 ml of light mineral oil, Group 4 air 38.5; 90 mm dishes containing 14 ml of culture medium, without oil.
ICSI; Intracytoplasmic sperm injection and PVP; Polyvinyl pyrrolidone.

Overall, the highest reductions of temperature were recorded with 90 mm culture dishes. There was a dramatic reduction of temperature under air flow, without heating; 3 minutes after removal from the incubator, the temperature was 23.62 ± 0.13°C and at 10 minutes it had almost reached the room temperature (22.42 ± 0.08°C).

When the heating stage was on, the lowest reductions were recorded with 50 mm ICSI dishes. After 3 minutes working on a heating stage at 37°C and without air flow, ICSI dishes reached 35.92 ± 0.17°C and remained above 35°C for the rest of the time until 10 minutes. At three time points on a heating stage working at 38.5°C and without air flow, ICSI dishes reached 36.71 ± 0.17°C and remained above 36°C up to 10 minutes. When there was air flow and the heating stage was at 38.5oC, ICSI dishes maintained a temperature of 36.32 ± 0.11°C at 3 minutes and 36.00 ± 0.04°C at 10 minutes outside the incubator.

Instead, when the heating stage was off, the lowest reductions were observed in 35 mm culture dishes, although in all cases they failed to maintain a temperature close to 37°C.

Discussion

The results of this study highlight the significance of time, stage conditions and most importantly the type of culture dishes. Regarding the time, it is obvious that a decline of temperature always happens when moving the dishes outside the incubator, but this decline becomes dramatic and detrimental for gametes and embryos as this time increases. In general, keeping the culture dishes for 5 to 10 minutes outside the incubator, even on a heating stage, should be avoided. During all the experiments, the room temperature was constantly in 22°C, nonetheless, we can speculate that even at a higher room temperature (e.g. 24°C) the decline of temperature in the culture dishes would have been similar, perhaps only slightly less significant.

Laminar air flow is another factor in increasing the speed of heat loss in culture dishes. However, working under laminar air flow, especially vertical air flow with class II cabinets, is essential for providing a clean and safe environment. Consequently, although the temperature decline increases in the presence of air flow, working without air flow is not considered an option in an embryological laboratory.

Heating stages are universally used to maintain the temperature of culture dishes outside the incubator. Here, it is worth underlining that heating stages cannot actually maintain the temperature of culture dishes at 37°C but rather lower the drop of it. According to the results of the present study, heating stages should be set at a temperature higher than 37°C in order to have better results. The exact working temperature should be decided in each laboratory according to their environmental conditions, specifically room temperature and air flow. In any case, a working temperature much higher than 38°C should be avoided even for a short time period, because it may heat the culture dishes above 37°C. Several studies have shown that temperatures higher than the core body temperature are more detrimental to embryos than temperatures lower than the core body temperature (12-14). In this study, by setting the heating stage at 38.5°C, we did not observe even a transient temperature increase above 37°C in the culture dishes.

The type of dishes is another factor influencing the temperature decline. For instance, 90 mm dishes showed the highest temperature decline in all experimental conditions. On the other hand, ICSI dishes had the best results when the heating stage was in use. Under laminar flow and with the heated stage at 38.5°C, ICSI dishes maintained a temperature of 36.32 ± 0.11°C, 36.20 ± 0.12°C and 36.00 ± 0.04°C at 3, 5 and 10 minutes outside the incubator respectively. It is worth mentioning that according to previous studies (6-7), 36-36.5°C is considered as an acceptable temperature for oocytes and embryos and while it does not provide any benefits, it seems that it does not cause any harms either. In our opinion, the better results associated with the ICSI dish are explained, at least partially, by its flat bottom. All the other types of dishes have a rim around the bottom that does not permit the bottom to touch the heating stage directly. For this reason, heat transfer is prevented by a thin air layer between the heating stage and the bottom of the culture dish. ICSI dishes with flat bottom are heated more effectively when placed on a heated stage and consequently maintain a safe temperature better than other types of dishes outside the incubator. It is strange why most IVF dishes are manufactured with a rim around the bottom. This is probably due to the safety issues in cell culture practice, as in cell culture dishes are usually stacked, one on top of the other, in the incubators and the rim around the dishes helps fix them on each other so they do not fall out easily. This practice, however, is not the case in most embryological laboratories.

Conclusion

The present study shows that time is a crucial factor for temperature decline in culture dishes outside the incubator. Keeping the culture dishes outside the incubator for more than 3 minutes results in a dramatic decline of temperature.

The golden rule here is: the quicker the better. Under air flow, the heat loss is greater and on a heating stage at 37°C or better at 38.5°C the loss is lower but still exists. The loss of heat is influenced by the type of dish as well. The best results, when heated stages were used, were recorded when flat bottom dishes were tested. Flat bottom dishes are heated effectively and maintain the temperature efficiently in a short time period. Based on our findings, the use of flat bottom dishes should become universal in IVF.

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Authors' Contributions

D.K.; Conducted experiments, data acquisition, and data analysis. S.M.; Contributed in the design of experiments and provided technical support. G.T.; Performed statistical analysis and data interpretation. B.A.; Contributed to conception and design of experiments, data analysis, and drafting. N.N.; Contributed to conception of experiments and extensively revised the manuscript. All authors read and approved the final manuscript.

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Short Communication

Psychiatric Disorders in Women Seeking Fertility Treatments: A Clinical Investigation in India

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Abstract

Fertility treatments began in several countries, including India, in the1970s. Despite various advancements in intra uterine insemination (IUI) and *in vitro* fertilization (IVF), empirical investigations on the psychological endurance and emotional tolerance of Indian women to such treatments are rather scarce. Thus, the aim of this study is to estimate the prevalence of psychiatric disorders in Indian women seeking fertility treatments. It is a cross-sectional study with three hundred women participants undergoing various treatments at the Manipal Assisted Reproductive Centre, Kasturba Medical College, Karnataka, India. Psychiatric disorders were assessed in women using the "ICD-10 Classification of Mental and Behavioural Disorders" followed by descriptive data analysis. The results show that 78% of women have psychological issues and 45% of them have a diagnosable psychiatric condition. Adjustment Disorders, Anxiety Disorders and Mixed Anxiety and Depression Disorder are established as the top three categories of diagnoses. The findings of this study suggest that women have a high emotional stake in infertility treatments. The data highlights the need for modification of the existing treatment protocol (in Indian clinics) in ways that ensure the emotional wellbeing of patients.

Keywords: Cross-Sectional Study, Distress, Infertility, Psychiatric Disorder, Women

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Globally, depression and anxiety are among the top causes for disease burden especially in middle-income countries such as India (1). WHO estimates for the period 2000-2015, show that together these psychiatric conditions contribute greatly to the total number of disability adjusted life years (1, 2), and their prevalence rates are estimated to rise by 2030 (3). These disorders are more common in women above the age of 18 years and in those with a co-morbid physical illness. Such ailments include hypertension, myocardial infarction, epilepsy, stroke, diabetes, cancer and tuberculosis, arthritis, chronic pain, back or neck problems, headaches, etc (4). However, their prevalence in those suffering from infertility is less documented.

Female infertility can be attributed to factors such as sexual dysfunctions, dyspareunia and vaginal causes, congenital defects in the genital tract, infections, chronic ill-health, cervical factors, uterine factors, tubal factors, ovarian factors, peritoneal and endocrinal factors (5). Psychiatric disorders in women undergoing infertility treatment are reported to be common (6). Review studies show that more than 50% of infertile patients face psychological problems. These are associated with variables like sex, number of cycles, type, length, costs of infertility evaluation and treatments (7-11). Other studies have revealed that a higher proportion of infertile women have psychiatric problems compared to fertile controls. Paranoid ideation, interpersonal sensitivity, and phobic anxiety are commonly found in childless women (9). Literature from the Indian context consists of studies that appear to have insufficient sample sizes and assessment biases. This literature indicates that both infertile men and women have subclinical as well as clinically significant psychiatric conditions (10). Evidenced based data emphasises that emotional distress in infertility makes a person vulnerable to complicated grief reactions, depression, dysthymia, reproductive mood disorders, anxiety disorders, adjustment disorders and sexual dysfunctions which compromise quality of life in men and women (11-20).The literature also suggests that there is an elevated risk in females in the age group 20-29 years (14).

Previous studies have suggested that adjustment disorders are more commonly found in women than men (11, 21). Additionally, evidence suggests that at the pre-treatment stage 16% of infertile couples and only 2% of

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fertile couples have significant adjustment problems. The clinical and subclinical features of emotional distress may be present in the couples during their 1st visit to the infertility expert (22). Mood disorders (depression and dysthymia) are prevalent in patients with infertility (23-25). These disorders are likely to worsen within the first three years of diagnosis and treatment (24-26). The latter sources also report that women are at a high risk for the emergence of reproductive mood disorders during the early child-bearing years. Researchers in Indian setups have also indicated that the prevalence of major depressive disorder is higher in women than in men. Men on the other hand are more often found to have mixed affective disorders (14, 15, 26-28). Depression is predicted by age, cause and duration of infertility, education, occupation and coping styles (8, 27, 29).

This research was planned knowing that: i. In India the rates of depression and anxiety rise in women above the age of 18 years, ii. Both these disorders contribute to considerable disease burdens and disability in work, family life and social functioning, and iii. There is a lack of empirical investigations estimating the prevalence of psychiatric disorders "in infertile women" in the Indian sub-continent. In this context, the aim of this study is to estimate the prevalence of psychiatric disorders in women seeking fertility treatments in a clinic based in Southern India.

The study is cross sectional and uses a convenience sample which is a part of a larger investigation of predictors of distress in infertile women (15). Sample size calculations were based on this larger investigation which included 300 Kannada/English/Hindi speaking women who were married, aged 22-50 years, had been diagnosed with primary infertility, and were seeking fertility treatments at Manipal Assisted Reproduction Centre (MARC), Kasturba Medical College, Manipal Academy of Higher Education (MAHE), Manipal. The study included all the women and their accompanying relative (spouse/family member) who consented to participate, and excluded women with secondary infertility and those unwilling to participate. Institutional Ethical clearance was taken with IEC number 275/2014.

The data in this study was collected using the following study tools. A brief form was compiled by the researchers for assessing socio-demographic variables. The second tool was the World Health Organization 'International Classification of Diseases-Clinical Descriptive and Diagnostic Guidelines, 10th revision' (ICD-10) (30). For the study, each woman's history of psychological problems/psychiatric disorders was collected during a detailed psychological consultation. It was conducted by the principal investigator, a licensed Clinical Psychologist trained in the use of ICD. The psychiatric history provided by the participants was corroborated for reliability and validity with the accompanying relative.

Data collection in this study involved the following steps. After ethical clearance, participants were enrolled on the basis that they met the inclusion criteria. The purpose of the study and its implications were explained, together with the participants' right to complete confidentiality and their right to withdraw from the study. Informed consent was taken from all of the women and their accompanying relatives who were willing to take part. Thereafter, the women completed the structured interview for the assessment of relevant socio-demographic and clinical variables. The assessment of the presence of psychiatric disorder was done using the ICD-10. Those found to have a significant psychiatric disorder were psycho-educated on their levels of distress and given the option of a consultation in the Department of Psychiatry and given a referral for the same.

All statistical analyses are carried out using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

Figure 1 presents the frequency counts and percentage of women with and without mental health problems. Table 1 presents details of various psychiatric disorders prevalent in the participants.

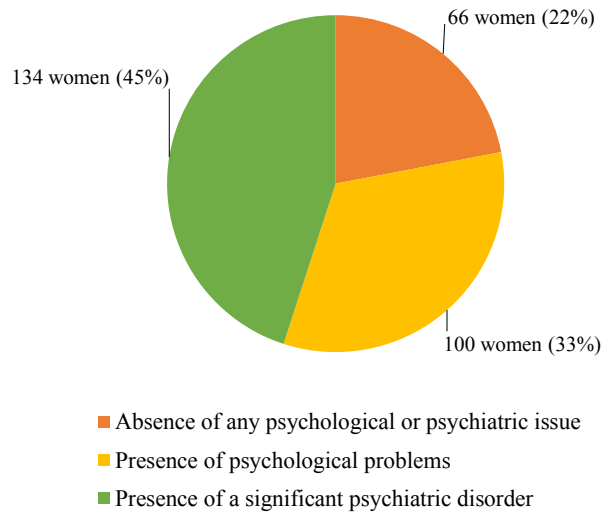

Fig.1: Frequency counts and percentage of women detected with and without mental health problems.

Table 1: Details of various psychiatric disorders prevalent in the participants

Details of various psychiatric disorders in infertile women (n=134)	Frequency data n (%)
i. Adjustment disorder (ICD 10 code F43.2)	49 (16)
ii. Anxiety NOS (ICD 10 code F41.9)	29 (10)
iii. Mixed anxiety and depressive disorder (ICD 10 code F41.2)	26 (9)
iv. Dysthymic disorder (ICD 10 code F41.2)	15 (5)
v. Major depressive disorder (ICD code 10 F 32)	12(4)
vi. Other anxiety disorders (social phobia, generalized anxiety disorder, obsessive compulsive disorder) (ICD 10 codes F40, F41.1, F42 respectively)	3 (1)

ICD; International classification of diseases and NOS; Not otherwise specified.

Results of this study reveal that the criterion for significant psychiatric disorder is met by 45% (134 out of 300) of the participants. This is followed by the 'off

and on' presence of psychological problems reported by 33% (100 out of 300) of the women who partially met the criteria for an ICD-10 diagnosis of disorder, but did not meet the time duration requirement). Lastly 22% (66 out of 300) of the women are found to be free from any psychological or psychiatric problem.

Based on the current data serious psychopathology is found to be quite widespread in infertility. Additionally, a sizeable proportion of women suffer from sub-threshold symptoms of anxiety and depression, although they did not fulfil the minimum duration for any specific psychiatric disorder in ICD-10. The present findings are somewhat similar to recent research which showed that 54% (27 out of 50) of infertile females have a significant psychiatric disorder (14). However, international estimates reveal a higher prevalence rate (6, 11-13).

The most common psychiatric diagnosis established in the present research is Adjustment Disorder with mixed affective features (found in 49 out of 300, roughly 16% of women). This is followed by anxiety disorder (unspecified) reported by nearly 10% (29 out of 134) of the participants and lastly mixed anxiety and depressive disorder reported by around 9% (26 out of 300) of women. Other psychiatric conditions, such as social phobia, generalized anxiety disorder and obsessive compulsive disorder were reported by 1% (3 out of 300) of the women. These findings are similar to those observed by other researchers who have also shown that adjustment disorders, anxiety disorders and mood disorders are frequently manifested in infertile women (11, 14, 23, 31-33). Additionally, the literature highlights the association between unmanageable infertility distress and occurrence of complicated grief reactions, depression, dysthymia, reproductive mood disorders, anxiety disorders, adjustment disorders, as well as sexual dysfunctions in patients undergoing intra uterine insemination (IUI) and *in vitro* fertilization (IVF). All of these are known to comprise quality of life in infertile men and women (18-25). Studies also reveal that 'anxiety features' are co-morbidly present with infertility distress, and depression is found in more than 50% of childless patients (11, 27, 28). Furthermore, less than 15% of participants in this study reported having consulted a professional mental health practitioner. Statistics from other nations also depict a similar trend (23). The present data is concordant with recent studies suggesting that psychiatric issues are often overlooked and undiagnosed in a majority of infertile patients in India (10, 15, 26).

Dysthymic features are reported by patients in the present study. Yet, severe depressive features, suicidal ideations or hopelessness are not reported. The results of this study are similar to other studies demonstrating that mild depression is more prevalent than moderate or severe depression, particularly in Indian contexts (10, 28).

This study has certain limitations. Firstly, is the lack of separate assessments of the husbands of the women who participated in this research. Secondly, an unstructured psychiatric interview schedule was included as a measure to tap psychiatric disorders in this study. This could have been supplemented with a structured tool for increasing the diagnostic validity of presence of a specific psychiatric disorder. Thirdly, reporting/recall biases of the participants could have crept in our data. Further studies conducted on this subject may consider drawing comparisons between mental health issues in i. Infertile women in comparison to fertile controls, ii. In women who conceive with treatments versus those who do not, and iii. In women who remain childless versus whose who go on to adopt. Additionally, psychiatric/psychological disorder is known to be associated with variables like age, gender, occupation, treatment type, length and history, duration of infertility, costs of evaluation or cure and other psychosocial variables (11, 12, 32, 33). Thus, the predictors and protective factors for psychiatric disorders in women seeking treatment for infertility can also be established in prospective investigations.

Over the past fifty years, most countries have come up with evidenced based committee reports on assessing the psychological endurance of couples prior to commencement of infertility treatments as well as protecting their overall wellbeing at all stages of the treatment process.

In conclusion, our data reveals that the prevalence of psychiatric disorders is high in infertile women. Ensuring the emotional wellbeing of patients seeking fertility treatments in India is an important component of comprehensive clinical care.

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Authors' Contributions

A.P.; Contributed in conceptualiation of topic, data collection, analysis, writing and editing of the final version. P.S.V.N.S.; Guided the project from its conception to the final completion as well as publication and review of literrature, and writing of the manuscript. P.K.; Contributed in conceptualization of topic, data collection and preparation of the final draft of the manuscript. All authors read and approved the final manuscript.

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Case Report

Endometriosis Presenting as Recurrent Haemorrhagic Ascites: A Case Report and Literature Review

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Abstract

Endometriosis is a common condition that occurs in 6-10% of all reproductive age women. This number increases to approximately 40% in women with infertility and nearly 75% in women with complaints of chronic pelvic pain. Endometriosis is characterized by the presence of endometrial glands and stroma outside the uterine cavity. The most common complaints associated with endometriosis are dysmenorrhea and pelvic pain; however, patients often present with a variety of symptoms and on occasion are asymptomatic. When presenting with haemorrhagic ascites, endometriosis mimics ovarian malignancy. Conservative medical treatment is a feasible management option, especially in young patients who desire to preserve fertility. This article aims to present an extremely rare presentation of endometriosis, haemorrhagic ascites, and a review of the associated literature.

Keywords: Endometriosis, Haemorrhagic Ascites, Pelvic Pain

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Introduction

Endometriosis is characterized by endometrial glands and stroma outside the uterine cavity. Endometriosis is a common condition that occurs in 6-10% of all reproductive age women (1-3). This number increases to approximately 40% in women with infertility and nearly 75% in women with complaints of chronic pelvic pain (4, 5). The pathogenesis of endometriosis is still debated. A well-founded theory postulates that it could be caused by retrograde menstruation of hormone-sensitive endometrial cells and tissues, which implant on peritoneal surfaces and cause an inflammatory response (6). The most common complaints associated with endometriosis are dysmenorrhea and pelvic pain; however, patients often present without pain and only with complaints of infertility, or there is an incidental finding of an ovarian mass on imaging (7). One exceedingly rare, and interesting, presentation is haemorrhagic ascites. Since its first description in 1954 by Dr. Brews, less than 100 cases of haemorrhagic ascites associated with endometriosis have been documented (8).

This article aims to present a case of a 32 year-old woman who presented with recurrent haemorrhagic ascites. We will discuss the patient's clinical course and surgical findings. A comprehensive review of the literature on medical/surgical management of patients with this rare finding will be presented.

Case Report

A 32-year-old nulligravida Hispanic female was referred to our department with complaints of general malaise, abdominal distention, loss of appetite, diffuse abdominal pain and difficulty breathing that had worsened over the last few days. She was known to have endometriosis that was diagnosed at the time of an exploratory laparotomy due to massive haemorrhagic ascites performed two years before. She was started on oral contraceptives at that time with poor response and was subsequently treated with monthly 3.75 mg leuprolide IM (Lupron®) but she self-discontinued the treatment due to the desire to conceive.

The patient provided consent for publication of the case report. The IRB was consulted and the IRB committee at Hospital Pedro Mallo, Buenos Aires, Argentina deemed this work exempt of approval.

Initial imaging with ultrasound and computed tomography (CT) scan revealed a large amount of intraperitoneal fluid. A paracentesis was performed that obtained 5 litres of thick bloody peritoneal fluid with a red blood cell count of >50000/μL that was negative for bacteria or malignant cells. The patient had symptomatic relief and was discharged home after the procedure. She then returned eight days later complaining of recurrence of the same symptoms. A repeat ultrasound was performed along with magnetic resonance

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imaging (MRI), which revealed massive ascites (Figs.1, 2). She was taken to the operating room for diagnostic laparoscopy and drainage of the hemoperitoneum. Upon entry of the peritoneal cavity, a large amount of bloody peritoneal fluid was identified. We removed ten litres of hemoperitoneum (Fig.3). Extensive pelvic adhesions with complete obliteration of surgical planes was noted (Fig.4). The pelvis was described as "frozen" due to encapsulating peritonitis that prevented the creation of surgical planes (Fig.5). Multiple peritoneal biopsies were taken which revealed endometriotic implants (Fig.6). The patient had an uneventful postoperative recovery and was treated with the gonadotropin-releasing hormone (GnRH) agonist triptorelin (3.75 mg intramuscular injection prior to discharge. At three months of the postoperative course, the patient was asymptomatic without recurrence of the disease.

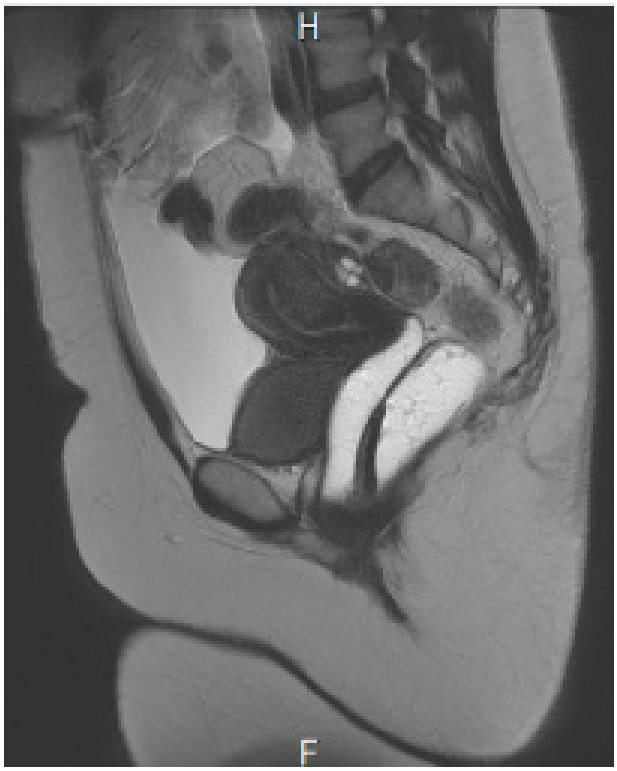

Fig.1: A large amount of intraperitoneal fluid is visualized on computed tomography (CT) of the abdomen and pelvis.

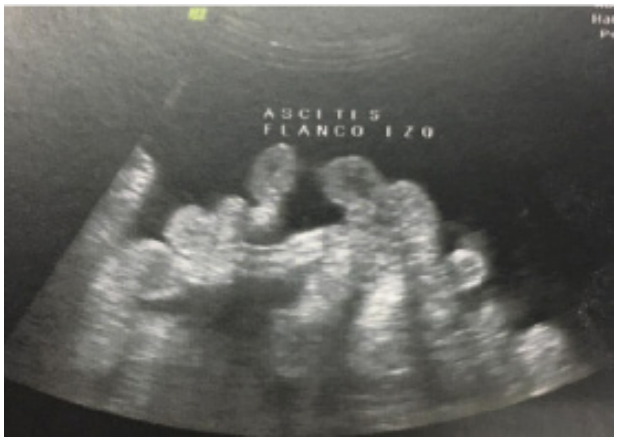

Fig.2: Massive ascites with small intestine floating inside the peritoneal cavity visualized on transabdominal ultrasound.

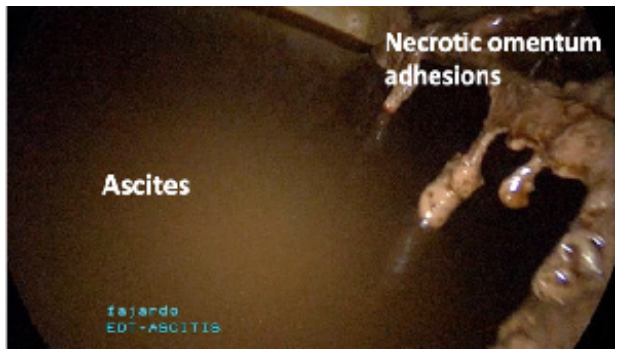

Fig.3: Bloody ascites filling the abdominopelvic cavity. Note necrotic omental adhesions on the anterior abdominal wall.

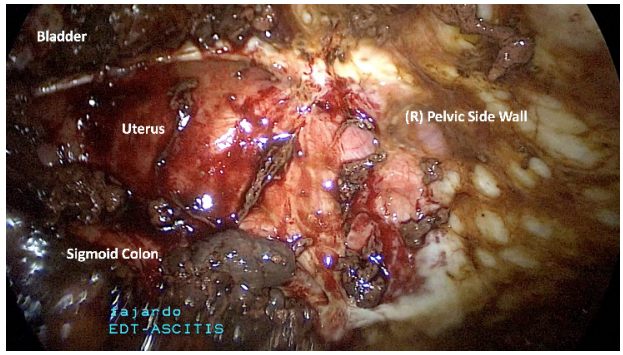

Fig.4: Note the complete obliteration of the vesicouterine space. The uterus is encapsulated from dense inflammatory plastic peritonitis and densely adheres to the pelvic side walls.

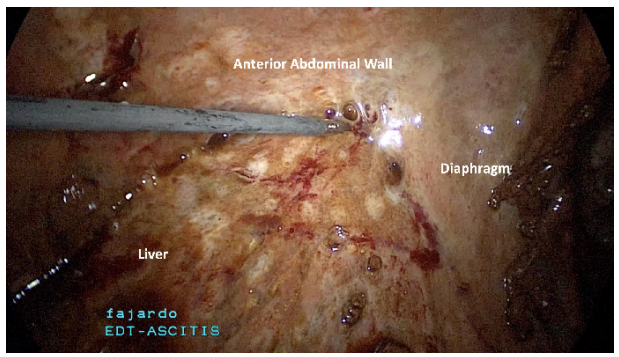

Fig.5: The liver is encapsulated by a dense parietal peritoneal inflammation. The liver is densely adherent to the anterior abdominal wall and the gallbladder is not visualized.

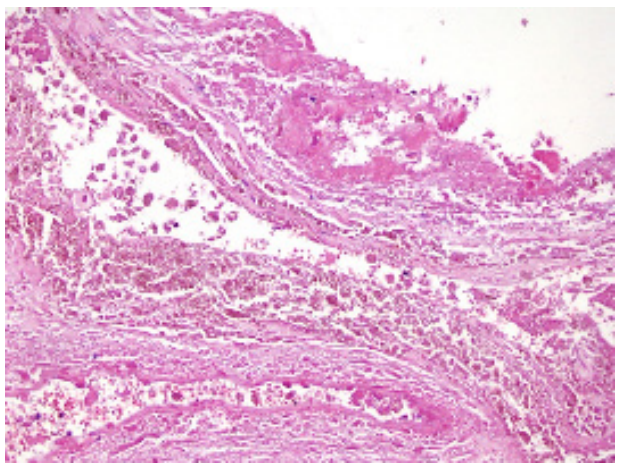

Fig.6: Peritoneal biopsy confirming the diagnosis of endometriosis. The endometriosis glands with periglandular endometriotic stroma that contain blood vessels are visualized.

Discussion

Massive ascites associated with endometriosis is extremely rare with less than 100 cases described in the literature (9). Endometriosis is a common challenging condition of reproductive-age women. The spectrum of the disease ranges from asymptomatic to complete debilitation, which requires both aggressive surgical and medical intervention. As stated above, the most common presenting symptoms of endometriosis are dysmenorrhea and pelvic pain. Our case of haemorrhagic ascites represents an incredibly rare complication associated with endometriosis. Patients with haemorrhagic ascites typically present with weeks to months of increasing abdominal pain, anorexia/weight loss, abdominal pain and dysmenorrhea. This presentation often leads to a workup for malignancy as ovarian cancer was the suspected diagnosis in more than half of the patients who presented with haemorrhagic ascites (8).

While the majority of patients with haemorrhagic ascites present with a gradual onset of symptoms, reports of acute onset of symptoms have been published. A 2013 case report described a 27-year-old who presented with a one day onset of neck and flank pain, abdominal distention, light-headedness and palpitations. She was initially stable, but progressively decompensated and required transfusion of numerous units of packed red blood cells. Ultimately, a diagnostic paracentesis was performed and 4.5 litres of grossly bloody ascitic fluid was removed (10). Our patient who presented with an acute recurrence following drainage via paracentesis provided evidence of how quickly the hemoperitoneum can accumulate.

Patients with haemorrhagic ascites often pose a difficult diagnostic dilemma on initial presentation. The different diagnosis must include large haemorrhagic ovarian cyst rupture, ovarian cancer, ectopic pregnancy, endometriosis, Meigs' syndrome, trauma, or other processes that could cause large hemoperitoneum. If necessary, initial stabilization measures with IV fluids and possible transfusion of blood products should be performed. As this presentation is so rare, no agreed upon workup is in place, but should be focused on ruling out the more common causes of hemoperitoneum. In a review of the literature, laboratory analyses that include complete blood count (CBC), basic metabolic panel (BMP), urine pregnancy and Ca-125 were typically performed, along with basic imaging with either ultrasound, CT scan or MRI (8). Choice of imaging is often physician dependent; however, MRI is being used more frequently in evaluation for patients with this presentation (11).

Haemorrhagic ascites has been treated both medically and surgically. Medical management was attempted in 97% of patients with the use of hormonal therapy (e.g., GnRH agonist, danazol, progesterone, combination oral contraceptive pills or a combination of these) (8). These medications aim to inhibit ovarian functions and have been well documented to successfully treat endometriosis. Although medical management was attempted, 89% of patients ultimately underwent a surgical procedure (8). The average volume of ascites was 4470 ± 2625 mL (12). A review of numerous case reports showed that patients underwent a variety of surgical procedures, which varied from exploratory laparotomy with excision of an adnexal mass, total abdominal hysterectomy, oophorectomy, ovarian wedge biopsy, lysis of adhesions, or a combination of these. Newer case reports have also been published that show successful management via a laparoscopic approach, and one via diagnostic and therapeutic paracentesis (9, 10, 13). Improvements were seen with both medical and surgical management; however, as in our patient, recurrence is possible. The most successful treatments were bilateral salpingo-oophorectomy or ovarian suppression therapy. Both treatments had no recurrence of ascites (12).

The exact cause of haemorrhagic ascites in patients with endometriosis is unknown. It has been suggested that the ascites is caused by a ruptured endometrioma or by exudation of widespread pelvic endometriosis. However, Ussia et al. (12) reported that endometriomas were only seen in 65% of cases, and that widespread superficial pelvic endometriosis was only associated with a minimal increase in peritoneal fluid and not with massive ascites. They have stated that the pathophysiology is ovarian in nature and due to excessive ovarian transudation (e.g., similar to Meigs' syndrome and Pseudo-Meigs' syndrome). Meigs' syndrome is based on the triad of an ovarian fibroma, pleural effusion and ascites with resolution of symptoms after resection of the fibroma. Pseudo-Meigs' syndrome is associated with a benign pelvic mass and a typical right-sided pleural effusion without ascites (14, 15). Their case is strengthened by a 50% recurrence rate in the setting of unilateral oophorectomy or cystectomy compared to no recurrences when a bilateral oophorectomy was performed. Patients placed on ovarian suppression therapy with a GnRH agonist also had no recurrence during the time they were taking the medication.

Management needs to take into account a patient's age, surgical history, medical history and future fertility plans. In patients who have no desire for future fertility and desire definitive surgical treatment, a bilateral salpingo-oophorectomy would be most effective. Subtotal surgical management (e.g., unilateral oophorectomy or cystectomy) alone should be avoided as the recurrence rate is high. Medical management with GnRH agonists are proven to be highly effective and should be used with a patient who desires future fertility, and for those who want to avoid surgical intervention.

Conclusion

Haemorrhagic ascites is a poorly understood and rare manifestation of pelvic endometriosis. The differential diagnosis includes a variety of benign conditions, but malignant pathology must be ruled out. There are no specific protocols for the treatment of this rare condition. Current theories regarding the pathophysiology point to the ovary and excessive ovarian transudation.

Management therefore involves surgical removal of bilateral ovaries or medical management with ovarian suppression. Patients who desire future fertility should be managed with a GnRH agonist. Clinicians should consider endometriosis in the differential diagnosis on female patients of reproductive age who present with haemorrhagic massive ascites.

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Authors' Contributions

A.G., S.A., F.E.; Participated in initial care and management of the patient upon presentation to the hospital. All authors participated in drafting, reading, editting, and approving the final manuscript. D.T., J.C.; Participated in the creation of the manuscript. All authors read, edited, and approved the final manuscript prior to submission.

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Endometriosis Classification and The Role of Tumor Necrosis Factor-Alpha Polymorphisms as A Therapeutic Target

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Abstract

In the recent original research published on International Journal of Fertility and Sterility the association between tumor necrosis factor-alpha (TNF-α) genetic polymorphisms and endometriosis in 150 Iranian patients suffered this disease. The authors notably found a lower frequency of TNF-α -863C/A allele A among the affected patients in comparison with healthy women, although this difference was not significant by adjusting multiple testing. We deem that the authors should specify, if these patients had peritoneal nodules, ovarian endometrioma/deep infiltrating endometriosis (DIE) nodules or combination of them, since it has been hypothesized that these phenotypes may represent three distinct pathogenetic entities of endometriosis.

Keywords: Endometriosis, Genetic Polymorphisms, Tumor Necrosis Factor-Alpha

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Dear editor of International Journal of Fertility and Sterility,

We recently read the article by Babaabasi et al. (1) entitled "The Association between TNF-alpha Gene Polymorphisms and Endometriosis in An Iranian Population" recently published in your journal. The aim of this study was to investigate association of some tumor necrosis factor-alpha (TNF-α) gene polymorphisms with risk of suffering endometriosis. The authors notably found a lower frequency of TNF-α -863C/A allele A among the patients with endometriosis compared to the healthy individuals, although this difference was not significant by adjusting multiple testing.

TNF-α is an inflammatory cytokine with a critical role in activating several transcription factors involved in inflammation, such as NF-Kappa-B and c-Jun N-terminal kinases (JNK) (2). Several literatures demonstrated that inflammation and particularly TNF-α may have a contributive role in genesis and establishment of implants of endometriosis (3).

In this letter, we would like to point out a methodological concern of this interesting study: in the "Materials and Methods" section, the authors indicated that analysis of some genotypes in the 5'-untranslated region of TNF-α was done on DNA obtained from peripheral blood samples of 150 Iranian women with confirmed endometriosis. In particular, the polymorphisms were genotyped by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). We deem that the authors should specify, if these patients had peritoneal nodules, ovarian endometrioma/deep infiltrating endometriosis (DIE) nodules or combination of them, since these three phenotypes of endometriosis may have different pathogenesis. Moreover, it would be of particular interest to know if the risk of having endometriosis in presence of the TNF-α specific -863 A allele was lower, regardless of such endometriosis phenotypes. In fact, we believe that different polymorphisms of TNF-α gene may lead to peculiar transcriptional activity and final protein function, definitively giving a different pathogenic contribution to each endometriotic phenotype (4).

Other genetic polymorphisms have been correlated with specific phenotypes of this disease: for example, it has been reported a significant association of progesterone receptor þ331G/A polymorphism with DIE, suggesting a potential role of this variant in the hormonal-dependent invasive behavior of endometrial cells (5). In a population of patients with histologically proved endometrioma without DIE, a specific polymorphism of DNA methyltransferase 3-like (DNMT3L) enzyme, which is responsible for DNA sub-telomeric hypomethylation, has been associated with the presence of endometrioma (6).

Overall, it has been described that nodules of DIE tend

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to have higher pro-inflammatory microenvironment and oxidative stress, with a more aggressive clinical behavior and different response to conventional therapies in comparison with the other endometriosis phenotypes (7, 8). Hormonal therapies, such as combined hormonal contraceptives and progestogens, should be regarded as the first-line treatment for DIE, considering that they are efficacious, safe and well-tolerated. Gonadotropin-releasing hormone agonists may be employed in women with refractory symptoms of the administration of first-line therapies (8). Surgery has been shown to be highly effective in ameliorating pain symptoms related to DIE implants; however, it may be particularly challenging and the benefits of performing surgical approach in terms of pain improvement should be always balanced with the risk of intraoperative complications (7). Until now, it is not clear if peritoneal endometriosis, ovarian endometrioma and DIE represent three distinct pathogenic entities (4).

Investigations on medical therapeutic approaches for treating endometriosis, represent one of our topics of research: in particular, we recently reviewed the role of experimental drugs targeting inflammation and immune system in this setting (9). Notably, *in vitro* studies have demonstrated that TNF-α is responsible for proliferation ectopic and eutopic endometrial cells as well as the cell adherence within the endometriotic lesions (10, 11). TNFRSF1A and c5N, two human recombinant TNF-α antagonists, have demonstrated to limit growth of endometriotic implants without altering the menstrual cycle in baboons (12, 13). More importantly, as a monoclonal antibody directed against TNF-α and largely used for treating chronic bowel inflammatory as well as rheumatologic disease, infliximab efficiently decreased the size of endometriotic lesions in rats with experimentally induced endometriosis (14). Until now, infliximab has been the only drug evaluated in the clinical setting for endometriosis, blocking TNF-α. However, contrary to the expectations, in a randomized placebo-controlled trial on 21 women, it did not modify number or size of DIE lesions (rectovaginal endometriosis of at least 1 cm in diameter); moreover, pain related to the disease did not ameliorate (15). Although a systematic review by Cochrane underscored that there is not enough evidence to support the use of anti-TNF-α drugs for endometriosis (16), the study of inflammatory-related pathways (including TNF-α polymorphisms) in pathogenesis of endometriosis, and particularly in DIE, appears extremely interesting.

Overall, findings of the study performed by Babaabasi and colleagues (1) are innovative and promising. Nevertheless, we deem that further genetic studies should be performed on this direction, in order to better clarify the role of TNF-α in the multiple aberrant pathways characterizing implants of endometriosis.

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Authors' Contributions

F.B., S.F.; Contributed to conception and design. U.L.M.R., V.G.V.; Were responsible for overall supervision. C.S.; L.F.D.; Were responsible for literature review, drafting the manuscript, which was revised by M.M. All authors read and approved the final manuscript.

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Letter to The Editor

Sexual Activity during Menstruation in The Holy Bible and Quran

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I read with great interest the manuscript entitled "Association between sexual activity during menstruation and endometriosis: a case-control study" published in the International Journal of Fertility and Sterility (1). According to the findings of this study, there was an association between sexual activities leading to orgasm during menstruation and endometriosis (1). These findings revealed the medical value and health benefit of the restriction of sexual contact during menstruation by major religions of the world. Particularly, in the third Book of the Pentateuch or Torah, known as Leviticus, it states that a woman undergoing menstruation is perceived as unclean for seven days and whoever touches her shall be unclean until evening (Leviticus 15:19). Moreover, "if a man actually lies with her so that her menstrual impurity is on him, he shall be unclean seven days, and every bed on which he lies shall be unclean" (Leviticus 15:24). In other words, if a man has sexual relations with a menstruating woman, he is not perceived as unclean only until evening, but for seven days. When seven days pass from the beginning of menstruation, the woman is regarded as clean and thus sexual contact is permitted. In another biblical passage it is stated that if a man has sexual relations with a woman during her menstrual period, both of them must be cut off from the community for they have both been exposed the source of her blood flow (Leviticus 20:18). The same biblical attitude to sexual relations during menstruation is also described in the following biblical passages: Leviticus 18:19 and 12:2, Ezekiel 18:5, 18:6, 18:9 and 22:10. These biblical sources were the reason for the attitudes of Judaism, as were the attitudes of subsequent religions that were influenced by Judaism, namely Christianity and Islam (2). In Christianity, sexual contact that occurs during menstruation is also considered as prostitution, since its unique purpose is usually the satisfaction of (man's mainly) sexual instinct and the achievement of pleasure (2). In addition to Judaism and Christianity, Islam also forbids men to have vaginal sexual intercourse with their wives during menstruation (Surah al-Baqarah 2:222). Other risks from sexual intercourse with a menstruating woman, except endometriosis, include the development of sexually transmitted diseases, increase in the flow of menstrual blood, and an undesirable pregnancy (2).

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

Aims and scope

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