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The Impact of Herpes Simplex Virus on Semen Parameters in Men with Idiopathic Infertility: A Systematic Review

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Abstract

Infertility due to the male factor is one of the major problems of infertile couples. One of the factors contributing to male infertility could be the herpes simplex virus (HSV). The aim of this systematic review was to evaluate the impact of HSV on semen parameters. This systematic review was performed according to the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA). Different English and Persian databases including Web of Science, PubMed, Scopus, Cochrane Library, EMBASE, ProQuest as well as SID, Magiran and Iranmedex were searched by two researchers, independently, without time limit until April 15, 2022. Observational studies that reported the relationship between HSV and semen parameters in men with idiopathic infertility were included in this review. The Newcastle-Ottawa Quality Assessment Scale was used for quality assessment of the included studies. Out of 356 retrieved articles, 12 observational studies comprising a total of 1460 patients were reviewed. Four studies examined the effect of HSV1 virus, two studies examined the effect of HSV2 virus and five studies examined the effect of both viruses on semen parameters. Seven studies reported at least one significant association between HSV infection and semen parameters. Sperm count and sperm motility were semen parameters further affected by the virus. In conclusion, HSV can be one of the risk factors for male infertility and it can affect semen parameters. However, due to the dearth of studies, further research with more robust designs are recommended.

Keywords: Herpes Simplex Virus, Infertility, Men, Semen, Sperm

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Introduction

Infertility is one of the problems of couples, and it is called, the lack of ability for couples to have children during one year of unprotected intercourse, affecting 10-15 percent of couples, and it causes a lot of problems for them (1-3). In keeping with the newest World Health Organization (WHO) statistics, 50-80 million people universally suffer from infertility (4, 5). A large number of research has found that about half of all instances of infertility occur due to female factors, 20-30 percent due to male factors, and 20-30 percent is unknown (5, 6). Recent meta-analyses by researchers showed that male factors are responsible for 20-70 percent of infertility instances (5, 7). Those findings are notably broader than outcomes previously observed. In various studies, several factors have been cited for male infertility (8-10). One of these factors is the herpes simplex virus (HSV), the effect of which some studies have shown on semen parameters (11-13).

HSV is a usual human pathogen, inflicting infections of orofacial membrane (HSV-1) and reproductive organ mucosal surfaces (HSV-2). Productive infection leads to the formation of vesicular lesions within the membrane epithelia, followed by unfolding of the virus to sensory neurons and institution of a latent infection, which will stay in the body throughout the lifetime of the host.

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Reactivation of the dormant virus results in recurrent disease at or contiguous to the location of the primary infection. During active infection, HSV takes over the host cell and a new code replication manages the viral gene (14, 15).

The HSV virus can stay for a long time with low copy numbers in the seminal fluid without presenting any symptoms (6, 11, 12). Recent studies have shown that HSV semen contamination is a possible risk factor for male infertility by affecting semen parameters such as sperm count, vitality, motility, and morphology by changing the pH of semen, changing the concentration or number of leukocytes, or increasing DNA fragmentation (11, 12, 16-18). Other later studies have not confirmed these findings (19-21), and extra analysis and synthesis of data is required to work out whether or not HSV infection contributes to male infertility.

Based on an extensive search among studies published in Persian and English on the reviewed topic, to the best of our knowledge, no systematic review has been published to evaluate the effect of HSV on semen parameters in male infertility. Previous reviews either were not systematic or have not specifically evaluated the effect of HSV on semen parameters to produce strong evidence; although there are some reviews on the impact of viral agents, as a whole, on the semen of infertile men such as, hepatitis B virus, human papilloma virus and epstein-barr virus (22-26). It should be noted that in the meta-analysis conducted by Malary et al. (27) in Iran, the prevalence of HSV virus in infertile men was reported, but the effect of the virus on semen parameters was not investigated. Also, since systematic reviews provide the highest level of evidence for evidence-based medicine (28), this systematic review was conducted to produce higher level of evidence with respect to the effects of HSV on semen parameters.Thus, due to the contradictory results that has been published about the effect of HSV on the quantity and quality of semen parameters, and considering that no systematic review has been conducted in this regard, the present systematic review was performed to identify the impact of HSV on semen parameters.

Materials and Methods

Data sources and search strategy

The present systematic review is based on the PRISMA 2020 guideline. Two researchers (AY, RLR), independently, searched English databases including Web of Science, PubMed, Scopus, Cochrane Library, EMBASE, ProQuest as well as SID, Magiran and Iranmedex databases to find Persian language articles without time limit until April 15, 2022. The search strategy was based on PICO: (P: men with idiopathic infertility and herpes simplex virus, I: none, C: fertile men, O: semen parameters including sperm count, sperm vitality, sperm motility, sperm volume and sperm morphology). The keywords used for searching included a combination of words: herpes simplex virus, HSV, men, infertility, sperm, semen, semen parameters with their equivalents in Mesh, and a combination of words with AND, OR functions. The type of study included cross-sectional and case-control studies. All the mentioned databases were searched to look for articles using the relevant keywords, in addition, the reference list of relevant articles, were manually searched to identify further studies missed by electronic search. The research question was as follows: What effect does the HSV has on semen parameters?

The PRISMA 2020 flowchart for systematic reviews was used to present the selection process of the studies (Fig.1).

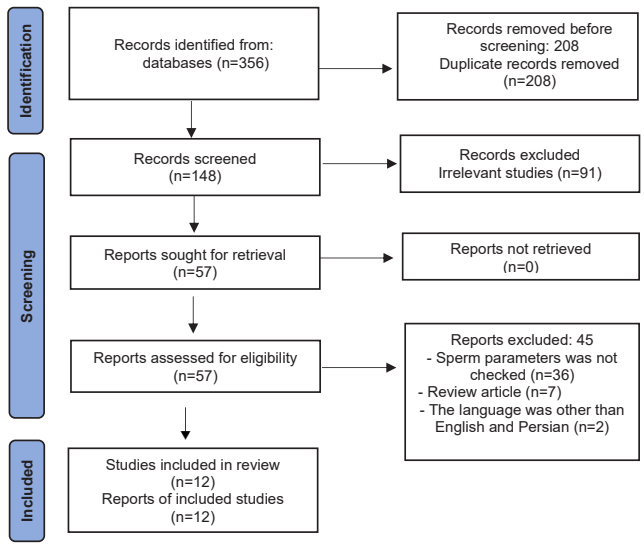

Fig.1: PRISMA 2020 Flow diagram of the study selection for systematic review.

Inclusion and exclusion criteria

Observational studies including cross-sectional and case-control studies that reported the relationship between HSV and semen parameters in men with idiopathic infertility were selected for review. Case reports, review articles, letters to the editor, and animal studies were excluded from the review. Also, the presence of anti-sperm antibodies, azoospermia, undescended testis, chromosomal abnormalities and a history of orchitis, epididymitis, varicocele and/or sexually transmitted infections in men were among the exclusion criteria.

Outcome measures

Outcome measures in this review included sperm motility, sperm vitality, sperm count, sperm morphology and semen volume.

Data extraction

Abstracts of articles were selected for screening using the search strategies described already. The title and

abstract of the articles were reviewed by two researchers, independently (AY, EI). If the article was related to the research topic, the full text of the article was reviewed. Articles with abstracts and texts other than English and Persian were excluded from the review. Also, articles with no published full text were removed from the review. Preliminary data were extracted by two researchers (AY, EMG) including the first author name, year of publication, country of publication, sample size, type of semen parameters studied and conclusion of the study. If two researchers disagreed in submitting an article to the study for review, they reviewed the inclusion and exclusion criteria and the research question and based on them, decided whether to include the article in the review process. The third researcher (AT) reviewed the article if the previous two researchers did not agree. However, disagreements between the two researchers were resolved through discussion with the third senior researcher (AT).

Quality assessment of studies

The type of articles extracted, included cross-sectional and case-control studies. Methodological quality assessment of studies was conducted by two researchers, independently (AY, EI) using the Newcastle Ottawa Quality Assessment Scale (29). Any disagreements between the two researchers were resolved through discussion with the third senior researcher (RLR).

Newcastle Ottawa Quality Assessment Scale has three sections for evaluating case-control studies: Selection (4 questions), Comparability (1 question) and Exposure (3 questions). The selection section receives a maximum of 4 points, the Exposure section receives a maximum of 3 points and the Comparability section receives a maximum of 2 points. Following assessment, a total of 9 points are awarded to the whole tool and the articles are rated as high, medium or poor quality (low quality ≤5 points, Medium=6-7 points, High=8-9 points).

To evaluate cross-sectional studies, this tool has three parts: Selection (4 questions), Comparability (1 question) and Outcome (2 questions). Selection gets a maximum of 5 points, Comparability gets a maximum of 2 points and Outcome gets a maximum of 3 points, and in total, the whole tool gets 10 points. Finally, the quality of the articles is rated as high, medium or poor (Low quality ≤5 points, Medium=6-7 points, High ≥ 8 points).

Results

In the first stage of searching the studies, 365 articles were retrieved. After removing 208 duplicate articles, 148 articles were assessed for title and abstract. Following removal of irrelevant articles, 57 articles were reviewed for the full text. Finally, 12 studies that examined the impact of HSV on semen parameters in men with idiopathic infertility were included in the systematic review (11-13, 16-21, 30-32). The characteristics of the comprised articles in this systematic review are summarized in Table 1.

Table 1: Basic characteristics of the included studies in the systematic review

ID	First author/ year of publication	Country	Study design	Age (Y)/sample size (n)	Cause of infertility	HSV type (HSV-1/ HSV-2)	Outcome measures	Main findings	Quality assessment score
1	Kapranos et al. (16), 2003	Greece	Cross-sectional	Not reported, (n=113 infertile men)	Idiopathic male infertility	HSV1	Sperm motility, Sperm count	HSV, by affecting the most important factors of semen quality, plays an important role in male infertility.	High
2	Bezold et al. (17), 2007	Germany	Cross-sectional	22-55 years, (n=241 infertile men)	Idiopathic male infertility	HSV-1, HSV-2	Sperm motility, Sperm count	HSV was associated with poor semen quality.	High
3	Kurscheidt et al. (12), 2018	Brazil	Cross-sectional	18 years or older, (n=279 infertile men)	Idiopathic male infertility (Semen analysis for fertility evaluation)	HSV-1, HSV-2	Sperm count, Vitality, Sperm motility, Sperm volume (mL)	HSV infections may have changes on the 2 equally important components of semen, spermatozoa, and seminal fluid, which may influence fertility.	High
4	Kotronias and Kapranos (11), 1998	Greece	Cross-sectional	Not reported, (n= 80 infertile men)	Idiopathic male infertility	HSV-1, HSV-2	Sperm count, Sperm motility	HSV seems to play an important role in male infertility.	Low
5	Pallier et al. (30), 2002	Praise	Case-control	-	HSV2	HSV2	Sperm motility	Slight difference was reported in the percentage of motile forms when seminal fluid –free sperm were incubated with HSV.	Medium

Table 1: Continued

ID	First author/ year of publication	Country	Study design	Age (Y)/sample size (n)	Cause of infertility	HSV type (HSV-1/ HSV-2)	Outcome measures	Main findings	Quality assessment score
6	Mergani et al. (19), 2019	Sudan	Case-control	Not reported, (n=90 infertile men, 45 control group, 45 case group)	HSV2	HSV2	Sperm count, Sperm motility	HSV type 2 antibodies had no objective impacts on sperm concentration or sperm motility among Sudanese infertile males.	High
7	Bocharova et al. (18), 2008	Russia	Cross-sectional	Not reported, (n=23 infertile men)	HSV	HSV	Spermatocytes I in pachytene and diplotene stage of meiosis	A partial spermatogenic arrest at the early stages of meiotic prophase I in HSV patients	Medium
8	el Borai et al. (31), 1997	Tokai	Case-control	Not reported, (n=169 Case=153 infertile men, control=16 fertile men)	HSV1	HSV1	-	No difference in the distribution of sperm quality within the 2 groups, herpes positive and negative	Low
9	Monavari et al. (13), 2013	Iran	Cross-sectional	Not reported, (n= 70 infertile men)	HSV1, HSV2	HSV1, HSV2	Sperm Count, Sperm motility, Sperm morphology	Asymptomatic seminal infection of HSV plays an important role in male infertility by adversely affecting sperm count	High
10	Neofytou and Sourvino (20), 2009	Greece	Case -control	Not reported, (n=172 infertile man, control=80 whit normal semen parameters, case=92 with abnormal semen parameters)	HSV1	HSV1	Sperm motility, Sperm count	The DNA of herpes viruses is frequently detected in the semen of asymptomatic fertile and infertile male patients. Further studies are required to investigate the role of herpes viruses in male factor infertility.	High
11	Moretti et al. (21), 2017	Italy	Cross-sectional	Not reported, (n=73 men)	HSV1	HSV1	Sperm count, Sperm motility, Sperm volume	The DNA of herpes viruses is frequently detected in the semen of asymptomatic fertile and infertile male patients. Further studies are required to investigate the role of herpes viruses in male factor infertility.	Medium
12	Tajedini et al. (32), 2017	Iran	Cross-sectional	34.1 ± 5.7 year (n=150 infertile men)	HSV1, HSV2	HSV1, HSV2	Sperm count, Sperm motility	HSV was associated with a decrease in the number of sperm in the semen.	Medium

HSV; Herpes symplex virus.

The design of the eight studies (11-13, 16-18, 21, 32) that were systematically reviewed in the present study was cross-sectional, four studies were case-control (19, 20, 30, 31), and their publication year was from 1997-2019. Half of these articles received a "High" score in quality assessment (12, 13, 16, 17, 19, 20), two of the articles received a "Low" score (11, 31) and the rest of articles received a "Medium" score (18, 21, 30, 32).

The total sample size was 1,460 infertile men with no obvious clinical signs of the virus. Also, the cause of infertility in these men was unknown. The sample size in the articles ranged from 23 (18) to 279 (12) people. WHO criteria was adopted for collecting, preparing, and analyzing semen sample according to the literature (33). In all studies, male infertility was referred to as the incapacity of couples to have children after twelve months of intercourse without the use of contraceptives. In all studies, semen sample was taken for sperm analysis after three days of sexual inactivity.

Four studies only measured the effect of HSV1 in

semen on semen parameters (16, 20, 21, 31). Two studies examined only the effect of HSV2 on semen parameters (19, 30). Five studies measured the effect of both HSV1 and HSV2 viruses on semen parameters (11-13, 17, 32). One study did not identify the type of HSV virus and HSV was generally mentioned in the article (18).

Out of the 12 studies included in the present review, in seven studies (11-13, 16-18, 32) at least one significant association was observed between semen contamination with HSV1 or HSV2 and a decrease in the quantity or quality of semen parameters. In these studies, sperm count and sperm motility were more affected by HSV and the virus had a small effect on the volume of semen, so that, in one study (12) it caused a significant reduction in the volume of semen.

Detection of HSV in semen: In the studies included in this review article, fluorescence in situ hybridization (FISH) or polymerase chain reaction (PCR) methods were used to identify the HSV virus in semen.

Studies reporting no association: In five (19-21, 30, 31) out of 12 studies, HSV virus had no impact on the quantity and quality of semen parameters. In one of these five studies, HSV was found in the semen of 24% of infertile men, but the virus was not detected in the semen of any of the fertile men (31).

In another study, HSV2 was injected into the semen of a number of infertile men and sperm motility was compared between the two groups. In the HSV2 positive group, sperm motility was lower than the other group, but this difference was not statistically significant (30).

Studies reporting association: Seven studies (11-13, 16-18, 32) in this review reported at least one significant association between semen contamination with HSV1or HSV2 virus and the quantity or quality of semen parameters. HSV1 in three studies (11, 13, 16) and HSV2 in two studies (11, 13) were associated with a significant reduction in sperm count and motility. Out of the five studies that measured the effect of HSV1 and HSV2 on semen parameters, three studies (12, 17, 32) just reported HSV in the results and did not examine semen parameters separately for each type of herpes simplex virus. In one of these studies (32), the HSV virus was related with a considerable reduction in sperm count. In two other studies (12, 17), the presence of HSV virus in semen was associated with decreased sperm count, sperm motility and sperm volume.

In the study by Kotronias and Kapranos (11), in HSV-positive men, sperm motility and sperm count were significantly lower than in HSV-negative men. Also, in the subsequent study of these authors, with a higher sample size, the presence of HSV in the semen of infertile men was associated with a significant reduction in sperm count and sperm motility (16). Bezold et al. (17), reported similar results. In their study, the presence of HSV virus in the semen of infertile men was associated with a significant reduction in sperm count, sperm motility and sperm concentration. In two other studies conducted in Iran by Tajedini et al. (32) and Monavari et al. (13), contamination of semen with HSV, significantly, reduced sperm count in infertile men. In the study of Bocharova et al. (18), HSV positive patients illustrated a significant reduction within the range of spermatocytes I, in the pachytene and diplotene stages of meiosis, reduction in the proportion of spermatocytes II and spermatids, and a two-fold increase in the number of unrecognizable premature germ cells. The data obtained demonstrate a partial spermatogenic detention at the early stages of meiotic prophase I in HSV patients. Sperm vitality and morphology were each examined in one study and HSV infection was not related with sperm morphological defects (13) and sperm vitality (12).

Semen parameters

Sperm mortality

The effect of semen contamination with HSV type one or two was investigated on sperm motility in ten studies (11-13, 16, 17, 19-21, 30, 32). In one study, HSV1 infection caused a significant decrease in sperm motility (16). But in three studies there was no significant relationship between HSV1 infection and decreased sperm motility (13, 19, 21). Also, in one study, a significant correlation was observed between HSV2 infection and decreased sperm motility (13), whereas no significant correlation was observed in the two other studies (19, 30).

In the articles by Bezold et al. (17) and Kotronias and Kapranos (11), which had not specified the type of virus in the results, a significant relationship was generally observed between HSV infection and decreased sperm motility and in the Kurscheidt et al. (12) and Tajedini et al. (32) articles, in general, no significant relationship was observed between HSV infection and decreased sperm motility.

Sperm count

In six studies, the relationship between sperm count and semen contamination with HSV type one or two was investigated (11, 13, 16, 19, 20, 32). In two studies, a significant correlation was observed between HSV1 infection and sperm count reduction (13, 16). However, in one study, no significant relationship was observed (20).

In one study, there was a significant relationship between HSV2 and decreased sperm count (13), and in another study, no significant relationship was observed (19). Kotronias and Kapranos (11) and Tajedini et al. (32) found a significant relationship between semen contamination with HSV1 and HSV2 with decrease in sperm count.

Sperm volume

In one study, a significant correlation was observed between semen infection with HSV1 and HSV2 with a decrease in the volume of the semen sample (12). In

another study, no significant relationship was observed between HSV1 and reduction of semen volume (21).

Sperm concentration

In the study by Moretti et al. (21), no significant difference was observed between HSV1 infection and changes in sperm concentration. But in the study by Bezold et al. (17) and Kotronias and Kapranos (11) a significant correlation was observed between HSV1 and HSV2 infection with changes in sperm concentration.

Sperm morphology

A significant relationship was observed between HSV2 infection and the reduction of normal sperm morphology, but no significant relationship was observed between HSV1 and sperm morphology (13).

Discussion

The present review was conducted to identify the impact of HSV on semen parameters. Twelve articles were included in the study, and in seven studies (11-13, 16-18, 32) at least one significant association was observed between semen contamination with HSV1 or HSV2 and a decrease in the quantity or quality of semen parameters. Sperm motility and sperm count were reduced in six studies due to virus infection (11-13, 16, 17, 32) and in one study (12) HSV caused a significant reduction in the volume of semen. In these studies, HSV was identified as the cause of male infertility, so that, acyclovir treatment in two studies improved semen parameters and led to a successful pregnancy (11, 16). Therefore, these authors recommended screening and treatment for HSV before assisted reproductive technologies in infertile men.

Akhigbe et al. (34), reported that HSV can infect almost all organs of the male reproductive system and it can cause direct damage to sperm and change sperm quality such as decreased sperm count, motility and morphology. Nawaz et al. (35), also mentioned that HSV DNA is mostly discovered in semen with low sperm count, indicating the connection of sperm HSV DNA and possible fertile problems. HSV infection affects the functions of the male genital glands and is related with male infertility and malformed semen parameters. Ochsendorf (36), inferred that infection of semen with the HSV may destroy sperm count and motility, but this has not yet been proven. It has been highlighted in two other studies that HSV infection may influence male infertility via harmful effects on semen parameters including sperm count, sperm motility and apoptotic loss of germ cells (37, 38). But the results of the study by Afrakhteh et al. (39), showed that semen samples of both fertile and infertile participants were negative for HSV1 and HSV2; therefore, infection of semen with HSV is not associated with impaired semen parameters. The results of the study by Elhadi et al. (40), in infertile men also showed no association between HSV2 DNA and low sperm count, which is inconsistent with the results of the mentioned studies and may be due to differences in sample size and virus detection methods.

In our systematic review, HSV infection was not related with sperm morphological defects (13) but in a study by Komijani, et al. (41), it was found that HSV infection resulted in abnormalities in the sperm head and neck, which could finally lead to infertility. Animal studies have also shown that infection of the testes of mice with HSV and retrograde ascent of the virus into the seminiferous tubules leads to irreversible atrophy of the germinal epithelium, sperm damage and infertility and the reduction of HSV thymidine kinase levels, which resulted in marked reduction of spermatozoa abnormalities and amendment of fertility (42, 43). A nearby ligand/receptor connection may exist between the virus and seminal cells, which cause the effect of the virus on the quantity and quality of sperm. These receptors mainly include: i. Glycosaminoglycans (GAGs), particularly heparan sulfate which is situated at the cell surface and can intervene with passage of numerous infections including HSV-1 and HSV-2 and ii. Nectin-1 and nectin-2, individuals from the immunoglobulin superfamily, are expressed in numerous organs, tissues, and cell lines, which are regularly contaminated by HSV. Nectin-1 articulation on murine spermatids, which would advance connection with the infection, has likewise been seen (38).

To the best of our knowledge, the present study is the first systematic review on the effect of HSV on semen parameters, in which articles both in Persian and English languages related to the purpose of the study were included in the review and this is considered as the strengths of the present study. One of the limitations of this study was that it was not possible to do meta-analysis. The reason for not conducting a meta-analysis and just carrying out a systematic review was that in the included articles in this review, the data were presented in some articles as continuous data and in others as percentage or median. Therefore, it was not possible to combine them and conduct a meta-analysis. Further studies are needed to make conduction of future meta-analysis possible. Also, since no meta-analysis was found regarding the effect of HSV on semen parameters, discussing the findings and comparing the results with other meta- analyses was limited.

Conclusion

According to the studies reviewed in this article, it can be concluded that HSV can be one of the risk factors for male infertility and affect the quantity and quality of sperm. Infection of semen with HSV reduces the parameters of sperm count, motility and volume and does not affect morphology and sperm vitality. In order to better assess the range of effects, future studies should include consideration of other semen parameters, including semen volume, sperm viability, as well as investigating how the HSV virus could damage the sperm production process.

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Authors' Contributions

A.Y., R.L.R., M.M.; Study design. A.Y., E.I.; Screening and quality assessment of the articles. A.Y., E.M.G.; Extraction of the preliminary data. A.Y., R.L.R., A.T.; Interpretation of data. A.Y., R.L.R., E.I., E.M.G., A.T., M.M.; Drafting and critical revision of the manuscript and final approval of the manuscript. All authors agreed to be accountable for all aspects of the study in ensuring that questions related to the accuracy or integrity of any part of the study are appropriately investigated and resolved. All authors read and approved the final manuscript.

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A Meta-Analysis of The Prevalence and Etiology of Infertility in Iran

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Abstract

Infertility is a serious health issue in the world affecting approximately 8-10% of couples worldwide. The meta-analysis study was performed to assess the prevalence and etiologic factors associated with infertility in Iran. We made sure that the implementation of study and reporting the results were consistent with the MOOSE and PRISMA guidelines, respectively. All stages of the research were conducted by two authors, and the disagreement at each stage of the research was resolved by consensus. On January 1, 2020, we started a detailed literature search on international online databases, and Iranian Online databases, as well as specialized journals, several authentic international publishers and Google Scholar. We reviewed the reference list of identified articles for missed articles and then searched online for them. Data analysis was performed to estimate the prevalence using a random effects model. The lifetime infertility prevalence was found to be 11.3% [95% confidence interval (CI): 8.6-14.7] and the current infertility was evaluated to be 3.7% (95% CI: 3.2-4.3). The prevalence of primary infertility (based on 45 articles consisting of 51,021 samples) as well as secondary infertility (based on 13 articles consisting of 35,683 samples) in Iran were estimated to be 18.3% (95% CI: 15.4-21.6) and 2.5% (95% CI: 1.6-4.0), respectively. The prevalence of female, male, both and unexplained causes was estimated to be 32.0% (95% CI: 27.6-36.8), 43.3% (95% CI: 38.2-48.6), 12.5% (95% CI: 9.6-16.2) and 13.6% (95% CI: 10.2-17.8), respectively. The prevalence of causes related to ovulation, uterine tubes, and endometriosis in infertile women was estimated to be 54.0% (95% CI: 45.6-62.2), 15.5% (95% CI: 11.3-21.0), 6.2% (95% CI: 3.5-10.6), and 5.4% (95% CI: 2.5-11.3), respectively. In summary, the estimate of infertility burden in Iran did not change between 1990 and 2017 and its prevalence remains high. This research presents a unified and up-to-date overview regarding the burden of infertility in Iran.

Keywords: Etiology, Infertility, Iran, Prevalence

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Introduction

Infertility is a serious worldwide health problem affecting almost 8-10% of couples global (1). According to the World Health Organization (WHO), 186 million ever-married women in sexual age in developing countries are affected by infertility (2). This serious problem requires urgent action, especially where most infertility cases are avoidable. According to the latest global described as failure to reach clinical pregnancy after regular unprotected intercourse for twelve months omore. Having regular sexual intercourse is a key factor in pregnancy. Primary infertility is the lack of ability to give birth either because of not having the ability to get pregnant or carry a baby till live birth, which can encompass miscarriage or stillbirth. Secondary infertility is the lack of ability to conceive or give birth despite a previous pregnancy or a live birth (3).

Infertility is a very important part of sexual health and efforts that have often been ignored (4) in this regard. Failure to give birth to an infant influences many couples around the world. The United State Centers for Disease Control and Prevention emphasize that infertility is beyond just a problem that affects the quality of life with significant consequences for public health, such as mental discomfort, social stigma, economic stress and marital separation. According to the results comparing stressful life events, after mother's death, father's death and spouse's infidelity, infertility is ranked as fourth stressful life event (5). Not considering the emotional afflictions of infertile men and women and secondary signs of infertility (interpersonal problems, marital discontent and

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loss of libido) may lead to a flawed cycle that increases infertility incidence (6). On the other hand, in recent years, factors such as changes in women's role in social activities, delaying marriage, changes in childbearing age, increasing use of contraceptive methods, liberal abortion laws, and undesirable economic status have decreased fertility rates and increased infertility (7).

There are over one million infertile couples living in Iran, and since childbearing is so important in the religious, historical, and cultural context of Iranian society, infertility can be one of the causes of divorce (8). Gaining accurate information about the prevalence, and infertility trends is the first main step in providing evidence-based measures and policies for decreasing the difficulties of this issue worldwide. There have been numerous studies in Iran on the prevalence and etiology of infertility and the results vary widely (9-43), and a systematic review of the original studies now seems necessary. The meta-analyses combine various studies with similar objectives, which involves increasing the number of samples and decreasing the confidence interval, and this can provide a more reliable estimate (44, 45). The purpose of this meta-analysis was to assess the prevalence and etiologic factors associated with infertility in Iran.

Materials and Methods

Study protocol

The protocol of the present study was posted on International Prospective Register of Systematic Reviews (PROSPERO 2020) prior to implementation (CRD42020170926 Available from: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020170926). We made sure that the implementation of study and reporting the results were consistent with the Meta-analyses Of Observational Studies in Epidemiology (MOOSE) (46) and Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines, respectively (47). All stages of the research were conducted by two authors, and the disagreement at each stage of the research was resolved by consensus.

Data sources and search strategy

On January 1, 2020, we started a detailed literature search on international databases of Web of Science (ISI), Ovid, Scopus, EMBASE, PubMed/Medline, Cochrane Library, EBSCO, CINAHL, and Iranian Online databases Scientific Information Database (SID), elmnet , Civilica, Regional Information Center for Science and Technology (RICST), , IranDoc , Magiran Barakat Knowledge Network System, and Iranian National Library, as well as specialized journals, several authentic international publishers including Wiley online library, Science Direct, and Springer and search engines such as Google Scholar. We reviewed the reference list of identified articles for missed articles and then searched online for them.

The following search strategy is an example of what was used in PubMed: ("prevalence" OR "frequency" OR "incidence" OR "rate" OR "epidemiology" OR "etiology" OR "cause") AND ("infertility") AND Iran.

Study selection

After excluding duplicate studies, the two writers reviewed the title and abstract of the studies independently. In case of disagreement, a third author was consulted or it resolved through consensus. Then, the full text of each study was reviewed based on the target inclusion criteria (Fig.S1, See Supplementary Online Information at www.ijfs.ir).

Inclusion and exclusion criteria

Eligible studies according to PECOS (population, exposure, comparison/comparator, outcome and study type) criteria: i. Population: Iranian population, ii. Exposure: infertility, iii. Comparison/comparator: type of infertility and its cause, iv. Outcome: prevalence of infertility, and v. Study type: cross-sectional study.

Definition

Infertility was defined as failure to achieve pregnancy after unprotected intercourse for 12 months. Primary infertility was defined as not experiencing a previous pregnancy and secondary infertility was defined as having a previous pregnancy (48). Infertility is considered as lifetime when a couple has experienced infertility in their whole lives. However, current infertility is defined as having the problem of infertility at present (49).

Data extraction

Two researchers extracted the required data from all studies based on data encryption list. The following variables were obtained for each article: i. Study information (authors, publication year, place of study and completion year), ii. Design of the study, iii. Sample selection, iv. Reliability coefficient of the instrument, v. Sample size, vi. Prevalence (lifetime infertility, current infertility, primary infertility, secondary infertility, and etiology of infertility).

Qualitative evaluation

Newcastle-Ottawa Scale adapted for cross-sectional research was used to assess the quality of articles (50). The highest achievable score was 9. Three categorizations were defined for the quality of studies with a score of less than 6, 6-7, and 8-9 as low, medium and high quality, respectively.

Data analysis

The program used here was Comprehensive Meta-analysis Software ver. 2, and the results were shown as forest plot. The heterogeneity of the data was evaluated using I^2 index. This test evaluates the percent of variability in estimating the effect of heterogeneity. Significant heterogeneity exists if I^2 values are above 50% (51). Meta-analysis was performed to estimate the prevalence using a random effects model by DerSimonian and Laird

for point estimation and 95% confidence interval (CI). Publication bias was assessed according to regression asymmetry tests (52). Sensitivity analysis was used to investigate the effect of individual studies on overall prevalence. Subgroup-analysis and meta-regression were conducted to determine the source of heterogeneity. Significance level was defined less than 0.05 in all tests.

Results

Study selection process and study characteristics

The infertility prevalence

The lifetime infertility prevalence (in 14 studies with 46,466 samples) (Table 1) was 11.3% (95% CI: 8.6-14.7) and current infertility (in 34 studies with 30,069 samples) estimated to be 3.7% (95% CI: 3.2-4.3) (Fig.1).

Subgroup analysis of infertility

The subgroup analysis regarding the prevalence of lifetime infertility according to the region (P=0.069), year (P=0.069) and studies quality (P=0.069) was insignificant (Fig.S2, See Supplementary Online Information at www.ijfs.ir). Subgroup analysis of current infertility prevalence was significant based on year (P<0.001) but not significant based on region (P=0.321) and studies quality (P=0.593, Fig.S3, See Supplementary Online Information at www.ijfs.ir).

Table 1: Summary of characteristics in studies into a meta-analysis

Ref.	First author, Published Year	Year	Place	Number	Lifetime infertility	Current infertility	Method	Quality
(9)	Badr et al., 2009	2004	Tabriz	3183	104	88	15- to 49-year-old couples	Moderate quality
(10)	Vahidi et al., 2006	2004-5	East Azarbaijan	610		14	19- to 49-year-old women	Moderate quality
			West Azarbaijan	502		5	19- to 49-year-old women	Moderate quality
			Ardabil	236		4	19- to 49-year-old women	Moderate quality
			Isfahan	675		18	19- to 49-year-old women	Moderate quality
			Ilam	114		8	19- to 49-year-old women	Moderate quality
			Bushehr	149		8	19- to 49-year-old women	Moderate quality
			Tehran	1730		58	19- to 49-year-old women	Moderate quality
			Chaharmahal and Bakhtiari	147		5	19- to 49-year-old women	Moderate quality
			Khorasan	1197		45	19- to 49-year-old women	Moderate quality
			Khuzestan	741		30	19- to 49-year-old women	Moderate quality
			Zanjan	205		10	19- to 49-year-old women	Moderate quality
			Semnan	86		3	19- to 49-year-old women	Moderate quality
			Sistan-Baluchestan	330		20	19- to 49-year-old women	Moderate quality
			Fars	742		26	19- to 49-year-old women	Moderate quality
			Qazvin	290		15	19- to 49-year-old women	Moderate quality
			Qom	136		8	19- to 49-year-old women	Moderate quality
			Kurdistan	254		8	19- to 49-year-old women	Moderate quality
			Kerman	371		8	19- to 49-year-old women	Moderate quality
			Kermanshah	384		7	19- to 49-year-old women	Moderate quality
			Kohkiluyeh and Boyer Ahmad	90		2	19- to 49-year-old women	Moderate quality

Table 1: Continued

Ref.	First author, Published Year	Year	Place	Number	Lifetime infertility	Current infertility	Method	Quality
			Golestan	261		8	19- to 49-year-old women	Moderate quality
			Guilan	427		19	19- to 49-year-old women	Moderate quality
			Lorestan	317		7	19- to 49-year-old women	Moderate quality
			Mazandaran	545		13	19- to 49-year-old women	Moderate quality
			Markazi	219		7	19- to 49-year-old women	Moderate quality
			Hormozgan	208		20	19- to 49-year-old women	Moderate quality
			Hamedan	292		7	19- to 49-year-old women	Moderate quality
			Yazd	148		2	19- to 49-year-old women	Moderate quality
(11)	Mohammad Baygi, 2002	2002	Sanandaj	902	166		15- to 49-year-old women	Moderate quality
(12)	Kamali et al., 2007	1995-2001	Tehran-Royan institute		2492		Infertile couples	High quality
(13)	Bakhtiari, 1999	1999	Babol –Fatematazah-ra		920		Infertile couples	High quality
(53)	Karimpour Malekshah et al., 2011	2003-8	Mazandaran- clinics		3734		consecutive couples	High quality
(15)	Moghaddam et al., 2000	1999	Mazandaran	2953	389		15- to 49-year-old couples	Moderate quality
(16)	Esmailzadeh et al., 2002	1996-8	Babol –Fatematazah-ra		2169		Infertile couples	Moderate quality
(17)	Sadegh Moghadam et al., 2008	2006	Gonabad	380	45		15- to 49-year-old women	Moderate quality
(18)	Ardalan et al., 2010	2004–2005	Iran	10783	1592		19- to 49-year-old women	High quality
(19)	Sedaghat Siahkal et al., 2003	2001	Tehran	1987	173	50	25- to 49-year-old women	High quality
(20)	Parsanezhad and Alborzi, 1998	1993	Shiraz	1430	159		15- to 49-year-old couples	Moderate quality
			Shiraz		693		20- to 49-year-old women	Moderate quality
(21)	Barouti et al., 1999	1997	Tehran	1784			19- to 49-year-old women	Moderate quality
(22)	Nojomi et al., 2002	2000	Tehran	1174	141	49	40- to 50-year-old women	High quality
(23)	Moini and Yazdan Panah, 1999	1990-5	Tehran-Royan Institute		4360			High quality
(14)	Karimpour et al., 2005	2001-3	Sari		657	37	Infertile couples	High quality
(24)	Delpishe et al., 2014	2013	Ilam- clinics	1013	117	44		High quality
(25)	Shagheibi et al., 2018	2014-15	Sanandaj-hospital		579		Infertile couples	High quality
(26)	Kazemijaliseh et al., 2015	1998	Tehran	1067			18- to 57-year-old couples	High quality
(27)	Noorbala, 2001	2001	Iran	10418		292		High quality
(28)	Shafi et al., 2016	2012	Babol	1081			20- to 45-year-old women	High quality

Table 1: Continued

Ref.	First author, Published Year	Year	Place	Number	Lifetime infertility	Current infertility	Method	Quality
(29)	Vizheh et al., 2015	2014	Tehran		123		infertile couples	High quality
(30)	Esfahani Shahsavari et al., 2010	2008-9	Jahrom		169		infertile couples	Moderate quality
(31)	Aflatoonian et al., 2009	2004-5	Yazd	5200	333		Couples	High quality
(32)	Rostami Dovom et al., 2014	2008-10	Iran	888	256	57	non-menopausal women age 18-49	High quality
(33)	Masoumi et al., 2015	2010-2011	Hamadan		1200		infertile couples	High quality
(35)	Akhondi et al., 2013	2010-2011	Iran	13750	2819		20- to 40-year-old women	High quality
(36)	Sepidarkish et al., 2016	2014-15	Iran-Royan Institute		405		infertile patients	High quality
(37)	Taghavi et al., 2011	2005-2010	Mashhad		2000		Infertile males	Moderate quality
(38)	Farmani et al., 2016	2016	Qom		100		Infertile males	Moderate quality
(39)	Hossein Rashidi B, 1998	1998	Tehran		1293		infertile couples	Moderate quality
(40)	Yousefi Z, 2001	1999-2000	Mashhad		1846		Infertile couples	Moderate quality
(41)	Natami M, 2016	2016	Bandar Abbas		151		Infertile males	Moderate quality
(42)	Mirzaei et al., 2018	2014-2015	Yazd	2611	135		20-69 years old people	High quality
(43)	Ershadi, 2006	2006	Gonabad	212	25	88	15- to 49-year-old women	Moderate quality

Prevalence of primary and secondary infertility

The primary infertility prevalence (in 45 studies with 51,021 samples) and secondary infertility (in 13 studies with 35,683 samples) in Iran was 18.3% (95% CI: 15.4-21.6) and 2.5% (95% CI: 1.6-4.0), respectively (Fig.2).

A

Study name	Event rate	Lower limit	Upper limit	Z-Value	p-Value	Relative weight
Ahmadi Asr Badr Y, 2006	0.033	0.027	0.039	-33.982	0.000	7.14
Mohammad baygi R, 2002	0.184	0.160	0.211	-17.332	0.000	7.19
Moghaddam A, 2000	0.132	0.120	0.144	-34.657	0.000	7.29
Sadegh Moghadam L, 2008	0.118	0.090	0.155	-12.644	0.000	6.82
Ardalan A, 2010	0.148	0.141	0.154	-64.584	0.000	7.34
Siahkal MS, 2003	0.087	0.075	0.100	-29.533	0.000	7.22
Parsanezhad ME, 1998	0.111	0.096	0.129	-24.711	0.000	7.20
Nojomi M, 2002	0.120	0.103	0.140	-22.182	0.000	7.18
Delpisheh A, 2014	0.115	0.097	0.137	-20.710	0.000	7.14
Aflatoonian A, 2009	0.064	0.058	0.071	-47.350	0.000	7.29
Rostami Dovom M. 2014	0.288	0.259	0.319	-12.198	0.000	7.23
Akhondi MM, 2013	0.205	0.198	0.212	-64.156	0.000	7.35
Mirzaei M, 2018	0.052	0.044	0.061	-32.916	0.000	7.18
Ershadi F, 2006	0.118	0.081	0.169	-9.449	0.000	6.44
	0.113	0.086	0.147	-13.483	0.000	

Statistics for each study | Event rate and 95% CI

0.00 0.50 1.00

Heterogeneity: I^2= 98.99%, P< 0.001

B

Study name	Event rate	Lower limit	Upper limit	Z-Value	p-Value	Relative weight
Ahmadi Asr Badr Y, 2006	0.034	0.027	0.041	-30.992	0.000	4.24
Vahidi S, 2006 (East Azarbaijan)	0.023	0.014	0.038	-13.874	0.000	3.01
Vahidi S, 2006 (West Azarbaijan)	0.010	0.004	0.024	-10.233	0.000	1.87
Vahidi S, 2006 (Ardabil)	0.017	0.006	0.044	-8.052	0.000	1.62
Vahidi S, 2006 (Isfahan)	0.027	0.017	0.042	-15.057	0.000	3.26
Vahidi S, 2006 (Ilam)	0.070	0.035	0.134	-7.048	0.000	2.34
Vahidi S, 2006 (Bushehr)	0.054	0.027	0.104	-7.895	0.000	2.36
Vahidi S, 2006 (Tehran)	0.034	0.026	0.043	-25.166	0.000	4.08
Vahidi S, 2006 (Chaharmahal and Bakhtiari)	0.034	0.014	0.079	-7.354	0.000	1.85
Vahidi S, 2006 (Khorasan)	0.038	0.028	0.050	-21.339	0.000	3.94
Vahidi S, 2006 (Khuzestan)	0.040	0.028	0.057	-16.983	0.000	3.68
Vahidi S, 2006 (Zanjan)	0.049	0.026	0.088	-9.161	0.000	2.62
Vahidi S, 2006 (Semnan)	0.035	0.011	0.103	-5.650	0.000	1.32
Vahidi S, 2006 (Sistan-Baluchestan)	0.061	0.039	0.092	-11.880	0.000	3.32
Vahidi S, 2006 (Fars)	0.035	0.024	0.051	-16.607	0.000	3.57
Vahidi S, 2006 (Qazvin)	0.052	0.031	0.084	-10.970	0.000	3.05
Vahidi S, 2006 (Qom)	0.059	0.030	0.113	-7.608	0.000	2.35
Vahidi S, 2006 (Kurdistan)	0.031	0.016	0.062	-9.536	0.000	2.38
Vahidi S, 2006 (Kerman)	0.022	0.011	0.043	-10.673	0.000	2.39
Vahidi S, 2006 (Kermanshah)	0.018	0.009	0.038	-10.450	0.000	2.24
Vahidi S, 2006 (Kohkiluyeh and Boyer Ahmad)	0.022	0.006	0.085	-5.292	0.000	0.98
Vahidi S, 2006 (Golestan)	0.031	0.015	0.060	-9.618	0.000	2.38
Vahidi S, 2006 (Guilan)	0.044	0.029	0.069	-13.067	0.000	3.29
Vahidi S, 2006 (Lorestan)	0.022	0.011	0.046	-9.918	0.000	2.24
Vahidi S, 2006 (Mazandaran)	0.024	0.014	0.041	-13.222	0.000	2.93
Vahidi S, 2006 (Markazi)	0.032	0.015	0.066	-8.878	0.000	2.23
Vahidi S, 2006 (Hormozgan)	0.096	0.063	0.144	-9.527	0.000	3.29
Vahidi S, 2006 (Hamedan)	0.024	0.011	0.049	-9.688	0.000	2.24
Vahidi S, 2006 (Yazd)	0.014	0.003	0.052	-6.026	0.000	0.99
Siahkal MS, 2003	0.025	0.019	0.033	-25.531	0.000	4.01
Nojomi M, 2002	0.042	0.032	0.055	-21.473	0.000	3.99
Delpisheh A, 2014	0.043	0.032	0.058	-20.060	0.000	3.93
Noorbala AA, 2001	0.028	0.025	0.031	-59.741	0.000	4.49
Rostami Dovom M. 2014	0.064	0.050	0.082	-19.570	0.000	4.05
Ershadi F, 2006	0.118	0.081	0.169	-9.449	0.000	3.47
	0.037	0.032	0.043	-40.851	0.000	

Heterogeneity: I^2= 77.40%, P< 0.001

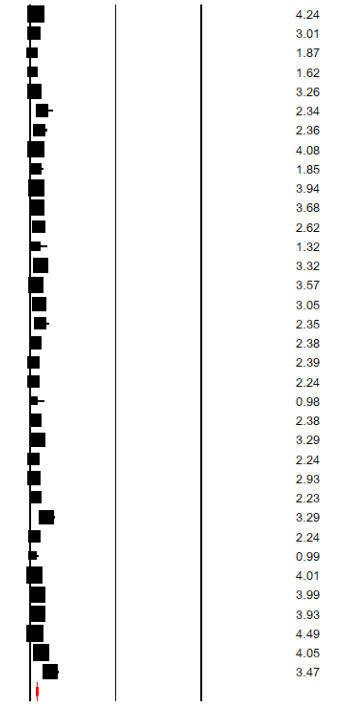


Fig.1: Prevalence of infertility. **A.** Lifetime infertility and **B.** Current infertility. Red rhombus; Overall estimate.

Subgroup analysis of primary and secondary infertility

Subgroup analysis of primary infertility prevalence was significant based on year (P<0.001) and studies quality (P=0.069) but not significant based on region (P=0.430) (Fig.S4, See Supplementary Online Information at www.ijfs.ir). Subgroup analysis of secondary infertility prevalence according to region (P=0.321), and studies quality (P=0.593) was not significant, but was significant based on year (P<0.001, Fig.S5, See Supplementary Online Information at www.ijfs.ir).

The etiology of infertility

The prevalence of female, male, both and unexplained causes was estimated to be 32.0% (95% CI: 27.6-36.8), 43.3% (95% CI: 38.2-48.6), 12.5% (95% CI: 9.6-16.2) and 13.6% (95% CI: 10.2-17.8), respectively (Fig.3).

Female etiology

The prevalence of causes related to ovulation, uterine tubes, and endometriosis in infertile women was estimated to be 54.0% (95% CI: 45.6-62.2), 15.5% (95% CI: 11.3-21.0), 6.2% (95% CI: 3.5-10.6), and 5.4% (95% CI: 2.5-11.3), respectively (Fig.4).

Semen analysis

Abnormal semen analysis was estimated to be 55.6% (95% CI: 45.7-65.2) among infertile men (Fig.S6, See Supplementary Online Information at www.ijfs.ir).

Meta-regression

Meta-regression based on year was not significant in terms of lifetime infertility prevalence (coefficient: - 0.000, 95% CI: -0.057 to 0.055, P=0.976) and current infertility (coefficient: 0.057, 95% CI: -0.005 to 0.119, P=0.073), primary infertility (coefficient: -0.021, 95% CI: -0.084 to 0.040, P=0.496), and secondary infertility (coefficient: -0.017, 95% CI: -0.106 to 0.071, P=0.700, Fig.5).

Sensitivity analysis

Sensitivity analysis with individual study elimination for all meta-analyzes showed a strong overall result (Fig.S7-S9, See Supplementary Online Information at www.ijfs.ir).

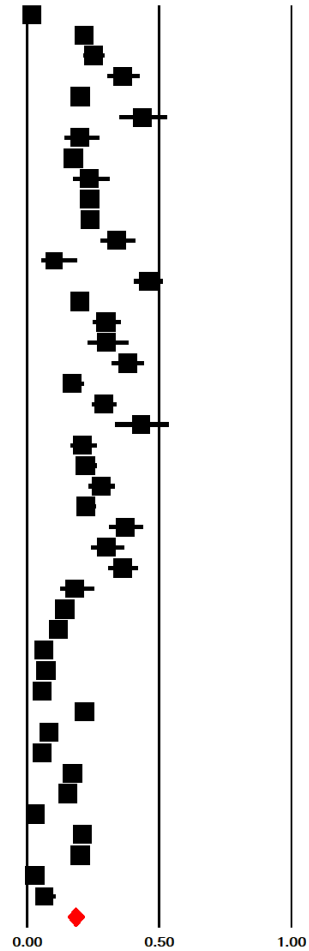
Publication bias

Publication bias tests were not significant for studies about the prevalence of lifetime, current, primary, and secondary infertility (Fig.S10, See Supplementary Online Information at www.ijfs.ir).

A

Study name	Event rate	Lower limit	Upper limit	Z-Value	p-Value	Relative weight
Ahmadi Asr Badr Y, 2006	0.020	0.016	0.026	-30.885	0.000	2.29
Vahidi S, 2006 (East Azarbaijan)	0.218	0.187	0.253	-13.025	0.000	2.32
Vahidi S, 2006 (West Azarbaijan)	0.253	0.217	0.293	-10.546	0.000	2.31
Vahidi S, 2006 (Ardabil)	0.364	0.305	0.428	-4.113	0.000	2.28
Vahidi S, 2006 (Isfahan)	0.203	0.174	0.235	-14.294	0.000	2.32
Vahidi S, 2006 (Ilam)	0.439	0.351	0.531	-1.308	0.191	2.20
Vahidi S, 2006 (Bushehr)	0.201	0.145	0.273	-6.745	0.000	2.17
Vahidi S, 2006 (Tehran)	0.177	0.160	0.196	-24.371	0.000	2.35
Vahidi S, 2006 (Chaharmahal and Bakhtiari)	0.238	0.176	0.314	-6.006	0.000	2.19
Vahidi S, 2006 (Khorasan)	0.239	0.216	0.264	-17.093	0.000	2.34
Vahidi S, 2006 (Khuzestan)	0.240	0.211	0.272	-13.391	0.000	2.33
Vahidi S, 2006 (Zanjan)	0.341	0.280	0.409	-4.459	0.000	2.26
Vahidi S, 2006 (Semnan)	0.105	0.055	0.189	-6.093	0.000	1.88
Vahidi S, 2006 (Sistan-Baluchestan)	0.461	0.407	0.515	-1.430	0.153	2.31
Vahidi S, 2006 (Fars)	0.201	0.174	0.231	-15.073	0.000	2.32
Vahidi S, 2006 (Qazvin)	0.300	0.250	0.355	-6.612	0.000	2.29
Vahidi S, 2006 (Qom)	0.301	0.230	0.384	-4.497	0.000	2.20
Vahidi S, 2006 (Kurdistan)	0.382	0.324	0.443	-3.729	0.000	2.29
Vahidi S, 2006 (Kerman)	0.173	0.137	0.214	-11.411	0.000	2.28
Vahidi S, 2006 (Kermanshah)	0.292	0.248	0.339	-7.903	0.000	2.30
Vahidi S, 2006 (Kohkiluyeh and Boyer Ahmad)	0.433	0.335	0.537	-1.261	0.207	2.16
Vahidi S, 2006 (Golestan)	0.211	0.165	0.264	-8.701	0.000	2.26
Vahidi S, 2006 (Guilan)	0.222	0.186	0.264	-10.754	0.000	2.30
Vahidi S, 2006 (Lorestan)	0.281	0.234	0.333	-7.526	0.000	2.29
Vahidi S, 2006 (Mazandaran)	0.224	0.191	0.261	-12.099	0.000	2.31
Vahidi S, 2006 (Markazi)	0.374	0.313	0.440	-3.676	0.000	2.27
Vahidi S, 2006 (Hormozgan)	0.303	0.244	0.369	-5.524	0.000	2.26
Vahidi S, 2006 (Hamedan)	0.363	0.310	0.420	-4.621	0.000	2.29
Vahidi S, 2006 (Yazd)	0.182	0.128	0.253	-7.047	0.000	2.16
Mohammad baygi R, 2002	0.144	0.123	0.169	-18.791	0.000	2.32
Moghaddam A, 2000	0.120	0.109	0.132	-35.189	0.000	2.35
Sadegh Moghadam L, 2008	0.066	0.045	0.096	-12.822	0.000	2.17
Siahkal MS, 2003	0.073	0.062	0.085	-29.470	0.000	2.33
Parsanezhad ME, 1998	0.058	0.047	0.071	-24.641	0.000	2.30
Barouti E, 1999	0.219	0.201	0.239	-22.200	0.000	2.35
Nojomi M, 2002	0.083	0.069	0.101	-22.708	0.000	2.31
Delpisheh A, 2014	0.059	0.046	0.076	-20.776	0.000	2.28
Kazemljaliseh H, 2015	0.173	0.152	0.197	-19.314	0.000	2.33
Shafi H, 2012	0.155	0.135	0.178	-20.164	0.000	2.33
Aflatoonian A, 2009	0.033	0.028	0.038	-43.438	0.000	2.34
Rostami Dovom M. 2014	0.211	0.185	0.239	-16.055	0.000	2.33
Akhondi MM, 2013	0.202	0.196	0.209	-64.610	0.000	2.36
Mirzaei M, 2018	0.031	0.025	0.038	-30.490	0.000	2.30
Ershadi F, 2006	0.066	0.039	0.108	-9.580	0.000	2.04
	0.183	0.154	0.216	-14.088	0.000	

Heterogeneity: I^2= 98.31%, P< 0.001



B

Study name	Event rate	Lower limit	Upper limit	Z-Value	p-Value	Relative weight
Ahmadi Asr Badr Y, 2006	0.012	0.009	0.017	-27.245	0.000	7.75
Mohammad baygi R, 2002	0.040	0.029	0.055	-18.698	0.000	7.72
Moghaddam A, 2000	0.012	0.009	0.016	-26.013	0.000	7.72
Sadegh Moghadam L, 2008	0.053	0.034	0.080	-12.581	0.000	7.49
Siahkal MS, 2003	0.014	0.010	0.020	-22.319	0.000	7.65
Parsanezhad ME, 1998	0.053	0.043	0.066	-24.432	0.000	7.87
Nojomi M, 2002	0.037	0.027	0.049	-21.044	0.000	7.77
Delpisheh A, 2014	0.037	0.027	0.050	-19.539	0.000	7.73
Aflatoonian A, 2009	0.021	0.017	0.025	-39.544	0.000	7.92
Rostami Dovom M. 2014	0.078	0.062	0.097	-19.736	0.000	7.85
Akhondi MM, 2013	0.003	0.002	0.004	-35.609	0.000	7.73
Mirzaei M, 2018	0.029	0.023	0.036	-30.050	0.000	7.87
Ershadi F, 2006	0.042	0.022	0.080	-9.147	0.000	6.94
	0.025	0.016	0.040	-14.935	0.000	

Heterogeneity: I^2= 97.05%, P< 0.001

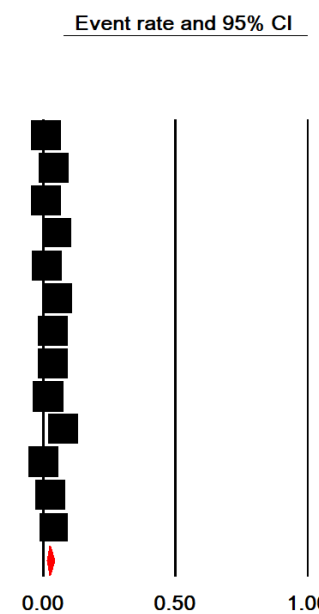
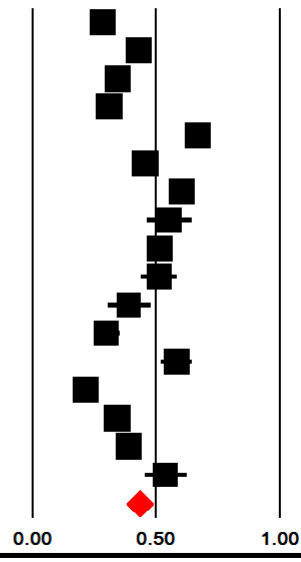


Fig.2: Prevalence of infertility. **A.** Primary infertility and **B.** Secondary infertility. Red rhombus; Overall estimate.

A

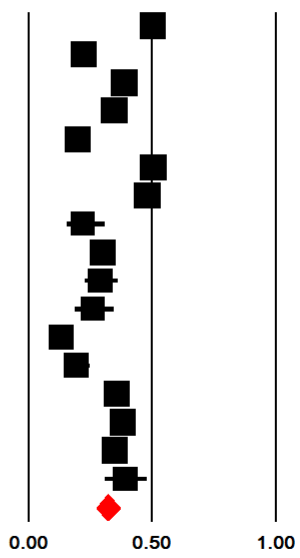
Study name	Event rate	Lower limit	Upper limit	Z-Value	p-Value	Relative weight
Kamali M, 2007	0.286	0.269	0.304	-20.628	0.000	6.16
Bakhtiari A, 1999	0.433	0.401	0.465	-4.076	0.000	6.09
Karimpour Malekshah AK, 2011	0.347	0.332	0.362	-18.412	0.000	6.19
Esmailzadeh S, 1999	0.312	0.293	0.331	-17.092	0.000	6.16
Parsanezhad ME, 1998	0.670	0.634	0.704	8.744	0.000	6.02
Moini A, 1999	0.459	0.444	0.474	-5.416	0.000	6.20
Karimpour A, 2005	0.604	0.566	0.641	5.305	0.000	6.03
Delpisheh A, 2014	0.556	0.465	0.643	1.199	0.230	5.27
Shagheibi Sh, 2017	0.518	0.477	0.559	0.873	0.383	6.01
Kazemijaliseh H, 2015	0.514	0.442	0.585	0.368	0.713	5.60
Vizheh M, 2015	0.390	0.308	0.479	-2.414	0.016	5.28
Aflatoonian A, 2009	0.300	0.253	0.352	-7.076	0.000	5.80
Rostami Dovom M. 2014	0.586	0.525	0.645	2.736	0.006	5.74
Sepidarkish M, 2016	0.217	0.180	0.260	-10.636	0.000	5.79
Hosseinrashidi B, 1998	0.345	0.320	0.371	-10.963	0.000	6.12
Yousefi Z, 2001	0.390	0.368	0.412	-9.371	0.000	6.15
Mirzaei M, 2018	0.541	0.456	0.623	0.946	0.344	5.39
	0.433	0.382	0.486	-2.460	0.014	



Heterogeneity: I^2= 97.85%, P< 0.001

B

Study name	Event rate	Lower limit	Upper limit	Z-Value	p-Value	Relative weight
Kamali M, 2007	0.505	0.485	0.524	0.481	0.631	6.26
Bakhtiari A, 1999	0.226	0.200	0.254	-15.613	0.000	6.11
Karimpour Malekshah AK, 2011	0.389	0.374	0.405	-13.436	0.000	6.27
Esmailzadeh S, 1999	0.348	0.328	0.368	-13.920	0.000	6.24
Parsanezhad ME, 1998	0.202	0.174	0.234	-14.520	0.000	6.03
Moini A, 1999	0.506	0.491	0.521	0.787	0.431	6.28
Karimpour A, 2005	0.482	0.444	0.521	-0.897	0.370	6.11
Delpisheh A, 2014	0.222	0.156	0.306	-5.634	0.000	5.00
Shagheibi Sh, 2017	0.302	0.266	0.341	-9.245	0.000	6.05
Kazemijaliseh H, 2015	0.292	0.231	0.361	-5.480	0.000	5.54
Vizheh M, 2015	0.260	0.190	0.345	-5.085	0.000	5.16
Aflatoonian A, 2009	0.132	0.100	0.173	-11.631	0.000	5.54
Rostami Dovom M. 2014	0.195	0.151	0.248	-8.981	0.000	5.58
Sepidarkish M, 2016	0.360	0.315	0.408	-5.539	0.000	5.97
Hosseinrashidi B, 1998	0.382	0.356	0.409	-8.401	0.000	6.20
Yousefi Z, 2001	0.350	0.329	0.372	-12.690	0.000	6.23
Mirzaei M, 2018	0.393	0.314	0.477	-2.476	0.013	5.42
	0.320	0.276	0.368	-6.902	0.000	

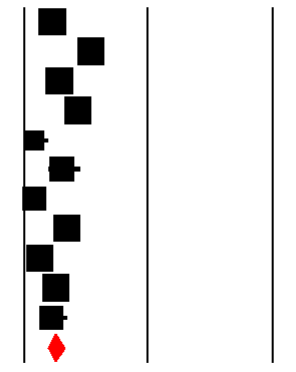


Heterogeneity: I^2= 97.93%, P< 0.001

Meta Analysis

C

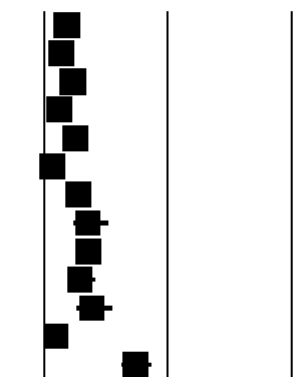
Study name	Event rate	Lower limit	Upper limit	Z-Value	p-Value	Relative weight
Kamali M, 2007	0.116	0.104	0.129	-32.466	0.000	10.26
Bakhtiari A, 1999	0.272	0.244	0.301	-13.302	0.000	10.19
Karimpour Malekshah AK, 2011	0.146	0.135	0.158	-38.115	0.000	10.34
Esmailzadeh S, 1999	0.219	0.202	0.237	-24.491	0.000	10.31
Delpisheh A, 2014	0.043	0.018	0.099	-6.802	0.000	5.37
Vizheh M, 2015	0.154	0.101	0.230	-6.814	0.000	8.15
Aflatoonian A, 2009	0.042	0.025	0.070	-11.448	0.000	7.81
Sepidarkish M, 2016	0.175	0.141	0.215	-11.849	0.000	9.69
Hosseinrashidi B, 1998	0.065	0.053	0.080	-23.634	0.000	9.87
Yousefi Z, 2001	0.130	0.115	0.146	-27.467	0.000	10.22
Mirzaei M, 2018	0.111	0.068	0.176	-7.593	0.000	7.80
	0.125	0.096	0.162	-12.767	0.000	



Heterogeneity: I^2= 96.79%, P< 0.001

D

Study name	Event rate	Lower limit	Upper limit	Z-Value	p-Value	Relative weight
Kamali M, 2007	0.093	0.082	0.105	-33.019	0.000	6.10
Bakhtiari A, 1999	0.070	0.055	0.088	-20.012	0.000	5.93
Karimpour Malekshah AK, 2011	0.118	0.108	0.129	-39.649	0.000	6.13
Esmailzadeh S, 1999	0.063	0.053	0.074	-30.536	0.000	6.05
Parsanezhad ME, 1998	0.128	0.106	0.155	-16.866	0.000	5.99
Moini A, 1999	0.035	0.030	0.041	-40.267	0.000	6.07
Karimpour A, 2005	0.142	0.117	0.170	-16.105	0.000	5.99
Delpisheh A, 2014	0.179	0.120	0.260	-6.309	0.000	5.44
Shagheibi Sh, 2017	0.180	0.150	0.213	-14.030	0.000	6.00
Kazemijaliseh H, 2015	0.146	0.102	0.204	-8.484	0.000	5.61
Vizheh M, 2015	0.195	0.134	0.275	-6.228	0.000	5.51
Aflatoonian A, 2009	0.048	0.030	0.077	-11.655	0.000	5.36
Rostami Dovom M. 2014	0.371	0.314	0.432	-4.078	0.000	5.93
Sepidarkish M, 2016	0.247	0.207	0.291	-9.677	0.000	5.98
Hosseinrashidi B, 1998	0.208	0.187	0.231	-19.511	0.000	6.10
Yousefi Z, 2001	0.130	0.115	0.146	-27.467	0.000	6.10
Mirzaei M, 2018	0.326	0.252	0.409	-3.957	0.000	5.72
	0.136	0.102	0.178	-11.264	0.000	



Heterogeneity: I^2= 97.82%, P< 0.001

Fig.3: The prevalence of infertility. **A.** Female, **B.** Male, **C.** Both, and **D.** Unexplained causes. Red rhombus; Overall estimate.

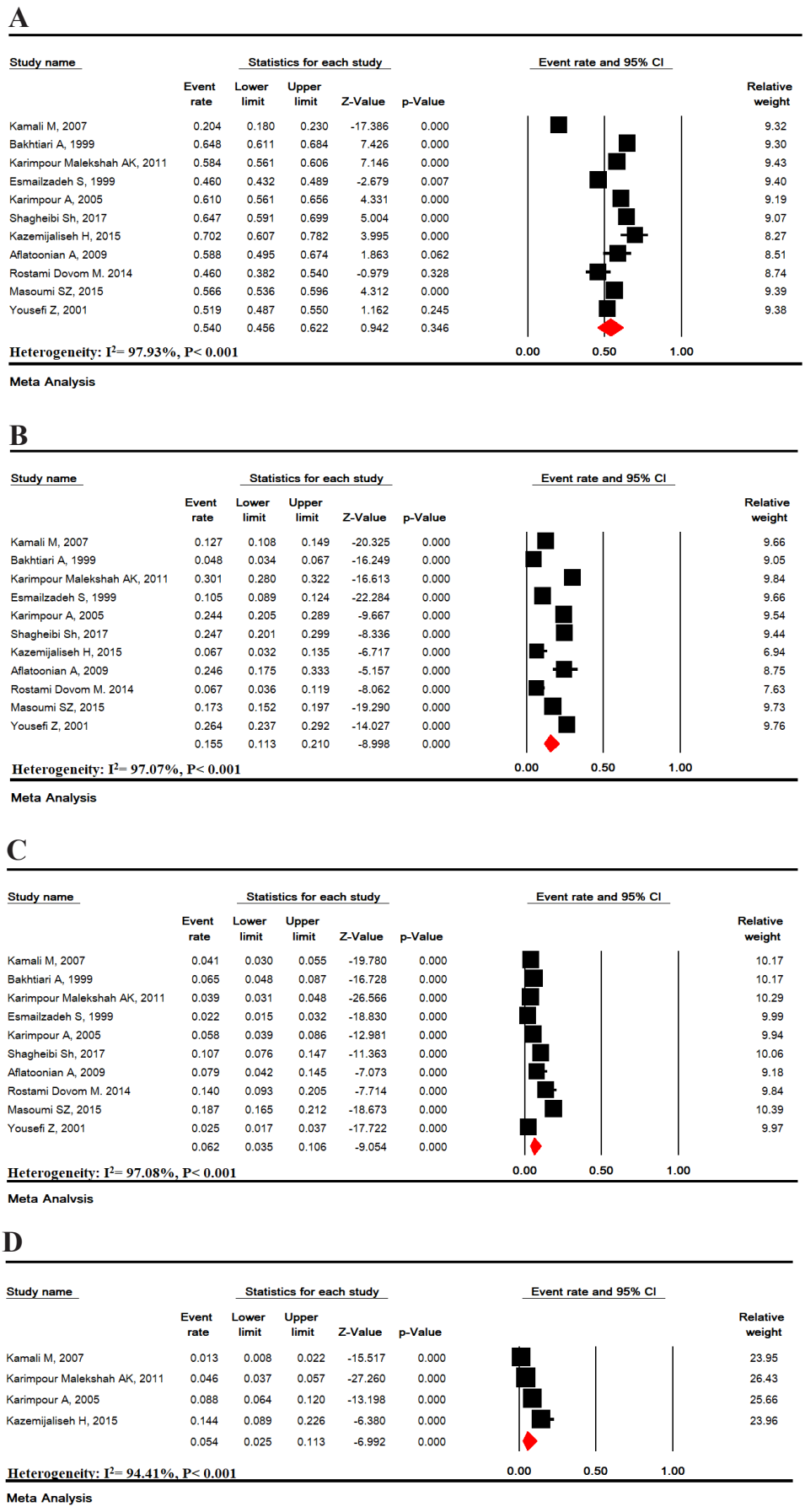


Fig.4: The prevalence of causes related to infertility. **A.** Ovulation, **B.** Uterine tubes, and **C.** Endometriosis in infertile women. Red rhombus; Overall estimate.

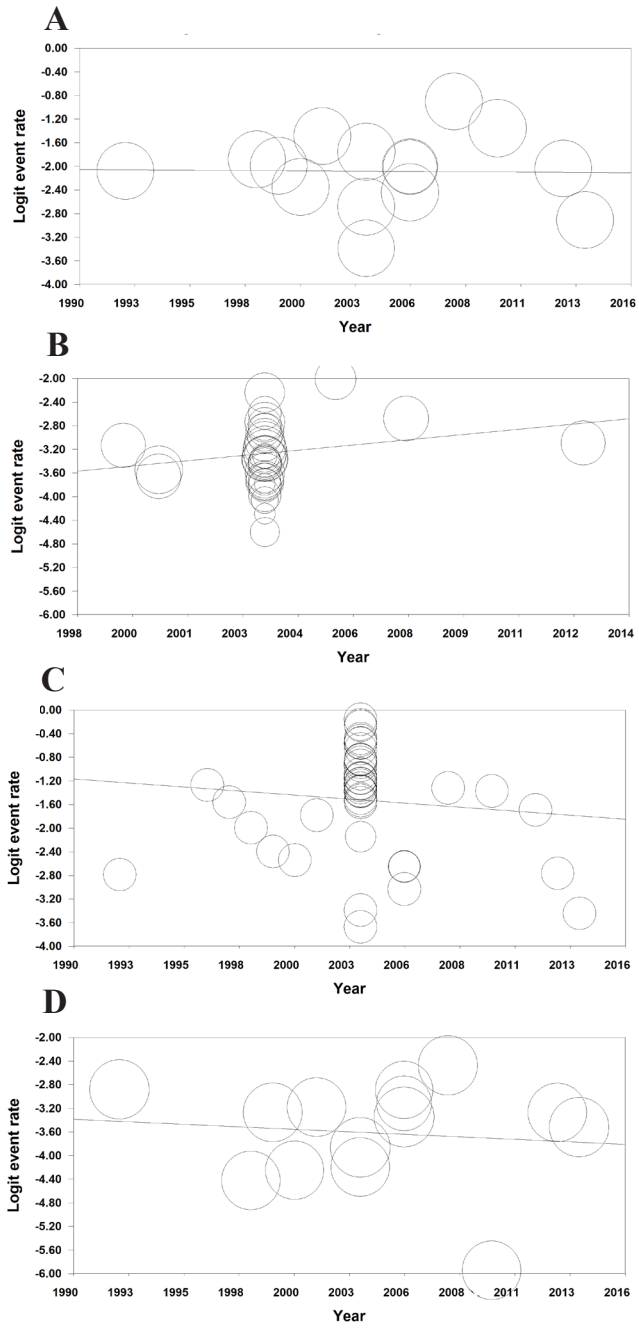


Fig.5: Meta-regression based on year for the prevalence of infertility. **A.** Lifetime, **B.** Current infertility, **C.** Primary infertility, and **D.** Secondary infertility.

Discussion

Infertility is a common situation with complex socio-economic and health outcomes for the individuals and the whole society. In spite of the important outcomes of infertility, estimating its prevalence is faced with limitations. In this systematic meta-analysis, the lifetime infertility prevalence was estimated at 11.1%, while 3.7% had current infertility. The estimated incidence of infertility is between 3.5 and 30% in various countries, and this variety is probably due to the population under study, the definitions of infertility and the estimation method (4). Therefore, the major challenges in evaluating the true infertility burden include the lack of population-based researches and the variety of definitions. In addition, advancements in diagnosing, treating and preventing infertility in recent decades have caused great changes in infertility prevalence worldwide. In least developed countries, the prevalence of 12-month infertility varies from 6.9 to 9.3%. Significant geographic diversities have been noted in its prevalence, and the diversities are mostly explained by the difference in cultural, environmental, and socioeconomic effects and having evaluate to the health-care system (54). The prevalence varies widely from 9% in Gambia to 11.8% in Ghana compared to 21.2% in northwest Ethiopia and between 20 and 30% in Nigeria (3, 8, 55-57). We do not have much information about Asian countries and Latin America. Based on the statistics provided by WHO, infertility prevalence in these areas varies from 8 to 12% in women of childbearing ages (2). Universally, the age-standardized female infertility prevalence has elevated by 14.96% (from 1366.85 per 100,000 in 1990 to 1571.35 per 100,000 in 2017) (58). Based on a previous meta-analysis in 2013, lifetime infertility and current infertility prevalence in Iran was reported to be 10.9 and 3.3%, respectively (59), and our estimate shows a slight increase compared to the previous estimate.

Searching for assistive behaviors related to infertility services is common in Iranians. In one study, more than 75% of people with fertility problems sought medical help. An international survey reported that 56% of women in more advanced countries seek help from infertility treatment systems (54), and a Trävä study (60) found that 57% of all infertile women did so. The popularity of seeking help for infertility treatment in Iran may be owing to the present perception that in Iran, having a child is the only way to improve one's position in the family and society (61). There have been several explanations for not seeking (or following up) infertility assistance, including lack of understanding or acknowledgment of the problem (62), fear of being labeled infertile, worrying about the cost of treatment , having no intention to provoke , and the physical and psychological burden of treatment.

Decrease in fertility rates does not imply that infertility has elevated. Despite the reports of increased infertility rates in some parts of the world, the evidence suggests the total prevalence of infertility has not changed significantly over the past thirty years. In this study, no significant change was found in the prevalence of infertility in Iran based on the meta-regression models over time (between 1990-2016). The important point is the noteworthy reduction in total fertility rate (TFR) in Iran; TFR in Iran was 7 children per woman in 1960, 6.5 in 1980, 2.06 in 2000, 1.83 in 2010, and 2.11 in 2017 (1). TFR is decreasing in the world. Europe has the lowest level of fertility rate among all regions of the world. Partnership instability, better cooperation of women in education and occupations, postponement of parenthood, changes in values and economic burdens impose significant effects on fertility rates (63). On the other hand, infertility

is more commonly experienced by married people, reflecting the fact that people in stable relationships are more likely to become pregnant and are hence aware of fertility problems (62). Much lower prevalence among young people somewhat indicates that they may never attempt to become pregnant. Previous studies have shown prolonged transition to adulthood and increase in the interval between important reproductive events such as first sexual relationship, marriage, and birth of first child (64). On the other hand, much of the literature describes the tendency of women in developed countries to delay having children (65) and it is suggested that this tendency is rapidly becoming a global phenomenon (66).

In the previous meta-analysis, the primary infertility prevalence was 10.6% and secondary infertility prevalence was 2.7% (59), which was higher in our study. A global study in 2010 among one hundred and ninety countries, among pregnant women aged 20-44, primary infertility was 1.9% and secondary one was 10.5%. Some regions have a high incidence of primary infertility, but secondary infertility is low, like North Africa and the Middle East, especially Adetoro and Ebomoyi (3). However, some areas have a high incidence of secondary infertility, but the incidence of primary infertility is low, such as in Central and Eastern Europe and Central Asia. In addition, several previous studies provided information on the prevalence of gender-related infertility. For instance, the prevalence of infertility in England was 12.5% in women, but it was 10.1% in men (62). It is worth noting that in these published studies, some only examined women (42). Others only evaluated the records of men in infertility clinics (67). Likewise, these articles are performed on fairly small groups which do not represent the majority of the infertile population (36).

Results of the causes of infertility in Iran showed that the causes of infertility were as follows: 32.0% male cause, 43.3% female cause, 12.5% both genders and 13.6% unknown. This calculated percentage is consistent with reported estimates of major causes of infertility in other studies (6).

Research shows that different studies provide different definitions of infertility, which may lead to misunderstanding of the actual situation (68). Some sources define infertility as lack of pregnancy after two years of unprotected intercourse , while others consider the couple infertility if pregnancy does not occur after unprotected intercourse for 12 months (69). We only provided studies with a 12-month definition. Therefore, we even excluded good-quality studies with more than 12 months of measurement (such as the study of Safarinejad) (70).

There was a high heterogeneity among articles across all of meta-analyses, and by considering the present data, we were able to ascribe this difference to the geographical area and year based on subgroup analysis. Other differences, including the partnership instability, increased cooperation of women in better education and occupations, postponement of parenthood, changes in values and economic burdens could not be investigated by using available data.

The strengths of the present study included the use of a comprehensive search strategy, selection of studies, extracting data and even analyzing the data by two independent researchers based on MOOSE guidelines, while the diversities were sorted out via group discussion. In case of any doubt of duplicate publications or more information that was required, we contacted the first or corresponding authors. We performed a conservative estimate using the random effects model, adopted a subgroup analysis and a meta-regression model to discover the heterogeneity causes. In the present study, in addition to updating previous meta-analyses to estimate the prevalence of lifetime and current infertility with a much higher sample size, we meta-analyzed the etiological details of infertility for the first time. The weaknesses of our study included the restricted search in internal databases and exclusion of studies with different infertility definitions.

Conclusion

In summary, the estimate of infertility burden in Iran did not change between 1990 and 2017 and its prevalence remains high. This study provides a comprehensive and up-to-date understanding of the that we need prevention and management interventions to alleviate infertility in Iran. Further research is required to evaluate the risk factors of infertility for developing effective prevention and management strategies to decrease the burden of this issue.

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Authors' Contributions

M.A., T.R., Gh.A., M.P.N.; Contributed to conception and design. M.A., Gh.A., M.P.N.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. M.A., M.P.N.; Were responsible for overall supervision. M.A.; Drafted the manuscript, which was revised by M.A., T.R., Gh.A., M.P.N. All authors read and approved the final manuscript.

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Role of Marital Relationship Quality in Emotional Disturbance and Personal Growth of Women with Infertility: A Cross-Sectional Study

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Abstract

Background: Infertility is a stressful condition that can lead to either emotional disturbance or personal growth. Marital relationship is one of the factors affecting the consequences of infertility. This study aimed to explore the role of marital relationship quality in development of women's personal growth after experiencing infertility.

Materials and Methods: In the cross-sectional study, 122 infertile women (mean age 28.79 ± 6.3) were invited to complete the survey, including ENRICH Marital Satisfaction Scale, Posttraumatic Growth, Fertility problem inventory, State-Trait Anxiety Inventory, and Beck Inventory Depression.

Results: Higher scores of quality of marital relationships were a protective factor against infertility stress and state/trait anxiety. Additionally, infertility stress was a strong negative predictor of personal growth. Furthermore, infertile women with a high level of marital relationships may have more chances to experience personal growth rather than stress in infertility treatments.

Conclusion: The study suggests that high quality of marital relationships may provide positive opportunities for women's personal growth after experiencing infertility.

Keywords: Infertility, Marital Relationships, Posttraumatic Growth, Stress, Women

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Introduction

Diagnosis of infertility is one of the greatest stressors for couples that not only causes psychological stress in infertile individuals but also leads them to achieve personal growth that is considered posttraumatic growth (PTG) (1). PTG is the positive legacy of experiencing a traumatic event (2). Considerable evidence confirms that the struggle with infertility may offer an opportunity for positive changes (3). Various factors are attributed to the development of PTG. Some studies have found the negative impact of anxiety and depression on PTG (4). However, a meta-analysis of PTG in cancer survivors found no significant association between PTG and anxiety/depression (5).

Marital relationships is one of the important factors influencing the mental health of couples dealing with infertility (6). It is estimated that 1 out of 8 couples lives with infertility worldwide (7). The evidence indicates that infertile couples having poor relationships are at higher risk of experiencing mental illness, such as depression and anxiety (8). The quality of marital relationships in infertile couples is controversial (1, 9). It appears that the relationship between infertility and marital relationships is reciprocal. Some research reported poor marital relationships in infertile couples due to the negative effects of infertility (1). However, positive effects of infertility on marital relationships have also been reported (10).

The quality of marital relationships is an important factor that may influence infertility growth and stress in people struggling to conceive (11). The quality of marital relationships is positively associated with psychological well-being and success in life. In addition, it is negatively

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related to mental illnesses like depression (12). Some evidence demonstrates that poor quality of marital relationships enhances infertility stress in infertile women (13). Furthermore, studies have documented that the high quality of marital relationships positively impacts PTG (14).

Although the role of marital relationships has been investigated in numerous studies, limited information has been published regarding the relations between the quality of marital relationships and PTG (14). Moreover, there is no evidence of an association between the quality of marital relationships and personal growth in infertility. To the best of our knowledge, this is the first study investigating the association of infertility stress with personal growth and marital relationships in women struggling with infertility. Considering the importance of PTG in contributing to individual progress and achieving positive new changes after facing stressful events, it is necessary to identify the factors related to this growth. Since PTG can result in positive changes, facilitating psychological growth and better adaptation to challenging conditions, this could benefit infertile women. Infertile women may experience emotional disturbances including anxiety, depression, and infertility stress. Therefore, identifying the factors contributing to PTG and emotional disturbance can guide health care practitioners in providing the appropriate conditions for promoting positive features. In the present study, we consider the quality of marital relationships as a factor and examine its impact on PTG and emotional disturbance.

Materials and Methods

Participants and study design

We conducted a cross-sectional study at the Fatemeh Azahra Infertility and Reproductive Health Research Center (Babol, Iran) from September 2018 to January 2020.

Convenience sampling was used to recruit infertile women. Women interested in participating were assessed for eligibility according to the inclusion and exclusion criteria in a clinic.

Inclusion criteria: Women who were at least 18 years old, had at least primary school educational, were not currently pregnant, and had no history of childbirth and adoption. However, in case of low literacy, the questionnaire questions were read by the questioners.

Exclusion criteria: In the study sample, infertility patients with a history of mental illness such as severe depression, psychotic disorders or any addiction, taking medications for psychiatric disorders and patients with a history of incurable disease, as well as women who underwent new stress for less than 6 months were excluded.

Procedure

A study research member performed an interview to obtain their infertility history and to evaluate the eligibility criteria. Furthermore, brief information about the aims of the study was explained to the participants. All of 122 eligible women, who provided written informed consent for study participation, entered the study.

The tools used in this study included the ENRICH marital satisfaction scale (EMSS), PTG, Anxiety Trait Inventory (STAI), Fertility Problems Inventory (FPI), and Beck Depression Inventory (BDI-II).

Instruments

ENRICH marital satisfaction scale

This is a 47-item scale consisting of 11 subscales. Topics that fall under the sub-category are ideal distortions, marital satisfaction, personality issues, marital communication, conflict resolution, financial management, leisure activities, sexual activities, children and parenting, family and friends and religious orientation. Items are scored on a 5-point scale, from 1 (strongly disagree) to 5 (strongly agree). The total possible score between 47 and 235 is calculated by adding up the score for each item. Higher scores reflect higher levels of marital satisfaction. Cronbach's alpha was 0.74 for the Persian version of the Scale (15).

Posttraumatic growth

This scale was created by Tedeschi and Calhoun (1996). The scale includes 21 items to assess positive psychological outcomes for people with a history of trauma (16). It contains 5 sub-fields including new abilities, relationships with others, personal strength, spiritual change, and life appreciation. The total score can be from 0 to 105. Higher scores indicate more positive psychological improvements. We use a PTG score ≥ 63 for personal growth. The computed Cronbach's alpha for the Persian version of PTG was 0.87 (17).

State-trait anxiety inventory

This scale is one of the self-report tools used in research to assess anxiety levels. It contains 20 items for anxiety characteristics and 20 items for anxiety status. Each item is rated on a four-point scale, from 1 (not at all) to 4 (almost always). The total score for each subscale ranged from 20 to 80. We identified anxiety symptoms with an anxiety threshold state ≥ 41 (18). Cronbach's alpha coefficients for the state anxiety and trait anxiety were 0.91 and 0.92, respectively (19).

Fertility problem inventory

This instrument was developed by Newton et al. (20) in 1999 to measure stress and problems related to infertility. This scale includes 5 child scales, including social concerns, sexual concerns, relationship concerns, parental rejection, and parenting needs. The total score is 46 to 276, as each of the 46 items can be scored on a six-point scale, from 1 (strongly disagree) to 6 (strongly agree). Higher scores indicate higher stress levels. The validity and reliability of the Persian version has been studied

(21). Cronbach's alpha for all sub-components was more than 0.70 and the overall integrity was found to be 87%.

Beck inventory depression

It is a self-assessment scale consisting of 21 questions which assesses the presence and severity of depressive symptoms. Items are scored on a 4-point scale from 0 to 3 and total score from 0 to 63. Higher scores indicate more severe depressive symptoms. In this study, depressive symptoms were defined as BDI-II threshold ≥ 14. The Cronbach's alpha value for the BDI-II-Persian was high (0.87) and the test-retest reliability was satisfactory (r=0.74) (22).

The study was approved by Babol University of Medical Sciences's Ethics Committee (IR.MUBABOL.HRI.REC.1398.077).

Statistical analysis

Data analysis was performed using SPSS 22.0 software (IBM, The United States). Descriptive statistics were conducted to describe the socio-demographic of the study sample. The mean comparison of psychological profiles was performed in infertile women using t tests. The associations between psychological factors were evaluated using Pearson correlation analysis.

Four stepwise multivariable logistic regression models were run to evaluate the relationship between variables. Model 1 is applied to investigate the role of six independent variables, namely quality of marital relationships, infertility stress, depression, anxiety, age, and duration of infertility as predictors of PTG (as dependent variables). In model 2, presence of depression symptoms was the dependent variable, and the total score quality of marital relationships, total score of infertility stress, anxiety score, age, and duration of infertility were independent variables. In Model 3, trait anxiety was the dependent variable, and the total score of quality of marital relationships, total score of infertility stress, age, and duration of infertility were independent variables. In Model 4, state anxiety was the dependent variable, and quality of marital relationships, total score of infertility stress, age, and duration of infertility were independent variables. The significance level was also set at $P<0.05$.

Results

Table 1 describes the demographic characteristics of the study population. The participants' mean age was 28.79 ± 6.3 years (range: 18-44 years), and the mean infertility duration was 3.9 ± 3.4 years.

According to the results, the total score of quality of marital relationships in infertile women was at a moderate level (M=180.14 ± 13.5) of the possible range of 47-235. In addition, the mean scores of all of nine subscales indicated that the quality of marital relationships was higher than the median of each subscale. Furthermore, the mean score of the PTG of infertile women was slightly higher than the median total score (M=63.1 ± 14.6 of the possible range of 0-105). Moreover, the total score of infertility stress was M=144.2 ± 27.5 (the possible range: 46-276), demonstrating that most infertile women had moderate infertility stress. The participants' mean scores in trait anxiety (M=41.5 ± 9.3, M=42.7 ± 7.8 of 0-80 range, respectively) and depression symptoms (M=14.58 ± 9.0 of 0-63 range) were higher than normal range.

Table 1: Characteristics of the study population

Variables	n (%) or Mean ± SD
Age (Y)	
18-30	72 (60.5)
≥31	47 (39.5)
Education	
Diploma/Under diploma	71 (59.7)
University	48 (40.3)
Job	
Employed	92 (77.3)
Unemployed	27 (22.7)
History of illness	
Yes	23 (19.2)
No	97 (80.8)
History of psychotic disorder	
Yesn	7 (5.8)
No	113 (94.2)
Smoking	
Yes	3 (2.5)
No	117 (97.5)
Duration of infertility	
≤4	78 (67.2)
≥5	38 (32.8)
Marital satisfaction	
Idealistic distortion	12.8 ± 1.6
Marital satisfaction	32.8 ± 3.2
Personality issues	14.6 ± 2.0
Marital communication	14.6 ± 1.9
Conflict resolution	17.9 ± 1.9
Financial management	11.8 ± 1.5
Leisure activities	14.8 ± 1.4
Sexual relationship	13.3 ± 1.2
Children and parenting	16.2 ± 1.6
Family and friends	15.9 ± 1.7
Religious orientation	14.9 ± 1.6
Total marital satisfaction	180.14 ± 13.5
PTG	
Relating to others	21.1 ± 5.6
New possibilities	14.7 ± 4.2
Personal strength	11.8 ± 3.7
Spiritual change	6.0 ± 2.0
Appreciation of life	9.3 ± 2.6
Total PTG	63.1 ± 14.6
FPI	
Social concern	25.7 ± 7.5
Sexual concern	20.8 ± 6.6
Relationship concern	24.4 ± 7.0
Rejection of childfree lifestyle	32.2 ± 6.9
Need for parenthood	40.9 ± 8.3
Total FPI	144.2 ± 27.5
Anxiety	
State anxiety	41.5 ± 9.3
Trait anxiety	42.7 ± 7.8
BDI	14.58 ± 9.0

Range of scores: Idealistic distortion (3-15), Marital satisfaction (8-40), Personality issues(4-20), Marital communication (4-20), Conflict resolution (5-25), Financial management (3-15), Leisure activities (4-20), Sexual relationship (4-20), Children and parenting (4-200), Family and friends (4-20), Religious orientation (4-20), Total Enrich marital satisfaction (47-235), PTG; Posttraumatic Growth (0-105), BDI; Beck depression inventory (0-63), State anxiety (0-80), Trait anxiety (0-80), and FPI; Fertility problem inventory (46-276).

Table 2 compares the mean scores of psychological variables regarding the participants' demographics. The results of the t test revealed no significant relationship between categories of age, education, and duration of infertility and the total score of PTG. However, some subscales of PTG, including new possibilities (P=0.004), personal strength (P=0.002), and spiritual change (P=0.02), were significantly higher in 31 years and older participants than in participants under the age of 30.

There were no significant relationships between psychological variables and duration of infertility. The total score of quality of marital relationship and all nine subscales were not significantly associated with age, education, and duration of infertility of women.

Additionally, the anxiety score was significantly higher in participants under the age of 30 than in the other age group (P=0.018). Women with primary/ high school education had significantly higher scores of depression than the ones with university education (P=0.006).

Table 3 lists the correlation matrix of psychosocial variables. The total score of the quality of marital relationships was positively associated with the total score of PTG. Furthermore, infertility stress was negatively correlated with the total score of quality of marital relationships (r=-0.454). Also, it has low to moderate negative correlation with most subscales.

Depression had a significant negative correlation with the total score of quality of marital relationships (r=-0.399). Depression has low to moderate negative correlation with subscales except leisure activities, sexual relationship, children and parenting.

State anxiety and trait anxiety had a significant negative correlation with all of the marital satisfaction subscales. However, both of them were not associated with sexual relationship.

Table 2: Comparison of mean ± SD scores of the psychological profile regarding demographic characteristic of infertile women

Variables	Age (Y)		P value	Education		P value	Duration of infertility		P value
	18-30	≥31		Diploma/ under diploma	University		≤4 years	≥5 years	
PTG									
Relating to others	20.9 ± 5.3	21.5 ± 5.1	0.586	20.8 ± 4.9	21.6 ± 5.6	0.398	21.2 ± 5.2	21.2 ± 5.4	0.982
New possibilities	13.8 ± 4.2	16.0 ± 3.7	0.004	14.5 ± 4.2	14.9 ± 4.1	0.592	14.4 ± 4.2	14.9 ± 4.1	0.516
Personal strength	11.0 ± 3.7	13.1 ± 3.2	0.002	11.9 ± 4.0	11.8 ± 3.1	0.930	11.6 ± 3.5	12.3 ± 3.9	0.324
Spiritual change	5.6 ± 2.0	6.5 ± 1.7	0.02	5.9 ± 1.8	6.1 ± 2.1	0.590	6.0 ± 1.9	5.8 ± 1.9	0.626
Appreciation of life	9.2 ± 2.5	9.4 ± 2.7	0.628	9.2 ± 2.6	9.3 ± 2.5	0.902	9.0 ± 2.7	9.8 ± 2.0	0.088
Total PTG	60.7 ± 14.7	66.6 ± 13.4	0.280	62.4 ± 14.0	63.9 ± 15.1	0.592	62.4 ± 14.8	64.2 ± 14.3	0.530
BDI	15.4 ± 9.7	12.6 ± 7.7	0.108	16.3 ± 9.3	11.6 ± 8.0	0.006	14.2 ± 8.8	14.1 ± 9.3	0.971
Anxiety									
State anxiety	42.4 ± 9.6	39.7 ± 8.9	0.124	42.0 ± 9.9	40.5 ± 8.74	0.403	42.0 ± 9.1	40.4 ± 9.6	0.404
Trait anxiety	44.1 ± 7.4	40.6 ± 8.1	0.018	43.3 ± 7.7	1.8 ± 7.9	0.300	42.9 ± 7.7	42.2 ± 8.2	0.639
FPI									
Social concern	26.2 ± 7.4	24.6 ± 7.5	0.257	26.3 ± 7.5	24.5 ± 7.2	0.185	26.0 ± 7.7	24.8 ± 6.7	0.418
Sexual concern	20.9 ± 6.5	20.5 ± 6.8	0.741	22.2 ± 6.7	18.7 ± 6.0	0.005	20.9 ± 7.0	20.4 ± 5.7	0.726
Relationship concern	24.7 ± 6.8	23.7 ± 7.3	0.474	25.7 ± 6.93	22.2 ± 6.7	0.009	24.6 ± 7.0	23.3 ± 6.9	0.377
Rejection of childfree	32.9 ± 6.5	30.9 ± 7.2	0.136	2.3 ± 6.2	31.8 ± 7.7	0.677	31.8 ± 7.2	33.0 ± 6.0	0.379
Need for parenthood lifestyle	41.9 ± 8.4	38.9 ± 8.0	0.055	41.4 ± 7.9	39.8 ± 8.9	0.293	40.7 ± 8.6	40.3 ± 8.0	0.824
Total FPI	146.8 ± 26.6	138.9 ± 27.8	0.123	148.1 ± 25.5	137.1 ± 28.5	0.031	144.2 ± 29.5	142.1 ± 22.7	0.679
Marital satisfaction									
Idealistic distortion	9.7 ± 1.8	10.1 ± 1.3	0.216	9.6 ± 1.6	10.14 ± 1.6	0.133	9.7 ± 1.5	9.9 ± 1.8	0.598
Marital satisfaction	24.7 ± 3.5	25.0 ± 2.7	0.617	24.3 ± 3.2	25.5 ± 3.1	0.056	24.8 ± 3.3	24.6 ± 2.9	0.800
Personality issues	10.5 ± 2.0	10.8 ± 1.9	0.391	10.4 ± 2.0	10.9 ± 1.9	0.192	10.5 ± 2.1	10.7 ± 1.9	0.640
Marital communication	10.5 ± 1.9	10.7 ± 1.8	0.481	10.4 ± 1.8	10.8 ± 1.8	0.246	10.6 ± 1.9	10.6 ± 1.6	0.849
Conflict resolution	12.6 ± 2.0	13.3 ± 1.8	0.062	12.9 ± 2.0	13.0 ± 2.0	0.730	12.7 ± 1.8	13.2 ± 2.2	0.256
Financial management	8.8 ± 1.4	8.8 ± 1.6	0.835	8.7 ± 1.41	8.8 ± 1.6	0.819	8.6 ± 1.5	9.0 ± 1.5	0.202
Leisure activities	10.8 ± 1.4	10.8 ± 1.4	0.846	0.6 ± 1.4	11.1 ± 1.3	0.072	10.8 ± 1.2	10.8 ± 1.6	0.992
Sexual relationship	9.2 ± 1.3	9.3 ± 1.1	0.649	9.1 ± 1.1	9.5 ± 1.3	0.099	9.3 ± 1.2	9.3 ± 1.1	0.998
Children and parenting	12.0 ± 1.51	12.3 ± 1.71	0.402	11.9 ± 1.6	12.4 ± 1.5	0.110	12.0 ± 1.4	12.3 ± 1.9	0.526
Family and friends	1.9 ± 1.9	1.8 ± 1.5	0.815	11.8 ± 1.7	12.0 ± 1.8	0.562	11.8 ± 1.7	11.8 ± 1.7	0.939
Religious orientation	11.0 ± 1.7	10.8 ± 1.3	0.579	10.8 ± 1.7	11.1 ± 1.3	0.384	10.9 ± 1.6	10.9 ± 1.5	0.858
Total marital satisfaction	132.1 ± 13.6	134.3 ± 13.3	0.388	131.1 ± 13.1	135.6 ± 13.6	0.073	132.3 ± 13.6	133.6 ± 13.6	0.614

Data are presented as mean ± SD. We used t tests to compare the means of the groups. Range of scores: Idealistic distortion (3-15), Marital satisfaction (8-40), Personality issues (4-20), Marital communication (4-20), Conflict resolution (5-25), Financial management (3-15), Leisure activities (4-20), Sexual relationship (4-20), Children and parenting (4-200), Family and friends (4-20), Religious orientation (4-20), Total enrich marital satisfaction (47-235), PTG; Posttraumatic growth (0-105), BDI; Beck depression inventory (0-63), State anxiety (0-80), Trait anxiety (0-80), and FPI; Fertility problem inventory (46-276).

Table 3: Correlation matrix of psychological variables

Variables	PTG	Depression	State anxiety	Trait anxiety	FPI
Idealistic distortion	r=0.144 P=0.115	r=-0.315 P=0.001	r=-0.362 P<0.001	r=-0.276 P=0.002	r=-0.325 P<0.001
Marital satisfaction	r=0.262 P=0.004	r=-0.295 P=0.001	r=-0.390 P<0.001	r=-0.394 P<0.001	r=-0.405 P<0.001
Personality issues	r=0.108 P=0.237	r=-0.281 P=0.002	r=-0.324 P<0.001	r=-0.387 P<0.001	r=-0.337 P<0.001
Marital communication	r=0.099 P=0.279	r=-0.326 P<0.001	r=-0.435 P<0.001	r=-0.402 P<0.001	r=-0.324 P<0.001
Conflict resolution	r=0.111 P=0.223	r=-0.332 P<0.001	r=-0.387 P<0.001	r=-0.435 P<0.001	r=-0.369 P<0.001
Financial management	r=0.120 P=0.188	r=-0.292 P=0.001	r=-0.360 P<0.001	r=-0.282 P=0.002	r=-0.308 P=0.001
Leisure activities	r=0.063 P=0.490	r=-0.210 P=0.24	r=-0.198 P=0.029	r=-0.198 P=0.029	r=-0.171 P=0.059
Sexual relationship	r=-0.087 P=0.339	r=-0.009 P=0.925	r=0.078 P=0.398	r=-0.056 P=0.540	r=-0.135 P=0.139
Children and parenting	r=0.167 P=0.066	r=-0.168 P=0.071	r=-0.250 P=0.006	r=-0.342 P<0.001	r=-0.282 P=0.002
Family and friends	r=0.124 P=0.174	r=-0.336 P<0.001	r=-0.390 P<0.001	r=-0.450 P<0.001	r=-0.335 P<0.001
Religious orientation	r=0.108 P=0.237	r=-0.273 P=0.003	r=-0.300 P=0.001	r=-0.318 P<0.001	r=-0.190 P=0.036
Total marital satisfaction	r=0.188 P=0.038	r=-0.399 P<0.001	r=-0.477 P<0.001	r=-0.503 P<0.001	r=-0.454 P<0.001

PTG; Posttraumatic growth, FPI; Fertility problem inventory, P; P value, and r= Pearson correlation coefficient.

Table 4 lists the results of stepwise multivariate logistic regression models based on four separate groups. Model 1 was applied to investigate the effect of six independent variables, including quality of marital relationships, infertility stress, depression, anxiety, age, and duration of infertility as the predictors of PTG. The results revealed that infertility stress (β=-0.25 ± 0.008, P=0.002) negatively predicted the PTG. However, individuals over 30 years old reported greater PTG than those under 30 years old (β=0.913, P=0.034).

In Model 2, the total score of the quality of marital relationships, total score of infertility stress, anxiety score, age, and duration of infertility were the predictors (independent variables) of depression symptoms. Depression was significantly predicted by infertility stress (β=0.038, P<0.001).

In Model 3, the total score of the quality of marital relationships, total score of infertility stress, age, and duration of infertility were the predictors (independent variables) of trait anxiety. Findings revealed that the quality of marital relationships was a significant negative predictor (β=-0.66, P=0.001), whereas infertility stress was a significant positive predictor of trait anxiety (β=0.027, P=0.006).

In Model 4, quality of marital relationships, total score of infertility stress, age, and duration of infertility were the predictors of state anxiety. Results showed that the total score of quality of relationships was a negative (β=-0.078, P<0.001), whereas infertility stress was a significant positive predictor of state anxiety (β=0.023, P=0.025).

Discussion

According to our results, infertile women had a moderate level of quality of marital satisfaction, similar to those in previous studies (11, 23). The mean of personal growth total scores in the present study was moderate and similar to that in the prior study (24). However, compared to our result, Zhang et al. (25) reported a lower mean of PTG among infertile women. This suggests that PTG differs across various social and cultural contexts. Similar to other previous studies, the participants' infertility stress scores were moderate (25, 26).

The results demonstrated that infertile women over the age of 30 experienced greater personal growth than those under the age of 30. On the contrary, another research found that younger individuals exhibited more PTG (27). In their study, it was suggested that younger individuals have a higher PTG due to their greater potential to make changes in their life. Different study populations, higher average age, and larger age range may be the reason for the different results. Suo et al. (28) reported that the participants' general demographic variables were not significantly associated with PTG. The reason may be the homogeneous participant characteristics of their study. Wang et al. (29) revealed that household income and educational level positively influenced PTG.

Table 4: Predictor of PTG, anxiety, depression in stepwise logistic regression

Models	Independent variables	Dependent	B (SE)	OR	95% CI	P value
Model 1	FPI Age	PTG	-0.25 (0.008) 0.913 (0.430)	0.975 2.492	0.960-0.99 01.072-5.791	0.002 0.034
Model 2	FPI	BDI	0.038 (0.009)	1.039	1.021-1.058	P<0.001
Model 3	Total marital satisfaction FPI	Trait anxiety	-0.66 (0.021) 0.027 (0.010)	0.936 1.027	0.898-0.975 1.008-1.047	0.001 0.006
Model 4	Total marital satisfaction FPI	State anxiety	-0.078 (0.022) 0.023 (0.010)	0.925 1.023	0.886-0.966 1.003-1.043	P<0.001 0.025

PTG; Posttraumatic growth, FPI; Fertility problem inventory, B; Beta, SE; Standard error, OR; Odds ratio, CI; Confidence interwall, PTG scores≥ 63, Depressive symptoms, BDI-II≥ 14; Anxiety symptom, State-anxiety≥41, Trait-anxiety≥41.

Furthermore, infertility stress and depression were higher in women with a lower education level than in those with a university level education, being consistent with previous studies conducted on infertile couples (26). Compared to individuals with a higher education level, those with an education level lower than high school graduation had a higher risk of depression (30). Higher education may lead infertile women to gain more knowledge about their infertility problems and have better ways to receive professional help. In terms of resilience and feeling of control, they have fewer psychological tools to cope with adversity. It is hypothesized that more educated infertile women have more social resources to cope with infertility stress and may be able to protect themselves against mental illnesses such as depression (31). Therefore, infertile women with lower levels of education should thus receive greater attention from health care providers.

The study revealed that the quality of marital relationships was positively correlated with personal growth while negatively correlated with infertility stress, anxiety, and depression. Previous studies supported the positive correlation between marital satisfaction personal growth (32, 33). Therefore, infertile couples with strong marital relationships use strategies that ultimately lead to personal growth. Maroufizadeh et al. (34) concluded that marital satisfaction in infertile patients was influenced by both their own anxiety and their spouses' anxiety. Another study found that total infertility stress scores and specific sub-rates of relationship concerns, social concerns, and rejection of a childless lifestyle, in particular, were significantly associated negatively with the marital satisfaction of infertile women (12). Moreover, longitudinal studies demonstrated that marital dissatisfaction was associated with subsequent depressive disorder (35, 36). High quality of marital relationships leads couples to spend time understanding their life conditions, such as infertility. Therefore, a satisfying marital relationship may improve an infertile woman's mental health by increasing positive emotions.

Our findings confirmed that a higher score of quality marital relationships was a protective factor against infertility stress, as well as state/trait anxiety. These findings were in agreement with the results obtained in previous studies (14, 32). Lee et al. (37) suggested that marital satisfaction positively influenced positive psychological adaptation to breast cancer among patients and their spouses. However, Dehle et al. (38) found that women's anxiety symptoms were not a significant predictor of changes in their or their partners' reports of marital satisfaction. A study implied that the trait anxiety strongly predicted marital satisfaction, but the role of state anxiety in predicting marital satisfaction was insignificant (39).

In our study, infertility stress was a negative predictor of personal growth. There is evidence supporting the effect of infertility stress on PTG (3). The results reported by Paul et al. (40) demonstrated that appreciation of the life factor was positively associated with infertility stress. Zhang et al. (25) stated that the husbands' infertility stress affected only their PTG, while the wives' infertility stress did not influence their own or their spouses' PTG.

It should be mentioned that this study has several limitations. The use of a convenience sample of infertile women limits the generalizability of the findings to all infertile patients. Therefore, in future studies, researchers should endeavor to recruit a larger sample size. The participants of our work included only women; therefore, it is recommended that future studies replicate the same study with the participation of men. Finally, this was a cross-sectional study; causal inferences are impossible with this kind of study.

Our results can have important clinical implications for clinicians working with infertile couples. The study proposed that clinicians working in infertility settings should pay further attention to the role of quality of marital relationships in reducing infertility stress. Clinicians should encourage infertile couples to enrich their marital relationships to better cope with stress. The study recommended that educating infertile couples and healthcare providers about the benefits of enhancing quality of couples' relationships might facilitate the ways of achieving personal growth rather than infertility stress or mental illness.

Conclusion

Our findings emphasized that higher scores of quality marital relationships were a protective factor against infertility stress and state/trait anxiety in infertile women. Additionally, higher infertility stress reduced personal growth. According to the results, infertile women with a high level of marital satisfaction may have more tendencies for personal growth than to stress.

The study suggests that all physicians of infertility centers, particularly gynecologists, should pay more attention to reduction of infertility stress in infertile couples, help them to improve marital satisfaction, and support them to have more positive opportunities for personal growth instead of negative consequences of infertility like mental illness.

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Authors' Contributions

M.F., Sh.Sh.; Conceptualization, Methodology and Conducted the project. H.Gh.; Data curation and Analyzed the data. M.F.; Wrote the primary draft of the paper

and Project administration. S.E.; Supervised the study, Resources and Project administration. F.Gh., Sh.Sh.; Collected the data and Investigation. All authors read and approved the final manuscript.

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The Predictive Value of Serum ß-HCG Levels in The Detection of Ectopic Pregnancy Sixteen Days after Embryo Transfer: A Cross-Sectional Study

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Abstract

Background: To detect the predictive value of beta human chorionic gonadotropin (β-hCG) levels 16 days post embryo transfer (ET) regarding detection of an ectopic pregnancy (EP) in assisted reproductive technology (ART) cycles.

Materials and Methods: In this cross-sectional study, we reviewed the database of Royan Institute from January 2011 to December 2014 and from January 2017 to December 2019 retrospectively. All cases with positive β-hCG levels sixteen days after ET were screened (n=4149). The pregnancies with oocyte or embryo donation and the multiple pregnancies based on the first ultrasound were excluded. All eligible singleton pregnancies with documented serum β-hCG levels at Royan institute laboratory (n=765) were included and then classified according to the type of pregnancy: EP (n=189) or non-EP (n=576). The data of the treatment cycle was extracted from the patients' files. A receiver operating characteristic (ROC) curve was used to detect the predictive power of the first measurement of β-hCG level in distinguishing EP from ongoing pregnancy in the ART and intrauterine insemination (IUI) cycles separately. Sensitivity, specificity, area under the ROC curve and 95% confidence intervals (CI) were calculated for each of the estimates.

Results: The mean levels of β-hCG 16 days after ET were remarkably higher in the ongoing pregnancy group than the EP group (1592.35 ± 87 IU/L vs. 369.69 ± 50.61 IU/L, P<0.001). The β-hCG thresholds predictive of ongoing pregnancy were 278 IU/L as the most suitable cut-off to predict viable pregnancy with a sensitivity of 72.8%, a specificity of 67.5%, a positive predictive value of 77.8%, standard error of 0.02, and a confidence interval of 73.8-81.7%. However, this relationship was not found in IUI cycles.

Conclusion: Based on these findings, if β-hCG levels 16 days after ET are below 278 IU/l, close follow-up is recommended, until either the diagnosis of EP or miscarriage is established.

Keywords: Beta Subunit, Chorionic Gonadotropin, Ectopic, Pregnancy, Reproductive Techniques Assisted

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Introduction

An ectopic pregnancy (EP) is a form of abnormal pregnancy occurs when a zygote implants and grows outside the main cavity of the uterus. EP constitutes 1-2% of all pregnancies and hemorrhage caused by EP because tubal rupture is the most common reason for maternal mortality in early pregnancy. Infertility affects 8-12% of couples in the whole world. There is a complex relationship between infertility and EP, as one of them could be a cause and the other a consequence. In pregnancies after infertility treatment, the rate of EP increases, which could be because of the effects of the treatment or the pre-existing disorder (1). A recent review has reported the rate of EP after assisted reproduction technologies (ART) cycles were between 2.1 and 8.6% of all clinical pregnancies (2).

The cause of the increased risk of EP in ART cycles is unclear; however, some risk factors such as tubal factor infertility, intracytoplasmic sperm injection (ICSI), assisted hatching, the fresh embryos transfer (ET) versus frozen ET,

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and the day of ET was reported in previous studies (3). EP rupture which occurred due to delay in its early diagnosis is responsible for more than a third of maternal mortality in the first trimester (4). Although unlike, a normal pregnancy, the day of ET in the *in vitro* fertilization (IVF) process is clear and follow-up such as assessment β- hCG titers and transvaginal sonography is performed routinely, Wang et al. (4), reported that delay in detection of EP following ART has occurred in 12.9% of cases.

Low levels of beta human chorionic gonadotropin (β-hCG) in early pregnancy can be associated with poor pregnancy outcomes such as miscarriage, ectopic pregnancy, which can be the result of abnormal placentation (5). Limited studies have shown that β-hCG levels in early pregnancy may be a predictor of IVF outcome (6), but the timing of β-hCG measurements varies in these studies (7), and ideally, each infertility center should provide its own data to determine the cut off for its center (8). Since early detection of pregnancy can reduce anxiety in couples and prevent dangerous consequences such as EP and considering the wide use of ART in the reproduction era. It is clinically valuable to determine EP in infertile patients as soon as possible, so high-risk women can be found at the early phase of abnormal pregnancy and be provided with targeted treatment to preserve their reproductive ability and lessen the incidence of ruptured EP. The present study was designed to detect the predictive value of β-hCG levels 16 days after ET regarding detection of ongoing pregnancy from an EP in ART cycles.

Materials and Methods

Study design

In this cross-sectional study, the Royan Institute database was reviewed that contained clinical and laboratory information on infertility treatment cycles carried out in the department of reproductive and endocrinology from January 2011 to December 2014 as well as from January 2017 to December 2019. The study protocol was approved by the Institutional Review Board and Ethics Committee of Royan Institute (IR.ACECR.ROYAN.REC.1398.156). The reason for selecting this period of time was that the patients' files were more complete based on the required information. The retrospective data was obtained from the registered database (Hakim and Rashen software). All cases with positive β-hCG levels (more than 10 mIU/mL), were screened (n=4149) sixteen days after ET. The pregnancies with oocyte or embryos donation and the multiple pregnancies were excluded from the study based on the first ultrasound. All eligible singleton pregnancies with documented serum β-hCG levels at Royan institute laboratory (n=765) were included and classified according to type of pregnancy (ectopic or ongoing). The diagnosis of all the ectopic pregnancies (EP group) (n=189) was confirmed by trans-vaginal ultrasound. All of eligible ongoing pregnancies (successful pregnancy with gestational age above 20 weeks) (n=576) were classified as non-EP group (Fig.1). The data of the treatment cycle was extracted from the patients' files and no patients' identifiable information was disclosed.

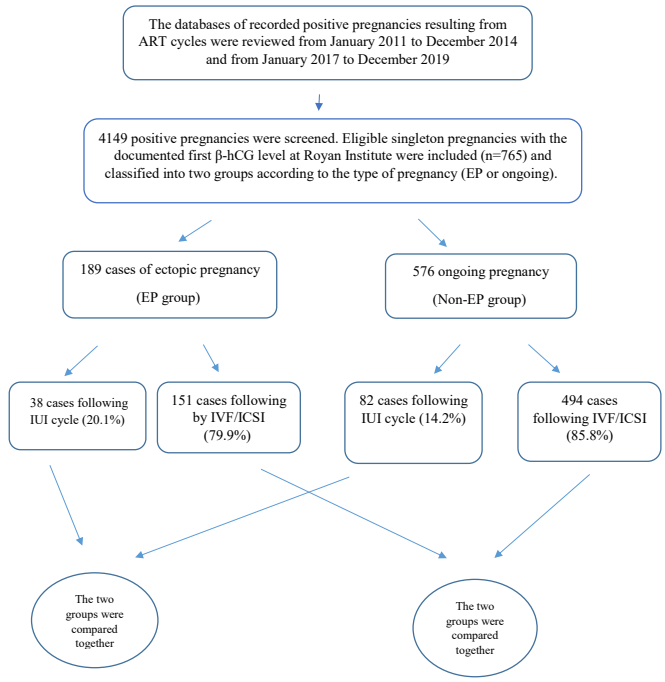

Fig.1: Sampling procedure and the distribution of the EP according to type of treatment cycles. EP; Ectopic pregnancies, ART; Assisted reproductive technology, β-hCG; Beta-human chorionic gonadotropin, IVF; *In vitro* fertilization, ICSI; Intra-cytoplasmic sperm injection, and IUI; Intrauterine insemination.

Treatment protocols and pregnancy follow up

The controlled ovarian stimulation (COS) protocol was selected based on the women's age, ovarian reserve, and previous cycle results. The standard ovarian stimulation protocols for patients included the standard gonadotropin-releasing hormone (GnRH) agonist protocol, GnRH antagonist. The descriptions of different COS protocols and standard procedures of IVF/ICSI as well as the qualification of ET cycles in Royan institute were stated elsewhere (9). Also, the verification method of embryo freezing and frozen ET cycles have been previously explained in detail (10). Luteal phase support in the fresh ET cycles was performed by a 400 mg vaginal progesterone suppository twice a day (Cyclogest®, Actavis, Barnstaple, UK) which was initiated on the evening of the oocyte retrieval day and continued until 10 weeks of pregnancy.

Because of FET cycles, endometrial preparation was done by using the artificial hormonal method with oral estradiol valerate (4-6 mg daily, Aboureihan Co., Iran) administrated from day 2-3 of the menstrual cycle, until the endometrial thickness reached ≥7 mm per two layers. Estradiol and progesterone treatment were prescribed until serum β-hCG level was checked and continued for 10 weeks in viable pregnancies. The serum β-hCG assay was performed routinely 16 days after ET and 17 days after IUI, respectively. It was measured by standard kits (Elecsys reagent kit, Roche Cobas). The demographic and infertility information such as body mass index (BMI), type of assisted reproduction method (IVF/ICSI or IUI), cause of infertility, type of infertility treatment protocol, number of transferred embryos, day of ET (cleavage or blastocyst), type of ET embryo (fresh or frozen) were entered into the statistical software.

Statistical analysis

The statistical analysis was performed by using SPSS Statistics Software (21.0, Statistical Package for the Social Sciences, Inc., Chicago, IL, USA). Also the power of study was measured by placing the mean and standard deviation of the first β-hCG in two groups in the post-hoc power calculator (ClinCalc software) and the analysis showed that the study has 90% power with α level 0.05. The distribution of variables was tested by the Kolmogorov Smirnov test. The Student's t test was used for variables with normal distribution. The comparison of proportions were carried out by using the Chi-square test. The presentation of continuous variables was done by mean ± standard deviation. A receiver operating characteristic (ROC) curve was applied to detect the predictive power of the first measurement of β-hCG level in distinguishing EP from ongoing pregnancy in the ART and IUI cycle that are determined separately. Sensitivity, specificity, positive and negative predictive values (PPV and NPV respectively), area under the ROC curve, and 95% confidence intervals (CIs) were computed for each of the estimates by MedCalc 11.1.1.0 (MedCalc® Statistical Software, Belgium). The multivariable logistic regression was applied for detection of significant predictive factors in the ectopic pregnancy.

Results

In this study, all cases with β-hCG levels >10 (mIU/mL), sixteen days after ET were determined in a specific period of time and then were divided into two groups on the basis of EP diagnosis. 189 pregnancies in the EP group and 3960 pregnancies in the non-EP group were identified. Out of 3960 who registered as ongoing pregnancies, data of 576 pregnancies were randomly matched in terms of maternal age compared with the EP group. Out of 576 cycles with ongoing pregnancy, 489 cycles (85.8%) were related to ART and 82 cycles (14.2%) were related to IUI. Out of the 189 cycles with EP, 151 cycles (79.9%) were ART, and 38 cycles (20.1%) were IUI (Fig.1).

The baseline and clinical characteristics of patients in the ART and IUI cycles were compared in two study groups in Tables 1 and 2, respectively. In 645 ART cycles, no statistically significant difference was observed between the two groups in terms of BMI, duration of infertility, duration of ovulation stimulation, endometrial thickness, number of previous IUI cycles, while the mean total dose of used gonadotropin in the ongoing pregnancy group was significantly lower than that of in the ectopic pregnancy group (P=0.052). The mean level of β-hCG 16 days after ET in the ongoing pregnancy group was statistically higher than that of the ectopic pregnancy group (P<0.0001). The previous history of EP was significantly higher in the ectopic pregnancy group compared to the control group (P=0.005). Regarding the type of ET, no statistically significant difference was noticed between groups (fresh or frozen) (P=0.07), fresh versus frozen. Most patients in the ongoing pregnancy group had primary infertility (76.3%) and there was a statistically significant difference between the two groups in the number of nulligravid cases (P<0.0001). Regarding the cause of infertility, a statistically significant difference was observed between the two groups (P<0.0001) and the number of patients with tuboperitoneal infertility in the EP pregnancy group (13.2%) was significantly higher than that of the ongoing pregnancy group (3.1%). In the ongoing pregnancy group, the number of blastocysts transferred was significantly higher than that of cleavage (77.6 compared to 22.4% respectively), whereas, in the ectopic pregnancy group, 56.2% of all transferred embryos were cleavage compared to 43.8% of the blastocyst transfers. Regarding the ovarian stimulation protocol between the groups under study, no statistically significant difference was seen (Table 1).

Table 1: The comparison of the baseline and clinical characteristics of patients between groups in ART cycles

Variables	Ectopic pregnancy (n=151)	Ongoing pregnancy (n=494)	P value*
BMI at the beginning of the treatment cycle (kg/m^2)	25.0 ± 3.5	25.6 ± 3.6	0.06
Duration of infertility (Y)	7.11 ± 4.44	6.64 ± 4.71	0.28
Duration of ovarian stimulation (Day)	11.05 ± 2.94	10.71 ± 2.54	0.27
Total dose of gonadotropin (IU)	2164.59 ± 144.65	1920.14 ± 80.52	0.052
Endometrial thickness on ET (mm)	9.47 ± 1.69	9.81 ± 1.97	0.14
Number of embryos transferred	2.3 ± 0.46	2.4 ± 0.45	0.1
Day of ET			0.0001>
Day 2 or 3 (cleavage stage)	85 (56.3)	112 (22.6)	
Day 4 or 5 (blastocyst stage)	66 (43.7)	382 (77.4)	
β-hCG level 16 days after ET (mIU/mL)	369.69 ± 61.50	1592.35 ± 87.00	0.0001>
Number of the previous IVF cycles	1.1 ± 0.11	0.78 ± 0.05	0.006
Number of previous IUI cycles	0.79 ± 0.09	0.70 ± 0.05	0.41
The number of previous miscarriages	0.3 ± 0.06	0.17 ± 0.02	0.017
Number of previous EP			0.0001>
No history	127 (84.1)	479 (96.9)	
1 time	19 (12.6)	12 (2.5)	
2 times	5 (3.3)	3 (0.6)	
Nulliparity			0.1
Yes	126 (83.4)	433 (87.7)	
No	25 (16.6)	61 (12.3)	
Nulligravidity			0.01
Yes	91 (60.2)	372 (75.3)	
No	60 (39.8)	122 (24.7)	
Type of embryo transfer			0.07
Fresh	76 (50.3)	290 (58.7)	
Frozen	75 (49.7)	204 (41.3)	
Type of infertility			0.0001>
Primary	91 (60.2)	376 (76.2)	
Secondary	60 (39.8)	118 (23.8)	
Causes of infertility			0.001
Ovulatory factor	48 (31.8)	179 (36.2)	
Tuboproteinal factor	20 (13.2)	15 (3.0)	
Unexplained factor	11 (7.3)	48 (9.7)	
Male factor	56 (37.1)	169 (34.3)	
Mixed factors	16 (10.6)	83 (16.8)	
Type of ovarian stimulation protocol			0.5
Long	97 (64.2)	254 (51.4)	
Antagonist	31 (20.5)	176 (35.6)	
Other protocols	23 (15.3)	64 (13)	

Data are presented as mean ± SD or n (%). ART; Assisted reproductive technology, BMI; Body mass index, ET; Embryo transfer, β-hCG; Beta-human chorionic gonadotropin, IVF; *In vitro* fertilization, IUI; Intra uterine insemination, EP; Ectopic pregnancy, and *; Obtained by independent sample t test and chi square test, statistically significant differences at 0.05.

Regarding the IUI cycles, there was no statistically significant difference between groups in terms of BMI, duration of infertility, duration of ovulation stimulation, gonadotropin dose, endometrial thickness, and the number of cycles. The significant differences were not observed in terms of the number of previous IUI cycles, abortion, and EP as well as the number of nulliparous and nulligravida cases between the two groups (Table 2).

Table 2: The comparison of the baseline and clinical characteristics of patients between groups in IUI cycles

Variables	Ongoing pregnancy (n=82)	Ectopic pregnancy (n=38)	P value*
BMI at the beginning of the treatment cycle (kg/m^2)	26.7 ± 3.2	26.2 ± 3.4	0.08
Duration of infertility (Y)	4.73 ± 3.97	5.7 ± 3.69	0.66
Duration of ovarian stimulation (day)	10.27 ± 2.49	10.31 ± 3.48	0.95
Total dose of gonadotropin (IU)	506.25 ± 71.26	702.2 ± 167.24	0.2
Endometrial thickness on IUI day (mm)	8.53 ± 1.18	8.23 ± 1.45	0.39
Level β-hCG 17 days after IUI (mIU/mL)	196.7 ± 58.1	215 ± 49.1	0.7
Number of the previous IUI cycles	0.62 ± 0.13	0.71 ± 0.17	0.67
The number of previous miscarriages	0.35 ± 0.09	0.35 ± 0.11	0.98
Number of previous EP			0.09
No history	82 (100)	35 (92.1)	
1 time	0 (0)	2 (5.2)	
2 times	0 (0)	1 (2.7)	
Nulliparity			0.41
Yes	68 (83)	34 (89.5)	
No	14 (17)	4 (10.5)	
Nulligravidity			0.81
Yes	54 (66.7)	25 (65.7)	
No	28 (33.3)	13(34.3)	0.88
Type of infertility			
Primary	58 (61.7)	24 (63.1)	
Secondary	24 (29.3)	14 (36.9)	
Causes of infertility			0.23
Ovulatory factor	30 (36.6)	15 (39.5)	
Unexplained factor	23 (28.1)	10 (26.3)	
Male factor	26 (31.7)	8 (21.1)	
Mixed factors	3 (3.6)	5 (13.1)	

Data are presented as mean ± SD or n (%). BMI; Body mass index, ET; Embryo transfer, β-hCG; Beta-human chorionic gonadotropin, IUI; Intra uterine insemination, EP; Ectopic pregnancy, and *; Obtained by independent sample t test and Chi square test, statistically significant differences at 0.05.

As shown in Figure 2, in ART cycles, the β-hCG threshold for distinguishing ongoing pregnancy from EP was 277 IU/l with a specificity of 72.9% (95% CI: 68.7-76.8) and a sensitivity of 67.5% (95% CI: 59. 5-74.9). The PPV was 40% (95% CI: 34.4-46.0) and the NPV was 93.8% (95% CI: 84.4-97.6). The accuracy of predicting the probability of ongoing pregnancy from non-ongoing pregnancy was 77.5% with a standard error of 0.02 and the confidence interval was 74.1 - 80.1% (P<0.0001, Fig.2).

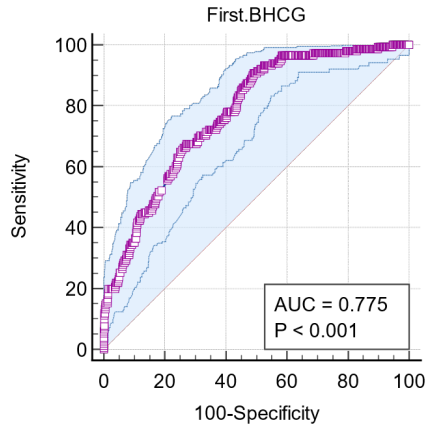

Fig.2: Receiver-operating characteristic curve of serum β-hCG levels 16 days after embryo transfer to distinguish between ongoing pregnancies and ectopic pregnancies in ART cycles [area under cure (AUC): 0.775, 95% CI: 0.74-0.80, P<0.001]. β-hCG; Beta-human chorionic gonadotropin and ART; Assisted reproductive technology.

In IUI cycles, the area under the curve was 0.57, which indicates the first measurement of β-hCG level had no significant power for differentiating ongoing pregnancy from EP (P=0.2, Cl: 0.45-0.69, Fig.3).

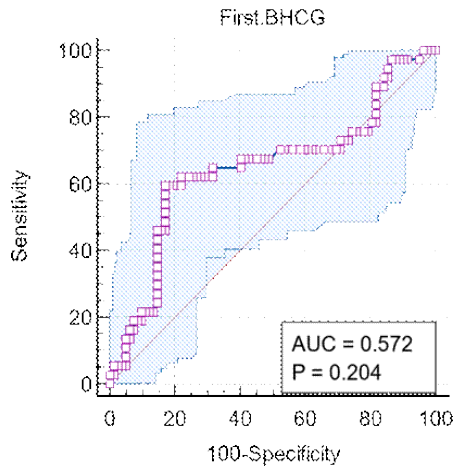

Fig.3: Receiver-operating characteristic curve of serum beta-human chorionic gonadotropin (β-hCG) levels 17 days after intrauterine insemination (IUI) to distinguish between ongoing pregnancies and ectopic pregnancies [area under curve (AUC): 0.57, 95% CI:0.55-0.73, P=0.2].

Discussion

Our results suggest that in IVF/ICSI cycles, the first detected β-hCG level (16 days after ET) can be predictive of ectopic pregnancy, and in cases with β-hCG level, less than 278 IU/L close follow-ups are recommended. However, this relationship was not found in IUI cycles.

β-hCG, as a sign of pregnancy progression, is detectable in the maternal blood serum as soon as day 8-9 of fertilization. Hence, detecting the level of β-hCG has been utilized as an index to note the IVF cycle outcome in order to ease the couple's anxiety and assist the physicians for assessing such high-risk pregnancies. The prevalence of pregnancy complications like ectopic pregnancy, blighted ovum, and missed abortion are more than that of

natural pregnancies (4, 11). It is well documented that the different hormonal milieu created by ovulation induction with gonadotropins may interfere with tubal function and embryo transport. Moreover, it is also confirmed that junctional zone contractions can be responsible for this as well. Lesny et al. (12) demonstrated that the contractions in the junctional zone and consequently the strong endometrial waves in the fundal area of the uterus caused by a difficult ET can shift mock embryos into the fallopian tubes in their studied oocyte donors.

The embryos are transferred directly into the uterus cavity through IVF treatment. In theory, the incidence of ectopic pregnancy after IVF treatment should be lower than that of natural pregnancies. Nevertheless, because of the higher rate of tubal infertility problems among those who are seeking IVF treatment, it is logical that the rate of EP is reported to be much higher in IVF cycles in comparison with spontaneous conception. IVF process by itself puts a lot of stress on couples. There is a challenging time between the initial positive pregnancy result and the first ultrasound revealing viable pregnancy for families. By the means of some serum markers or tests, we can predict the pregnancy outcome to help the clinician to make a better decision for the patient's situation and relieve the couple (7).

For the ongoing pregnancy, the threshold value to exclude patients with safety is 278 IU/L (sensitivity 72.8%, specificity 67.5%). The standard error of our study at this cut-off level was around 0.02 and its confidence interval was 73.8-81.7.% The study conducted by Kim have taken a cut-off value of serum β-hCG on day 11 after ET is significantly lower in the early pregnancy loss group, and 50 mIU/mL was considered a cut-off for the group with promising pregnancy results (11). Naredi et al. (13), showed that a satisfactory pregnancy outcome (beyond 12 weeks) can be achieved by higher levels of initial β-hCG while they were analyzing the outcomes of two different levels of β-hCG.

The present study is consistent with the worldwide experience and emphasizes the need for prognostic information to be given to patients as soon as pregnancies are diagnosed. In this study, we report evidence that day 16 hCG values routinely obtained on IVF-GIFT patients hold significant prognostic information. β-hCG shows placental functional activity and a low level of β-hCG is related to poor pregnancy outcomes. The comparison among causes of infertility in our study demonstrates that the number of patients with the diagnosis of tuboperitoneal infertility was significantly higher (13.2%) in the EP group compared with those of the ongoing pregnancy group (3.1%). This was consistent with the study of Ribic-Pucelj et al. (14) conducted on 8083 cycles, and the most frequent cause of ectopic pregnancies in IVF-ET cycles was (95.4%) tubal factor, followed by (2.3%) unexplained.

In the study of Bu et al. (15) in 2016, the risk factors for EP following IVF in 712 women were examined. They reported an odds ratio (OR) of 3.99 for women with tubal factor infertility compared to those with other infertility causes. Regarding the infertility category, the rate of ectopic pregnancy in tuboperitoneal group was higher than that of ongoing pregnancy. We presume that in such cases the most likely cause is the underlying pathology, which could not necessarily be diagnosed with routine diagnostic procedures such as HSG and laparoscopy.

It is possible that the freezing and thawing process may damage the trophoblast, thereby adversely affecting implantation, embryonic development, and β-hCG production. However, in comparison between FET and fresh ET, there was no statistically significant difference in β-hCG levels between ongoing and ectopic pregnancy groups in our study. Reljič et al. (16) performed a detailed analysis on 775 (51.5 %) cycles; 568 after fresh ET and 207 after FET in which statistics demonstrate comparable results in biochemical pregnancy, spontaneous abortion, and rate of ectopic pregnancy. On the other hand, the live birth rate was statistically higher after fresh ET than that of FET. There was no statistically significant difference at the level of mean β-hCG between these two groups. Moreover, 496 IU/L was determined as an ideal threshold level with good sensitivity and specificity for anticipating live birth post ET compared to 527 IU/L for the FET group (10). On the other hand, Poikkeus et al, compared the β-hCG level of 290 fresh versus 72 frozen embryo cycles that resulted in viable singleton pregnancies that was found no difference in median HCG values (114 versus 115 IU/I) between groups (17). Studies investigating β-hCG levels after fresh ET and FET cycles present inconsistent results so it is impossible to use these findings in predicting pregnancy outcomes after FET. It has been hypothesized that several factors may affect β-hCG level after transfer. In method of freezing (vitrification compared to slow freezing) in FET cycles, and treatment protocol (the effect of gonadotropin level on endometrial maturation) are some factors that may have an impact on β-hCG levels in a transfer cycle.

One time measuring of β-hCG on predicting pregnancy outcome has been investigated. The study conducted by Bjercke et al. (18) showed that the level of β-hCG of more than 150 IU/L, 14 days after ET, with a sensitivity and specificity of 79 and 78% respectively identifying between viable and non-viable pregnancies. Comparably, in the study conducted by Bjercke et al. (18), β-hCG levels more than 55 IU/l, 12 days after ET had a 90% association with viable pregnancy results.

The present study was the first study in Iran on this topic which was its strength. Our study is limited by its retrospective nature. Whether the population under study represents the general IVF women is not clear. However, there were considerable similarities in terms of demographics and clinical characteristics between the participants in our study compared to that of other studies. Moreover, it is assumed that the different assays we applied to measure the level of β-hCG may have an impact

on the β-hCG level reported. Therefore, physicians need to be aware of this in their consultations with patients. Prospective studies are needed to predict pregnancy outcomes for IUI and IVF/ICSI based on reliable cut-off values.

Conclusion

This study implies that the higher the initial production of β-hCG, the more biologically efficient and ultimately, a better chance to get as far as the stage of ongoing pregnancy, and we found that, a one-time measurement of β-hCG at an early stage can significantly aid to spot pregnancies that extend to a minimum 12 weeks of gestation. Based on our study, if β-hCG levels were above 278 IU/l on day 16, intensive follow-up is not required in the absence of danger signs, and if βHCG levels are below 278 IU/l on day 16, close follow-up is recommended, such as weekly β-hCG titers and an ultrasound evaluation until either the diagnosis of EP or miscarriage is established. This particular outcome can aid physicians with analyzing early βhCG, assist with calming stressed patients until they receive their IVF cycle treatment results, and help comfort couples about whether it is more likely for the pregnancy to be successful or not.

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Authors' Contributions

S.H.H., F.Gh.; The conception and interpretation of data. S.H.H., S.V., A.A.; Design of the work. Z.Z., S.V., S.K.; The acquisition and analysis. S.H.H., A.A., F.Gh.; Have drafted the manuscript and revised it. All authors have read and approved the manuscript.

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The Effects of Clove Oil on The Biochemical and Histological Parameters, and Autophagy Markers in Polycystic Ovary Syndrome-Model Rats

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Abstract

Background: This study aimed to determine whether syzygium aromaticum (clove) could help polycystic ovary syndrome (PCOS) rats.

Materials and Methods: In this experimental study, forty adult female Wistar rats (weighing 250 ± 10 g) were divided randomly into five groups; G1: control, G2: PCOS group, G3: PCOS+clove (30 mg/kg/ orally/daily) group, G4: PCOS+clove (60 mg/kg/orally/daily) group, and G5: PCOS+gonadectomy group. The PCOS was induced by a single dose injection of estradiol valerate (16 mg/kg/IM). Following PCOS induction, the rats were treated for 14 days. Histological parameters, follicle apoptosis, mRNA expression of autophagy markers (*Lc3, Beclin1*), oxidative stress markers, insulin and blood glucose levels, as well as serum levels of aromatase and testosterone were evaluated in these rats. Finally, the ratio of serum luteinizing hormone (LH) to follicle-stimulating hormone (FSH) levels was also calculated.

Results: The autophagy markers (*Lc3, Beclin1*), histological parameters, oxidative stress, insulin, and hormone levels changed significantly in the PCOS rats (G2). In G3 and G5 groups, it was observed that the levels of LH/FSH and testosterone decreased significantly in comparison to the PCOS group, and inhibition of autophagy was also observed in these groups. Treatment with cloves in the G3 group significantly improved oxidative stress, histological parameters, and insulin levels.

Conclusion: These findings demonstrated that oxidative stress, apoptosis, and excessive autophagy could be improved by treatment with low doses of clove and gonadectomy. Cloves may help to improve these parameters by regulating and inhibiting excessive autophagy. However, discovering the direct role of this extract in regulating the parameters such as oxidative stress, insulin, and androgens requires further investigation. In the present study, $P<0.05$ was considered statistically significant.

Keywords: Apoptosis, Autophagy, Oxidative Stress, Polycystic Ovary Syndrome, Syzygium Aromaticum (Clove)

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Introduction

One of the prevalently complex endocrinopathy disorders is "polycystic ovary syndrome" (PCOS), affecting up to 17.8% of women of reproductive age. The etiology of this syndrome is still unclear, although environmental, genetic, and hormonal factors may be necessary for its development (1, 2). PCOS has been associated with numerous reproductive, metabolic, and biochemical abnormalities such as elevated androgens, oxidative stress, insulin resistance disruption of the hypothalamic-pituitary-gonadal axis (the ratio of luteinizing hormone (LH)/follicle-stimulating hormone (FSH) ≥ 1) (3, 4), and finally, dysregulation of ovarian steroidogenesis, folliculogenesis (5). In PCOS, the PI3K/AKT/MTORE signaling pathway plays a vital role in regulating the interactions among androgens, insulin, and growth factors. Autophagy, which is induced by oxidative stress (6), is one of the main pathways of destruction and is essential

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for cellular homeostasis, as well as maintaining energy production and synthesis of new macromolecules. This process is vital for separating natural components from inefficient and unnecessary substances. Additionally, the protective function of this process has been proven during proliferation, differentiation and regulation of reproduction (7, 8). Although the autophagy process typically occurs in the ovary and is essential for folliculogenesis, this process has a detrimental effect on the ovary through prolonged activation (5). The importance of autophagy in PCOS-related disorders is well known, including dysregulation of androgen, gonadotropins, insulin levels, and most importantly, increased follicular atresia, which leads to decreased ovarian reserve (9). Due to the role of autophagy in metabolic disorders of PCOS, the use of antioxidant inhibitors of autophagy is a reasonable treatment (10).

The *Syzygium aromaticum*, generally known as clove, is originally from the Asiatic region. Clove essential oil (eugenol, phenols, flavonoids, oleanolic acid, and tannins) has been shown to minimize damage caused by toxicity or oxidative stress in the brain, liver, heart, kidneys, and testicles (11, 12). Clove oil is a useful and valuable herb with anti-apoptotic, antioxidant, anti-diabetic, anti-hyperlipidemic properties, and is effective in treating sexual disorders (12). Furthermore, it inhibits autophagy as an antiviral therapy (12, 13). Since the typical clinical symptoms of this syndrome are associated with autophagy disorder, in this study, clove extract was used as an autophagy regulator to improve PCOS rat model parameters, including apoptosis, oxidative stress, and and the levels of androgen, gonadotropins, aromatase, and insulin. Accordingly, the present study was conducted with the aim of investigating the potential ability of clove extract in regulating parameters related to autophagy in PCOS rats.

Materials and Methods

The current experimental study used 40 adult female Wistar rats (weighing 250 ± 10 g). All animals were purchased from Razi Institute in Mashhad, and then they were intently maintained in an animal facility under standard conditions (12/12 hours light/dark cycles, 23 ± 2°C, and 60-70% humidity). Rats in all groups had free access to food and water. We considered ethical considerations according to the regulations of the "Gonabad University of Medical Science". The Ethical Code of the present research was specified as IR.GMU.REC.1394.32.

We randomly divided the rats into 5 experimental groups: (30 mg/kg/orally/daily)

G1) Control group, that received normal saline only (daily oral) (n=8).

G2) PCOS group, that received a single dose injection of estradiol valerate for PCOS induction only with no other treatments (n=8).

G3) PCOS+clove (30 mg/kg) group, PCOS rats that received 30 mg/kg of clove extract daily by oral gavage (n=8).

G4) PCOS+clove (60 mg/kg) group, PCOS rats that received 60 mg/kg of clove extract daily by oral gavage (n=8).

G5) PCOS+gonadectomy group, PCOS rats that underwent gonadectomy (n=8).

Experimental protocol

In the experimental process, the vaginal smear was examined daily and confirmed that all rats had regular cycles (about 8-10 days). The PCOS was induced by an intramuscular injection of a single dose (1) of estradiol valerate (16 mg/kg) dissolved in 0.2 ml of sesame oil (Riedeldehaen, Germany) (14). After confirming the induction of ovarian cysts (by evaluation with estrous cycle without ovulation), clove oil was administered orally for 14 days (30-60 mg) in G3 and G4 groups (15). Clove oil contains 13 identified compounds, among which eugenol 76.8% is considered as the main component. Other components incluse beta caryophyllene17.4%, alpha humulene 1.1%, and eugenol acetate 1.1%, which use gas chromatography-spectrum mass measurement. Finally, at the end of treatment on day 14, the rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). Blood samples (1 cc) were taken from the cardiac apex and transferred into sterile test tubes for biochemical assessment (measure the serum levels of LH, FSH, testosterone, aromatase, insulin, and oxidative stress marker). The samples were centrifuged at 3000 rpm for 15 minutes at room temperature to separate the serum (16). Then, the separated sera without red blood cells were kept at -80°C until testing (17). In addition, all groups except for G5 were operated on for ovariectomy to examine both ovaries. Ovariectomy was performed as follows: initially, the animals were anesthetized, and a longitudinal incision was created in the midline behind the animals. Two cm incision was made below the last rib. Then, Fallopian tubes with ovaries were pulled out. The ovaries were removed, and the fallopian tubes were returned to the abdominal cavity. Finally, the incision site was sutured (3, 13). On the fourteenth day (the end of the treatment period), the rats deeply anesthetized using ketamine-xylazine and then underwent ovariectomy. The ovaries on one side were fixed in formalin for histological studies, and the other was used for molecular studies.

Vaginal smear

The PCOS model evaluation with an ovulatory estrous cycle was confirmed by examination of vaginal smears. Vaginal smears of all the rats were inspected daily at the same time to estimate the stage of the 4-day ovarian cycle. Accordingly, at first, a cotton swab was moistened and after placing it in the animal's vagina, the swab was rotated on the vaginal wall. The swab was carefully removed from the vagina and the sample on the swab was spread on a microscope slide. After fixing the sample with alcohol,

the prepared smear was stained with hematoxylin-eosin (H&E).

The observation of the slides by an optical microscope (Olympus BX51, Japan) with a magnification of 400× shows the different stages of the estrous cycle as follows:

- Proestrus: the presence of a large number of epithelial cells with round and large nuclei.
- Estrus: the presence of cornified epithelial cells without nuclei.
- Metestrus: the presence of both types of epithelial cells, nucleated and non-nucleated, along with a small number of leukocytes.
- Diestrus: the presence of leukocytes along with a small number of non-nucleated epithelial cells.

Observing a pause in the estrous cycle for two weeks (at least three periods) indicates the induction of PCOS (18, 19).

Histological evaluation

At the end of the experimental period, the rats were anesthetized with ketamine and xylazine and then sacrificed. The sample collection method was that after opening the abdominal wall, the ovaries were carefully separated from the fallopian tube and then kept in 10% formalin for 48 hours. After ensuring the stabilization of the ovaries, the process of dehydration and clarification was done by alcohol and xylene, respectively. After embedding in paraffin, the ovaries were cut into 5 µm-thick in coronal serial sections at 50-60 µm intervals using a microtome. Ten sections were prepared from each ovary. Then, the samples are spread on the slide to be prepared for H&E staining and to identify the types of follicles in the ovary. The follicles were counted according to the following definitions:

Primordial follicle: A single layer of squamous follicular cells surrounding the oocytes.

Primary follicle: One or more layers of follicular cells that have become cubic.

Secondary follicle: This is characterized by a single, sizeable antral cavity.

Atretic follicles: The granulosa cells undergo apoptosis, and consequently, the oocyte degenerates (19).

Finally, the number of primordial, primary, antral, atretic, and cystic follicles and corpus luteum were counted by an optical microscope (Olympus BX51, Japan) with a magnification of 400×.

Apoptotic cell evaluation

In the sections prepared from the ovarian tissues, apoptotic cells were identified by the TUNEL technique. To perform this technique, first, serial sections of the sample with a thickness of 5 micrometers were obtained. The sample sections were placed on a slide, and then deparaffinization and hydration were done with xylene and decreasing concentrations of ethanol, respectively. In the next step, the slides were washed twice in PBS for ten minutes each time. After that, the slides were exposed to protein kinase for 20 minutes. After washing the slides again, the TUNEL reaction was performed according to the manufacturer's protocol (Roche, Germany). At the end of the experiment, the slides were incubated with DAB for ten minutes. Next, they were exposed to hematoxylin dye for 30 seconds to create a background color. In this staining technique, dark brown color indicates apoptotic cells (20). For calculatingthe apoptotic index (AI) in each type of follicles, we counted the number of TUNEL-positive cells, then divided that number by the total number of granulosa cells and expressed the results in percentages. Then the mean AI of each case was calculated (21).

Malondialdehyde level evaluation

The serum level of malondialdehyde was calculated by transferring 0.20 cm^3 of each serum sample into a separate microtube that contained 3.0 cm^3 of glacial acetic acid, followed by addition of 1% thiobarbituric acid (TBA) in 2% NaOH to the tube. It was then placed into the boiling water bath for 15 minutes. After cooling the vial in cold water to room temperature, the absorbance of the pink-colored product was read at 532 nm. From the standard solution obtained from malondialdehyde tetrabutylammonium salt (Sigma-Aldrich, Germany), different concentrations were prepared. A calibration curve was made using different concentrations of the standard solution (22).

Superoxide dimutase and glutathione peroxidase level evaluation

The levels of superoxide dimutase and glutathione peroxidase in serum were evaluated by an ELISA Reader (Model: ABER-2, China) - AccuBioTech) according to the manufacturer's protocols (Randox, and Ransod, UK). GPX level measurement is based on the Paglia-Valentine method. GPX catalyzes the oxidation of glutathione (GSH) by cumene hydroperoxide. The SOD level assay uses xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazoliumchloride (I.N.T) to form a red formazan dye (23).

Evaluation of testosterone, aromatase, and the ratio of luteinizing hormone to follicle-stimulating hormone levels

Testosterone serum levels were measured according to the protocol of ELISA kits (Demeditec Diagnostics, Germany). Likewise, the serum levels of aromatase, FSH, and LH were also evaluated according to the ELISA kit protocol (ZelBio, Italy) (3).

Blood glucose and insulin level evaluation

At the end of the treatment period, the levels of serum blood sugar were measured by using a glucometer

(Bayer Corporation, USA). Serum insulin concentration was measured by enzyme-linked immunosorbent assay and it was determined using ELISA kits (Antibody and Immunoassay Services, HKU). The level of serum insulin and blood sugar were expressed as mIU/ml and mg/dl, respectively (21).

Real time analysis evaluation

Total RNA was extracted from the ovarian tissue of each rat with a RNX-plus (ParsTous, Iran) kit according to the manufacturer's protocol. The purity of RNA was defined by electrophoresis on an agarose gel. The total RNA (1 µg) was reverse-transcribed with a cDNA synthesis kit (ParsTous, Iran). Reverse transcription polymerase chain reaction (RT-PCR) was performed with ABI PRISM® 48-well optical reaction plate (Applied Biosystems StepOne, FosterCity, USA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous gene control. The relative gene expression levels were compared with those in control, and all real-time quantitative PCR were performed in triplicates (24). The primers were as follows:

Beclin1-F: CGAAAGGTGGTGGCAGAAAAC
R: ACTATATTCTCGCTGGTACTGAGC
Lc3β-F: TCAGTGAGAGCTGCCTCTGTC
R: AGCAGTGGGGATTTACACAGTG
Gapdh-F: GGTCTACATGTTCCAGTATGACTC
R: CATTTGATGTTAGCGGGATCTCG

Statistical analysis

Statistical analysis was performed using SPSS 20 software (IBM, USA). Kolmogorov-Smirnov test was used to measure the normal distribution of data. All data were presented in mean ± SE. The one-way ANOVA test followed by the Tukey posthoc test were used to compare histopathological values. The independent t test was used to compare and analyze the data from the real-time PCR. A P≤0.05 was considered statistically significant.

Results

Superoxide dimutase, glutathione peroxidase and malondialdehyde level analysis

The serum levels of GPX and SOD decreased significantly in the PCOS group as compared to the control group (P=0.001). In the PCOS+clove (30 and 60 mg/kg/orally/daily) and PCOS+gonadectomy groups, these levels significantly increased when compared with the PCOS group (P=0.001, Fig.1). The level of malondialdehyde (MDA) in the PCOS group was significantly higher than the control group; on the other hand, it was significantly lower in PCOS+clove (30 and 60 mg/kg/ orally/daily) and PCOS+gonadectomy groups compared to the PCOS group (P=0.001, Fig.1). No significant differences were observed in the serum levels of GPX, SOD, or MDA between the clove treatment and gonadectomy groups compared with the control group.

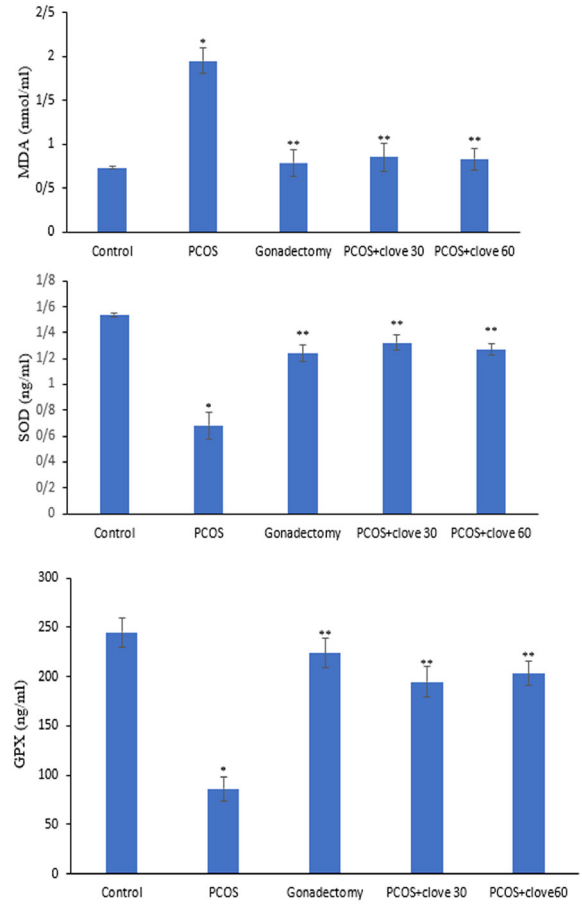

Fig.1: A comparison of the oxidative stress markers MDA, SOD, GPX in control, PCOS, PCOS+gonadectomy; PCOS+clove 30, and PCOS+clove 60 groups. Data values are mean ± SD and P<0.05 is significant. Analyzed by ANOVA test. MDA; Malondialdehyde, SOD; Superoxide dismutase, GPX; Glutathione peroxidase, PCOS; Polycystic ovary syndrome, *; P<0.05 vs. the control group, and **; P<0.05 vs. the PCOS group.

Analysis of hormones in serum

The serum levels of testosterone showed a significant enhancement in the PCOS group compared to the control group. In the PCOS+clove (30 mg/kg/orally/daily) and PCOS+gonadectomy groups, the testosterone levels decreased significantly when compared with the PCOS group (P=0.001, Fig.2). In comparison with the control group, the LH/FSH levels illustrated a considerable increase in the PCOS group (LH/FSH ≥1). The ratio of serum levels of LH to FSH in the PCOS+clove (30 mg/kg/orally/daily) and PCOS+gonadectomy groups were significantly decreased compared to the PCOS group (P=0.01, LH/FSH <1). Interestingly, there was no significant difference in the serum levels of testosterone and the ratio of the serum levels of LH to FSH in when comparing PCOS+clove (60 mg/kg/orally/daily) and PCOS groups (Fig.2).

Insulin level analysis

The insulin level improved remarkably in the PCOS group compared to the control group. In the PCOS+clove (30 mg/kg/orally/daily) and PCOS+gonadectomy groups, it significantly decreased in comparison with the PCOS group (P=0.001, Fig.3).

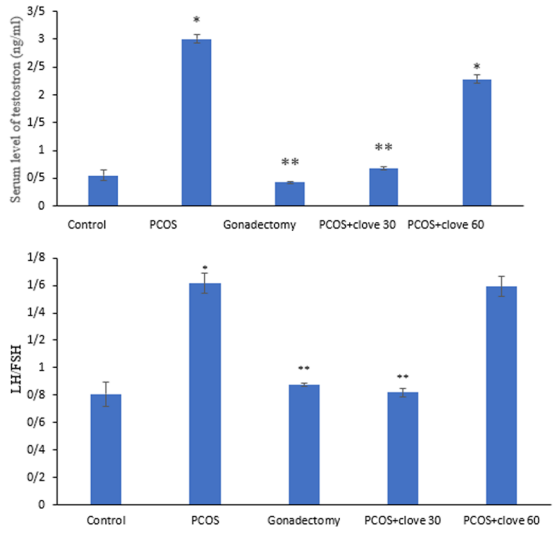

Fig.2: A comparison of the LH/FSH and testosterone in control, PCOS, PCOS+gonadectomy; PCOS+clove 30, and PCOS+clove 60 groups. Data values are mean ± SD and P<0.05 is significant. Analyzed by ANOVA test. LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, PCOS; Polycystic ovary syndrome, *; P<0.05 vs. the control group, and **; P<0.05 vs. the PCOS group.

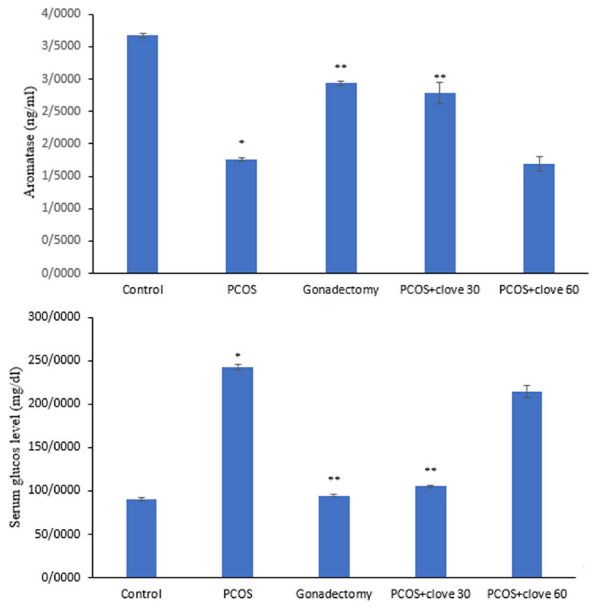

Fig.3: A comparison of the levels of aromatase, insulin, and glucose in control, PCOS, PCOS+gonadectomy; PCOS+clove 30, and PCOS+clove 60 groups. Data values are mean ± SD and P<0.05 is significant. Data were analyzed by ANOVA test. PCOS; Polycystic ovary syndrome, *; P<0.05 vs. the control group, and **; P<0.05 vs. the PCOS group.

Blood glucose level analysis

The PCOS group showed a significant increase in blood glucose levels compared to the control group. There was a significant decrease in the blood glucose of the PCOS+clove (30 mg/kg/orally/daily) and PCOS+gonadectomy groups as compared to the PCOS group (P=0.001, Fig.3).

Aromatase level analysis

The aromatase levels decreased significantly in the PCOS group compared to the control group (P=0.001). But, in the PCOS+clove (30 mg/kg/orally/daily) and PCOS+gonadectomy groups, it significantly increased compared with the PCOS group (P=0.001). In this regard, there was no significant difference between the PCOS+clove (60 mg/kg /orally/daily) and the PCOS group (Fig.3).

Apoptosis index analysis

In this study we evaluated apoptosis indicators in the primary and antral follicles. The rats displayed a standard apoptosis index in the control group. Compared with the controls, PCOS rats exhibited an increased apoptosis index in both the primary and antral follicles. Clove (30 mg/kg/ orally/daily) treatment reduced the apoptosis index as compared with the PCOS group (P<0.001). In this regard, we did not observe a significant difference between the PCOS+clove (60 mg/kg /orally/daily) and PCOS groups (Fig.4).

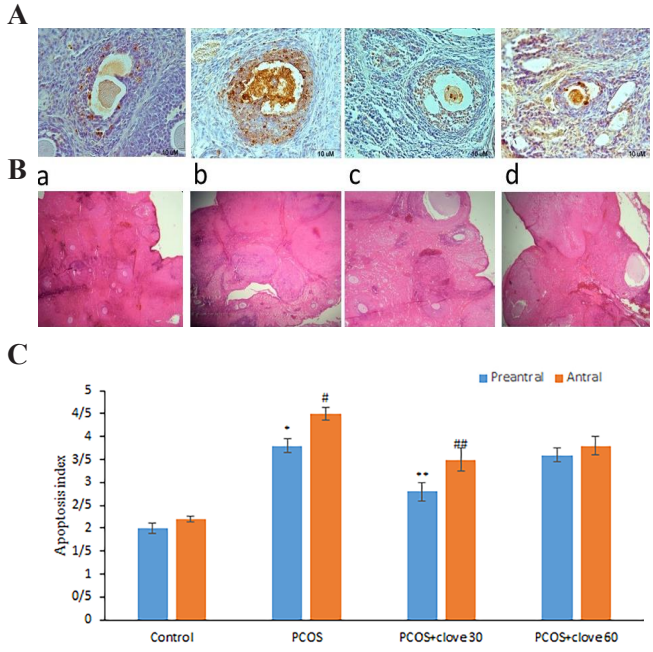

Fig.4: Effect of clove oil on PCOS symptoms in control, PCOS, PCOS+clove 30, and PCOS+clove 60 groups. **A.** Ovarian follicle apoptosis findings (scale bar: 10 µM), **B.** Ovarian histological findings, a; Control, b; PCOS group, c; PCOS+clove (30 mg/kg/ orally/daily) group, d; PCOS+clove (60 mg/kg /orally/daily) group (scale bar: 40 µM). **C.** Comparison of the apoptosis index in control, PCOS, PCOS+clove 30, and PCOS+clove 60 groups. Data values are mean ± SD and P<0.05 is significant. Analyzed by ANOVA test. PCOS; Polycystic ovary syndrome, *; P<0.05 vs. the control group, **; P<0.05 vs. the PCOS group in prenatal follicles, #; P<0.05 vs. the control group, and ##; P<0.05 vs. the PCOS group in antral follicles.

Histological parameters analysis

Rats in the control group displayed normal ovary histology. In the PCOS group the number of corpus luteum and all the follicles significantly decreased. However, the number of follicular cysts increased, and cell debris in the antrum was detected in this group. Treatment with clove (30 mg/kg/ orally/daily) exhibited well-developed antral follicles, a standard granulosa cell layer, and minimal cell debris. Also, the number of follicular cysts decreased following clove treatment. In this regard, there was no significant difference between the PCOS+clove (60 mg/kg/ orally/daily) and PCOS groups (P=0.001, Fig.4B, Table 1).

Table 1: A comparison of the primordial, primary, antral, cystic follicles, and corpus luteum in control, PCOS, PCOS+clove 30, and PCOS+clove 60 groups

Group	Primordial follicles	Primary follicles	Antral follicles	Cystic follicles	Corpus Leuteum
Control	49.6 ± 2.07	18.4 ± 1.14	15.6 ± 1.14	0	10.2 ± 0.83
PCOS	32.2 ± 1.87*	7.6 ± 1.14*	3.8 ± 0.83*	6.6 ± 0.54*	1.6 ± 0.89*
PCOS+clove 30	40 ± 1.78	15.4 ± 1.14†	13 ± 1.58†	1.1 ± 0.05	6.8 ± 0.83†
PCOS+clove 60	35.2 ± 1.78*	7.8 ± 0.83*	4.6 ± 1.14*	5.1 ± 0.83*	1.4 ± 0.05

Data values are mean ± SD and P<0.05 is significant. Control group; Injection of normal saline only; PCOS group; PCOS was induced by injection of estradiol valerate, PCOS+clove 30; PCOS induction along with clove extract (30 mg/ kg) treatment, PCOS+clove 60; PCOS induction along with clove extract (60 mg/kg) treatment of clove, PCOS; Polycystic ovary syndrome, *; P<0.05 vs. the control group, and †; P<0.05 vs. the PCOS group. Analyzed by ANOVA test.

Real-time analysis

Real-time PCR results showed that the Beclin1 and Lc3β mRNA levels increased significantly in the PCOS groups compared with the control group (P=0.001). However, there was a significant decrease in Beclin1 and Lc3β mRNA levels in the PCOS+clove (30 mg/kg/ orally/daily) group compared to the PCOS group (P≤0.001). However, the comparison of mRNA of the mentioned genes showed no significant difference between the PCOS+clove group (60 mg/kg/orally/day) and PCOS (Fig.5).

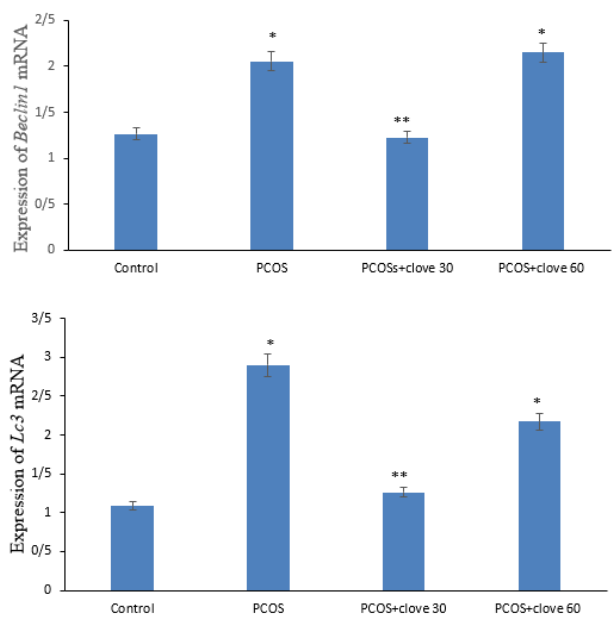

Fig.5: mRNA expression on *Beclin1* and *Lc3* in control, PCOS, PCOS+clove 30, and PCOS+clove 60 groups, as measured by the real-time PCR. Data values are mean ± SD and P<0.05 is significant. PCOS; Polycystic ovary syndrome, PCR; Polymerase chain reaction, *; P<0.05 vs. the control group, and **; P<0.05 vs. the PCOS group.

Discussion

In the present study, autophagy markers (Lc3β, Beclin1), histological parameters, oxidative stress, insulin levels, and hormone levels all changed significantly in PCOS rats following PCOS induction when compared to the control rats receiving only saline. Testosterone levels and the ratio of LH to FSH in G3 and G5 rats were significantly lower than in the rats with PCOS only (G2 group). Autophagy inhibition was also observed in these two groups. Clove treatment in the G3 group improved oxidative stress, histological parameters, and insulin levels significantly. PCOS is an endocrinological disorder. Internationally, the Rotterdam criterion describes PCOS as follows: i. Low or no ovulation, ii. Hyper-androgenic, iii. Polycystic ovaries (25). This syndrome is associated with impaired insulin levels, obesity, and diabetes in most women. In this syndrome, some morphological ovarian markers such as a few preantral follicles, antral follicles, atretic follicles, and degenerative ovarian cysts may be shown. Although this study showed all the above markers, some studies have suggested that these markers are not exclusive to PCOS (3-5).

In the present study, following the induction of PCOS in adult rats, parameters such as hyper-androgenemia, hyperinsulinemia, hyperglycemia, increased LH/FSH ratio, increased aromatase level, oxidative stress, and ovarian tissue apoptosis were observed. Autophagy is considered as an intracellular homeostasis process. During this process, autophagy-related proteins, including Lc3 and Beclin, form a structure called the autophagosome, which destroys the dysfunctional and stressful components of the cell by attaching to the lysosome (26). Autophagy has been shown to regulate primary follicle growth and atresia. It is a key part of each follicle's defense mechanism in response to various intracellular and extracellular stimuli (9). As mentioned above, autophagy dysregulation has been reported in PCOS. Molecular findings of a recent study showed an increase in autophagy markers (LC3 and Beclin1) following induction of PCOS (6). Other features of PCOS include increased oxidative stress, such as intracellular ROS, which is one of the main pathways preoccupied in PCOS pathogenesis (26-28). This study showed that the MDA level significantly increased while the SOD and GPX levels significantly decreased as a result of induction of PCOS in rats. Other reports have shown that oxidative stress may lead to induction of autophagy and eventually follicular atresia through the PI3K/AKT/mTOR pathway (6, 29). The biochemical findings of this paper show an increase in androgens in rats with PCOS, which makes it possible to induce autophagy by hyperandrogenism. Studies have shown that androgens induce autophagy by regulating Beclin expression (30). On the other hand, autophagy in the PCOS ovarian theca layer has been shown to lead to hyperandrogenism (31). Autophagy and hyperandrogenism seem to have a synergistic effect.

Other biochemical results of the present study include an increased LH/FSH ratio, which provides a significant value in diagnosing PCOS (32). Studies have shown that the formation of autophagosomes have an effect on

FSH levels (33, 34). The role of autophagy in regulating the insulin associated with this syndrome is well known (35, 36). In this study, impaired insulin levels may have resulted from disruption of normal autophagic function. Due to the impairment of autophagy-related parameters, by considering the above data, *S. aromaticum* (Clove) has been suggested as a potent antioxidant and an autophagy inhibitor (37) for managing PCOS. This plant enhances antioxidant enzymes by increasing the levels of MDA, SOD, and GPX in the treatment groups. We found the highest reduction in oxidative stress in a dose of 60mg clove, but this dose could not increase aromatase and did not reduce the levels of androgen and gonadotropins. In contrast, 30mg of cloves improved insulin, androgen, and gonadotropin levels. In this regard, other studies have suggested the opposite effects of high doses over low doses of clove oil on the reproductive system (15). The findings of our study also indicated an increase in apoptosis index in antral and preantral follicles in PCOS rats. In the study, treatment with 30 mg, unlike 60 mg clove extract, improved the number of follicles and reduced the apoptosis index. The results of the treatment with 30 mg may be due to an improvement in the hypothalamic-pituitary-ovarian axis and an approximate return to normal levels of gonadotropins. Since clove has an inhibitory effect on the expression of autophagic genes, including Beclin and Lc3, it has been reported to be effective as an anti-autophagic drug in the treatment of influenza, a viral infection (38).

In this study, there was a decreased autophagy activity, with special significance in the clove-treated group. This decrease in autophagy activity was associated with normalized insulin and blood glucose levels. The present results further suggest that clove may indicate a good therapeutic strategy in the treatment of autophagy-related PCOS parameters. In addition, we found that an orally administered clove reduces LH/FSH levels as well as the abnormal secretion of androgen levels. Since autophagy plays an important role in PCOS-dependent metabolism, this study was designed to determine whether the improvement of PCOS parameters was the result of inhibition of autophagy by cloves. As one control group, we used a gonadectomy group (to eliminate the source of autophagy). We found that improvement in PCOS parameters (oxidative stress, insulin, blood glucose, gonadotropins, and testosterone levels) occurred after the removal of the source of autophagy (ovarian tissue). Therefore, clove may help to improve the parameters by regulating autophagy and inhibiting excessive autophagy. However, the direct role of this extract in regulating the parameters such as oxidative stress, insulin, and androgens cannot be ignored (39, 40).

Conclusion

This study suggests that gonadectomy may be an appropriate method for women who are not prone to fertility; however, pharmacological properties of low-dose clove as a metabolic regulator may be beneficial for women who are prone to fertility.

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Authors' Contributions

M.S., M.M., M.Sh.; Planned and designed the experiments. S.-H.A.-E., M.Sh.; Performed the experiments. S.-A.E., M.Sh., M.S.; Analyzed the data. M.Sh.; Wrote the manuscript. M.S., M.Sh., S.-H.A.-E., M.M., R.M.; Reviewed and discussed the data. All authors read and approved the finan manuscript.

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Evaluation of Early Frozen Blastocyst Transfer in A True Natural Cycle Protocol in Comparison to A Hormone Replacement Protocol: A Single-Center Cohort Study

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Abstract

Objective: Timing of frozen embryo transfer (FET) within a purported window of implantation is of increasing interest, and there is a paucity of evidence surrounding the transfer of frozen embryos early within these frozen embryo transfer protocols. This study aimed to evaluate whether live birth rates were equivalent after FET of blastocysts 4 days after luteinizing hormone (LH) surge in a true natural cycle protocol, compared to a hormone replacement (HR) protocol.

Materials and Methods: Single-centre, retrospective cohort study involving patients undergoing autologous frozen blastocyst transfer from January 1st, 2013, to December 31st, 2016. Cycles were grouped according to their protocol: true natural cycle (hormonal detection of LH surge with FET scheduled four days later) versus HR cycle (luteal phase gonadotropin-releasing hormone agonist suppression, oral or vaginal estradiol and intramuscular progesterone starting five days before FET). A total of 850 cycles were included, 501 true natural cycles and 349 HR cycles. The primary outcome was the live birth rate, secondary outcomes included clinical pregnancy rate and miscarriage. Log-binomial regression models were performed adjusting for a priori selected variables.

Results: Adjusted resulted in live birth rates of 38.7 and 40.4%, [adjusted risk ratio (aRR): 0.96, 95% confidence interval (CI): 0.76-1.22, P=0.729] in the natural cycle and HR groups, respectively. The secondary outcome analyses did not demonstrate any statistically significant difference in the rate of positive human chorionic gonadotropin (hCG), clinical intrauterine pregnancy rate, or miscarriage rate.

Conclusion: The timing of the FET four days after LH surge in a true natural cycle protocol results in equivalent live birth rates compared to a HR protocol. Results of this study suggest that the window of implantation within the natural cycle may be less finite than currently believed and further prospective studies evaluating the timing of frozen embryo transfer are warranted.

Keywords: Blastocyst, Embryo Transfer, Hormone Replacement, Natural Cycle

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Introduction

Overall rates of frozen embryo transfer (FET) have increased over time, likely as a result of more efficient cryopreservation strategies, increased number of good quality embryos following elective single embryo transfer policies, and elective freeze-all protocols (1). Many publications, including a recent meta-analysis, have reported that FET pregnancy rates may be superior to fresh, however, two recent large randomized control trials produced conflicting results and this remains to be elucidated (1-4). Several protocols exist for FET and it is not possible to identify one method as superior to another (5).

The two most employed protocols include the natural cycle (NC) and hormone replacement (HR) approach-

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es. In the NC approach, the FET is timed to ovulation in the patients' own cycle, often divided into 'true NC', where ovulation is allowed to occur spontaneously, or 'modified NC' (mNC) where ovulation is triggered with human chorionic gonadotropin (hCG) administration. In the HR approach, the patient is administered exogenous hormones and the FET is timed to the duration of exogenous progesterone. Typical HR protocols use progesterone supplementation for the equivalent number of days before transfer as the stage of development of the embryo is transferred (ie. 5 days for a day 5 blastocyst) (6). Pregnancy rates are lower, and the risk of early pregnancy loss is higher when transfer and implantation occur after greater than 6 days of progesterone administration for a day 5 blastocyst transfer (7-9). Conversely, there is a paucity of evidence evaluating the shorter duration of progesterone exposure. Given the relative importance of the outcomes associated with differing durations of progesterone exposure, it is of critical importance that this factor should be taken into consideration (10, 11).

We present a retrospective cohort analysis of an NC protocol with FET 4 days after luteinizing hormone (LH) surge and an HR protocol with transfer on the 5th day of progesterone administration from a single centre. Our study aimed to evaluate if live birth rates are equivalent between these two protocols. We will comment on the comparability of pregnancy and live birth rates to those reported after more standard FET protocols, given the paucity of evidence surrounding the early transfer.

Materials and Methods

Study population

Patients who started a frozen embryo transfer cycle between January 1st, 2013, and December 31st, 2016, at the Ottawa Fertility Centre in Ottawa, Ontario, Canada, were eligible for inclusion. The average age of patients at the time of FET was 34.7 years and the average body mass index (BMI) of patients included in this study was 24.4. Patients were identified through an in-house medical record system, and clinic linkage to the Canadian Assisted Reproductive Technologies Register (CARTR Plus) provided birth outcome data, which has been previously validated (12). The study protocol was reviewed by the Ottawa Health Science Network Research Ethics Board (OHSN-REB) and deemed exempt from OHSN-REB review as a quality improvement initiative. Data was housed on a local secure server and analysis was available only to study authors.

Patients were included in the analysis if they underwent FET with blastocysts cryopreserved by vitrification, created from their oocytes with either partner or donor sperm, whether embryos cryopreserved were surplus after fresh embryo transfer or were cryopreserved in a freeze-all cycle. Patients were excluded if donor oocytes or a gestational carrier were utilized. Vitrification of blastocysts occurred on day 5 unless the cycle included pre-implantation genetic testing at which point blastocysts were vitrified on days 5 and 6. The vitrification-warming method was carried out using RapidVit and RapidWarm Blast kits (Vitrolife) and the Rapid-i vitrification system (Vitrolife) in accordance with the manufacturer's instructions (13).

Blastocysts were graded based on Gardner's scoring system (14). At our clinic, only good and best quality blastocysts (B1-3 and greater) were selected for cryopreservation, unless exceptional circumstances prevailed. During the duration of the study period, approximately 35% of *in vitro* fertilization (IVF) cycles performed at our clinic had resultant embryos to freeze (whether as surplus after a fresh embryo transfer or in a 'freeze-all' protocol to avoid ovarian hyperstimulation syndrome or in the case of PGT). The number of embryos transferred in the cycle was at the discretion of the physician in discussion with the patient and was pre-determined at a follow-up appointment before the FET cycle. The decision to transfer 1 vs. 2 embryos was made with the patient by considering the patient's age, the number of prior embryo transfers, and patient factors posing an additional risk in pregnancy given multiple gestations, with a tendency at our clinic toward elective single embryo transfer.

Natural cycle frozen embryo transfer protocol

The "true NC" approach was employed at our centre throughout this study period, whereby ovulation occurs spontaneously and was not triggered with exogenous hormones. Women were considered candidates for NC protocol if they had regular menstrual cycles, ranging in length between 27-32 days, a mid-luteal phase serum progesterone ≥30 nmol/L typically measured 6-8 days post urinary LH surge, and there was no luteal phase concern (ie. luteal phase spotting, or evidence of a short luteal phase). A patient's age and BMI were not considered as inclusion or exclusion criteria. The protocol involved daily serial morning bloodwork sampling for estradiol and LH, typically started 3-4 days prior to the expected LH surge, until the LH surge was observed. The LH surge was defined as the attainment of a serum LH ≥30 IU/L with a dropping estradiol, or the highest-level LH ≥30 IU/L given that a dropping serum estradiol was not a strict criterion. The day on which this was observed was considered day 0 of the cycle, as is standard within the FET literature (15).

Once a surge was identified, a pelvic ultrasound was performed to obtain a measurement of endometrial thickness. After a documented LH surge and endometrial thickness ≥7 mm, embryo transfer was scheduled on day 4. Exogenous progesterone was not administered for luteal phase support. If a patient did not meet these criteria, the cycle was cancelled, and the patient was scheduled for a follow-up with their physician to discuss

either another attempt at the NC protocol or switching to an HR protocol.

Hormone replacement frozen embryo transfer protocol

Patients were selected for HR FET if they did not meet the criteria for NC as outlined above, or if they elected to proceed with this approach for other reasons (ie. ease of scheduling and fewer visits for bloodwork and ultrasound). Gonadotropin-releasing hormone (GnRH) agonist pre-treatment was employed as a standard of care throughout this study period (Abbvie, Lupron depot, leuprolide acetate 3.75 mg intramuscular), which was administered prior to the onset of menses. Estrogen priming with an escalating oral or vaginal micronized estradiol (Acerus Pharmaceuticals Corportation, Estrace, 17β-estradiol tablets) administration was started between menstrual cycle days 3-5. Transvaginal estrogen administration proceeded as follows: 0.5 mg twice daily for 6-10 days, 1mg twice daily for 5-8 days, and 2mg three times daily for 5 days for a total of 16-23 days of estrogen prior to the ultrasound evaluation of endometrial thickness and serum estradiol and progesterone assessment. If patients met the requirements of the endometrial lining of ≥7 mm, serum estradiol ≥650 pmol/L, and progesterone <5 nmol/L, they were advised to start progesterone in oil IM 50 mg daily. The embryo transfer was scheduled for four days after the progesterone was begun. In cases of inadequate endometrial thickness or serum estrogen, ongoing estrogen supplementation, typically for an additional week at the same or higher doses, was employed. Endometrial thickness and serum estradiol were re-checked after additional estrogen and if adequate, progesterone was commenced, and FET scheduled. If inadequate, the cycle was either cancelled, or the patient could elect to proceed with progesterone and scheduling of FET after a discussion with the physician.

Embryo transfers were typically done between the hours of 10h00 – 13h00. The total number of hours of progesterone exposure with this protocol was 85-92. Estrogen and progesterone supplementation were then continued until either a negative serum pregnancy test or until 10 weeks' gestational age.

Outcome assessment

The primary outcome was live birth after FET. A live birth was defined as an infant born showing any signs of life, or at least ≥ 20 weeks' gestational age, or weighing 500 grams. Secondary outcomes included rate of positive serum hCG, clinical intrauterine pregnancy, miscarriage, ectopic and stillbirth pregnancy. Serum hCG was measured approximately 14 days after ET, and measurements ≥ 5 IU/L were considered positive. Clinical intrauterine pregnancy was defined as the presence of a gestational sac and yolk sac on transvaginal ultrasound. Miscarriage was defined as a birth outcome where a clinical pregnancy was diagnosed but no fetus development could be seen at <20 weeks' gestation. Stillbirth was defined as a pregnancy loss at ≥ 20 weeks' gestation.

Statistical analysis

Patient and cycle characteristics were described using frequencies and proportions for categorical variables and statistical comparisons were done with Fisher Exact test for non-parametric data and Chi-square for parametric data. We described normal continuous variables using means and standard deviations and compared groups using a two-sided t test. Overall live birth, positive hCG, clinical intrauterine pregnancy, miscarriage, ectopic and stillbirth pregnancy rates were compared between the two groups. We fit a multivariable log-binomial regression model with a priori variables for the primary and secondary outcomes, adjusting for patient age at oocyte retrieval, body mass index, polycystic ovarian syndrome (PCOS) or other ovulatory disorder as an indication for treatment, and the number of blastocysts transferred. Adjusted risk ratios with 95% confidence intervals were performed.

To detect a difference of 10% in the live birth rate between the two groups from a baseline of 35%, a sample size of 329 was required per group, with a power of 80% and an alpha of 0.05. A P<0.05 was considered statistically significant. Statistical analyses were performed using SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC).

Results

There were 850 frozen embryo transfer cycles from 614 patients between January 1st, 2013, and December 31st, 2016 meeting the inclusion criteria for this study. Demographic characteristics are presented in Table 1. Of the included cycles, 501 were from 354 patients within the NC group and 349 were from 267 patients within the HR group (there was a small amount of crossover between the two groups). The difference in the number of patients with more than 1 cycle included within each group was not statistically significant. There was a greater average BMI (P=0.023) and a higher percentage of patients with a diagnosis of the PCOS in the HR group [risk ratio (RR): 1.32, 95% confidence interval (CI): 1.24-1.41, P<0.001]. There were also more patients with a diagnosis of tubal factor (RR: 1.39, 95% CI: 1.01-1.92, P=0.048) and endometriosis (RR: 1.68, 95% CI: 1.02-2.79, P=0.018) within the NC group. The groups did not differ with respect to the number of prior embryo transfers.

Adjusted risk ratios for the primary outcome of live birth and the secondary outcomes are presented in Table 2. We found no significant difference between the NC and HR groups for the primary outcome or any of the secondary outcomes.

We performed a post-hoc sensitivity analysis stratifying each group by age. We found no statistically significant difference in the primary outcome of live birth rate between the two groups (P=0.729, Table 3).

Table 1: Baseline characteristics for the NC group versus the HR group

Demographic	NC group (n=501)	HR group (n=349)	P value
Age at FET (Y)	34.9 ± 3.7	34.4 ± 3.9	0.051
Age at oocyte retrieval (Y)	33.9 ± 3.7	33.5 ± 3.9	0.096
Body mass index (kg/m^2)	24.1 ± 4.1	24.8 ± 4.4	0.023
Prior pregnancies			0.048
0	198 (39.5)	114 (32.7)	
1	189 (37.7)	127 (36.4)	
≥2	114 (22.8)	108 (30.9)	
Prior births			0.123
0	314 (62.7)	233 (66.8)	
1	165 (32.9)	96 (27.5)	
≥ 2	22 (4.4)	20 (5.7)	
Indication for treatment			
Unexplained infertility	40 (8.6)	25 (7.6)	0.590
Male factor	349 (75.4)	234 (70.9)	0.160
Tubal factor	91 (19.7)	47 (14.2)	0.048
Endometriosis	47 (10.2)	18 (5.5)	0.018
PCOS/Other ovulatory disorder	6 (1.2)	102 (29.2)	<0.001
Other	12 (2.6)	7 (2.1)	0.669
Missing	38 (7.6)	19 (5.4)	0.220
Number of prior fresh cycles of IVF			0.849
1	355 (70.9)	241 (69.0)	
2	88 (17.6)	61 (17.5)	
≥3	58 (11.5)	47 (13.5)	
Number of prior ETs			0.059
0	70 (14.0)	69 (19.8)	
1	213 (42.5)	141 (40.4)	
≥2	218 (43.5)	139 (39.8)	
The number patients within the group with more than 1 FET cycle included	107 (21.4)	63 (18.1)	0.067
Use of PGT-A	7 (1.4)	5 (1.4)	1.000
Number of embryos transferred			
1	420 (83.8)	284 (81.4)	0.597
2	79 (15.8)	64 (18.3)	
3	2 (0.4)	1 (0.3)	
Number of embryos transferred (continuous outcome)	1.2 (0.4)	1.2 (0.4)	0.389
Endometrial thickness	9.6 (2.0)	9.5 (2.1)	0.433

Continuous data are presented as mean ± standard deviation (SD), and categorical data are presented as number (%). NC; Natural cycle, HR; Hormone replacement, FET; Frozen embryo transfer, PCOS; Polycystic ovarian syndrome, IVF; *In vitro* fertilization, and PGT-A; Preimplantation genetic testing for aneuploidy.

Ninety-nine patients were age 40 or over at the time of embryo transfer (in contrast to at the time of egg retrieval). Within this group, 60 (60.6%) and 39 (39.4%) utilized the NC and HR protocols, respectively (P=0.698). Seventeen of 60 (28.3%) patients within the NC protocol group achieved a live birth, and 8 of 39 (20.5%) patients within the HR protocol group achieved a live birth, which was not statistically significant (P=0.382).

Table 2: Pregnancy outcomes for NC versus HR group

Outcome	NC group (n=501)	HR group (n=349)	aRR [95% CI]	P value
Live birth	194/501 (38.7)	141/349 (40.4)	0.96 [0.76-1.22]	0.729
Positive hCG	273/501 (54.5)	205/349 (58.7)	1.05 [0.86-1.29]	0.617
Clinical intrauterine pregnancy	236/501 (47.1)	174/349 (49.9)	0.98 [0.82-1.16]	0.781
Miscarriage	39/236 (16.5)	33/174 (19.0)	0.95 [0.51-1.77]	0.876
Ectopic pregnancy	5/273 (1.8)	7/205 (3.4)	0.85 [0.21-3.39]	0.813
Stillbirth (>20 weeks)	3/236 (1.3)	0/174 (0)	---	---

Values are numbers (%). All analyses were performed using log-binomial regression adjusted for age at the time of retrieval, body mass index, diagnosis of PCOS or other ovulatory disorder, and the number of embryos transferred. NC; Natural cycle, HR; Hormone replacement, PCOS; Polycystic ovarian syndrome, aRR; Adjusted risk ratio, CI; Confidence interval, and hCG; Human chorionic gonadotropin.

Table 3: Live birth outcomes for NC versus HR group, according to patient age at the time of oocyte retrieval

Age group (Y)	NC group (n=501)	HR group (n=349)	aRR (95% CI)	P value
<35 (n=494)	118/284 (41.6)	99/210 (47.1)	0.83 [0.66-1.05]	0.122
35-37 (n=208)	49/127 (38.6)	26/81 (32.1)	1.12 [0.70-1.80]	0.629
38-39 (n=90)	20/57 (35.1)	13/33 (39.4)	0.98 [0.46-2.09]	0.967
≥40 (n=58)	7/33 (21.2)	3/25 (12.0)	-	-

Values are numbers (%). All analyses were performed using log-binomial regression adjusted for age at the time of retrieval, body mass index, diagnosis of PCOS or other ovulatory disorder, and the number of embryos transferred. NC; Natural cycle, HR; Hormone replacement, aRR; Adjusted risk ratio, and CI; Confidence interval.

Discussion

Patients who underwent a true natural cycle FET with transfer four days after LH surge did not have lower live birth rates compared to patients who underwent a hormone replacement FET with transfer on the 5th day of progesterone administration. This study demonstrates similar live birth outcomes when embryo transfer occurs relatively early within a true NC protocol, compared to the literature recommendation of transfer timing within this protocol (15). Results of this study suggest that it is likely that the purported 'window of implantation' may therefore include timing with a shorter duration of progesterone exposure.

The control group in this study was a HR protocol employing FET on the 5th day of progesterone administration. Standard HR embryo transfer protocols recommend transfer after progesterone exposure equivalent to the development of the embryo (6 days when considering a blastocyst) or less 1 day, as evidence

suggests these are equivalent (7, 8). The utilization of this HR protocol is additionally supported by the fact that pregnancy and live birth rates are similar to recent reports of FET on the 6th day of progesterone (16-18). There is a possibility that a shorter duration of progesterone exposure may be associated with an increased risk of miscarriage (19), however, within this study, we observed a low risk of miscarriage which did not differ between the two protocols.

We observed a slightly higher average BMI and a greater percentage of patients with a diagnosis of ovulatory disorder (PCOS) within the HR group, which was expected given that irregular menstrual cycles are an indication of a medicated FET cycle. While the higher average BMI in the HR group was statistically significant, this difference of 0.7 between the two groups may arguably not be clinically relevant. We do know that differences in BMI are linked to pregnancy outcomes, and BMI was taken into account as a confounder during the log-binomial regression analysis. Patients in the NC group were more likely to have a diagnosis of tubal factor or endometriosis, which was also expected given that these are anatomical factors that do not impact cycle regularity. We do not feel that these differences would have had a clinically important impact on the study outcomes.

The results of this study are highly generalizable given the limited exclusion criteria, representation of patients from all infertility diagnoses, and comparable proportions of natural cycles and HR protocols utilized. Additional strengths of this study include the large sample size, adjustment for important confounders including age, BMI, endometrial thickness, and the inclusion of a relatively large number of women over the age of 40 at the time of embryo transfer.

Our data suggest that a true NC may be a reasonable approach among women over the age of 40. This contrasts studies demonstrating a lower chance of live birth among patients greater than 40 years of age undergoing natural cycle FET compared to hormone replacement FET, and recent recommendations for a modified natural protocol (using hCG to trigger ovulation) in women over the age of 40 (16, 20). We need to interpret these last results with caution, as this was a secondary analysis of a much smaller sample size.

The main limitation of this study is its retrospective nature, the inherent selection bias, and confounding not addressed by statistical analysis. Additionally, it would be ideal to compare early transfer within a natural cycle FET protocol to more 'standard' transfer timing within the NC protocol for optimal evaluation of the early timing, however as this is not our standard practice this control group was not available. Finally, the outcomes of both groups in this study may represent a 'better prognosis' patient population given that only good and best quality embryos, based on the Gardner scoring criteria, are selected for freezing at our institution. However, this study does add to the literature given the substantial paucity of outcomes surrounding any transfer early within the purported window of implantation.

As a result of evidence indicating possible increased pregnancy rates and decreased maternal and neonatal morbidity among pregnancies conceived through FET relative to fresh transfer, it is likely we will continue to see an increase in frozen embryo transfer cycles (1, 21, 22). The NC approach is purported to have several benefits as it involves less (or no) medication, lower cost, and less discomfort for the patient (15, 23). Additionally, emerging evidence suggests that NC transfers, related to the presence of the corpus luteum, are associated with lower rates of pregnancy complications including hypertensive disorders of pregnancy, postpartum hemorrhage, macrosomia, and post-term birth (24, 25). Given that the optimal protocol within the NC has yet to be elucidated, further research in this area is required.

Conclusion

Timing of the FET four days after LH surge in a true NC protocol results in equivalent live birth rates compared to a HR protocol. The results of this study suggest that the window of implantation for frozen embryo transfer within the NC may be less finite than currently believed. When considering the probable future increase in the use of natural cycle FET protocols to optimize patient experience and pregnancy outcomes, these results fuel further important queries, specifically the need for prospective studies surrounding transfer timing within the NC protocol.

Acknowledgments

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Authors' Contributions

J.G., D.Sh., V.B., M.-C.L.; Involved in the conception, and design of the manuscript, data acquisition, or review, contributed to the interpretation of the data, as well as drafting and revising the manuscript and approved the final draft. J.G., V.B.; Completed the data analysis. All authors read and approved the final manuscript.

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Effects of Date Palm Pollen Supplementations on The Expression of *PRDX1* and *PRDX6* Genes in Infertile Men: A Controlled Clinical Trial

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Abstract

Background: Accumulating evidences suggest that date palm pollen (DPP) induces antioxidant activity and improves semen parameters in male rats. However, there is a few scientific evidences in support of the DPP effects on human male fertility. Hence, the effect of oral consumption of DPP on sperm parameters and expression pattern of *Peroxiredoxin-1* (*PRDX1*) and *Peroxiredoxin-6* (*PRDX6*) genes was evaluated in men with infertility.

Materials and Methods: The current controlled clinical trial included 40 men with infertility (DPP group) and 10 normospermic fertile men as controls. The DPP group received gelatinous capsules of DPP (400 mg/kg) for 74 days. Semen sampling was done before and after treatment in the both groups. Semen analysis and 8-isoprostane concentration assessments were performed by computer-assisted sperm analysis and ELISA methods, respectively. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays were employed to explore expression of *PRDX1* and *PRDX6* genes.

Results: DPP consumption significantly improved semen volume (P=0.030), count (P<0.001) and morphology of sperm (P=0.023). Concentration of 8-isoprostane was significantly decreased after intervention in the DPP group (P<0.001). DPP consumption led to a significant elevation in the expression of *PRDX1* and *PRDX6* genes (P<0.001). Elevated gene expression of *PRDX6* and *PRDX1* was positively correlated with improved parameters of sperm including count, volume, motility and morphology.

Conclusion: Taken together, DPP seems to promote sperm quality through a decrease in reactive oxygen species (ROS) by increasing expression of antioxidant genes. Further large-scale studies are required to challenge this hypothesis (registration number: IRCT2015021221014N2)

Keywords: 8-Isoprostane, Male Infertility, *PRDX1*, *PRDX6*

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Introduction

Oxidative stress (OS) has been described as an important factor in the etiology of male infertility resulting from the excess generation and/or defective scavenging of reactive oxygen species (ROS) in the reproductive system (1). Spermatozoa have a finely balanced oxidant-antioxidant system to maintain ROS levels within the normal range (2). Under physiological conditions, an ROS generating enzymatic system provides low amounts of ROS required for sperm maturation and function (2, 3). It appears that the mitochondrial respiratory chain in mature and immature semen spermatozoa, leukocytes and environmental pro-oxidants are the primary sources of the excessive amount of ROS which in turn affects plasma membrane fluidity and DNA integrity through oxidation and peroxidation (4). An array of antioxidant enzymes and free radical scavengers neutralized the excessive ROS and enabled sperms to tolerate OS (5).

Peroxiredoxins are a family of thiol-specific enzymes that can function as antioxidants by eliminating harmful effect of excessive peroxynitrite (ONOO-) and hydrogen peroxide (H_20_2) (6). To date, six isoforms of peroxiredoxins have been identified in spermatozoa which are abundantly expressed in various subcellular compartments of these

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cells (5, 7). Peroxiredoxin-1 (PRDX1) enzyme contains two cysteine residues at the active site which are needed for its catalytic function (5). PRDX1 localized in the nucleus, the equatorial region and the flagellum of spermatozoa that highlights the antioxidant potential of PRDX1 in protecting the mitochondrial complex structures against OS (7). Peroxiredoxin-6 (PRDX6) is the most abundant isoform of peroxiredoxins found in all subcellular parts of the sperm and contains only one cysteine residue at its active site (5). Glutathione is the physiological electron donor to PRDX6 contributing to the neutralization of H2O2 and ONOO- in human sperms (8). Moreover, it has been documented that peroxiredoxins are involved in different cell signaling cascades (9). Owing to these critical roles, it is not surprising that dysregulation of *PRDX1* and *PRDX6* gene expressions and/or their function may alter normal sperm function and lead to male infertility (7, 10-13). In the past decades, accumulated evidences have demonstrated that date palm pollen (DPP) supplementation could positively impact sperm parameters and contribute to the improvement of fertility in male rats (14-17). Moreover, due to the presence of phenolic compounds, flavonoids and anthocyanins, DPP can induce expression and activity of antioxidant genes (18-20).

However, antioxidant effect of DPP on human sperm parameters and its mode of action to improve semen parameters have not been understood and well-documented. This hypothesis raised the point that perhaps DPP supplementation can reduce ROS levels, particularly through peroxiredoxins genes. Thus, deciphering this link likely provides insight into one of the molecular mechanisms of DPP on improvement of semen parameters in men with infertility. We designed the present attempt to explore influences of DPP on semen parameters and reduction of ROS level by quantifying expression of peroxiredoxins genes as well as 8-isoprostane, a marker for oxidative stress.

Materials and Methods

Ethics statement

We received approval for our study from the Institutional Review Board/Independent Ethics Committee (IRB/IEC) of Hormozgan University of Medical Sciences, Bandar Abbas, Iran (IR.HUMS.REC.1394.201). All clinical trial protocols were registered in Iranian Registry of Clinical Trials (IRCT2015021221014N2). According to the Declaration of Helsinki of World Medical Association, all participants signed an informed written consent after receiving full explanation on the study procedures and objectives.

Study design

This comparative clinical trial (before-after clinical trial study) consisted of infertile men admitted to the Omm-e-Leila Fertility and Infertility Center of Bandar Abbas (Iran) between January 21 and June 22, 2016. A total of 40 men with infertility were recruited by convenience sampling (the DPP group). Men with systemic or genetic conditions, abnormalities in reproductive tracts, alcohol and substance abuse, testicular trauma, and use of fertility drugs in the past six months were excluded from the study. The control group (n=10) were selected from fertile volunteers fathered a child during the past two years, with normal semen analysis to comparably evaluate rate of changes in expression of the studied genes, in order to reach this answer how much expression of the genes in DPP-treated infertile men averagely became near to normal men.

Details of participant enrollment, allocation and analysis are demonstrated in the CONSORT flow diagram (Fig.1). The DPP group were treated with 400 mg/kg DPP powder (21) in gelatinous capsules every day for 74 consecutive days and all participants completed the treatment course. Semen samples were obtained twice from participants in both groups: before and after the treatment period. Expression of the studied genes, ROS measurement and semen analysis were blindly accomplished by another one, who did not aware about the samples.

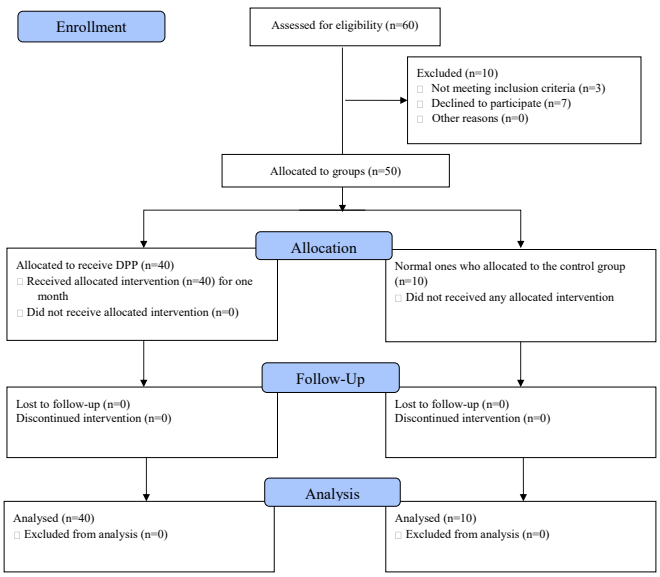

Fig.1: Consort flow chart.

Semen analysis and isolation of spermatozoa from seminal fluid

After a 3-5 days abstinence cycle, fresh semen was sampled via masturbation in sterile plastic vessels, followed by liquefying for half an hour. Initial analysis of semen was done immediately according to the sperm quality analyzer IIC (United Medical Systems Inc., USA) according to the world health organization (WHO) protocols (22, 23). The spermatozoa were purified by Goodrich protocol (24), and then the samples were washed twice with BSA-free Ham's-F10 medium and stored in Qiagen RNA later solution (Germany) at -80°C awaiting extraction of RNA.

Purification and measurement of free 8-Isoprostane

Isoprostane is a free radical-catalyzed peroxidation of essential fatty acids. It was measured as marker for OS in sperms. Free 8-isoprostane was purified in duplicate with the aid of Affinity Column (Cayman Chemical, USA). For precipitate isolation, specimens were all initially centrifuged with 15000 g, followed by diluting the supernatant in the presence of column buffer (at 1:5); the next steps were performed on the basis of manufacturer's instructions. Fifty mili-liters of specimens were applied to assess concentration of 8-isoprostane via an enzyme-linked immunosorbent assay reader (STAT FAX 2100; Awareness Technology Inc, USA) at a wavelength of 405 nm.

cDNA synthesis and quantitative reverse transcription polymerase chain reaction

The manufacturer's protocol was followed to extract total RNA from the specimens through the NucleoSpin® RNA Midi kit (Macherey-Nagel, Germany) and RNA yield was exposed to Thermo Scientific RNase-free DNase I (USA). The extracted RNA quantity and quality were evaluated by electrophoresis on agarose gel and NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, USA), respectively. Subsequently, based on the manufacturer's protocol, 1 µg of total RNA was reverse transcribed to cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Quantitative reverse transcription PCR (qRT-PCR) was run in duplicate with the aid of gene-specific primers and Tli RNaseH Plus SYBR® Premix Ex Taq™ II kit (Takara Bio Inc., Japan) through the Rotor-GeneTM 6000 system (Corbett Research, Australia) based on the guidelines of manufacturer. The utilized primers for qRT-PCR assays were:

PRDX1-
F: 5'-CAAAGCCACAGCTGTTATGC-3'
R: 5'-GAGAATCCACAGAAGCACC-3′
PRDX6-
F: 5'-CTTTGAGGCCAATACCACCG-3'
R: 5'-AGATGGTCCTCAACACTGTC-3′
and *β-actin*-
F: 5'-ATGGAATCCTGTGGCATCCA-3'
R: 5'-CGCTCAGGAGGAGCAATGAT-3′.

Primary and secondary endpoints

Our primary endpoint was the impact of DPP on the gene expression of *PRDX1* and *PRDX6*, while our secondary endpoint was the impact of DPP on the parameters of sperm.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences version 22.0 (SPSS Inc., USA) software and $P<0.05$ was considered statistically significance level. Chi-square and paired t test were performed to compare sperm parameters before and after treatment. mRNA expression levels of *PRDX1* and *PRDX6* genes were compared in the DPP group before and after treatment with healthy individuals using the qRT-PCR. Gene expression ratio was determined via the method of $2^{-\Delta\Delta ct}$. Kolmogorov Smirnov normality test showed the abnormal distribution of *PRDX1* and *PRDX6* gene expression levels and thus, non-parametric tests were used for comparison. The Wilcoxon test was run to compare expression levels of *PRDX1* and *PRDX6* before and after treatment within the groups. Expression level of the genes was compared after treatment between groups, by a two-tailed Mann-Whitney U-test. Association of variable sperm indices and free 8-isoprostane levels with expression levels of *PRDX1* and *PRDX6* genes were clarified via chi-square and one-way ANOVA tests. Spearman's correlation test was used to determine correlations of *PRDX1* and *PRDX6* expressions with the variable sperm parameters and free 8-isoprostane levels.

Results

Clinical and demographic profiles of the DPP group and healthy controls are shown in Table 1.

Table 1: Demographic and clinical characteristics of the cases and healthy controls

Demographic variable	Cases		Controls	
	n (%)	Mean ± SD	n (%)	Mean ± SD
Age (Y)				
≤30	30 (75)	29.3 ± 3.21	4 (40)	27.25 ± 1.70
>30	10 (25)	33.25 ± 3.65	6 (60)	32.83 ± 2.04
Weight (Kg)	40 (100)	88.1 ± 11.47	10 (100)	86.40 ± 10.80
Height (M)	40 (100)	1.66 ± 0.08	10 (100)	1.67 ± 0.08
BMI (kg/m^2)				
Normal (<25)	4 (10)	23.45 ± 0.86	2 (20)	23.22 ± 0.43
Overweight (25≤ - <30)	8 (20)	27.65 ± 1.19	2 (20)	27.50 ± 0.73
Moderately obese (30≤ - <35)	19 (75.5)	32.57 ± 1.57	4 (40)	32.30 ± 1.58
Severely obese (35≤ - <40)	4 (10)	37.09 ± 1.31	2 (20)	37.64 ± 1.85
Very severely obese (≥40)	5 (12.5)	43.75 ± 2.50	-	-

BMI; Body mass index.

DPP supplementation significantly improved count and morphology of sperm, as well as semen volume

The findings of this study demonstrated a significant enhancement in semen indices like semen volume, sperm count and sperm morphology after treatment with DPP (Table 2), while differences of the other parameters including viscosity, liquefaction, pH, appearance and motility of sperm before and after DPP treatment were not statistically significant. Comparison of sperm parameters of the DPP group with healthy controls after treatment revealed significant differences in semen parameters including count, volume, motility and morphology (Table 3).

Table 2: The comparison of sperm parameters and free 8-Isoprostane of the infertile participants before and after treatment

Variable	Before treatment		After treatment		P value†
Sperm parameters	**n (%)**	**Mean ± SD**	**n (%)**	**Mean ± SD**	
Count					**<0.001**
Normal (≥15 million/ml)	10 (25)	16.75 ± 1.35	31 (77.5)	45.90 ± 28.79	
Abnormal (<15 million/ml)	30 (75)	5.29 ± 3.95	9 (22.5)	5.98 ± 3.59	
Volume					**0.030**
Normal (≥1.5ml)	35 (87.5)	3.32 ± 1.27	40 (100)	3.72 ± 1.30	
Abnormal (<1.5ml)	5 (12.5)	1.18 ± 0.16	-	-	
Appearance					0.320
Normal	39 (97.5)	-	40 (100)	-	
Abnormal	1 (2.5)	-	-	-	
Viscosity					0.320
Normal	39 (97.5)	-	40 (100)	-	
Abnormal	1 (2.5)	-	-	-	
Liquefaction					0.562
Normal	39 (97.5)	-	39 (97.5)	-	
Abnormal	1 (2.5)	-	1 (2.5)	-	
pH					0.166
Normal	39 (97.5)	7.72 ± 3.22	40 (100)	7.64 ± 0.16	
Abnormal	1 (2.5)	7.00	-	-	
Motility					0.154
Normal (≥40%)	13 (32.5)	46.69 ± 12.01	13 (32.5)	53.15 ± 11.67	
Abnormal (<40%)	27 (67.5)	23.55 ± 8.42	27 (67.5)	27.51 ± 7.48	
Morphology					**0.023**
Normal (≥30%)	5 (12.5)	42.40 ± 4.27	8 (20)	46.25 ± 5.82	
Abnormal (<30%)	35 (87.5)	23.40 ± 7.75	32 (80)	27.09 ± 6.71	
Free 8-Isoprostane (ηg/ml)	40 (100)	3.84 ± 3.90	40 (100)	1.76 ± 1.64	**<0.001‡**

Bold values indicate statistically significant differences (P<0.05). †; P value were calculated by chi-square test and ‡; P value were calculated by t test.

DPP supplementation significantly reduced OS marker concentration

Mean free 8-isoprostane concentration before treatment in the DPP group and healthy controls were 3.84 ± 3.89 and 1.62 ± 0.65, respectively. Seminal plasma concentrations of free 8-isoprostane were significantly higher in the DPP group before treatment, by comparing with the control group (P<0.001, Tables 2, 3). Free 8-isoprostane concentration was significantly decreased in the DPP group after treatment with DPP (Table 2). Moreover, after treatment, concentration of this marker was higher in the DPP group, compared to the control group, while it was not statistically significant (P=0.087, Table 3). The probable correlation was explored between 8-isoprostane concentration and seminal parameters. The reduced concentration of 8-isoprostane displayed a negative correlation with sperm count after treatment with DPP (r=-0.360, P=0.001), while it indicated no correlation with the other parameters of semen.

Table 3: The comparison of sperm parameters and free 8-Isoprostane levels of the date palm pollen (DPP)-treated patient group (after treatment DPP-group) with healthy controls

Variable	DPP-treated patient	Normal Controls	P value†
Sperm parameters			
Count (million/ml)	36.92 ± 30.41	81.90 ± 22.18	**<0.001**
Volume (ml)	3.72 ± 1.30	2.75 ± 0.95	**0.032**
pH	7.64 ± 0.16	7.67 ± 0.27	0.685
Motility	35.85 ± 15.07	61.50 ± 12.86	**<0.001**
Morphology	30.92 ± 10.10	61.60 ± 10.41	**<0.001**
Free 8-Isoprostane (ηg/ml)	1.76 ± 1.64	1.62 ±0.65	0.087

Data presented as mean ± SD. Bold values indicate statistically significant differences (P<0.05). †; P value were calculated by independent t test.

DPP supplementation significantly elevated the antioxidant gene expression

Comparison of the gene expression levels demonstrated that *PRDX1* and *PRDX6* mRNA was significantly

overexpressed in infertile individuals compared to the healthy controls (P<0.001, Fig.2A, B). By comparing the expression level of *PRDX1* in the DPP group before and after treatment, a significant increase was seen after treatment (P<0.001, Fig.2A); nevertheless, the difference between the study groups after treatment was not statistically significant, regarding mean PRDX1 expression levels (P=0.188, Fig.2A). The mRNA expression level of *PRDX6* was increased significantly in the DPP group after treatment (P<0.001, Fig.2B). Comparison of *PRDX6* gene expression between the DPP group and healthy individuals after treatment indicated a lower (but not statistically significant) mRNA expression level in the healthy controls, compared to DPP group (P=0.577, Fig.2B).

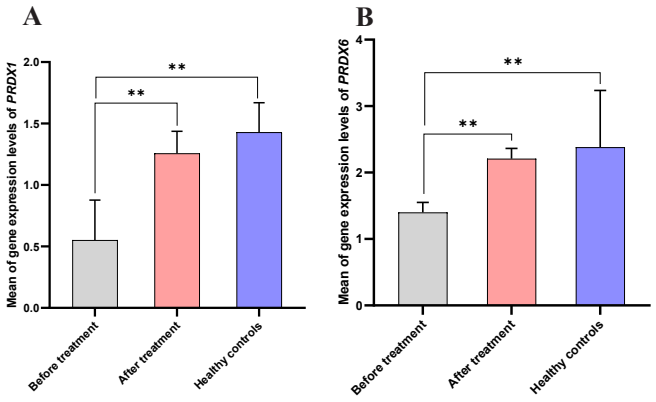

Fig.2: Mean of gene expression levels of *PRDX1* and *PRDX6* in cases before and after treatment, and the healthy controls. **A.** Upregulation of *PRDX1* expression in cases before treatment compared with cases after treatment and healthy controls. **B.** Upregulation of *PRDX6* expression in cases before treatment compared with cases after treatment and healthy controls. **; Indicate statistically significant differences(P<0.05).

The increased expression of PRDX1 and PRDX6 genes were correlated with improvement of semen parameters

To find a possible relationship between the altered gene expression and semen parameters in the participants after treatment, we evaluated correlation of *PRDX1* and *PRDX6* fold changes with sperm parameters and seminal plasma levels of free 8-isoprostane. We observed a significant association between the increased *PRDX1* expressions and improvement of semen parameters including count, motility, volume and morphology. Similarly, the increased expression of PRDX6 was significantly associated with the increased count, motility, volume and improved morphology of sperm (Table 4). Our findings showed a significant correlation of the altered *PRDX1* expression level with sperm motility and morphology. In addition, altered expression of the PRDX6 gene was significantly related to count, appearance, pH and morphology. Nonetheless, we did not find any significant correlation of the altered expression of *PRDX1* and *PRDX6* genes with decreased concentration of 8-isoprostane levels (Table S1, See Supplementary Online Information at www.ijfs.ir).

Table 4: Association of *PRDX1* and *PRDX6* gene expressions fold change with seminal fluid parameters

Variable	Expression of the genes		P value†
Sperm parameters	Before treatment	After treatment	
Mean expression level of *PRDX1*			
Count (million/ml)	0.5403	1.1813	**<0.001**
Volume (ml)	0.5600	2.4780	**0.036**
Motility	0.4504	1.0550	**0.001**
Morphology	0.5334	1.3189	**0.001**
Mean expression level of *PRDX6*			
Count (million/ml)	1.3627	2.2410	**0.003**
Volume (ml)	0.9600	2.5180	**0.028**
Motility	1.5370	2.2145	**0.047**
Morphology	1.3623	2.1518	**0.002**

Bold values indicate statistically significant differences (P<0.05). †; P value were calculated by Wilcoxon test.

Discussion

In the current study, we found that DPP supplementation led to significant improvement of sperm morphology, semen volume and sperm count in infertile men. We also found that expression of *PRDX1* and *PRDX6* genes was significantly upregulated in the DPP group compared to the healthy controls. Significant improvement in the sperm parameters of our study was consistent with several previously published studies (14, 15, 17, 25). In a study conducted by Rasekh et al. (25), a significant improvement in count, morphology and forward progressive motility of sperm was identified after consumption of DPP. Bahmanpour et al. (15) reported that DPP consumption led to a significant improvement of count, motility, morphology and DNA quality of sperm in male rate. Similar results were obtained from studies performed by Mehraban et al. (14) and Iftikhar et al. (17); they demonstrated a beneficial impact of DPP on sperm indices in male rats.

The observed positive effects of DPP on the parameters of sperm in our work and the other reports can be attributed to gonad-stimulating compounds such as estradiol, estrone and estriol present in DPP (26). Accordingly, it has been demonstrated that DPP consumption can improve testosterone, estradiol, follicle-stimulating hormone and luteinizing hormone levels, which led to increased weight of testis, epididymis, and sexual behaviors (15, 16, 27). Therefore, it seems that efficacy of DPP with respect to sperm parameters is at least in part due to the presence of its estrogenic components.

Since OS is a major contributor to male infertility, an antioxidant-based treatment strategy can be beneficial to restore fertility. In the past decades, there has been great interest in the use of herbal remedies, as natural antioxidant sources with minimum side-effects, to treat male infertility in developing countries (14, 28). From ancient times, DPP was used in traditional medicine of Egypt for enhancement of male fertility (28). Phytochemical screening revealed that DPP was a natural source of

minerals such as zinc, calcium and selenium, vitamins including A, B, C and niacin, phenolic components such as gallic, coumaric, ferulic, and protocatechuic acids, and flavonoids like luteolin, quercetin and apigenin (28-30). Having these components altogether, DPP can be considered as an antioxidant agent. Hence, we examined effect of DPP supplementation on sperm parameters. We also assessed its efficacy on 8-isoprostane concentrations and expression pattern of *PRDX1* and *PRDX6* genes in infertile men.

We found that concentration of 8-isoprostane (a marker for lipid peroxidation) was significantly higher in the infertile individuals compared to the normospermic healthy controls. Our finding were in agreement with those of Khosrowbeygi and Zarghami (31) who reported a high concentration of 8-isoprostane in males with asthenozoospermia, oligoasthenoteratozoospermia and asthenoteratozoospermia, by comparing with normospermic healthy controls.

In the current attempt, DPP-treated infertile males showed a significant reduction of 8-isoprostane concentration and this was correlated with a significant increase in sperm count. In line with our observation, previous investigations demonstrated a protective effect of DPP against cadmium and electromagnetic fields-induced OS in male rats (26, 32). Selenium is required for normal spermatogenesis and it serves as an antioxidant via the selenoprotein glutathione peroxidase which catalyzes reduction of lipid hydroperoxides and protects sperm cell membrane against oxidative damage (33). It has been reported that selenium supplementation led to improvement of sperm motility and successful induction of conception (33, 34). Antioxidant efficacy of zinc micronutrient in the improvement of sperm parameters of infertile men has also been previously reported (35). Therefore, containing selenium, zinc and vitamins, DPP has potential antioxidant properties to counteract excessive ROS. On the other hand, the aforementioned phenolic components and flavonoids can induce expression of nuclear factor-erythroid factor 2 (Nrf2) (19, 20, 36). Nrf2 encodes a transcription factor regulating the antioxidant gene expression via binding to the response elements in their promoters (37). In accordance with these data, it can be suggested that DPP induces high expression of antioxidant genes through Nrf2 transcription pathway and protects sperms from OS.

We found that expression level of PRDX1 and PRDX6 genes were significantly lower in the DPP group, by comparing with the healthy control group. In line with our finding, a decreased amount of PRDX1 and especially PRDX6 was detected in the men with idiopathic infertility in comparison with normospermic controls (13). Bumanlag et al. (38) demonstrated that absence of PRDX6 gene induced oxidization of lipid, proteins and DNA of mice spermatozoa which in turn led to an impairment of sperm motility and function. In the present study, DPP consumption resulted in significantly increased expression of the *PRDX1* and *PRDX6* genes in the infertile men. Increased expression of PRDXs was positively correlated with improved parameters of semen including count, motility, morphology and liquefaction in our study. Similarly, a significant association of *PRDX1* and *PRDX6* modifications with motility of sperm, DNA damage and lipid peroxidization was reported (13). There was no direct evidence to unveil the correlation between DPP consumption and increased expression of the PRDXs observed in our study, suggesting that phenolic and flavonoids components presented in DPP were responsible for the altered expression pattern of PRDXs. For example, Miyamoto et al. showed that quercetin could induce expression of Nrf2 and subsequently upregulate antioxidant *PRDX3* and *PRDX5* genes expression (37). However, further research are needed to disclose other potential pathways.

The current study limitation was its small sample size. Due to the limited resources for evaluation of gene expressions and the relatively unpleasant semen sampling for the participants, we were not able to include more individuals in the study. This limits the generalizability of our findings.

Conclusion

We found that DPP consumption has a positive effect on semen parameters in men with infertility. Moreover, our study was the first to demonstrate that DPP consumption could result in an increased expression of antioxidant *PRDX1* and *PRDX6* genes. However, the reason for this remains inexplicable and it should be further investigated.

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Authors' Contributions

K.M.; Supervisor, participated in study design, data collection, and drafting and statistical analysis. A.M.F.; Conducted data collection and contributed to all experimental work and manuscript draft preparation. S.F.; Contributed extensively in interpretation of the data and conclusion. Z.A., M.I.R.; Contributed to data collection and cooperate in molecular experiments. All authors are responsible for the final approval of the version to be published.

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Levels of DNA, Protein, Lipid Oxidation and Apoptosis Biomarkers in Semen of Men with Hyperviscous Semen: A Cross-Sectional Study

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Abstract

Background: Semen hyperviscosity is a threatening cause of abnormal spermatozoa and infertility in men. We aimed to evaluate oxidative stress, antioxidants depletion and sperm apoptosis as main reasons for poor quality of spermatozoa in men with hyperviscous semen.

Materials and Methods: In this cross-sectional study, ejaculate specimens were collected from fertile (n=102) and infertile men with hyperviscous semen (n=123) and without semen hyperviscosity (n=143). Total antioxidant capacity (TAC), glutathione (GSH), malondialdehyde (MDA), protein carbonyl (PC), 8-hydroxydeoxyguanosine (8-OHdG), and were measured in semen samples to estimate oxidative stress status. Gene expression pattern of *BAX*, *CASPASE-9*, *CASPASE-3*, and *BCL2* was assessed to estimate sperm apoptosis.

Results: The average of sperm count, normal morphology, normal motility, and sperm vitality in men with hyperviscous semen was significantly lower than infertile subjects without hyperviscous semen (P<0.01). Men with hyperviscous semen exhibited higher levels of PC (8.34 ± 1.03 nmol/mg vs. 6.01 ± 0.93 nmol/mg, P=0.008), MDA (1.14 ± 0.27 nmol/ml vs. 0.89 ± 0.22 nmol/ml, P=0.031), 8-OHdG (259.71 ± 24.59 ng/ml vs. 197.13 ± 18.47 ng/ml, P=0.009), but lower TAC contents (1250.44 ± 66.23 µM/L vs. 1784.31 ± 89.87 µM/L, P=0.018) and GSH (3.82 ± 1.05 µM vs. 5.89 ± 0.87 µM, P=0.021) than men with non-viscous semen. The expression of *BAX*, *CASPASE-3* and *CASPASE-9* genes in men with hyperviscous semen was significantly increased by 1.39-fold (P=0.041), 1.47-fold (P=0.046), 1.29-fold (P=0.048), respectively, as compared with the infertile subjects without hyperviscous semen. However, *BCL2* expression in infertile men without hyperviscous semen was higher compared to those with hyperviscous semen (1.36-fold, P=0.044).

Conclusion: Hyperviscous semen is markedly associated with depletion of seminal plasma antioxidants, sperm membrane lipid peroxidation, DNA and protein oxidation, and sperm apoptosis. Antioxidant therapy might be considered as a valuable strategy to protect sperm cells against oxidative damage in cases with seminal fluid hyperviscosity.

Keywords: DNA Oxidation, Lipid Peroxidation, Protein Oxidation

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Introduction

Human infertility is a main health problem that has affected many couples worldwide (1). Approximately 40-50% of all infertility cases are due to male factor infertility (2). In general, impaired spermatogenesis and poor sperm quality are important causes of male infertility (3). Therefore, identification of factors that impose either direct or indirect effects on human spermatogenesis and spermatozoa functioning, is essential. A wide range of risk factors from environmental pollutants and toxicants to epigenetic changes and genetic disorders have been identified that affect human spermatozoa and cause male infertility (4, 5). However, there are many risk factors with underlying mechanisms on sperm function and fertility potency that have remained unclear (3).

Semen hyperviscosity or failure in liquefaction of semen is an important cause of idiopathic male infertility (6). It is characterized by an unusual and very thick appearance of semen that can cause serious impairment of sperm maturation and function (7). Hyperviscous semen can also reduce sperm movement and subsequently inhibits normal sperm

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progression through the female genital tract (8). Until now, some studies have reported the adverse effects of semen hyperviscosity on sperm motility and count, but the underlying mechanisms, in which it affects sperm function remain unclear (9, 10). Since semen viscosity is a critical factor to achieve fertilization, in-depth studies are essential to consider the effect of semen hyperviscosity and its underlying mechanisms on sperm function (11). Recent evidence has recommended that oxidative stress caused by massive production of reactive oxygen species (ROS) and antioxidant depletion may be a main mechanism of action, disturbing hyperviscous sperm function and spermatozoa quality (7, 12); however, there are very limited studies to support this theory.

Oxidative stress is a pathological condition that occurs as the loss of balance between ROS generation and the contents of antioxidants defense systems against them (13). ROS like hydroxyl radicals ($OH^{\cdot}$) and superoxide anion ($O2^{\cdot}$) are highly reactive oxidizing agents that easily react with biomolecules to compensate their deficit electron (14). However, human semen contains various antioxidants such as glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), vitamins E, C, copper, zinc and glutathione (GSH) that are referred to as total antioxidant capacity (TAC) (15). These antioxidant defense systems neutralize the detrimental effects of ROS. During uncontrolled generation of ROS, the effective concentration of seminal antioxidants will be reduced and consequently ROS impair normal sperm function, causing sperm apoptosis by peroxidation of sperm membrane unsaturated fatty acids, as well as oxidation of sperm DNA and proteins (16, 17). ROS may also attack the membrane fluidity of spermatozoa, which is subsequently associated with reduced ability of spermatozoa for oocyte fusion and fertilization (18). We assume that oxidative stress and antioxidant depletion may be a major mechanism, by which semen hyperviscosity affects sperm quality and function, and enhances sperm apoptosis. Since the relationship between seminal fluid viscosity and changes in levels of oxidative stress and sperm apoptosis is not well-elucidated, we designed this research to determine whether the hyperviscous semen is associated with seminal plasma antioxidant depletion, oxidative stress and sperm apoptosis.

Material and Methods

Semen samples

In this cross-sectional study, 368 ejaculate samples were collected from normal individuals (n=102), infertile men with hyperviscous semen (n=123) and infertile men without semen hyperviscosity (n=143) at Behta Clinical Laboratory in Rasht (Guilan, Iran) from April 2019 to May 2021. After obtaining institutional review board approval (IR.BMSU.REC.1396.75) and informed consent, semen samples were collected by masturbation within a sexual abstinence for three days. Before samples collection, a checklist was provided, in which basic demographic information of men, including age, weight, smoking habits or opiate using were recorded. Men with history of opiate using, testicular damage or developmental disorders in sex organs, infectious diseases, varicocele, cryptorchism, as well as those using antioxidant supplementation during at least 3 weeks prior to sample collection, were excluded from the study. Only healthy individuals with normal sperm parameters (normozoospermia) and men with idiopathic infertility and hyperviscous semen were entered into the study. The consistency of semen was assessed by placing a glass rod into the liquefied semen samples and measuring the length of the thread on withdrawal of the rod. Semen with a thread length above 2 cm were considered as normal consistency, but hyperviscous ejaculates showed a thread length >2 cm (19).

Semen analysis

Following liquefaction of ejaculates at room temperature (after 25-30 minutes), semen volume was measured. One hundred microliter (µl) of each semen sample was applied to examine the sperm count, sperm motility, morphology, and viability under a light microscopic (Nikon TS100, USA). Sperm quality parameters (sperm counts, motility and viability) were evaluated according to the World Health Organization (WHO) criteria (20), but normal morphology of spermatozoa was examined according to Kruger's strict criteria (21). Sperm viability was determined via eosin staining method (22). Briefly, 10 µl of semen samples were placed on sterile slides and each sample was mixed with 10 µl of 5% eosin and white cells were counted to determine the live cells.

Total antioxidant capacity measurement

Seminal plasma TAC was assessed by Ferric Reducing of Antioxidant Power method (FRAP) described by Benize (1996) (23). FRAP is a colorimetric assay that uses antioxidants as reductants for the conversion of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion and estimates the antioxidant capacity of a particular sample. Approximately, 500 µl of ejaculates were centrifuged at 1400 × g, at 4°C for 7 minutes, and the supernatants were diluted with distilled water (10-fold). The solution was urgently used for TAC assay using the FRAP reagent [300 mM Acetate buffer, pH=3.6, 10 mM 2,4,6-Tri(2-pyridinyl)-S-triazine (TPTZ) and 20 mM Ferric chloride].

Glutathione assessment

Reduced GSH was determined using the Tietz method (24). Approximately, 400 µl of NaH2PO4 (3 mM) with 50 µl of 0.04% 5,5-dithiobis 2-nitrobenzoic acid (DTNB) in 0.1% sodium citrate was added to seminal plasma samples. The absorbance of the solution was recorded at 410 nm using a UV/vis spectrophotometer (UV-1600PC, USA).

Malondialdehyde measurement

Seminal plasma malondialdehyde (MDA) level was measured as a biomarker of membrane lipid peroxidation. It was determined using the thiobarbituric acid reaction method

(TBAR) previously described by Hosseinzadeh Colagar et al. (25). Briefly, semen specimens were centrifuged at 2000 × g for 7 minutes. Supernatants (100 µl) were diluted with distilled water (900 µl) and then transferred into a sterilized glass tube containing 500 µl of TBA reagent. The mixture was then incubated for 1 hour in boiling water and then centrifuged at 4000 × g for 8 minutes following cooling at room temperature. The absorbance of each supernatant was read in a UV/vis spectrophotometer at 534 nm.

8-hydroxydeoxyguanosine measurement

8-hydroxydeoxyguanosine (8-OHdG) level was measured as a biomarker of sperm DNA oxidation (26). It was assessed using an enzyme-linked immunosorbent assay kit (ELISA kit, China). The severity of the reaction product color was assessed by ELISA reader (Beckman Coulter, USA) at 450 nm.

Protein carbonyl measurement

Protein carbonyl (PC) was assessed as a biomarker of protein oxidation. Its level in seminal fluids (50 µL) was detected according to a previous method (27, 28). The concentration of carbonyle was measured based on the molar extinction coefficient of 2,4-dinitrophenylhydrazine (DNPH) and expressed as nmol/mg of the protein.

Gene expression analysis

In this study we evaluated the expression of *CASPASE-3*, *BAX*, and *CASPASE-9* genes (as biomarker of apoptosis), as well as *BCL2* (as an anti-apoptosis biomarker). Briefly, 1 ml of liquefied ejaculate specimens were centrifuged at 2500 × g for 10 minutes and then the pellets containing spermatozoa were used for RNA extraction. RNX-Plus Kit (SinaClon, RN7713C) was used to extract the total RNAs of spermatozoa. The quantity of the extracted RNAs was measured by Nanodrop ND-1000 spectrophotometer. For cDNA synthesis at 42°C for 1 hour, a revert aid reverse transcriptase (Thermo Fisher Scientific, USA) along with random hexamer primers were applied. A Rotor Gene 6000 thermocycler in 41 cycles was used for amplifications. Each reaction contained 5 µl master mix and 100 nmol primers. The specific primer sequences for candidate genes are presented in Table 1. The mRNA levels of the study genes were normalized relative to the *GAPDH* mRNA levels. Eventually, the $2^{-\Delta Ct}$ method was applied to calculate the relative expression of the studied genes (29).

Statistical analysis

In this study, parametric data are shown as means ± SD. Comparison of the means of each of the quantitative parameters between the three groups was completed using one-way ANOVA: Post Hoc-Tukey test. SPSS software (IBM, USA, version 22) was applied for data analysis. A $P<0.05$ was considered as significant.

Table 1: Primer sequences for the genes used in the study

Genes	Primer sequence (5′-3′)
BAX	F: GAGGATGATTGCTGATGTGGATA
	R: CAGTTGAAGTTGCCGTCTG
BCL2	F: GGAGCGTCAACAGGGAGATG
	R: ACAGCCAGGAGAAATCAAACAGA
CASP-2	F: AAGCCGAAACTCTTCATCATTCA
	R: GCCATATCATCGTCAGTTCCAC
CASP-9	F: ATGACCACCACAAAGCAGTCC
	R: CGTGACCATTTTCTTGGCAG
GAPDH	F: AAGTTCAACGGCACAGTCAAGG
	R: CATACTCAGCACCAGCATCACC

Results

The comparison results of the mean of semen parameters among different groups are summarized in Table 2. No significant differences were found in the means of age and body mass index (BMI) among the three groups. The means of sperm count, normal morphology, vitality and normal morphology were significantly higher in healthy individuals than the infertile men with or without hyperviscous semen ($P<0.001$). While there was no significant difference in the mean of ejaculate volume between men with and without hyperviscous semen, a significant decrease was observed in the mean of sperm count (1.69-fold, $P=0.038$), sperm vitality (1.68-fold, $P=0.041$), sperm motility (1.44-fold, $P=0.045$) and normal morphology of spermatozoa (2.70-fold, $P=0.018$) in men with hyperviscous semen as compared to infertile individuals without hyperviscous semen (Table 2).

Table 2: Comparison of the semen analysis between three groups

Sperm parameters	Control	Infertile men		P value
		Hyperviscous semen	Non-hyperviscous semen	
Age (Y)	34.67 ± 2.48	32.79 ± 3.59	35.12 ± 3.86	0.76
BMI (Kg/m^2)	24.18 ± 1.09	23.72 ± 1.48	24.77 ± 1.22	0.27
Semen volume (ml)	4.82 ± 1.26	3.14 ± 1.12*	3.26 ± 1.33*	0.043
Sperm count (×10^6/ml)	78.63 ± 18.35	18.41 ± 9.67*	31.27 ± 18.42*	<0.001
Total sperm count (×10^6)	378.99 ± 23.12	57.80 ± 10.83*	98.18 ± 20.63*	<0.001
Sperm vitality (%)	67.44 ± 9.37	21.17 ± 5.39*	35.57 ± 6.82*	<0.001
Sperm motility (%)	62.32 ± 8.72	19.77 ± 5.14*	28.64 ± 6.54*	<0.001
Normal morphology (%)*	14.84 ± 4.65	3.22 ± 2.24*	8.72 ± 1.48*	<0.001

Results are presented as mean ± SD. BMI; Body mass index. According to Kruger's criteria, One-Way ANOVA; Post Hoc-Tukey test was applied to compare mean value of all parameters between all groups, *; P<0.001, compared to control group.

Comparison results of the mean of FRAP value, PC, MDA and 8-OHdG contents in seminal plasma of all groups are shown in Table 3. A significant difference was observed among the mean values of the oxidative biomarkers in the three groups. Collectively, the control fertile group had significantly higher FRAP and GSH mean levels, but lower contents of seminal PC, MDA and 8-OHdG, when compared to both infertile groups (P<0.001). Infertile men without hyperviscous semen exhibited higher FRAP values (1.42-fold, P=0.047) and GSH (1.54-fold, P=0.037) in their seminal plasma when compared to those with hyperviscous semen. In contrast, men with hyperviscous semen showed significantly higher mean levels of MDA (1.28-fold, P=0.048), PC (1.39-fold, P=0.041) and 8-OHdG (1.32-fold, P=0.046) in their seminal plasma than those without semen hyperviscosity (Table 3).

There was a significant difference in expression of *BAX*, *BCL2*, *CASPASE-3* and *CASPASE-9* genes among the groups (P<0.001). Men with hyperviscous semen had significantly higher degree of *BAX* (3.52-fold, P<0.001, Fig.1A), *CASPASE-3* (2.81-fold, P<0.001, Fig.1B) and *CASPASE-9* (2.56-fold, P<0.001, Fig.1C) genes expression, but lower expression of *Bcl2* gene (2.41-fold, P<0.001, Fig.1D) when compared to the control. Moreover, the expression of *BAX*, *CASPASE-3* and CASPASE-9 genes in men with hyperviscous semen was significantly increased by 1.39-fold (P=0.041), 1.47-fold (P=0.046), 1.29-fold (P=0.048), respectively, as compared with infertile subjects without hyperviscous semen. The expression of *BCL2* in infertile men without hyperviscous semen was greater than that in men with hyperviscous semen (1.36-fold, P=0.044, Fig.1).

Table 3: Comparison of oxidative stress biomarkers between three groups

Sperm parameters	Control	Infertile men		P value
		Hyperviscous semen	Non-hyperviscous semen	
FRAP (µM/L)	2651.8 ± 67.38	1250.44 ± 66.23*	1784.31 ± 89.87*	<0.001
GSH (µM/L)	9.42 ± 1.18	3.82 ± 1.05*	5.89 ± 0.87**	<0.001
PC (nmol/mg)	3.89 ± 1.09	8.34 ± 1.03*	6.01 ± 0.93**	<0.001
MDA (nmol/ml)	0.53 ± 0.11	1.14 ± 0.27*	0.89 ± 0.22*	<0.001
8-OHdG (ng/ml)	136.41 ± 16.12	259.71 ± 24.59*	197.13 ± 18.47*	<0.001

Results are presented as mean ± SD. One-Way ANOVA: Post Hoc-Tukey test was applied to compare mean value of all parameters between all groups. FRAP; Ferric reducing of antioxidant power, GSH; Reduced glutathione, PC; Protein carbonyl, MDA; Malondialdehyde, 8-OHdG; 8-hydroxydeoxyguanosine, *; P<0.001, and **; P<0.01 compared to control group.

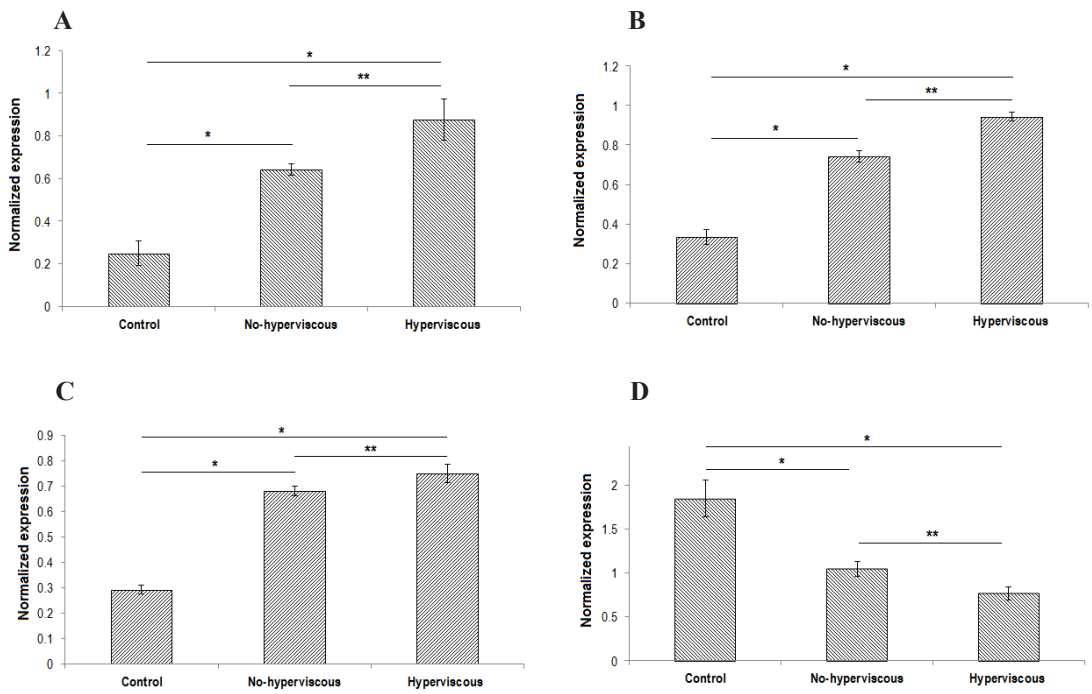

Fig.1: Comparison of the expression of apoptotic genes between the study groups. **A.** mRNA expression of *BAX*, **B.** mRNA expression of *CASPASE-3*, **C.** mRNA expression of *CASPASE-9* and **D.** mRNA expression of *BCL2*. One-Way ANOVA: Post Hoc-Tukey test was applied to compare mean value of all parameters between all groups. *; P<0.001 and **; P<0.01 compared to control group.

Discussion

Hyperviscosity semen can be considered as a leading cause of male infertility as it traps spermatozoa in the coagulated semen and prevents sperm migration through the female reproductive tract. Moreover, it may decrease the success rate of assisted reproductive techniques (ART) as it leads to difficulties in proper separation of spermatozoa during ART (30). In the current research, we investigated the relationship between hyperviscous semen and oxidative stress, antioxidant systems and apoptosis status. Our data illustrated that hyperviscosity is significantly correlated with depletion of TAC and GSH contents in the semen. In contrast, a significant enhancement was found in the mean contents of MDA, PC and 8-OHdG, as the biomarkers of lipid peroxidation, and oxidation of proteins and DNA, respectively. More importantly, we observed overexpression of apoptosis-related genes, *BAX*, and *CASPASES*, while down-regulation of the anti-apoptotic gene, *BCL2*, in spermatozoa of infertile men with hyperviscous semen.

Although oxidative stress, antioxidant depletion and apoptosis were found in both infertile groups, men with hyperviscous semen exhibited a significantly higher degree of these pathological conditions when compared to the non-hyperviscous group. Besides, we found that severe hyperviscosity was closely associated with higher levels of oxidative stress and antioxidant depletion. Therefore, these data emphasize that oxidative stress and apoptosis are among the major underlying mechanisms, by which seminal fluid viscosity leads to production of poor quality of spermatozoa, and thus male infertility. To support this hypothesis, there are some studies that have reported increased oxidative stress in seminal fluids of men with hyperviscous semen. For instance, in a previous study, Siciliano et al. (31) showed that both enzymatic and non-enzymatic antioxidants in semen of men with hyperviscosity are impaired. They concluded that the poor sperm motility in these patients is likely due to oxidative stress caused by depletion of antioxidants. In another study, Aydemir et al. (32) reported enhanced MDA mean contents in seminal fluids of men with hyperviscous semen compared to non-viscous samples. Layali et al. (19) demonstrated depletion of TAC and increased contents of MDA in seminal fluids of infertile men with hyperviscous semen. More recently, Barbagallo et al. (12) reported that seminal fluid viscosity is associated with impairment of antioxidant systems and consequently increases oxidative stress. Our research is in line with these studies; however, our study has advantages compared to previous work. In our study, not only we evaluated antioxidant depletion and lipid peroxidation status, but also, we evaluated oxidation of DNA and proteins, and performed expression analysis of apoptosis-related genes in men with hyperviscous semen, that are not reported in previous studies. According to our findings, men with hyperviscous semen exhibit lower TAC mean value, but higher MDA, 8-OHdG, and PC mean levels. In addition, we observed that overexpression of apoptosis-related genes were found in these cases compared to the non-hyperviscous group.

Therefore, our results indicate that antioxidant defense system in seminal plasma of men with hyperviscous semen is impaired. This defect is associated with increased levels of sperm membrane lipid peroxidation, DNA and proteins oxidation, and subsequently sperm apoptosis and poor quality of spermatozoa. The exact mechanism, in which oxidative stress in the seminal fluids of men with hyperviscous semen is increased has remained unclear at this point. We anticipate that overproduction of ROS is the main reason for antioxidant deficiency and oxidative damage in hyperviscous semen. Previous studies demonstrated that morphologically abnormal spermatozoa and leukocytes are important sources of ROS generation in human ejaculates (11).

It has been reported that the percentage of leukocytes is higher in men with hyperviscous semen than those without hyperviscous semen (12, 18). Leukocytes may be involved in developing and progressing hyperviscous semen, because their numbers are dramatically increased during infection, and produce massive amounts of ROS (33). To support this statement, some studies have demonstrated a significant relationship between leukocytospermia and hyperviscous semen (34). For example, Mahran and Saleh (34) have reported that the prevalence of leukocytospermia in infertile men with hyperviscous semen is about 37.5%. Moreover, they showed that an elevation in the number of leukocytes in men with semen hyperviscosity significantly decreases the percentage of sperm with normal motility and vitality. These researchers concluded that hyperviscous semen may be resulted from a previous ongoing infection or inflammation in 75% of these cases.

In another study, Elia et al. (8) recommended that administration of anti-inflammatory agents might successfully treat patients with mild semen hyperviscosity. Reduced levels of seminal plasma zinc is likely another main reason for poor sperm quality in men with hyperviscous semen. Hyperviscous seminal fluid contains a low level of zinc, which acts as an important cofactor for several antioxidants such as Cu/Zn-superoxide dismutase. Moreover, it has a great contribution to homocysteine trans-sulfuration into glutathione. So, there is a possibility that reduced Zn leads to decreased GSH production as well as SOD activity and consequently elevated oxidative stress (12). Therefore, these data indicate that overproduction of ROS and the subsequent oxidative stress and depletion of antioxidants such as Zn are important underlying mechanisms, in which hyperviscous semen causes sperm apoptosis and poor sperm quality. Given the fact that hyperviscous semen is associated with the risk of sperm DNA oxidation, some epigenetic or genetic abnormalities may be transmitted to offspring through the ART. Therefore, it is very important to evaluate the relationship between hyperviscous semen and the risk of some mutations in certain genes. Furthermore, antioxidant therapy might be helpful in mitigating these abnormalities and protecting sperm cells

against oxidative damages in patients with hyperviscous semen. However, further clinical trials are necessary to investigate the effects of antioxidant supplementation on mitigating oxidative stress and fertility potency in men with hyperviscous semen.

Conclusion

In summary, our findings showed that semen hyperviscosity triggers some cellular events associated with male infertility. Oxidative stress, antioxidant depletion and subsequent apoptosis of spermatozoa are likely the main mechanisms, by which hyperviscous semen induces poor sperm quality, and thus infertility in men. Impairment of seminal plasma antioxidants can be associated with oxidation of sperm DNA, protein, membrane lipids, and consequently low fertilization rate. Antioxidants therapy may be helpful in treating patients presenting with hyperviscous semen.

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Authors' Contributions

N.K., F.G.B., F.H., T.K.Z., H.B.D., S.R.; Clinical samples collection and laboratory phases. A.Sh., S.S.S.; Study concept and design, preparing data and manuscript writing. All authors read and approved the final manuscript.

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Mosaic Ring Chromosome 13 Presented with Isolated Male Infertility: Case Report

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Abstract

Ring chromosomes are the result of breakage and re-union of distal ends of chromosomal arms. They have a general frequency of 1 in 50,000 and 1 in 58,000 for chromosome 13. Ring chromosome 13 is usually presented as a syndromic situation stigmatized by particular features, including developmental delay, mental retardation and CNS, skeletal or organ anomalies. As an experimental study, here we report a 31 years old male with no major phenotypic manifestation who was evaluated for azoospermia, while his karyotype revealed presence of a mosaic ring chromosome 13. He had a history of bilateral varicocelectomy and no other major finding in his routine infertility work up was determined. Genetic counseling did not provide any clue for mental disability or dysmorphic features. Pathology examination of the testicular tissue revealed very scarce number of spermatid/spermatozoa within the tubules in conjunction with degrees of maturation arrest mostly in spermatocyte stage. In our knowledge, this is the first report of a ring chromosome 13, manifested by an isolated male infertility.

Keywords: Azoospermia, Case Report, Male Infertility, Ring Chromosome

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Introduction

Ring chromosomes are the result of breakage and re-union of distal ends of chromosomal arms. They have a general frequency of 1 in 50,000 (1) and 1 in 58,000 for chromosome 13 (2). For ring chromosome 13, the clinical syndrome is broad and dependent on the amount of deleted segment in chromosome 13 as well as the stability of the ring structure (3). Inherent instability within the ring structure may lead to creation of various clones with secondary aberrations. That is why ring structure are similarly called "dynamic mosaicism" (4). The full spectrum of clinical manifestation in ring chromosome 13 syndrome may include delay in growth and developmental steps, mental retardation, microcephaly, facial dysmorphic features, gastrointestinal atresia, genital anomaly, eye abnormalities (retinoblastoma) and skeletal dysmorphologies (5). Isolated infertility, as a consequence of ring chromosome 13, was previously reported only in a Chinese female with premature ovarian failure (6). To our knowledge, this is the first report of a case of ring chromosome 13 presented with isolated male infertility. The aim of this study is to evaluate association of mosaic ring chromosome 13 and male infertility.

Editorial policies and ethical considerations

The study was approved by the Ethical Review Board of the Royan Reproductive and Biomedicine Research Centre (Tehran, Iran, IR.ACECR.ROYAN.REC.1401.013). A written informed consent was provided by the patient. The study was performed in accordance with the ethical standards, as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Case report

A 31 years old man was married to a 42 years old woman and referred to infertility clinic, while their unconsanguineous marriage could not lead to a successful pregnancy after more than six months of unprotected sexual intercourse. This was his second marriage, while the first was terminated after less than 10 months. He had a primary level education (grade 5) and was employed as a scaffolding worker. He had a history of bilateral varicocelectomy and his two latest consecutive spermograms revealed severe oligospermia and azoospermia respectively (Table 1).

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Table 1: Spermogram details

Spermogram	Volume (CC)	Color	pH	Liquefaction Time	Count
First	5.7	Opaque grey	8-9	30-60	Only 5 immotile and 2 motile sperms in total
Second	6.2	Opaque grey	7-8	15-30	0

There was no history of infertility in his first degree relatives. No positive finding was detected in his physical examination, except for the incisional scars due to varicocelectomy, while external genitalia seemed to be normal. Genetic counseling did not provide any clues for mental disability or dysmorphic features. Hormonal assessments showed no impairment as total testosterone=5.19 ng/mL, luteinizing hormone (LH)=4.63 mIU/mL and follicle-stimulating hormone (FSH)=6.79 mIU/mL were all in normal ranges. In ultrasound scan of scrotom, size of the right and left testes were respectively 35×17 mm and 27×16 mm, while some varicose veins were also observed in the left side. The patient also went under diagnostic biopsy of the testis. Microscopic evaluation revealed unsatisfactory for the quality as well as the quantity of sperm content. Pathology examination of the testicular tissue revealed very scarce number of spermatid/spermatozoa within the tubules in conjunction with degrees of maturation arrest mostly in spermatocyte stage. There were a few spermatid/spermatozoa with abnormal morphology within only three tubules. The patient was also nominated for testicular sperm extraction (TESE) procedure which resulted in 0-1 sperm in each high power field (HPF). Testicular extracts were then cryopreserved for future assisted reproductive treatments (ARTs). For blood karyotyping, GTG banding of peripheral blood lymphocytes was carried out. Findings revealed mosaic ring chromosome 13 as the following; 46, XY, r(13) (p11.2q34) [24]/45, XY, -13[4]/46, XY, dicr (13;13) (p11.2q34;p11.2q34) [2].

Three different lines were observed with dominancy of a simple ring chromosome 13 with breakpoints at p11.2 and q34, a line with deletion of chromosome 13 and finally a minor line with double ring chromosome 13 at the same break points (Fig.1). The patient was briefed with the possible assisted reproductive technology (ART) outcomes, necessity of undergoing preimplanation genetic testing for aneuploidy (PGT-A) of their own biologic child and the other reproductive options, including embryo donation as well as adoption.

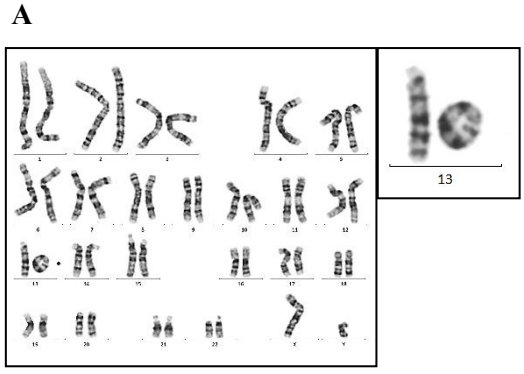

Fig.1: The karyograms prepared from peripheral blood of the patient with ring chromosome 13 including three different lines. **A.** 46,XY,r(13)(p11.2q34): the line with a ring chromosome 13 with breakpoints at p11.2 and q34. **B.** 45,XY,-13: a line with a missing chromosome 13. **C.** 46,XY,dicr(13;13)(p11.2q34;p11.2q34): the line with a double ring of chromosome 13 at the same breakpoints.

Discussion

Ring chromosome 13 is a rare genetic syndrome, whilst it is still considered as the most frequent ring chromosome abnormality (7). The spectrum of clinical features is wide and basically dependent on the deleted material from chromosome 13 (especially the long arm) and therefore it is related to the breakpoints in the chromosomal arms (8). In addition, stability of the ring structure is an important indicator for the clinical consequences (9). The smaller deleted region of the 13q causes milder clinical abnormalities. In addition, more proximal deletions, particularly those with intact 13q32 usually show mild to moderate phenotypes, while extension of deleted materials to 13q32 present severe mental retardation, growth delay and major abnormalities, such as microcephaly, gastrointestinal defects, dysmorphic extremities. Finally, very distal deletions affecting q33-34 have severe mental retardation, but usually do not show growth impairments or major organ abnormalities (10). With regards to the reproductive system, there are reports of ring chromosome 13 accompanied by genitourinary tract malformations or ambiguous genitalia (11, 12), while isolated infertility was only previously reported

in a woman evaluated for premature ovarian failure (6). To the best of our knowledge, the current case is the first report of a ring chromosome 13 presented with isolated male infertility. He was 31 years old with normal mental function, and no other detectable abnormal or dysmorphic feature. He was referred to infertility clinic for evaluation of azoospermia. The only positive finding was a history of bilateral varicocelectomy. Testis tissue assessment also revealed maturation arrest in spermatocytic stage.

Conclusion

With regards to the inheritance of ring chromosome 13, most of the cases are sporadic and they are usually the result of breakage and re-union of the chromosomal ends within the egg or sperm which usually happens during meiosis. Whilst there have been rare reports of transmission of ring chromosome from a parent to the offspring. Therefore, parental karyotyping in our case may help determine the origin of abnormal structure, while for reproductive management, they were recommended to use egg donation.

Acknowledgements

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Authors' Contributions

M.T.; Performed the cytogenetic experiments. H.S.; Performed the clinical consultation for the patient to enter the study and he was also the scientific consultant. P.B.B.; Collected the data and revised the manuscript. N.A.; Did the genetic consultation and draw pedigree. M.R.Z.; Designed the case study, collected and interpreted the data, drafted the manuscript, and revised and edited the paper. All authors approved revisions and the final paper.

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