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

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Web: www.ijfs.ir
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Printing Company:

Naghsh e Johar Co.
NO. 103, Fajr alley, Tehranpars Street, Tehran, Iran

INTERNATIONAL JOURNAL OF FERTILITY AND STERILITY

Int J Fertil Steril, Vol 14, No 2, July-September 2020, Pages: 79-160

Contents

Review Article

Male Equivalent Polycystic Ovarian Syndrome: Hormonal, Metabolic and Clinical Aspects

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Abstract

Recent studies identified the presence of a male polycystic ovarian syndrome (PCOS), which mainly affects men whose female relatives are afflicted with PCOS, caused by genes responsible for the susceptibility of this syndrome in women. Similar hormonal, metabolic, and clinical alterations occurring in PCOS women have also been reported in their male relatives, suggesting a association between the male and female forms of the syndrome. Although the remarkable clinical manifestation of the male equivalent PCOS is diagnosed by the early-onset androgenetic alopecia, characterized by hair recession, pronounced hypertrichosis, insulin resistance, biochemical and hormonal abnormalities, the hormonal/metabolic profile is still controversial. Men affected by early-onset androgenetic alopecia (AGA) are at risk of developing hyperinsulinemia, insulin-resistance, dyslipidaemia, and cardiovascular diseases. However, there is no consensus on the association of male equivalent PCOS with hypertension and obesity. Moreover, reduced levels of sex hormone-binding globulin have been detected in these male patients, accompanied by increased free androgens. Conversely, literature reported lower concentrations of testosterone in male equivalent PCOS when compared with the normal range, indicating a crucial role for the conversion of cortical androgens. Finally, further studies are warranted to investigate a possible link among AGA, metabolic/hormonal alterations, and acne. Our study assessed the hormonal, metabolic and clinical aspects of male equivalent PCOS syndrome reported in the literature to evaluate similar and divergent elements involved in the female version of the syndrome.

Keywords: Androgenetic Alopecia, Insulin Resistance, Polycystic Ovarian Syndrome

Citation: Di Guardo F, Ciotta L, Monteleone M, Palumbo M. Male equivalent polycystic ovarian syndrome: hormonal, metabolic and clinical aspects. Int J Fertil Steril. 2020; 14(2): 79-83. doi: 10.22074/ijfs.2020.6092.

Introduction

Polycystic ovarian syndrome (PCOS) is a well-known endocrine disorder characterized by the changes in menstrual cycle, altered ultrasound of the ovaries, and clinical and/or biochemical abnormalities resulting from hyper-androgenism (1). Although the alteration of gonadotropin levels, in terms of luteinizing hormone (LH) elevation respect follicle stimulating hormone with respect to follicle stimulating hormone (FSH), seems to be the most representative hormonal sign, PCOS develops in a more complex metabolic background determined by obesity and insulin resistance. These last two elements play a fundamental role in disease pathogenesis, leading to the development of the long-term complications, such as cardiovascular diseases (CVDs) and type II diabetes mellitus (DM II) (2). The scientific scenario has been recently focused on the involvement of a genetic component in the etiology of PCOS, identifying the existence of a male equivalent PCOS resulting from the heredity of susceptibility genes responsible for the pathogenesis of the disease in male relatives of women with PCOS (3). Although similar clinical characteristics of PCOS observed in women have been found in male subjects affected by male equivalent syndrome, the precise mechanism of the hormonal and metabolic backgrounds in these patients has not been yet established (4). The current study aimed to highlight the hormonal, metabolic and clinical aspects of male equivalent PCOS, trying to point out similar and divergent elements from the female syndrome.

Data sources

The presented study represents a review about male equivalent PCOS. We searched research articles published in MEDLINE (PubMed), EMBASE, IBECS, BIOSIS, Web of Science, SCOPUS, and Grey literature (Google Scholar; British Library) from 2000 to May 2019. We used the terms ‘‘PCOS’’, ‘‘PCOS male equivalent syndrome’’, and “Androgenic alopecia” as appropriate medical subject headings or equivalent subject heading/ thesaurus terms. These terms mentioned earlier were combined with ‘male’, ‘metabolic alterations’, ‘endocrine’, ‘symptoms’

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Royan Institute
International Journal of Fertility and Sterility
Vol 14, No 2, July-September 2020, Pages: 79-83

and 'signs'. The reference list of the available primary studies was reviewed to eventually find the additional relevant citations. We considered the following outcomes: hormonal pattern, metabolic profile, disease symptoms, and signs.

Screening of abstracts for eligibility

Original papers, meta-analyses, and published reviews were considered. In case of duplicate publications produced by the same team, the latter study was included. Case reports were not considered. Two authors (F.D.G and L.C.) independently extracted the data from the remaining studies. Disagreements about the inclusion or exclusion of articles were solved by consensus, and in the case of, a third reviewer (M.P.) was involved.

Finally, all the authors were involved in decisional process for including the relevant studies and a decision was made in the case of all authors' approval.

Study selection and eligibility criteria

A set of explicit criteria were used for the selection of the literature: (1) original articles, (2) meta-analysis conducted on human population, (3) adult males at the age range of 18 to 80 years old, and (4) being written in English language.

The investigation identified a total of 48 papers, of which 35 were potentially relevant following an initial evaluation. These met the inclusion criteria and were analyzed. The included studies were divided into three different issues: "Hormonal pattern", "Metabolic pattern", and "Clinical signs".

Hormonal pattern

Several studies identified the association of male equivalent PCOS with the hormonal markers of insulin resistance and metabolic syndrome. Although the hormonal pattern of female and male individuals is too challenging to compare, the literature reported a similar hormonal background to PCOS in men affected by the early-onset androgenetic alopecia (AGA). This event has been considered a sign of male equivalent PCOS, since it was found that its characteristic (androgenetic alopecia before 35 years of age) is mostly present in men belonging to families where female members have been affected by PCOS, supporting the idea of a genetic transmission (5). A recent case-control study analyzing the hormonal pattern of 57 young men (19–30 years) with early onset AGA, found hormonal parameters similar to the ones of female PCOS, when compared to the agedmatched controls. Besides, as far as the gonadotropin levels are concerned, the results showed a significant increase in LH, accompanied by reduced FSH. With regards to prolactin (PRL) and dehydroepiandrosterone sulfate (DHEAS), higher values were reported, while FSH and sex hormone-binding globulin (SHBG) were lower than in controls (6). In addition, similar to what happens in women with PCOS, an increased function of adrenal glands has been supposed to occur in men with early-onset AGA considering the augmented presence of two markers of adrenal activity (17αOH-P and DHEAS) found in these subjects (7) (Table 1). Considering the recent literature about hormonal assessment of earlyonset AGA, these studies showed results in accordance with the pattern discussed above. The first study conducted by Starka et al. (8) focused on the levels of SHBG, FSH, testosterone, and epitestosterone in men, showing a higher percentage of men affected by earlyonset AGA with the lower concentrations of the abovementioned values compared with the normal range. In the same way, in a successive study, they found lower levels of FSH, as well as lower concentrations of serum SHBG and higher levels of free androgen index (FAI) (Table 1).

Table 1: Hormonal/metabolic pattern of early onset AGA

Studies	Hormonal and metabolic alterations in men with early onset AGA
Sanke et al.(6)	Increased levels of LH, PRL, and DHEAS Reduced levels of FSH and SHBG
Stárka et al.(7)	Increased levels of 17αOH-P and DHEAS Reduced levels of SHBG
Starka et al.(8)	Low levels of SHBG with consequently elevation of the free androgen index Insulin resistance
Arias-Santiago et al.(9), Golden et al.(10)	Lower levels of SHBG and metabolic imbalance
Hirsso et al.(11), Matilainen et al.(12), Su et al.(13)	Higher BMI and risk of developing hyperinsulinemia

Hormonal/metabolic pattern the early-onset AGA considering the results of the most significant literature studies. LH; Luteinizing hormone, PRL; Prolactin, DHEAS; Dehydroepiandrosterone sulfate, FSH; Follicle stimulating hormone, SHBG; Sex hormone binding globulin, 17αOH-P ; 17α hydroxyprogesterone, BMI; Body mass index, and AGA; Androgenetic alopecia.

Metabolic pattern

Higher frequency of reduced insulin sensitivity was reported in men affected by the early-onset AGA associated with the low level of SHBG, introducing the concept of insulin unbalance in these patients (8). Although the relationship between lower SHBG levels and metabolic alterations has not been yet established (14), some studies reported the combination of lower level of SHBG and metabolic unbalance in the earlyonset AGA men, in virtue of the fact that this protein should be involved in mediation of the signaling pathways responsible for glycaemia maintenance (9, 10) (Table 1). According to this, lower SHBG levels may be regarded as a risk factor for the development of hyperglycaemia, insulin-resistance, and/or DM II in young patients with AGA. However, among metabolic abnormalities, a higher prevalence of hyperinsulinemia, hypertriglyceridemia, and hypertension has been reported in siblings with PCOS; more specifically they seem to occur frequently in brothers of women with PCOS (11, 15, 16).

In accordance with the last concept, an increased prevalence of metabolic disorders, such as insulin resistance (IR) and obesity, as well as, the early-onset AGA has been reported in first degree of male and female relatives of women with PCOS. Moreover, scientific evidence reported that men with the early-onset AGA and higher body mass index (BMI) have higher risk of developing hyperinsulinemia (11-13). Finally, in contrast with the expectations, the association among higher BMI values, insulin resistance, and higher blood pressure is still controversial (17). However, a study investigating the risk of developing cardiovascular disease (CVDs) among men with the early-onset AGA showed a higher incidence of metabolic syndrome and carotid atherosclerotic plaques, suggesting the importance of the routinely use of color Doppler ultrasound to scan the plaques in these patients (18). Considering all these elements, patients with the early-onset AGA should be screened for metabolic parameters in order to eventually adopt pharmacological and nutritional therapeutics strategies to reduce the risk of developing DM II and CVDs (2, 18, 19).

Clinical signs

According to the genetic hypotheses on the development of male equivalent PCOS as a result of the heredity of susceptibility genes in women, Duskovà and colleagues described the syndrome in men for the first time (4). They characterized the clinical characteristic of male equivalent PCOS manifested by the early-onset AGA and identified several featured elements, such as hypertrichosis, IR, biochemical, and hormonal abnormalities (4, 20, 21) (Table 2). However other authors had previously hypothesized its existence (20-22); for example, Lunde et al. (5) reported the occurrence of the early-onset androgenetic alopecia characterized by the hair recession of the frontotemporal region before 35 years of age. This condition is defined by a grade of alopecia higher than III according to the Hamilton–Norwood scale (23, 24) in male members of families in which a considerable number of women had PCOS (5). AGA is determined by a progressive reduction in size of the hair follicles until the vellus transformation of the terminal hair. This event is the consequence of the alteration in hair cycle dynamics, in particular the telogen phase, increasing with a subsequent decrease in the anagen phase duration. Considering that the duration of anagen is responsible for the hair length, the new anagen hair becomes shorter, eventually leading to baldness (5, 24). As discussed in the previous section, the suspicion that the early-onset AGA may represent a clinical marker of IR founded on the results of a case-control study reporting an increased prevalence of hyperinsulinemia and insulin-resistance-associated disorders و such as dyslipidaemia, hypertension و and obesity, in men with the-early onset of alopecia (<35), compared with age-matched controls (8). Moreover, several studies revealed that the condition of IR is also associated with acne in young males and post-adolescent men (25, 26).

Additionally, a study by Del Prete et al. (25) found a significant increase in plasma insulin levels, higher BMI, and waist circumference in young men with acne compared to healthy control subjects. Although the investigation of the androgenic profiles in males with acne was normal, this finding let us suppose an independent role of IR in the pathogenesis of the acne in the absence of hyperandrogenism. This mechanism may explain the presence of acne in men affected by the early-onset AGA either in the presence or absence of hyperandrogenism. With regard to the metabolic abnormalities in men with the early-onset AGA, it seems there is high prevalence of metabolic syndrome reflecting the phenotypical pattern of PCOS women (6, 27). According to this result, a meta-analysis reported an increased risk of metabolic syndrome development about 2.3 folds in AGA patients compared to healthy controls (28). However, other studies did not confirm this relation that is strongly well documented in the case of female pattern alopecia (29, 30). What is certainly known is the association of the early-onset AGA with hyperglycemia/ DM II and low levels of SHBG (31) (Table 2).

Table 2: Clinical elements of Early onset AGA

Studies	Hormonal and metabolic alterations in men with early onset AGA
Carey et al.(20), Legro et al.(21), Dusková et al.(4)	Hypertrichosis, insulin resistance biochemical, and hormonal abnormalities hyperglycemia/T2DM, and IR
Arias-Santiago et al.(31), Arias-Santiago et al.(18)	Increased risk of CVD due to the metabolic abnormalities increased risk of atheromatosis
Arias-Santiago et al.(31)	Higher levels of aldosterone contributing to the development of hypertension and increased prevalence of hypertension, lipid-lowering medication use, and obesity.

Symptoms and Signs of the early-onset AGA considering the results of the most significant literature studies. IR; Insuline resistence, T2DM; Type II diabetes mellitus, CVDs; Cardiovascular disease .

Discussion

Considering the evidence mentioned above, male equivalent PCOS may be considered a well-defined entity involving specific hormonal and metabolic patterns, as well as, the signs and symptoms. Although the syndrome was taken into account by more than ten years (21), it has been changeling to recognize its typical elements in order to the fact that the androgens-related alopecia have been considered normal in the male phenotype. Moreover, it is not common that the male category recurs to medical consultation for signs involved in the virilization process (12). In this case, symptoms of PCOS, such as acne, defluvium, and hypertricosis/hirsutism do not affect men as much as women. This may be explained by the fact that in women these symptoms are accompanied by the irregular cycles which, in most of the cases catch the women attention, letting them decide to consult a gynecologist (32). According to the lines above, this syndrome has to be considered a possible pathology in the case of men with PCOS-positive family history and

hyper-androgenism signs accompanied by the metabolic alterations and hormonal PCOS pattern.

Although the association of the early-onset AGA and acne with male equivalent PCOS is well documented, less clear data are available for hypertricosis that has to be investigated in detail. On the other hand, in opposed to what we expect, hypertestosteronemia does not represent one of the characteristic element of male equivalent PCOS. According to this, scientific literature reported testosterone values lower than the normal range and higher FAI in men with the early-onset AGA (8). This should be explained by the conversion of cortical androgens, such as DHEAS, which is increased in these subjects, and converted into other compounds and stronger androgens responsible for syndrome sings. Moreover, the difference in testosterone values between men and women affected by the two syndromes is due to the different action mediated by insulin on Theca and Leydig cells, stimulating and inhibiting the steroidogenesis, respectively (33, 34).

What is certain is that men with clinical aspect of the early-onset AGA are at risk of developing CVDs, metabolic syndrome and carotid atherosclerotic plaques (18). Similarly, first degree relatives of women with PCOS report an increased prevalence of insulin resistance, obesity, and early-onset AGA. Moreover, although the association with male equivalent PCOS and hyperinsulinemia is still controversial, several studies demonstrated that men with the early-onset AGA and higher BMI had higher risk of developing insulin resistance (11-13).

In contrast to the expectations, the presented paper highlights that high BMI, obesity, and pathologic-related symptoms, such as hypertension and high cholesterol levels, do not seem to be typical elements of male equivalent PCOS. In particular, the literature shows only one study conducted on men with the early-onset AGA, reporting a positive correlation between the levels of diastolic blood pressure and insulin resistance, excluding the BMI parameter. Accordingly, it has been identified a correlation among the levels of diastolic blood pressure, total cholesterol, insuline resistance, and fasting insulinresistance index (FIRI). All these parameters were higher in patients with the early-onset AGA than in controls (35). In addition, a case control-study conducted on 80 men with the early-onset AGA reported higher values of diastolic blood pressure and a frequent family history of AGA in non-obese cases than BMI-matched counterparts (36). According to this, we may affirm that high BMI and obesity cannot be defined as typical elements of male equivalent PCOS but when presented, they may contribute to the development of the hormonal, metabolic, and clinical scenario. Finally, an important mention may be reserved to the fertility potential of men affected by AGA; the literature shows that men with moderate to severe AGA have poorer semen quality than patients affected by moderate to mild AGA (36). In this context, it would be interesting to evaluate the potential fertility of men affected by male equivalent PCOS according to typical metabolic and hormonal alterations mentioned earlier.

Conclusion

Male equivalent PCOS may be defined as a disorder that occurs in male members of a family with a PCOS history, characterized by the clinical signs of androgenism, complete hair loss, and the same hormonal pattern seen in PCOS, except for testosterone levels that seems to be in the subnormal range. The metabolic pattern should be represented by hyperinsulinemia and insulin resistance with a side role for overweight and obesity in the case of occurrence. However, these patients have high risk of developing CVDs, metabolic syndrome, and carotid atherosclerotic plaques. According to this, the early diagnosis of the disease would be necessary to permit the patients to adopt healthy lifestyle preventing the risk of metabolic and cardiovascular events.

Acknowledgements

This research received no financial support. The authors declare no conflict of interest.

Authors' Contributions

D.G.F.; Contributed to the design, implementation of the research, and writing the initial draft of the manuscript. D.G.F., C.L.; Collection and analyzing were performed. The revision process was entirely made by D.G.F. Improving the analysis and the structure of the paper. P.M., M.M.; Contributed to the English editing and approved the final draft of the paper. All authors read and approved the final manuscript.

References

1. Fratantonio E, Vicari E, Pafumi C, Calogero AE. Genetics of polycystic ovarian syndrome. Reprod Biomed Online. 2005; 10(6): 713-720.
2. Di Guardo F, Cerana MC, D'urso G, Genovese F, Palumbo M. Male PCOS equivalent and nutritional restriction: are we stepping forward? Med Hypotheses. 2019; 126: 1-3.
3. Cannarella R, Condorelli RA, Mongioì LM, La Vignera S, Calogero AE. Does a male polycystic ovarian syndrome equivalent exist? J Endocrinol Invest. 2018; 41(1): 49-57.
4. Dušková M, Čermáková I, Hill M, Vaňková M, Šamalíková P, Stárka L. What may be the markers of the male equivalent of polycystic ovary syndrome. Physiol Res. 2004; 53(3): 287-294.
5. Lunde O, Magnus P, Sandvik L, Høglo S. Familial clustering in the polycystic ovarian syndrome. Gynecol Obstet Invest. 1989; 28(1): 23-30.
6. Sanke S, Chander R, Jain A, Garg T, Yadav P. A comparison of the hormonal profile of early androgenetic alopecia in men with the phenotypic equivalent of polycystic ovarian syndrome in women. JAMA Dermatol. 2016; 152(9): 986-991.
7. Stárka L, Čermáková I, Dušková M, Hill M, Doležal M, Poláček V. Hormonal profile of men with premature balding. Exp Clin Endocrinol Diabetes. 2004; 112(1): 24-28.
8. Starka L, Duskova M, Vrbikova J, Hill M. Premature androgenic

alopecia and insulin resistance. Male equivalent of polycystic ovary syndrome? Endocr Regul. 2005; 39(4): 127-131.
9. Arias-Santiago S, Gutiérrez-Salmerón MT, Buendía-Eisman A, Girón-Prieto MS, Naranjo-Sintes R. Sex hormone-binding globulin and risk of hyperglycemia in patients with androgenetic alopecia. J Am Acad Dermatol. 2011; 65(1): 48-53.
10. Golden SH, Dobs AS, Vaidya D, Szklo M, Gapstur S, Kopp P, et al. Endogenous sex hormones and glucose tolerance status in postmenopausal women. J Clin Endocrinol Metab. 2007; 92(4): 1289-1295.
11. Hirsso P, Laakso M, Matilainen V, Hiltunen L, Rajala U, Jokelainen J, et al. Association of insulin resistance linked diseases and hair loss in elderly men. Finnish population-based study. Cent Eur J Public Health. 2006; 14(2): 78-81.
12. Matilainen V, Koskela P, Keinänen-Kiukaanniemi S. Early androgenetic alopecia as a marker of insulin resistance. The Lancet. 2000; 356(9236): 1165-1166.
13. Su LH, Chen TH. Association of androgenetic alopecia with metabolic syndrome in men: a community-based survey. Br J Dermatol. 2010; 163(2): 371-377.
14. Narad S, Pande S, Gupta M, Chari S. Hormonal profile in Indian men with premature androgenetic alopecia. Int J Trichology. 2013; 5(2): 69-72.
15. Norman RJ, Masters S, Hague W. Hyperinsulinemia is common in family members of women with polycystic ovary syndrome. Fertil Steril. 1996; 66(6): 942-947.
16. Kaushal R, Parchure N, Bano G, Kaski JC, Nussey SS. Insulin resistance and endothelial dysfunction in the brothers of Indian subcontinent Asian women with polycystic ovaries. Clin Endocrinol (Oxf). 2004; 60(3): 322-328.
17. Mumcuoglu C, Ekmekci TR, Ucak S. The investigation of insulin resistance and metabolic syndrome in male patients with early-onset androgenetic alopecia. Eur J Dermatol. 2011; 21(1): 79-82.
18. Arias-Santiago S, Gutiérrez-Salmerón MT, Castellote-Caballero L, Buendía-Eisman A, Naranjo-Sintes R. Male androgenetic alopecia and cardiovascular risk factors: a case-control study. Actas Dermosifiliogr. 2010; 101(3): 248-256.
19. Guardo FD, Currò JM, Valenti G, Rossetti P, Di Gregorio LM, Conway F, et al. Non-pharmacological management of gestational diabetes: the role of myo-inositol. J Complement Integr Med. 2019 (ahead of print).
20. Carey AH, Chan KL, Short F, White D, Williamson R, Franks S. Evidence for a single gene effect causing polycystic ovaries and male pattern baldness. Clin Endocrinol (Oxf). 1993; 38(6): 653-658.
21. Legro RS. Is there a male phenotype in polycystic ovary syndrome families? J Pediatr Endocrinol Metab. 2000; 13 Suppl 5: 1307-1309.
22. Starka L, Hill M, Polacek V. Hormonal profile in men with premature androgenic alopecia. Sb Lek. 2000; 101(1): 17-22.
23. Hamilton JB. Patterned loss of hair in man: types and incidence. Ann N Y Acad Sci. 1951; 53(3): 708-728.
24. Norwood OT. Male pattern baldness: classification and incidence. South Med J. 1975; 68(11): 1359-1365.
25. Del Prete M, Mauriello MC, Faggiano A, Di Somma C, Monfrecola G, Fabbrocini G, et al. Insulin resistance and acne: a new risk factor for men? Endocrine. 2012; 42(3): 555-560.
26. Nagpal M, De D, Handa S, Pal A, Sachdeva N. Insulin resistance and metabolic syndrome in young men with acne. JAMA Dermatol. 2016; 152(4): 399-404.
27. Banger HS, Malhotra SK, Singh S, Mahajan M. Is early onset androgenic alopecia a marker of metabolic syndrome and carotid artery atherosclerosis in young Indian male patients? Int J Trichology. 2015; 7(4): 141-147.
28. Wu DX, Wu LF, Yang ZX. Association between androgenetic alopecia and metabolic syndrome: a meta-analysis. Zhejiang Da Xue Xue Bao Yi Xue Ban. 2014; 43(5): 597-601.
29. Ozbas Gok S, Akin Belli A, Dervis E. Is there really relationship between androgenetic alopecia and metabolic syndrome? Dermatol Res Pract. 2015; 2015: 980310.
30. Yi SM, Son SW, Lee KG, Kim SH, Lee SK, Cho ER, et al. Gender-specific association of androgenetic alopecia with metabolic syndrome in a middle-aged Korean population. Br J Dermatol. 2012; 167(2): 306-313.
31. Arias-Santiago S, Gutiérrez-Salmerón MT, Buendía-Eisman A, Girón-Prieto MS, Naranjo-Sintes R. Hypertension and aldosterone levels in women with early-onset androgenetic alopecia. Br J Dermatol. 2010; 162(4): 786-789.
32. Reyes-Muñoz E, Guardo FD, Ciebiera M, Kahramanoglu I, Sathyapalan T, Lin LT, et al. Diet and nutritional interventions with the special role of myo-inositol in gestational diabetes mellitus management. An evidence-based critical appraisal. Curr Pharm Des. 2019; 25(22): 2467-2473.
33. Kurzrock R, Cohen PR. Polycystic ovary syndrome in men: Stein–Leventhal syndrome revisited. Med hypotheses. 2007; 68(3): 480-483.
34. Cadagan D, Khan R, Amer S. Thecal cell sensitivity to luteinizing hormone and insulin in polycystic ovarian syndrome. Reprod Biol. 2016; 16(1): 53-60.
35. Ahn SW, Gang GT, Kim YD, Ahn RS, Harris RA, Lee CH, et al. Insulin directly regulates steroidogenesis via induction of the orphan nuclear receptor DAX-1 in testicular Leydig cells. J Biol Chem. 2013; 288(22): 15937-15946.
36. Güngör ES, Güngör Ş, Zebitay AG. Assessment of semen quality in patients with androgenetic alopecia in an infertility clinic. Dermatologica Sinica. 2016; 34(1): 10-13.

Original Article

The Effects of *Calligonum* Extract and Low-Intensity Ultrasound on Motility, Viability, and DNA Fragmentation of Human Frozen-Thawed Semen Samples

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Abstract

Background: The study aimed to evaluate the impact of *Calligonum* extract and US radiation on sperm parameters of cryopreserved human semen samples.

Materials and Methods: In this experimental study, twenty-five semen specimens were obtained from healthy semen donors and incubated in human tubal fluid (HTF) medium supplemented with 10% human serum albumin (HSA) for 45 minutes. Samples were treated with *Calligonum* extract (10 µg/ml) alone (CGM group) and US radiation (LIPUS-exposed group) alone or a combination of both treatments (CGM+LIPUS). The US group received US stimulation (in both continuous and pulsed wave modes) at a frequency of 1 MHZ and intensity of 200 mW/cm2 for 200 seconds. Sperm morphology was assessed by Diff-Quik staining. The DNA fragmentation was evaluated the Halo sperm kit. Sperm parameters was analyzed by a computer-assisted semen analysis system. Reactive oxygen species (ROS) was assessed by flow cytometry.

Results: The results showed that the treatment with *Calligonum* extract significantly ($P<0.05$) increased the progressive motility of spermatozoa in the CGM group as compared with the control group. The application of low-intensity US significantly ($P<0.05$) decreased the motility and viability of spermatozoa in the US group when compared with the control group. Our findings also indicated that the use of both low-intensity US in continuous mode and *Calligonum* extract slightly increased progressive motility; however, such an increase was not statistically significant. The rate of DNA fragmentation was considerably higher ($P<0.05$) in control and LIPUS-exposed groups than the other groups.

Conclusion: Treatment of spermatozoa with *Calligonum* extract slightly improved the sperm parameters due to its antioxidant activity, on the other hand, according to our results, US radiation did not improve sperm parameters which may be due to interference with the motility of sperm, as well as its physical effects on spermatozoa.

Keywords: Antioxidants, *Calligonum*, Cryopreservation, Low-Intensity Ultrasound, Spermatozoa

Citation: Ekrami H, Movahedin M, Koosha F, Mazaheri Z, Mokhtari-Dizaji M. The effects of calligonum extract and low-intensity ultrasound on motility, viability, and DNA fragmentation of human frozen-thawed semen samples. Int J Fertil Steril. 2020; 14(2): 84-90. doi: 10.22074/ijfs.2020.5896.

Introduction

Cryopreservation of human semen is considered one of the most vital and essential strategies for the preservation and maintenance of spermatozoa, and it is broadly applied in malignancies or other therapies which could damage to the functionality of testicles (1). During the cryopreservation process, the viability, motility and the integrity of chromatin might be adversely influenced, usually accompanied by the elevation of oxidative stress and excessive production of reactive oxygen species (ROS) (2). In the presence of increased ROS production, free radical oxygen molecules can target and attack to bis-allylic methylene groups of phospholipid-bound polyunsaturated fatty acids which are present in the plasma membrane of spermatozoa, leading to lipid peroxidation (3). The impact of lipid peroxidation on the sperm function include irreversible loss of motility, discharge of intracellular enzymes, sperm DNA damage, impairment of oocyte penetration and sperm-oocyte fusion, and apoptosis of spermatozoa in frozen media (4).

Many efforts have been made to minimize the rate of cryodamage in frozen spermatozoa during the cryopreservation process. For instance, the addition of antioxidant agents or cryoprotectants to the extender is one of the promising tools for the increase of sperm quality during the freeze-thaw process (5-7). Various types

Received: 3/March/2019, Accepted: 28/Jan/2020
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Royan Institute
International Journal of Fertility and Sterility
Vol 14, No 2, July-September 2020, Pages: 84-90

of antioxidants, such as vitamin C or E (8), genistein (5), and resveratrol (9) have been employed for cryopreservation. However, the yield of the cryopreservation procedure for human spermatozoa is not yet satisfactory, and new strategies are still warranted to improve the fidelity of cryopreservation. *Calligonum comosum (C. comosum)* is a medicinal plant abundantly found in the Egyptian desert, containing numerous polyphenol antioxidant agents. The beneficial roles of *C. comosum* may stem from its antioxidant activity, which has been extensively assessed *in vitro* (10). Some studies have demonstrated some biological properties of *C. comosum* extract on rodent models, such as the anti-inflammatory and anti-gastric ulcer activities (11).

Additionally, *C. comosum* showed the anticancer potential in mice inoculated with Ehrlich ascites carcinoma cells (12). It has been implicated that low-intensity pulsed ultrasound (LIPUS) at the incident power density from 0.5 to 3000 mW/cm^2 have been used for bone healing (13). LIPUS can promote the growth of human skin fibroblasts, thereby the activation of the integrin receptors, RhoA(Ras homolog gene family, member A)/ROCK, and Src-ERK signaling pathways (14, 15). The biological action of LIPUS occurs when the mechanical wave is converted into a biochemical signal within the cell as the mechanoreceptors and integrin are thought to be involved in this process. A number of studies have highlighted the formation of focal adhesions on the surface of the cells treated with LIPUS, which is mediated by the activation of integrin-associated signaling pathways (16, 17). Focal adhesions are large multi-protein complexes that could serve as a transmembrane bridge between the extracellular matrix (ECM) and the actin cytoskeleton. They could be identified in specific sites within the cell where clustered integrin receptors can interact with the ECM components on the outside and with the actin cytoskeleton on the inside of the cells. One of the main components of focal adhesion proteins involved in the transduction of the LIPUS signal from a mechanical force to a chemical messenger is focal adhesion kinase (FAK), which is phosphorylated when the cells are exposed to LIPUS (17, 18).

Hence, regarding the above statements, it would be plausible that LIPUS can enhance the penetration of herb extraction into the sperm via an increase in motility of spermatozoa. Therefore, the primary goal of the present study was to determine the impact of *C. comosum* extract alone, LIPUS signal, and the combination of both on the count, viability, total motility, progressive motility, DNA fragmentation, and morphology of spermatozoa during the freeze-thawing process.

Materials and Methods

Study design

In this experimental study, we evaluated the effects of *Calligonum* extract and LIPUS at a frequency of 1 MHZ (in pulsed and continues wave modes) on the cryopreservation of human spermatozoa. After the preparation of semen samples, each sample was liquated into 5 parts included; washed spermatozoa (control group), frozen-thawed spermatozoa, frozen-thawed spermatozoa treated with *Calligonum* extract at a concentration of 10 µg/ml (CGM group), frozen-thawed spermatozoa exposed to the US radiation (LIPUS-exposed group) at a duty cycle of 40% (pulsed mode, at a frequency of 1 MHz, at the incident power density of 200 mW/cm2 for 200 seconds), and frozen-thawed spermatozoa treated with the combination of Calligonum extract and the US radiation with continues mode (CGM+LIPUS group). The present research was approved by Ethical Committee of Tarbiat Modares University (No. 52/6757 dated 30.11.92).

Herb extraction

The identification of the herb

The plant (*Calligonum comosum* L.) was collected by Dr. Hosein Batooli from the desert located at the proximity of Kashan, Iran, (33.9850°N, 51.4100°E). The taxonomic identity of the collected plant was confirmed by Dr. Abdoalrasool Haghir Ebrahim Abadi, Faculty of Science, Kashan University, Iran. Freshly collected plant materials were air-dried in the shade at room temperature. The aerial parts (stem, flowers, and leaves) of this herb were used for further investigations.

The extraction protocol

Two kilograms of the fresh aerial parts of the plant (equal to 50% of the weight of a wildly-growing plant) were air-dried in the shade at room temperature, grounded, and exhaustively extracted by cold maceration with aqueous methanol (70%). The extract was evaporated under reduced pressure at 40°C to yield 80 g residue. The residue was suspended in distilled water and successfully fractionated with n-hexane, CH_2Cl_2, EtOAc, and n-BuOH (Thermo Fisher, USA) saturated with H_2O. Each extract was evaporated under reduced pressure to yield 3, 7, 12 and 22 g residues, respectively.

The antioxidant activity

The activity of free radical-scavenging of the methanolic extract of C. comosum was determined concerning the potential to neutralize the free radical-producing [2,2-diphenyl-1-picrylhydrazyl (DPPH)] according to a method, as described previously (19, 20). Concisely, the level of DPPH was calculated by the measurement of the absorbance at the wavelength of 517 nm prior to and after the addition of a specific amount of the herb extract. Afterward, the inhibitory percentage of radical formation was estimated using the following equation: % inhibition= $(A_{control} - A_{sample})/A_{control} \times 100$, where $A_{control}$ is considered the absorbance of DPPH alone and $A_{,sample}$ is regarded as the absorbance of DPPH in combination with the C. comosum extract.

Semen sample collection

In this experimental study, semen samples were obtained from twenty-five fertile men with the average age of 34 (range, 25-50 years) who referred to the *in vitro* fertilization (IVF) center of Gandhi Hospital according to the World Health Organization (WHO) criteria. The process of semen collection was carried out by masturbation into a sterile, wide-mouthed, calibrated glass container after five days of sexual abstinence. The collected specimens were allowed to liquefy at 37°C for 30 minutes prior to the semen analysis. The entire samples were collected from individuals who referred for medical procedures during assisted reproductive technology (ART). We used the remaining samples obtained from patients, with their permission, for research purposes.

Sperm preparation by the swim-up method

A fraction of motile spermatozoa was selected to be analyzed by the swim-up method. For this purpose, 1 ml of each semen sample (kept at 37°C and 5% CO_2) was added to 3 ml of human tubal fluid (HTF, Genocell Co., Iran) supplemented with 10% human albumin serum (HAS, Biotest, Germany) and centrifuged at 2000 ×g for 3 minutes. Afterward, 0.5 ml of HTF supplemented with 10% HAS was gently added on the resultant pellets. The samples were then incubated at 37°C, 5% CO_2, and inclined at a 45° angle to incubator for 45 minutes. Consequently, 0.5 ml of the uppermost medium was recovered, and the swim-up method was performed (21).

Sperm freezing and thawing

Each processed semen sample was cryopreserved according to the standard protocol for sperm freezing. According to Li et al. (7) and Ibrahim et al. (22), semen samples were gently mixed with an equal volume of modified cryoprotective medium (Global Media, USA) supplemented with 10 µg/ml of *Calligonum* extract. The samples were kept at 4ºC for 30 minutes and then frozen by placing the straws horizontally at 10 cm above the surface of liquid nitrogen (nitrogen vapor) at -80ºC for 20 minutes. Finally, all frozen straws were stored in liquid nitrogen until use.

At the thawing stage, the cryo-straws were removed from liquid nitrogen and immediately immersed in a water bath at 37ºC for at least 1-2 minutes. The thawed straws containing semen samples were flicked and inverted to mix the contents before sampling thoroughly and then washed with a culture medium HTF supplemented with 10% HAS (The samples were then treated with *Calligonum* extract (10 µg/ml)). At the end step, semen samples were centrifuged at 2000 ×g for 3 minutes to remove any trace of cryoprotectant in the freezing medium. The samples were analyzed for motility, viability, morphology, and DNA fragmentation (9, 23).

Preparation of ultrasound device

The US device (Physiomed, Germany) was set up at the frequency of 1 MHz, incident power density of 200 mW/cm^2, 200-second time period, and a 45-minute period as the duration of the experiment. These fine-tuned parameters were chosen based on our previous study published in this regard (24). The US stimulation was carried ou in which the transducer was put at the focal distance (3.5 cm) of a cell culture plate incubated in 5% CO_2 at 32˚C. It was transmitted through the bottom of the well via coupling gel between the transducer and the tube. The device was transmitted through the bottom of the well via coupling the gel between the transducer and cell culture plate. Notably, no temperature change more than 1ºC was recorded during the US stimulation.

Sperm stimulation using low-intensity pulsed ultrasound

Sperms maintained in the HTF medium supplemented with 10% HAS (Gibco, Germany). The samples were exposed to low-intensity pulsed ultrasound (LIPUS) (1 MHz, 200 mW/cm2 and 40% DC) alone or in combination with C. comosum coined as experimental groups. The control group was also cultured in the HTF medium supplemented with 10% HAS. After the US stimulation, spermatozoa were incubated for 45 minutes in 5% CO_2 at 32˚C ,similar to other experimental groups. To investigate the sperm parameters, the mean number of whole cells per volume, viability, morphology, and motility were examined after the incubation process.

Semen samples analysis

The sperm count, motility, morphology, viability, and DNA fragmentation were evaluated according to the guidelines introduced by the WHO (25). The sperm motility analysis was conducted using light microscopy (at ×400 magnification), combined with a computer-assisted semen analysis system (CASA, Video Test Sperm 1.2, Russia). A Makler chamber was used for the scoring of the sperm motility at room temperature. A minimum of 100 spermatozoa from at least five different fields was analyzed from each aliquot. Sperm morphology was assessed by the Diff-Quik staining method according to WHO guidelines by light microscopy (Labomed, USA). The sperm viability was determined by Eosin B (5% in saline) (Merck, Germany) staining technique at one-step. In this procedure, viable or dead spermatozoa are recognized by white (unstained) and pink (or red) coloration in the head region of the sperm cells, respectively.

Sperm DNA fragmentation

The Halotech DNA G2 kit (Spain) was used to study the DNA fragmentation in frozen-thawed spermatozoa. Based on the sperm chromatin dispersion test (SCD), the intact frozen-thawed spermatozoa were diluted in a culture medium HTF supplemented with 10% HAS to achieve sperm concentration of 15-20 million per milliliter. In this method, 50 µl of the semen sample was added to 100µl of dissolved agarose (0.7%) (Sigma, US). Afterward, 25 µl of the cell suspension was transferred to slides, pre-coated with

0.65% agarose and covered with a coverslip without any air bubbles, and then incubated at 4ºC for 5 minutes. After the removal of coverslips, the slides were horizontally immersed in a tray with freshly prepared acid denaturation solution (0.08 N HCl) for 7 minutes at 22°C in a dark place to create restricted single-stranded DNA from DNA breaks. The slides were then immersed in lysis Solution I [0.4 M Tris (Sigma, USA), 0.4 M 2-Mercaptophenol (Sigma, USA), 1% sodium dodecyl sulfate (SDS, Sigma, USA), and 50 mM Ethylenediaminetetraacetic acid (EDTA, Sigma, USA), pH=7.5 for 20 minutes and lysis Solution II (0.4 M Tris, 2 M NaCl, and 1% SDS, pH=7.5) for 5 minutes to remove nuclear proteins. Slides were then rinsed with distilled water for 5 minutes, followed by dehydration through an ascending gradient of ethanol (70, 90, and 100%) for 2 minutes. The slides were then placed at room temperature to be air-dried.

For Diff-Quik staining, the slides were incubated in eosin solution for 6 min; then, transferred into Azur B solution, for another 6 minutes. The nucleoid of spermatozoa with fragmented DNA did not develop a dispersion halo, or the halo was minimal. From each slide, a minimum of 500 spermatozoa was scored under an oil-immersion objective (×100 magnification) by light microscopy (Labomed, USA). The sperm cells showing no halo, small halo, medium halo, large halo, or fragmentation were separately scored. Spermatozoa indicating no halo/fragmentation were considered to have damaged chromatin, and the results were expressed as a percentage of sperm cells with DNA damage.

Intracellular reactive oxygen species level measurement

Spermatozoa were rinsed with phosphate-buffered saline (PBS, Atocel, Austria) and incubated with 20 µM 2, 7- dichlorofluorescein diacetate (DCFH-DA, Life Technologies, USA), diluted in serum-free medium at 37°C for 45 minutes. The intracellular ROS level was immediately analyzed with flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm (26).

Statistical analysis

The analysis of the values obtained in this study was performed by the SPSS version 19 (SPSS Inc., IBM company, USA), while the level of significance was set at $P<0.001$. The difference between the values of each group was analyzed by one-way analysis of variance (one-way ANOVA), followed by Tukey's test. The data were expressed as the means ± standard deviation (means ± SD).

Results

The activity of radical scavenging of DPPH

The *C. comosum* extract showed free radical-scavenging activity in a dose-dependent manner when examined by the DPPH assay. The inhibitory concentration (IC_{50}) value (the concentration of a given substance causing 50% inhibition of the DPPH) of the herb extract was 29.2 µg/ml when compared with ascorbic acid (positive control).

Effect of *Calligonum* extract and/or LIPUS on sperm parameters

Motility assessment

The demographic characteristics of patients are demonstrated in Table 1.

Table 1: The demography of semen samples

Characteristics	Average	SD
Age (Y)	34	3.041
Total motility (%)	80.7795	1.69
Grade A (%)	33.1496	1.74
Grade B (%)	30.7443	1.56
Grade C (%)	16.8856	1.39
Grade D (%)	19.4599	1.58
Grade A+B (%)	64.0651	2.08
Viability (%)	87	1.27
Normal morphology (%)	28.6397	4.26
SDF (%)	10.63	2.15
ROS (%)	11.0804	2.16

Grade A; Linear progressive motility, Grade B; Progressive motility, Grade C; Non-progressive motility, Grade D; Immotile, SDF; Sperm DNA fragmentation, ROS; Reactive oxygen species, and SD; Standard Diviation.

All of the sperm parameters, including viability, motility, and morphology in all experimental groups, are shown in Table 2. Accordingly, the percentage of total motility of spermatozoa in the fresh group was 89.37 ± 3.74, while this value was 81.30 ± 5.72 in the frozen-thawed group. After the addition of 10 µg/ml of Calligonum extract in the freezing medium increased the total motility; yet, such an increase was not statistically significant when compared with the control group ($P\geq0.05$). LIPUS (in pulsed and continues mode waves) alone decreased the motility of spermatozoa, compared with the control group ($P\leq0.038$), but the combination of *Calligonum* extract and LIPUS increased the motility to (81.90 ± 3.93) and (81.65 ± 5.18) respectively when used in pulsed mode and continuous modes waves. Also, there was an improvement in progressive motility of spermatozoa treated with the combination of *Calligonum* extract and LIPUS, compared with the frozen-thawed group; however, the difference between two groups was not statistically significant ($P\geq0.05$).

Viability assessment

As shown in Table 2, the viability of spermatozoa in all groups undergone the freeze-thawing process was significantly reduced as compared with the fresh group ($P\leq0.026$). Such a reduction was more pronounced in the LIPUS-exposed and CGM-treated groups, compared with other groups. There was no significant difference between the CGM+LIPUS and control groups (frozen-thawed spermatozoa) (frozen-thawed spermatozoa, $P\geq0.05$).

Table 2: Comparison of sperm parameters (± SD) between the experimental groups after frozen-thawed and treatment with 10 µg/ml Calligonum (CGM) extract and LIPUS (pulsed mode and continues wave)

Groups Sperm parameters	Control	Frozen - thawed	Frozen thawed+CGM extract	Frozenthawed+ continues wave of ultrasound	Frozenthawed+ continues wave of trasound+CGM extract	Frozenthawed+ pulsed mode of ultrasound	Frozenthawed+ pulsed mode of ultrasound +CGM extract
Viability (%)	$94.17^{c} \pm 5.97$	87.52 ± 4.75	90.11 ± 6.12	$82.76^{b} \pm 5.68$	86.70 ± 7.12	$81.82^{b} \pm 2.87$	87.94 ± 4.25
Total motility (%)	89.37 ± 3.74	81.30 ± 3.74	85.42 ± 4.28	$76.76^{ab} \pm 5.29$	81.65 ± 5.18	$75.86^{ab} \pm 3.47$	81.90 ± 3.93
Progressive motility (%)	38.80 ± 2.57	34.81a ± 3.28	37.81 ± 4.13	$33.04^{ab} \pm 2.98$	37.24 ± 5.11	$32.60^{ab} \pm 3.58$	$36.18^{a} \pm 4.78$
Normal morphology (%)	30.11 ± 3.16	26.58 ± 3.54	28.41 ± 4.27	27.58 ± 3.95	30.82 ± 2.75	28.35 ± 4.82	30.05 ± 3.66

[a]; Significant difference with control group in the same row (P≤0.05), [b]; Significant difference with freeze and thawed group + CGM extract, in the same row P≤0.026, and [c]; Significant difference with other groups in the same row (P≤0.047).

Table 3: Assessment of fragmented DNA and free radicals percentage in all experimental groups

Groups	Control	Frozen - thawed	Frozen - thawed + CGM extract	Frozen - thawed + continues wave of ultrasound	Frozen - thawed + continues wave of ultrasound+CGM extract	Frozen - thawed + pulsed mode of ultrasound	Frozen - thawed + pulsed mode of ultrasound+CGM extract
Fragmented DNA (%) ± SD	24.5 ± 7.23	$46.5^{a} \pm 9.26$	37 ± 4.29	38 ± 5.37	34 ± 5.17	$51^{a} \pm 11.85$	$42.5^{a} \pm 7.39$
Free radicals(ROS) (%) ± SD	-	12.46 ± 7.06	9.18 ± 2.57	12.52 ± 8.14	7.77 ± 2.06	14.75 ± 4.67	10.05 ± 1.68

CGM; Calligonum extract, ROS; Reactive oxygen species, and [a]; significant difference with control group in the same row (P≤0.042).

Morphology assessment

According to Table 2, the normal morphology score was 30.11 ± 3.16 in the fresh group, while the normal morphology score was 26.58 ± 3.54 i\n the control group. The statistical analysis revealed that there was no significant difference in the score of normal morphology among all treated groups, namely, the CGM-treated, LIPUS-exposed, and CGM+LIPUS groups.

Effect of Calligonum extract and/or LIPUS (pulsed or continuous mode waves) on DNA fragmentation

Table 3 shows the rate of DNA fragmentation in all experimental groups. The percentage of spermatozoa undergone DNA fragmentation was considerably higher in all groups than the fresh group. The rate of DNA fragmentation was significantly (P≤0.042) elevated in the frozen-thawed (46.5 ± 9.26) and LIPUS-exposed groups (51 ± 11.85) in comparison with the control group.

Effect of Calligonum extract and/or LIPUS on reactive oxygen species level

According to Table 3, the level of ROS in the control group receiving no treatment was 12.46 ± 7.06, while the level of ROS was decreased to 9.18 ± 2.57 in the CGM group. The statistical analysis demonstrated that the difference in the amount of ROS was not significant in all experimental groups as compared with the control group (P≥0.05).

Discussion

Cryopreservation of human semen provides a valuable therapeutic opportunity for the management of patients who are at risk of infertility (27). However, during cryopreservation, spermatozoa experience physical and chemical stress that could result in detrimental changes in lipid composition of the cell membrane, leading to the excessive amount of ROS production, as well as a decrease in sperm motility and viability (28, 29). Notably, the osmotic stress and the alterations in the temperature may cause mechanical stress to the cell membrane of spermatozoa. Therefore, the changes mentioned above could reduce the fertilization capability of human spermatozoa after the cryopreservation process (30).

It has been implicated that antioxidant therapy can increase the quality of cryopreserved spermatozoa when employed both *in vitro* and *in vivo* (31). Numerous antioxidant agents have been indicated to have beneficial roles in the protection against cellular damages caused by cryopreservation-induced ROS, affecting sperm motility and viability. Hence, the application of antioxidant compounds to neutralize the deleterious impacts of oxidative damage would be of asset to improve the sperm parameters (32). Therefore, in this study, we examined the effects of *Calligonum* extraction, as an antioxidant agent, to annul the harmful effects of free radical molecules generated during the freeze-thawing process. We also applied LIPUS (in continuous and pulsed mode waves) to induce

physical stimulation to spermatozoa for possible inciting the motility and viability of sperm cells after cryopreservation.

Our data revealed *Calligonum* extract at a concentration of 10 µg/ml slightly increased the sperm motility and viability, whereas it decreased DNA fragmentation and ROS level in human spermatozoa. The combination therapy using LIPUS and *Calligonum* extract could enhance sperm parameters when compared with the LIPUS-exposed group. Our findings were consistent with the previous studies performed in this area. For instance, Martinez-Soto et al. (29) evaluated the effects of extender supplemented with genistein on frozen-thawed human spermatozoa. They found that genistein, known as isoflavone compound, had antioxidant properties on cryopreserved spermatozoa. Their results also showed that the ROS production was decreased and the sperm motility was slightly improved in response to treatment with genistein. In another study, Banihani et al. (33) found that L-carnitine had positive effects on the improvement of sperm motility and viability during cryopreservation but had no effect on the reduction of DNA oxidation. Regarding our results, the addition of *Calligonum* extract to the freezing medium led to a significant increase in progressive motility.

On the other hand, LIPUS at the frequency of 1 MHZ and incident power density of 200 mW/cm^2, in both pulsed and continuous mode waves, had adverse impacts on the sperm parameters. In the LIPUS-exposed group, the viability, as well as total and progressive motility was decreased while the number of none-motile spermatozoa was increased as compared with the fresh group. The US radiation alone increased intracellular ROS level and disrupted the balance of pro-oxidant and antioxidant contents in human spermatozoa, led to the elevated rate of DNA fragmentation. Also, our data demonstrated that LIPUS did not alter the morphology of frozen spermatozoa; however, the combinatory treatment of spermatozoa with *Calligonum* extract and LIPUS was capable of enhancing the sperm parameters during cryopreservation.

Previous studies have highlighted that LIPUS, as mechanical energy, could have therapeutic effects on bone and wound healing (34, 35). Also, it has been reported that it could expedite the process of tissue repair by the stimulation of the proliferation of fibroblasts and osteoblasts (17, 36). Furthermore, Xu et al. (37) have shown that LIPUS can stimulate the viability of freeze-thawed spermatozoa and it can increase the proliferation and differentiation of hematopoietic stem cells, obtained from fresh and cryopreserved peripheral blood leukapheresis product. However, inconsistent with other studies, our findings failed to show the beneficial effects of this therapeutic approach on the sperm parameters.

In the present study, the adverse effects of LIPUS on the sperm parameters may be due to the changes in the frequency of waves as a result of the sperm motility, leading to the reduction in the effectiveness of LIPUS penetration. Since the distribution of the US field is not homogenous, and it is susceptible to the reflection and attenuation once the field passes through the boundary separating two different media (38), the potency of US waves might be mitigated. So further examinations on applying other techniques for irradiating ultra sound on motile sperms is needed.

Conclusion

Our results showed that *Calligonum* extract, at a concentration of 10 µg/ml, slightly enhanced the cellular parameters of cryopreserved spermatozoa diminished the rate of DNA fragmentation, and decreased the intracellular ROS level. Contrary to our expectation, LIPUS, in both pulsed and continuous mode waves, had the adverse effects on the sperm parameters which may stem from alterations in the temperature of the medium as a result of LIPUS treatment. It should be noted that the sperm motility might influence the frequency of the US waves, lowering the effectiveness of LIPUS treatment. This phenomenon can weaken the impact of LIPUS on the sperm parameters during cryopreservation.

Acknowledgements

This study was financially supported by Research Deputy of Tarbiat Modares University, Tehran, Iran. There is no conflict of interest in this study.

Authors' Contributions

M.M.; Contributed to the conception and design of the work and acquisition, analysis and interpretation of the data; H.E.; Participated in study design, data collection and evaluation, drafting and statistical analysis. Z.M., K.F.; Contributed extensively in interpretation of the data and the conclusion. M.M.D.; Designed and conducted ultrasound irradiation part and read early draft and gave some corrections. All authors read and approved the final manuscript.

References

1. Meamar M, Zribi N, Cambi M, Tamburrino L, Marchiani S, Filimberti E, et al. Sperm DNA fragmentation induced by cryopreservation: new insights and effect of a natural extract from Opuntia ficus-indica. Fertil Steril. 2012; 98(2): 326-333.
2. Aitken RJ, Baker MA. Oxidative stress, sperm survival and fertility control. Mol Cell Endocrinol. 2006; 250(1-2): 66-69.
3. Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. Am J Reprod Immunol. 2008; 59(1): 2-11.
4. Gadea J, Molla M, Selles E, Marco MA, Garcia-Vazquez FA, Gardon JC. Reduced glutathione content in human sperm is decreased after cryopreservation: effect of the addition of reduced glutathione to the freezing and thawing extenders. Cryobiology. 2011; 62(1): 40-46.
5. Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang J-A, Clark AM. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod. 2009; 24(9): 2061-2070.
6. Kalthur G, Raj S, Thiyagarajan A, Kumar S, Kumar P, Adiga SK. Vitamin E supplementation in semen-freezing medium improves the motility and protects sperm from freeze-thaw–induced DNA damE-

krami et al. Int J Fertil Steril, Vol 14, No 2, July-September 2020 90 age. Fertil Steril. 2011; 95(3): 1149-1151.
7. Li Z, Lin Q, Liu R, Xiao W, Liu W. Protective effects of ascorbate and catalase on human spermatozoa during cryopreservation. J Androl. 2010; 31(5): 437-444.
8. Askari HA, Check JH, Peymer N, Bollendorf A. Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. Arch Androl. 1994; 33(1): 11-15.
9. Garcez ME, dos Santos Branco C, Lara LV, Pasqualotto FF, Salvador M. Effects of resveratrol supplementation on cryopreservation medium of human semen. Fertil Steril. 2010; 94(6): 2118-2121.
10. Abdo W, Hirata A, Shukry M, Kamal T, Abdel-Sattar E, Mahrous E, et al. Calligonum comosum extract inhibits diethylnitrosamine-induced hepatocarcinogenesis in rats. Oncol Lett. 2015; 10(2): 716-722.
11. Liu XM, Zakaria MN, Islam MW, Radhakrishnan R, Ismail A, Chen HB, et al. Anti-inflammatory and anti-ulcer activity of Calligonum comosum in rats. Fitoterapia. 2001; 72(5): 487-491.
12. Badria FA, Ameen M, Akl MR. Evaluation of cytotoxic compounds from Calligonum comosum L. growing in Egypt. Z Naturforsch C J Biosci. 2007; 62(9-10): 656-660.
13. Khanna A, Nelmes RT, Gougoulias N, Maffulli N, Gray J. The effects of LIPUS on soft-tissue healing: a review of literature. Br Med Bull. 2009; 89: 169-182.
14. Iwashina T, Mochida J, Miyazaki T, Watanabe T, Iwabuchi S, Ando K, et al. Low-intensity pulsed ultrasound stimulates cell proliferation and proteoglycan production in rabbit intervertebral disc cells cultured in alginate. Biomaterials. 2006; 27(3): 354-361.
15. Kobayashi Y, Sakai D, Iwashina T, Iwabuchi S, Mochida J. Low-intensity pulsed ultrasound stimulates cell proliferation, proteoglycan synthesis and expression of growth factor-related genes in human nucleus pulposus cell line. Eur Cell Mater. 2009; 17: 15-22.
16. Mahoney CM, Morgan MR, Harrison A, Humphries MJ, Bass MD. Therapeutic ultrasound bypasses canonical syndecan-4 signaling to activate rac1. J Biol Chem. 2009; 284(13): 8898-8909.
17. Zhou S, Schmelz A, Seufferlein T, Li Y, Zhao J, Bachem MG. Molecular mechanisms of low intensity pulsed ultrasound in human skin fibroblasts. J Biol Chem. 2004; 279(52): 54463-54469.
18. Tang CH, Yang RS, Huang TH, Lu DY, Chuang WJ, Huang TF, et al. Ultrasound stimulates cyclooxygenase-2 expression and increases bone formation through integrin, focal adhesion kinase, phosphatidylinositol 3-kinase, and Akt pathway in osteoblasts. Mol Pharmacol. 2006; 69(6): 2047-2057.
19. Loganayaki N, Siddhuraju P, Manian S. Antioxidant activity and free radical scavenging capacity of phenolic extracts from Helicteres isora L. and Ceiba pentandra L. J Food Sci Technol. 2013; 50(4): 687-695.
20. Kedare SB, Singh RP. Genesis and development of DPPH method of antioxidant assay. J Food Sci Technol. 2011; 48(4): 412-422.
21. Saylan A, Duman S. Efficacy of hyaluronic acid in the selection of human spermatozoa with intact DNA by the swim-up method. Cell J. 2016; 18(1): 83-88.
22. Ibrahim SF, Osman K, Das S, Othman AM, Majid NA, Rahman MP. A study of the antioxidant effect of alpha lipoic acids on sperm quality. Clinics (Sao Paulo). 2008; 63(4): 545-550.
23. Donnelly ET, McClure N, Lewis SE. Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. Fertil Steril. 2001; 76(5): 892-900.
24. Mohaqiq M, Movahedin M, Mokhtari Dizchi M, Mazaheri Z. Investigation on the effect of low intensity ultrasound stimulation on mouse spermatogonial stem cell proliferation and colonization. Anat Sci J. 2013; 10(3): 119-124.
25. Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, et al. World Health Organization reference values for human semen characteristics. Hum Reprod Update. 2010; 16(3): 231-245.
26. Chang JC, Lien CF, Lee WS, Chang HR, Hsu YC, Luo YP, et al. Intermittent Hypoxia prevents myocardial mitochondrial Ca2+ overload and cell death during ischemia/reperfusion: the role of reactive oxygen species. Cells. 2019; 8(6). pii: E564.
27. Oehninger S, Duru NK, Srisombut C, Morshedi M. Assessment of sperm cryodamage and strategies to improve outcome. Mol Cell Endocrinol. 2000; 169(1-2): 3-10.
28. O'connell M, McClure N, Lewis SE. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod. 2002; 17(3): 704-709.
29. Martinez-Soto JC, de Dioshourcade J, Gutiérrez-Adán A, Landeras JL, Gadea J. Effect of genistein supplementation of thawing medium on characteristics of frozen human spermatozoa. Asian J Androl. 2010; 12(3): 431-441.
30. Câmara DR, Silva SV, Almeida FC, Nunes JF, Guerra MM. Effects of antioxidants and duration of pre-freezing equilibration on frozenthawed ram semen. Theriogenology. 2011; 76(2): 342-350.
31. Shi X, Hu H, Ji G, Zhang J, Liu R, Zhang H, et al. Protective Effect of sucrose and antioxidants on cryopreservation of sperm motility and DNA integrity in C57BL/6 mice. Biopreserv Biobank. 2018; 16(6): 444-450.
32. Yousef MI, Abdallah GA, Kamel KI. Effect of ascorbic acid and vitamin E supplementation on semen quality and biochemical parameters of male rabbits. Anim Reprod Sci. 2003; 76(1-2): 99-111.
33. Banihani S, Agarwal A, Sharma R, Bayachou M. Cryoprotective effect of L-carnitine on motility, vitality and DNA oxidation of human spermatozoa. Andrologia. 2014; 46(6): 637-641.
34. Heckman JD, Ryaby JP, McCabe J, Frey JJ, Kilcoyne RF. Acceleration of tibial fracture-healing by non-invasive, low-intensity pulsed ultrasound. J Bone Joint Surg Am. 1994; 76(1): 26-34.
35. El-Bialy T. Therapeutic ultrasound applications in craniofacial growth, healing and tissue engineering. Rejuvenation Res. 2007; 10(3): 367-371.
36. Doan N, Reher P, Meghji S, Harris M. In vitro effects of therapeutic ultrasound on cell proliferation, protein synthesis, and cytokine production by human fibroblasts, osteoblasts, and monocytes. J Oral Maxillofac Surg. 1999; 57(4): 409-419.
37. Xu P, Gul-Uludag H, Ang WT, Yang X, Huang M, Marquez-Curtis L, et al. Low-intensity pulsed ultrasound-mediated stimulation of hematopoietic stem/progenitor cell viability, proliferation and differentiation in vitro. Biotechnol Lett. 2012; 34(10): 1965-1973.
38. Patel US, Ghorayeb SR, Yamashita Y, Atanda F, Walmsley AD, Scheven BA. Ultrasound field characterization and bioeffects in multiwell culture plates. J Ther Ultrasound. 2015; 3: 8.

Original Article

Long-Term Effect of Aspartame on Male Reproductive System: Evidence for Testicular Histomorphometrics, Hsp70-2 Protein Expression and Biochemical Status

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Abstract

Background: Aspartame is one of the most commonly consumed artificial sweeteners that is widely used in foodstuffs. There are many debatable reports about aspartame toxicity in different tissues; however, on the subject of its effects on the reproductive system, few literatures are available. The present study was carried out for evaluating effects of aspartame on the reproductive system in male mice.

Materials and Methods: In this experimental study, a total of 36 adult male mice were randomly divided into four groups of nine animals each. Three groups received aspartame at doses of 40, 80 and 160 mg/kg (gavage) for 90 days; also, a control group was considered. Twenty-four hours after the last treatment, animals were sacrificed. Then, body and testis weights, sperm parameters, serum testosterone concentration, total antioxidant capacity, and malondialdehyde (MDA) levels, antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px)] activities in blood, histomorphometrical indices and histochemical changes in testis were evaluated; also, mRNA and immunohistochemical expression of Hsp70-2 was measured in testis tissue.

Results: The results revealed remarkable differences in sperm parameters, testosterone and oxidative stress biomarkers levels, and histomorphometrical indices, between the control and treatment groups. Also, in 80 and 160 mg/kg aspartametreated groups, expression of Hsp70-2 was decreased. Besides, in the aspartame receiving groups, some histochemical changes in testicular tissue were observed.

Conclusion: The findings of the present study elucidated that long-term consumption of aspartame resulted in reproductive damages in male mice through induction of oxidative stress.

Keywords: Aspartame, *Hsp70-2*, Mice, Testis

Citation: Anbara H, Sheibani MT, Razi M. Long-term effect of aspartame on male reproductive system: evidence for testicular histomorphometrics, Hsp70-2 protein expression and biochemical status. Int J Fertil Steril. 2020; 14(2): 91-101. doi: 10.22074/ijfs.2020.6065.

Introduction

Recently, food consumers are increasingly concerned about the quality and safety of many foodstuffs produced by industrialized countries; in particular, the usage of artificial sweeteners, flavorings, dyes, preservatives and food supplements has raised concerns. Many non-nutrient sweeteners have been used in foods and beverages to help people enjoy a sweet taste without raising body calories. One of these sweeteners is aspartame (1). Aspartame (Laspartyl-L-phenylalanine methyl ester) is a synthetic nonnutritional sweetener that was firstly discovered in 1965 and approved in 1981 for use in the United States (2). This sweetener is a dipeptide derived from the combination of two non-aromatic amino acids namely, aspartic acids and phenylalanine. Sweetening power of aspartame is 160 to 180 times more than that of sucrose, it has the same number of calories as sugar, and it does not smell and lacks metallic taste (3).

After oral intake, aspartame is hydrolyzed in the gastrointestinal tract by esterases and peptidases into amino acids (aspartic acid and phenylalanine) and methanol. Also, it is possible that aspartame is absorbed by the mucosal cells of the intestines and metabolized before hydrolysis (3).

Methanol is not metabolized in the enterocytes; it immediately enters the portal circulation and is then oxidized in the liver into formaldehyde (4). Metabolism of methanol into formaldehyde and formic acid is associated with formation of superoxide anion and hydrogen peroxide (5). Development of oxidative stress through methanol oxidation results in structural and functional impairments of proteins responsible for regulating and maintaining the lower temperature of the testes (7). In fact, the testicular temperature must be 2-4°C lower than the rest of the body for maintaining optimal sperm quality. Even a slight increase in temperature could lead to rapid disrupts

Received: 9/August/2019, Accept: 28/December/2019
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in spermatogenesis due to inducing protein denaturation (8). Hsp70 proteins besides increasing the RNA-binding protein stability in haploid cells, are able to take part in recovering DNA and RNA damages through improvement of DNA integrity. During early meiosis and/or mitosis, Hsp70-2, which is considered the main expressed chaperone, has capability to induce folding/refolding in proteins during different phases of cell cycles. Also, there is evidence about more than 20 chaperone families, which are influenced by some biochemical stressors, including oxidative and nitrosative stresses, and become well up regulated (8).

Considering the fact that the majority of these chaperone families are cell stress responders or heat shock proteins (HSPs), chaperones have important roles in raising the cellular resistance against environmental stressors, although the HSPs are known to be involved in regulating spermatogenesis (8, 9). Researchers have reported that long-term consumption of aspartame up-regulates the expression of Hsp70 in the brain, heart, liver and immune organs (6, 10-12). Beside having cytoprotective effects, Hsp70 has a role in regulating spermatogenesis (8, 9). It was indicated that long-term consumption of aspartame leads to reproductive toxicity in male rats (13). In the present study, we investigated Hsp70-2 expression following long-term consumption of aspartame in male mice. Also, in order to confirm induction of oxidative stress and reproductive toxicity by aspartame, levels of oxidative stress biomarkers were measured in blood. Moreover, concentration of testosterone in serum was measured, and histochemical and histopathological evaluations in testis tissue were performed in order to confirm reproductive toxicity of aspartame. In the literature, there is no study done in the male genital system that investigated Hsp70, or performed histomorphometerical assessment in this context, which implies the novelty of the present experiment.

Materials and Methods

Chemicals

Aspartame was purchased from Sigma-Aldrich (St Louis, MO, USA, CAS No. 22839-47-0). Acridine orange was purchased from sigma chemical Co. (St. Louis, MO, USA). All other chemicals used were commercial products of analytical grade. The rabbit anti-mouse primary antibodies for *Hsp70-2* (Cat NO. SKU: 407), were obtained from Biocare (Biocare, California, USA).

Animals

All experimental protocols were conducted on the basis of the proofed principles for laboratory animal care (7506025.6.24), approved by the Ethical Committee of the University of Tehran. For this study, a total number of 36 NMRI mature male mice (8-10 weeks of age), weighing 25-35 g were used. The animals were provided from the Laboratory Animal Sciences Center, Pasteur Institute of Iran, Karaj, Iran. Before initiation of the treatment period, the mice were maintained for two weeks in order to acclimatize. The mice were housed in special cages under well-ventilated conditions at normal temperature (22 ± 5°C) with 12:12-hour light-dark cycles and fed standard pellet diet (Tehran pellet, Iran).

Chemical administration and grouping

In this experimental study, The European Food Safety Authority has confirmed acceptable daily intake (ADI) for 40 mg/kg bodyweight/day of aspartame. This ADI was approved by the food and drug administration (FDA) for the European countries (EFSA Journal 2013). After labeling the mice, they were randomly divided into four groups of nine mice. The treatment groups received aspartame for 90 days by gavage as follows:

1. The first group (control): The animals of this group received normal saline at the dosage of 0.5 ml.

2. The second group was called low dose aspartame and it received 40 mg/kg bodyweight/day of aspartame.

3. The third group was called medium dose aspartame and it received 80 mg/kg bodyweight/day of aspartame.

4. The forth group was called high dose aspartame and it received 160 mg/kg bodyweight/day of aspartame.

Thereafter, the animals were kept under standard conditions and monitored for 90 days. On the basis of the fact that the duration of the chronic dose of aspartame is ninety days to have probable pathogenicity, this period was chosen for this experiment. The dosages and duration of the treatment in the present study were chosen on the basis of earlier studies (13, 14) (Fig.S1, See Supplementary Online Information at www.ijfs.ir).

Serum and tissue samples preparation

Following the 90-day period, all animals were anesthetized using a mixture of ketamine and xylazine cocktail (0.10 ml xylazine and 1 ml ketamine and 8.90 ml distilled water), with the dose of 0.1 ml/10 g BW (15). In order to obtain serum, 15 minutes after anesthesia induction, the blood samples were centrifuged at 3000 g for 10 minutes at room temperature (RT) and stored at -70°C for further analyses. The testicular specimens were removed and rinsed with chilled normal saline. One of the testes from each individual mouse was snap frozen in liquid nitrogen and then kept in -70°C until further biochemical analyses and the other testes were fixed in Bouin's solution for histological examinations.

Histomorphometrical and histochemical assay

The testes were quickly dissected out, cleared of adhered connective tissue and weighed on a digital scale (with a minimum accuracy of 0.001 g). For Histomorphometrical study, Dino-Lite digital lens and Dino Capture 2 Software were used. Furthermore, histometrical structures of the testes, including testicular capsule thickness, germinal

epithelium height and diameter of seminiferous tubules, as well as the number of Sertoli and Leydig cells were evaluated. In order to classify spermatogenesis, Johnsen's criteria were used. This classification is based on graded scoring between 1-10 for each tubule cross-section, according to presence or absence of main cell types organized in the order of maturity:10, complete spermatogenesis exists and tubules are normal in arrangement; 9, there are many spermatozoa with disorganization in germinal epithelium; 8, only a few spermatozoa are observed; 7, lacking spermatozoa while many spermatids exist; 6, only a few spermatids are present; 5, absence of spermatozoa and spermatids but existence of many spermatocytes; 4, only a few spermatocytes exist; 3, only spermatogonia are observed; 2, presence of only Sertoli cells and the absence of germ cells, and 1, no germ cells or Sertoli cells are present. Tubule cross-sections with scores of 9 and 10 were considered mature tubules (15).

Paraffin blocks were sectioned at 5-6 μm and stained with Hematoxylin and Eosin (H&E), Periodic acid-Schiff (PAS) and Masson's trichrome. Masson's trichrome staining was used to show the amount of collagen fibers and fibrosis in testicular tissue. In order to analyze carbohydrate ratio in testicular germinal epithelium, PAS was conducted on specimens. Also, for the purpose of histochemical evaluations, frozen sectioning method was carried out. The samples were embedded using optimal cutting temperature compound (OCT gel) and sections of testicular tissues were prepared at 15-20 μm levels at -40°C using cryostat (SLEE, Germany). Also, the Sudan black B (SB) staining was performed to evaluate the rate of lipid foci supplement in treatment and control animals and identify the Leydig cells cytoplasmic bio-steroid supplement. The alkaline phosphatase staining (ALP) was conducted to demonstrate the ratio of this enzyme as a biomarker for inflammation. The photomicrographs were taken by a SONY on-board camera (Zeiss, Cyber-Shot, Japan).

Sperm preparation and DNA damage assessment

Epididymides were carefully refined from their surrounding tissues under 10X magnification provided by a Stereo Zoom Microscope (TL2, Olympus Co., Tokyo). The caudate part of the epididymis was trimmed and minced in 5 ml TCM199 medium for 30 minutes, with 5% CO_2, at 36.5°C in a CO_2-equipped incubator (LEEC Co., England). After centrifugation, the sperm pellet was re-suspended in 0.5 ml of TCM199 medium. A small aliquot (20 μl) of sperm suspension was glass-smeared. The slides were air-dried and then fixed overnight in Carnoy's solution (methanol/acetic acid, 3:1). Next, they were stained for 5 minutes with a freshly-prepared acridine orange stain (AO). After washing and drying, the slides were examined using a fluorescent microscope (Leitz, Germany; excitation of 450-490 nm). On each slide, an average of 200 sperms were evaluated and two types of staining patterns were identified including yellow (single-stranded DNA) sperms and green (double-stranded DNA) (16).

The percentage of spermatozoa with single-stranded DNA was calculated from the ratio of spermatozoa with red, orange, or yellow fluorescence, to the total spermatozoa counted per sample.

Sperm count, motility and viability

Sperm count was assessed by a standard hemocytometer method (15). The motility of the sperm was evaluated according to the WHO (WHO, 2010) standard method for manual examination of sperm motility (17). Accordingly, the sperm samples were diluted 1:8 in TCM199 before the assessment. A 20-μl sample of the sperm was placed on a sperm test area and evaluated under 1,000X magnifications. Only the motile sperms with forward progression were counted within 10 boxes and recorded. Finally, motility was calculated based on the following equation:
Motility (%) = [motile sperm/(motile+non-motile sperm)]×100.

The Eosin-nigrosin staining method was performed to assess the sperm viability. For this purpose, 50 μl of sperm was mixed with 20 μl of eosin in a sterile test tube. After 5 seconds, 50 μl of nigrosin was added and mixed thoroughly. Then, the mixture of the stained sperm was smeared on the slide and examined under a light microscope (1,000X magnification, Olympus, Germany). The colorless sperms were considered live and the yellow to pink stained sperms were marked as dead. The sperm count was performed according to the standard hemocytometric method previously described by Pant and Srivastava (16). The sperm viability and motility are reported in percentage and compared between groups.

Assessment of serum levels of testosterone

Following 90 days, blood samples were obtained directly from the heart under light anesthesia (induced using diethyl ether). After 15 minutes, the samples were centrifuged at 3000×g for 10 minutes at RT to obtain serum. Serum concentration of testosterone was measured by enzyme-linked immunosorbent assay (ELISA) as described by the manufacturer (Demeditec Diagnostics GmbH, Germany). In brief, 100 μl of serum sample and control (from the kit) were dispensed into the ELISA wells, and 100 μl of Enzyme conjugate was added into the wells and thereafter, incubated 60 minutes at RT. Next, the content of the wells was discarded and rinsed 4 times with diluted Wash Solution (300 μl per well), and 200 μl of Substrate Solution was added to each ELISA well. The samples were thereafter incubated in the dark for 30 minutes. Finally, 50 μl of Stop Solution was added to each well and the absorbance of each sample was determined at 450 nm.

Assessment of oxidative stress biomarkers

Some important detectable oxidative stress biomarkers, including total antioxidant capacity (TAC), and activities of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) and nitric oxide (NO) content were measured in the blood samples as described previously (15, 18).

Determination of GSH-Px activity was performed by GSH-Px detection kit (Ransel, RanDox Co, UK) based on manufacturer's instructions. One unit of GSH-Px was defined as μM of oxidized NADPH per minute mg-1 of protein. A decrease in absorbance was recorded by spectrophotometry against blank, at 340 nm.

The SOD activity was evaluated at 505 nm using a standard curve. The SOD activity was determined by the SOD detection kit (RanSod, RanDox Co, UK) based on the manufacturer's instructions. Serum NO level was measured according to the Griess reaction (17) and expressed as μM/l. CAT activity, on the basis of a previously described method, was evaluated. Here, the blood samples were homogenized in Triton X-100 1% (Merck, Germany) and then diluted using phosphate buffer (pH=7.0). For initiation of the reaction, hydrogen peroxide was added to the mixture and the level of enzyme activity on the basis of the competency of the CAT in decompensation of hydrogen peroxide, was determined. This was gained through scanning the decrease in absorbance at 240 nm against a blank containing phosphate buffer instead of the substrate. The value of log A1/A2 of a measured interval was used for unit definition due to the initial reaction of the enzyme, where the value of A1 refers to the absorbance at 240, at time 0 seconds and A2 is the absorbance at 240, at second 15. These enzyme activities were expressed as U g-1 Hb in blood. Then the measurement of the protein level in supernatant took place using the colorimetric method of Lowry with bovine serum albumin (BSA) as standard (15).

The MDA level as an indicator of lipid peroxidation in serum was determined according to the procedure described by Buege and Aust. Here, 100 μl of serum specimens using a glass homogenizer was homogenized in 0.15 M/l KCl at a ratio of 1 to 9 ml. One volume of homogenate was blended thoroughly with two volumes of a stock solution of 15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, and 0.25 M/l hydrochloric acid. After heating and cooling cycles, the solution was clarified by centrifugation at 1000 ×g for 10 minutes. The absorbance of the clear solution was read at 535 nm and MDA content was figured out using 1.56×10^5 M-1 cm-1 as molar absorbance coefficient. MDA levels are presented as mM per ml protein (15).

Evaluation of TAC was carried out on the basis of the manual of the kit (TAS test kit, Randox Laboratories Ltd, GB).

Immunohistochemical analysis for Hsp70-2

Immunohistochemical staining was done in order to analyze Hsp70-2 positive cells distribution. For this, before beginning the staining process, 5-μm tissue sections were heated at 60°C for approximately 25 minutes in a hot-air oven (Venticell, MMM, Einrichtungen, Germany). After deparaffinization in two changes of xylene, the sections were rehydrated using an alcohol gradient (96, 90, 80, 70, and 50%). The antigen retrieval process was performed in 10 mM sodium citrate buffer (pH=7.2). Immunohistochemical staining was conducted according to the manufacturer's protocol (Biocare, USA). In brief, endogenous peroxidase was blocked in a peroxidase blocking solution (0.03% hydrogen peroxide containing sodium azide) for 5 minutes. Washing the sections was done with phosphate-buffered saline (PBS, DNAbiotech, Iran, pH=7) and subsequently incubation was performed with Hsp70-2 (1:600) biotinylated primary antibodies (Biocare, USA) at 4°C in humidified chamber overnight. After rinsing with PBS, the sections were incubated with streptavidin–HRP (streptavidin conjugated to horseradish PBS containing an anti-microbial agent) for 20 minutes. Followed by rinsing in washing buffer and adding a 3,3' Diaminobenzidine (DAB) chromogen, they were incubated for 10 minutes and counter stained with hematoxylin for 10 seconds. Then, the sections were dipped in ammonia (0.037 Ml), rinsed with distilled water, and cover slipped. Positive immunohistochemical staining could be observed as brown stains under a light microscope (8).

RNA isolation, cDNA synthesis and reverse transcription-polymerase chain reaction

For RNA extraction, the collected testicles and those previously stored at -70°C, were used; RNA extraction was performed on the basis of the standard TRIzol method (8). For this, 20-30 mg of testicular tissue from an individual animal of each group was homogenized in 1 ml of TRIZOL. Then, in order to avoid genomic DNA contamination, the colorless aqueous phase was collected carefully. The quantitative assessment of RNA was performed using a nanodrop spectrophotometer (260 nm and A260/280=1.8-2.0), followed by storage of the samples at -70°C. For reverse transcriptionpolymerase chain reaction (RT-PCR), the cDNA was synthesized in a 20-μl reaction mixture containing 1 μl of oligo (dT) primer, 1 μl of RNAse inhibitor, 4 μl of 5X reaction buffer, 1 μg of RNA, 1 μl of M-MuLV reverse transcriptase and 2 μl of a 10 mM dNTP mix, on the basis of the manufacturer's protocol (Fermentas, GmbH, Germany). The cycling protocol for 20 μl reaction mix was 5 minutes at 65°C, followed by 60 minutes at 42°C, and 5 minutes at 70°C to finalize the reaction. For evaluating the PCR reaction, a total volume of 27 μl containing primers pair's sequences (each 1 μl), 13 μl of PCR master mix and cDNA as a template (1.5 μl) and 10.5 μl of nuclease free water, were used. The following PCR conditions were considered; general denaturation at 95°C for 3 minutes for 1 cycle, followed by 35 cycles of 95°C for 20 seconds; annealing temperature (62°C for *Hsp70-2*, and 58°C for *GAPDH*) for 60 which participate in antioxidant defense system (6). Hsp70 functions as a cell supportive factor against many stresses that induce the production of reactive oxygen species(ROS), which in turn affect cellular molecules including DNA, proteins and lipids. Also, Hsp70 protein is known to be seconds; elongation: 72°C for 1 minute and 72°C for 5 minutes. Final PCR products were analyzed on 1.5% agarose gel electrophoresis and densitometric analysis of the bands were done by using PCR Gel analyzing software (ATP, Iran). The control was set at 100% and experimental samples were compared to the control. Specific primers were designed and manufactured by Sinaclon (Sinaclon Co., Iran). The primers pair's sequences and product size for primers used in RT-PCR are presented in Table 1.

Table 1: Nucleotide sequences and product size of primers used in reverse transcription-polymerase chain reaction

Target gene	Primer sequence (5'-3')	Product size (bp)
Hsp70	F: CAGCGAGGCTGACAAGAAGAA R: GGAGATGACCTCCTGGCACT	340
GAPDH	F: TGAAGCAGGCATCTGAGGG R: CGAAGGTGGAAGAGTGGGAG	320

Statistical analysis

The data was analyzed using SPSS program version 19.0 (SPSS Inc, Chicago, IL, USA). All results are presented as mean ± SD. Differences between quantitative histological and biochemical data were analyzed by one-way ANOVA, followed by Tukey test, using Graph Pad Prism, 4.00. The $P<0.05$ were considered statistically significant.

Results

Histomorphometrical parameters

The results of histomorphometric studies showed that the thickness of testicular capsule in the high-dose group of aspartame, had a significant increase compared to the control group, whereas, the number of Sertoli and Leydig cells showed a significant decrease ($P<0.05$) in this group. Also, in medium- and high-dose aspartame-treated groups, a significant decrease ($P<0.05$) was observed in the diameter of the seminiferous tubules, the height of the germinal epithelium and the Johnsen's score (Table 2).

Histological and histochemical findings

Our histological observations revealed that aspartame, in a dose-dependent manner, could increase disarrangement and produce severe edema in connective tissue. An increase in germinal epithelium dissociation (GED) and tubular depletion in medium- and high-dose aspartame-treated groups, was observed. Especially in the high-dose group, aspartame could induce drastic morphologic changes in the testes. There were some atrophied seminiferous tubules indicating severe reduction in the number of germ cells and intensive immune cells infiltration, edematous fluid accumulation and intertubular space widening in interstitial connective tissue. Moreover, Sertoli cells lost their junction with germ cells and looked amorphous with irregular and smaller nuclei (Fig.1).

Also, concerning the histochemical features observed following Masson's trichrome staining, it was found that the groups do not differ in the density of collagen fibers. Histochemical analyses of the PAS-stained specimens elucidated that the cells in three first layers of spermatogenesis cell series, Sertoli and Leydig cells faintly reacted with PAS in medium- and high-dose aspartame-treated groups and the carbohydrate ratio was severely decreased in their cytoplasm. In Sudan black B staining, in seminiferous tubules, brown to black particles which contain lipid were clearly seen inside the cytoplasm of the cells close to the lumina of seminiferous tubules and Leydig cells. No cytoplasmic lipids in Leydig cells and spermatogenesis series cells in the control group, were observed. Animals in the aspartame receiving groups showed high lipid-stained sites in the cytoplasm of the Leydig cells and spermatogenesis series cells. In testicular tissue section, alkaline phosphates staining indicated the highest rate of small brown particles in the cytoplasm of Leydig cells and spermatogenesis cells in the high-dose group, compared to the other groups. In addition, it should be noted that the level of alkaline phosphatase reaction in the groups treated with aspartame was dose-dependently increased (Fig.1).

Table 2: Comparison of sperm parameters (± SD) between the experimental groups after frozen-thawed and treatment with 10 µg/ml Calligonum (CGM) extract and LIPUS (pulsed mode and continues wave)

Parameters	Control	Low dose	Medium dose	High dose
TBW (g)	36.12 ± 2.82^{a}	36.31 ± 3.06^{a}	36.38 ± 3.67^{a}	37.31 ± 3.01^{a}
TW (g)	0.12 ± 0.011^{a}	0.12 ± 0.009^{a}	0.12 ± 0.008^{a}	0.10 ± 0.010^{b}
BWA (g)	4.62 ± 0.67^{a}	5.24 ± 1.56^{ab}	5.58 ± 2.53^{ab}	6.97 ± 1.15^{b}
Testosterone (ng/ml)	6.88 ± 0.32^{a}	6.44 ± 0.30^{a}	6.22 ± 0.53^{a}	5.10 ± 0.57^{b}
STsD (µm)	194.38 ± 4.33^{a}	187.48 ± 5.56^{a}	173.32 ± 5.78^{b}	161.96 ± 5.45^{c}
GEH (µm)	58.97 ± 3.48^{a}	57.36 ± 2.36^{a}	50.02 ± 1.79^{b}	43.69 ± 3.28^{c}
TCT (µm)	13.47 ± 1.27^{a}	14.07 ± 2.09^{a}	16.41 ± 1.93^{a}	20.70 ± 2.58^{b}
LCs (No/mm^2)	37.35 ± 2.79^{a}	36.82 ± 2.11^{a}	33.57 ± 2.30^{a}	28.72 ± 2.97^{b}
SCs (No/one tubule)	22.76 ± 1.37^{a}	22.79 ± 1.64^{a}	20.72 ± 1.83^{a}	16.68 ± 1.55^{b}
Johnsen's score	9.42 ± 0.26^{a}	9.35 ± 0.30^{a}	8.64 ± 0.39^{b}	7.52 ± 0.47^{c}
Sperm count ($\times 10^6$)	34.66 ± 1.65^{a}	31.55 ± 1.42^{b}	27.44 ± 1.81^{c}	19.22 ± 1.48^{b}
Sperm motility (%)	85.06 ± 2.32^{a}	81.95 ± 3.32^{a}	74.34 ± 1.25^{b}	62.40 ± 2.98^{c}
Sperm viability (%)	89.22 ± 1.56^{a}	86.66 ± 2.73^{a}	79.33 ± 1.80^{b}	72.55 ± 2.12^{c}
DNA damage sperms (%)	5.11 ± 1.36^{a}	7.55 ± 1.94^{a}	11.22 ± 2.16^{b}	19.33 ± 2.12^{c}
Abnormal sperms (%)	10.33 ± 0.86^{a}	11.66 ± 1.22^{a}	15.44 ± 1.87^{b}	19.88 ± 1.69^{c}

All data are presented as mean ± SD. Low dose; 40 mg/kg aspartame-treated, Medium dose; 80 mg/kg aspartame-treated, High dose; 160 mg/kg aspartame-treated. TBW; Total body weight, TW; Testicular weight, BWA; Body weight alternations, STsD; Seminiferous tubules diameter, GEH; Germinal epithelium height, TCT; Testicular capsule thickness, LCs; Leydig cells, and SCs; Sertoli cells. Different superscripts in the same row show significant differences between groups ($P<0.05$).

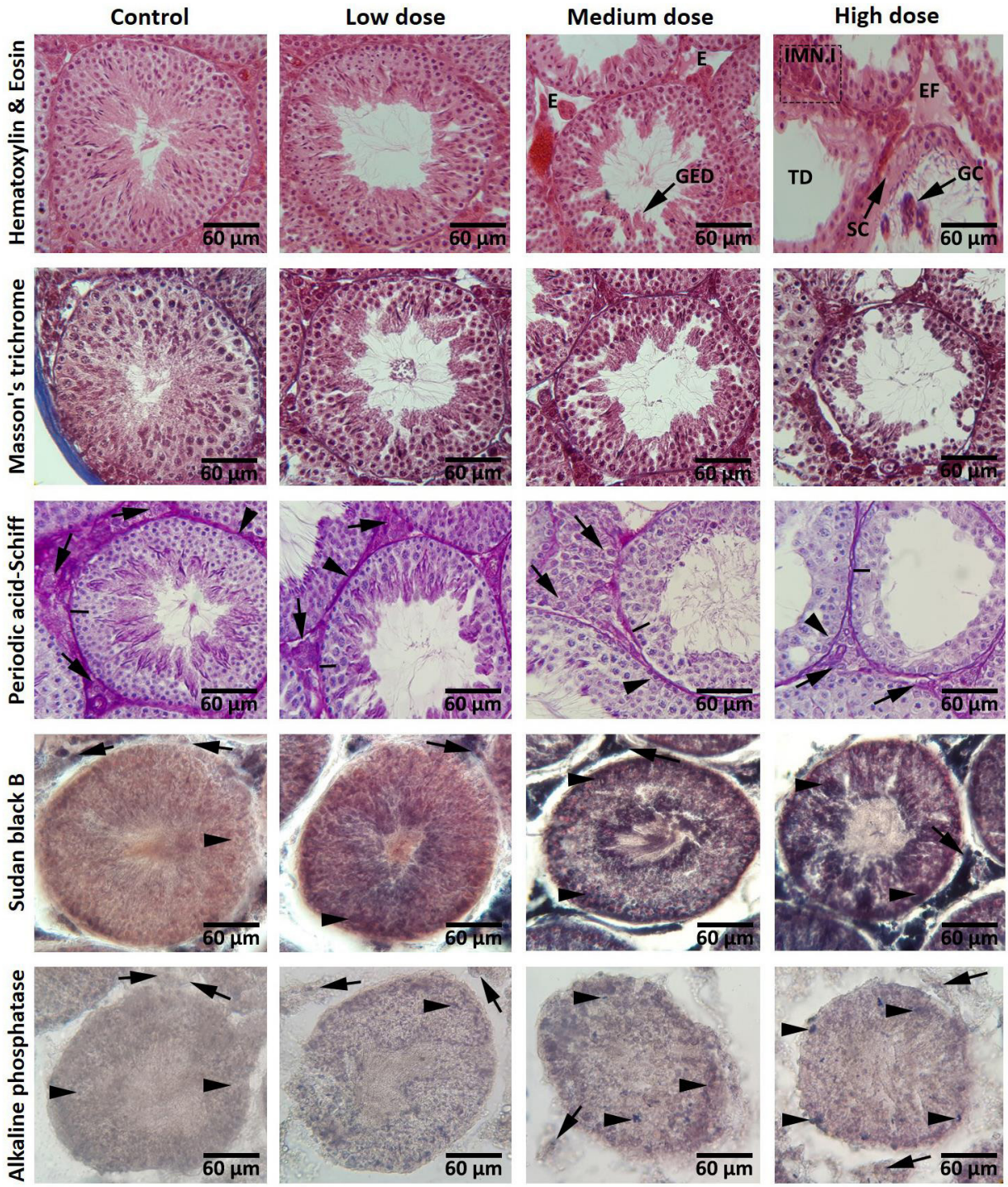

Fig.1: Cross sections from testes: Hematoxylin & Eosin staining; intact spermatogenesis is seen in the control group. Cross sections from medium- and high-dose groups present reduced epithelial height as well as germinal epithelium dissociation (GED), edema (E) and oedematous fluid accumulation (EF) of interstitial connective tissue, immune cells infiltration (IMN.I), atrophic and depletion seminiferous tubules (TD), giant cell (GC), detachment of Sertoli cell (SC) and spermatogenesis. Masson's trichrome staining; there was no difference in the amount of collagen fibers between the control group and the aspartame-treated groups. Periodic acid-Schiff staining; Control group with the Leydig cells (arrows), Sertoli cells (head arrows) and the first three cell layers (lines) with normal Periodic acid-Schiff (PAS) reaction. Low-dose group with light germinal cell dissociation and moderated PAS reaction are present in seminiferous tubules. Medium- and high-dose groups with negative PAS reaction in Leydig cells (arrows), Sertoli cells (head arrows) and the first three cell layers (lines) with faint PAS-stained cytoplasm. Sudan black B staining; Frozen sections from testes. Control group with spermatogenesis series cell lineage with negative Sudan black B-stained cytoplasms (arrows) and Leydig cells area (head arrows) are appeared with dense reaction sites. Comparing aspartame-treated groups with the control group indicates that in low-dose group, spermatogenesis series cells are presented with faint lipid stained cytoplasms (arrows) and Leydig cells area (head arrows) stained densely, while the medium- and high-dose groups are manifested with darkly stained spermatogenesis series cells (arrows) and Leydig cells area (head arrows). Alkaline phosphates staining; Frozen sections from testes. All germinal epithelium cells (head arrows) and Leydig cells area (arrows) in the control group are presented with the negative alkaline phosphatase (ALP) reaction. Comparing the aspartame-received groups reveals that there are numbers of cells in the germinal epithelium (head arrows) and Leydig cells (arrows) with ALP-stained cytoplasms (scale bar: 60 µm).

Sperm characteristics

Observations showed that aspartame in a dose-dependent manner, significantly (P<0.05) reduced the sperm count. Survival rate and sperm motility in medium- and high-doses aspartame-treated groups were significantly decreased (P<0.05) compared to the control group. Also, the average percentage of abnormal sperms as well as the percentage of sperms with damaged DNA, in medium- and high-dose aspartame-treated groups was significantly increased (P<0.05, Fig.2, Table 2).

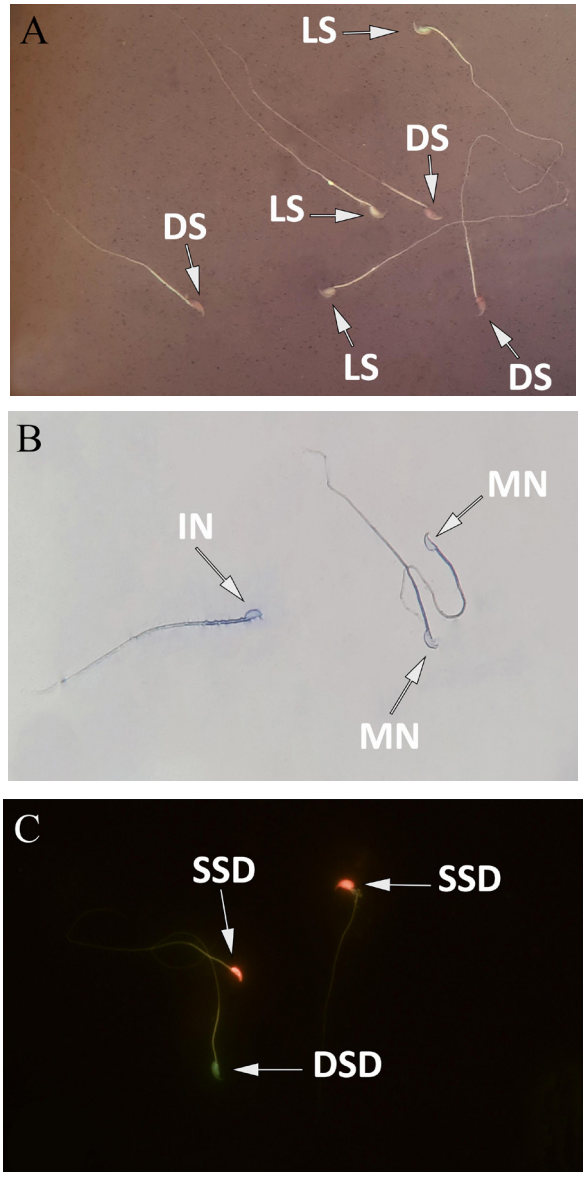

Fig.2: Photomicrographs of mice epididymal spermatozoa. **A.** Eosin-nigrosin staining, **B.** Aniline-blue staining, and **C.** Acridine-orange staining, (1,000X).
DS; Dead sperms, LS; Live sperms, MN; Mature nucleus, IN; Immature nucleus, SSD; Single-strand DNA, and DSD; Double-strand DNA.

Effect of aspartame on oxidative stress parameters

Aspartame effects on various parameters of oxidative stress biomarkers in serum and blood samples are shown in Figure 3. As can be seen, aspartame administration resulted in a significant increase (P<0.05) in MDA levels in the high-dose group as well as NO in medium- and high-dose aspartame-treated groups compared to the control group. Also, our observations showed that aspartame could induce a significant decrease (P<0.05) in TAC and CAT activity in the high-dose group and consequently led to a significant decrease (P<0.05) in the level of GSH-Px and SOD in both medium- and high-dose aspartame-treated groups compared to the control group.

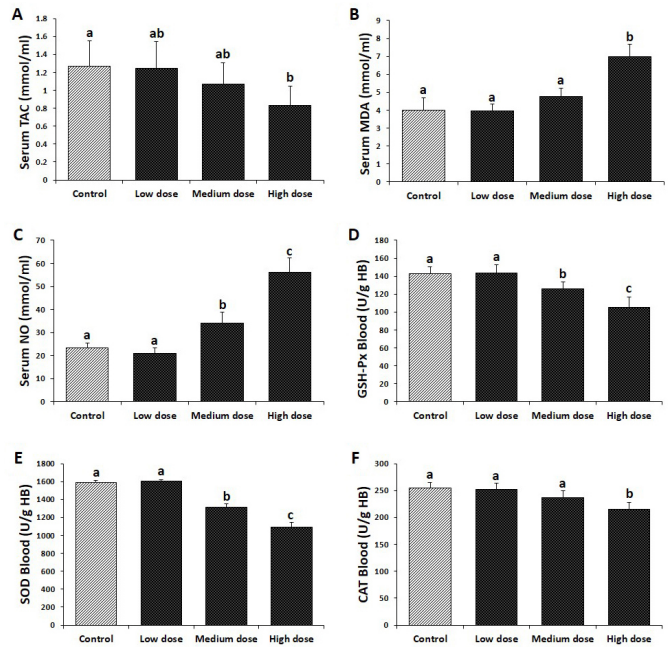

Fig.3: Effect of aspartame on antioxidant status. **A.** Serum total antioxidant capacity (TAC), **B.** Serum malondialdehyde (MDA) level, **C.** Serum nitric oxide (NO) level, **D.** blood glutathione peroxidase (GSH-Px) activity, **E.** Blood superoxide dismutase (SOD) activity, and **F.** Blood catalase (CAT) activity in different groups. All data are presented as mean ± SD. The different superscripts are representative of significant differences (P<0.05) between groups. Low dose; 40 mg/kg aspartame-treated, Medium dose; 80 mg/kg aspartame-treated, and High dose; 160 mg/kg aspartame-treated.

Aspartame diminished Hsp70-2 expression

The mRNA and protein levels of Hsp70-2 were analyzed. In order to clarify Hsp70-2 expression in different cellular layers of germinal epithelium, immunohistochemical analyses were done. Our finding revealed that, biosynthesis of Hsp70-2 increased in low-dose aspartame-treated group (especially at spermatocytes and spermatids cell lineages) versus the control group. However, it was significantly decreased in medium- and high-dose aspartame-treated groups. The immunohistochemical results were confirmed by the semiquantitative RT-PCR analysis. A significant (P<0.05) increase in the mRNA level of Hsp70-2 was observed in the animals treated with low-dose aspartame. However, the mRNA levels of Hsp70-2 decreased in medium- and high-dose aspartame-treated groups (Fig.4).

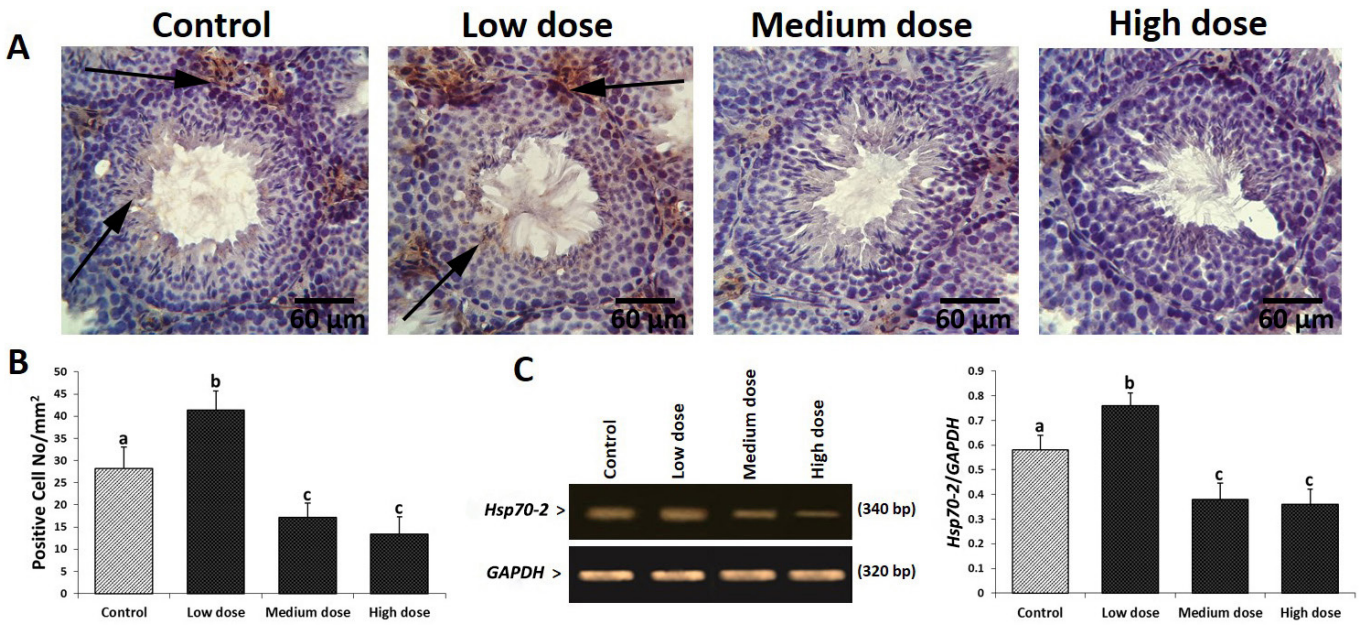

Fig.4: Effect of aspartame on Hsp70-2 protein expression in different groups. **A.** Immunohistochemical staining for Hsp70-2; see arrows indicating positive reaction for Hsp70-2 in two cell lines of genital cells in the control group, which is elevated in all cellular layers of low-dose group and significantly decreased in medium- and high-dose groups, respectively. **B.** See cell count results for Hsp70-2 (+) cells/1 mm2 of tissue in different groups. **C.** The mRNA levels of Hsp70-2 and GAPDH were evaluated by using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). The density of Hsp70-2 mRNA levels in testicular tissue was measured by densitometry and normalized to GAPDH mRNA expression level. Data are presented as mean ± SD. The different superscripts are representative of significant differences (P<0.05) between groups.

Discussion

Aspartame which is extensively used in food and medicinal products as a low-calorie sweetener, is mostly consumed by people trying to lose weights, patients with diabetes, and athletes (13). In recent decades, increased human infertility caused by toxic materials has raised concerns in human societies. In the same way, food additives and nutrition are important and influential factors in the entry of these toxic substances into the body and affect the reproductive capacity of the male sex (19). Effects of aspartame on the male reproductive parameters might be a consequence of the metabolites derived from aspartame hydrolysis during digestive and absorptive processes in the body. Studies showed that aspartame toxicity induced following oral intake is mainly related to the digestive metabolites and intestinal absorption of this substance which occurs during the metabolism of aspartame in the gastrointestinal tract by esterases and peptidases. Methanol is not metabolized in enterocytes and is rapidly introduced into the portal system of the liver and oxidized to formaldehyde by the alcohol-dehydrogenase enzyme; formaldehyde causes toxicity in most cells and tissues of the body (20). It was reported that aspartame and its metabolites potentially disturb a wide range of body processes, including amino acid metabolism, and affect the structure and metabolism of proteins, structural integration of nucleic acids and endocrine equilibria (20, 21). Many reports declared that the most destructive toxic effects of aspartame are probably related to methanol oxidation following aspartame metabolism. It was obviously indicated that receiving aspartame and subsequently the increased levels of methanol, formaldehyde and formic acid could damage the mitochondrial membrane through formation of superoxide anion and hydrogen peroxide, leading to higher levels of ROS and oxidative stress (13).

It was determined that aspartame has an effect on weight loss in humans and it can reduce weight and control obesity (22, 23). It was declared that weight loss occurs because aspartame reduces the brain's neuropeptide Y and reduction of this neuropeptide, which plays a vital role in metabolism, could reduce body weight (24). In this study, aspartame increased body weight in the high-dose aspartame group, which does not match with the results of the mentioned research (22, 23). In some other studies, it was reported that aspartame inhibits an intestinal enzyme called intestinal alkaline phosphatase (IAP) which can prevent obesity, type 2 diabetes and metabolic syndrome. The results of these experiments showed that the mice that took aspartame-containing water compared to the mice without aspartame, became overweight (25).

Evidence indicates that oxidative stress can cause sperm abnormalities through various mechanisms such as inducing lipid peroxidation in sperm plasma membrane, sperm motility disorder, sperm abnormal morphology and fracture in sperm DNA (16). Also, literature shows that sperm DNA damage caused by oxidative stress increases apoptosis in immature sex cells leading to a decreases in the concentration of sperm (26). In this regard, our study showed that the use of aspartame increases sperm DNA damage by the mechanisms involved in oxidative stress induced by medium- and high-dose aspartame. Previously, it was shown that using as-

partame could cause an increase in the morphologically abnormal sperms that it is consistent with the results we obtained following treatment with medium- and high-dose aspartame, but does not match with the effects of low-dose aspartame (27). Earlier studies showed that aspartame reduced sperm count, viability and motility in rats, which are in accordance with the findings of this study related to decreases in sperm viability and motility following administration of medium- and high-dose aspartame (13, 27).

The mechanism of action of aspartame may also be mediated via its effect on Leydig cells, which leads to a decrease in testosterone levels. With degradation and atrophy of Leydig cells under the influence of formaldehyde produced from aspartame, the levels of synthesis and secretion of testosterone decrease (28), which perfectly matched with the findings of this study that presented a significant decrease in serum testosterone level in the high-dose group of aspartame.

Besides, in order to achieve insight into the delicate in vivo oxidants/antioxidants balance, measurement of TAC could be proper. High polyunsaturated acid ratio in testes and sperm causes the male reproductive system to be susceptible to oxidative stress. The collaboration of antioxidant enzymes, SOD, CAT and GSH-Px, in cleansing ROS causes a protection of tissues and cells from oxidants' harmful effects. So, even minor changes in normal contents of the mentioned enzymes could result in susceptibility of biomolecules to oxidative damages and so disturbances in the defense shield of the body (15). In this study, aspartame could reduce the levels of CAT, SOD and TAC in high-dose group which is supported by earlier reports (1, 11). In the defense against oxidative damages, GSH-Px has an important role by using glutathione as the reducing substrate and through catalyzing the reduction in a variety of hydroperoxides (15). We observed that administration of aspartame to mice for 90 days could dose-dependently reduce GSH-Px activity which is compatible with some earlier reports showing the ability of aspartame in reduction of GSH-Px through possessing oxidizing power (1, 11). Receiving aspartame and then increased levels of methanol and creation of formaldehyde could induce the formation of superoxide anion and hydrogen peroxide which can cause damage to the mitochondrial membrane and by inducing lipid peroxidation, could induce damage to the cell membranes (13). Increased levels of NO and MDA in the mice receiving aspartame were shown in some earlier reports (1, 11, 13), which are consistent with the results of medium- and high-dose aspartame but not the low dose, in this study.

Under different stress conditions, Hsp70-2 plays an important role in homeostasis although under physiological conditions, it is usually involved in assembling intracytoplasmic proteins. Also, biosynthesis of Hsp70-2 protein could be directly changed depending on the free radicals generation ratio in testicular tissue and depending on androgen withdrawal, it might be altered indirectly. In our study, immunohistochemical and semi-quantitative RT-PCR assessment indicated that in low-dose aspartame-exposed animals, the expression of *Hsp70-2* increased against the control group. However, medium- and high-dose aspartame-treated animals revealed significantly reduced expression of Hsp70-2 both at immunohistochemical and mRNA levels. To better understand the molecular changes at Hsp70-2 level, one should note that Hsp70-2 protein is a stress responder, and based on the intensity of the stress, it exerts homeostatic role. Therefore, in case of increasing stressors, based on its protein nature, Hsp70-2 can be peroxidated. Taking together, minding the increased Hsp70-2 expression in low-dose group, we can suggest that it exerted a homoeostatic characteristic, and based on its reduction in medium- and high-dose aspartame-administered groups, it can be concluded that due to increasing impact of stressors, pre-existing and newly synthetized Hsp70-2 proteins were peroxidated and the immunohistochemical technique failed to detect the protein. Concerning mRNA content, it is well-established that free radicals degenerate the DNA and mRNA backbones. Therefore, it is possible to suggest that due to the increasing amount of stressors, the DNA and mRNA contents of the cell were attacked and through this mechanism, the RT-PCR analysis showed diminished Hsp70-2 mRNA (29). Based on the obtained results, it could be deduced that with low-dose aspartame-induced NOS/ROS stress and androgen depletion or lower stress, in order to control the stress-induced derangements in testicular tissue, the over expression of Hsp70-2 happened. Nevertheless, the mechanisms of aspartame action at higher doses, were different. In fact, Hsp70-2 and different stimulant agents, such as NO, free radicals and superoxide affect the cellular protein structures adversely (30). Also, it could be concluded that significant reductions in total RNA and protein levels besides decreasing biosynthesis and mRNA levels of Hsp70-2, could prove that in the animals receiving high-dose aspartame, its alone-induced damage in association with ROS/NOS-induced impairments could result in such damages to Hsp70-2 at the protein and RNA levels. In a study, it was shown that lacking Hsp70-2 in spermatocytes, caused interruption in their meiosis and they were deleted by apoptosis subsequently (29). So, it might be suggested that severe damages which were observed at spermatocyte cell levels (marked with diminished Johnsen's criteria), induced by the aspartame were induced through affecting the expression and/or biosynthesis of Hsp70-2. Besides, expression of Hsp70-2 and its function are altered during late stages of spermiogenesis process and it could be associated with spermatid-specific-DNA-packing proteins. In fact, synthesis of protamines 1 and 2 and DNA-packing transition proteins 1 and 2, often depends on *Hsp70-2* chaperones expression (29, 31). They could provide cytoprotection against a great number of stressors and stress hormones, including corticosterone and protect cells from stress or harmful conditions (32).

While Hsps are considered regulators of apoptosis, because of this fact that the oxygen radical induced synthesis of stress proteins could result in oxidative stress tolerance, it seems that Hsp plays a role in protecting of the oxyradical-induced changes (33). Based on the obtained results, it might be concluded that the reduction induced by aspartame during spermatogenesis could be due to induction of apoptosis in spermatogenic germ cells. These results confirm apoptotic effects of aspartame, which were reported in earlier studies (7, 12, 29).

In several studies, assessment of histomorphometric parameters of testicular tissue is considered an appropriate approach for evaluating the extent of damage to this organ (15, 16). Aspartame and its metabolites such as formaldehyde, appear to change the histomorphometric parameters of testicular tissue through inducing oxidative stresses (13, 34). In this study, aspartame caused a decrease in histomorphometric parameters of testicular tissue in medium- and high-dose aspartame. In this regard, and in confirmation of the findings of this study, recent investigations also showed that aspartame and formaldehyde could induce a reduction in the Johnsen's criteria, the diameter of the seminiferous tubules, the height of germinal epithelium and the number of Leydig and Sertoli cells (15, 35, 36).

The alkaline phosphatase enzyme plays an important role in cellular processes. Cell membrane damage results in the release of this enzyme in the cell and ultimately, in the serum. Thus, alkaline phosphatase enzyme measurement is used as an indicator for testicular tissue changes (37). Consistent with some earlier reports, in this study, dose dependent aspartame intake could increase the amount of alkaline phosphatase enzyme in testis tissue sections. Under healthy conditions, spermatogenesis series cells on the basal lamina of seminiferous tubules, possess carbohydrate sources, while the cells near the luminal space of the seminiferous tubules use lipids for their metabolism. In cases where the metabolic cycle is impaired, subsequently, cell metabolism also changes. In these circumstances, the cells use other food sources in the environment for metabolism. The results of this study showed that in testicular tissue of the mice receiving aspartame, PAS reaction (carbohydrate particles) decreases in Leydig cells and spermatogenesis series cells. These results indicate an imbalance in the metabolism of testicular tissue cells under the influence of aspartame which is consistent with other investigations in this field. In Sudan black B staining, in the present study, plenty of dense and dark granules were observed in the cytoplasm of Leydig cells, Sertoli cells and spermatogenesis series cells especially in medium- and high-dose aspartame groups. Presence of dark brown granules in the cytoplasm of Leydig and Sertoli cell adjacent to the basement membrane of the atrophied seminiferous tubules, were more obvious in Sudan black B staining which is compatible with some other studies (37, 38). Collagen fibers are studied by Masson's trichrome staining in various tissues; this study also showed that in the control group, testicular capsule had the lowest density of collagen fibers and the lamina propria in the vicinity of seminiferous tubules, showed some bundles of collagen fibers as a blue layer. The amount of these collagen strands in lamina properia of seminiferous tubules did not show any obvious changes in aspartame-received groups, compared to the control group. Nevertheless, earlier studies indicated that formaldehyde increased the amount of collagen fibers in rats testicular tissue (34, 39). The effects of aspartame consumption result in excessive free radicals (ROS/RNS) production through different ways. The sperm abnormalities occurring due to induction of oxidative stress could affect different features of the involved cells. Consumption of aspartame affects the mitochondrial membrane integrity and leads to oxidative stress. Also, aspartame could induce some cellular disorders such as a reduction in their distribution as well as decrease in Hsp70-2 expression, damage to the cellular protein, severe damage to DNA and homeostasis contents including chaperones that in turn leads to severe oxidative stress. Aspartame, affects the Leydig cells, which induces a considerable decrease in testosterone level, and consequent dysfunction of Sertoli cells through impairing their physiological activities leading to oxidative stress, by increasing cellular apoptosis. Finally, all of the mentioned pathways will result in; increasing damage to sperm DNA, reducing sperm motility and viability and also impairing chromatin condensation (Fig.S2, See Supplementary Online Information at www.ijfs.ir).

Conclusion

The findings of this study suggest that aspartame due to increased production of free radicals, induction of oxidative stresses and weakening the antioxidant defense system, could induce some disorders related to histomorphometric and serum parameters, increasing oxidative and nitrosative stress and down-regulating chaperone Hsp70-2 expression/biosynthesis, sperm quality and histochemical changes in medium- and highdose groups of mice. However, the results of the low-dose aspartame did not significantly differ from the control group's results and did not show any damages observed in the two other groups. Nonetheless, confirmation of the toxicity of aspartame in male reproductive system requires more extensive experimental studies, as well as clinical trials.

Acknowledgements

The authors wish to appreciate Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tehran. Moreover, they deeply thank the Department of Histology and Embryology, Faculty of Veterinary Medicine, Urmia University for scientific supports. The current manuscript is related to thesis for post-graduate (Ph.D) degree No. 7506025/6/24 which was approved by University of Tehran. There is no conflict of interest in this study.

Authors' Contributions

H.A.; Designed experiments, analyzed data and co-wrote the manuscript. M.T.S.; Performed experiments, analyzed data and co-wrote the manuscript. M.R.; Analyzed data and co-wrote the manuscript. All authors read and approved the final manuscript.

References

1. Choudhary AK, Sheela Devi R. Longer period of oral administration of aspartame on cytokine response in Wistar albino rats. Endocrinol Nutr. 2015; 62(3): 114-122.
2. Leme FAGdL, Azoubel R. Effects of aspartame on the exocrine pancreas of rat fetuses. Int J Morphol. 2006; 24(4): 679-684.
3. Abhilash M, Paul MV, Varghese MV, Nair RH. Effect of long term intake of aspartame on antioxidant defense status in liver. Food Chem Toxicol. 2011; 49(6): 1203-1207.
4. Iman MM. Effect of aspartame on some oxidative stress parameters in liver and kidney of rats. Afr J Pharm Pharmacol. 2011; 5(6): 678-682.
5. Datta NJ, Namasivayam A. In vitro effect of methanol on folate-deficient rat hepatocytes. Drug Alcohol Depend. 2003; 71(1): 87-91.
6. Ashok I, Sheeladevi R. Oxidant stress evoked damage in rat hepatocyte leading to triggered nitric oxide synthase (NOS) levels on long term consumption of aspartame. J Food Drug Anal. 2015; 23(4): 679-691.
7. Kregel KC. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol. 2002; 92(5): 2177-2186.
8. Shamsi-Gamchi N, Razi M, Behfar M. Testicular torsion and reperfusion: evidences for biochemical and molecular alterations. Cell Stress Chaperones. 2018; 23(3): 429-439.
9. Rerole AL, Jego G, Garrido C. Hsp70: anti-apoptotic and tumorigenic protein. Methods Mol Biol. 2011; 787: 205-230.
10. Iyaswamy A, Kammella AK, Thavasimuthu C, Wankupar W, Dapkupar W, Shanmugam S, et al. Oxidative stress evoked damages leading to attenuated memory and inhibition of NMDAR-CaMKII-ERK/CREB signalling on consumption of aspartame in rat model. J Food Drug Anal. 2018; 26(2): 903-916.
11. Choudhary AK, Sundareswaran L, Sheela Devi R. Aspartame induced cardiac oxidative stress in Wistar albino rats. Nutr Clin et Metab. 2016; 30(1): 29-37.
12. Choudhary AK, Devi RS. Effects of aspartame on hsp70, bcl-2 and bax expression in immune organs of Wistar albino rats. J Biomed Res. 2016; 30(4): 427-435.
13. Ashok I, Poornima PS, Wankhar D, Ravindran R, Sheeladevi R. Oxidative stress evoked damages on rat sperm and attenuated antioxidant status on consumption of aspartame. Int J Impot Res. 2017; 29(4): 164-170.
14. Onaolapo AY, Onaolapo OJ, Nwoha PU. Aspartame and the hippocampus: Revealing a bi-directional, dose/time-dependent behavioural and morphological shift in mice. Neurobiol Learn Mem. 2017; 139: 76-88.
15. Asri-Rezaei S, Nourian A, Shalizar-Jalali A, Najafi G, Nazarizadeh A, Koohestani M, et al. Selenium supplementation in the form of selenium nanoparticles and selenite sodium improves mature male mice reproductive performances. Iran J Basic Med Sci. 2018; 21(6): 577-585.
16. Anbara H, Shahrooz R, Razi M, Malekinejad H, Najafi G. The effect of vitamin C on mice hemolytic anemia induced by phenylhydrazine: an animal model study using histological changes in testis, pre-implantation embryo development, and biochemical changes. Iran J Basic Med Sci. 2018; 21(7): 668-677.
17. Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, et al. World Health Organization reference values for human semen characteristics. Hum Reprod Update. 2010; 16(3): 231-245.
18. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem. 1982; 126(1): 131-138.
19. Yuet-Wan Lok K, Chung WY, Benzie IF, Woo J. Colour additives in snack foods consumed by primary school children in Hong Kong. Food Addit Contam Part B Surveill. 2010; 3(3): 148-155.
20. Oyama Y, Sakai H, Arata T, Okano Y, Akaike N, Sakai K, et al. Cytotoxic effects of methanol, formaldehyde, and formate on dissociated rat thymocytes: a possibility of aspartame toxicity. Cell Biol Toxicol. 2002; 18(1): 43-50.
21. Trocho C, Pardo R, Rafecas I, Virgili J, Remesar X, Fernandez-Lopez JA, et al. Formaldehyde derived from dietary aspartame binds to tissue components in vivo. Life Sci. 1998; 63(5): 337-349.
22. De La Hunty A, Gibson S, Ashwell M. A review of the effectiveness of aspartame in helping with weight control. Nutr Bull. 2006; 31(2): 115-128.
23. Blackburn GL, Kanders BS, Lavin PT, Keller SD, Whatley J. The effect of aspartame as part of a multidisciplinary weight-control program on short- and long-term control of body weight. Am J Clin Nutr. 1997; 65(2): 409-418.
24. Beck B, Burlet A, Max JP, Stricker-Krongrad A. Effects of long-term ingestion of aspartame on hypothalamic neuropeptide Y, plasma leptin and body weight gain and composition. Physiol Behav. 2002; 75(1-2): 41-47.
25. Gul SS, Hamilton AR, Munoz AR, Phupitakphol T, Liu W, Hyoju SK, et al. Inhibition of the gut enzyme intestinal alkaline phosphatase may explain how aspartame promotes glucose intolerance and obesity in mice. Appl Physiol Nutr Metab. 2017; 42(1): 77-83.
26. Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril. 2003; 79(4): 829-843.
27. Ikpeme EV, Udensi OU, Ekerette EE, Okon UH. Potential of ginger (Zingiber officinale) rhizome and watermelon (Citrullus lanatus) seeds in mitigating aspartame-induced oxidative stress in rat model. Res J Med Plants. 2016; 10(1): 55-66.
28. Hozayen WG, Soliman HAE, Desouky EM. Potential protective effects of rosemary extract, against aspartame toxicity in male rats. J Inter Acad Res Multidisc. 2014; 2(6): 111-125.
29. Rezazadeh-Reyhani Z, Razi M, Malekinejad H, Sadrkhanlou R. Cytotoxic effect of nanosilver particles on testicular tissue: Evidence for biochemical stress and Hsp70-2 protein expression. Environ Toxicol Pharmacol. 2015; 40(2): 626-638.
30. Kaur P, Bansal MP. Effect of oxidative stress on the spermatogenic process and hsp70 expressions in mice testes. Indian J Biochem Biophys. 2003; 40(4): 246-251.
31. Govin J, Caron C, Escoffier E, Ferro M, Kuhn L, Rousseaux S, et al. Post-meiotic shifts in HSPA2/HSP70.2 chaperone activity during mouse spermatogenesis. J Biol Chem. 2006; 281(49): 37888-37892.
32. Sun L, Chang J, Kirchhoff SR, Knowlton AA. Activation of HSF and selective increase in heat-shock proteins by acute dexamethasone treatment. Am J Physiol Heart Circ Physiol. 2000; 278(4): H1091-1097.
33. Beere HM, Green DR. Stress management - heat shock protein-70 and the regulation of apoptosis. Trends Cell Biol. 2001; 11(1): 6-10.
34. Hegazy AA, Elsayed NE, Ahmad MM, Omar NM. Effect of formaldehyde on rat testis structure. Acad Anat Int. 2017; 3(2): 15-23.
35. Razi M, Malekinejad H, Sayrafi R, Hosseinchi MR, Feyzi S, Moshtagion SM, et al. Adverse effects of long-time exposure to formaldehyde vapour on testicular tissue and sperm parameters in rats. Vet Res Forum. 2013; 4(4): 213-219.
36. Askaripour M, Hasanpour A, Hosseini F, Moshrefi M, Moshtaghi G, Hasannejad M, et al. The effect of aqueous extract of Rosa damascena on formaldehyde-induced toxicity in mice testes. Pharm Biol. 2018; 56(1): 12-17.
37. Razi M, Sadrkhanloo RA, Malekinejad H, Sarrafzadeh-Rezaei F. Testicular biohistochemical alterations following experimental varicocele in rats. Iran J Reprod Med. 2012; 10(3): 209-218.
38. Zobeiri F, Sadrkhanlou RA, Salami S, Mardani K. Long-term effect of ciprofloxacin on testicular tissue: evidence for biochemical and histochemical changes. Int J Fertil Steril. 2013; 6(4): 294-303.
39. El-Hak HNG. The protective effects of panax ginseng extract on fertility of albino rats treated with formaldehyde vapours. Egypt J Zool. 2017; 68(68): 221-238.

Original Article

Prediction of 3D Protein Structure Based on The Mutation of *AKAP3* and *PLOD3* Genes in The Case of Non-Obstructive Azoospermia

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Abstract

Background: The present study has been designed with the aim of evaluating A-kinase anchoring proteins 3 (*AKAP3*) and Procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase 3 (*PLOD3*) gene mutations and prediction of 3D protein structure for ligand binding activity in the cases of non-obstructive azoospermic male.

Materials and Methods: Clinically diagnosed cases of non-obstructive azoospermia (n=111) with age matched controls (n=42) were included in the present case-control study for genetics analysis and confirmation of diagnosis. The sample size was calculated using Epi info software version 6 with 90 power and 95% confidence interval. Genomic DNA was isolated from blood (2.0 ml) and a selected case was used for whole exome sequencing (WES) using Illumina Hiseq for identification of the genes. Bioinformatic tools were used for decode the amino acid sequence from biological database (www.ncbi.nlm.nih.gov/protein). 3D protein structure of *AKAP3* and *PLOD3* genes was predicted using I-TASSER server and binding energy was calculated by Ramachandran plot.

Results: Present study revealed the mutation of *AKAP3* gene, showing frameshift mutation at rs67512580 (ACT → -CT) and loss of adenine in homozygous condition, where, leucine changed into serine. Similarly, *PLOD3* gene shows missense mutation in heterozygous condition due to loss of guanine in the sequence AGG→A-G and it is responsible for the change in post-translational event of amino acid where arginine change into lysine. 3D structure shows 8 and 4 pockets binding site in *AKAP3* and *PLOD3* gene encoded proteins with MTX respectively, but only one site bound to the receptor with less binding energy representing efficient model of protein structure.

Conclusion: These genetic variations are responsible for alteration of translational events of amino acid sequences, leading to protein synthesis change following alteration in the predicted 3D structure and functions during spermiogenesis, which might be a causative "risk" factor for male infertility.

Keywords: *AKAP3*, Infertility, Iterative Threading ASSEmbly Refinement, *PLOD3* gene, Whole Exome Sequencing

Citation: **Saxena AK, Tiwari M, Agarwal M, Aniket Kumar A. Prediction of 3D protein structure based on the mutation of AKAP3 and PLOD3 genes in the case of non-obstructive azoospermia. Int J Fertil Steril. 2020; 14(2): 102-109. doi: 10.22074/ijfs.2020.6028.**

Introduction

Globally, infertility is a serious problem in the world, which affects more than 15% of the couples amounting to 48.5 million people. The genetic landscape of male infertility is extremely heterogeneous due to molecular interactions that exist between primary spermatocytes, spermatids and finally sperm. More than 2000 genes work together in synchronous way to form single mature and healthy sperm under highly complex procedure -spermatogenesis. Amongst more than 15% of infertile populations, males alone contribute to 20-30% cases of infertility. It has been suggested by various studies that genetic mutations are responsible for dysregulation of spermatogenesis leading to male infertility (1-3). The highest frequency (25%) was observed in the nonobstructive azoospermic category. Simultaneously, the other anomalies were also evaluated on the basis of semen analysis in the cases of oligozoospermia (4). The cellular morphogenetic events undergo drastic changes including chromatin condensation, acrosome formation and maturation of sperm tail (5). Whole exome sequencing (WES) is one of the most sensitive and powerful technique to generate mutational spectra of unidentified gene(s) and their regulation in disease condition such as infertility. DNA sequencing analysis help identify nucleotide changes such as insertion, deletion or frameshift/non-frameshift mutation that alters post-translational event resulting in modifications in proteins structure and function. They might interfere in the process of spermatogenesis relevant role in male infertility. Identifying such changes might help determine the causative factors involved in unexplained infertility. Our candidate genes namely A-kinase anchoring pro-

Received: 8/July/2019, Accepted: 31/December/2019
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teins 3 (*AKAP3*) and Procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase 3 (*PLOD3*) were collected from the affected "gene pool" of infertile cases after whole genome sequencing and predicted 3D model protein structure for ligand binding receptor site.

In human, investigations showed several isoforms of AKAP gene family express in testicular tissue, among them sperm-specific AKAP3 were found to localize in the sperm tail and regulate sperm motility. The main functions of *AKAPs* are to modulate protein kinase A (PKA) signalling, during germ cell proliferation and further development of the gamete. *In vitro* studies suggested that *AKAP3* interacts with other isoforms of AKAPs and plays an important role during assembly of fibrous sheath and spermatid morphogenesis (6). However, the roles of *AKAP3* gene expression to modulate PKA functions are still confusing due to the lack of define structure during spermiogenesis. *In vitro* studies on protein interaction indicated that *AKAP3* gene has been associated with numerous signalling proteins, like PDE4A, Ga13 and Ropporin, which participate in the regulation of sperm motility (7, 8).

Similarly, *PLOD3* gene plays an important role in spermatogenesis and mutation in this gene has been associated with connective tissue disorder and congenital malformations (6). *PLOD3* gene encodes Lysyl hydroxylase 3 (LH3), as an enzyme with multiple functions that leads to hydroxylation of lysyl residues and O-glycosylation of hydroxylysyl. Such reactions leads to production of monosaccharide or disaccharide derivatives that play role in post translational modifications involved in collagen biosynthesis. Previous studies have suggested the significance of *PLOD3* gene in biosynthesis of glycosylated type IV and VI collagens required for normal formation of basement membranes, however, the functional role of this enzyme is not clear yet (7). In testis, Sertoli cells and germ cells are in close contact with the basement membrane which is a modified form of extracellular matrix (ECM). These cells are relaxed on the basement membrane of the seminiferous tubule for hormonal supports at different stages of the seminiferous epithelial cycle. However, the role of ECM is poorly understood in regulating spermatogenesis (9).

There is lack of information in the literatures, regarding the *AKAP3* and *PLOD3* genes, their structural and functional interaction on the proliferating germ cells during translational events and how to play a significant role in reproductive dysfunction. However, WES data analysis confirms the mutation types in male infertility. Therefore, this study is quite important to understand the molecular pathogenesis of pre- and post-translational events during spermiogenesis, in addition to help predict 3D model structure of protein after bioinformatics tools like molecular docking to methotrexate with respect to controls. Hence, the present study explores knowledge of functional genomics in reproductive medicine.

Materials and Methods

The present study has been performed in clinically diagnosed patients (n=111) of non-obstructive azoospermia (NOA) classified after semen analysis, according to WHO guidelines (2010) with respect to the age matched controls (n=42) (3). Inclusion criteria for present study is that none of them had any history of childhood disease, radiation exposure or prescription of continuation of drug and the median age of patients was 35.4 years old (age group range 21-45 years). The sample size was calculated using Epi info software version 6 with 90 power and 95% confidence interval (CI), with alpha error 0.05%; beta 0.02 taking into account the normal population have prevalence of gene mutation in male infertile cases that varies from 1.0 to 3.5%. This study was further extended in the case of NOA to identify further "novel" mutations. The genetics analyses were carried out in the Department of Pathology/Lab Medicine, All India Institute of Medical Sciences, Patna. Blood samples (2.0 ml) were collected from the proband after written informed consent, and the study was approved by the Institutional Ethical Committee (IEC) (code: dean/2008-09/384). The bioinformatics tool were used for the prediction of 3D protein helical structure and their functional binding site to the ligand (drug) with calculated energy using docking (server) system.

Identification of *AKAP3* and *PLOD3* genes from whole exome sequencing

Genomic DNA was isolated from the clinically diagnosed cases of male infertility for characterization of Y-chromosome microdeletion using STS markers (3). A selected case was used for further characterization of small insertions/deletions (In/Dels) and single nucleotide variants (SNVs) using WES by Illumina Hiseq 2000 (Illumina, USA). These variants were further characterized using filters covering position of the gene variants excluded non-coding and repetitive regions (10). The information of *AKAP3* and *PLOD3* genes and translational events were further verified on the basis of availability of sequence database (https://www.ncbi.nlm.nih.gov/protein). Hence, prediction of 3D structure of *AKAP3* gene becomes quite relevant as it is not available in the structural database (https://www.rcsb.org/). Similarly, *PLOD3* protein 3D structure was further predicted on the basis of sequencing data for remodelling of chromatin during spermiogenesis into ligand binding sites to explore the pathogenesis of infertility.

Homology modelling of 3D structure

I-TASSER (Iterative Threading ASSEmbly Refinement) is used to evaluate the structure and function of protein, after prediction in scientific research based on state of the-art of algorithms (11). First structural template is identified by using local meta threading server (LOMETS) from the construction of full-length atomic models and iterative template-based fragment assembly from protein data bank (PDB). I-TASSER have five models for prediction of large clusters of protein structure. For prediction of protein structure, in each model C-score is calculated (-1.19) on the basis of significance of alignment and con-

vergence parameters. Higher value of C-score range (-5 to 2) signify the best structure of protein. Structural similarity is calculated by template modelling score (TM; 0.57 ± 0.15) between query and template protein using root mean square deviation (RMSD; 11.4 ± 4.5Å0) between amino acid residues and protein length following the correlation observed between these qualities to improve the predicted 3D model (12,13).

Identification of the binding site of 3D model structure

The ligand binding sites are active site of enzyme during assembly of protein structure and become relevant to explore the functional interaction to other molecule. During structural analysis, the strategy was initiated with identification of the target molecule to ligand binding sites (pocket) including donors and acceptors of potential hydrogen bond that are hydrophobic in nature. In protein structure, there are well accepted target bind sites to ligand which are highly specific in different disease conditions. Prediction of 3D protein structure is developed from the sensitive template library (http://raptorx.uchicago.edu/ bindingsite/) and arranged the target sequence based on neural networking (https://playmolecule.org/deepsite/) (14-16).

Selection of methotrexate as ligand binding molecule

Activity of methotrexate (MTX), as an antagonist of folate that inhibits tetrahydrofolate dehydrogenase enzyme, is essential for DNA synthesis (https://www. drugbank.ca/drugs/DB00563). Selection of MTX has been developed not only due to the commonly used as an antineoplastic agent for the management of malignancy, but also used as an immunosuppressive drug. MTX is highly toxic in nature and entered into the S-phase of cell-cycle affecting rapidly dividing cells, which leads to inhibit DNA replication followed by cell-death (17). The present study becomes relevant as it proposes an approach to reduce the cellular toxicity by structural remodelling of ligand binding sites during gene-protein or drug-protein interaction in rapid dividing cells e.g. germ cells.

Molecular docking of *AKAP3* and *PLOD3* protein

The iGEMDOCK v2.1 software (BioXGEM Lab, Taiwan) was used for evaluation of protein structural and functional activities, based on algorithm and scoring efficiency between standalone. iGEMDOCK v2.1 is an integrated software used for structural analysis and pharmacological interaction with the corresponding ligand molecules. Findings of the software showed interaction of the biological active compounds involved in biological mechanisms. This software has embedded statistical application for calculation of minimum energy to binding sites. It automatically generates pharmacological interaction and calculates the preference between hydrogen atoms and ligand binding site with the help of compound library. Furthermore, RasMol (visualization tool for protein-ligand interactions) displays interactions with conserved residues of amino acid and the functional groups of compound. Thus, iGEMDOCK provides an interactive boundary for visualizing the active compound by combining the pharmacological interactions in the energy-based scoring function (18). The visualization properties (analyses) inside the helical structure of protein to the ligand binding sites (enlarge view) are concluded by using UCSF Chimera technique (19).

Statistical Analysis

Chi square (x^2) test (two-tailed) was applied to find out significant differences (P values) between the infertile cases and controls.

Results

In our recent study, we identified that deletion frequency of AZFa region is 1.0%, while AZFb and AZFc regions respectively showed 6% and 19% in non-obstetric azoospermic cases. We further extended our study to identify novel gene mutations followed by bioinformatics analysis in the cases of NOA. In the present study our candidate genes, *AKAP3* and *PLOD3* were selected after sequencing analysis and these mutations further characterized translational event after using bioinformatics tools in the case of infertility. Figures 1 A and B show location of *AKAP3* gene mapped on chromosome 12p13.3 (variant table NC_000012.12) with the loss of adenine resulting in modifications of translation process of amino acid (i.e. leucine change into serine) due to "frameshift mutation" at rs67512580 (ACT → -CT) in homozygous condition. Similarly, PLOD3 gene locus is on chromosome 11p13.4 (variant table GCF_000001405.39) showing "missense mutation" at rs536496296 in heterozygous condition, where the nucleotide 'G' is missed in sequence AGG→A-G. This results in changes in translational event of spermiogenesis (i.e. amino acid arginine is changed into lysine). *PLOD3* gene mapped on chromosome 11, showing "missense mutation", where, guanine is changed into adenine (G → A), followed by changes in the amino acid arginine into lysine.

Due to the lack of normal structure for AKAP3 protein in the structural data bank, iTASSER server was used for protein homology modelling using 6BFIA.pdb as a template. Similarly, PLOD3 normal protein structure prediction was based on 3E0J.pdb template as shown in supplementary Figures 1A and B. The predicted 3D modelled of targeted protein showed different binding sites to α-chain and β-sheets (magenta). This also reveals eight and four pocket binding pocket (golden) as predicted in Figures 2 A and B for both of the AKAP3 and PLOD3 proteins, respectively. Table 1 shows molecular docking between normal and mutated protein with MTX as shown to the active binding sites with their residues through VDW and H-bonding, with one active site (represented in bold letter) which gives significant binding site to the receptor and inhibits unbalanced functions of the protein molecule. The gene coded 3D modelled protein structure and their

binding energy with favoured regions lies in phi (Φ) and psi (Ψ), which further confirms active stability of residue after construction of Ramachandran plot, as shown in Figures 3.A and B for AKAP3 and PLOD3 proteins, respectively. Molecular docking was done using iGEMDOCK to study the protein drug interaction. Interestingly, free binding energy in the mutated protein structure of AKAP3 and PLOD3 with MTX was between 0 to -10 Kcal/mol, showing significant binding energy whereas the normal protein structures of AKAP3 and PLOD3 showed low free binding energy of -85.33 Kcal/mol and -132.5 Kcal/mol respectively. This indicates weak binding efficiency between normal protein structures and MTX, as depicted in Table 1. Furthermore, findings of iGEMDOCK revealed less binding energy (-10.03 and -8.40), VDW (Van der Waals forces) (-21.89 and -23.07), H-bond (-12.82 and -23.07), and Z-Score (4.07 and 3.10) were required to bind both mutated protein and ligand molecule. Although, less binding energy represent good potential to develop 3D structure based on drug designing and reduced mutagenic properties of AKAP3 and PLOD3 gene coded protein. Figures 4.A and B showed binding sites of MTX into the target protein and interaction with altered amino acid residues.

Table 1: Comparison of sperm parameters (± SD) between the experimental groups after frozen-thawed and treatment with 10 µg/ml Calligonum (CGM) extract and LIPUS (pulsed mode and continues wave)

Docking Compound	Binding Sites (BS)	Binding Site (Residues & Position)	Residues Binding Energy (kj/mol-1)	-19.63vdm	H-bond	Z-Score
AKAP3 Normal Protein Structure with MTX Binding	BS	M286 T287 A289 K324 Y288 D290	-85.33*	-65.61	-19.72	1.645
AKAP3 Mutated Protein Structure with MTX Binding	BS 1	K429 L430 E442 E443 T444 C445 E451 D521 S522 W523 A524 S760 N761 N763 L764 T765 D766 T767 G794	-10.03*	-21.89	-12.82	4.07
	BS 2	D697 D698 S704 R705 D698 D702 A703 S704 P792	+8.13	-18.22	-6.82	6.09
	BS 3	E123 S150 H342 S343 T345	+12.35	-10.48	-16.70	3.15
	BS 4	S343 M349 T350	+17.65	-17.33	-14.81	8.19
	BS 5	E671	+28.12	-30.75	-11.07	2.27
	BS 6	T444 C445 A446	+81.35	-09.34	-9.56	7.48
	BS 7	Y435 E614 P615 K616	+72.58	-32.59	-19.32	5.67
	BS 8	F246 N250 S280 V281 I285 L378 Y382	+48.42	-45.63	-36.63	9.43
PLOD3 Normal Protein Structure with MTX Binding	BS	T50 T305 P307 P379 D380 T381 T390 D391 F393	-132.5*	-92.8	-39.76	1.650
PLOD3 Mutated Protein Structure with MTX Binding	BS 1	K38 S97 I98 H99 Y101	-8.40*	-23.07	-18.15	3.10
	BS 2	V34 N35 K38 Q39 Y42	-01.46	-11.25	-15.09	2.15
	BS 3	V77	+20.38	-17.49	-12.27	4.01
	BS 4	V34 N35 A134	+67.42	-19.63	-15.32	1.15

***; Standard molecular docking binding energy ranges from 0.0 to - 10.0 in mutated protein showing significant interaction with drug (MTX). MTX; Methotrexate , and VDW; Van der waals forces.**

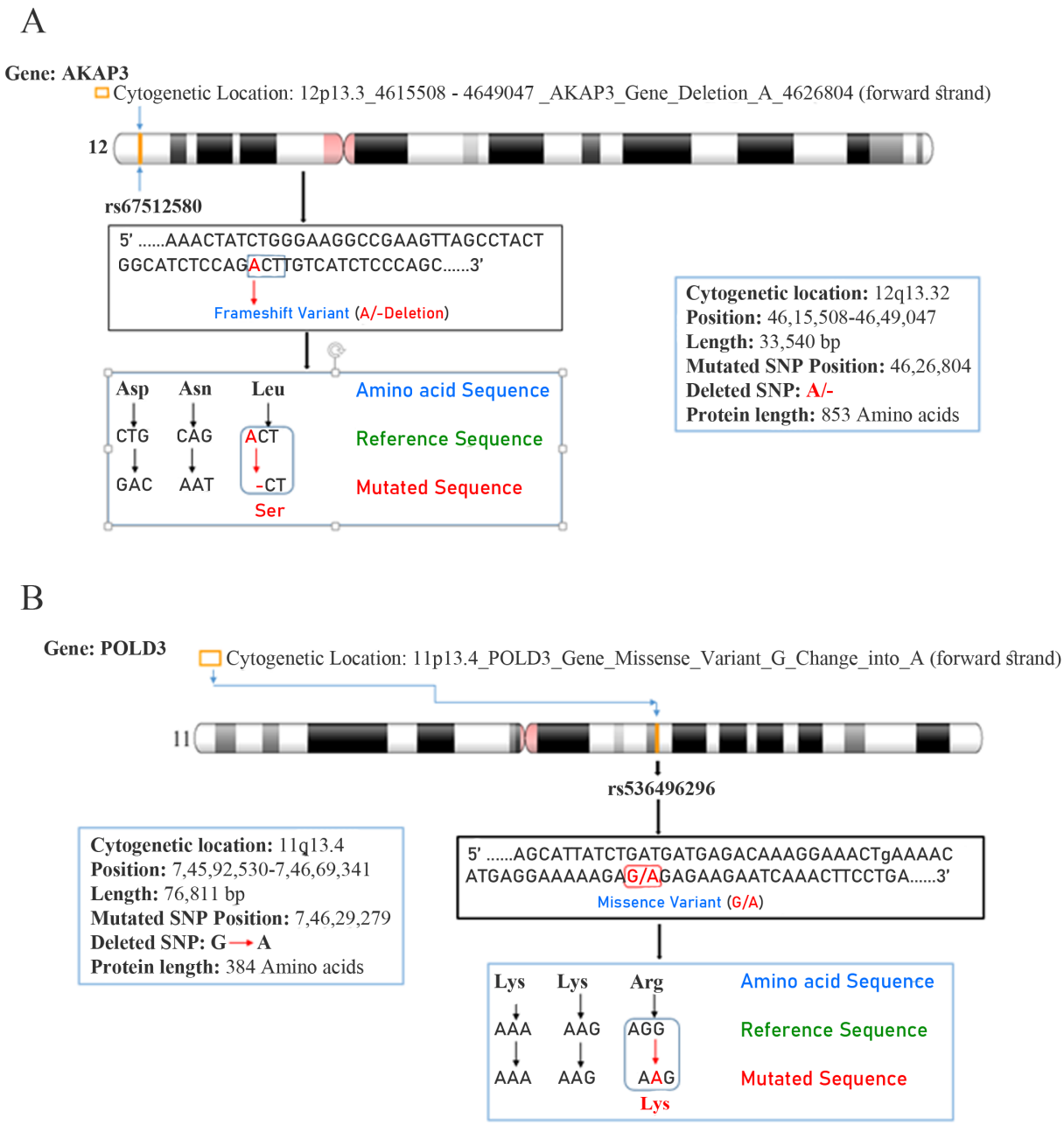

Fig. 1: Gene mapping-chromosomal location. Cytogenetic location and mutational site of the **A.** *AKAP3* and **B.** *PLOD3* genes mapped on chromosomes 12p13.32 and 11q13.4, respectively (https://www.ncbi.nlm.nih.gov/genome/tools/gdp).

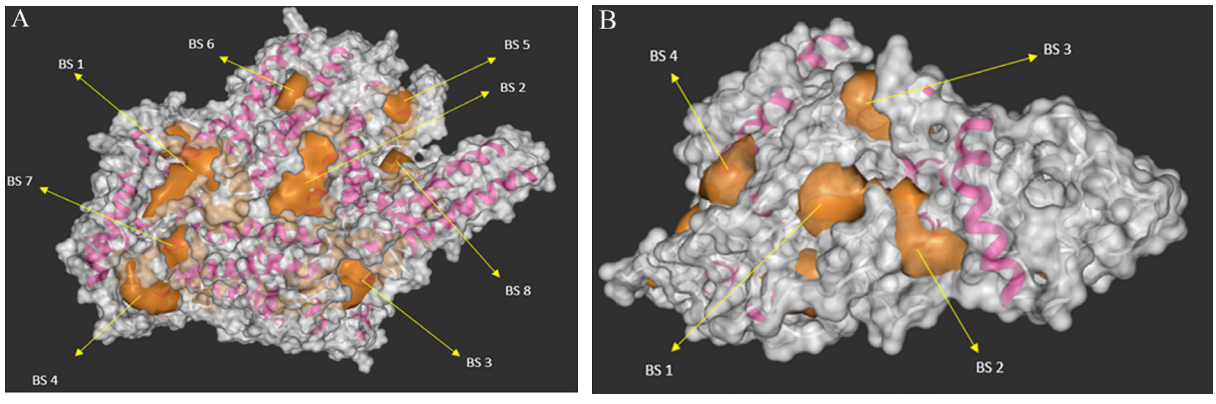

Fig. 2: 3-D protein structure of *AKAP3* and PLOD3 genes. Illustration of the modelled 3D structure and available target protein of different binding sites with α-chain and β-sheets (magenta), binding pocket (golden) and surface structure visualization (grey) of **A.** *AKAP3* and **B.** *PLOD3* protein structure binding sites are represented by arrow (→).

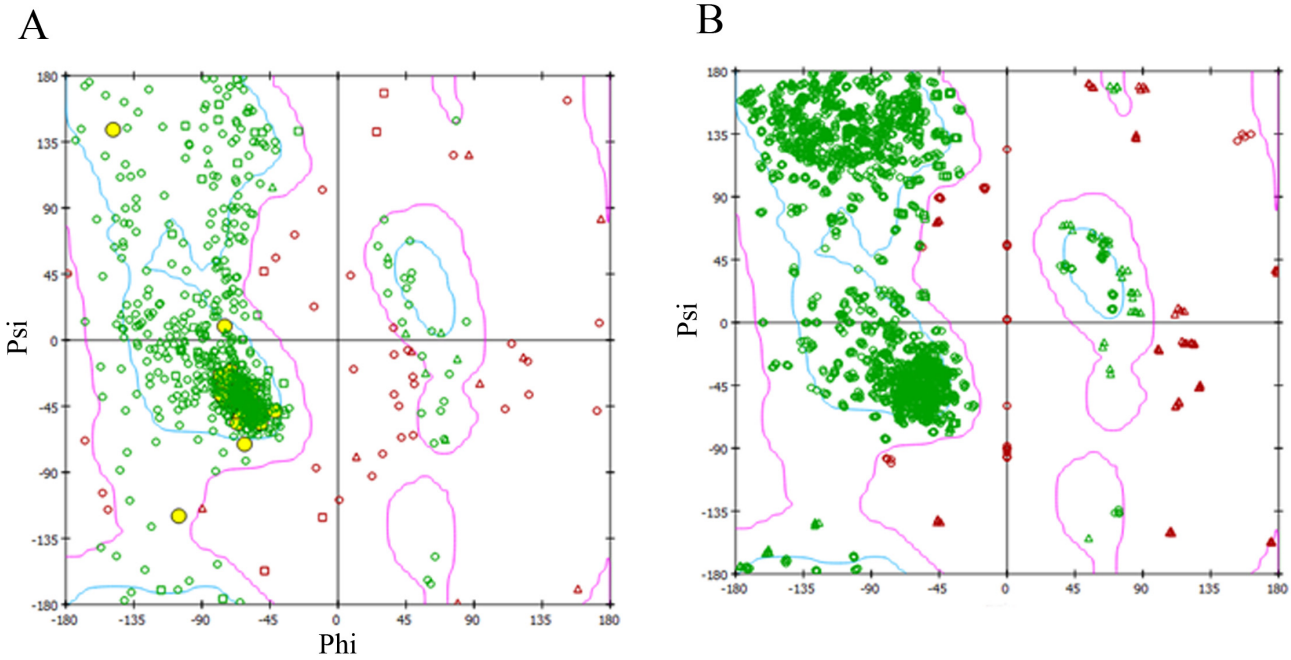

Fig. 3: Ramachandran plot. Homology modelled structure between **A.** AKAP3 and **B.** *PLOD3* gene coded proteins, supported by Ramachandran plot.

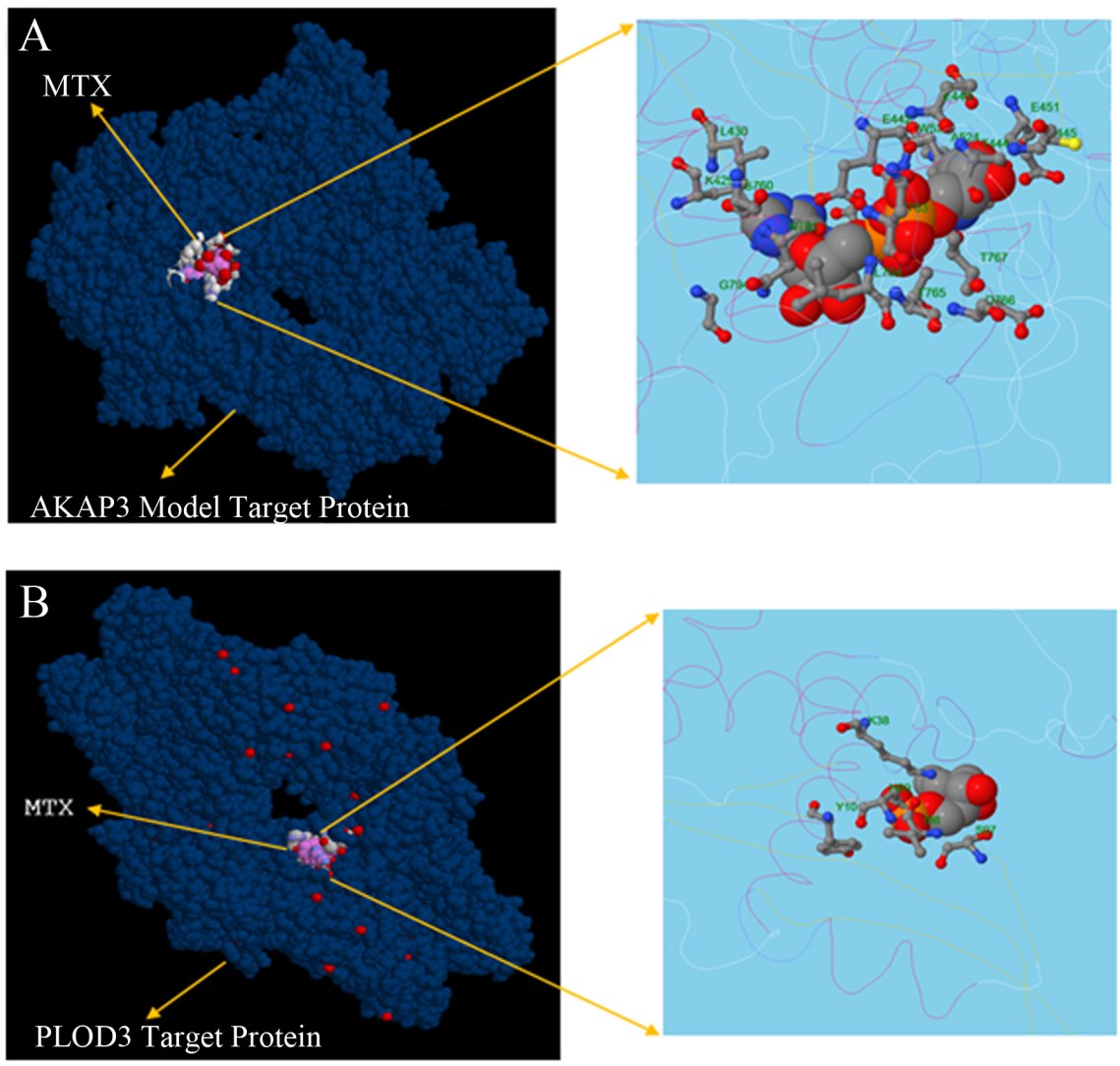

Fig. 4: MTX binding with AKAP3 and PLOD3 protein structures. Structure showing the binding site with MTX molecule superimposed on target protein. Structure models of **A.** AKAP3 and **B.** PLOD3 are showed in enlarged view as visualized by UCSF chimera.

Discussion

The present study explores acquaintance between changes of nucleotides (frameshift and non-frameshift mutation) in *AKAP3* and *PLOD3* gene and their transcriptional events (amino acids) in the cases of infertility. Human spermatogenesis is highly sensitive process that involves complex interactions between genetic and environmental factors. Such pathways regulate proliferation and differentiation of germ cells (spermatocytes, spermatids, sperm) and Sertoli cells inside the seminiferous tubules of testes. WES is one of the most powerful and sensitive techniques utilized for identification of new mutations in genome. Bioinformatics tools play a significant role in structural designing and modelling of drugprotein interactions based on pharmacogenomics and personalized medicine. Earlier study, based on WES and bioinformatics analysis gives new insight into discovery of gene(s) and disease(s). Additionally, role of single nucleotide polymorphism (SNP) mutations, such as In/Del, were identified using the ensemble database (https://www.ensembl.org/Multi/Search/ Results q=snp;site=ensembl_all) during germ cell differentiation and proliferation in spermatogenesis (10).

With the help of bioinformatics tools, we are able to trace our finding in various biological databases across the countries. However, there is lack of AKAP3 protein 3D structure availability in structural database (Protein Data Bank) (https://www.rcsb.org). Here, we used virtual protein modelling iTASSER server based on the principle of X-ray crystallography and Nuclear Magnetic Resonance. Hence modelling 3D structure of the mutated *AKAP3* gene coded protein with the help iTASSER online server and predicted the model. There are several available computational procedures for determination of protein structure in homologous modelling, but using this model we could perform the most accurate structural and functional predictions based on the algorithms (11). It firstly identified structural templates from the PDB and compared template-target based modelling (TTBM) with powered detection and alignment accuracy. With no doubt, another TTBM has become an extremely useful approach for the prediction of protein complex structure based on BLAST alignment methods. Prediction of 3D protein structure based on *AKAP3* and *PLOD3* gene sequences provide knowledge of structural and functional activities of the encoded protein compared to the normal protein. Ligand binding with specific active sites has been predicted with MTX, which is known to interfere with spermatogenesis. *AKAP3* gene plays significant role in sperm motility after stimulating effect of bicarbonate, which activates soluble adenylyl cyclase followed by triggering signalling cascade of tyrosine phosphorylation. *AKAP3* activates PI3K/Akt pathway, and leucine influences sperm motility. But in the present study, leucine change into serine might be one of the causative factors for infertility by interfering motility of sperm during process of fertilization (21). Another relevant *PLOD3* gene associated to infertility is a member of family of lysyl hydroxylase that catalyses hydroxylation of proline and lysine at the time of collagen synthesis. Collagen, as a major component of the ECM, plays an essential role in embryo implantation (22). Similarly, *PLOD3* gene mutation might interfere with translational event due to the change of arginine into lysine and failing catalysis of hydroxylation event during collagen synthesis, resulting in alteration of sperm function. Previously it was reported that oral supplement of arginine enhanced sperm count and motility in the majority of oligospermia cases and prevented infertility (23).

Interestingly, the bioinformatics tools using molecular docking studies help explore the structural (integrity) and functional prediction of protein structure to ligand binding sites affecting sperm morphology during spermiogenesis. However, it is not clear how the gene(s) interact with protein and protein interact with the ligand (drug), like methotrexate which known to function during germ cell proliferation and modify defined 3D structure followed by loss of function. Drugs bind to protein and specific binding target sites where they are firstly absorbed, secondly transported and finally distributed to their respective sites, if not mutated. Using iGEMDOCK v2.1, ligand binding site was predicted and based on the minimum energy to bind with MTX, the best model was chosen as shown in Table 1. Thus, present findings justify relevance of normal and mutated protein interactions with MTX during prediction of 3D model structure (*AKAP3* gene) developed and reported for the first time in the field of reproductive medicine (Fig. S1 A and B) (See Supplementary Online Information at www.ijfs.ir). However, our efforts are evolved to predict 3D structure and their affinities based on the binding energy to ligand after penetrance of mutated gene into the genome. This increases genetic susceptibility risk of the disease either in homozygous or heterozygous condition. WES is a highly sensitive and most reliable technique to identify new gene mutations in clinical samples. However, further validations are required to incorporate in large sample size, in order to make the study more significant.

Conclusion

The findings of present study are quite interesting, as predicted structural and functional activities based on genomic alterations and germ cells proliferation during spermiogenesis. Such type of study widens the scope of developing new derivatives based on pharmacogenomics and personalized medicine for the management of infertility.

Acknowledgements

Thankfully acknowledges the Director, AIIMS Patna for the valuable suggestions. Financial support of this study was provided by the Department of Biotechnology (Govt. India), grant code: No.BT/PR14671/MED/12/487/2010) to carry out this research work. We also thankfully acknowledge the participants of the study and their families. The authors declare no conflict of interest.

Authors' Contributions

A.K.S.; Genetic analysis. M.A.; Clinical diagnosis. A.K.; and M.T.; Preparation of samples and genetic analysis, A.K.; Structural data analysis and functional genomics. All of the authors have equally participated in preparation of the manuscript.

References

1. Pereira R, Oliveira J, Sousa M. A molecular approach to sperm immotility in humans: a review. Medicina Reproductiva y Embriología Clínica. 2014; 1(1): 15-25.
2. Gungor-Ordueri NE, Tang EI, Celik-Ozenci C, Cheng CY. Ezrin is an actin binding protein that regulates sertoli cell and spermatid adhesion during spermatogenesis. Endocrinology. 2014; 155(10): 3981-3995.
3. Saxena AK, Tiwari M, Kumar A. Penetrance of de novo mutation of USP9Y and PCDH11Y gene in AZF regions of non-obstructive Azoospermic population in India. Int J Curr Res. 2019; 11(2): 1373-1379.
4. Krausz C, Riera-Escamilla A. Genetics of male infertility. Nat Rev Urol. 2018; 15(6): 369-384.
5. Li YF, He W, Mandal A, Kim YH, Digilio L, Klotz K, et al. CABYR binds to AKAP3 and Ropporin in the human sperm fibrous sheath. Asian J Androl. 2011; 13(2): 266-274.
6. Bajpai M, Fiedler SE, Huang Z, Vijayaraghavan S, Olson GE, Livera G, et al. AKAP3 selectively binds PDE4A isoforms in bovine spermatozoa. Biol Reprod. 2006; 74(1): 109-118.
7. Salo AM, Cox H, Farndon P, Moss C, Grindulis H, Risteli M, et al. A connective tissue disorder caused by mutations of the lysyl hydroxylase 3 gene. Am J Hum Genet. 2008; 83(4): 495-503.
8. Carr DW, Fujita A, Stentz CL, Liberty GA, Olson GE, Narumiya S. Identification of sperm-specific proteins that interact with A-kinase anchoring proteins in a manner similar to the type II regulatory subunit of PKA. J Biol Chem. 2001; 276(20): 17332-17338.
9. Maor-Sagie E, Cinnamon Y, Yaacov B, Shaag A, Goldsmidt H, Zenvirt S, et al. Deleterious mutation in SYCE1 is associated with non-obstructive azoospermia. J Assist Reprod Genet. 2015; 32(6): 887-891.
10. Saxena AK, Tiwari M, Agarwal M. Single nucleotide polymorphism of Arylsulfatase D gene (ARSD) and their association with male infertility. J Clin Gen Genomics. 2018; 1(2): 11-13.
11. Zhang Y. I-TASSER: fully automated protein structure prediction in CASP8. Proteins. 2009; 77 Suppl 9: 100-113.
12. Xu F, Wu H, Katritch V, Han GW, Jacobson KA, Gao ZG, et al. Structure of an agonist-bound human A2A adenosine receptor. Science. 2011; 332(6027): 322-327.
13. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. The I-TASSER Suite: Protein structure and function prediction. Nat Methods. 2015; 12(1): 7-8.
14. Scietti L, Chiapparino A, De Giorgi F, Fumagalli M, Khoriauli L, Nergadze S, et al. Molecular architecture of the multifunctional collagen lysyl hydroxylase and glycosyltransferase LH3. Nat Commun. 2018; 9(1): 3163.
15. Källberg M, Wang H, Wang S, Peng J, Wang Z, Lu H, et al. Template-based protein structure modeling using the RaptorX web server. Nat Protoc. 2012; 7(8): 1511-1522.
16. Jiménez J, Doerr S, Martínez-Rosell G, Rose AS, De Fabritiis G. DeepSite: protein-binding site predictor using 3D-convolutional neural networks. Bioinformatics. 2017; 33(19): 3036-3042.
17. Saxena AK, Singh D, Singh G. Structural interaction between drug-DNA protein-A novel approach for bioinformatics in medicine. Biomed Res. 2009; 20(1): 28-34.
18. Hsu KC, Chen YF, Lin SR, Yang JM. iGEMDOCK: a graphical environment of enhancing GEMDOCK using pharmacological interactions and post-screening analysis. BMC Bioinformatics. 2011; 12 Suppl 1: S33.
19. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera-a visualization system for exploratory research and analysis. J Comput Chem. 2004; 25(13): 1605-1612.
20. Haraksingh RR, Snyder MP. Impacts of variation in the human genome on gene regulation. J Mol Biol. 2013; 425(21): 3970-3977.
21. Luconi M, Carloni V, Marra F, Ferruzzi P, Forti G, Baldi E. Increased phosphorylation of AKAP by inhibition of phosphatidylinositol 3-kinase enhances human sperm motility through tail recruitment of protein kinase A. J Cell Sci. 2004; 117(Pt 7): 1235-1246.
22. Zhang J, Zhang X, Liu Y, Su Z, Dawar FU, Dan H, et al. Leucine mediates autophagosome-lysosome fusion and improves sperm motility by activating the PI3K/Akt pathway. Oncotarget. 2017; 8(67): 111807-111818.
23. Brincat D, Catania S, Wismayer PS, Calleja-Agius J. Male factors in ART outcome prediction. Gynecol Endocrinol. 2015; 31(3): 169-175.

Original Article

Interfering Effects of *In Vitro* Fertilization and Vitrification on Expression of *Gtl2* and *Dlk1* in Mouse Blastocysts

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Abstract

Background: Embryo vitrification is a key instrument in assisted reproductive technologies (ARTs). However, there is increasing concern that vitrification adversely affects embryo development. This study intends to assess the effect of vitrification on developmental competence, in addition to expressions of long non-coding RNA (lncRNA) gene trap locus 2 (*Gtl2*) and its reciprocal imprinted gene delta-like homolog 1 (*Dlk1*), in mouse blastocysts.

Materials and Methods: In this experimental study, we have designed three experimental groups: control (fresh blastocysts collected from superovulated mice), *in vitro* fertilization (IVF; blastocysts derived from IVF) and vitrification (IVF derived blastocysts subjected to vitrification/warming at the 2-cell stage). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to assess the expression levels of *Gtl2* and *Dlk1* in the blastocysts.

Results: The results showed that vitrification group had significantly lower blastocyst and hatching rates compared to the IVF group ($P<0.037$) and ($P<0.041$), respectively. Gtl2 was down-regulated and Dlk1 was up-regulated following the IVF and vitrification ($P<0.05$).

Conclusion: These results suggested that IVF and vitrification disturbed genomic imprinting and lncRNA gene expressions, which might affect the health of IVF children.

Keywords: IVF, Mouse, Preimplantation Embryo, Vitrification

Citation: **Movahed E, Shabani R, Hosseini S, Shahidi S, Salehi M. Interfering effects of IVF and vitrification on expression of Gtl2 and Dlk1 in mouse blastocysts. Int J Fertil Steril. 2020; 14(2): 110-115. doi: 10.22074/ijfs.2020.5984.**

Introduction

Embryo cryopreservation, an important component of assisted reproductive technologies (ARTs), has considerably improved the clinical results of this technology (1). Vitrification and slow freezing are two routine methods for embryo cryopreservation. Vitrification is routinely used in ART clinics because of its higher survival rate post-warming, in addition to its simple and inexpensive technique in comparison with slow freezing. However, it is still not known whether vitrification affects the health of adults who were conceived by ART, with respect to the cytotoxicity of high concentrations of cryoprotectants used for vitrification and stresses from high cooling and warming rates (2).

Long non-coding RNAs (lncRNAs) are transcripts with more than 200 up to several thousand nucleotides. Although most of these molecules do not have protein coding capacity, some of them code small peptides of less than 100 amino-acids (3). It is anticipated that thousands of lncRNAs exist in the mammalian transcriptome and, until now, nearly 15000 human lncRNAs have been characterized (4, 5).

lncRNAs have important regulatory roles in many cellular processes such as gene expression, imprinting, cytoplasmic scaffolds and intracellular trafficking. They affect cell function during development and differentiation (4, 6). In addition, correlated with the expression of pluripotency markers, lncRNAs play role in the embryonic stem cell regulatory (5). lncRNAs are involved in the numerous pathological conditions, including oncogenesis (4). On the other hand, a regulatory epigenetic mechanism (genomic imprinting) causes asymmetric parental allele expressions in a series of mammalian genes (7, 8). In imprinting genes, one of the parental alleles is expressed whereas another allele is methylated and silenced. Disruption in genomic imprinting results in pathological conditions such as Beckwith-Wiedemann Syndrome (BWS) and Angelman Syndrome (AS) (8). Gene trap locus 2 (*Gtl2*; approved symbol: *Meg3*) and delta-like homolog 1 (*Dlk1*) are reciprocally imprinted gene located on mouse distal chromosome 12. *Gtl2* is a chromatin-interacting lncRNA expressed from the maternal allele, whereas *Dlk1*

Received: 19/May/2019, Accepted: 4/November/2019
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Royan Institute
International Journal of Fertility and Sterility
Vol 14, No 2, July-September 2020, Pages: 110-115

is a paternally expressed gene. The *Dlk1/Gtl2* imprinting locus has an momentous role in embryonic development and growth (9). Previous researches have established that epigenetic disruption of this imprinted locus is related to facial dysmorphisms, skeletal abnormalities and muscular hypertrophy. Additionally, loss of imprinting in *DLK1/GTL2* has been reported in pheochromocytoma, neuroblastoma and Wilms' tumour (10-12).

A review of the literature showed no data that pertained to an association between embryo vitrification and lncRNA expressions. Thus, considering the importance of *Dlk1* and *Gtl2* in embryo development, we sought to investigate their expressions *in vitro* fertilization (IVF) pre-implanted embryos, embryos subjected to vitrification and warming, and fresh blastocysts. Here, we made use of a mouse embryo model because of the ethical issues that pertain to research on human embryos.

Materials and Methods

This experimental study, approved by and Ethical Committee of Shahid Beheshti University of Medical Sciences (Tehran, Iran, Ethical permission number: IR.SBMU. RETECH.REC.1396.997). All animal experiments were conducted in compliance with the guidelines established by this university for the keeping and manipulate of laboratory animals.

Materials

All chemicals and reagents were obtained from Sigma Chemical Company (St. Louis, USA) unless otherwise noted.

Animals

We obtained 6-8 weeks old female and 10-weeks old male NMRI mice from Royan Institute (Tehran, Iran) to use in this study. The mice were accommodated under the controlled conditions of 12 hours light: 12 hours dark photoperiod at room temperature (22 ± 2°C) and 50 ± 10% humidity with ad libitum use of food and water. The animals were killed by cervical dislocation.

Experimental design

Female mice were superovulated by intraperitoneal (IP) injection of 10 IU pregnant mare serum gonadotropin (PMSG; Pregnecol®, Australia), followed 48 hours later by 10 IU human chorionic gonadotropin (hCG; Pregnyl). The experiment was carried out on three treatment groups: control, IVF, and vitrification as shown in Figure 1.

In the control group, after hCG injection, female mice were mated with male mice. Successful mating was verified by the detection of a vaginal plug, the next day morning. Fresh blastocysts were collected from the mice uteri by flushing the uterine horns with FHM flushing media 94 hours posthCG, according to the previous study (13). The blastocysts were used for RNA extraction and reverse transcription.

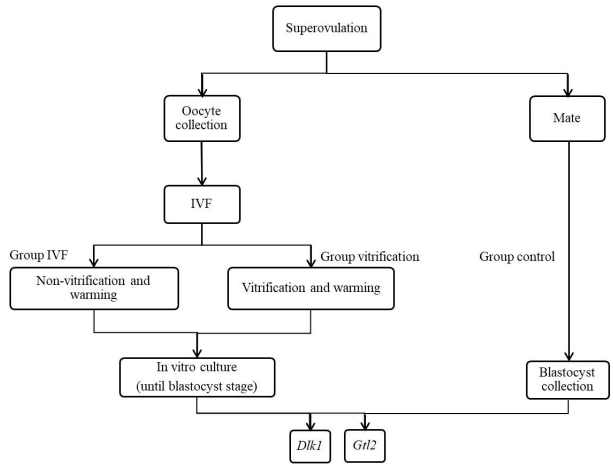

Fig.1: Experimental design and IVF; *In vitro* fertilization, Dlk1; Delta-like homolog 1, and Gtl2; Gene trap locus 2.

In the IVF and vitrification groups, we collected the cumulus oocyte complexes containing metaphase II (MII) oocytes from the oviduct ampullae 14-16 hours after hCG injection. The oocytes were released into FHM medium and then transferred to 50 µl droplets of human tubal fluid medium (HTF) supplemented with 4 mg/ml bovine serum albumin (BSA).

In vitro fertilization

IVF was performed as formerly explained (14). Sperms were collected from the male mice. The cauda epididymides and vas deferens were isolated and placed in a petri dish containing previously equilibrated HTF medium (37°C, 5% CO_2 in air). The sperms were passively released into the culture by using pointed forceps and a razor blade. The suspended sperms were incubated at 37°C for 45 minutes to allow capacitation. Capacitated motile spermatozoa were added to 50 µl IVF drops to reach 1×10^6 sperm/ml concentrations. Subsequently, they were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C under mineral oil for 5-6 hours. Next, the in vitro-derived zygotes were washed in FHM medium and cultured in potassium simplex optimized medium (KSOM) supplemented with 4% BSA under the same conditions to allow for further development. After 24 hours, we divided the 2-cell embryos into two groups. In the IVF group, the embryos were maintained in KSOM for 72 hours until the blastocyst stage. In the vitrification group, the 2-cell embryos were vitrified/warmed and then cultured under the same conditions as the IVF group, for 72 hours, to reach the blastocyst stage. Finally, the rates of development at the 4-cell, 8-cell, morula and blastocyst stages were assessed in both groups. The blastocysts were used for RNA extraction and reverse transcription.

Vitrification and warming

In the vitrification group, the 2-cell embryos were vitrified by the cryotop method with Kitazato Vitrification Kit (Kitazato Biopharmaceuticals, Japan), as previously described (15). Briefly, embryos were equilibrated in equilibration solution (ES) with 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO). After 3 minutes, the embryos were exposed to the vitrification solution (VS) containing 15% (v/v) EG, 15% (v/v) DMSO and 0.5

mol/l sucrose for less than 1 minute. Next, 3-5 embryos with minimal VS were loaded onto the inner surface of the cryotop and immediately submerged in liquid nitrogen (LN2), followed by capping and storing in LN2 for up to 2 weeks. Vitrification processes were carried out at room temperature. For warming, the embryos were exposed to decrease concentrations of sucrose on a 37°C hot plate, as follows: 0.5 M sucrose for 1 minute, 0.25 M sucrose for 3 minute and 0.125 M sucrose for 5 minute. Finally, the embryos were placed for 3 minute in a 0 M washing solution and they were assessed for survival by observing the intactness of zona pellucida and blastomeres. The surviving 2-cell embryos were cultured in KSOM medium in an incubator at 37°C and 6% CO2 to allow further development to the blastocyst stage. All media used for warming were incubated at 37°C for 30 minutes before warming.

RNA extraction and complementary DNA synthesis

RNA extraction, complementary DNA (cDNA) synthesis, and quantitative reverse-transcription PCR (qRT-PCR) analysis were carried out according to the previous study protocols (16). Briefly, two blastocysts in each replicate of each experiment were pipetted into microtubes containing 1.5 µl lysis buffer. We added 5 µl nuclease-free water and 2 µl random hexamer to each sample and then placed the samples in a BioRad thermocycler for 5 minutes at 75°C. Immediately afterwards, the microtubes that contained the reaction product were placed on ice, followed by the addition of 5x RT buffer, 200 u RT enzyme, 10 mM dNTP, and 10 U RNase inhibitor to each reaction for cDNA synthesis. Reverse transcription (RT) reaction was performed in the thermocycler with the following amplification program: 25°C for 10 minutes, 37°C for 15 minutes, 42°C for 45 minutes and 72°C for 10 minutes. The samples were left at 4°C overnight. PCR mixture, consisted of 5 µl Master Mix (Taq DNA Polymerase Mix Red-MgCl; Amplicon, Denmark), 3 µl nuclease-free water, 1 µl cDNA, and 1 µl specific primer (Table 1) was added to each PCR microtube to amplify cDNA product. The endogenous control (β2m) and the investigated genes were amplified according to the following PCR cycle: 94°C for 3 minues (denaturation), 94°C for 30 seconds (denaturation), 60°Cfor 45 seconds (annealing) and 72°C for 45 seconds (extension), followed by 40 cycles. A final elongation step was carried out at 72°C for 10 minutes. The amplification products were loaded and run alongside a DNA ladder on a 2% agarose gel in TAE and, after 25 minutes, they were observed under short-wave UV.

Quantitative reverse transcription PCR (qRT-PCR) analysis

qRT-PCR was executed to evaluate the amount of Dlk1 and Gtl2 expressions by using a Rotor Gene Q instrument (Qiagen, USA). Table 1 lists the primer sequences applied for qRT-PCR. qRT-PCR reaction were conducted in a total volume of 13 µl reaction containing 1 µM of each primer for the indicated genes and 1 µM of the synthesized cDNA based on the manual for the DNA Master SYBR Green 1 mix (Roche Applied Sciences, Germany). Cycling program for the RT-PCR was as follows: 2 minutes at 95°C, and 40 cycles of 5 seconds at 95°C, 30 seconds at 60°C, 10 seconds at 72°C. Melting curve examination for all amplification reactions confirmed the particular amplification peaks and lack of primer-dimer formation. *β2m* was the endogenous internal house-keeping gene for RT-PCR data normalization. We used the Relative Expression Software Tool (REST, version 2009) for qRTPCR data analysis.

Statistical analysis

Statistical analyses were performed by applying the Statistical Package for the Social Science software, version 16 (SPSS, USA). Cleavage and developmental ratio to blastocysts stage between IVF and vitrification groups were compared by the non-parametric Mann- Whitney test. The relative gene expression levels of Gtl2 and Dlk1 were analyzed by REST software (Qiagen). $P<0.05$ was regarded as statistically significant.

Results

Embryo development

We assessed the effect of vitrification on developmental competence of preimplantation embryos. The 2-cell embryos obtained from IVF in three runs were divided into two groups. Totally, for the IVF group, there were 170 cultured 2-cell embryos. In the vitrification group, 166 embryos (2-cell) were vitrified/ thawed. The vitrification group had a survival rate of 96.72% ± 2.93, after vitrification and warming. We compared the percentage rates of the 4-cell, 8-cell and morula stages between the IVF and vitrification groups. There was no significant difference between these two groups, in terms of cleavage rate. The blastocyst (64.04% ± 10.16) and hatching (48.51% ± 10.92) rates in the vitrification group were significantly lower than the blastocyst (82.63% ± 2.56; $P<0.037$) and hatching (69.22% ± 5.20; $P<0.041$) rates in the IVF group (Table 2).

Table 1: Details of primers applied for RT-PCR and qRT-PCR

Genes	Nucleotide sequences (5′–3′)	Tempreture (°C)	GC%	Self-complementarity	Accession number
Gtl2	F: CTGAAGAAAAGAAGACTGAGGAC	56.83	43.48	3.00	NR_003633.3
	R: CGATTTACAGTTGGAGGGTC	55.86	50.00	3.00	
Dlk1	F: CTGCGAAATAGACGTTCGG	56.56	52.63	4.00	XM_006515457.3
	R: GTACTGGCCTTTCTCCAGG	57.14	57.89	4.00	
β2m	F: AGACTGATACATACGCCTGC	57.20	50.00	3.00	M_009735.3
	R: ATCACATGTCTCGATCCCAG	56.80	50.00	6.00	

RT-PCR; Reverse transcriptio polymerase chain reaction, and qRT-PCR; Quantitative reverse transcription polymerase chain reaction. GC; Guanine - Cytosine Percent.

Table 2: Development of 2-cell mouse embryos *in vitro* fertilization and vitrification groups

Group	2-cell embryos (n)	Survival rate	4-cell rate	8-cell rate	Morula rate	Blastocysts rate	Hatched rate
IVF	177	100% (170/170)	95.36% ± 1.17 (162/170)	92.19% ± 2.83 (157/170)	88.04% ± 2.59 (150/170)	82.63% ± 2.56*(141/170)	69.22% ± 5.20** (117/170)
Vitrification	166	96.72% ± 2.93 (160/166)	92.32% ± 2.64 (148/160)	84.49% ± 6.92 (135/160)	76.6% ± 7.58 (123/160)	64.04% ± 10.16* (102/160)	48.51% ± 10.92** (77/160)

Data are presented as mean ± SD or n (%). *Significant difference (P<0.037), **Significant differences (P<0.041)

Dlk1 and *Gtl2* expression levels

qRT-PCR was implemented to appraise the expression levels of the lncRNA *Gtl2* and *Dlk1* gene in blastocysts. *Gtl2* expression was down-regulated in the IVF and vitrification groups compared to the control group. *Gtl2* was less expressed in the vitrification group compared to the IVF group (P<0.05, Fig. 2A). *Dlk1* was up-regulated in the IVF and vitrification groups compared to the control group (P<0.05). There was no difference between the IVF and vitrification groups, in terms of *Dlk1* expression (P<0.05, Fig.2B).

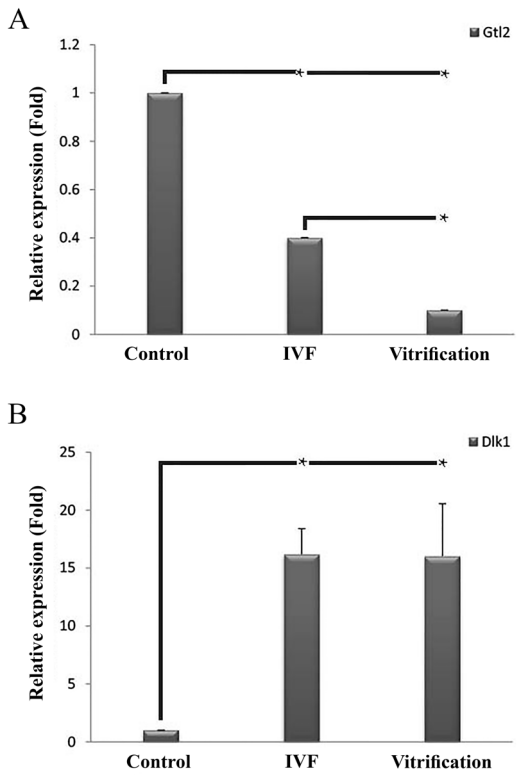

Fig.2: Relative expression levels of mouse of gene trap locus 2 (Gtl2) and delta-like homolog 1 (Dlk1) in the blastocysts of the experimental groups. **A.** The expression levels of Gtl2 and **B.** Dlk1, *; P<0.05.

Discussion

Vitrification is an encouraging technology to cryopreserve gametes and embryos in ART clinics. The main challenge faced by researchers is to evaluate the consequences of this process on healthy and affectedadults conceived by IVF and optimization of this important technology (2, 17, 18). In this study, we assessed the influence of vitrification using cryotops on developmental competence and expression levels of the lncRNA *Gtl2* and *Dlk1* gene in pre-implanted mouse embryos.

We assessed the embryonic developmental potential after vitrification by comparing cleavage, blastocysts and hatching rates of the non-vitrified embryos (IVF group) compared to the vitrified embryos (vitrification group). The results showed that vitrification/warming at the 2-cell stage significantly decreased blastocysts and hatching rates in mouse preimplantation embryos. This finding provided evidence of the adverse effects of vitrification on development of preimplantation embryos. This result supported earlier observations where vitrification negatively impacted development of preimplantation mouse embryos (2, 19, 20). Vitrification generates increased levels of reactive oxygen species (ROS). ROS leads to interrupted cell function and division. Thus, to some extent, high ROS levels are in charge of lower developmental competence in embryos subjected to vitrification (20, 21). Most likely, antioxidant enzymes such as SOD and catalase, which are responsible for cell defense against ROS in normal conditions, are destroyed during vitrification (20). Additionally, it has been shown that vitrification leads to zona hardening of preimplantation embryo. Thus, zona hardening could be the explanation of the decrease in hatching rate subsequent to vitrification. Difficulty in hatching process could have negative effect on implantation potential of embryo (22).

Recent evidence suggests that ART, including superovulation, IVF and vitrification cause a disturbance in genetic and epigenetic mechanisms in the pre-implanted embryo affecting health of the children conceived by ART (2, 17, 18). However, previous studies have not addressed lncRNA changes in embryos derived from ART. lncRNA Gtl2 and its reciprocal imprinted gene, *Dlk1*, are important for normal development of embryo tissues such as the brain and bones, in addition to the postnatalregulation of neural system and metabolism (23). *Gtl2* has also a major anti-tumor activity mediated through p53- dependent and p53-independent pathway in humans (4). Through RNA–DNA triplex structures, Gtl2 takes part in the regulation of TGF-b signaling pathway genes (24). *Dlk1* codes a transmembrane protein and it is fundamental to normal cellular differentiation. It plays a major role in

carcinogenesis. Therefore, the central thesis of this paper is whether IVF and embryo vitrification interfere with the expression of lncRNA *Gtl2* and its reciprocal imprinted gene, *Dlk1*, in mouse blastocysts. In the maternal allele, the intergenic differentially methylated region (IG-DMR) of *Dlk1/Gtl2* is unmethylated and there is expression of *Gtl2*. However, in the paternal allele, the IG-DMR of *Dlk1/Gtl2* is methylated, and *Dlk1* is expressed (9). In this study, we observed decreased *Gtl2* expression and increased Dlk1 following IVF and vitrification. Disruption in the imprinting of other imprinted genes following IVF and vitrification have been shown in the previous papers (25, 26).A possible explanation for our result might be decline in level of DNA methylation. Prior studies noted that IVF and vitrification decreased DNA methylation in blastocysts (2, 13, 26). Decreased DNA methylation might be attributed to disturbances in DNA methyltransferases (Dnmts) expressions following IVF and vitrification, as the previous study revealed that IVF and vitrification result in increased relative expression levels of miR-29a and miR-29b and consequently decrease in Dnmt3a and Dnmt3b relative expression levels, as the target genes of *miR-29a* and *miR-29b* and responsible for de novo DNA methylation (13).

Conclusion

In conclusion, vitrification at the 2-cell stage adversely affected preimplantation mouse embryo development. In addition, IVF and vitrification interrupted the expressions of lncRNA *Gtl2* and its reciprocal imprinted gene, *Dlk1*, in mouse blastocysts. This study was the first to assess expression of lncRNAs following ART manipulation. Due to the importance of lncRNAs in embryo development, more research would be needed to evaluate lncRNA expressions in embryos conceived by ART.

Acknowledgements

This study was financially supported by Iran University of Medical Sciences (Tehran, Iran) and Shahid Beheshti University of Medical Sciences (Tehran, Iran). There is no conflict of interest in this study.

Authors' Contributions

E.M., M.S., R.Sh.; Contributed to the conception and study design. E.M., S.H., S.Sh.; Performed all experimental work, contributed to data and statistical analysis, and interpretation of data. E.M., M.S.; Drafted the manuscript. All authors read and approved the final draft of the manuscript.

References

1. Derakhshan-Horeh M, Abolhassani F, Jafarpour F, Moini A, Karbalaie K, Hosseini SM, et al. Vitrification at day3 stage appears not to affect the methylation status of H19/IGF2 differentially methylated region of in vitro produced human blastocysts. Cryobiology. 2016; 73(2): 168-174.
2. Bakhtari A, Rahmani HR, Bonakdar E, Jafarpour F, Asgari V, Hosseini SM, et al. The interfering effects of superovulation and vitrification upon some important epigenetic biomarkers in mouse blastocyst. Cryobiology. 2014; 69(3): 419-427.
3. Fatima R, Akhade VS, Pal D, Rao SM. Long noncoding RNAs in development and cancer: potential biomarkers and therapeutic targets. Mol Cell Ther. 2015; 3: 5.
4. Cao J. The functional role of long non-coding RNAs and epigenetics. Biol Proced Online. 2014; 16: 11.
5. Caballero J, Gilbert I, Fournier E, Gagné D, Scantland S, Macaulay A, et al. Exploring the function of long non-coding RNA in the development of bovine early embryos. Reprod Fertil Dev. 2014; 27(1): 40-52.
6. He Y, Meng XM, Huang C, Wu BM, Zhang L, Lv XW, et al. Long noncoding RNAs: novel insights into hepatocelluar carcinoma. Cancer Lett. 2014; 344(1): 20-27.
7. Song Y, Wu N, Wang S, Gao M, Song P, Lou J, et al. Transgenerational impaired male fertility with an Igf2 epigenetic defect in the rat are induced by the endocrine disruptor p, p′-DDE. Hum Reprod. 2014; 29(11): 2512-2521.
8. Denomme MM, Mann MR. Genomic imprints as a model for the analysis of epigenetic stability during ARTs. Reproduction. 2012; 144(4): 393-409.
9. Geuns E, De Temmerman N, Hilven P, Van Steirteghem A, Liebaers I, De Rycke M. Methylation analysis of the intergenic differentially methylated region of DLK1-GTL2 in human. Eur J Hum Genet. 2007; 15(3): 352-361.
10. Kalish JM, Jiang C, Bartolomei MS. Epigenetics and imprinting in human disease. Int J Dev Biol. 2014; 58(2-4): 291-298.
11. Georges M, Coppieters W, Charlier C. Polymorphic miRNAmediated gene regulation: contribution to phenotypic variation and disease. Curr Opin Genet Dev. 2007; 17(3): 166-176.
12. Feinberg AP. Phenotypic plasticity and the epigenetics of human disease. Nature. 2007; 447(7143): 433-440.
13. Movahed E, Soleimani M, Hosseini S, Akbari Sene A, Salehi M. Aberrant expression of miR-29a/29b and methylation level of mouse embryos after in vitro fertilization and vitrification at two-cell stage. J Cell Physiol. 2019; 234(10): 18942-18950.
14. Zare Z, Masteri Farahani R, Salehi M, Piryaei A, Ghaffari Novin M, Fadaei Fathabadi F, et al. Effect of L-carnitine supplementation on maturation and early embryo development of immature mouse oocytes selected by brilliant cresyle blue staining. J Assist Reprod Genet. 2015; 32(4): 635-643.
15. Dehghani-Mohammadabadi M, Salehi M, Farifteh F, Nematollahi S, Arefian E, Hajjarizadeh A, et al. Melatonin modulates the expression of BCL-xl and improve the development of vitrified embryos obtained by IVF in mice. J Assist Reprod Genet. 2014; 31(4): 453-461.
16. Haghpanah T, Salehi M, Ghaffari Novin M, Masteri Farahani R, Fadaei-Fathabadi F, Dehghani-Mohammadabadi M, et al. Does sperm DNA fragmentation affect the developmental potential and the incidence of apoptosis following blastomere biopsy? Syst Biol Reprod Med. 2016; 62(1): 1-10.
17. Chen H, Zhang L, Deng T, Zou P, Wang Y, Quan F, et al. Effects of oocyte vitrification on epigenetic status in early bovine embryos. Theriogenology. 2016; 86(3): 868-878.
18. Jahangiri M, Shahhoseini M, Movaghar B. H19 and MEST gene expression and histone modification in blastocysts cultured from vitrified and fresh two-cell mouse embryos. Reprod Biomed Online. 2014; 29(5): 559-566.
19. Zhang Y, Qu L. Non-coding RNAs and the acquisition of genomic imprinting in mammals. Sci China C Life Sci. 2009; 52(3): 195-204.
20. Gao C, Han HB, Tian XZ, Tan DX, Wang L, Zhou GB, et al. Melatonin promotes embryonic development and reduces reactive oxygen species in vitrified mouse 2-cell embryos. J Pineal Res. 2012; 52(3): 305-311.
21. Gupta MK, Uhm SJ, Lee HT. Effect of vitrification and etamercaptoethanol on reactive oxygen species activity and in vitro development of oocytes vitrified before or after in vitro fertilization. Fertil Steril. 2010; 93(8): 2602-2607.
22. Balaban B, Urman B, Yakin K, Isiklar A. Laser-assisted hatching increases pregnancy and implantation rates in cryopreserved embryos that were allowed to cleave in vitro after thawing: a prospective randomized study. Hum Reprod. 2006; 21(8): 2136-2140.
23. Wilkinson LS, Davies W, Isles AR. Genomic imprinting effects on brain development and function. Nat Rev Neurosci. 2007; 8(11):

832-843.
24. Mondal T, Subhash S, Vaid R, Enroth S, Uday S, Reinius B, et al. MEG3 long noncoding RNA regulates the TGF-β pathway genes through formation of RNA–DNA triplex structures. Nat Commun. 2015; 6: 7743.
25. Chatterjee A, Saha D, Niemann H, Gryshkov O, Glasmacher B, Hofmann N. Effects of cryopresevation on the epigenetic profile of cells. Cryobiology. 2017; 74: 1-7.
26. Yao J, Geng L, Huang R, Peng W, Chen X, Jiang X, et al. Effect of vitrification on in vitro development and imprinted gene Grb10 in mouse embryos. Reproduction. 2017; 154(3): 97-105.

Original Article

Effect of Different Concentrations of Leukemia Inhibitory Factor on Gene Expression of Vascular Endothelial Growth Factor-A in Trophoblast Tumor Cell Line

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Abstract

Background: Several studies have shown that leukemia inhibitory factor (LIF) is one of the most important cytokines participating in the process of embryo implantation and pregnancy, while, the role of this factor on vascular endothelial factor-A (VEGF-A), as one of the most important angiogenic factor, has not been fully investigated yet. The aim of this study was to evaluate the effect of LIF on gene expression of *VEGF* in the choriocarcinoma cells (JEG-3).

Materials and Methods: In this experimental study, JEG-3 choriocarcinoma cells were treated with different concentrations of LIF (1, 10, and 50 ng) for 6, 12, 24, 48 and 72 hours. Expression of *VEGF* was analyzed by real-time PCR. Delta CTs were subjected to one-way analysis of variance (ANOVA) and a post hoc Tukey's test by SPSS version 25.0 software for data analyzing.

Results: In the stimulated cells, different concentrations of LIF caused significant decrease of *VEGF* gene expression ($P<0.05$) at 12, 24 and 48 hours. In contrast, it was increased after 72 hours ($P<0.001$). Analysis of data after 6 hours also showed that level of *VEGF* gene expression was significantly decreased by increasing LIF concentration ($P<0.001$).

Conclusion: Expression level of *VEGF* gene was decreased in trophoblast cells (except after 72 hours) under the effect of different concentrations of LIF in a time-dependent manner. So, this study showed that further studies are needed to determine the effect of LIF on other angiogenic factors in trophoblast cells.

Keywords: Leukemia Inhibitory Factor, Trophoblast, Vascular Endothelial Growth Factor-A

Citation: Jahanbin Kh, Ghafourian M, Rashno M. Effect of different concentrations of leukemia inhibitory factor on gene expression of vascular endothelial growth factor-A in trophoblast tumor cell line. Int J Fertil Steril. 2020; 14(2): 116-121. doi: 10.22074/ijfs.2020.6058.

Introduction

Leukemia inhibitory factor (LIF) is a glycoprotein cytokine with a molecular weight of 38-67 kDa. That is a member of the interleukin 6 family. LIF receptor is a heterodimer composed of two chains, gp130 and leukemia inhibitory factor receptor-β (LIFR-β) expressing on the surface of trophoblast cells (1, 2). LIF induce tyrosine phosphorylation in signal transducers and transcription factors of several trophoblast cell types, like choriocarcinoma cell line (JEG-3) (3, 4). Phosphorylation and signal transduction lead to migration, invasion, stimulation or suppression of various categories of genes in trophoblast cells (5, 6). Janus kinase 1 (JAK-1) and Signal transducer and activator of transcription 3 (STAT-3), play important roles in the signal transduction factors and activation of transcription in the LIF signaling (7, 8). VEGF is a homodimer glycoprotein which can stimulate angiogenesis and vasculogenesis by two types of its receptors like Fms-like tyrosine kinase 1 (Flt1) and kinase insert domain receptor (KDR) (9, 10). VEGF has many roles in early pregnancy, such as oocyte maturation and development, trophoblast proliferation, placenta angiogenesis, embryo implantation, maternal blood vessel growth and development of the embryonic blood vessels (11). Formation of the placenta in uterus depends on differentiation of extravillous cytotrophoblast (EVT) for invasion to the uterine stroma and forming endovascular trophoblast (12, 13). Incorrect differentiation of EVT cells leads to disruption of spiral artery remodeling, and this impairment in spiral artery remodeling can lead to preeclampsia and defective development of the fetus (13). Trophoblast invasion is a localized and temporary process. That is the main factor in the regulation of implantation and supply of oxygen to the fetus. By *VEGF* gene inactivation, invasion and migration of trophoblast cells are reduced (14). VEGF-A is one of the main factors of EVT differentiation to the

Received: 1/August/2019, Accepted: 11/December/2019
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endovascular trophoblast (15). Anti-angiogenic factors that reduce the amount of VEGF-A is one of the factors inhibiting formation of spiral arteries, which eventually associated with the creation of preeclampsia (16). VEGF-A is one of the factors encoded by *VEGF* gene. Studies have shown that among all growth factors encoded by this gene, VEGF-A is the most potent type in stimulating angiogenesis (17). During formation of placenta, EVTs, involving in vascular reconstruction, acquire the features associated with epithelial cells, following the production of VEGF and its receptor expression on the surface (12, 18). These cells migrate to decidua, followed by replacement of the endothelial cells in the spiral arteries to form spiral arteries (19). In this study, a choriocarcinoma cell line JEG-3 (derived from fetal trophoblast tumor) was used as EVTs (20). This cell line has many biological and biochemical features of EVTs (Cells lining the blood vessels of villus in the placenta) (21). This cell line is able to produce progesterone, hCG, steroids and other hormones in the placenta (22). In this study we aimed to evaluate *VEGF* gene expression levels in trophoblast tumor cell line (JEG-3) at different times, while these cells were treated by different concentrations of LIF.

Materials and Methods

This experimental study approved by Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Ethics code: IR.AJUMS.REC.1395.577).

Cell culture and treatment

JEG-3 choriocarcinoma cells were purchased from the Pasteur Institute of Iran (Tehran, Iran). These cells were maintained in Dulbecco's modified Eagle's medium-F12 (DMEM-F12; GIBCO, Ireland) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO, Ireland) along with penicillin (BioIdea, Iran;100 units/ml) and streptomycin (BioIdea; 100µg/ml). All JEG-3 cultures were commenced at 106 cells/175-cm^2 flask and maintained under standardized conditions (37˚C, 5% CO_2, humidified atmosphere). The cells were trypsinized twice a week when confluence was estimated at over 75%. For all assays, JEG-3 cells were adjusted to 10^5 cells/ml. The cells (10^5cell/ml) were seeded in six-well plates, following the resuspension in complete growth media. Before adding the stimuli, the cells were starved for 2 hours in medium without FBS. The cells were cultured per well in the presence and absence of different concentrations (1 ng/ml, 10 ng/ml, 50 ng/ml) (23, 24) of human LIF (Sigma-Aldrich, Germany), while non-stimulated cells were included as controls. Treated and non-treated cells were incubated for 3, 6, 12, 24, 48 and 72 hours at 37°C with 5% CO_2. The cell culture supernatants were then collected by aspiration and centrifugation at 1000 g for 5 minutes and they were stored at -70°C until cytokine analysis. JEG-3 cells were harvested and kept at -70°C until total ribonucleic acid (RNA) extraction.

Ribonucleic acid (RNA) isolation and real-time polymerase chain reaction (PCR) analysis

RNA was isolated using TRI Reagent (SinaClonCo., Iran). According to the manufacturer's protocol, and the purity of extracted RNA was determined by the A260/A280 ratio (A260/A280 ratio was 1.8). 50-100 ng RNA was reverse transcribed using cDNA synthesis kit (SinaClonCo.) and relative changes in *VEGF* mRNA level was quantified by real-time reverse transcription PCR (RT-PCR). Expression level of *VEGF* was determined by quantitative RT-PCR (qRT-PCR) using SYBR Green ® Premix Ex Taq (Takara, Japan) dye detection method on ABI StepOne PCR instrument (Applied Biosystems, USA), compared to *GAPDH* as an internal control. Initial denaturation at 95˚C for 10 minutes, 40 cycles of annealing at 95˚C for 15 seconds and extension at 68˚C for 60 seconds. Rest 2009 and Excel software were used for the analysis of gene expression ratio. Gene-specific primers for *VEGF* and *GAPDH* are summarized in Table1. The fold change for target genes normalized by internal control was determined by the formula $2^{-\Delta\Delta Ct}$. All reactions were run in duplicate.

Statistical analysis

All of the experiments were repeated in triplicates and data were demonstrated as means ± standard error (SE). Statistical software SPSS 25.0 and Graphpad Prism 8.0.1 were used for data analysis. Delta CTs were subjected to one-way ANOVA and a post hoc Tukey's test, while the non-parametric Kruskal-Wallis test was used to compare the results of different experimental days. P values lower than 0.05 were considered statistically significant.

Results

Effects of different concentrations of LIF on VEGF gene expression level This study evaluated the effects of different concentrations of LIF on *VEGF* gene expression in different time periods, compared to untreated cells. The results are described (Fig.1, 2) in more details.

Table 1: Gene specific primers used for RT-PCR

Primer (accession)	Sequence (5'-3')	T_m	Amplicon size
VEGF (NM_001287044.1)	F: AGGAGGAGGGCAGAATCATCA R: CTCGATTGGATGGCAGTAGCT	60	76 bp
GAPDH (NM_002046.5)	F: TGGGCTACACTGAGCACCAG R: CAGCGTCAAAGGTGGAGGAG	60	72 bp

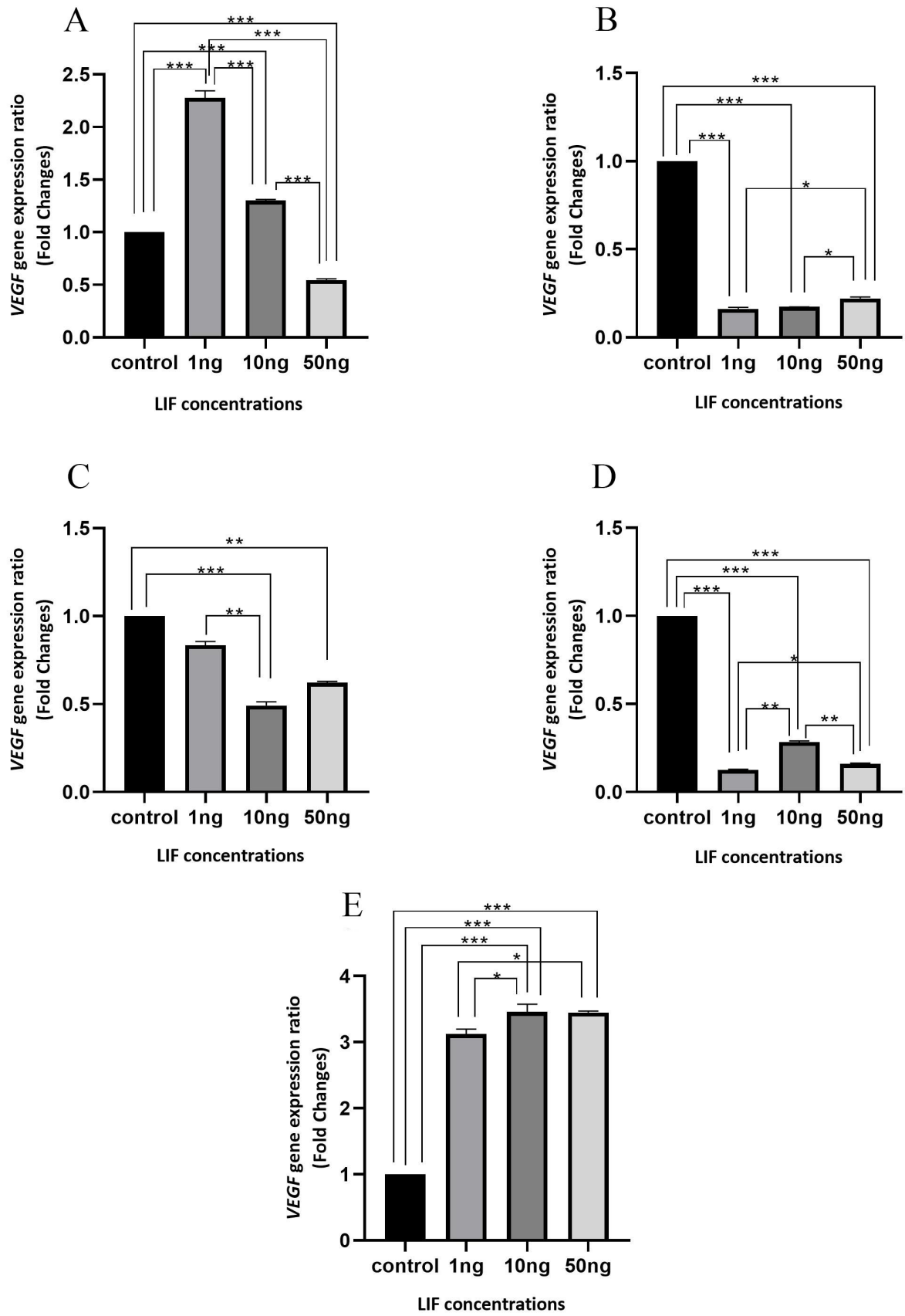

Fig.1: *VEGF* gene expression level at different time points, under treatment with different concentrations of LIF. The effect of different concentrations of LIF (1, 10 and 50 ng) on *VEGF* gene expression after **A.** 6 hours; **B.** 12 hours; **C.** 24 hours; **D.** 48 hours; and E. 72 hours. Cells that did not treated by LIF were considered at any time as control, and the *VEGF* gene expression was measured in treated cells relative to these untreated cells. *; P<0.05, **; P≤0.01, and ***; P≤0.001

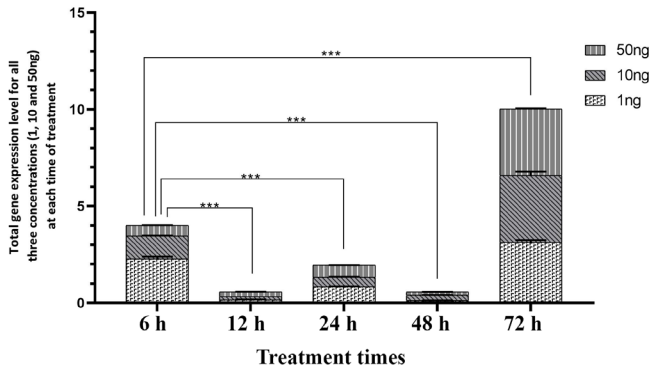

Fig.2: Comparing total *VEGF* gene expression at different time (6, 12, 24, 48 and 72 hours) under the treatment of different concentrations of LIF. *; P<0.05, **; P≤0.01, ***; P≤0.001) , and h; Hours.

Six hours treatment

An analysis of 6 hours data showed that by increasing LIF concentration, level of *VEGF* gene expression was decreased. In this time point, there is a significant difference (P<0.001) between the rate of *VEGF* gene expression in comparison with each other at different concentrations and control (Fig.1A).

Twelve hours treatment

After 12 hours, there was a significant reduction in the *VEGF* gene expression in all three concentrations of LIF treatment than control (P<0.001). The lowest *VEGF* gene expression level was observed at 1 ng concentration of LIF. The results of 10 ng concentration of LIF were almost similar to the 1 ng (the difference between 1 and 10 ng was not significant). At 50 ng concentration of LIF, *VEGF* expression level was higher than the both concentrations of 1 and 10 ng (P<0.05, Fig.1B).

Twenty-four hours treatment

Twenty-four hours after cells treatment with different concentrations of LIF, the results showed lowest expression of the *VEGF* gene at the concentration of 10 ng (P<0.001). Using 10 ng (P<0.001) and 50 ng (P<0.01) concentrations, there was a significant decrease in gene expression compared to control, but at 1 ng concentration, there was no significant decrease in the gene expression (P=0.324). Comparing gene expression between different concentrations of LIF showed a significant difference between the concentration of 1 ng and 10 ng (P=0.004, Fig.1C).

Forty-eight hours treatment

After 48 hours, like 12 and 24 hours, *VEGF* gene expression was decreased by treating with different concentrations of LIF, compared to control (P<0.001), and the lowest gene expression was observed at 1 ng in comparison with 10 ng (P<0.01) and 50 ng (P<0.05). *VEGF* gene expression was more in 10 ng than the other two concentrations (1 and 50 ng) of LIF (P<0.01; Fig. 1D).

Seventy-two hours treatment

After 72 hours, effect of LIF on the *VEGF* gene expression was reversed, and contrary to the previous times, in all three concentrations of LIF, we observed a dramatic increased expression of the *VEGF* gene, in comparison with control (P<0.001; Fig.2). The maximum *VEGF* gene expression was observed at 10 ng of LIF concentration, which had significant difference in comparison with 1 ng concentration of LIF (P<0.05). But, the difference between 50 ng and 10 ng LIF concentrations was not significant (Fig.1E).

VEGF gene expression at different time points

As shown in Figure 3, *VEGF* gene expression was dramatically decreased (P<0.001) at 12, 24, and 48 hours after cell treatment with LIF, in comparison with 6 hours treatment. In contrast to decrease in the *VEGF* gene expression at 12, 24 and 48 hours, we determined a significant increase (P<0.001) in *VEGF* gene expression at 72 hours compared to other time points.

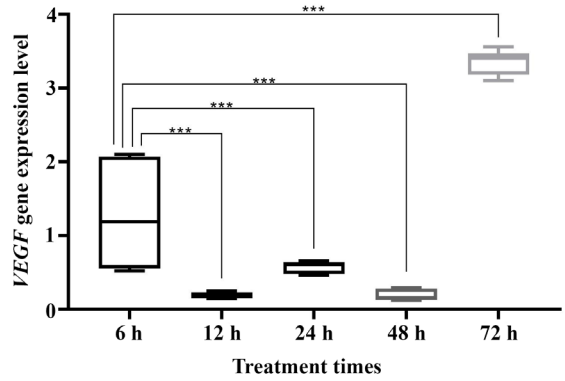

Fig.3: Changes in *VEGF* gene expression at different time points of inducting LIF to the cells. *; P<0.05, **; P≤0.01, ***; P≤0.001.

Discussion

Pregnancy is a complex process that depends on many factors. Studies have shown that cytokines, growth factors and several transcription factors play important roles in embryo implantation. For example, production of LIF by endometrial cells is essential for the beginning of implantation (25, 26). Data obtained from mice and humans have shown that among the all molecules expressed in uterus, LIF plays the most important role in embryo implantation (27). Formation of new blood vessels is called angiogenesis and it accompanies with migration, growth and differentiation of endothelial cells (28). Angiogenesis usually occurs during the menstrual cycle or estrus to convert the ovulation follicles to corpus luteum which leads to the synthesis of progesterone and restructure of the endometrium. This culminates in maintenance of embryo implantation (29). Angiogenesis and vasculogenesis are essential processes for increasing blood flow to the fetus and, consequently, supplying the nutrients and oxygen needed by fetus (13, 30, 31). Several growth factors con-

trol angiogenesis and vasculogenesis during pregnancy. Among these factors, VEGF plays a critical role in the development of the placenta and formation of vesJahanbin sels. Carmeliet et al. (32) showed that deactivation of onlyone VEGF allele leads to fetal death through angiogenesis disruption. Shalaby et al. (33) by disrupting Vascular endothelial growth factor receptor 1 (VEGFR1), Fong et al.(34) by disrupting VEGFR2 and Tsoi et al. (35) by disruption of neuropilin-1 and -2 (all of them are VEGF receptors) determined similar results to Carmeliet et al. (32). Adequate blood supply to the placenta is highly dependent on regulated invasion and trophoblast vascular remodeling in uterus (36, 37). Extravillous trophoblast (EVT) is a subset of trophoblasts that play the most important role in invasion (the same mechanism as cancerous cells for invasion) to the mother's uterus and vascular remodeling. This eventually acquires the phenotype of endothelial cells and improves artery formation (37). Previous studies have shown that EVTs have receptors for VEGF at their surface and message through these receptors which stimulate invasion, switch phenotype to endovascular cells and tube formation in EVT cells (37, 38). Defects in EVT invasion and angiogenesis have been observed in disorders, such as preeclampsia and intrauterine growth restriction (IUGR) (37). Due to the vital role of vascular formation by trophoblast cells (especially EVTs) in pregnancy and implantation, in this study, we decided to investigate the effect of LIF on one of the most important angiogenic factors, VEGF, in EVTs. For this purpose, we had to select an appropriate cell line with similar features to EVT cells. According to the previous studies (20, 39), JEG-3 cell line was selected. The results of this study showed that LIF could have a dual effect on *VEGF* gene expression with respect to time. So that at 12, 24, and 48 hours, *VEGF* gene expression was decreased, while it was increased at 6 and 72 hours (the increase of *VEGF* gene expression at 6 hours depended on the concentration of LIF showing a significant decrease at 50 ng concentration of LIF in contrast to 1 and 10 ng).

Considering the mentioned roles for VEGF during pregnancy and relevant disorders, as well as the important role of LIF during pregnancy, we decided to investigate the effect of LIF on the level of *VEGF* gene expression in trophoblast cells. Regarding to the results, it was found that the expression of the *VEGF* gene in trophoblast tumor cells treated by LIF was reduced in concentration- and time-dependent manners. Although expression of the *VEGF* gene was significantly increased after 72 hours, a study has previously shown that half-life of the LIF attachment to its receptor is slightly more than 24 hours (40). It can be concluded that after 72 hours, interactions between LIFs and their receptors are broken-down and the LIF signaling from their receptors are ended in trophoblast cells. As the results of this study showed. different concentrations of LIF can reduce the rate of *VEGF* gene expression depending on the time. So given the fact that *VEGF* gene expression level was decreased in LIF-treated cells, assessment of the production and secretion of VEGF protein in treated trophoblast cells is vital. Further investigations have to be performed on the other angiogenic factors to clarify the role of LIF on angiogenesis procedure in trophoblast cells.

Conclusion

In conclusion, recent studies have shown that both LIF and VEGF are essential for maintaining and initiating the pregnancy process. It has also been found that angiogenesis process is a critical procedure in embryonic trophoblast cells for a normal pregnancy. VEGF-A is one of the most important angiogenic factors. Therefore, in this study we investigated the effect of LIF on *VEGF* gene expression in JEG-3 cell line as extravillous trophoblast cells. According to the results of this study, LIF causes a significant decrease in gene expression level of VEGF-A in JEG-3 cells. Further studies are needed to determine the action mechanism of LIF in angiogenesis of trophoblast cells.

Acknowledgements

Ahvaz Jundishapur University of Medical Sciences has financially supported this study. This article is the result of thesis for Khodakaram Jahanbin to obtain a master degree in Immunology (Grant number: FIRC-9507). There is no conflict of interest in this study.

Authors' Contributions

M.G., M.R.; Contributed to concept and design and were responsible for overall supervision. K.J.; Contributed to all experimental work, data and statistical analysis, as well as interpretation of data. M.G.; Drafted the manuscript, which was revised by K.J. All authors read and approved the final manuscript.

References

1. Sun X, Bartos A, Whitsett JA, Dey SK. Uterine deletion of Gp130 or Stat3 shows implantation failure with increased estrogenic responses. Mol Endocrinol. 2013; 27(9): 1492-1501.
2. Nicola NA, Babon JJ. Leukemia inhibitory factor (LIF). Cytokine Growth Factor Rev. 2015; 26(5): 533-544.
3. Suman P, Malhotra SS, Gupta SK. LIF-STAT signaling and trophoblast biology. JAKSTAT. 2013; 2(4): e25155.
4. Morales-Prieto DM, Barth E, Murrieta-Coxca JM, Favaro RR, Gutiérrez-Samudio RN, Chaiwangyen W, et al. Identification of miRNAs and associated pathways regulated by Leukemia Inhibitory Factor in trophoblastic cell lines. Placenta. 2019; 88: 20-27.
5. Chen X, Tong C, Li H, Peng W, Li R, Luo X, et al. Dysregulated expression of RPS4Y1 (Ribosomal Protein S4, Y-Linked 1) impairs STAT3 (Signal Transducer and Activator of Transcription 3) signaling to suppress trophoblast cell migration and invasion in preeclampsia. Hypertension. 2018; 71(3): 481-490.
6. Massimiani M, Vecchione L, Piccirilli D, Spitalieri P, Amati F, Salvi S, et al. Epidermal growth factor-like domain 7 promotes migration and invasion of human trophoblast cells through activation of MAPK, PI3K and NOTCH signaling pathways. Mol Hum Reprod. 2015; 21(5): 435-451.
7. Takahashi Y, Takahashi M, Carpino N, Jou ST, Chao JR, Tanaka S, et al. Leukemia inhibitory factor regulates trophoblast giant cell differentiation via Janus kinase 1-signal transducer and activator of transcription 3-suppressor of cytokine signaling 3 pathway. Mol

Endocrinol. 2008; 22(7): 1673-1681.
8. Murphy MJ, Halow NG, Royer PA, Hennebold JD. Leukemia inhibitory factor is necessary for ovulation in female rhesus macaques. Endocrinology. 2016; 157(11): 4378-4387.
9. Xiao Z, Li S, Yu Y, Li M, Chen J, Wang F, et al. VEGF-A regulates sFlt-1 production in trophoblasts through both Flt-1 and KDR receptors. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. Mol Cell Biochem. 2018; 449(1-2): 1-8.
10. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. J Clin Oncol. 2002: 4368-43890.
11. Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M, et al. Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. J Clin Invest. 2004; 114(6): 744-754.
12. Pollheimer J, Vondra S, Baltayeva J, Beristain AG, Knöfler M. Regulation of placental extravillous trophoblasts by the maternal uterine environment. Front Immunol. 2018; 9: 2597.
13. Maynard SE, Karumanchi SA. Angoing factors and preeclampsia. Semin Nephrol. 2011; 31(1): 33-46.
14. Dubinsky V, Poehlmann TG, Suman P, Gentile T, Markert UR, Gutierrez G. Role of regulatory and angiogenic cytokines in invasion of trophoblastic cells. Am J Reprod Immunol. 2010; 63(3): 193-199.
15. Li Y, Zhu H, Klausen C, Peng B, Leung PC. Vascular endothelial growth factor-A (VEGF-A) mediates activin A-induced human trophoblast endothelial-like tube formation. Endocrinology. 2015; 156(11): 4257-4268.
16. Helmo FR, Lopes AMM, Carneiro ACDM, Campos CG, Silva PB, dos Reis Monteiro MLG, et al. Angiogenic and antiangiogenic factors in preeclampsia. Pathol Res Pract. 2018; 214(1): 7-14.
17. Arutyunyan I, Fatkhudinov T, Kananykhina E, Usman N, Elchaninov A, Makarov A, et al. Role of VEGF-A in angiogenesis promoted by umbilical cord-derived mesenchymal stromal/stem cells: in vitro study. Stem Cell Res Ther. 2016; 7: 46.
18. Babischkin JS, Aberdeen GW, Lindner JR, Bonagura TW, Pepe GJ, Albrecht ED. Vascular endothelial growth factor delivery to placental basal plate promotes uterine artery remodeling in the primate. Endocrinology. 2019; 160(6): 1492-1505.
19. Tessier DR, Yockell-Lelièvre J, Gruslin A. Uterine spiral artery remodeling: the role of uterine natural killer cells and extravillous trophoblasts in normal and high-risk human pregnancies. Am J Reprod Immunol. 2015; 74(1): 1-11.
20. Olivares EG, Muñoz R, Tejerizo G, Montes MJ, Gómez-Molina F, Abadía-Molina AC. Decidual lymphocytes of human spontaneous abortions induce apoptosis but not necrosis in JEG-3 extravillous trophoblast cells. Biol Reprod. 2002; 67(4): 1211-1217.
21. Drwal E, Rak A, Gregoraszczuk EL. Differential effects of ambient PAH mixtures on cellular and steroidogenic properties of placental JEG-3 and BeWo cells. Reprod Toxicol. 2019; 86: 14-22.
22. Hu W, Gao F, Zhang H, Hiromori Y, Arakawa S, Nagase H, et al. Activation of peroxisome proliferator-activated receptor gamma and disruption of progesterone synthesis of 2-ethylhexyl diphenyl phosphate in human placental choriocarcinoma cells: comparison with triphenyl phosphate. Environ Sci Technol. 2017; 51(7): 4061-4068.
23. Fitzgerald JS, Tsareva SA, Poehlmann TG, Berod L, Meissner A, Corvinus FM, et al. Leukemia inhibitory factor triggers activation of signal transducer and activator of transcription 3, proliferation, invasiveness, and altered protease expression in choriocarcinoma cells. Int J Biochem Cell Biol. 2005; 37(11): 2284-2296.
24. Suman P, Gupta SK. STAT 3 and ERK 1/2 cross-talk in leukaemia inhibitory factor mediated trophoblastic JEG-3 cell invasion and expression of Mucin 1 and Fos. Am J Reprod Immunol. 2014; 72(1): 65-74.
25. Cheng J, Rosario G, Cohen TV, Hu J, Stewart CL. Tissue-specific ablation of the LIF receptor in the murine uterine epithelium results in implantation failure. Endorinology. 2017; 158(6): 1916-1928.
26. Bulun SE. Steroids, cytokines, and implantation. Endocrinology. 2017; 158(6): 1575-1576.
27. Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M, et al. Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. J Clin Invest. 2004; 114(6): 744-754.
28. Chun CZ, Sood R, Ramchandran R. Vasculogenesis and angiogenesis. In: North PE, Sander T, editors. Vascular tumors and developmental malformations. New York: Springer; 2016; 77-99.
29. Tamanini C, De Ambrogi M. Angiogenesis in developing follicle and corpus luteum. Reprod Domest Anim. 2004; 39(4): 206-216.
30. Lang U, Baker RS, Braems G, Zygmunt M, Künzel W, Clark KE. Uterine blood flow-a determinant of fetal growth. Eur J Obstet Gynecol Reprod Biol. 2003; 110(Suppl 1): S55-S61.
31. Tal R, Dong D, Shaikh S, Mamillapalli R, Taylor HSJBor. Bone-marrow-derived endothelial progenitor cells contribute to vasculogenesis of pregnant mouse uterus. Biol Reprod. 2019; 100(5): 1228-1237.
32. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 1996; 380(6573): 435-439.
33. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995; 376(6535): 62-66.
34. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature. 1995; 376(6535): 66-70.
35. Tsoi SC, Wen Y, Chung JY, Chen D, Magness RR, Zheng J. Co-expression of vascular endothelial growth factor and neuropilin-1 in ovine feto-placental artery endothelial cells. Mol Cell Endocrinol. 2002; 196(1-2): 95-106.
36. Burton GJ, Woods AW, Jauniaux E, Kingdom JC. Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy. Placenta. 2009; 30(6): 473-482.
37. Lala PK, Nandi P. Mechanisms of trophoblast migration, endometrial angiogenesis in preeclampsia: The role of decorin. Cell Adh Migr. 2016; 10(1-2): 111-125.
38. Lala N, Girish GV, Cloutier-Bosworth A, Lala PK. Mechanisms in decorin regulation of vascular endothelial growth factor-induced human trophoblast migration and acquisition of endothelial phenotype. Biol Reprod. 2012; 87(3): 59.
39. Zygmunt M, Hahn D, Münstedt K, Bischof P, Lang U. Invasion of cytotrophoblastic JEG-3 cells is stimulated by hCG in vitro. Placenta. 1998; 19(8): 587-593.
40. Hilton DJ, Nicola NA. Kinetic analyses of the binding of leukemia inhibitory factor to receptor on cells and membranes and in detergent solution. J Biol Chem. 1992; 267(15): 10238-10247.

Original Article

Phosphodiesterase 8B Polymorphism rs4704397 Is Associated with Infertility in Subclinical Hypothyroid Females: A Case-Control Study

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Abstract

Background: Subclinical hypothyroidism (SCH) remains largely unnoticed as a major cause of infertility due to asymptomatic. Polymorphisms of phosphodiesterase 8B gene (*PDE8B*) have been linked with various diseases, including female infertility. Hence, we aimed to study prevalence of SCH, in infertile females, explore association of *PDE8B* rs4704397 A/G and rs6885099 G/A polymorphisms with infertility in females suffering from SCH and genotype-phenotype correlation of the polymorphisms with thyroid stimulating hormone (TSH) levels in Gujarat population.

Materials and Methods: In this retrospective study, TSH level was estimated from plasma of 230 infertile and 100 control females by enzyme-linked fluorescence immunoassay (ELFA) to find out the prevalence of SCH. Further, based on TSH levels, thyroid function test (TFT) was performed in controls and infertile females with subclinical hypothyroidism (IF-SCH). *PDE8B* rs4704397 and rs6885099 polymorphisms were genotyped by PCR-RFLP and ARMS-PCR, respectively in 74 controls and 60 IF-SCH females.

Results: We observed i. significantly high prevalence of SCH (32%) in the infertile females, ii. significantly lower frequency of 'G' allele (P=0.006), while the frequency of 'A' allele (P<0.0001) was higher in IF-SCH females, compared to the controls, for rs4704397 A/G SNP, iii. no significant difference in the genotype (P=0.214; OR=2.51; CI=0.74–8.42) and the allele frequency (P=0.129; OR=1.51; CI=0.92-2.47) of rs6885099 G/A SNP, iv) low linkage disequilibrium for the polymorphisms, v. significantly higher frequency of 'AA' haplotype (P=0.0001; OR=3.84; CI=1.86-8.01),while the 'GG' haplotype (P=0.0023; OR=0.33; CI=0.16-0.69) was significantly lower in IF-SCH females and vi. no significant difference in the TSH level of IF-SCH females with respect to the genotypes.

Conclusion: The present study reports an association of *PDE8B* rs4704397 polymorphism with infertility in SCH females. The study categorically shows a higher prevalence of SCH in infertile females of Gujarat and advocates the importance of screening for SCH in infertility management.

Keywords: Genetic Polymorphisms, Infertility, Thyroid

Citation: Mansuri T, Jadeja Sh.D, Singh M, Begum R, Robin P. Phosphodiesterase 8B polymorphism rs4704397 is associated with infertility in subclinical hypothyroid females: a case-control study. Int J Fertil Steril. 2020; 14(2): 122-129. doi: 10.22074/ijfs.2020.6015.

Introduction

Apart from its multiple functions, thyroid hormones play crucial role in reproduction. Hence, altered thyroid hormone levels can greatly affect reproductive function (1). Thyroid diseases in women with reproductive age are very common due to the complex interplay of various hormones (2). Abnormal thyroid functions of hyper or hypothyroidisms are symptomatic and they may have an adverse effect on the reproductive health contributing to infertility (3-4). However, subclinical hypothyroidism (SCH) is silent and hence it is often undiagnosed. It is a common thyroid disorder often found to coexist with various other morbidities. It is an asymptomatic condition where the patient has a normal serum free T_4 (fT_4/thyroxin) levels, but high thyroid stimulating hormone/thyrotropin (TSH) levels (5). TSH is considered as a sensitive indicator of the thyroid status and SCH. Normal TSH levels in serum are finely regulated in humans. Nevertheless, serum thyroid parameters show substantial inter- individual variability (6), in which genetic variations are proved as the major factors in several populations. It has been shown that altered TSH levels are related to genetic factors in up to 65% of the cases (7-9).

Received: 14/June/2019, Accepted: 22/December/2019
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Different cohort studies reported phosphodiesterase 8B (*PDE8B*) as a genetic modulator of TSH levels. PDE8B gene encodes a cyclic adenosine monophosphate (cAMP) specifi c phosphodiesterase (PDE) enzyme (10). *PDE8B* affects cAMP levels in the thyroid gland resulting in changes in the levels of thyroid hormones, which in turn affects the release of TSH from the pituitary gland. *PDE8B* is mainly expressed in thyroid and brain (11, 12). Several single nucleotide polymorphisms (SNPs) for *PDE8B* have been demonstrated to associate with increased levels of serum TSH. More than 360,000 SNPs were tested for their associations with serum TSH levels with an additive model. The obtained results revealed three SNPs (i.e. rs4704397, rs6885099 and rs2046045) with genome-wide significance ($P<10^{-10}$). These three SNPs were reported to be in strong linkage disequilibrium. Of the three SNPs, rs4704397 showed strongest association and it could explain 2.3% of the variations in TSH levels (13). *PDE8B* rs4704397 polymorphism has been found to associate with myocardial infarction, height (14), pregnancy (15, 16), recurrent miscarriage (17) and obesity in children (18), apart from thyroid function. Another *PDE8B* polymorphism, rs6885099 has also been shown to increase TSH levels, but to a lesser extent, in different populations (13). The relevance of human reproduction to PDE has been well-documented (19-22). While the underlying mechanism regulating oocyte maturation is not clearly known yet, the second messenger cyclic adenosine monophosphate (cAMP) role in oocyte maturation is well known (23) and thus research investigating the role of rs4704397 in the oocyte maturation might give an insight to primary infertility caused by hypothyroidism.

Numerous studies have reported the importance of screening for SCH, and the worldwide prevalence of SCH in infertile-females has been reported to be as high as 26.7% in various populations (24-27). In India, prevalence of SCH is high and reported to be 25% (28-33). However, there is no study on the status of SCH per se or its prevalence amongst infertile females in western part of India. Furthermore, there is no report on the role of PDE8B polymorphisms in female infertility. We therefore, aimed to estimate the prevalence of SCH in infertile females and explore association of *PDE8B* rs4704397 and rs6885099 polymorphisms in infertile females of Gujarat population.

Materials and Methods

Study subjects

The present retrospective study is a matched, case-control study. Two hundered and therty infertile females were recruited from Dr. Mahesh Pandya's Ghanshyam Clinic (a fertility management center; Vadodara, India) along with 100 control females recruited from various health check-up camps. Random sampling method was followed for selection of the groups. The study protocol was explained and informed consent was obtained from all participants of the study. Seventy four out of 230 infertile females were found to have (IF) for the TSH level with the inclusion criteria of primary infertility diagnosis and duration of more than one year of unprotected intercourse without pregnancy, while 76 out of 100 controls were found to be euthyroid (with normal thyroid hormone levels). Exclusion criteria were male factor infertility, any tubal anomaly congenital or urogenital tract anomaly and history of thyroid disease/medication/surgery.

For this study, IF-SCH females/case group are defined as the infertile females who have subclinical hypothyroidism with no other clinical difficulty. In addition, they should not be under any type of medication, including thyroid disorder. Whereas, the control group includes fertile, perous, healthy euthyroid females with no medical history for thyroid or any other disorder. Control group does not include any subclinical hypothyroid female.

Sample size for the present study was calculated using G-Power software with Alpha 0.05 and effect size of 0.9. The effect size was calculated based on the observed genotype frequencies (34).

Thyroid function test

Five ml blood samples was collected by venous puncture from fasting individuals and serum was separated for thyroid function test (TFT). Estimation of serum TSH, free T_3 (fT_3) and fT_4 were carried out by enzyme-linked fluorescence immunoassay (ELFA) on mini VIDAS® immuno-analyzer (BioMérieux India Pvt. Ltd., India). Females having TSH values between 3.5 and 10 μIU/ml with normal fT_4, along with an opinion from gynecologist and endocrinologist were considered as IF-SCH females. Fertile females having TSH values within the normal/euthyroid range (i.e. 0.35-3.5 μIU/ml) and fT4 levels within the normal range were included as controls in the present study. The reference range for serum thyroid hormones (fT_3 and fT_4) and TSH levels for different conditions are shown in Table S 1(See Supplementary Online Information at www.celljournal.org). The confounding variables such as age, body mass index (BMI), smoking and hemoglobin (Hb) levels showed no significant difference between control and IF-SCH females (Table S2, See Supplementary Online Information at www.celljournal.org).

Genotyping *PDE8B* rs4704397 and rs6885099 polymorphisms

DNA was extracted from peripheral blood mononuclear cells (PBMCs) using 'IAamp DNA Blood Kit (QIAGEN Inc., USA) as per manufacturer's instructions. *PDE8B* rs4704397 A/G genotyping was done by polymerase chain reaction-restriction fragment length polymorphism

(PCR-RFLP) while PDE8B rs6885099 (G/A) genotyping was done by amplification refractory mutation system (ARMS)-PCR. Amplification was performed using Mastercycler Gradient PCR (Eppendorf, Germany) according to the following protocol: initial denaturation at 94°C for 10 minutes, followed by 30 cycles of denaturation at 94°Cfor 45 seconds, annealing at 60°C for 45 seconds and 72°C for 1 minute. The amplified products were analyzed by electrophoresis in a 2.0% agarose gel stained with ethidium bromide. The respective primers and restriction enzyme (RE) used for genotyping are shown in Table S3. 15 µl of the amplified products was digested for 16 hours at 37°C, using 1 U restriction enzyme. For PCR-RFLP based genotyping, the digested products (300 bp and 219 bp) with 100 bp DNA ladder (Bioron, Germany) were loaded in 3.5% agarose gels stained with ethidium bromide and visualized under UV transilluminator. Furthermore, genotyping of PDE8B rs6885099 G/A was done by Amplification refractory mutation system (ARMS-PCR) in 60 IF-SCH females and 76 control females. Human growth hormone (HGH) was used, as a reaction control in the ARMS-PCR (35). Amplification was performed using Mastercycler Gradient PCR according to the following protocol: initial denaturation at 94°C for 10 minutes, followed by 35 cycles of 94°C for 30 seconds, primer dependent annealing for 30 seconds and 60°C for 1 minute. The amplified products were analyzed by electrophoresis in a 3.5% agarose gel stained with ethidium bromide using 100 bp DNA ladder.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) test was evaluated for the polymorphisms using chi-square test equating the observed and expected genotype frequencies. The genotype and allele risk associations were calculated by chi-square test using Prism 5 software (GraphPad Software Inc, USA; 2007). For genetic analysis, Bonferroni's correction was applied and statistical significance was considered at P-value less than 0.025. The linkage disequilibrium (LD) and haplotype analysis were carried out using http://analysis.bio-x.cn/myAnalysis.php (36). Levels of TSH and thyroid hormones were analyzed by non-parametric unpaired t-test and one-way ANOVA using Prism 5 software (GraphPad Software Inc.; 2007).

In-silico analysis

Web-based in-silico prediction tool HaploReg v4.1 (https://www.pubs.broadinstitute.org/mammals/haploreg/haploreg.php) was employed to predict the effect of non-coding rs4704397 polymorphism. Tissue specific effect of rs4704397 was assessed by an eQTL database-GTeX portal (https://www.gtexportal.org).

Ethical consideration

It was ensured that the study design complies with the ethical standards of the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda,Vadodara, Gujarat, India (FS/IECHR/BC/PR/1) and with the 1964 Hel inki declaration.

Results

Estimation of thyroid stimulating hormone, free T3 and free T4 levels

Analysis of TSH, fT3 and fT4 levels in the studied subjects revealed that among 230 females with primary infertility, 58% (n=133) were euthyroid, 32% (n=74) were SCH, 6% (n=14) were overt hypothyroid and the rest 4% (n=9) females were hyperthyroidism (Fig.1 A, Table S3) (See Supplementary Online Information at www. celljournal.org). IF-SCH females had significantly higher (P<0.0001; Fig.1B) TSH levels (mean ± SEM: 5.34 ± 0.21 µIU/ml) compared to the control females (mean ± SEM: 1.91 ± 0.08 µIU/ml) and they had no significant difference in fT3 levels (P=0.1159, mean ± SEM: 3.036 ± 0.0462pg/ml; Fig.1C) compared to the controls (mean ± SEM: 2.935 ± 0.0436). There was no significant difference between fT4 levels (P=0.0741, mean ± SEM: 1.22 ± 0.0249) in IF-SCH females compared to controls (mean ± SEM: 1.195±0.0318 ng/dl).

PDE8B rs4704397 SNP in infertile females with subclinical hypothyroidism females

Genotyping *PDE8B* rs4704397 polymorphism was carried out in 60 IF-SCH females and 76 healthy fertile females (Fig.2A). Other variables such as age (P=0.419), BMI (P=0.309), smokers (0%) and Hb (P=0.117) levels were not significantly different between the subjects of each genotypes (Table S4). The observed genotype frequencies of *PDE8B* rs4704397 SNP in IF-SCH females were slightly deviated from HWE (P=0.049; Table 1), whereas the control population was under HWE (P=0.062; Table 1). Ancestral allele 'A' and genotype 'AA' were considered as the reference allele and genotype respectively. The frequency of AG and GG genotypes were significantly lower in IF-SCH females, compared to controls (P=<0.0001 and P=0.006 respectively; Table 1). The frequency of 'G' allele was also significantly lower in IFSCH females, compared to the control females (23% vs. 47%, P<0.0001, OR=0.34). Hence, "G" allele was identified to have a protective effect and 'A' allele was identified as the risk allele for SCH and infertility in females.

PDE8B rs6885099 SNP in infertile females with subclinical hypothyroidism

Genotyping of *PDE8B* rs6885099 polymorphism was carried out in 60 IF-SCH and 76 control females (Fig.2B). The observed genotype frequencies of *PDE8B* rs6885099 polymorphism among the control and IF-SCH females were in accordance with HWE (P=0.248 and P=0.134 respectively; Table 2). Distribution of genotype as well as allele frequencies revealed no significant difference among the IF-SCH and control females (Table 2).

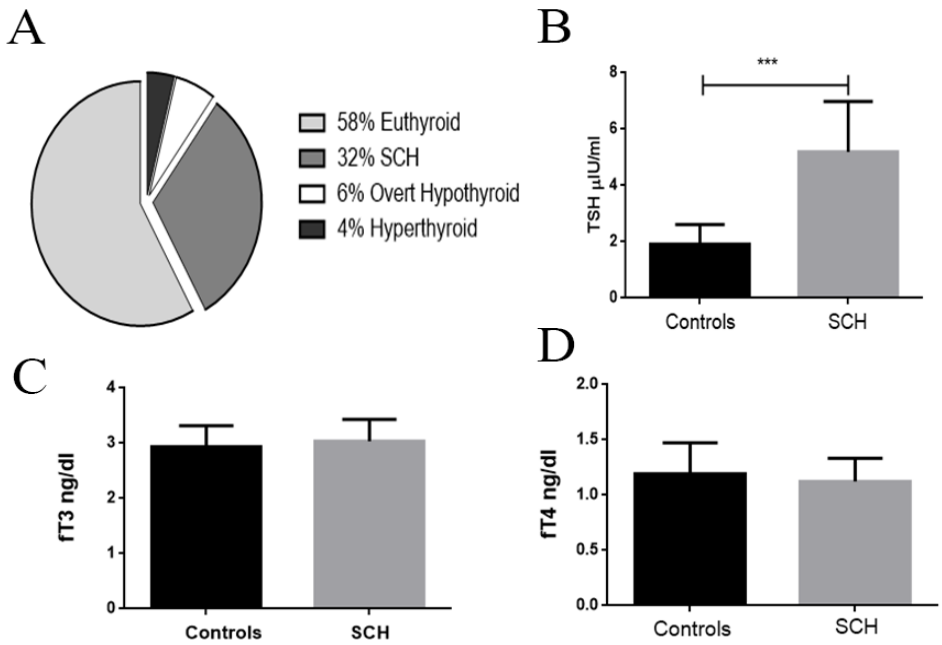

Fig.1: Estimation of TSH and thyroid hormone levels. **A.** Prevalence of thyroid dysfunction among the infertile females. **B.** TSH level in controls and IF-SCH females. **C.** fT_3 levels in the controls and IF-SCH females. **D.** fT_4 levels in controls and IF-SCH females. TSH; Thyroid stimulating hormone, IF-SCH; Infertile females with subclinical hypothyroidism, fT_3; Free T_3, fT_4; and Free T_4.

Table 1: Distribution of genotype and allele frequencies for PDE8B rs4704397 A/G polymorphism

Genotype or allele	IF-SCH females (Freq. %)	Control females (Freq. %)	P value	Odds Ratio	95% CI	P value HWE
Genotype	n= 60	n=76				
AA	38 (63%)	17 (22%)	R	1	0.07-0.35	0.062 (C)
AG	16 (27%)	46 (61%)	<0.0001[a]	0.16	0.07-0.63	
GG	06 (10%)	13 (17%)	0.006[a]	0.21		0.049 (P)
Allele						
A	92 (77%)	80 (53%)	R	1	-	
G	28 (23%)	72 (47%)	<0.0001[b]	0.34	0.19-0.57	

n; number of IF-SCH females/control females, R; reference group, Freq.; Frequency, CI; Confidence interval, P; IF-SCH females, C; Control females, [a] IF-SCH female vs. control females (genotype) using chi-squared test with 2×2 contingency table, and [b] IF-SCH females vs. control females (allele) using chi-squared test with 2×2 contingency table, and IF-SCH; Infertile females with subclinical hypothyroidism.

Table 2: Distribution of genotypes and alleles for PDE8B rs6885099 G/A polymorphism

Genotype or allele	IF-SCH females (Freq. %)	Control females (Freq. %)	P value	Odds Ratio	95% CI	P value HWE
Genotype	n= 60	n=76				
GG	17 (28%)	32 (42%)	R	1	-	-
GA	35(58%)	38 (50%)	0.1914[a]	1.73	0.82-3.65	0.248 (C)
AA	08 (13%)	06 (8%)	0.2145[a]	2.51	0.74-8.42	
Allele						0.134 (P)
A	69 (58%)	102 (67%)	R	1	-	
G	51 (42%)	50 (33%)	0.1292[b]	1.51	0.92-2.47	

F-SCH; Infertile females with subclinical hypothyroidism; n; number of IF-SCH females/Control females, R; reference group, Freq.; Frequency, CI; Confidence interval, P; IF-SCH females and C; Control females, [a] IF-SCH female vs. control females (genotype) using chi-squared test with 2×2 contingency table, and [b] IF-SCH females vs. control females (allele) using chisquared test with 2×2 contingency table.

Table 3: Distribution of haplotype frequencies for PDE8B rs4704397 and rs6885099 polymorphisms

Haplotype [rs4704397(A/G): rs6885099 (G/A)]	IF-SCH Female Freq. (%)	Control females Freq. (%)	P value for association	P value (Global)	Odds Ratio [95% CI]
AG	48 (46%)	49 (21%)	0.4434		1.230 [0.72-2.09]
AA	31 (30%)	12 (10%)	0.0001	7.5×10^{-5}	3.84 [1.86-8.01]
GG	12 (12%)	34 (28%)	0.0023		0.33 [0.160-0.69]
GA	13 (12%)	25 (21)	0.0876		0.53 [0.25-11.10]

Freq.; Frequency, CI; Confidence interval (Frequency <0.03 in both control and case has been dropped and it was ignored in the analysis), and IF-SCH; Infertile females with subclinical hypothyroidism

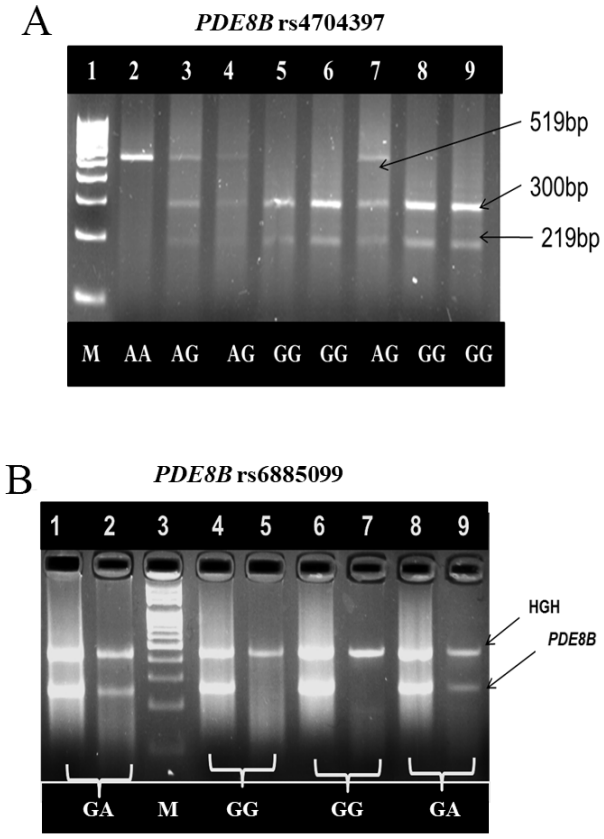

Fig.2: Representative gel images for PDE8B rs4704397 and rs6885099 genotyping. **A.** PCR-RFLP analysis of *PDE8B* rs4704397 SNP on 3.5% agarose gel. Lane 1 shows 100 bp ladder, lane 2 shows homozygous (AA) genotype, lanes 3, 4 and 7 show heterozygous (AG) genotypes, lanes 5, 6, 8 and 9 show heterozygous (GG) genotypes. **B.** ARMS-PCR analysis of *PDE8B* rs6885099 SNP on 3.5% agarose gel. Lanes 1 and 2 show homozygous (GA); lane 4, 5, 6 and 7 show homozygous (GG) genotypes and lane 3 shows 100 bp ladder, lanes 8 and 9 show heterozygous (GA) genotypes. PCR-RFLP; Polymerase chain reaction-restriction fragment length polymorphism.

Linkage disequilibrium and haplotype analysis

Linkage disequilibrium (LD) analysis revealed that two investigated PDE8B polymorphisms (i.e. rs4704397 and rs6885099) were in low LD association (D'=0.060, r2=0.003). Haplotype analysis revealed that the frequency of 'AA' haplotype was significantly higher in the patients and risk of IF-SCH females was increased by 3.84 fold (P=0.0001, OR=3.84; CI=1.86-8.01; Table 3). The frequency of 'GG' haplotype was significantly lower in IF-SCH females, compared to the controls suggesting its protective effect (P=0.0023, OR=0.33; CI=0.16-0.69; Table 3).

Genotype-phenotype correlation analysis

TSH levels in IF-SCH females were analyzed with respect to the genotypes of *PDE8B* rs4704397 A/G and rs6885099 G/A. No significant difference in TSH levels was observed with respect to genotypes of the both SNPs (Fig.3).

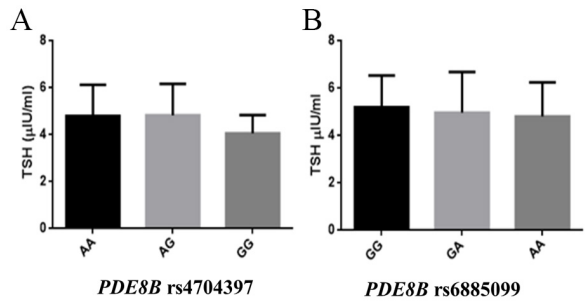

Fig. 3: Correlation of *PDE8B* rs4704397 and rs6885099 with TSH levels in IF-SCH females. No significant difference of TSH levels was observed with respect to PDE8B polymorphisms **A.** rs4704397 and **B.** rs6885099. TSH; Thyroid stimulating hormone, IF-SCH; Infertile females with subclinical hypothyroidism

In-silico analysis

Analysis of functional consequences of PDE8B rs4704397 by HaploReg v4.1 predicted that PDE8B rs4704397 could alter heat shock factor-type (HSF) motif and enhancer state by H3K27 acetylation (H3K27ac) in inferior temporal lobe of brain (https://www.pubs.broadinstitute.org/mammals/haploreg/detail_v4.1.php?query=&id=rs4704397).eQTL database GTEx portal showed significantly elevated PDE8B transcripts in thyroid tissue of individuals carrying 'A' allele, compared to 'G' allele (https://www.gtexportal.org/home/snp/rs4704397).

Discussion

The present study shows a high prevalence rate of SCH in infertile females (32%) in comparison with the healthy controls (Table S1) and the association of rs4704397 SNP with infertility in IF-SCH females of Gujarat region. In developing countries, one among four couples suffers from infertility and in these couples, hypothyroidism is one of the key perpetrators. In a study performed by Verma et al. (28), out of 394 infertile women, 23.9% were hypothyroid (TSH>4.2 μIU/ml). An intervention to rectify the hypothyroidism resulted in 76.6% of the conceived infertile women. Primary health caregivers most often pick up overt hypothyroidism easily; however, SCH with its subtle symptoms most often goes unnoticed. The prevalence of SCH amongst infertile females is common, but there is a scarcity on available data. However, there are a few studies reporting the prevalence of hypothyroidism, ranging from 15-25% in Indian population (28-33). As SCH is largely asymptomatic, it goes undiagnosed, resulting in infertility. It is essential to include evaluation of thyroid related hormones as a standard practice along with other tests to ascertain the causes of infertility.

SCH occurs due to multiple factors. Some of them include congenital agenesis, defect in synthesis due to iodine deficiency or anti-thyroid drugs, autoimmune diseases, post-surgery, hypopituitarism, TSH deficiency, environmental pollutants, mutations and SNPs (37). Of these factors, the present study focuses on the SNPs. To evaluate possible correlation between the polymorphisms associated with increased TSH levels and infertility, two SNPs (rs4704397 and rs6885099) of the *PDE8B* were studied in healthy controls and IF-SCH females. Higher frequency of the "A" allele for *PDE8B* rs4704397 polymorphism in SCH related infertile patients which revealed "A" as a risk allele for infertility in IF-SCH females. However, PDE8B rs6885099 was not associated with infertility. Earlier, *PDE8B* rs4704397 was also found to associate with recurrent miscarriage (17). PDE8B is found in the thyroid but not pituitary. In addition, given the importance of cAMP activity in TSH signaling, it is suggested that the *PDE8B* rs4704397 polymorphism could reduce cAMP levels in the thyroid resulting in a decreased response of thyroid gland to TSH stimulation, which leads to an increase of TSH set point for the same free T3 and T4 levels (18). Polymorphism in *PDE8B*, rs4704397 results in an increase in PDE8B enzyme expression. We propose that this could result in a faster degradation of cAMP, which decreases the synthesis and release of T3 and T4. In such a scenario, the negative inhibition of Thyrotropin-releasing hormone (TRH) will not take place and this will result in increased levels of TRH and hence TSH. As a consequence, T3 and T4 levels become normal. The increased level of TSH results in development of SCH. PDE8B rs4704397 polymorphism might induce phosphodiesterase activity in PDE8B, thereby reducing the ability of thyroid gland to generate free T4 when stimulated by TSH. This results in SCH, which can be the cause of infertility in IF-SCH patients. Arnaud et al. in a GWAS study reported that *PDE8B* rs4704397 could affect plasma TSH levels. Each copy of the minor allele "A" may lead to a mean increase of 0.13 mU/l TSH levels (13). However, we did not observe significant correlation of the *PDE8B* rs4704397 SNP with circulating TSH levels. This might be due to the limited sample size in the present study. *PDE8B* rs4704397 SNP was also found to be associated with various conditions like cardiovascular, body height, pregnancy, recurrent miscarriage, obesity in children, etc. (14-18). Though the exact underlying mechanism of *PDE8B* rs4704397 SNP affecting TSH levels is not clear, in-silico tools predicted that this variation might lead to enhancement ofPDE8B expression by influencing epigenetic level. The role of PDE8B in human placenta and ovaries is still to be understood, while human reproduction relevance to PDE has been proposed (19-22). The underlying mechanism of regulating oocyte maturation is not clearly documented yet, but the second messenger cAMP role in oocyte maturation is well known (23). Thus, investigating the role of rs4704397 in the oocyte maturation could be an interesting area of research as far as female infertility is concerned.

On the other hand, medications given to alter the levels of reproductive hormones have serious repercussions on the health of females with long-term implications (38). Treatment of infertility is usually done by direct targeting the reproductive system, instead of looking for the involvement of other factors, such as genetic polymorphisms, as a cause of infertility. This genetic approach could be used to identify IF-SCH patients and treat infertility with greater success and fewer side-effects without disturbing the reproductive system. Since, small sample size was a limiting factor for the present study, we suggest investigating larger number of infertile females in different populations. This might provide a significant insight into understanding the role of *PDE8B* in infertility.

Conclusion

The present study establishes an association of *PDE8B rs4704397* with infertility in IF-SCH females and reiterates the importance of screening SCH, as a diagnostic tool in infertility management.

Acknowledgements

We thank all the subjects for their participation in the study. We thank Dr. Mahesh Pandya, Dr. Jigisha Pandya and Dr. Jignesh Pandya of Ghanshyam clinic (A Fertility management center), Vadodara, India for their wholehearted support and permission to carry out counseling

and blood collection for recruitment of the subjects. This work financially was supported by a grant to Tabassum Mansuri from the Department of Science and Technology, Government of India, New Delhi under Women Scientist Scheme-A (SR /LS-117 /2012). The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Authors' Contributions

T.M., P.R. R.B.; Contributed to conception and design. T.M.; Contributed to all experimental works and drafted the manuscript. T.M., S.D.J. and M.S.; Contributed to data collection, statistical analysis and interpretation of data. R.B. P.R.; Were responsible for overall supervision. All authors read and approved the final manuscript.

References

1. Jefferys A, Vanderpump M, Yasmin E. Thyroid dysfunction and reproductive health. Obstet Gynaecol. 2015; 17(1): 39-45.
2. Silva JF, Ocarino NM, Serakides R. Thyroid hormones and female reproduction. Biol Reprod. 2018; 99(5): 907-921.
3. Weiss RV, Clapauch R. Female infertility of endocrine origin. Arq Bras Endocrinol Metab. 2014; 58(2): 144-152.
4. Saran S, Gupta BS, Philip R, Singh KS, Bende SA, Agroiya P, et al. Effect of hypothyroidism on female reproductive hormones. Indian J Endocrinol Metab. 2016; 20(1): 108-113.
5. Stavreus Evers A. Paracrine interactions of thyroid hormones and thyroid stimulation hormone in the female reproductive tract have an impact on female fertility. Front Endocrinol (Lausanne). 2012; 3: 50.
6. Practice Committee of the American Society for Reproductive Medicine. Subclinical hypothyroidism in the infertile female population: a guideline. Fertil Steril. 2015; 104(3): 545-553.
7. Bernadette Biondi. The Normal TSH reference range: what has changed in the last decade? J Clin Endocrinol Metab. 2013; 98(9): 3584-3587.
8. Panicker V. Genetics of thyroid function and disease. Clinical Biochem Rev. 2011; 32(4): 165-175.
9. Malinowski JR, Denny JC, Bielinski SJ, Basford MA, Bradford Y, Peissig PL, et al. Genetic variants associated with serum thyroid stimulating hormone (TSH) levels in European Americans and African Americans from the eMERGE Network. PLoS One. 2014; 9(12): e111301.
10. Medici M, Visser WE, Visser TJ, Peeters RP. Genetic determination of the hypothalamic-pituitary-thyroid axis: where do we stand? Endocr Rev. 2015; 36(2): 214-244.
11. Vezzosi D, Bertherat J. Phosphodiesterases in endocrine physiology and disease. European journal of endocrinology. Eur J Endocrinol. 2011; 165(2): 177-188.
12. Lakics V, Karran EH, Boess FG. Quantitative comparison of phosphodiesterase mRNA distribution in human brain and peripheral tissues. Neuropharmacology. 2010; 59(6): 367-374.
13. Arnaud-Lopez L, Usala G, Ceresini G, Mitchell BD, Pilia MG, Piras MG, et al. Phosphodiesterase 8B gene variants are associated with serum TSH levels and thyroid function. Am J Hum Genet. 2008; 82(6): 1270-1280.
14. Jorde R, Schirmer H, Wilsgaard T, Joakimsen RM, Mathiesen EB, Njølstad I, et al. The phosphodiesterase 8B gene rs4704397 is associated with thyroid function, risk of myocardial infarction, and body height: the Tromsø study. Thyroid. 2014; 24(2): 215-222.
15. Shields BM, Freathy RM, Knight BA, Hill A, Weedon MN, Frayling TM, et al. Phosphodiesterase 8B gene polymorphism is associated with subclinical hypothyroidism in pregnancy. J Clin Endocrinol Metab. 2009; 94(11): 4608-4612
16. Yang S, Tao J, Zhang J, Fan J, Qian W, Shu K. Genetic association study of phosphodiesterase 8B gene with subclinical hypothyroidism in pregnant women. Endocrine Res. 2015; 40(4): 199-203.
17. Granfors M, Karypidis H, Hosseini F, Skjöldebrand-Sparre L, Stavreus-Evers A, Bremme K, et al. Phosphodiesterase 8B gene polymorphism in women with recurrent miscarriage: a retrospective case control study. BMC Med Genet. 2012; 13: 121.
18. Grandone A, Perrone L, Cirillo G, Di Sessa A, Corona AM, Amato A, et al. Impact of phosphodiesterase 8B gene rs4704397 variation on thyroid homeostasis in childhood obesity. European J Endocrinol. 2012; 166(2): 255-260.
19. Hayashi M, Shimada Y, Nishimura Y, Hama T, Tanaka T. Genomic organization, chromosomal localization, and alternative splicing of the human phosphodiesterase 8B gene. Biochem Biophys Res Commun. 2002; 297(5): 1253-1258.
20. Soderling SH, Bayuga SJ, Beavo JA. Cloning and characterization of a cAMP-specific cyclic nucleotide phosphodiesterase. Proc Natl Acad Sci USA. 1998; 95(15): 8991-8996.
21. Gamanuma M, Yuasa K, Sasaki T, Sakurai N, Kotera J, Omori K. Comparison of enzymatic characterization and gene organization of cyclic nucleotide phosphodiesterase 8 family in humans. Cell Signal. 2003; 15(6): 565-574.
22. Horvath A, Giatzakis C, Tsang K, Greene E, Osorio P, Boikos S. A cAMP-specific phosphodiesterase (PDE8B) that is mutated in adrenal hyperplasia is expressed widely in human and mouse tissues: a novel PDE8B isoform in human adrenal cortex. Eur J Hum Genet. 2008; 16(10): 1245-1253.
23. Shu YM, Zeng HT, Ren Z, Zhuang GL, Liang XY, Shen HW, et al. Effects of cilostamide and forskolin on the meiotic resumption and embryonic development of immature human oocytes. Hum Reprod. 2008; 23(3): 504-513.
24. Papi G, degli Uberti E, Betterle C, Carani C, Pearce EN, Braverman LE, et al. Subclinical hypothyroidism. Curr Opin Endocrinol Diabetes Obes. 2007; 14(3): 197-208.
25. Orouji Jokar T, Fourman LT, Lee H, Mentzinger K, Fazeli PK. Higher TSH levels within the normal range are associated with unexplained infertility. J Clin Endocrinol Metab. 2017; 103(2): 632-639.
26. Deeba F, Fatima P, Banu J, Ishrat S, Begum N, Anwary SA. Thyroid status and treatment response of hypothyroid infertile women in tertiary care center of bangladesh. Bangladesh J Obstet Gynaecol. 2016; 31(2): 86-89.
27. Feldthusen AD, Pedersen PL, Larsen J, Toft Kristensen T, Ellervik C, Kvetny J. Impaired fertility associated with subclinical hypothyroidism and thyroid autoimmunity: the Danish general suburban population study. J Pregnancy. 2015; 2015: 132718.
28. Verma I, Sood R, Juneja S, Kaur S. Prevalence of hypothyroidism in infertile women and evaluation of response of treatment for hypothyroidism on infertility. Int J Appl Basic Med Res. 2012; 2(1): 17-19.
29. Priya DM, Akhtar N, Ahmad J. Prevalence of hypothyroidism in infertile women and evaluation of response of treatment for hypothyroidism on infertility. Indian J Endocrinol Metab. 2015; 19(4): 504-506.
30. Pushpagiri N, Gracelyn LJ, Nagalingam S. Prevalence of subclinical and overt hypothyroidism in infertile women. Int J Reprod Contraception Obstet Gynecol2015; 4(6): 1733-1738.
31. Bharti G, Singh K, Kumari R, Kumar U. Prevalence of hypothyroidism in subfertile women in a tertiary care centre in North India. Int J Res Med Sci. 2017; 5(5): 1777-1780.
32. Malaiarasi N, Santhanalakshmi L. The association of thyroid dysfunctions with infertility in females. Int J Adv Res. 2016; 4 (7): 1017-1024.
33. Abdul R, Seema M. Effect of clinical/sub-clinical hypothyroidism on fertility in infertility case and the response of treatment for hypothyroidism on fertility in cases of infertility. IOSR Journal of Dental and Medical Sciences (IOSR-JDMS). 2015; 14(2): 5-8.
34. Faul F, Erdfelder E, Lang AG, Buchner A. G*power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007; 39(2): 175-191.
35. Jadeja SD, Mansuri MS, Singh M, Dwivedi M, Laddha NC,

Begum R. A case-control study on association of proteasome subunit beta 8 (PSMB8) and transporter associated with antigen processing 1 (TAP1) polymorphisms and their transcript levels in vitiligo from Gujarat. PLoS One. 2017; 12(7): e0180958.
36. Barrett JC, Fry B, Maller JD, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2004; 21(2): 263-265.
37. Biondi B, Cooper DS. The clinical significance of subclinical thyroid dysfunction. Endocr Rev. 2008; 29(1): 76-131.
38. Reigstad MM, Storeng R, Myklebust TÅ, Oldereid NB, Omland AK, Robsahm TE, et al. Cancer risk in women treated with fertility drugs according to parity status-a registry-based cohort study. Cancer Epidemiol Biomarkers Prev. 2017; 26 (6): 953-962.

Original Article

Cross-Border Reproductive Care: Psychological Distress in A Sample of Women Undergoing *In Vitro* Fertilization Treatment with and without Oocyte Donation

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Abstract

Background: Cross-border reproductive care (CBRC) refers to the movement of patients to foreign countries for fertility treatment. Limited evidence indicates that this phenomenon is associated with a risk of psychological distress, but few studies on the psychological impact of CBRC are currently available. The aim of this study was to compare the anxiety and depression levels of a group of cross-border patients with a local Spanish patient group, both of which underwent *in vitro* fertilization (IVF) treatment. We also sought to explore the clinical, sociodemographic and personality profiles of the CBRC group and local women.

Materials and Methods: This present cross-sectional study was conducted on 161 infertile females (71 CBRC patients and 90 local women) who were undergoing IVF treatment. The following questionnaires were used to collect data: Spielberger State Anxiety Inventory (STAI-S), the Beck Depression Inventory-II (BDI-II) and the Zuckerman-Kuhlman Personality Questionnaire (ZKPQ). Sociodemographic, clinical, reproductive and CBRC variables were also recorded.

Results: CBRC patients, specifically CBRC oocyte recipients, showed higher levels of anxiety compared to local women. However, no significant differences in depression scores were found between both groups. Finally, when analysing personality, the Activity scale scores of the ZKPQ were found to be higher in CBRC oocyte recipients, which indicated a greater tendency for general activity and higher energy levels.

Conclusion: CBRC oocyte recipient women may have greater vulnerability to anxiety than local women prior to infertility treatment. Screening and psychological support protocols for anxiety in this population should be considered.

Keywords: Assisted Reproductive Technologies, *In Vitro* Fertilization, Oocyte Donation, Personality, Psychopathology

Citation: Lasheras G, Mestre-Bach G, Clua E, Rodríguez I, Farré-Sender B. Cross-Border Reproductive Care: Psychological Distress in a sample of women undergoing in vitro fertilization with and without oocyte donation. Int J Fertil Steril. 2020; 14(2): 129-135. Int J Fertil Steril. 2020; 14(2): 130-136. doi: 10.22074/ijfs.2020.5997.

Introduction

Infertility is defined as the failure to establish a clinical pregnancy after 12 months of regular, unprotected sexual intercourse. The prevalence of infertility is between 17% and 28% in industrialized countries (1). Cross-border reproductive care (CBRC), also known as reproductive tourism, refers to a phenomenon in which infertile patients travel to other countries to obtain specific assisted reproduction treatments (ART), mainly intrauterine insemination, *in vitro* fertilization (IVF) and oocyte donation (2). Several clinical, social and legal issues can make the treatment more difficult in the patients' country of origin, which leads patients to seek CBRC. In most cases, the treatment is forbidden by law in the country of origin for the following reasons: it is considered an unsafe technique (e.g., oocyte freezing); ethical considerations (e.g., gamete donation, preimplantation genetic diagnosis), or patient characteristics (e.g., postmenopausal or homosexual women). Likewise, excessively long waiting lists in one's home country, lack of expertise (e.g., preimplantation genetic diagnosis) and high financial costs also block access to reproductive treatments (3). Consequently, there has been a progressive increase in migration to obtain ART. With regards to Europe, countries in which CBRC is most prevalent are France, Italy and Germany, 79% of those applicants are treated in Spain mostly due to the absence of legal restrictions in gamete donation. In 2016, the Spanish Fertility Society (SEF) Registry documented

Received: 4/June/2019, Accepted: 13/October/2019
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Royan Institute
International Journal of Fertility and Sterility
Vol 14, No 2, July-September 2020, Pages: 130-136

12 939 ART cycles. The majority of these patients were from Italy and France. A total of 86% of cases had gamete donation, which was mostly from an oocyte source (53.3%) (4).

Infertility can have a negative impact on quality of life (5-7), and the IVF treatment process is associated with stress (8), depression, anxiety and psychosomatic symptoms that interfere with fertility treatment (9). In terms of personality traits, some studies have found that 'sensation seeking' profiles are less prevalent in infertile women, who tend to engage in more experiential avoidance and self-judgment coping mechanisms (10). Evidence suggests that neuroticism, the relatively stable tendency to respond with negative emotions (hostility, sadness, anxiety, etc.) to threat, frustration, or loss (11) is related to the presence and maintenance of anxiety and depressive symptoms/disorders (12). It remains unclear, however, if the same holds true in infertile women and in women who seek CBRC.

In women who seek CBRC, it appears that multiple elements combine to increase vulnerability to psychopathological conditions. According to data from the SEF Registry, more than half of CBRC women in Spain seek oocyte donation. This, in turn, entails resorting to what may be considered as illegal fertility techniques in their respective countries (13). In addition, these women are usually older; have a longer duration of infertility; experience an increased conception failure rate; and have more difficulties in attaining healthy and normal births (14). In addition, further stress results from living in and adapting to unfamiliar environments, seeking justification for their work absence, the economic expense of treatment (15), and the travel costs involved.

Numerous studies carried out from a medical-legal perspective mainly considered the ethical aspects of this practice (16,17). However, only some studies have highlighted the fact that the adaptation to a different medical care context together with the associated psychological/economic discomfort caused by displacement can interfere with the quality of life of these patients. Therefore, studies relating CBRC to psychopathological consequences are needed.

In order to improve treatment interventions for patients who receive CBRC, a better understanding of the mechanisms underpinning its associated psychiatric symptomatology is required. To our knowledge, no empirical study has yet explored the association between CBRC and psychopathology in women. As such, we aimed to determine if there were differences in emotional states and personality profiles of women who receive CBRC in comparison to local women. Our study aims were twofold: a) to compare anxiety and depression levels between the CBRC patient group and the local patient group and b) to explore the sociodemographic, clinical and personality profiles of both groups. We hypothesized that CBRC patients: 1. would show higher anxiety and depression levels derived from factors associated with displacement and 2. would show a distinct personality profile in comparison with local women.

Materials and Methods

Sample

The present cross-sectional study was conducted at a hospital in Barcelona, Spain between October, 2015 and March, 2016. A total of 163 women were recruited through the Department of Reproductive Medicine in a hospital of Barcelona (Spain) at the beginning of IVF treatment with either their own or donated oocytes through convenience sampling, so that the samples were selected based on availability. The local group comprised 90 local Spanish women who underwent IVF treatment. Initially, the CBRC group was comprised of 73 women from other countries who sought IVF treatment in Spain; however, only two women were not Italian. In order to homogenize the sample, these two non-Italian women were excluded.

Inclusion criteria for both groups were: infertile female aged between 18 and 50 years, need for IVF treatment with or without oocyte donation, completed primary school as the minimum level of education, agreed to participate in the study, and signed the informed consent. The local group included women from Spain, while the CBRC group only included women from other countries initially, and finally just Italy, who travelled to Spain for IVF treatment.

Exclusion criteria in the local group was: an insufficient level of Spanish needed to complete the self-administered questionnaires and, in the CBRC group, an insufficient level of Italian needed to complete the self-administered questionnaires.

Procedure

Comprehensive clinical and psychological evaluations were carried out the week prior to the transfer along with the collection of additional reproductive, clinical and demographic data. The week prior to the transfer was considered a homogeneous moment for all patients and was not influenced by ongoing treatment variables, nor did it interfere with the CBRC group's return to Italy. Two staff biologists from the Reproductive Medicine Department in our hospital explained the basis of the study to the participants and, if they agreed to participate, they were required to sign the informed consent forms. At the gynaecology offices, staff asked the patients to complete four study questionnaires - the Spielberger State Anxiety Inventory (STAI-S), Beck Depression Inventory-II (BDI-II), Zuckerman-Kuhlman Personality Questionnaire (ZKPQ), and a socio-demographic, clinical and reproductive questionnaire.

Instruments

Spielberger State Anxiety Inventory

This 20-item questionnaire was used to assess the current mood of the respondent. All items were rated on a 4-point scale, from “Almost Never” 1. to “Almost Always” 2. which resulted in total scores from 20 to 80, with

higher scores indicative of greater levels of anxiety. Internal consistency coefficients for the original scale ranged from 0.86 to 0.95, whilst test-retest reliability coefficients ranged from 0.65 to 0.75 over a two month interval. The STAI-S, a widely used sub-scale, was the only variable from this questionnaire used in the present study. Two different validated translations were used for each sample population, Spanish (18) and Italian.

Beck Depression Inventory-II

The beck depression inventory-II (BDI-II) is an instrument for rating the severity of depressive symptoms. The BDI-II contains 21 items with four statements rated on a 0-3 scale from "Almost Never" to "Almost Always", and a total score from 0 to 63. This instrument categorizes depression using a low 14-19, moderate 20-28, or severe 29-63 stratum. Internal consistency for the original BDI scale ranges from 0.73 to 0.92 with a mean of 0.86 (19). Two different validated translations were used for each sample population of the study: Spanish and Italian (20).

Zuckerman-Kuhlman Personality Questionnaire

This 99-item questionnaire has a true/false format and assesses personality traits according to five personality factors: Neuroticism-Anxiety (19 items), Activity (17 items), Sociability (17 items), Impulsive Sensation Seeking (19 items), and Aggression-Hostility (17 items). Additionally, it has an Honesty scale (10 items) in order to ensure the reliability of the results. The original version features favourable psychometric properties of a high internal consistency (Cronbach's alpha range: 0.77 to 0.91), in addition to satisfactory convergent, discriminant, and consensual validity (21). Two different validated translations were used for the study: Spanish (22) and Italian (23).

Socio-demographic, clinical and reproductive variables interview

Additional clinical, demographic and reproductive variables were measured via a self-administered structured questionnaire created socio-demographic, clinical and reproductive variables interview (ad hoc) for this study. We included clinical and demographic variables of age, community origin, partner gender, education level, occupation and psychiatric history. In addition, the questionnaire explored cross-border issues such as: causes of movement; companions; perceived psychological discomfort; relevant difficulties in cross-border infertility treatment; and an evaluation of help received from language facilitation institutions during the process. Reproductive history was also taken into account, e.g., quantifying the number of living children; duration of infertility, previous failure(s) with assisted reproduction technology cycles (intrauterine insemination, IVF, oocyte donation); and previous miscarriages.

Ethics

The study was carried out in accordance with the latest version of the Declaration of Helsinki. Signed informed consent was obtained from all participants, and approval was granted from the Hospital Institutional Review Board.

Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA). Comparison between categorical variables was carried out using the chi-square tests ($\chi 2$) and the t-test. All tests were bilateral with a significance level set to $\alpha=0.05$.

Results

Descriptive for the sample

Table 1 shows participants' descriptives at intake (baseline values) and a comparison between the CBRC and local patients. Both groups had similar sociodemographic characteristics and no observed significant differences. No statistical differences were found in personal psychiatric history between the groups except for a higher than average incidence of previous IVF with the patients' own oocytes in the CBRC group.

Cross-border reproductive care issues

In the present study the women were accompanied by either their partners (89%), their partners and family/friends (9.6%), or by only family/friends (1.4%). Frequency distribution of the main causes for CBRC are represented in Figure 1, which shows that the primary reason for foreign fertility treatment was the difficulty to access the desired treatment technique (66.2%). Generally, CBRC respondents felt supported by the international department (87.7%). The most positive aspects listed were linguistic-communication support (49.3%) and personalized monitoring (22%). Job-related problems and financial costs were the main relevant difficulties within the CBRC group (Fig.2). A total of 32.9% of CBRC respondents reported significant psychological discomfort, and most reported significant and relevant difficulties in cross-border infertility treatment (55.9%).

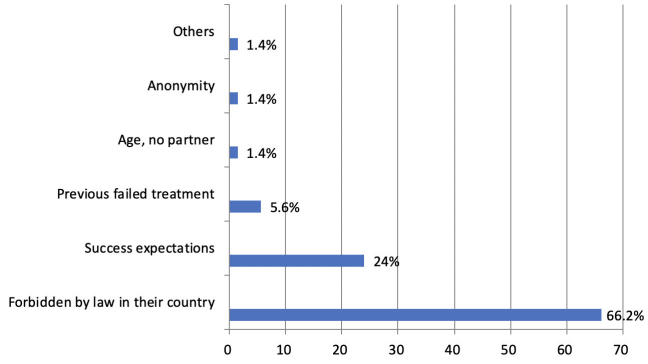

Fig.1: Mean reasons for patients choosing CBRC (Cross-border reproductive care).

Table 1: Sample description

Sociodemographic and clinical variables	CBRC group (n=71)	Local group (n=90)	P value
Age (Y)	39.9 ± 5.08	38.8 ± 5.04	0.201
Origin			
Spain	0 (0)	90 (100)	
Italy	71 (100)	0 (0)	
Education level			
Primary	9 (12.7)	11 (12.2)	0.835
Secondary	29 (40.8)	33 (36.7)	
University	33 (46.5)	46 (51.1)	
Civil status			
Single	0 (0.00)	1 (1.10)	0.373
Married-partner	71 (100)	89 (98.9)	
Partners' gender			
male	71 (100)	97 (96.7)	0.299
Employment status			
Employed	65 (91.5)	78 (86.7)	0.052
Duration of infertility (months)	48.8 ± 32.4	46.1 ± 39.6	0.657
Previous infertility treatments: IUI	2.07 ± 2.47	1.77 ± 1.89	0.392
Previous infertility treatments: IVFO	2.21 ± 2.75	1.03 ± 1.30	0.001*
Previous infertility treatments: IVFD	0.21 ± 0.71	0.32 ± 0.85	0.378
Recurrent pregnancy loss	26 (36.6)	24 (26.7)	0.143
Current treatment			
IVFO	36 (50.7)	54 (60.0)	0.238
IVFD	35 (49.3)	36 (40.0)	
Personal psychiatric history	7 (9.9)	19 (21.1)	

Data are presented as mean ± SD or n (%) (n=161). SD; standard deviation, CBRC; Cross-border reproductive care, IUI; Intrauterine insemination, IVFO; *In vitro* fertilization with own oocytes, IVFD; *In vitro* fertilization with donated oocytes, and *; significant at P<0.05.

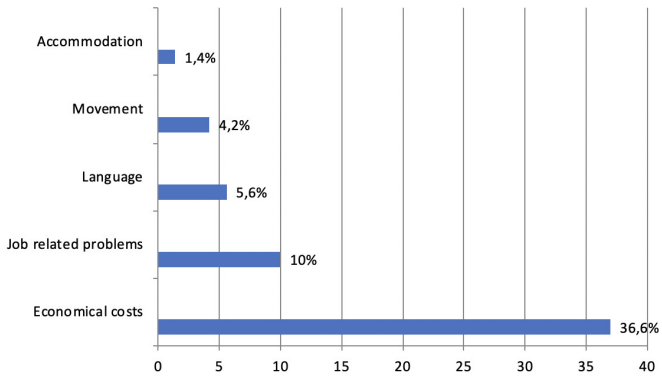

Fig.2: Main relevant difficulties related to CBRC (Cross-border reproductive care) referred by patients care) referred by patients

Anxiety and depression levels of cross-border reproductive care women and local women

Table 2 shows the results obtained from analysis of variance (ANOVA) for comparing clinical scores between CBRC patients and local women, controlled for the IVF technique. CBRC women reported higher STAI-S scores, but this difference was only relevant (P<0.001) and statistically significant in receptor woman (IVF with donated oocytes). No differences in depression scores were found between CBRC women and local women.

Personality profiles in cross-border reproductive care women and local women

Personality results are shown in Table 3 in a comparison of clinical scores between CBRC patients and local women, controlling for the IVF technique. In both groups, means in all subscales were within normal levels (+ 1 SD with regards to the general population) (21). No significant differences were found between groups, except for the Activity subscale with higher scores in the CBRC receptor group (IVF with donated oocytes; P=0.002).

Table 2: Comparison of STAI-S and BDI-II scores between the CBRC and local groups according to *in vitro* fertilization technique

Clinical assessment	CBRC group	n	Local grou	n	P value	MD (95% CI)
STAI-S $_{IVFO}$	22.6 ± 9.70	36	20.8 ± 10.1	54	0.383	1.85 (-2.35; 6.06)
STAI-S $_{IVFD}$	27.4 ± 6.85	35	18.8 ± 10.5	36	<0.000*	8.62 (4.42; 12.82)
BDI-II $_{IVFO}$	8.06 ± 6.30	36	9.81 ± 7.15	54	0.234	-1.76 (-4.67; 1.16)
BDI-II $_{IVFD}$	4.26 ± 4.04	35	5.36 ± 6.46	36	0.392	-1.10 (-3.66; 1.45)

Data are presented as mean ± SD or n (%); STAI-S; Spielberger State Anxiety Inventory, BDI-II; Beck Depression Inventory-II, IVFO; *In vitro* fertilization with own oocytes, IVFD; *In vitro* fertilization with donated oocytes, CBRC; Cross-border reproductive care, SD; Standard deviation, MD; Mean difference, CI; Mean difference confidence interval, *; significant at P<0.05.

Table 3: ZKPQ score comparison between the CBRC and local groups according to IVF technique

ZKPQ personality factors	CBRC group	n	Local group	n	P value	MD (95% CI)
Neuroticism-Anxiety $_{IVFO}$	-0.44 ± 1.13	36	-0.33 ± 0.91	53	0.619	-0.109 (-0.54; 0.32)
Neuroticism-Anxiety $_{IVFD}$	0.06 ± 0.74	35	0.04 ± 0.92	36	0.059	-0.435 (-0.88; 0.02)
Activity $_{IVFO}$	0.16 ± 0.92	36	0.07 ± 1.10	53	0.702	0.086 (-0.36; 0.53)
Activity $_{IVFD}$	0.45 ± 0.90	35	-0,21 ± 0.95	36	0.002*	0.711 (0.27; 1.15)
Impulsive Sensation Seeking $_{IVFO}$	-0.27 ± 0.7	36	-0.66 ± 0.78	53	0.069	0.385 (0.06; 0.70)
Impulsive Sensation Seeking $_{IVFD}$	-0.16 ± 0.84	35	-0.27 ± 1.11	36	0.624	0.115 (-0.35; 0.58)
Aggression-Hostility $_{IVFO}$	-0.22 ± 0.74	36	-0.08 ± 1.02	53	0.452	-0.14 (-0.51; 0.23)
Aggression-Hostility $_{IVFD}$	-0.08 ± 0.96	35	-0.01 ± 1.07	36	0.755	-0.076 (-0.56; 0.40)
Infrequency $_{IVFO}$	0.63 ± 1.06	36	0.54 ± 1.22	53	0.725	0.08 (-0.41; 0.58)
Infrequency $_{IVFD}$	1.26 ± 1.23	35	0.67 ± 1.60	36	0.086	0.592 (-0.08; 1.26)

Data are presented as mean ± SD or n (%); ZKPQ; Zuckerman-Kuhlman Personality Questionnaire, IVFO; *In vitro* fertilization with own oocytes, IVFD; *In vitro* fertilization with donated oocytes, SD; Standard deviation, CBRC; Cross-border reproductive care, *; Significant at P<0.05.

Discussion

This study analysed whether there were psychopathological differences between CBRC women and local women undergoing IVF. We explored CBRC issues and the clinical, sociodemographic and personality profiles of both groups.

In a similar way to another Spanish study (24), the majority of women in the CBRC group were from Italy where Spain's CBRC treatment approach is particularly well-perceived (13). The main cause of cross-border travel in the CBRC group was the illegality/difficulty of access to the practice in their home country, which explained why all of the participants originated from Italy, a country with some of the most highly restrictive laws in Europe in terms of medically assisted procreation (25).

Both clinical groups showed a similar profile with respect to sociodemographic and clinical features. The only difference was that the CBRC group had previously undergone more ART, specifically more IVF with their own oocytes. This was in line with the causes of CBRC described by these patients in our study, most of whom had had experienced failed treatments and came to Spain to receive infertility treatment that was illegal in Italy.

Regarding psychopathology, our study finds higher anxiety-state levels in CBRC oocyte recipient patients in comparison with the local group. These findings suggest that anxiety is simultaneously associated with the migratory process and the type of ART used. These findings cannot be compared with other results as there have been no similar previous investigations. In terms of depression, no significant differences were found between the groups, which was in line with other research where women who underwent CBRC IVF in Spain had low levels of depression (24). Therefore, the present data has supported the position that the migratory process, when an oocyte donation is needed, exacerbates anxiety symptomatology. Previous studies described high and moderate levels of anxiety in women requiring donor oocytes, assessed immediately prior to the IVF (26). Before treatment, many oocyte recipients expressed concern about the lack of a genetic tie to a child born after the donor procedure and doubts about whether or not to disclose the donation (27). These fears could increase anxiety levels; thus, a psychological consultation prior to treatment with gamete donation is recommended (28). In addition, Italian women who undergo treatment with oocyte donation endure a technique that was illegal in Italy until 2013 (13, 29). Currently, despite being legal, oocyte donation presents with greater difficulties in terms of access compared to other countries, which potentially increases patients' anxiety levels. All of the above factors add to the anxiety generated by infertility itself and support the observation that anxiety-related symptoms of infertile women are more prominent than those of fertile females (30). Furthermore, repeated fertility treatments and the accumulation of unsuccessful IVF treatments generate a sensation

of vulnerability-based anxiety (31). The CBRC women in our study had more previous infertility treatments with their own oocytes. The psychological burden of conceiving through the donation of oocytes is added to the process of undergoing the treatment outside their country of origin. CBRC poses new challenges and difficulties for patients, such as having to live away from home and to adapt to an unknown country with possible language barriers and little social support (32). In fact, a third of the women in the CBRC group endorsed significant psychological significant discomfort, and more than half reported significant and relevant difficulties in cross-border infertility treatment. As reported in other studies (33), the majority of psychological discomfort in our CBRC sample arose from economical costs, with absence from the participant's workplace listed as the second most relevant difficulty.

In light of these results, assisted reproduction centres that assist CBRC patients should be prepared to identify patients' anxiety levels prior to treatment, especially with oocyte donation and, if necessary, facilitate patient access to psychological support. Previous CBRC research in Spain (24) showed that couples with a history of oocyte donation treatments were more likely to perceive psychosocial support as useful and to desire it. Psychosocial interventions for couples under treatment for infertility, particularly cognitive behaviour therapy, has proven to be effective, both in reducing psychological distress and in improving clinical pregnancy rates.

On the other hand, no significantly different personality profiles were obtained between both groups, other than the Activity characteristic. This means that the CBRC group, specifically when an oocyte donation was required, was characterized by a greater tendency for general activity, an inability to relax and do nothing when the opportunity arises, a preference for hard and challenging work, a busy life, and a high energy level (34). CBRC patients who need oocyte donations tend to face more difficulties accessing reproductive treatment, thus making this personality tendency coherent in this subgroup of CBRC women given that CBRC recipient women must develop a proactive attitude towards infertility. These women must overcome the barriers and regulations of their countries to be able to carry out the reproduction treatment necessary for them to become mothers.

Finally, regarding CBRC issues and in accordance with previous studies (35), the main cause of reproductive tourism in our sample was the legal prohibition in the country of origin. This finding stresses the importance of taking a rapidly changing legal environment into consideration and to promote the adequate regulation of ART (36).

The present study is not without its limitations. First, all data were collected only from women who sought ART treatment. Future studies should aim to assess their partners in in order to obtain a more comprehensive view of CBRC effects (24). Second, the CBRC patients were from Italy, which limited the external validity of our analysis and comparisons with other countries of origin. The existence of country-based differences in the mental health of couples who undergo CBRC has been reported in previous studies (24), which suggests that this kind of analysis could be of interest. Third, the evaluation was carried out only through questionnaires, without a complementary clinical interview. Fourth, the cross-sectional perspective of this study did not permit the detection of differences between both groups after clinical intervention. It would be of interest to determine if a relationship existed between anxiety levels prior to fertility treatment and during pregnancy or postpartum. Finally, despite having been identified in infertile women (10), we did not assess coping strategies, cognitive style, quality of life or other psychopathologies of interest, such as somatic disorders.

Conclusion

This study provides further information about the existence of increased anxiety in CBRC women, specifically those who receive oocyte donations. The findings suggest that screening systems and psychological support for anxiety in this population should be considered in order to improve the quality of care in CBRC.

Acknowledgements

Dr. Mestre-Bach is supported by a postdoctoral grant of the Fundación Ciudadanía financially Valores. There is no conflict of interest in this study.

Authors' Contributions

G.L., E.C.; Designed the study, contributed to the data collection, and involved in developing the research aims. G.M-B., B.F-S.; Aided in the literature search and the framing of the introduction and discussion section. I.R., B.F-S., G.M.B.; Conducted the statistical analysis and interpretation of the results. G.M.B., B.F-S. ; Involved in writing and proofreading the manuscript. All authors have read and approved the final manuscript.

References

1. Schmidt L. Social and psychological consequences of infertility and assisted reproduction - what are the research priorities? Hum Fertil (Camb). 2009; 12(1): 14-20.
2. Ahuja KK. Patient pressure: is the tide of cross-border reproductive care beginning to turn? Reprod Biomed Online. 2015; 30(5): 447-450.
3. Couture V, Drouin R, Tan SL, Moutquin JM, Bouffard C. Crossborder reprogenetic services. Clin Genet. 2015; 87(1): 1-10.
4. Sociedad Española de Fertilidad. Informes Registro Nacional de Actividad-Registro SEF. Sociedad Española de Fertilidad. 2016.
5. Fekkes M, Buitendijk SE, Verrips GH, Braat DD, Brewaeys AM, Dolfing JG, et al. Health-related quality of life in relation to gender and age in couples planning IVF treatment. Hum Reprod. 2003; 18(7): 1536-1543.
6. Verhaak CM, Smeenk JM, Nahuis MJ, Kremer JA, Braat DD. Longterm psychological adjustment to IVF/ICSI treatment in women. Hum Reprod. 2007; 22(1): 305-308.
7. Chachamovich JR, Chachamovich E, Zachia S, Knauth D, Passos EP. What variables predict generic and health-related quality of life in a sample of Brazilian women experiencing infertility? Hum Reprod. 2007; 22(7): 1946-1952.

8. Turner K, Reynolds-May MF, Zitek EM, Tisdale RL, Carlisle AB, Westphal LM. Stress and anxiety scores in first and repeat IVF Cycles: a pilot study. PLoS One. 2013; 8(5): e63743.
9. Aarts JW, van Empel IW, Boivin J, Nelen WL, Kremer JA, Verhaak CM. Relationship between quality of life and distress in infertility: a validation study of the Dutch FertiQoL. Hum Reprod. 2011; 26(5): 1112-1118.
10. Cunha M, Galhardo A, Pinto-Gouveia J. Experiential avoidance, self-compassion, self-judgment and coping styles in infertility. Sex Reprod Healthc. 2016; 10: 41-47.
11. Lahey BB. Public health significance of neuroticism. Am Psychol. 2009; 64(4): 241-256.
12. Brown TA, Barlow DH. A proposal for a dimensional classification system based on the shared features of the DSM-IV anxiety and mood disorders: implications for assessment and treatment. Psychol Assess. 2009; 21(3): 256-271.
13. Zanini G. Abandoned by the State, betrayed by the Church: Italian experiences of cross-border reproductive care. Reprod Biomed Online. 2011; 23(5): 565-572.
14. Liu K, Case A, Cheung AP, Sierra S, AlAsiri S, Carranza-Mamane B, et al. Advanced reproductive age and fertility. J Obstet Gynaecol Can. 2011; 33(11): 1165-1175.
15. Chambers GM, Sullivan EA, Ishihara O, Chapman MG, Adamson GD. The economic impact of assisted reproductive technology: a review of selected developed countries. Fertil Steril. 2009; 91(6): 2281-2294.
16. Pennings G, De Wert G, Shenfield F, Cohen J, Tarlatzis B, Devroey P. ESHRE task force on ethics and law 15: cross-border reproductive care. Hum Reprod. 2008; 23(10): 2182-2184.
17. Millbank J. Responsive regulation of cross-border assisted reproduction. J Law Med. 2015; 23(2): 346-364.
18. Spielberger CD, Gorsuch RL, Lushene RE. STAI. Cuestionario de ansiedad estado-rasgo. 7th ed. Madrid: TEA Ediciones; 2008.
19. Beck AT, Ward CH, Mendelson M, Mock J, Erbaugh J. An inventory for measuring depression. Arch Gen Psychiatry. 1961; 4: 561-571.
20. Beck AT, Steer RA, Brown GK. BDI-II Manual. In: Ghisi M, Flebus GB, Montano A, Sanavio E, Sica C, editors. Firenze: Giunti OS Organizzazioni Speciali; 2007; 1-79.
21. Zuckerman M, Kuhlman DM, Joireman J, Teta P. A comparison of three structural models for personality: the big three, the big five, and the alternative five. J Pers Soc Psychol. 1993; 65(4): 757-768.
22. Gomà-i-Freixanet M, Valero Ventura S. Spanish normative data of the Zuckerman-Kuhlman Personality Questionnaire in a general population sample. Psicothema. 2008; 20(2): 324-330.
23. De Pascalis V, Russo PM. Zuckerman-Kuhlman Personality Questionnaire: preliminary results of the Italian version. Psychol Rep. 2003; 92(3 Pt 1): 965-974.
24. Madero S, Gameiro S, García D, Cirera D, Vassena R, Rodríguez A. Quality of life, anxiety and depression of German, Italian and French couples undergoing cross-border oocyte donation in Spain. Hum Reprod. 2017; 32(9): 1862-1870.
25. Boggio A. Italy enacts new law on medically assisted reproduction. Hum Reprod. 2005; 20(5): 1153-1157.
26. Lisovskaya TV, Zakhezina EA, Filippova GG, Ambartsumyan EM, Portnov IG, Mayasina EN. Mental state assessment of recipients in the IVF donor programs and psychotherapeutic methods of its correction. Gynecol Endocrinol. 2017; 33(sup 1): 28-31.
27. Hammarberg K, Carmichael M, Tinney L, Mulder A. Gamete donors' and recipients' evaluation of donor counselling: a prospective longitudinal cohort study. Aust N Z J Obstet Gynaecol. 2008; 48(6): 601-606.
28. Boivin J, Griffiths E, Venetis CA. Emotional distress in infertile women and failure of assisted reproductive technologies: meta-analysis of prospective psychosocial studies. BMJ. 2011; 342: d223.
29. Shenfield F, de Mouzon J, Pennings G, Ferraretti AP, Andersen AN, de Wert G, et al. Cross border reproductive care in six European countries. Hum Reprod. 2010; 25(6): 1361-1368.
30. Lakatos E, Szigeti JF, Ujma PP, Sexty R, Balog P. Anxiety and depression among infertile women: a cross-sectional survey from Hungary. BMC Womens Health. 2017; 17(1): 48.
31. Greil AL, McQuillan J, Lowry M, Shreffler KM. Infertility treatment and fertility-specific distress: A longitudinal analysis of a population-based sample of U.S. women. Soc Sci Med. 2011; 73(1): 87-94.
32. Blyth E, Thorn P, Wischmann T. CBRC and psychosocial counselling: assessing needs and developing an ethical framework for practice. Reprod Biomed Online. 2011; 23(5): 642-651.
33. Blyth E. Fertility patients' experiences of cross-border reproductive care. Fertil Steril. 2010; 94(1): e11-e15.
34. Gomà-i-Freixanet M, Valero S, Muro A, Albiol S. Zuckerman-Kuhlman Personality Questionnaire: psychometric properties in a sample of the general population. Psychol Rep. 2008; 103(3): 845-856.
35. Van Hoof W, Provoost V, Pennings G. Reflections of Dutch patients on IVF treatment in Belgium: a qualitative analysis of internet forums. Hum Reprod. 2013; 28(4): 1013-1022.
36. Jackson E, Millbank J, Karpin I, Stuhmcke A. Learning from cross-border reproduction. Med Law Rev. 2017; 25(1): 23-46.

Original Article

Rescue *In Vitro* Maturation in Polycystic Ovarian Syndrome Patients Undergoing *In Vitro* Fertilization Treatment who Overrespond or Underrespond to Ovarian Stimulation: Is It A Viable Option? A Case Series Study

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Abstract

Background: This study intends to present the role of rescue *in vitro* maturation (IVM) in polycystic ovarian syndrome (PCOS) patients undergoing *in vitro* fertilization (IVF) treatment who have inappropriate responses to ovarian stimulation.

Materials and Methods: This was a retrospective case series study of five PCOS patients undergoing IVF treatment considered for cycle cancellation due to increased risk of ovarian hyperstimulation syndrome (OHSS) as group A or poor response to ovarian stimulation as group B. Patients in group A had high oestradiol levels and recruitment of high numbers of small/intermediate sized follicles that did not meet the criteria for human chorionic gonadotropin (hCG) triggering. Patients in group B responded inadequately to hormonal stimulation despite high gonadotropin dosage. Treatment was changed to rescue IVM cycles after the patients provided consent.

Results: In group A, three IVF patients deemed to have high chances of developing OHSS as evidenced by high oestradiol levels were converted to IVM. A total of the 58/68 oocytes retrieved were mature or matured *in vitro*. There were 26 cleaving embryos obtained. Two patients had live births and one patient suffered a miscarriage. In group B, rescue IVM was implemented in two patients due to poor ovarian response (POR). A total of 22/26 oocytes retrieved were mature or matured *in vitro*. There were 13 cleaving embryos obtained. One patient had a live birth, whilst the other suffered a miscarriage.

Conclusion: Rescue IVM could be a viable option in PCOS patients undergoing IVF treatment who are unable to safely meet the criteria for hCG triggering due to overresponse to ovarian stimulation or ovarian resistance to high doses of stimulation. Conversion to IVM can still result in reasonable oocyte retrieval and lead to clinical pregnancy and live births without the risks of OHSS.

Keywords: Infertility, *In Vitro* Fertilization, *In Vitro* Maturation Techniques, Oocytes

Citation: **Fatum M, Bergeron ME, Ross C, Ding A, Bhevan A, Turner K. Rescue in vitro maturation in polycystic ovarian syndrome patients undergoing in vitro fertilization treatment who overrespond or underrespond to ovarian stimulation: is it a viable option? a case series study. Int J Fertil Steril. 2020; 14(2): 137-142. doi: 10.22074/ijfs.2020.6025.**

Introduction

Ovarian superovulation with gonadotropin stimulation is still the mainstay of *in vitro* fertilization (IVF) (1). The aim of ovarian stimulation is to induce multifollicular recruitment with as much synchronized cytoplasmic and nuclear maturation as possible, and to safely obtain a higher number of mature eggs at the time of egg collection (2). Side effects of ovarian stimulation can include breast tenderness, abdominal bloating, nausea and vomiting (3). More importantly, it can lead to ovarian hyperstimulation syndrome (OHSS), particularly in women with polycystic ovarian syndrome (PCOS) (4, 5).

PCOS is probably the most frequently encountered endocrinopathy in women of reproductive age (6). It is characterized by irregular menses, hyperandrogenism, and polycystic ovaries (PCO) on ultrasound findings. The prevalence of PCOS may be as high as 15-20% (7). It is believed that harvesting more eggs would compensate for subfertility in these patients. However, ovarian responses to the same stimulation protocols may vary considerably among different PCOS patients and even among different cycles in the same patient (8).

In some cycles, patients may be overstimulated, resulting

Received: 25/June/2019, Accepted: 3/November/2019
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in a very high number of growing follicles and increased levels of oestradiol. This group of patients is at higher risk of developing OHSS (9-11). In addition, a large cohort of antral and preantral follicles are recruited in these overstimulated cycles, which are asynchronous and heterogeneous in their growth and development (1). Consequently, immature and mature eggs are retrieved in these cycles. In some cases, this may prove to be a complex conundrum that needs much consideration, particularly when the patient is at high risk of OHSS, as demonstrated by high hormone levels, and there is an insufficient number of large-sized follicles. In these cases, cancellation could be the only option. Coasting may not be effective or plausible, as oestradiol production may increase further (12).

On the other end of the spectrum, management of PCOS women with poor ovarian response (POR) can be an equally frustrating challenge. Despite the high number of small follicles per ovary (2-3 times that of normal) (13), there is poor follicular growth and development in response to gonadotropin stimulation. This adversely affects mature oocyte retrieval and, more importantly, pregnancy success. Like patients at high risk of developing OHSS, these women also face the prospect of cycle cancellation.

We report a cohort of overstimulated IVF patients, as indicated by their rapidly increasing oestradiol levels and the large number of follicles, and a cohort of poor responders to ovarian stimulation who converted to rescue *in vitro* maturation (IVM) treatment. The aim of this study is to examine the rate of immature oocyte recovery and their potential for IVM from cancelled IVF cycles due to an abnormal response to gonadotropin stimulation.

Materials and Methods

Eligible patients

Unplanned IVM rescue cycles were undertaken for five PCOS patients who had abnormal responses to gonadotropin stimulation as part of their IVF treatment between 2007 and 2010 at the Oxford Fertility Clinic.

PCOS was defined according to the modified Rotterdam criteria (14) . Women who were considered to have overresponded had either high levels of oestradiol and/ or a high number of growing follicles (>20 at an early stage). Conversely, women who were considered as resistant to gonadotropin stimulation either responded poorly biochemically with low oestradiol levels or had poor follicular growth as evidenced by scans. Women aged over 40 and who had more than three previous failed IVF cycles were excluded from the study. In accordance with Oxford University Ethics Committee, the study was not registered and Ethical approval was not required as data were anonymised, not identifiable by researchers and were collected before the study was formulated.

In vitro fertilization and in vitro maturation

Our standard protocol for IVF and IVM treatments were described previously (15).

Statistical analysis

This was a case series study produced as part of an IVM programme at Oxford Fertility Unit, UK. Statistical analysis was carried out by a biostatistician at Oxford University. Statistical analyses were done using Microsoft Excel (Microsoft Office 365). Table was produced using Microsoft Excel (Microsoft Office 365). Graphs were produced using GraphPad Prism 8.0.0 on Mac OSX (Apple Inc. USA). The case series was reported using the case report (CASE) guidelines checklist (16).

Results

We present five cases of PCOS patients (see criteria above) aged between 31 and 39 years who each underwent an unplanned rescue IVM cycle due to an abnormal ovarian response to gonadotropin stimulation at Oxford Fertility Clinic between 2007 and 2010. They agreed to undergo immature oocyte maturation retrieval with subsequent IVM of oocytes to rescue their IVF treatment. Prior to the treatment, they all had normal ovarian reserves according to their early follicular phase follicle stimulating hormone (FSH) and antral follicle counts (AFC). The main results examined were biochemical pregnancy [beta human chorionic gonadotropin (βhCG) positive], clinical pregnancy rate (defined as heart activity at 8 weeks on an ultrasonography scan) and live birth rate.

Three patients (group A) were offered the option of converting to IVM rather than cancelling their IVF cycles as they were deemed to be at risk of developing severe OHSS. Average oestradiol on the day of cancellation was 11 078 ± 5141.9 pmol/L (Table 1). Nevertheless, none of these patients actually developed OHSS. Oocyte retrieval rate per aspirated follicle was 35%. A total of 68 oocytes were retrieved between the three patients in each group, and 58 of the 68 oocytes reached metaphase I (MI) or metaphase II (MII, Fig.1). Twenty-six cleaving embryos were obtained in group A (Table 1).

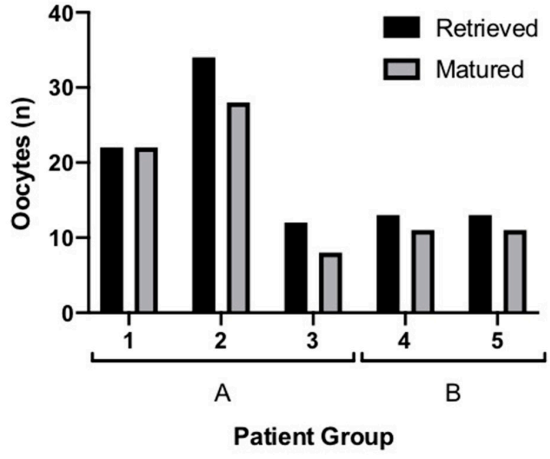

Fig.1: Numbers of oocyte retrieved and matured. Bar chart shows the numbers of oocytes retrieved and matured for each patient. Patients 1-3 represent group A and patients 4-5 represent group B.

Table 1: ZKPQ score comparison between the CBRC and local groups according to IVF technique

Pt no.	Age	BMI	E2 on day of cancellation	Oocytes retrieved	Oocytes reaching MI or MII (% of total)	No. ooc ytes injected	Fertilization rate	No. cleaving embryos	Embryos transferred	Pregnancy test	Cycle outcome
1	32	23	6065	22	22 (100)	22	17 (77)	12	2	+	Live birth
2	31	21	16340	34	28 (82)	28	15 (54)	12	2	+	Miscarriage 21 weeks
3	34	23	10830	12	8 (67)	8	4 (50)	4	2	+	Live birth
4	32	23	1800	13	11 (85)	11	6 (55)	5	2	+	Live birth
5	39	24	2483	13	11 (85)	11	8 (73)	8	2	+	Biochemical pregnancy

Table showing baseline characteristics of each patient, oestradiol levels on the day of cancellation of IVF treatment, as well as parameters on oocytes and embryos obtained in each case. Patients 1-3 represent group A. Patients 3-4 represent group B. Pt; Patient, no; Number, MI; Metaphase I, MII; Metaphase II, BMI; Body mass index, and IVF; In vitro fertilization.

In group B, two patients were offered the option of rescue IVM cycle because they had POR to gonadotropin stimulation. Average oestradiol level of the day of cycle cancelation was 2141.5 ± 482.9 pmol/L (Table 1). Despite their disappointing response to ovarian stimulation, 13 oocytes were retrieved from each patient. In fact, oocytes could be obtained in 33% of all follicles identified and aspirated. Eleven oocytes were mature or matured in vitro for each patient (Table 1). A total of 13 cleaving embryos were obtained in this group.

In both groups, all patients had two fresh cleavage embryos transferred on day 3 of development and all (100%) had positive pregnancy tests two weeks later. Three of the five patients (60%) gave birth to healthy singletons at term (38 and 40 weeks) or near term (35 weeks). Unfortunately, one patient in group A had a late second trimester miscarriage and one patient in group B had an early first trimester miscarriage (Table 1). Moreover, three patients had the opportunity to store their embryos. Two patients returned for a total of three frozen embryo replacement cycles, but they were all unsuccessful.

Discussion

Our case series study shows that rescue IVM could be a viable option in PCOS patients undergoing IVF treatment but failing to safely meet the criteria for hCG triggering because of either ovarian overresponse or underresponse to hormonal stimulation.

In our study, we did not use the conventional definition of POR as defined by the European Society of Reproduction and Embryology (ESHRE) (17). Instead, POR in our study referred specifically to PCOS patients with normal ovarian reserve and high AFC, yet showed poor hormonal and follicular response despite controlled ovarian hyperstimulation (COH). POR patients have reduced oocyte production, cycle cancellation and, most importantly, a reduced probability of pregnancy. It is unclear why women with PCOS can have such contrasting responses to gonadotropin stimulation, although it has been suggested that certain PCOS phenotypes may be correlated with adverse assisted reproductive outcomes (8). There is no test that can reliably predict outcome of ovarian stimulation in women with PCOS. However, anti-Müllerian hormone (AMH) on day 3 of the IVF stimulation cycle may positively predict ovarian response to gonadotropin stimulation. Oestradiol levels on the day of hCG administration and oocyte retrieval rate positively correlate with increasing AMH levels during IVF cycles in PCOS patients (18). As there is no way to reliably predict poor responders to gonadotropin stimulation, we cannot immediately identify these women for IVM. However, rescue IVM after failed IVF may provide these women with a chance of pregnancy within the same cycle of treatment.

There have been efforts to identify an algorithm based on the woman's age and markers of ovarian reserve to optimise the FSH starting dose in assisted reproductive techniques (ARTs). A recent study suggested that the application of a nomogram could lead to a more tailored approach, increasing the cost-effectiveness of infertility treatment. In general, the starting dose of FSH as calculated by the nomogram was lower than the actual prescribed dose, which might reduce the risk of OHSS. However, the authors also suggested the inadequacy of the nomogram in PCOS patients, especially in those with high AMH levels (19). Further studies are required to assess the utility and generalisability of such nomograms. The risk of OHSS may also be reduced by the administration of adjuvant medication. Administration of D-chiro-inositol (DCI) in PCOS patients resulted in a higher ovulation rate compared to placebo (20, 21). Myo-inositol and DCI may improve many of the metabolic and hormonal dysregulations characteristic of PCOS (22), and myo-inositol seems to be able to increase oocyte quality, decrease the days of FSH stimulation before hCG administration and, hence, the risk for OHSS (23, 24).

OHSS is an iatrogenic, systemic condition secondary to gonadotropin stimulation that occurs either during the luteal phase or during pregnancy. The most common form happens a few days after the induction of follicular rupture via injection of hCG when follicular growth has been medically induced (25). Fundamentally, in OHSS, an increase in vascular permeability results in third-space fluid loss, leading to intravascular volume depletion and haemoconcentration (9). Thromboembolism is a potentially serious consequence of OHSS, and can sometimes be fatal despite treatment (26). Additionally, OHSS been reported to be linked to hepatic and renal dysfunction (27, 28), but the link between COH and renal/ liver dysfunction are still debated. A study by Romito et al. (29) examined 426 patients undergoing IVF treatment and found that COH did not significantly alter renal and hepatic functions. In contrast, Giugliano et al. (30) reported a case of hepatic failure after four cycles of COH in a patient that developed severe haemolysis, elevated liver enzymes and low platelets (HELLP) syndrome. Various preventative strategies of OHSS during IVF have been suggested, such as coasting (31), co-treatment with cabergoline (32) or metformin (33), cryopreservation of embryos (34), or the administration of gonadotropin releasing hormone agonists (GnRH-agonist) instead of hCG in women treated in antagonist protocols (35). However, the only absolute way of preventing OHSS is to avoid ovarian stimulation, as in IVM. Given the evidence between COH and renal/ liver dysfunction is still debated, avoiding ovarian stimulation by using IVM may have the added advantage of preventing such complications, especially when many women have already gone through multiple cycles of IVF and may be at higher inherent risk for developing renal/ hepatic dysfunction.

Despite the advances in ARTs, one of the main challenges is the management of patients who have POR. To this end, luteal phase ovarian stimulation and dehydroepiandrosterone (DHEA) supplementation have shown promising results in improving outcomes in PORs. Preliminary results from a single centre pilot study by Lin et al. have demonstrated that luteal phase ovarian stimulation significantly improved oocyte retrieval and quality when compared to follicular phase ovarian stimulation in patients undergoing IVF (36). In a similar finding, Chern et al. (37), in their retrospective study, reported a potential benefit of DHEA supplementation pre-IVF cycle in PORs by showing improved oocyte retrieval rate, quality of embryos and live birth rate compared to the control group.

The success rate with IVM is associated with the number of immature oocytes obtained, which is predicted by the AFC. Women with PCOs have higher AFCs (13) and, therefore, have a comparatively increased rate of success than those with normal ovaries. Women with PCO are at significantly higher risk of developing OHSS (4, 5). In our previous study, we have reported that IVM is a simpler, safer, although less successful alternative, for women with PCO or PCOS (15). Balancing the higher success rate of IVF in PCO/PCOS women with the risk of potentially developing OHSS can be a complex dilemma. With the possibility of initial IVF treatment, and then rescue IVM if they are at significant risk of developing OHSS, we may be able to make a compromise between success rate and safety that neither IVF nor IVM alone can achieve in PCOS patients. One of the strengths of our study is the corroboration of previous findings, not only from our own group but that of others. The concept of rescue IVM began approximately two decades ago. Coskun et al. (38) have demonstrated that immature oocytes can be recovered from cancelled human gonadotropin cycles and these oocytes can be matured *in vitro*. Later, in a related publication, Jaroudi et al. (39) reported on 18 patients who underwent IVF but were then deemed to be at significant risk of developing OHSS. These women had cycle cancellation and underwent immature oocyte retrieval with subsequent IVM. On average, 8.1 immature oocytes were retrieved from each patient and 44 embryos were transferred in 17 cycles. There were two live births; however, one baby was delivered preterm and died shortly after. The study suggested that oocytes matured in vitro from incomplete IVF cycles could be fertilised by intracytoplasmic sperm injection (ICSI) and the those embryos could result in pregnancies. However, at the time, the low success rate could not justify recommendation of more widespread use without further research. In our study, the average number of oocytes retrieved per patient in both groups was higher than reported by Jaroudi et al. (39).

There are a number of potential explanations for this. First, the study by Jaroudi et al. (39) included not only PCOS patients, but also those with other types of infertility, such as anovulatory and unexplained cases. It is known that PCOS patients have higher numbers of follicles from which immature oocytes may be retrieved. It is also plausible that the improvements in both the IVF and IVM protocols have contributed to the higher numbers of immature oocytes picked up in our study. The live birth rate (60% overall) in our study was also higher. Again, improvements in techniques and protocols may have contributed to results; however, we are aware that our cohort is very small. In our study, the maturation rate (reaching MII) in group B (27%) was lower than that in group A (58%), which was comparable with our previous study (65%) (40). Whilst this seems to be a significant difference, it is noteworthy that the cohort size in our previous study was 94, which is considerably larger than that of our current study. It is possible that there a genuine difference exists in the ability of oocytes to mature between poor responders and overresponders, which may share the same aetiology as ovarian resistance to hormonal stimulation. The fertilization rate for both groups is similar to that reported in our previous study, which is promising as it suggests that oocytes in rescue IVM are not adversely affected by their previous exposure to gonadotropin sti mulation, regardless of the ovarian response.

The main limitation of our study is the sample size the high clinical pregnancy rate and live birth rate requires caution. Whilst a biostatistician carried out the data analy-

sis, we did not calculate the sample size required before the start of the study. This was due to logistical reasons of finding cases of cancelled IVF with subsequent agreement of undergoing IVM. Arguably this affects the generalisability of our study and the ability to draw definitive conclusions based on the findings of this mini case series. However, our aim is to highlight the possibility of IVM success in a proportion of PCOS patients who fail IVF treatment in a field that has the scope for further study and research.

IVM has an inherent advantage over conventional IVF by utilising the natural menstrual cycle, and bypassing the need for ovarian stimulation and pituitary suppression, albeit at the cost for reduced chances of success. Conventionally, IVM has been considered an alternative to IVF in women at risk of OHSS or in those who may have a POR to gonadotropin stimulation. Here, we present IVM as a potential add-on treatment, which is not considered as an alternative to IVF, but rather alongside it as a rescue strategy. The advantage is that potentially recoverable immature oocytes in cancelled cycles are not wasted and the emotional stress associated with facing a potentially cancelled cycle is reduced. Additionally, it may help prevent these patients from undergoing another costly, lengthy stimulation protocol.

Conclusion

We conclude that rescue IVM could be a viable option in PCOS patients undergoing IVF treatments who fail to safely meet the criteria for hCG triggering, either due to overresponse to ovarian stimulation or ovarian resistance to high doses of stimulation. Conversion to IVM can still result in reasonable oocyte retrieval and lead to clinical pregnancy and live births without the risks of OHSS. Further research is needed to determine the aetiology of POR and OHSS, and identify markers that will allow us to reliably predict which patients for whom IVF is less appropriate than IVM. Larger studies are needed to determine whether rescue IVM is a widely applicable strategy for women who respond inappropriately to ovarian stimulation and its success rate.

Acknowledgements

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

Authors' Contributions

M.F., C.R., T.C., K.T.; Participated in study design. T.C.; Participated in patient recruitment. A.B., K.T., C.R.; Performed IVM laboratory procedures. M.E.B., C.R., A.B., K.T., A.D.; Performed data collection. A.D., M.E.B., M.F.; Performed data analysis and interpretation, and drafted the manuscript. All authors performed editing and finalization of the manuscript.

References

1. Fanchin R, Salomon L, Castelo-Branco A, Olivennes F, Frydman N, Frydman R. Luteal estradiol pre-treatment coordinates follicular growth during controlled ovarian hyperstimulation with GnRH antagonists. Hum Reprod. 2003; 18(12): 2698-2703.
2. Blockeel C, Devroey P. Optimisation of the follicular phase in IVF/ICSI. Facts Views Vis ObGyn. 2012; 4(3): 203-212.
3. Rizk B, Smitz J. Ovarian hyperstimulation syndrome after superovulation using GnRH agonists for IVF and related procedures. Hum Reprod. 1992; 7(3): 320-327.
4. MacDougall MJ, Tan SL, Balen A, Jacobs HS. A controlled study comparing patients with and without polycystic ovaries undergoing in-vitro fertilization. Hum Reprod. 1993; 8(2): 233-237.
5. Swanton A, Storey L, McVeigh E, Child T. IVF outcome in women with PCOS, PCO and normal ovarian morphology. Eur J Obstet Gynecol Reprod Biol. 2010; 149(1): 68-71.
6. Carmina E, Lobo RA. Polycystic ovary syndrome (PCOS): arguably the most common endocrinopathy is associated with significant morbidity in women. J Clin Endocrinol Metab. 1999; 84(6): 1897-1899.
7. Sirmans SM, Pate KA. Epidemiology, diagnosis, and management of polycystic ovary syndrome. Clin Epidemiol. 2013; 6: 1-13.
8. Ramezanali F, Ashrafi M, Hemat M, Arabipoor A, Jalali S, Moini A. Assisted reproductive outcomes in women with different polycystic ovary syndrome phenotypes: the predictive value of anti-Müllerian hormone. Reprod Biomed Online. 2016; 32(5): 503-512.
9. Corbett S, Shmorgun D, Claman P; Reproductive Endocrinology Infertility Committee; Special Contributor. The prevention of ovarian hyperstimulation syndrome. J Obstet Gynaecol Can. 2014; 36(11): 1024-1033.
10. Humaidan P, Nelson SM, Devroey P, Coddington CC, Schwartz LB, Gordon K, et al. Ovarian hyperstimulation syndrome: review and new classification criteria for reporting in clinical trials. Hum Reprod. 2016; 31(9): 1997-2004.
11. Smith V, Osianlis T, Vollenhoven B. Prevention of Ovarian hyperstimulation syndrome: a review. Obstet Gynecol Int. 2015; 2015: 514159.
12. Rabinovici J, Kushnir O, Shalev J, Goldenberg M, Blankstein J. Rescue of menotrophin cycles prone to develop ovarian hyperstimulation. Br J Obstet Gynaecol. 1987; 94(11): 1098-1102.
13. Pigny P, Jonard S, Robert Y, Dewailly D. Serum anti-müllerian hormone as a surrogate for antral follicle count for definition of the polycystic ovary syndrome. J Clin Endocrinol Metab. 2006; 91(3): 941-945.
14. Azziz R. Controversy in clinical endocrinology: diagnosis of polycystic ovarian syndrome: the Rotterdam criteria are premature. J Clin Endocrinol Metab. 2006; 91(3): 781-785.
15. Gremeau AS, Andreadis N, Fatum M, Craig J, Turner K, Mcveigh E, et al. In vitro maturation or in vitro fertilization for women with polycystic ovaries? A case-control study of 194 treatment cycles. Fertil Steril. 2012; 98(2): 355-360.
16. Gagnier JJ, Kienle G, Altman DG, Moher D, Sox H, Riley D, et al. The CARE guidelines: consensus-based clinical case reporting guideline development. BMJ Case Rep. 2013; 2013. pii: bcr2013201554.
17. Ferraretti AP, La Marca A, Fauser BC, Tarlatzis B, Nargund G, Gianaroli L, et al. ESHRE consensus on the definition of "poor response" to ovarian stimulation for in vitro fertilization: the Bologna criteria. Hum Reprod. 2011; 26(7): 1616-1624.
18. Xi W, Gong F, Lu G. Correlation of serum anti-müllerian hormone concentrations on day 3 of the in vitro fertilization stimulation cycle with assisted reproduction outcome in polycystic ovary syndrome patients. J Assist Reprod Genet. 2012; 29(5): 397-402.
19. Di Paola R, Garzon S, Giuliani S, Laganà AS, Noventa M, Parissone F, et al. Are we choosing the correct FSH starting dose during controlled ovarian stimulation for intrauterine insemination cycles? Potential application of a nomogram based on woman's age and markers of ovarian reserve. Arch Gynecol Obstet. 2018; 298(5): 1029-1035.
20. Unfer V, Orrù B, Monastra G. Inositols: from physiology to rational therapy in gynecological clinical practice. Expert Opin Drug Metab Toxicol. 2016; 12(10): 1129-1131.
21. Nestler JE, Jakubowicz DJ, Reamer P, Gunn RD, Allan G. Ovulatory and metabolic effects of D-chiro-inositol in the polycystic ovary syndrome. N Engl J Med. 1999; 340(17): 1314-1320.
22. Paul C, Laganà AS, Maniglio P, Triolo O, Brady DM. Inositol's and other nutraceuticals' synergistic actions counteract insulin resist ance in polycystic ovarian syndrome and metabolic syndrome:

state-of-the-art and future perspectives. Gynecol Endocrinol. 2016; 32(6): 431-438.
23. Laganà AS, Sapia F, La Rosa VL, Vitale SG. Comment on "Inositols: from physiology to rational therapy in gynecological clinical practice." Expert Opin Drug Metab Toxicol. 2016; 12(12): 1527.
24. Reyes-Muñoz E, Sathyapalan T, Rossetti P, Shah M, Long M, Buscema M, et al. Polycystic ovary syndrome: implication for drug metabolism on assisted reproductive techniques-a literature review. Adv Ther. 2018; 35(11): 1805-1815.
25. Delvigne A, Rozenberg S. Epidemiology and prevention of ovarian hyperstimulation syndrome (OHSS): a review. Hum Reprod Update. 2002; 8(6): 559-577.
26. Cluroe AD, Synek BJ. A fatal case of ovarian hyperstimulation syndrome with cerebral infarction. Pathology. 1995; 27(4): 344-346.
27. Obrzut B, Kuczyński W, Grygoruk C, Putowski L, Kluz S, Skret A. Liver dysfunction in severe ovarian hyperstimulation syndrome. Gynecol Endocrinol. 2005; 21(1): 45-49.
28. Selter J, Wen T, Palmerola KL, Friedman AM, Williams Z, Forman EJ. Life-threatening complications among women with severe ovarian hyperstimulation syndrome. Am J Obstet Gynecol. 2019; 220(6): 575. 575.e1-575.e11.
29. Romito I, Gulino FA, Laganà AS, Vitale SG, Tuscano A, Leanza G, et al. Renal and hepatic functions after a week of controlled ovarian hyperstimulation during in vitro fertilization cycles. Int J Fertil Steril. 2017; 11(1): 15-19.
30. Giugliano E, Cagnazzo E, Pansini G, Vesce F, Marci R. Ovarian stimulation and liver dysfunction: Is a clinical relationship possible? A case of hepatic failure after repeated cycles of ovarian stimulation. Clin Exp Reprod Med. 2013; 40(1): 38-41.
31. Nardo LG, Cheema P, Gelbaya TA, Horne G, Fitzgerald CT, Pease EH, et al. The optimal length of "coasting protocol" in women at risk of ovarian hyperstimulation syndrome undergoing in vitro fertilization. Hum Fertil (Camb). 2006; 9(3): 175-180.
32. Kılıç N, Özdemir Ö, Başar HC, Demircan F, Ekmez F, Yücel O. Cabergoline for preventing ovarian hyperstimulation syndrome in women at risk undergoing in vitro fertilization/intracytoplasmic sperm injection treatment cycles: a randomized controlled study. Avicenna J Med. 2015; 5(4): 123-127.
33. Costello MF, Chapman M, Conway U. A systematic review and meta-analysis of randomized controlled trials on metformin coadministration during gonadotrophin ovulation induction or IVF in women with polycystic ovary syndrome. Hum Reprod Oxf Engl. 2006; 21(6): 1387-1399.
34. D'Angelo A. Ovarian hyperstimulation syndrome prevention strategies: cryopreservation of all embryos. Semin Reprod Med. 2010; 28(6): 513-518.
35. Herrero L, Pareja S, Losada C, Cobo AC, Pellicer A, Garcia- Velasco JA. Avoiding the use of human chorionic gonadotropin combined with oocyte vitrification and GnRH agonist triggering versus coasting: a new strategy to avoid ovarian hyperstimulation syndrome. Fertil Steril. 2011; 95(3): 1137-1140.
36. Lin LT, Vitale SG, Chen SN, Wen ZH, Tsai HW, Chern CU, et al. Luteal phase ovarian stimulation may improve oocyte retrieval and oocyte quality in poor ovarian responders undergoing in vitro fertilization: preliminary results from a single-center prospective pilot study. Adv Ther. 2018; 35(6): 847-856.
37. Chern CU, Tsui KH, Vitale SG, Chen SN, Wang PH, Cianci A, et al. Dehydroepiandrosterone (DHEA) supplementation improves in vitro fertilization outcomes of poor ovarian responders, especially in women with low serum concentration of DHEA-S: a retrospective cohort study. Adv Ther. 2018; 35(6): 847-856.
38. Coskun S, Jaroudi KA, Hollanders JM, Atared AM, Roca GL. Recovery and maturation of immature oocytes in patients at risk for ovarian hyperstimulation syndrome. J Assist Reprod Genet. 1998; 15(6): 372-377.
39. Jaroudi KA, Hollanders JM, Elnour AM, Roca GL, Atared AM, Coskun S. Embryo development and pregnancies from in-vitro matured and fertilized human oocytes. Hum Reprod. 1999; 14(7): 1749-1751.
40. Gremeau A-S, Andreadis N, Fatum M, Craig J, Turner K, McVeigh E, et al. In vitro maturation or in vitro fertilization for women with polycystic ovaries? A case-control study of 194 treatment cycles. Fertil Steril. 2012; 98(2): 355-360.

Original Article

Effect of Vitamin D3 on Mitochondrial Biogenesis in Granulosa Cells Derived from Polycystic Ovary Syndrome

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Abstract

Background: Polycystic ovary syndrome (PCOS) is an endocrine disorder diagnosed by anovulation hyperandrogenism. Hyperandrogenism increases apoptosis, which will eventually disturb follicular growth in PCOS patients. Since mitochondria regulate apoptosis, they might be affected by high incidence of follicular atresia. This may cause infertility. Since vitamin D3 has been shown to improve the PCOS symptoms, the aim of study was to investigate the effects vitamin D3 on *mtDNA* copy number, mitochondrial biogenesis, and membrane integrity of granulosa cells in a PCOS-induced mouse model.

Materials and Methods: In this experimental study, the PCOS mouse model was induced by dehydroepiandrosterone (DHEA). Granulosa cells after identification by follicle-stimulating hormone receptor (FSHR) were cultured in three groups: 1. granulosa cells treated with vitamin D3 (100 nM for 24 hours), 2. granulosa cells without any treatments, 3. Non-PCOS granulosa cells (control group). Mitochondrial biogenesis gene (TFAM) expression was compared between different groups using real-time PCR. *mtDNA* copy number was also investigated by qPCR. The mitochondrial structure was evaluated by transmission electron microscopy (*TEM*). Hormonal levels were measured by an enzymelinked immunosorbent assay (ELISA) kit.

Results: The numbers of pre-antral and antral follicles increased in PCOS group in comparison with the non-PCOS group. Mitochondrial biogenesis genes were downregulated in granulosa cells of PCOS mice when compared to the non-PCOS granulosa cells. However, treatment with vitamin D3 increased *mtDNA* expression levels of these genes compared to PCOS granulosa cells with no treatments. Most of the mitochondria in the PCOS group were spherical with almost no cristae. Our results showed that in the PCOS group treated with vitamin D3, the *mtDNA* copy number increased significantly in comparison to PCOS granulosa cells with no treatments.

Conclusion: According to this study, we can conclude, vitamin D3 improves mitochondrial biogenesis and membrane integrity, mtDNA copy number in granulosa cells of PCOS mice which might improve follicular development and subsequently oocyte quality.

Keywords: Granulosa Cell, Mitochondrial Biogenesis, Mitochondrial DNA, Polycystic Ovary Syndrome, Vitamin D3

Citation: **Safaei Z, Bakhshalizadeh Sh, Nasr Esfahani MH, Akbari Sene A, Najafzadeh V, Soleimani M, Shirazi R. Effect of vitamin D3 on mitochondrial biogenesis in granulosa cells derived from polycystic ovary syndrome. Int J Fertil Steril. 2020; 14(2): 143-149. doi: 10.22074/ijfs.2020.6077.**

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women (1). Menstrual dysfunction, anovulation, hyperandrogenism, hirsutism, and polycystic ovaries are considered as the symptoms of PCOS (2). Hyperandrogenism stimulates follicular atresia in granulosa cells via apoptosis. As a result, apoptosis and oxidative stress can interfere with follicular growth in women suffering from PCOS (3). Therefore, disruption of follicular growth in patients suffering from PCOS is related to granulosa cells apoptosis and oxidative stress caused by high production of reactive oxygen species (ROS). Since mitochondria can regulate apoptosis and ROS production, these organelles may be affected by high rates of follicular atresia in the PCOS patients (4). Changes in the mitochondrial function might cause insulin resistance,

Received: 23/August/2019, Accepted: 05/January/2020
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Royan Institute
International Journal of Fertility and Sterility
Vol 14, No 2, July-September 2020, Pages: 143-149

oxidative stress, hyperandrogenism, and glucose intolerance, leading to the appearance of PCOS symptoms (5-7). The disturbance of mitochondrial function in granulosa cells may cause some disorders in oocyte function, maturation, and fertilization. This may also affect the fertility of PCOS patients by reducing the oocyte quality. Therefore, the proper functionality of mitochondria is of importance in this regard (5). Mitochondria play a key role in the determination of numerous factors involved in the reproduction, such as oocyte quality, follicular growth, development, and granulosa cell proliferation (6). Mitochondria also mediate various cellular processes, including apoptosis, ROS production, calcium signaling, adenosine triphosphate (ATP) synthesis, pyrimidine synthesis, and Fe-S protein synthesis (3, 8). Furthermore, mitochondria, as the main organelle for cellular ROS production, can also impair mitochondrial DNA (*mtDNA*), which may subsequently be the cause of different diseases. Being more susceptible to oxidative damage and attaining high rates of mutations are more common in the mitochondrial genome than nuclear DNA due to the adjacency to the electron transport chain (ETC), lack of sheltering histones and inefficient DNA repair capabilities (9). *mtDNA* of mammals is nearly 16 kb in size, encrypting 13 proteins of the oxidative phosphorylation (OXPHOS) complexes, 22 ribosomal RNAs (*rRNA*), and transfer RNAs (*tRNA*) that are required for mitochondrial mRNA translation. *mtDNA*, like nuclear DNA, can influence mitochondrial gene expression, biogenesis, and function through epigenetic modifications (10). It is of note to mention that mitochondrial biogenesis can also influence mtDNA and nuclear-encoded protein synthesis, the congregation of the double genetic origin derived proteins, *mtDNA* replication as well as cell growth and proliferation (11). Mitochondrial biogenesis is hard to understand and needs several processes, such as synthesis of *mtDNA* and nuclear genes (12). The main gene in mitochondrial biogenesis that is critical for *mtDNA* transcription and maintenance is mitochondrial transcription factor A (*TFAM*). Mitochondrial biogenesis is also regulated by nuclear genes such as NRF2, which controls the other factors in mitochondria (11).

Since oocyte quality is a crucial factor for conception in PCOS patients and that depends on mitochondrial function and structure, prescription of an appropriate medication may improve fertility rate as a consequence of improved oocyte quality (13). Different treatments have been trialed, and vitamin D3 is one of which to have shown signs of improvement in PCOS patients (6). Vitamin D3 has been used before to alleviate signs of insulin resistance, hyperandrogenism, and oxidative stress in PCOS patients and other metabolic disorders (14). Vitamin D3 also has an important role in calcium homeostasis, cellular proliferation, and differentiation(15). Recently it has been demonstrated that the low level of vitamin D can result in excessive androgen secretion, insulin resistance, and follicular growth interruption in the patients suffering from PCOS. These occur through the decline of sex hormone-binding globulin (SHBG) levels, insulin receptors, and calcium dysregulation (8). The serum concentration of 25-hydroxyvitamin D in women with PCOS is less than 20 ng/ml, which can exacerbate PCOS symptoms (16). Therefore, in this study, we aimed to investigate the effects of vitamin D3 on the mitochondrial biogenesis, membrane integrity, and *mtDNA* copy numbers in the granulosa cells isolated from PCOS-induced mice.

Materials and Methods

PCOS animal model and assessment of morphology

This is an experimental study that the effect of vitamin D3 on mitochondrial biogenesis in a PCOS mouse model was investigated. Androgen excess and other symptoms of PCOS were induced by the injection of DHEA (Sigma, Austria), 6 mg/100 g body weight. DHEA was dissolved in 95% ethanol (0.01 mL) and mixed with sesame oil (0.09 mL). Subsequently, it was injected subcutaneously into female BALB/C mice (25 days old) for 20 consecutive days before reaching puberty (PCOS group, n=20). As a vehicle control, 0.1 mL of sesame oil (Sigma, Austria) and 0.01 mL of 95% ethanol (Sigma, Austria) were injected into another group of the same mouse strain for 20 consecutive days (n=20). A Control group of the same mouse strain without any treatment was also considered (n=20). The mice were kept at room temperature (25 ± 1°C, RT), with enough food and water, and under diurnal modulation by daily light. All the animal trials were performed in agreement with the Institutional Animal Care Committee of Iran University of Medical Sciences and Health Services for animal welfare. (ethics code: IR.IUMS.REC 1396.29969). The weight changes in mice were measured every day. Vaginal smears were also taken every day over the 20-day course of treatment. The mice were sacrificed by cervical dislocation. For histological assessments, the ovaries were subsequently fixed with 10% formalin (Merck, Germany). Next, 5-µm sections were made with a microtome, and the sections were immersed in xylene and ethanol with different grades for deparaffinization and rehydration, respectively (Merck, Germany). The ovaries were then stained with hematoxylin and eosin (DAKO, USA). For morphology assessment, the ovaries assessed by a Nikon microscope (Nikon, Japan), and photographs were taken.

Sex hormones assessments

For the analysis of sex hormones, cardiac blood samples were collected using needles. Blood serum was subsequently separated using a centrifuge machine at (300 rpm, 4°C, 10 minutes) and follicle-stimulating hormone (FSH), luteinizing hormone (LH), 17β-estradiol (E2) and progesterone levels were measured by an ELISA kit (Abcam, Cambridge, UK) according to the manufacturer's guidelines.

Isolation and culture of granulosa cells

The ovaries of 45-day BALB/C mice (DHEA-reated

and the vehicle group) were removed after the mice were sacrificed via cervical dislocation. For aspiration of the follicles, 25-gauge needles were used, and the follicles were aspirated in a solution made of phosphate buffer saline (PBS) and 1.0% bovine serum albumin (BSA) (Invitrogen, USA). 70-µm cell strainers (BD Falcon, MA, USA) were used to isolate granulosa cells from the other cells and tissues. Subsequently, granulosa cells were separated from the oocytes with a 40-µm cell strainer (BD Falcon, MA, USA). Blood cell contamination was removed by RBC lysis buffer after centrifugation at 1000 rpm (4°C, 10 minutes). Then, the pellet was mixed with phenol red-free DMEM/F12 medium containing 10% fetal bovine serum (Sigma, Austria). The medium was centrifuged at 1000 rpm (4°C, 10 minutes). Next, the pellet was removed and transferred to cell culture dishes containing DMEM-F12, 10% FBS (Sigma, Austria), 100 mg/mL streptomycin (Sigma, Austria), 100 IU/mL penicillin (Sigma, Austria), 2 mM glutamine (Sigma, Austria), 1 mM sodium pyruvate (Sigma, Austria). The culture dishes were then incubated at 37°C, with 5% CO_2 and 95% humidity.

Identification of isolated GCs

To identify granulosa cells, an antibody against FSHR, a specific marker of granulosa cells, was used. Affinity-purified rabbit anti-follicle stimulating hormone receptor (FSHR) polyclonal antibody was purchased from antibodies-online (ABIN1872743). First, the cells were spread on a slide using a cytospin centrifugation device, and the slides were then immersed in a cold normal buffered formalin (NBF) solution to be fixed. Subsequently, the cells were washed with PBS and blocked using PBS-Triton/BSA. Afterward, the primary FSHR antibody was added to granulosa cells overnight. The following morning the cells were washed three times with PBS and were subsequently treated with the secondary FSHR antibody for 30 minutes. DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Preparations were washed in PBS before mounting on glass slides. Slides were viewed on an epifluorescence microscope and captured with a digital camera.

Experiment design

The treatment groups for granulosa cells were as follows:

1. PCOS granulosa cells treated with vitamin D3 (100 nM) for 24 hours (17, 18)

2. PCOS granulosa cells without any treatments

3. Non-PCOS granulosa cells (control group)

Reverse transcription- polymerase chain reaction and quantitative reverse transcription- polymerase chain reaction

The Trizol reagent (Sigma, Austria) was used to extract the total RNA of granulosa cells in all groups. Then, chloroform was added to the mixture of granulosa cells and the Trizol reagent. Afterward, the mixture was centrifugated at 1000 rpm (4 C, 10 minutes). The upper phase containing the total RNA was collected. Next, the total RNA was washed with 75% ethanol, allowed to air dry, and then reconstituted in diethylpyrocarbonate (DEPC) water. Using a cDNA synthesis kit (Thermo Scientific, USA), the total RNA was reverse-transcribed according to the manufacturer's guideline. In summary, a mixture of the random hexamer, first-strand buffer (all from Fermentas), DNase-(Fermentas Inc, MD, USA) treated RNA, RiboLockTM RNase inhibitor, dNTP Mix, Dithiothreitol (0.1M) and SuperScriptTM II Reverse Transcriptase was made for reverse transcription of each sample. The thermocycler (company) was set at 25°C for 10 minutes, 43°C for 40 minutes, and 75°C for 15 minutes. Quantitative PCR was performed using 1 µl of cDNA in a reaction consisting of ROXTM Reference Dye, SYBR Premix EX TaqTM (Takara, Japan), and 1 µl of the desired primer. The β-actin gene was utilized as a housekeeping gene. The reactions were amplified with StepOne™ Real- Time PCR System (Applied Biosystems, MA, USA) as following: denaturation at 95 °C for 10 seconds, 35 cycles of amplification (95°C for 5 seconds and 60°C for 30 seconds), separation stage at 95°C for 15 seconds, 60°C for 1 minutes, and 95°C for 15 seconds. Using oligo 7.60 software to design primers. The TFAM forward primer was CCG AGC TCC TCC TCC TTT GC and the TFAM reverse primer was CCT ACA ACG CAG CGA CCG AG.

Mitochondrial DNA quantification

For the measurement of the *mtDNA* copy number, quantitative polymerase-chain-reaction (qRT-PCR) was used. Forward primer and reverse primer were used to analyze *mtDNA*. SYBR Green I Master Mix (10 µl) (Sigma, Austria), which contains 10 pmol of reverse primer and 10 pmol of forward primer was mixed with DNA (10 ng). The qPCR set-up consisted of 4 segments: 50°C for 2 minutes, 95°C for 10 seconds followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing at 59°C for 35 seconds, and extension at 72°C for 1 minutes. For each qPCR reaction, the copy number of the *mtDNA* and the threshold cycle number (Ct) of the β-actin gene were measured. The runs were replicated at least two times, and the normalization was performed against the housekeeping gene, β-actin. For the quantification of the *mtDNA* copy number, the double delta Ct analysis was applied.

Electron microscopy of mitochondria

Isolated granulosa cells were fixed using 2.5% glutaraldehyde in PBS and then treated with 1.0% osmium tetroxide in the same buffer for the post-fixation procedure. For performing the dehydration process, ethanol and propylene oxide were used. Then inserted in epoxy resin, and sectioned. Using ethanolic uranyl acetate to contrast the sections and lead citrate and observed under a transmission electron microscope (Zeiss LEO 906 (TEM), 100 kV, Germany).

Statistical analysis

The data in this experiment are expressed as the means and standard error of the mean (19) for three independent biological replicates. Statistical significance between different groups was evaluated and analyzed by on-way analysis of variance (ANOVA), followed by Tukey's post hoc test. The level of statistical significance was set at $P<0.05$. The SPSS software (version 21.0) was utilized for the data analysis.

Results

Hormonal levels and cycle alteration in mice

In the PCOS group induced by DHEA, the serum level of estradiol and LH were higher when compared to control and vehicle groups, respectively (Table 1). The lower amount of FSH serum was detected in the PCOS group versus the control group and vehicle group due to estradiol negative feedback. The ratio of LH/FSH was significantly increased in the PCOS group in comparison to the vehicle group (Table 1). The estrous cycle was irregular in the PCOS group and ultimately stopped, whereas, in the control and vehicle group, normal cycles (nearly 5-7 days) continued as normal. Using mice in the control and vehicle groups that were just in the estrous cycle to exclude the influence of the estrous cycles on other measurements.

Table 1: Hormonal levels

Hormone	Control	Vehicle	DHEA
Estradiol (pg/mL)	132 ± 9.10	142 ± 8.52	3786 ± 13.1**
FSH (IU/L)	6. 59 ± 0.82	6.84 ± 0.29	4.11 ± 0.64
LH (IU/L)	5.60 ± 0.11	6.13 ± 0.38	18.58 ± 0.82*
LH/FSH (IU/L)	0.84 ± 0.13	0.89 ± 1.31	4.52 ± 1.28**
Progesterone (pg/mL)	3.647 ± 0.69	3.268 ± 0.54	2.369 ± 0.19

Data are presented as mean ± SD. *; P<0.05, **; P<0.005, FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, DHEA; dehydroepiandrosterone.

Histological analysis for characterization of PCOS ovaries

Upon H&E staining, the specimen was analyzed under the light microscope. Normal follicles were detected at various developmental stages in the vehicle group. Corpus luteum was also observed in the control group, which was an indicator of normal ovulation (Fig.1A). Due to the seizure of the estrus cycle, no corpus luteum was detected in the PCOS group (Fig.1B).

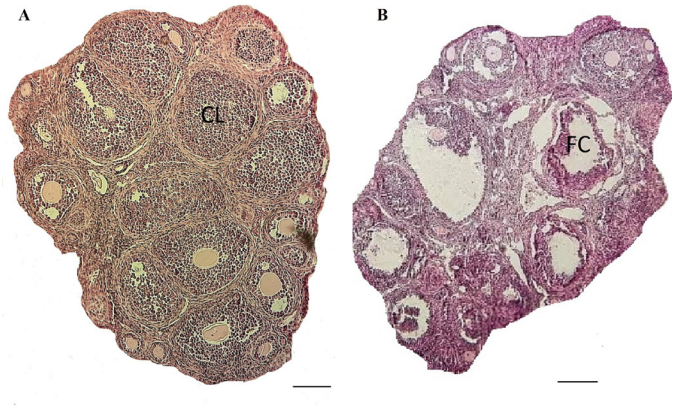

Fig. 1: Histological assessment of ovaries. **A.** Follicles of normal ovaries represented follicles at different stages, and corpus luteum (CL) and **B.** Ovaries of the polycystic ovary syndrome (PCOS) model induced by dehydroepiandrosterone (DHEA) revealed antral and pre-antral follicles and some cysts (FC) were observed in H&E staining. No corpus luteum was observed in the PCOS ovary .Scale bar: 50 µm.

Mitochondrial biogenesis gene expression

To assure that the cells being experimented on granulosa cells, a granulosa cell antibody was used against FSHR. Photograph analysis showed that the target cells were stained with this antibody, indicating that they were granulosa cells (Fig.2). Thus, for the treatment of granulosa cells, vitamin D3 (100 nM) was used for 24 hours. Subsequently, RNA was extracted, and Reverse transcription- polymerase chain reaction (RT-PCR) performed to measure the expression of mitochondrial biogenesis gene (TFAM) in different groups. Vitamin D3 increased the expression of TFAM in the PCOS group (Fig.3) by 5-fold compared to the PCOS group without any vitamin D3 treatment. This indicates that vitamin D3 might stimulate mitochondrial biogenesis in PCOS-induced granulosa cells.

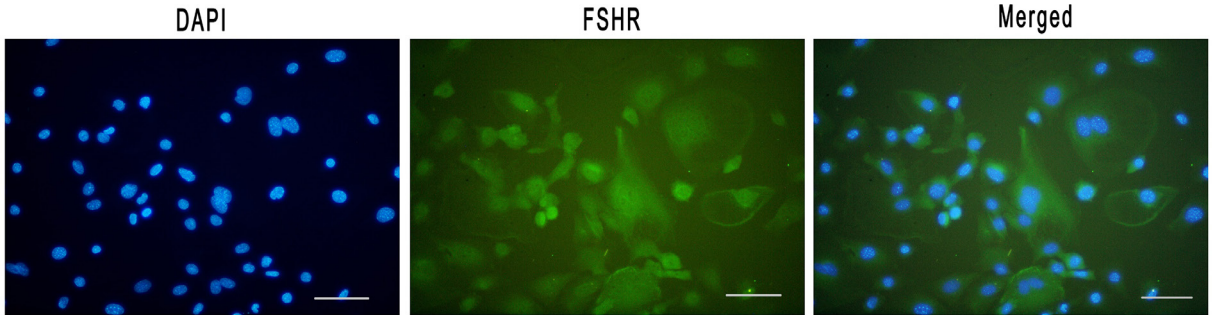

Fig. 2: Follicle-stimulating hormone receptor (FSHR) (specific markers of granulosa cells) was investigated. The FSHR expression in isolated granulosa cells (green) was observed. Nuclei (blue) were stained by 4',6-diamidino-2-phenylindole (DAPI). Scale bar: 100 µm.

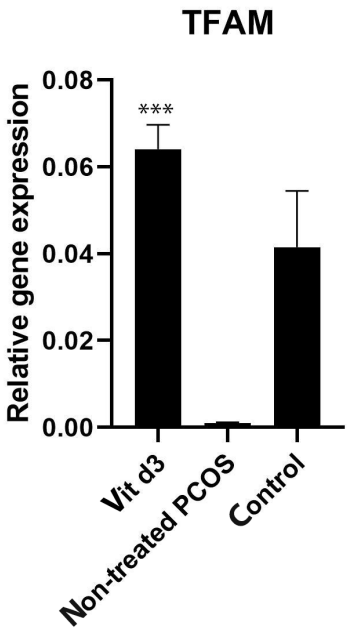

Fig. 3: The expression of TFAM (mitochondrial biogenesis gene) in cultured granulosa cells of DHEA-induced PCOS BALB/C mice was compared between three groups. Granulosa cells were pre-incubated in the serumfree medium in the presence or absence of vitamin D3. The expression of the mitochondrial biogenesis gene was upregulated in the vitamin D3 group. It is also revealed that the gene expression was declined in PCOS granulosa cells in comparison with non-PCOS healthy granulosa cells (control group), ***; P<0.05, DHEA; Dehydroepiandrosterone, and PCOS; Polycystic ovary syndrome.

Mitochondrial DNA

For the analysis of the *mtDNA*, qPCR was performed. Our results revealed that in the PCOS group treated with vitamin D3, the *mtDNA* copy number increased significantly in comparison to the non-treated PCOS group (Fig.4). Data analysis by the quartile distribution of *mtDNA* copy number in the non-treated PCOS group showed an association between *mtDNA* copy number and PCOS risk.

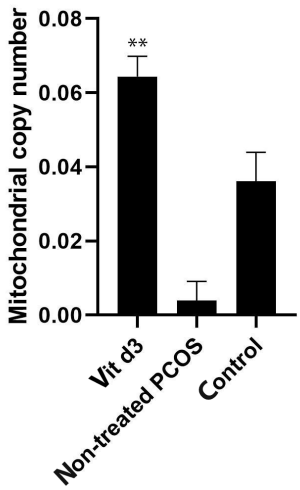

Fig. 4: The mitochondrial DNA copy number (mtDNA) in cultured granulosa cells of DHEA-induced PCOS BALB/C mice was compared between three groups. Granulosa cells were pre-incubated in the serum-free medium in the presence or absence of vitamin D3. The mitochondrial DNA copy number was significantly increased in the vitamin D3 group in comparison with the non-treated PCOS group (**; P<0.05). It is also revealed that the mitochondrial DNA copy number was declined in the non-treated PCOS granulosa cells in comparison with the non-PCOS healthy granulosa cells (control group). DHEA; Dehydroepiandrosterone, PCOS; Polycystic ovary syndrome.

Transmission electron microscopy of mitochondria structure

For the evaluation of the alterations of the mitochondria structure, transmission electron microscopy was employed. Most of the mitochondria in the PCOS group without any treatment were spherical, with almost no cristae; however, in the PCOS group treated with vitamin D3 as well as in the non- PCOS group (control group) intact inner and outer membrane and a clear intermembrane space was observed (Fig.5).

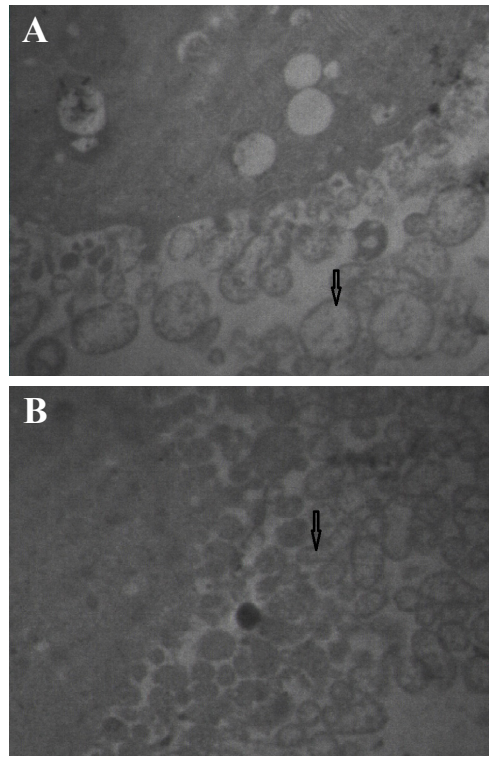

Fig. 5: Mitochondria membrane structure (TEM). **A.** PCOS group without any treatments were spherical with almost no cristae (arrow: abnormal mitochondria). **B.** PCOS group treated with vitamin D3 and also in the non-PCOS group (control group) include undamaged mitochondria (arrow: normal mitochondria).

Discussion

The present study demonstrated that vitamin D3 affected *mtDNA* copy number, mitochondrial structure, and mitochondrial biogenesis in granulosa cells of a PCOS-induced mouse model in comparison with healthy normal ovaries. PCOS is regularly described by oligomenorrhea, chronic anovulation, hyperandrogenism, and hyperinsulinemia (20). Androgenic hormones, such as DHEA, testosterone, and androstenedione, cause some problems in the patients suffering from PCOS (2). According to previous studies, in the PCOS ovaries, the atretic follicles increased that caused by hyperandrogenism, which is critical in the pathogenesis of PCOS. High levels of androgen in women that suffer from PCOS might intensify follicular atresia and follicular development disruption that might cause subfertility (4). According to our previous study (17), to mimic the hyperandrogenism condition, for induction of the PCOS model and also confirmation of

the abnormal hormonal level and ovarian morphological features in PCOS mice, DHEA was injected into the 25-day old female mice intraperitoneally. Androgen excess, insulin resistance, and disturbed follicular development are some symptoms of this disorder that might interfere with female fertility (21-23). The levels of LH, estradiol, along with the ratio of LH to FSH were increased in the PCOS-induced mice compared to the vehicle group. The level of FSH was comparatively decreased in the PCOS group caused by the estradiol level feedback.

Disrupted ovulation and oocyte quality induced by hyperandrogenism can be improved by different treatments such as metformin and spironolactone (7). Besides hormonal treatments, various supplements such as vitamin D3 have been shown to improve the PCOS symptoms (24). Moreover, several lines of evidence demonstrate the positive role of vitamin D3 in some disorders, such as premature ovarian failure (POF), endometriosis, PCOS, and male infertility (17, 25). It has been demonstrated that vitamin D3 might stimulate follicular development in patients with PCOS; however, it could not alleviate disrupted lipid and glucose metabolism (26-28). A large body of studies has shown that vitamin D3 has constructive effects on alleviating the symptoms of ovulation disorders and insulin resistance in women suffering from PCOS disorder (17, 29). Hormonal fluctuations in PCOS women can be improved by vitamin D3; however, the duration of treatment can influence the degree of symptom alleviation (14, 26). Underlying mechanisms as to how vitamin D3 exerts its effects are yet to be elucidated.

For the assessment of the effect of vitamin D3 on mitochondrial biogenesis, isolated granulosa cells from PCOS ovaries were treated with vitamin D3. Mitochondrial membrane integrity and alteration in mtDNA copy numbers were also evaluated. It has been demonstrated that mitochondria, as the powerhouse of the cell, are of importance for optimum oocyte quality and fertilization. Poor oocyte quality and subsequent embryonic development could be attributed to mitochondrial dysfunction (10, 15). We hypothesized that vitamin D3 may improve *mtDNA* copy number, mitochondrial membrane integrity, and biogenesis.

In the present study, we demonstrated that mitochondrial biogenesis could be upregulated after 24 hours of treatment with vitamin D3. The findings showed that vitamin D3, as a supplementation, improves the main mitochondrial biogenesis marker (*TFAM*) in the granulosa cells of PCOS ovaries. According to some evidence, *TFAM* plays an important role in mitochondrial biogenesis (12, 30-33). It is revealed that total antioxidant capacity (TAC) raise by vitamin D3 and also vitamin D3 may alleviate the hormonal disturbances in women with PCOS (34, 35). Since our results showed that vitamin D3 has an improvement effect on ovulation problems and follicular disruption, we could understand that vitamin D3 might have an important role in declining the atretic follicles and alleviating the development of follicles via upregulating the mitochondrial biogenesis main gene and mitochondrial membrane integrity.

In this study, for the first time, we have shown the vitamin D3 effect on mtDNA copy number and mitochondrial membrane integrity in a mouse model of PCOS granulosa cells. Our results revealed that most of the mitochondria in the PCOS group were spherical with almost no cristae. In line with our study, Longfei et al. revealed a distorted mitochondrial structure and diminished membrane integrity in the PCOS group (10). Oocytes of the PCOS mouse model, induced with DHEA, have demonstrated disrupted mitochondrial biogenesis, decreased *mtDNA* copy number, and distorted mitochondrial ultrastructure that is in agreement with our findings in this study (10, 36). Ding et al. also observed that mitochondrial dysfunction, due to mtDNA mutation, has a role in the manifestation of PCOS symptoms that is in line with our results (37). Reduced *mtDNA* copy number is associated with poor oocyte quality and subsequent compromised embryo development and implantation (10) (15). Our findings showed decreased *mtDNA* copy number in the PCOS group (non-treated), which is increased upon treatment with vitamin D3.

In line with this finding, researchers showed reduced mtDNA copy number in the PCOS patients (37, 38). Also, in agreement with our findings, a case-control study showed reduced *mtDNA* copy number in Korean women suffering from PCOS (9, 10, 39). Bhanoori et al. (9) also demonstrated that mtDNA copy number severely decreased in PCOS patients.

Conclusion

According to our results, *mtDNA* copy number, the biogenesis might be affected by vitamin D3 in PCOS granulosa cells. We nominate that mitochondrial biogenesis genes expression might be increased by vitamin D3. Therefore, vitamin D3 can have a significant role in the alleviation of mitochondria and follicular damages in PCOS ovaries. However, extensive studies are needed to determine the optimal dose and duration of treatment with vitamin D3 in PCOS women.

Acknowledgements

This study was supported by a grant from Iran University of Medical Sciences and Health Services, Tehran, Iran (grant no.29969). Authors would like to acknowledge Prof. Dr. Erich Gnaiger, Medical University of Innsbruck, Austria, Department of Visceral, Transplant and Thoracic Surgery, D. Swarovski Research Laboratory, Mitochondrial Physiology.

Authors' Contributions

Z.S.; Performed the experiments, analyzed the data, and wrote the manuscript. S.H.B., M.S., M.H. N-E.; Assisted in performing the study. R.S., Z.S., A.A.S., V.N.; Designed the study, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

References

1. Finsterer J, Zarrouk-Mahjoub S. Polycystic ovary syndrome in mitochondrial disorders due mtDNA or nDNA variants. Am J Transl Res. 2018; 10(1): 13-15.
2. Badawy A, State O, Abdelgawad S. N-Acetyl cysteine and clomiphene citrate for induction of ovulation in polycystic ovary syndrome: a cross-over trial. Acta Obstet Gynecol Scand. 2007; 86(2): 218-222.
3. Sheu SS, Nauduri D, Anders MW. Targeting antioxidants to mitochondria: a new therapeutic direction. Biochim Biophys Acta. 2006; 1762(2):256-265.
4. Cai L, Ma X, Liu S, Liu J, Wang W, Cui Y, et al. Effects of upregulation of Hsp27 expression on oocyte development and maturation derived from polycystic ovary syndrome. PloS One. 2013; 8(12): e83402.
5. Zhao H, Zhao Y, Li T, Li M, Li J, Li R, et al. Metabolism alteration in follicular niche: The nexus among intermediary metabolism, mitochondrial function, and classic polycystic ovary syndrome. Free Rad Biol Med. 2015; 86: 295-307.
6. Victor VM, Rocha M, Banuls C, Sanchez-Serrano M, Sola E, Gomez M, et al. Mitochondrial complex I impairment in leukocytes from polycystic ovary syndrome patients with insulin resistance. J Clin Endocrinol Metab. 2009; 94(9): 3505-3512.
7. Speroff L, Fritz MA. The Clinical gynecologic endocrinology and infertility. 7th ed. Philadelphia: lippincott Williams & wilkins; 2005.
8. May-Panloup P, Boucret L, Chao de la Barca J M, Desquiret-Dumas V, Ferre-L'Hotellier V, Moriniere C, et al. Ovarian ageing: the role of mitochondria in oocytes and follicles. Hum Reprod Update. 2016; 22(6): 725-743.
9. Reddy TV, Govatati S, Deenadayal M, Sisinthy S, Bhanoori M. Impact of mitochondrial DNA copy number and displacement loop alterations on polycystic ovary syndrome risk in south Indian women. Mitochondrion. 2019; 44:35-40.
10. Jia L, Li J, He B, Jia Y, Niu Y, Wang C, et al. Abnormally activated one-carbon metabolic pathway is associated with mtDNA hypermethylation and mitochondrial malfunction in the oocytes of polycystic gilt ovaries. Sci Rep. 2016; 6: 19436.
11. Wenz T. Regulation of mitochondrial biogenesis and PGC-1α under cellular stress. Mitochondrion. 2013; 13(2): 134-142.
12. Jornayvaz FR, Shulman GI. Regulation of mitochondrial biogenesis. Essays Biochem. 2010; 47: 69-84.
13. Jain P, Jain M, Haldar C, Singh TB, Jain S. Melatonin and its correlation with testosterone in polycystic ovarian syndrome. J Hum Reprod Sci. 2013; 6(4): 253258.
14. Akbari M, Ostadmohammadi V, Lankarani KB, Tabrizi R, Kolahdooz F, Heydari ST, et al. The Effects of vitamin D supplementation on biomarkers of inflammation and oxidative stress among women with polycystic ovary syndrome: A systematic review and metaanalysis of randomized controlled trials. Horm Metab Res. 2018; 50(04): 271-279.
15. Wang Q, Frolova AI, Purcell S, Adastra K, Schoeller E, Chi MM, et al. Mitochondrial dysfunction and apoptosis in cumulus cells of type I diabetic mice. PLoS One. 2010; 5(12): e15901.
16. Bakhshalizadeh S, Amidi F, Alleyassin A, Soleimani M, Shirazi R, Shabani Nashtaei M. Modulation of steroidogenesis by vitamin D3 in granulosa cells of the mouse model of polycystic ovarian syndrome. Syst Biol Reprod Med. 2017; 63(3): 150-161.
17. Lee CT, Wang JY, Chou KY, Hsu MI. 1, 25-Dihydroxyvitamin D3 increases testosterone-induced 17beta-estradiol secretion and reverses testosterone-reduced connexin 43 in rat granulosa cells. Reprod Biol Endocrinol. 2014; 12: 90.
18. Barrett G, Greenwood R, Ross K. Integrating interprofessional education into 10 health and social care programmes. J Interprof Care. 2003; 17(3): 293-301.
19. Diamanti-Kandarakis E. Polycystic ovarian syndrome: pathophysiology, molecular aspects and clinical implications. Expert Rev Mol Med. 2008; 10:e3.
20. Sander V, Luchetti CG, Solano ME, Elia E, Di Girolamo G, Gonzalez C, et al. Role of the N, N′-dimethylbiguanide metformin in the treatment of female prepuberal BALB/c mice hyperandrogenized with dehydroepiandrosterone. Reproduction. 2006; 131(3): 591-602.
21. Anderson E, Lee MT, Lee GY. Cystogenesis of the ovarian antral follicle of the rat: ultrastructural changes and hormonal profile following the administration of dehydroepiandrosterone. Anat Rec. 1992; 234(3): 359-382.
22. Henmi H, Endo T, Nagasawa K, Hayashi T, Chida M, Akutagawa N, et al. Lysyl oxidase and MMP-2 expression in dehydroepiandrosterone- induced polycystic ovary in rats. Biol Reprod. 2001;64(1):157-162.
23. Wang R, Kim BV, van Wely M, Johnson NP, Costello MF, Zhang H, et al. Treatment strategies for women with WHO group II anovulation: systematic review and network meta-analysis. BMJ. 2017; 356: j138.
24. Pál É, Hadjadj L, Fontányi Z, Monori-Kiss A, Mezei Z, Lippai N, et al. Vitamin D deficiency causes inward hypertrophic remodeling and alters vascular reactivity of rat cerebral arterioles. PloS One. 2018; 13(2): e0192480.
25. Çelik LS, Kuyucu Y, Yenilmez ED, Tuli A, Dağlıoğlu K, Mete UÖ. Effects of vitamin D on ovary in DHEA-treated PCOS rat model: A light and electron microscopic study. Ultrastruct Pathol. 2018; 42(1): 55-64.
26. Hadjadj L, Várbíró S, Horváth EM, Monori-Kiss A, Pál É, Karvaly GB, et al. Insulin resistance in an animal model of polycystic ovary disease is aggravated by vitamin D deficiency: Vascular consequences. Diab Vasc Dis Res. 2018; 15(4):294-301.
27. Fang F, Ni K, Cai Y, Shang J, Zhang X, Xiong C. Effect of vitamin D supplementation on polycystic ovary syndrome: A systematic review and meta-analysis of randomized controlled trials. Complement Ther Clin Pract. 2017; 26: 53-60.
28. Rashidi H, Ghaderian SB, Moradi L. The effect of vitamin D3 on improving lipid profile, fasting glucose and insulin resistance in polycystic ovary syndrome women with vitamin D deficiency. Middle East Fertil Soc J. 2018; 23(3):178-183.
29. Babayev E, Seli E. Oocyte mitochondrial function and reproduction. Curr Opin Obstet Gynecol. 2015; 27(3): 175-181.
30. Fernandez-Marcos PJ, Auwerx J. Regulation of PGC-1α, a nodal regulator of mitochondrial biogenesis. Am J Clin Nutr. 2011; 93(4): 884S-90.
31. Brenmoehl J, Hoeflich A. Dual control of mitochondrial biogenesis by sirtuin 1 and sirtuin 3. Mitochondrion. 2013; 13(6): 755-761.
32. Oyewole AO, Birch-Machin MA. Mitochondria-targeted antioxidants. FASEB J. 2015; 29(12): 4766-4771.
33. Ge H, Zhang F, Shan D, Chen H, Wang X, Ling C, et al. Effects of mitochondrial uncoupling protein 2 inhibition by genipin in human cumulus cells. Biomed Res Int. 2015; 2015.
34. Turner N, Heilbronn LK. Is mitochondrial dysfunction a cause of insulin resistance? Trends Endocrinol Metab. 2008; 19(9): 324-330.
35. Lee CT, Wang JY, Chou KY, Hsu MI. 1, 25-dihydroxyvitamin D3 modulates the effects of sublethal BPA on mitochondrial function via activating PI3K-Akt pathway and 17β-estradiol secretion in rat granulosa cells. J Steroid Biochem Mol Biol. 2019;185: 200-211.
36. Weghofer A, Munne S, Chen S, Barad D, Gleicher N. Lack of association between polycystic ovary syndrome and embryonic aneuploidy. Fertil Steril. 2007; 88(4):900-905.
37. Ding Y, Jiang Z, Xia B, Zhang L, Zhang C, Leng J. Mitochondria-targeted antioxidant therapy for an animal model of PCOS-IR. Int J Mol Med. 2019; 43(1): 316-324.
38. Ilie IR. Advances in PCOS Pathogenesis and progression—mitochondrial mutations and dysfunction. Adv Clin Chem. 2018; 86: 127-155.
39. Lee SH, Chung DJ, Lee HS, Kim TJ, Kim MH, Jeong HJ, et al. Mitochondrial DNA copy number in peripheral blood in polycystic ovary syndrome. Metabolism. 2011; 60(12): 1677-1682.

Short Communication

The Viability of Human Testis-Derived Cells on Human Serum Albumin-Based Scaffold as An Artificial Male Germ Cell Niche

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Abstract

Azoospermia is one of the challenging disorders affecting couples who are afflicted with infertility. Human testis-derived cells (hTCs) are suitable candidates for the initiation of in-vitro spermatogenesis for these types of patients. The current study aimed to assess the proliferation of hTCs through the cell culture on the three-dimensional (3D) porous scaffolds. Cells harvested from the testicular sperm extraction (TESE) samples of the azoospermic patients were cultured on the 3D porous scaffolds containing human serum albumin (HSA)/tri calcium phosphate nanoparticles (TCP NPs) for two weeks. The proliferation/viability of the cells was assessed using the MTT assay, along with H&E histological staining method. The MTT assay showed that hTCs could stay alive on this scaffold with 50 and 66.66% viability after 7 and 14 days, respectively. Such viability was not significantly different when compared with cells grown on monolayer flask culture ($P>0.05$). Therefore, 3D HSA/TCP NPs scaffolds could be used for the reconstitution of the artificial human somatic testicular niche for future applications in regenerative medicine for male infertility.

Keywords: Azoospermia, Human Serum Albumin, Scaffold, Spermatogenesis, Testis

Citation: Borzouie Z, Hekmatimoghaddam SH, Jebali A, Aflatoonian B. The viability of human testis-derived cells on human serum albumin-based scaffold as an artificial male germ cell niche. Int J Fertil Steril. 2020; 14(2): 150-153. doi:10.22074/ijfs.2020.6086.

Introduction

Spermatogenesis is a vital developmental phenomenon in which the production of haploid male gametes from diploid spermatogonia occurs in mammalian testes. It starts from spermatogonial stem cells (SSCs) in the seminiferous tubules with gradual differentiation toward spermatocytes, spermatids, and spermatozoa (1).

Azoospermia is defined as the lack of spermatozoa in semen, and it is one of the challenging disorders in male infertility. Approximately 1 per 200 men in any population is diagnosed as azoospermic. Although treatments, such as percutaneous epididymal sperm aspiration (PESA) and testicular sperm extraction (TESE) followed by intracytoplasmic spermatozoa injection (ICSI), are available for azoospermic patients, there is still need to improve the therapeutic approaches. As of 2004, several studies have shown that embryonic stem cells may restore the spermatogenesis and functional sperms in mouse and human, known as *in vitro* gametogenesis (IVG) (2-4).

Moreover, in parallel, several groups have demonstrated the pluripotency of germ-line stem cells (GSCs) following SSCs culture in rodents, though, there is a debate about pluripotency of GSCs in primates and humans (5).

Beside mitotic and meiotic divisions of SSCs for the production of mature spermatozoa, there are different significant factors that play roles in this process. These elements include somatic cells (such as Leydig cells, myoid cells, and Sertoli cells), extracellular matrix (ECM) components (including laminin, collagen type IV and collagen type I), as well as growth factors and hormones [including bFGF, glial cell-derived nerve factor, glial cellderived nerve factor (GDNF), and testosterone] that are capable of forming a complex microenvironment where spermatogenesis occurs (6).

By means of scaffolds, cells, and growth factors, tissue engineering has provided enormous hope and interest in academia, industry, and the public to cure various disorders (7). A recent review article by Del Vento et al. (8) indicates that tissue engineering might be helpful for the

Received: 5/September/2019, Accepted: 24/December/2019
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Royan Institute
International Journal of Fertility and Sterility
Vol 14, No 2, July-September 2020, Pages: 150-153

transplantation of germ cells by improving the cellular environment using scaffolds to enhance graft outcomes for prepubertal boys exposed to gonadotoxic treatments. Following our previous animal studies performed on mice (9, 10), the aim of this study was to evaluate the viability and proliferation of the cells derived from human TESE samples, which were cultivated on a novel three-dimensional (3D) nano-scaffold containing human serum albumin (HSA)/tri calcium phosphate nanoparticles (TCP NPs), as examined by MTT and H&E histological staining assays. Advantages of using HSA include its low price, availability as a sterile solution, and numerous binding sites for bioactive molecules. This artificial niche could be a step forward to fertility restoration for male infertility.

TESE samples were taken after obtaining written informed consent from two non-obstructive azoospermic patients (with the ages of 27 and 36 years) who had rare immotile spermatozoa in testicular biopsies with complete spermatogenic arrest, unremarkable spermatogonia, normal Leydig cells, and normal serum hormones. The Ethics Committee of Shahid Sadoughi University of Medical Sciences in Yazd, Iran (IR.SSU.REC.1394.226). The chemicals used in this study were all purchased from Sigma-Aldrich (Poole, UK). Culture media and supplements were procured from Invitrogen (UK) unless otherwise stated. As described previously (9), in brief, 36 g of calcium nitrate [Ca $(NO_3)_2$] and 12 g of diammonium phosphate [$(NH_3)_2$ HPO_4] were dissolved in 525 mL and 375 mL of distilled water (DW), respectively. Then, 25 mL of calcium nitrate was added to 25 mL of the diammonium phosphate solution, adjusted to pH=13, and kept for 6 hours at room temperature. After mild shaking, the synthesized product was washed with DW and allowed to dry. All dried TCP NPs were ball-milled for 1 hour. Then, 12.5 mg of TCP NPs were separately added to 4 mL of 500 mg/mL HSA (available as sterile injectable vials) and mixed for 1 minute. The resulting HSA/TCP NPs mixture was kept at 100°C water for 30 minutes. After the construction of solid matter, HSA/TCP NPs scaffold was frozen at -20°C, followed by the incubation at 37°C water for 30 minutes.

As explained previously (5), fresh TESE samples were placed in 2 mL of the Dulbecco's Modified Eagle Medium (DMEM, Gibco, UK) supplemented with 5% fetal bovine serum (FBS, Gibco, UK) and transferred to the laboratory within 15 minutes. The TESE biopsies were rinsed in a Petri dish using a 19-gauge needle. TESE specimens were enzyme-dissociated overnight by the incubation in 0.1% collagenase type I in DMEM/10% FBS at 37ºC, with 5% CO_2. Cells were subsequently recovered by aspiration and washed by centrifugation at 200 g for 3 minutes. The supernatant was discarded, and the pellet was recovered for culturing human testis-derived cells (hTCs). The obtained hTCs were incubated in flasks containing DMEM/10% FBS. Trypsin/EDTA (Gibco, UK) enzymatic method was used to passage hTCs. All cell culture experiments were performed at least in triplicate.

To sterilize the scaffolds, UV-irradiation was used for 1 hour. Following the expansion of hTCs by five passages using trypsin/EDTA, hTCs were detached from the flasks, counted, and plated on the scaffolds at a concentration of 5000 cells per well in 96-well plate culture dishes and incubated at 34°C with 5% CO2.

After 7 and 14 days, the cell-coated scaffolds were fixed by 4% paraformaldehyde (Sigma-Aldrich Chemie GmbH, Germany). The H&E staining method was carried out to detect arrays of hTCs within the porous scaffold.

Three cell-coated scaffolds were checked for cell proliferation/viability by the MTT [3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl -2H- tetrazolium bromide] assay on days 7 and 14, and the average of 3 cultures was determined. The optical densities (ODs) at 570 nm with background subtraction at 630 nm were evaluated using an enzyme-linked immunosorbent assay (ELISA) reader (Tajhizat Sanjesh, Iran). The percentage of viability/proliferation was determined by the below formula:

Viability (%)=(OD of the test sample/OD of the control sample)×100.

For each of the 3 scaffolds, OD measurement was performed in triplicate. The statistical analysis was analyzed by the Statistical Package for the Social Sciences (SPSS) software version 22 (IBM, USA). Two-tailed bivariate (Pearson) correlations were calculated for the test and control samples based on their OD. Data are presented as mean ± SD. Differences with $P<0.05$ were considered statistically significant.

The TESE samples stained by H&E are shown in Figure 1A. hTCs were initially cultured and expanded in flasks as monolayer cell culture, and they showed mostly elongated shapes (Fig.1B). The 3D porous scaffolds containing HSA/TCP NPs were successfully established, as described earlier. The size of pores (~10-300 μm) was checked using an inverted microscope (Fig.1C). The scaffolds were sectioned and stained (Fig.1D) before the 3D cell cultures.

Following the five passages of culture flasks, hTCs were cultured for 14 days on the 3D HSA/TCP NPs scaffolds. For the assessment of the homing and viability of the cells, cell-seeded scaffolds were sectioned and stained after 7 (Fig.1E) and 14 days (Fig.1F). H&E staining revealed the biocompatibility of scaffolds for hTCs; nevertheless, the number of cells within the pores was dependent on the size of pores. Interestingly, there is a similarity between histological sections of TESE (Fig.1A) and hTC-seeded scaffolds (Fig.1E, F); however, the latter exhibits disarrangement and possesses fewer cells.

The OD of monolayer cultures did not significantly ($P>0.05$) differ from the 3D cultures for 2 weeks (Fig.2). On day 7, the ratio of viable cells in the 3D culture was about half of that observed in the monolayer culture (0.1 ± 0.06 vs. 0.2 ± 0.10), which became 66.7% after 14 days (0.2 ± 0.08 vs. 0.3 ± 0.10). This implies the nontoxic nature of the 3D scaffold for hTCs.

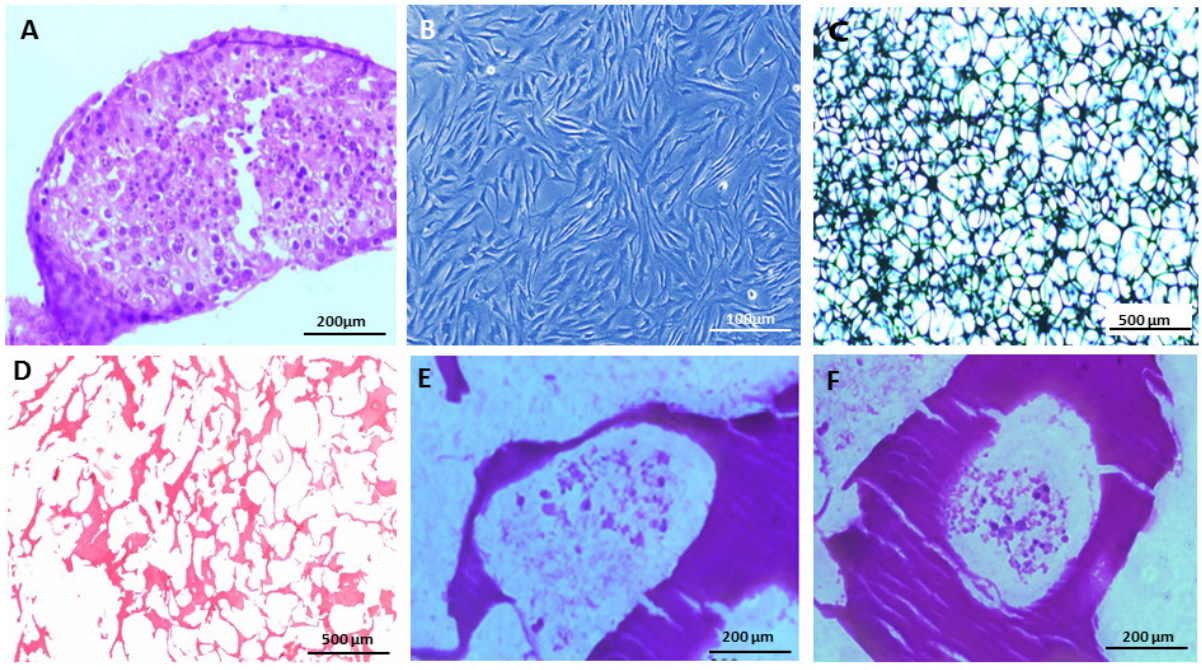

Fig.1: The main steps and procedures. **A.** The H&E-stained sections of TESE samples (scale bar: 200 µm), **B.** Culture of hTCs in flasks (scale bar: 100 µm), **C, D.** HSA/TCP NPs scaffolds (scale bar: 500 µm), **E.** Sections of 3D culture of hCs after 7 days (scale bar: 200 µm), and **F.** Sections of 3D culture of hTCs after 14 days (scale bar: 200 µm).
TESE; Testicular sperm extraction, hTCs; Human testis-derived cells, HSA/TCP NPs; Human serum albumin/tricalcium phosphate nanoparticles, and 3D; Three-dimensional.

Recently, successful IVG was shown in mouse embryonic stem cells for the production of sperms (11), oocytes (12), and offspring. Some potential was indicated in human embryonic stem cells but without success in achieving actual spermatozoa or oocytes (3, 4, 13). On the other hand, fertile mouse spermatozoa (14) and eggs (15) were produced using GSCs in vitro. In humans, oocytes were claimed to be produced from GSCs in women who are in the reproductive age (16), but there is still debate regarding the origin and pluripotency of GSCs and their potential for in vitro spermatogenesis (IVS) (5). One of the strategies for IVS in mouse (other than adding exogenous growth factors to the culture medium) is the transplantation of GSCs into seminiferous tubules (14). There are several challenges regarding human IVS. First of all, there are ethical and technical difficulties for the isolation of SSCs as well as the generation and expansion of putative male pluripotent GSCs. Besides, even if it becomes feasible to generate putative pluripotent GSCs from human samples in boys undergoing chemotherapy, ethical issues remain for the transplantation of GSCs in recipient testes. Tissue engineering can help to reconstitute the human somatic niche for IVS (8). In the present study, has, as an abundant source of proteins in the blood (35-50 g/L of human serum) was used for designing a homemade scaffold. HSA is a soluble globular molecule with an average half-life of 19 days. Correspondingly, it is extremely stable in a pH range of 4-9. One of the major benefits of scaffolds made from HSA is the lack of immunogenicity. Additionally, HSA produces amino acids upon the breakdown, providing nutrition for the cells in culture media. Altogether, HSA is available, cheap, biodegradable, biocompatible, and ideal candidate compound for scaffold construction (17). TCP NPs are biocompatible and biodegradable with high absorption capacity. The viability of mouse (9) and rat (10) SSCs was shown previously using HSA-based scaffolds. Our data reveal that 3D HSA/TCP NPs scaffolds support the survival and proliferation of hTCs with 70% viability for two weeks, as compared with monolayer culture. This 3D culture system could be further studied as an artificial niche for human IVS derived from either GSCs or pluripotent stem cells.

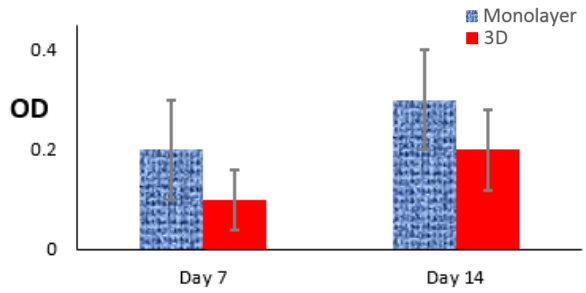

Fig.2: The MTT assay of hTCs cultured on monolayer versus 3D scaffold after 7 and 14 days. hTCs; Human testis-derived cells, OD; Optical density, and 3D; Three-dimensional.

Acknowledgements

This study was financially supported by the Yazd Repro- nReconstitution of Artificial Testicular Somatic Niche Fig.1: The main steps and procedures. A. The H&E-stained sections of TESE samples (scale bar: 200 µm), B. Culture of hTCs in flasks (scale bar: 100 µm), C, nD. HSA/TCP NPs scaffolds (scale bar: 500 µm), E. Sections of 3D culture of hCs after 7 days (scale bar: 200

μm), and F. Sections of 3D culture of hTCs after 14 days (scale bar: 200 μm). TESE; Testicular sperm extraction, hTCs; Human testis-derived cells, HSA/TCP NPs; Human serum albumin/tricalcium phosphate nanoparticles, and 3D; Three-dimensional. 153 Int J Fertil Steril, Vol 14, No 2, July-September 2020 ductive Sciences Institute, Yazd, Iran, as a Ph.D. grant for Miss Zahra Borzouie's thesis. There is no conflict of interest in this study.

Authors' Contributions

Z.B.; Performed the majority of tests and procedures, interpretation of the data, and the conclusion. S.H.; Designed and supervised the study and is responsible for the scientific integrity of the manuscript. A.J.; Assisted with nanomaterial preparations and gave consultation on data gathering. B.A.; Gave consultation about the whole study, helped with cell culture, imaging and performed the final revision of the manuscript. All authors read and approved the final manuscript.

References

1. Lokman M, Moore H. An artificial sperm-next year or never? Hum Fertil (Camb). 2010; 13(4): 272-276.
2. Imamura M, Hikabe O, Lin ZY, Okano H. Generation of germ cells in vitro in the era of induced pluripotent stem cells. Mol Reprod Dev. 2014; 81(1): 2-19.
3. Aflatoonian B, Ruban L, Jones M, Aflatoonian R, Fazeli A, Moore HD. In vitro post-meiotic germ cell development from human embryonic stem cells. Hum Reprod. 2009; 24(12): 3150-3159.
4. Hendriks S, Dancet EA, van Pelt AM, Hamer G, Repping S. Artificial gametes: a systematic review of biological progress towards clinical application. Hum Reprod Update. 2015; 21(3): 285-296.
5. Sadeghian-Nodoushan F, Aflatoonian R, Borzouie Z, Akyash F, Fesahat F, Soleimani M, et al. Pluripotency and differentiation of cells from human testicular sperm extraction: An investigation of cell stemness. Mol Reprod Dev. 2016; 83(4): 312-323.
6. Moore H, Aflatoonian B. From stem cells to spermatozoa and back. Soc Reprod Fertil Suppl. 2007; 65: 19-32.
7. Borzouie Z, Naghibzadeh M, Talebi AR, Pourrajab F, Jebali A, Nikukar H, et al. Development of an artificial male germ cell niche using electrospun poly vinyl alcohol/human serum albumin/gelatin fibers. Cell J. 2019; 21(3): 300-306.
8. Del Vento F, Vermeulen M, de Michele F, Giudice MG, Poels J, des Rieux A, et al. Tissue engineering to improve immature testicular tissue and cell transplantation outcomes: one step closer to fertility restoration for prepubertal boys exposed to gonadotoxic treatments. Int J Mol Sci. 2018; 19(1): Pii: E286.
9. Yadegar M, Hekmatimoghaddam SH, Nezami Saridar S, Jebali A. The viability of mouse spermatogonial germ cells on a novel scaffold, containing human serum albumin and calcium phosphate nanoparticles. Iran J Reprod Med. 2015; 13(3): 141-148.
10. Nezami Saridar S, Hekmatimoghaddam S, Yadegar M. The homing of spermatogonial cells in the cavities of a novel nanoscaffold. IJML. 2015; 2(2): 134-142.
11. Nayernia K, Nolte J, Michelmann HW, Lee JH, Rathsack K, Drusenheimer N, et al. In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. Dev Cell. 2006; 11(1): 125-132.
12. Hayashi K, Saitou M. Generation of eggs from mouse embryonic stem cells and induced pluripotent stem cells. Nat Protoc. 2013; 8(8): 1513-1524.
13. Rombaut C, Mertes H, Heindryckx B, Goossens E. Human in vitro spermatogenesis from pluripotent stem cells: in need of a stepwise differentiation protocol? Mol Hum Reprod. 2018; 24(2): 47-54.
14. Sato T, Katagiri K, Yokonishi T, Kubota Y, Inoue K, Ogonuki N, et al. In vitro production of fertile sperm from murine spermatogonial stem cell lines. Nat Commun. 2011; 2: 472.
15. Hikabe O, Hamazaki N, Nagamatsu G, Obata Y, Hirao Y, Hamada N, et al. Reconstitution in vitro of the entire cycle of the mouse female germ line. Nature. 2016; 539(7628): 299-303.
16. White YA, Woods DC, Takai Y, Ishihara O, Seki H, et al. Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. Nat Med. 2012; 18(3): 413-421.
17. Weszl M, Skaliczki G, Cselenyák A, Kiss L, Major T, Schandl K, et al. Freeze-dried human serum albumin improves the adherence and proliferation of mesenchymal stem cells on mineralized human bone allografts. J Orthop Res. 2012; 30(3): 489-496.

Case Report

Which One Is More Prominent in Recurrent Hydatidiform Mole, Ovum or Sperm?

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Abstract

Recurrent hydatidiform mole is defined as episodes of two molar pregnancies in a female. Often, complete moles only derive androgenic nuclear genome. We described two cases with repeated molar pregnancies attempted to prevent future episodes by performing intracytoplasmic sperm injection (ICSI) and preimplantation genetic diagnosis (PGD) to assess genetic disorders. The first patient had previously six complete molar pregnancies and advised to carry out ICSI with ovum donation to achieve a normal pregnancy. The second case had previously five molar pregnancies and no XY embryos from the ICSI/PGD process. We had to (at the insistence of the patient) transfer XX embryos in this patient which resulted in a complete hydatidiform mole (CHM). Hence, available data based on our patients and previous studies demonstrated that oocyte might play a critical role in the pathophysiology of recurrent hydatidiform mole, while it has not been often considered.

Keywords: Hydatidiform Mole, Intracytoplasmic Sperm Injections, Ovum Donation, Preimplantation Genetic Diagnosis

Citation: **Hafezi M, Chekini Z, Zamanian M. Which one is more prominent in recurrent hydatidiform mole, ovum or sperm? Int J Fertil Steril. 2020; 14(2): 154-158. doi:10.22074/ijfs.2020.6017.**

Introduction

Complete hydatidiform mole (CHM) is characterized by diffuse chorionic villi hyperplasia and generalized hydatidiform villous swelling (1). Recurrent hydatidiform mole is an extremely rare occurrence. Rate of that is more than 23% after two molar pregnancies in the same woman (2, 3). Recurrent CHM is related to a higher malignancy risk (4). Early abortions in approximately 10-20% after one hydatidiform mole show the genetic origin of molar pregnancies in some of these patients (1, 5). Furthermore, there is 44-66% chance of live births at future pregnancies (6).

Molar pregnancy has a multifactorial etiology related to several environmental and genetic factors (7). Complete moles usually have their nuclear genome from the paternal (androgenesis). In such cases, chromosomal material from the ovum is lost or becomes inactive, whereas the mitochondrial DNA has maternal origin (1, 8).

Preliminary investigation for prevention of CHM was based on the morphological manifestation of embryos during *in vitro* fertilization (IVF) (9). However, according to genetic composition and pathogenesis of molar pregnancies, intracytoplasmic sperm injection (ICSI) and preimplantation genetic diagnosis (PGD) with fluorescent in situ hybridization (FISH) provide a diploid 46, XY complement which is appropriate for prevention of an additional event in patients with repeated molar pregnancies (6). Defective oocytes can be a predisposing factor as the main cause of abnormal fertilization thus an oocyte or embryo donation is considered for achieved normal pregnancy (10, 11).

In the present study, we described two cases of recurrent molar pregnancy which were advised to have ICSI/PGD to prevent repeated CHM. This led to their molar pregnancies. Oocyte donation in the current cases resulted in normal pregnancies and live births.

Case Report

Case 1

A 30-years-old woman presented with six molar pregnancies and five suction curettages in the last nine years. All pregnancies had complete molar pathology. The patient underwent subfertility treatment for nine years and had conceived by ovarian stimulation with clomiphene in all pregnancies, which led to histopathological diagnoses of hydatidiform mole. She had regular menstrual cycles and a body mass index (BMI) of 29kg/m^2. The patient had no history of blood transfusions and no addictions.

Received: 15/June/2019, Accepted: 14/January/2020
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Her blood group was B positive and she had normal thyroid hormone profile. Immunological assessments such as anti-phospholipid (IgG and IgM) and anti-cardiolipin antibodies, anti-ds-DNA, anti-nuclear antibody (ANA), lupus anti-coagulant and CHso were performed due to her recurrent abortion history. She was negative for any autoimmune disorders. Pelvic examination showed remarkable deformation and scarring in the cervix, resulted from tenaculum lesions. Her husband was 37 years old with the following semen indices: concentration of 120×10^6/ml with normal motility (40%) and normal morphology (21%) according to Kruger's criteria evaluation. Both of the patient and her partner had normal karyotypes. It was not consanguine marriage.

Genetic counseling recommendations included ICSI/PGS. The first ovarian stimulation was achieved by the use of oral contraceptive pill (OCP) long GnRHa. Combined low-dose (LD) contraceptive pills (Abureyhan Pharmaceutical Company, Iran) starting on day 2 of the menstrual cycle then buserelin (Suprefact; Hoechst, Denmark) was initiated from day 17th of the cycle. After pituitary down-regulation was achieved, Gonal-F was subcutaneously injected for nine days at a dose of 150 IU/day (Serono, Switzerland). Ovarian response was monitored by vaginal ultrasound when two follicles had diameters of more than 17 mm and ovulation was induced by administration of 10000 IU of human chorionic gonadotropin (hCG; Pregnyl, Germany). A total of 12 oocytes were retrieved 36 hours post-hCG administration. The number of metaphase II (MII) oocytes was evaluated and the patient underwent insemination procedures. Ten embryos were obtained at the cleavage stage. An advanced cleavage status of an 8-cell embryo at 72 hours after insemination resulted in four embryos with two-pronuclear (PN), three embryos with 1.PN, one embryo without PN and 2 embryos with three-PN and good and fair grading. All embryos proceeded to the cleaving stage (day 3 after retrieval) and were biopsied for single-cell PGS by FISH. FISH probes were specific for chromosomes 13, 18 and 21 to determine aneuploidy. Two technologists were evaluated and construed the signals of FISH probes. We observed micronuclei and extra nucleus in all embryos that were not transferred due to the presence of multi-micronuclei embryos (Table 1).

After three years and based on the patient's history, the couple was advised to have an oocyte donation and ICSI procedure. Ovarian stimulation of the donors was performed using a standard oral contraceptive pills (OCP) long GnRHa stimulation protocol and pituitary-ovarian suppression by buserelin. There were 21 out of 26 oocytes at the MII stage.

ICSI procedure was routinely performed to prevent dispermia. We obtained 20 2.PN good grade embryos from insemination. Three cleaving excellent embryos were transferred 2 days after ICSI procedure without any positive result. Other frozen embryos were transferred three times in three years. The second cycle resulted in pregnancy and live birth, by transferring three good embryos.

One year after delivery of the first live birth, an endometrial adhesion was detected by ultrasonography examination. A hysteroscopy was subsequently performed. During the second donor cycle, four oocytes were retrieved according to the previous donor protocol and three excellent stage 2.PN embryos were transferred. Pregnancy was confirmed by a persistent rise in serum β-hCG levels on the 14th day after embryo transfer. Ultrasonography at six weeks gestation was remarkable for a singleton pregnancy with a positive fetal heart rate. She eventually had vaginal delivery of a healthy infant. The recruited patient gave her consent to participate.

Table 1: Details of biopsy and spreading of embryos from the second case and the respective results of FISH analysis

Embryo Grade	No. Cells biopsied	Nucleus	Fragmentation	PGD FISH results	Whole embryo FISH results	Embryos status in transfer day
B	3/8	3.PN	+	?#	-	5B
B	1/8	2.PN	+	[13][18] [13][18][21×3]	-	8B
B-C	1/10	1.PN	+	[13×2][18][21×2]	-	Compact
B	1/10	1.PN	+	[13][18×2][21]	-	Compact
C	1/8	2.PN	+	[13][18×3][21×2] [13][21]	-	7C
C	1/6	NO*	+	[13][18][21]	-	5C
C	1/4	2.PN	+	[13×3][18×3][21×4]	-	6C
C	1/6	2.PN	+	No signal detected	-	8BC
C	1/4	1.PN	+	[13×2][18×2][21×2]	-	3C
C-D	1/4	3.PN	+	[13][18][21]×2 [13][18]	-	4CD

*; No nucleus was observed, # ; Not available, PGD; Genetic diagnosis, FISH; Fluorescent in situ hybridization, PN; Pronucleus, and ?; Unkhnown.

Case 2

A 24-years-old woman had complaints of consecutive CHM verified by histopathology assessment of the evacuated uterine contents. Her medical history included five previous molar pregnancies occurred over 8 years following spontaneous conceptions that did not continue beyond the first trimester. The latest molar pregnancy occurred one year before admission. The patient married 12 years ago and she had no infertility history. Her BMI was 36 kg/m2 and the blood group was O positive. She had regular menstrual cycles and normal thyroid profile. All infection tests were also normal. Hormonal assessment on day 3 of the menstrual cycle indicated an FSH level of 7.5 mIU/ml and LH level of 4.93 mIU/ml. Abdominal ultrasonography revealed a 13 mm diameter isoechoic myometrial fibroid without any pressure effect on the endometrium.

Semen analysis showed a concentration of 70×106/ml according to Kruger criteria, along with 30% motility and 14% morphology. The patient's husband was a smoker and allergic to plastic supplies due to his employment at the plastics production plant. The couple had normal karyotypes with no gross abnormalities. They had no consanguine marriage.

A nutritional counselor advised the patient to lose weight. After extensive counseling, the couple underwent ICSI-PGS, which resulted in 46, XY embryo transfers to prevent sperm chromosome duplication. After confirmation of satisfactory down-regulation with subcutaneous buserelin, she underwent ovarian stimulation using a standard long protocol induced by daily Gonal-F (150 IU; Serono, Switzerland) administration. When two dominant follicles reached greater than 18mm in diameter, she received hCG (Ovitrelle; Serono, Switzerland). There were 11 mature MII oocytes and one immature oocyte transvaginally retrieved 36 hours after hCG injection. There were 2.PN confirmed in 4 embryos and one embryo with 3.PN observed 18 hours after insemination. The remaining 4 embryos showed no evidence of fertilization (1.PN). The embryos developed to the eight-cell stage 72 hours after insemination and a single-cell PGS was performed. No evidence of blastomere fragmentation or irregularity was observed. FISH probes were specific for the 18, X, Y chromosomes to determine the ploidy and sex selection. In the first FISH round, two XY embryos were detected; however, the second analysis showed 45 Y and 23 XX embryos. Because the insistence of the patient, two XX embryos were transferred, because the 46, XY embryo was not acquired (Table 2).

Two weeks after transfer, the patient had a positive serum β-hCG test. Ultrasonography after 6 weeks revealed one gestational sac and regions of low echogenicity that became multicystic and hydropic chorionic villi suggestive of a CHM. Despite the use of ICSI/PGS procedure, histologic examination after suction evacuation confirmed diagnosis of CHM for the sixth time. Two recruited patients gave their consent to participate. Informed consent form was obtained and completed by participant.

Discussion

It appears to be a crucial relationship within CHM, immature ovum and delayed fertilization. Another possibility is altered integrity of the zona pellucida prone to entering double spermatozoa (12). CHM is predominantly 46,XX due to androgenic duplication, whereas only rare cases present 46,XX or 46,XY conditions as a result of dispermia (6, 10). The 46,YY constitution has yet to be described, most likely because such embryos will not be developed beyond a few cells (13).

IVF is predisposed to multi-sperm fertilization as a mechanism, which most likely causes the formation of hydatidiform moles (12). ICSI can prevent polyploidy by assurance arrival of a single spermatozoa to oocyte, resulting in more reduced triploidy; however, probability of molar pregnancy still remains (14). Avoidance of triploidy and ensuring delivery of haploid spermatozoa by ICSI might be of the benefits for patients who have a history of

Table 2: Details of biopsy and spreading of embryos as well as the results of FISH analysis

Embryo Grade	No. Cells biopsied	Nucleus	Fragmentation	PGD FISH results	Whole embryo FISH results	Embryos status in transfer day
B	1/8	2.PN	-	XX[18]×2	Transferred	Compact
B	1/4	2.PN	-	XX[18]×2	Transferred	Compact
C	1/8	2.PN	-	XY[18] ?*	-	Compact
C	1/4	2.PN	-	Y ?	-	5C
B	1/8	1.PN	+	Y[18]	-	Compact
B	2/8	1.PN	+	Y[18]	-	Compact
C	1/6	-	-	?	-	Compact
C	2/8	3.PN	+	X[18] X ?	-	Compact

*; Not available, #; Binucleated cell with a normal-size nucleus and additional smaller nucleus, PGD; Genetic diagnosis, FISH; Fluorescent in situ hybridization (FISH results summarize both nuclei), PN; Pronucleus, and ?; Unkhnown.

the recurrent trophoblastic disorder (12). However, during ICSI cycles, morphological assessment of the embryo before transfer cannot prevent CHM (14). An alternative genetic approach to prevent recurrent CHM is ICSI/PGD via FISH technique that complies with prior knowledge of the pathogenesis of hydatidiform mole. ICSI is likely to result in diploid and monospermic fertilization, as well as PGD for male sex selection embryos that confirm diploidy and the chromosomal contribution of both parents during fertilization (6).

In both cases, the couples were advised by genetic counselors to undergo ICSI/PGS. In the first case, no embryo was transferred because all embryos had multimicronuclei. In the second case, there was no XY embryo resulted from the ICSI/PGS process, presenting the restricted problem of this technique. Because the insistence of the patient, we had to transfer XX embryos with the appearance of normal nuclei, which resulted in a CHM by triploidy of origin. Therefore, the ICSI/PGS process was not effective in preventing molar pregnancy.

Recent advances have shown androgenesis in at least 80% of CHM, however, one of the remaining pathophysiologies of cases may be diploid biparental (15). ICSI/PGD is an appropriate method for androgenetic avoidance and triploid dispermic in origin CHM due to its pathogenesis that occurs at the time of fertilization. However, this approach does not completely prevent a recurrent diploid biparental hydatidiform mole (10).

Biparental diploids have one maternal set of chromosomes and one set of the chromosome complement from father (8, 10). Although maternal defect is more involved, it seems that both of the partners are involved in molar pregnancy (10). Considering the biparental origin of CHM and the implicated disturbance in oocyte meiosis (10, 11), donor oocyte IVF/ICSI might be a therapeutic modality to prevent recurrent CHM and achieve a normal pregnancy.

In the current study, the first patient was advised to conceive via ICSI with ovum donation. This case had two normal pregnancies and a healthy child after a two embryo transfer cycle from donated oocytes. Nevertheless, the patient who underwent ICSI/PGS with her oocytes did not obtain a normal embryo nucleus, suggesting a critical role of the ovum in the pathophysiology of molar pregnancy.

As mentioned above, maternal mitochondrial DNA of the entire oocyte genome contributes only to the fertilization in CHM (8). Altered expression of mitochondrial genes has been associated with gestational trophoblastic disease (GTD) (16). The involvement of multiple somatic mtDNA mutations in GTD have been proposed in the pathogenesis of CHM and tumor development (17).

Pan et al. have described maternally derived mtDNA in CHM based on the polymorphic D-loop region. However, since mtDNA is highly polymorphic and heteroplasmic, limited reports have described the mtDNA-transmission pattern in hydatidiform moles (18). Therefore, most likely in the current cases, maternal mtDNA was involved in the pathophysiological events of CHM.

One important limitation of our study is the rare occurrence of GTD and insufficient achievement cases. However, further studies with larger sample size would be needed to understand the designation of mtDNA and maternal genetics predisposing to GTD.

Conclusion

With respect to the established ICSI/PGS strategy for prevention of recurrent molar pregnancy, this technique has restrictions of sperm duplication, defective oocytes and avoidance from XX embryos. It seems that ovum donation is better treatment option to achieve normal pregnancy in such cases. Available data based on our patients and previous studies indicate that oocytes might have a critical role in pathophysiology of recurrent HM, while it has not been considered in most of the studies.

Acknowledgements

There is no financial support and the authors declare no conflict of interest.

Authors' Contributions

M.H.; Contributed to the study concept and design as well as the administrative support. Z.C.H.; Contributed to collection and assembly of data. M.H., Z.C.H., M.R.Z.; Contributed to literature review and assembly of data. All authors read and approved the final version of the manuscript.

References

1. Berkowitz RS, Goldstein DP. Clinical practice. Molar pregnancy. N Engl J Med. 2009; 360(16): 1639-1645.
2. Eagles N, Sebire NJ, Short D, Savage PM, Seckl MJ, Fisher RA. Risk of recurrent molar pregnancies following complete and partial hydatidiform moles. Hum Reprod. 2015; 30(9): 2055-2063.
3. Sebire NJ, Seckl M. Gestational trophoblastic disease: current management of hydatidiform mole. BMJ. 2008; 337: a1193.
4. Ogilvie CM, Renwick PJ, Khalaf Y, Braude PR. First use of preimplantation genotyping in prevention of recurrent diandric complete hydatidiform mole. Reprod Biomed Online. 2009; 19(2): 224-227.
5. Nguyen NM, Slim R. Genetics and epigenetics of recurrent hydatidiform moles: basic science and genetic counselling. Curr Obstet Gynecol Rep. 2014; 3: 55-64.
6. Reubinoff BE, Lewin A, Verner M, Safran A, Schenker JG, Abeliovich D. Intracytoplasmic sperm injection combined with preimplantation genetic diagnosis for the prevention of recurrent gestational trophoblastic disease. Hum Reprod. 1997; 12(4): 805-808.
7. Muminhodzic L, Bogdanovic G, Ljuca D, Babovic A. Epidemiological factors and pathomorphologic characteristics of hydatidiform mole. J Health Sci. 2013; 3(2): 129-137.
8. Hui P, Buza N, Murphy KM, Ronnett BM. Hydatidiform moles: genetic basis and precision diagnosis. Annu Rev Pathol. 2017; 12: 449-485.
9. Edwards R, Crow J, Dale S, Macnamee M, Hartshorne G, Brinsden P. Preimplantation diagnosis and recurrent hydatidiform mole. Lancet. 1990; 335(8696): 1030-1031.
10. Fisher RA, Khatoon R, Paradinas FJ, Roberts AP, Newlands ES. Repetitive complete hydatidiform mole can be biparental in origin and either male or female. Hum Reprod. 2000; 15(3): 594-598.
11. Huang X, Wang H, Zhao X, Xu X, Chen Q. Gestational trophoblastic disease following in vitro fertilization. Arch Gynecol Obstet. 2007; 275(4): 291-293.

12. Pal L, Toth TL, Leykin L, Isaacson KB. High incidence of triploidy in in-vitro fertilized oocytes from a patient with a previous history of recurrent gestational trophoblastic disease. Hum Reprod. 1996; 11(7): 1529-1532.
13. Ngan HY, Kohorn EI, Cole LA, Kurman RJ, Kim SJ, Lurain JR, et al. Trophoblastic disease. Int J Gynaecol Obstet. 2012; 119 Suppl 2: S130-S136.
14. Hamanoue H, Umezu N, Okuda M, Harada N, Ohata T, Sakai H, et al. Complete hydatidiform mole and normal live birth following intracytoplasmic sperm injection. J Hum Genet. 2006; 51(5): 477-479.
15. Li HW, Tsao SW, Cheung AN. Current understandings of the molecular genetics of gestational trophoblastic diseases. Placenta. 2002; 23(1): 20-31.
16. Durand S, Dumur C, Flury A, Abadie P, Patrito L, Podhajcer O, et al. Altered mitochondrial gene expression in human gestational trophoblastic diseases. Placenta. 2001; 22(2-3): 220-226.
17. Chiu PM, Liu VW, Ngan HY, Khoo US, Cheung AN. Detection of mitochondrial DNA mutations in gestational trophoblastic disease. Hum Mutat. 2003; 22(2): 177.
18. Pan Z, Usui H, Sato A, Shozu M. Complete hydatidiform moles are composed of paternal chromosomes and maternal mitochondria. Mitochondrial DNA A DNA Mapp Seq Anal. 2018; 29(6): 943-950.

Letter to Editor

The COVID-19 Pandemic: Is It A Wolf Consuming Fertility?

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Citation: Napolitano L, Barone B, Crocetto F, Capece M, La Rocca R. The COVID-19 pandemic: is it a wolf consuming fertility? Int J Fertil Steril. 2020; 14(2): 159-160. doi: 10.22074/ijfs.2020.6302.

Nowadays, male infertility is regarded as a global problem. Male infertility occurs due to abnormal sperm production or function that prevent the delivery and/or the quality of sperm. Infections, chronic illnesses, lifestyle choices and other factors can play a role in male infertility (1, 2).

Most of viral infections indeed, are able to affect both the reproductive tract tissue and the semen of humans and animals, impairing sperm parameters and DNA integrity by different pathogenetic mechanisms that are responsible for temporary or permanent infertility in males and females (1).

Considering the well-known evidence on relations between human immunodeficiency virus (HIV), human papilloma virus (HPV), cytomegalovirus (CMV), adenoviruses, parvovirus, mumps and fertility, the real question is "Can the SARS-CoV-2 pandemia possibly influence male patency?" Coronaviruses are enveloped, positive single-stranded large RNA viruses that infect both animals and humans (3).

The first example is the avian infectious bronchitis virus (AIBV), a coronavirus known to cause an acute respiratory infection in chickens. In addition to that, AIBV causes the formation of epididymal stones in roosters, with detrimental effects on sperm production and fertility. The presence of such calcium carbonate stones in the efferent duct of roosters causes the erosion of the epithelial lining, the reduction of testis weight and impairment of sperm production lowering the fertility for 35-40%.

Secondly, porcine reproductive and respiratory syndrome virus (PRRSV), a small enveloped RNA virus, shows a negative impact on fertility. In fact, the PRRSV primarily attacks pulmonary alveoli to further replicate in epithelial germ cells (mainly spermatids and spermatocytes) inducing apoptosis and increasing the number of immature sperm cells. The overall effect is a diminished quality of the semen. Moreover, this kind of replication permits sexual transmission of the virus that could be easily identified in ejaculate.

Thirdly, the blue tongue virus (BTV) - a double stranded RNA virus that affects rams – represent another example of viral infection-caused semen impairment. The BTV RNA was detected in 75-100% of semen samples obtained 25-57 days post clinical signs with notable impact on semen motility, concentration and vitality. Although very little is known regarding the pathogenic mechanisms, the increasing number of germ cell at different maturation stages and early signs of germinal epithelial regeneration suggest a previous severe degeneration and sloughing of germ cells.

Recent evidence reports that SARS coronavirus could also lead to a fertility damage, despite the absence of a direct mumps-like orchitis. It was hypothesized that SARS coronavirus may utilize the ACE-2 receptor expressed on testicular tissue. SARS patients' testes displayed indeed a widespread germ cell destruction with thickened basement membrane and leukocyte infiltration, mainly macrophages, compatible with findings of previous animal studies (3). The presence of components of the renin angiotensin system (RAS) and specific receptors of angiotensin in the female and male reproductive tract supports the hypothesis that reproductive functions may be controlled by RAS (4). Angiotensin converting enzyme (ACE) is also involved in the regulation of blood pressure. One of the most active components of RAS system is angiotensin II (Ang II) that regulates cardiovascular and electrolyte homeostasis. Since Ang II was also found in seminal plasma, it might be able to act on mammalian spermatozoa where it is implicated in the maintenance of sperm function and fertility. Exposure of human spermatozoa to Ang II increases the percentage of motile spermatozoa and stimulates sperm linear velocity. Moreover, Ang II is involved in capacitation and acrosomal reaction. This ligand binds to two different receptors, AT1R and

Received: 31/March/2020, Accepted: 20/May/2020
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Royan Institute
International Journal of Fertility and Sterility
Vol 14, No 2, July-September 2020, Pages: 159-160

AT2R. These receptors were detected either in the male reproductive system and in spermatozoa. AT1R was detected in the tail and along the whole flagellum of spermatids and mature spermatozoa of humans and animals and found to be involved in capacitation and acrosome reaction. AT2R is mainly present in human semen at the equatorial/post acrosomal region of the head (5). The AT2R is located in the same region of μ-opioid receptor, pro-enkephalin, estrogen receptor-α, and γ-aminobutyric acid A receptor. Such proteins are all involved in cell transduction and sperm motility. It was suggested that AT2R may be involved in the control of sperm motility as well (6). Coronaviruses isolated from bats since 2005 showed a particular propension to cross species barriers, infecting the lung cells of mammals utilizing the ACE-2 receptors and exerting a potential zoonotic-reverse zoonotic cycle that allow the virus to maintain viral population in multiple hosts (7). The reported different spillover episodes, the well-established reproductive problems related to coronaviruses in mammals and birds and finally the evidence regarding the presence of ACE-2 receptors in human genital tract does not let us excluding potential reproductive issues in humans. Particular attention should be given to asymptomatic patients who are often the major carriers of the Covid-19 infection (8). It is also necessary to identify all potential clinical presentations and the possible, long-term consequences of Covid-19 -infection. According to the literature data, a possible reproductive system localization and, particularly spermatozoa localization with possible implications for male fertility, cannot be excluded. Further studies are needed to better define the physiopathology and clinical implications of respiratory virus infections on male fertility.

Acknowledgments

There is no financially support and conflict of interest.

Authors' Contribution

L.N., B.B., F.C.; Drafted the manuscript. M.C., R.L.; Revised the manuscript. All authors read and approved the final manuscript.

References

1. Punab M, Poolamets O, Paju P, Vihljajev V, Pomm K, Ladva R, et al. Causes of male infertility: a 9-year prospective monocentre study on 1737 patients with reduced total sperm counts. Hum Reprod. 2017; 32(1): 18-31.
2. Arcaniolo D, Favilla V, Tiscione D, Pisano F, Bozzini G, Creta M, et al. Is there a place for nutritional supplements in the treatment of idiopathic male infertility? Arch Ital Urol Androl. 2014; 86(3): 164-170.
3. Xu J, Qi L, Chi X, Yang J, Wei X, Gong E, et al. A complication of severe acute respiratory syndrome (SARS). Biol Reprod. 2006; 74(2): 410-416.
4. Gianzo M, Muñoa-Hoyos I, Urizar-Arenaza I, Larreategui Z, Quintana F, Garrido N, et al. Angiotensin II type 2 receptor is expressed in human sperm cells and is involved in sperm motility. Fertil Steril. 2016; 105(3): 608-616.
5. V Rago, F Giordano, E Brunelli, D Zito, S Aquila, A Carpino. Identification of G protein-coupled estrogen receptor in human and pig spermatozoa. J Anat. 2014; 224(6): 732-736.
6. Vinson GP, Ho MM, Puddefoot JR. The distribution of angiotensin II type1 receptors, and the tissue renin-angiotensin systems. Mol Med Today 1995; 1(1): 35-39.
7. Huynh J, Li S, Yount B, Smith A, Sturges L, Olsen JC, et al. Evidence supporting a zoonotic origin of human coronavirus strain NL63. J Virol. 2012; 86(23): 12816-12825.
8. Guo YR, Cao QD, Hong ZS, Tan YY, Chen SD, Jin HJ, et al. The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak - an update on the status. Mil Med Res. 2020; 7(1): 11.

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

Aims and scope

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

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