

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

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Printing Company:

Jurband Ghaemprint Co.
NO. 5, Jalil khoob alley, Niroom Havaei Street, Tehran, Iran

INTERNATIONAL JOURNAL OF FERTILITY AND STERILITY

Int J Fertil Steril, Vol 12, No 4, Jan-Mar 2019, Pages: 267-342

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Possible Mechanisms for The Effects of Calcium Deficiency on Male Infertility

Asghar Beigi Harchegani, M.Sc.¹, Ali Irandoost, M.Sc.¹, Mahdiyeh Mirnamniha, M.Sc.², Hamid Rahmani, M.Sc.¹, Eisa Tahmasbpour, Ph.D.^{3*}, Alireza Shahriary, Ph.D.^{1*}

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Abstract

Calcium (Ca) is a significant element that acts as an intracellular second messenger. It is necessary for many physiological processes in spermatozoa including spermatogenesis, sperm motility, capacitation, acrosome reaction and fertilization. Although influences of Ca deficiency on sperm function and male infertility have been widely studied, mechanisms for these abnormalities are not well considered. Poor sperm motility, impairment of chemotaxis, capacitation, acrosome reaction and steroidogenesis are the major mechanisms by which Ca deficiency induces male infertility. Therefore, an optimal seminal Ca concentration is required to strengthen sperm function and all steps leading to successful fertilization. Furthermore, identification of these mechanisms provides valuable information regarding the mechanisms of Ca deficiency on male reproductive system and the way for developing a better clinical approach. In this review, we aim to discuss the proposed cellular and molecular mechanisms of Ca deficiency on male reproductive system, sperm function and male fertility. Also we will discuss the valuable information currently available for the roles of Ca in male reproduction.

Keywords: Acrosome Reaction, Calcium, Capacitation, Fertility, Sperm Motility

Citation: Beigi Harchegani A, Irandoost A, Mirnamniha M, Rahmani H, Tahmasbpour E, Shahriary A. Possible mechanisms for the effects of calcium deficiency on male infertility. *Int J Fertil Steril.* 2019; 12(4): 267-272. doi: 10.22074/ijfs.2019.5420.

Introduction

According to World Health Organization (WHO), infertility refers to the biological inability of an individual to achieve pregnancy following at least 12 month of unprotected intercourse (1). It has been estimated that approximately 15% of couples face some form of infertility (2, 3) and among them, male factor infertility plays a role in nearly 30-50% of all infertile couples (4). Many studies have reported that different factors including: varicocele, testicular failure, testicular cancer, endocrinal disorders, hormonal disturbances, genital tract infection, ejaculatory disorders, immunological factors, prolonged exposure to heat, obesity, older age, tobacco smoking, alcohol consumption, pesticides, the effect of radiation and magnetic waves, chemotherapy, occupational exposure, electronic devices, heavy metals, reactive oxygen species (ROS), genetic and chromosomal defects, dietary and lifestyle factors and different environmental pollutants (5-11) can play a role in the etiology of male infertility.

Nutritional deficiency of some traces elements is another significant factor that affects semen quality and plays important roles in the male reproductive process. Human semen contains several trace elements such as calcium

(Ca), copper (Cu), manganese (Mn), magnesium (Mg), zinc (Zn), and selenium (Se) that are necessary for metabolic processes, normal spermatogenesis, sperm maturation, motility and capacitation, as well as sperm normal function (5, 12). Therefore reduced level of these trace elements can be considered as one of the significant factors for impaired spermatogenesis, poor semen quality and male fertility (5, 13, 14). For example, numerous studies reported decreased levels of Zn, Cu and Se in seminal plasma of infertile men compared to infertile individuals (5, 15). Furthermore, seminal Zn concentration was reported to be significantly correlated with sperm counts, motility and viability (5). Another study demonstrated declined level of zinc in seminal plasma and serum of azoospermic patients compared to normospermic men (16).

Physiological concentrations of Fe and Cu in sperm and seminal plasma have exhibited a positive correlation with a variety of antioxidant markers and a negative association with lipid peroxidation (17). Potassium and sodium are also present in high levels in human seminal plasma and have a significant role in acrosome reactions (18). Mg and Ca maintain the osmotic balance and contribute in nutrient transfer. The presence of Mg²⁺ and Ca²⁺ ions is essential for sperm capacitation, acrosome reaction and hy-

Received: 12/Oct/2017, Accepted: 29/Jan/2018

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 4, Jan-Mar 2019, Pages: 267-272

peractive motility of spermatozoa. It has been suggested that rise in concentrations of Ca, Mg and inorganic phosphate may be effective in treatment of accessory gland function of the male genitalia (19, 20).

Ca is one of the most extensively studied elements of mammalian semen (21). It is well-known that Ca, as an intracellular and universal second messenger, is crucial for maximum motility of sperm cells, capacitation, hyperactivation, acrosome reaction, chemotaxis and fertilization processes (22). Human spermatozoa are unable to fertilize an oocyte before their maturation through the female reproductive tract. The process of fertilization and maturation is tightly modulated by some signaling cascades and Ca, which plays a critical dynamic role in this process as an intracellular second messenger. Therefore, there may be a close relationship between Ca, and human sperm function and fertility outcome.

Understanding the mechanisms in which Ca deficiency can affect sperm function and fertility is a matter of utmost importance. Although different studies have demonstrated decreased level of Ca, in semen of infertile men, mechanisms of Ca, deficiency on male infertility are not well-considered. In the following sections we will discuss critical roles of Ca, in sperm function and consequences of Ca, deficiency in semen of the both experimental animal models and human subjects. Additionally, we will review the possible mechanisms by which Ca deficiency induces impaired spermatogenesis and male infertility.

Relationship between Ca and male infertility

Ca serves as a regulatory factor in different biological processes such as cell proliferation, protein secretion and muscle contraction (22). Most of these biological events are modulated by an intracellular Ca receptor known as calmodulin. Upon binding to Ca, calmodulin can activate different enzymes, especially protein kinases (PK), phosphatases, and phosphodiesterases (23). Na/K-ATPase and inositol 1,4,5-triphosphate receptor (IP3R), as an intracellular Ca store receptor, increase intercellular Ca concentration (24).

Prostate gland is a major source of Ca in human semen (25). Since Ca concentration in the prostate, seminal vesicles and epididymis is very high, numerous studies have investigated the association between Ca and male infertility (26). Many studies have reported a relationship between seminal Ca and male infertility (20, 23). The Ca channel blockers were also reported to be associated with male infertility. For example, Prien et al. observed that semen of men with hypomotility had significantly lower Ca level than men with normal motility (27).

Similarly, Wong et al. (28) reported that seminal Ca level in patients with hypomotility of sperm was significantly lower than those in fertile subjects. In another study, it

has been suggested that infertile men with and without varicocele have a significant lower Ca in their seminal plasma compared to fertile men (29). Other studies have also demonstrated a positive relationship between high Ca levels and fertility in men (30). In another research, a significant reduction was found in mean of Ca concentration in seminal plasma of normozoospermic infertile men compared with fertile men; however, it was not related to infertility classification (normo-, oligo-, and azoospermic) (31). Nishida et al. (32) have shown that in vitro exposure of human sperm to low Ca level increases fertilizing ability. Therefore, these data suggest that decreased level of seminal plasma Ca can be a reason for infertility in men. Recent studies have illustrated that there is a close relationship between vitamin D (VD) and seminal Ca concentration. Blomberg Jensen et al. (21, 33) indicated that VD deficiency can be associated with decreased level of intracellular Ca and subsequently poor sperm motility, deficiency of sperm acrosome reaction and increased risk of male infertility.

Although these studies showed relationship between reduced levels of Ca and increased risk of male infertility, the mechanism by which Ca deficiency affects fertilization rate of spermatozoa is not well-elucidated. The possible mechanisms in which Ca deficiency can affect sperm function and male infertility are discussed in the following sections. Impaired spermatogenesis, deficiency of steroidogenesis, poor sperm motility, abnormality in sperm chemotaxis, capacitation and acrosome reaction as well as reduced fertilization rate can be considered as possible mechanisms of the Ca deficiency effects on male infertility (Fig.1).

Role of Ca in spermatogenesis

Spermatogenesis is a process in which diploid spermatogonial stem cells undergo meiosis and produce differentiated haploid spermatozoa (34). Several studies have pointed out that Ca has important role in the regulation of spermatogenesis and fertilization processes as well as growth, differentiation, proliferation and cell death in spermatogonium and spermatocyte cells (34, 35). Ultrastructural distribution of intracellular Ca has been illustrated within the various germ cells during different developmental stages of gametogenesis in rat testis (35). Interestingly, the Ca level increased significantly from early to late stages of spermatogenesis (spermatogonia>spermatocyte>spermatids>spermatozoa) and probably reflects changes in its homeostasis and specific function during the formation of spermatozoa (34-36). Similar results have been observed in rat seminiferous tubules that showed significant increase of Ca from spermatogonia to early spermatids and gradually at advanced stages of mouse spermatogenesis (37). Recent investigations have shown that Ca deposits are observed in the Sertoli, myoid, and Leydig cells (35). Interestingly, calmodulin is particularly abundant in the testis, suggesting the importance of Ca for normal spermatogenesis (38).

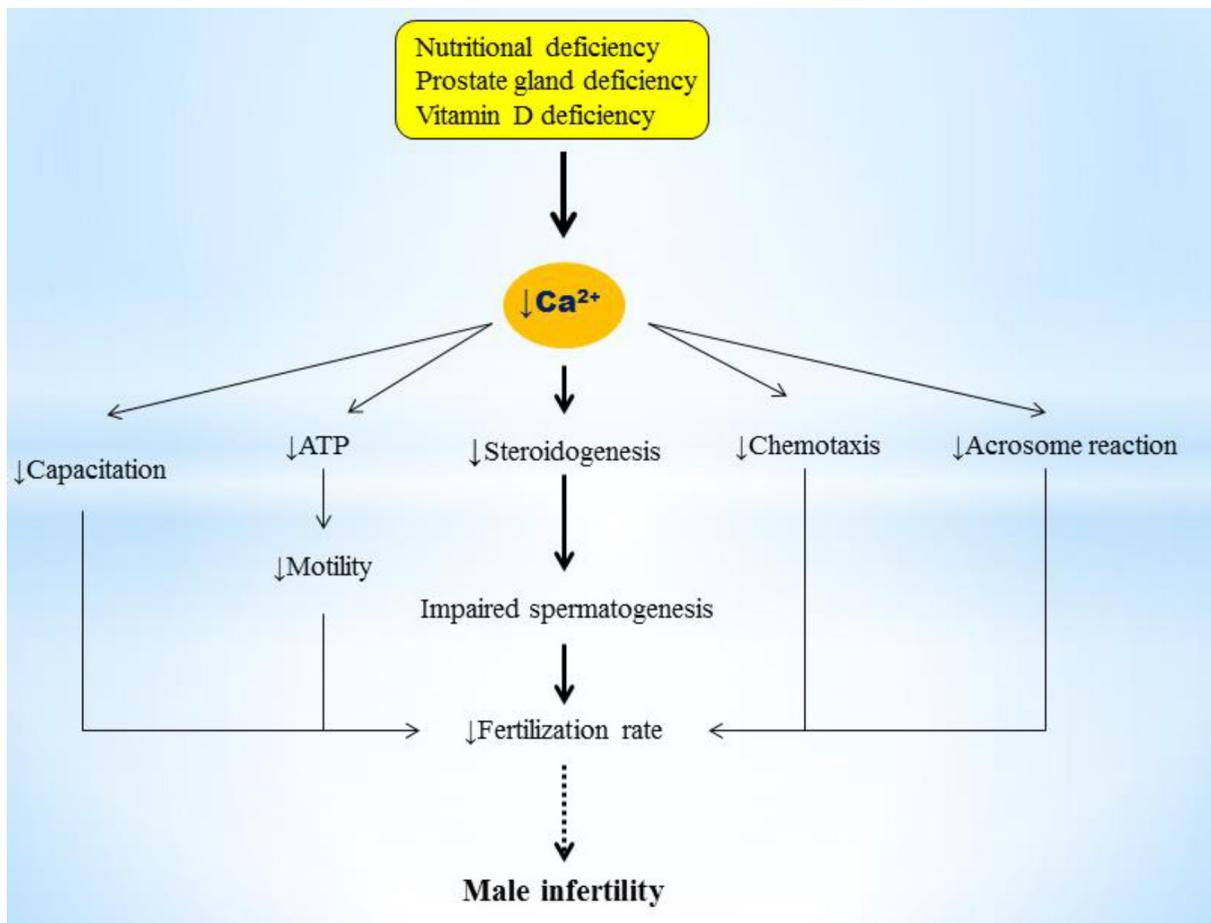


Fig.1: A schematic for the effects of Ca^{2+} deficiency on male infertility. Calcium deficiency decreases spermatogenesis and fertilization rate through several mechanisms including: reduced sperm chemotaxis, motility, capacitation, acrosome reaction, and steroidogenesis (21, 24, 39-42).

There are many Ca channels in germ cells of spermatogonia and spermatozoa which likely are responsible in the regulation of Ca signaling process (23). Ca signaling is necessary to shift from a proliferating phase of spermatogonia to the other advanced phases of spermatogenesis (43). Therefore, activation of the different Ca channels may induce changes in the Ca balance and homeostasis during different developmental stages of spermatogenesis (44). These changes are important in maintenance of spermatocyte function; otherwise any prolonged increase in the free Ca levels is toxic for cell function including chromatin condensation, mitochondria damage and activation of degradative enzymes so that ultimately can leads to cell death (45).

Role of Ca in Leydig and Sertoli cells

Recent evidences have reported that Ca is essential for stimulation of steroidogenesis in Leydig cells of the testis (42). An increased Ca level has been shown to be associated with an increased production and secretion of testosterone in stimulated Leydig cell, while Ca chelators inhibit steroid synthesis (24). Ca also plays a critical role in Sertoli-Sertoli junction dynamics. Previous studies reported that testosterone-mediated enhancement of Ca is required for the influx of extracellular Ca in Sertoli cells, indicating that Ca channels in plasma membrane play

crucial role in testosterone-Ca signaling (46). Therefore, given the regulatory function of testosterone in gonadal formation, differentiation, maturation and spermatogenesis, Ca deficiency can be associated with depletion of testosterone, inhibition of spermatogenesis and subsequently male infertility (Fig.1).

Ca and sperm parameters

There are evidences that changes in intracellular and seminal plasma Ca can affect sperm function and motility. Spermatozoa modulate their movement in response to an alteration in the intracellular Ca concentration, depending on the pH of the medium (47). Numerous studies demonstrated a relationship between Ca and sperm motility (48). Given the biochemical requirements for Ca by adenosine triphosphate (ATP) to drive the flagella, the relationship between Ca and sperm motility seems to be logical (Fig.1). For example, a pervious study showed that addition of Ca with calsemin to isolated ram caudal spermatozoa caused stimulation of flagellar beat activity (49). Morton et al. (50) demonstrated that reduced seminal Ca concentrations are positively correlated with decreased human sperm motility in the epididymal. Similarly, Schmid et al. (14) reported that declined level of intracellular Ca in sperm is significantly associated with increased risks of poor sperm motility, independent of male age.

Banjoko and Adeseolu (51) observed that men with hypomotile sperm (<60%) exhibited lower Ca concentrations compared to men with normal motility. Furthermore, seminal plasma Ca was negatively correlated with sperm motility and count. Bassey et al. (48) observed that seminal plasma Ca level was significantly lower in oligospermic, azospermic and asthenoligospermic infertile men compared to normospermic men. A more recent study has found a positive correlation between seminal plasma Ca concentration and semen parameters including pH, volume, sperm counts, and HOST% (52). Experimental evidences showed that use of EDTA, as a Ca chelator, causes a reduction in Ca concentration and significant loss of sperm motility (53). This suggests the regulatory effect of Ca on sperm motility and destructive effect of EDTA on spermatozoa. In another experimental study, Uhland et al. (54) showed that 1,25(OH)₂D₃ treatment increased intracellular Ca concentration in human spermatozoa from an intracellular Ca storage, increased sperm motility and induced the acrosome reaction.

Sperm chemotaxis

Sperm chemotaxis is a process in which spermatozoa are attracted toward egg (55). This is an important event in the fertilization process and may provide an insight into the mechanism underlying sperm-egg interaction. Recent studies have indicated that Ca may play a central role in sperm chemotaxis because it regulates sperm flagellar beating (56). When the spermatozoon ejaculated into the female reproductive tract, progesterone stimulates Ca entry from extracellular spaces into the spermatozoon. The increased intracellular Ca induces the beating of sperm flagella, and subsequently causes to the chemotactic turn and 'turn-and-straight' movements (35).

Role of Ca in sperm capacitation

Capacitation is a process in which sperm cells undergo a series of consecutive biochemical and molecular events during their movement across the female reproductive tract before reaching and fusing with oocyte. Sperm capacitation is modulated by several molecules present in the female reproductive tract. Ca is now considered as one of the significant factors that regulate sperm capacitation. Increased level of intracellular Ca as well as membrane hyperpolarization has been reported during the spermatozoon capacitation (36). Recent studies have indicated that Ca modulates sperm capacitation via the regulation of sperm cAMP-dependent signaling and tyrosine phosphorylation pathways in a biphasic manner (34). It has been also shown that the distribution of intracellular Ca is remodeled during post-mating capacitation in the male gamete of animals (57). In addition, recent proteomics studies identified some proteins such as ryanodine receptor, troponin, and sarcoplasmic Ca-binding protein that may contribute to the processes of Ca signaling in the male gamete of animals during different stages of reproduction such as capacitation (58, 59).

Sperm acrosome reaction

The acrosome reaction is a critical step during sperm interaction. This is a process in which spermatozoa penetrate and fuse with the oocyte membrane. Many studies indicated that Ca influx through the Ca channels of the sperm plasma membrane is necessary to initiate the acrosomal reaction and sperm fertility (37). This process is associated with the release of enzymes and membrane modifications, which are required for sperm-egg communication (60).

In a study, Walensky and Snyder (61) showed that acrosome is a source of internal Ca storage. Additionally, it has been demonstrated that both the plasma and the acrosomal membranes of mammalian spermatozoa contain Ca pumps which serve as a Ca storage during acrosome reaction (62). Experimental studies have shown that addition of ionophore A23187, which actively transports Ca from extracellular to intracellular space, induces sperm acrosome reaction (63). Another experimental study showed that the initiation of acrosome reaction with the ionophore is possible in the presence of Ca in the extracellular space (64). De Jonge (65) reported that exposure of capacitated spermatozoa to progesterone caused Ca influx from extracellular to intracellular space and initiation of sperm acrosome reaction. In another research, Benoff et al. (66) indicated that Cadmium (Cd) reduces sperm ability to undergo acrosome reaction via inhibition of Ca channels. Arabi and Mohammadpour (67) found that Cd can change the integrity and fluidity of acrosomal membranes of spermatozoa and induce abnormal acrosome reaction.

Role of Ca in fertilization

A great number of studies demonstrated the critical role of Ca in the regulation of egg activation and successful fertilization (68). Upon fertilization an increase in intracellular Ca induces sperm movement across the egg in a global wave as a sign of egg activation. During the spermatozoon-egg interaction, Ca is injected into the egg that triggers signal transduction pathway and initiates egg activation (39). A more recent study has shown that a large amount of Ca is concentrated in the head of mature spermatozoa that facilitates Ca injection into the egg (38). The Ca wave triggered by the spermatozoon can be generated even when the egg or oocyte is placed in Ca-free media, suggesting that it is because of release from internal sources (69). Some studies demonstrated that changes in intracellular Ca levels are involved in both oocyte maturation and egg activation at fertilization process (70). Ca signalling also plays a key role in the development of patterning in early embryos (69).

Conclusion

Ca deficiency can cause male infertility via several cellular and molecular mechanisms. Given the regulatory function of Ca in steroidogenesis, sperm motility, chemotaxis, capacitation and acrosome reaction within the women reproductive tract, Ca deficiency can be as-

sociated with reduced fertilization rate and male infertility. These data indicate that infertile patients may benefit from Ca supplementation. Therefore, Ca level should be monitored in semen of men with idiopathic infertility.

Acknowledgements

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

Authors' Contributions

A.B.H., A.I., H.R., M.M.; Participated in data collection and manuscript writing. E.T.; Contributed extensively in interoperation of the data collection, study concept and design, manuscript writing and review. A.S.; Contributed in manuscript writing, review and submission. All authors read and approved the final manuscript.

References

1. La Vignera S, Vicari E, Condorelli RA, D'Agata R, Calogero AE. Male accessory gland infection and sperm parameters (review). *Int J Androl*. 2011; 34(5 Pt 2): e330-e347.
2. Thoma ME, McLain AC, Louis JF, King RB, Trumble AC, Sundaram R, et al. Prevalence of infertility in the United States as estimated by the current duration approach and a traditional constructed approach. *Fertil Steril*. 2013; 99(5): 1324-1331. e1.
3. De Braekeleer M, Dao TN. Cytogenetic studies in male infertility: a review. *Hum Reprod*. 1991; 6(2): 245-250.
4. Kovac JR, Pastuszak AW, Lamb DJ. The use of genomics, proteomics, and metabolomics in identifying biomarkers of male infertility. *Fertil Steril*. 2013; 99(4): 998-1007.
5. Colagar AH, Marzony ET, Chaichi MJ. Zinc levels in seminal plasma are associated with sperm quality in fertile and infertile men. *Nutr Res*. 2009; 29(2): 82-88.
6. Mehrpour O, Karrari P, Zamani N, Tsatsakis AM, Abdollahi M. Occupational exposure to pesticides and consequences on male semen and fertility: a review. *Toxicol Lett*. 2014; 230(2): 146-156.
7. Kovac JR, Khanna A, Lipshultz LI. The effects of cigarette smoking on male fertility. *Postgrad Med*. 2015; 127(3): 338-341.
8. Colagar AH, Marzony ET. Ascorbic Acid in human seminal plasma: determination and its relationship to sperm quality. *J Clin Biochem Nutr*. 2009; 45(2): 144-149.
9. Colagar AH, Jorsaraee GA, Marzony ET. Cigarette smoking and the risk of male infertility. *Pak J Biol Sci*. 2007; 10(21): 3870-3874.
10. Hosseinzadeh Colagar A, Pouramir M, Tahmasbpour Marzony E, Jorsaraei SGA. Relationship between seminal malondialdehyde levels and sperm quality in fertile and infertile men. *Braz Arch Biol Technol*. 2009; 52(6): 1387-1392.
11. Tahmasbpour E, Balasubramanian D, Agarwal A. A multi-faceted approach to understanding male infertility: gene mutations, molecular defects and assisted reproductive techniques (ART). *J Assist Reprod Genet*. 2014; 31(9): 1115-1137.
12. Guzikowski W, Szykowska MI, Motak-Pochrzest H, Pawlaczyk A, Sypniewski S. Trace elements in seminal plasma of men from infertile couples. *Arch Med Sci*. 2015; 11(3): 591-598.
13. Marzec-Wroblewska U, Kaminski P, Lakota P. Influence of chemical elements on mammalian spermatozoa. *Folia Biologica*. 2012; 58(1): 7-15.
14. Schmid TE, Grant PG, Marchetti F, Weldon RH, Eskenazi B, Wyrobek AJ. Elemental composition of human semen is associated with motility and genomic sperm defects among older men. *Hum Reprod*. 2013; 28(1): 274-282.
15. Wdowiak A, Bakalczuk G, Bakalczuk S. Evaluation of effect of selected trace elements on dynamics of sperm DNA fragmentation. *Postepy Hig Med Dosw (Online)*. 2015; 69: 1405-1410.
16. Sultan J, Khan SH, Rashid F. Infertile men; a comparative study of trace elements in serum and seminal plasma in infertile men in an industrial area of lahore, punjab. *Professional Med J*. 2016; 23(3): 274-278.
17. Trvdá E, Lukáč N, Schneidgenová M, Lukáčová J, Szabó C, Goc Z, et al. Impact of seminal chemical elements on the oxidative balance in bovine seminal plasma and spermatozoa. *J Vet Med*. 2013; 2013: 125096.
18. Vickram AS, Ramesh Pathy M, Sridharan TB. Effect of various biomolecules for normal functioning of human sperm for fertilization: a review. *Int J Pharm Pharm Sci*. 2012; 4(4): 18-24.
19. Alvarez L, Dai L, Friedrich BM, Kashikar ND, Gregor I, Pascal R, et al. The rate of change in Ca(2+) concentration controls sperm chemotaxis. *J Cell Biol*. 2012; 196(5): 653-663.
20. Valsa J, Skandhan KP, Gusani P, Khan PS, Amith S, Gondalia M. Effect of daily ejaculation on semen quality and Ca and magnesium in seme. *Rev Int Androl*. 2013; 11(3): 94-99.
21. Blomberg Jensen M, Gerner Lawaetz J, Andersson AM, Petersen JH, Nordkap L, Bang AK, et al. Vitamin D deficiency and low ionized Ca are linked with semen quality and sex steroid levels in infertile men. *Hum Reprod*. 2016; 31(8): 1875-1885.
22. Valsa J, Skandhan KP, Khan PS, Avni KP, Amith S, Gondalia M. Ca and magnesium in male reproductive system and in its secretion. I. Level in normal human semen, seminal plasma and spermatozoa. *Urologia*. 2015; 82(3): 174-178.
23. Darszon A, Nishigaki T, Beltran C, Treviño CL. Ca channels in the development, maturation, and function of spermatozoa. *Physiol Rev*. 2011; 91(4): 1305-1355.
24. Costa RR, Varanda WA, Franci CR. A Ca-induced Ca release mechanism supports luteinizing hormone-induced testosterone secretion in mouse Leydig cells. *Am J Physiol Cell Physiol*. 2010; 299(2): C316-C323.
25. Valsa J, Skandhan KP, Sumangala B, Amith S, Skandhan Avni KP. Effect of different timings of the day on semen and Ca and magnesium in it. *Urologia*. 2016; 83(4): 207-210.
26. Hommonai ZT, Fainman N, Matzkin H, Paz(Frenkel) G, Kraicer PF. Zinc, magnesium and Ca in human semen and in prostatic function. *Harefuah*. 1978; 94(11): 361-363.
27. Prien SD, Lox CD, Messer RH, DeLeon FD. Seminal concentrations of total and ionized Ca from men with normal and decreased motility. *Fertil Steril*. 1990; 54(1): 171-172.
28. Wong WY, Flik G, Groenen PM, Swinkels DW, Thomas CM, Copius-Peereboom JH, et al. The impact of Ca, magnesium, zinc, and copper in blood and seminal plasma on semen parameters in men. *Reprod Toxicol*. 2001; 15(2): 131-136.
29. Hamada AJ, Esteves SC, Agarwal A. A comprehensive review of genetics and genetic testing in azoospermia. *Clinics (Sao Paulo)*. 2013; 68 Suppl 1: 39-60.
30. Logoglu G, Kendirci A, Ozgunen T. The role of seminal Ca in male infertility. *Journal of Islamic Academy of Sciences*. 1997; 10(1): 25-27.
31. Abou-Shakra FR, Ward NI, Everard DM. The role of trace elements in male infertility. *Fertil Steril*. 1989; 52(2): 307-310.
32. Nishida S, Yamano S, Aono T. Exposure of human sperm to low Ca medium enhances fertilizing ability. *Arch Androl*. 1996; 36(2): 145-153.
33. Blomberg Jensen M, Bjerrum PJ, Jessen TE, Nielsen JE, Joensen UN, Olesen IA, et al. Vitamin D is positively associated with sperm motility and increases intracellular Ca in human spermatozoa. *Hum Reprod*. 2011; 26(6): 1307-1317.
34. Golpour A, Pšenička M, Niksirat H. Subcellular distribution of Ca during spermatogenesis of zebrafish, *Danio rerio*. *J Morphol*. 2017; 278(8): 1149-1159.
35. Golpour A, Psenicka M, Niksirat H. Ultrastructural Localization of Intracellular Ca During Spermatogenesis of Sterlet (*Acipenser ruthenus*). *Microsc Microanal*. 2016; 22(6): 1155-1161.
36. Geybels MS, McCloskey KD, Mills IG, Stanford JL. Ca Channel Blocker Use and Risk of Prostate Cancer by TMPRSS2:ERG Gene Fusion Status. *Prostate*. 2017; 77(3): 282-290.
37. Santi CM, Martínez-López P, de la Vega-Beltrán JL, Butler A, Alisio A, Darszon A, et al. The SLO3 sperm-specific potassium channel plays a vital role in male fertility. *FEBS Lett*. 2010; 584(5): 1041-1046.
38. Abdou HS, Villeneuve G, Tremblay JJ. The Ca signaling pathway regulates leydig cell steroidogenesis through a transcriptional cascade involving the nuclear receptor NR4A1 and the steroidogenic acute regulatory protein. *Endocrinology*. 2013; 154(1): 511-520.
39. Navarrete FA, García-Vázquez FA, Alvau A, Escoffier J, Krapf D, Sánchez-Cárdenas C, et al. Biphasic role of Ca in mouse sperm capacitation signaling pathways. *J Cell Physiol*. 2015; 230(8): 1758-1769.
40. Jeon BG, Moon JS, Kim KC, Lee HJ, Choe SY, Rho GJ. Follicular fluid enhances sperm attraction and its motility in human. *J Assist Reprod Genet*. 2001; 18(8): 407-412.

41. Marquez B, Suarez SS. Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. *Biol Reprod.* 2004; 70(6): 1626-1633.
42. Chung JJ, Navarro B, Krapivinsky G, Krapivinsky L, Clapham DE. A novel gene required for male fertility and functional CATSPER channel formation in spermatozoa. *Nat Commun.* 2011; 2: 153.
43. Amemiya K, Hirabayashi M, Ishikawa H, Fukui Y, Hoichi S. The ability of whale haploid spermatogenic cells to induce Ca oscillations and its relevance to oocyte activation. *Zygote.* 2007; 15(2): 103-108.
44. Chiarella P, Puglisi R, Sorrentino V, Boitani C, Stefanini M. Ryanodine receptors are expressed and functionally active in mouse spermatogenic cells and their inhibition interferes with spermatogonial differentiation. *J Cell Sci.* 2004; 117(Pt 18): 4127-4134.
45. Lizama C, Alfaro I, Reyes JG, Moreno RD. Up-regulation of CD95 (Apo-1/Fas) is associated with spermatocyte apoptosis during the first round of spermatogenesis in the rat. *Apoptosis.* 2007; 12(3): 499-512.
46. Gorczyńska-Fjälling E. The role of Ca in signal transduction processes in Sertoli cells. *Reprod Biol.* 2004; 4(3): 219-241.
47. Giroux-Widemann V, Jouannet P, Pignot-Paintrand I, Feneux D. Effects of pH on the reactivation of human spermatozoa demembrated with Triton X-100. *Mol Reprod Dev.* 1991; 29(2): 157-162.
48. Bassey IE, Essien OE, Udoh AE, Imo IU, Effiong IO. Seminal plasma, selenium, magnesium and zinc levels in infertile men. *J Med Sci.* 2013; 13: 483-487.
49. Bradley MP, Forrester IT. Human and ram seminal plasma both contain a Ca-dependent regulator protein, calsemin. *J Androl.* 1982; 3(5): 289-296.
50. Morton BE, Sagadraca R, Fraser C. Sperm motility within the mammalian epididymis: species variation and correlation with free Ca levels in epididymal plasma. *Fertil Steril.* 1978; 29(6): 695-698.
51. Banjoko SO, Adeseolu FO. Seminal Plasma pH, Inorganic Phosphate, Total and Ionized Ca Concentrations In The Assessment of Human Spermatozoa Function. *J Clin Diagn Res.* 2013; 7(11): 2483-2486.
52. Talluri TR, Mal G, Ravi SK. Biochemical components of seminal plasma and their correlation to the fresh seminal characteristics in Marwari stallions and Poitou jacks. *Vet World.* 2017; 10(2): 214-220.
53. Lee CH, Anderson M, Chien YW. Characterization of in-vitro spermicidal activity of chelating agent against human sperm. *J Pharm Sci.* 1996; 85(6): 649-654.
54. Uhland AM, Kwiecinski GG, DeLuca HF. Normalization of serum Ca restores fertility in vitamin D-deficient male rats. *J Nutr.* 1992; 122(6): 1338-1344.
55. Eisenbach M. Sperm chemotaxis. *Rev Reprod.* 1999; 4(1): 56-66.
56. Yoshida M, Yoshida K. Sperm chemotaxis and regulation of flagellar movement by Ca²⁺. *Mol Hum Reprod.* 2011; 17(8): 457-465.
57. Niksirat H, Kouba A. Subcellular localization of Ca deposits in the noble crayfish *Astacus astacus* spermatophore: Implications for post-mating spermatophore hardening and spermatozoon maturation. *J Morphol.* 2016; 277(4): 445-452.
58. Niksirat H, Andersson L, James P, Kouba A, Kozák P. Proteomic profiling of the signal crayfish *Pacifastacus leniusculus* egg and spermatophore. *Anim Reprod Sci.* 2014; 149(3-4): 335-344.
59. Niksirat H, James P, Andersson L, Kouba A, Kozak P. Label-free protein quantification in freshly ejaculated versus post-mating spermatophores of the noble crayfish *Astacus astacus*. *J Proteomics.* 2015; 123: 70-77.
60. Zaneveld LJ, De Jonge CJ, Anderson RA, Mack SR. Human sperm capacitation and the acrosome reaction. *Hum Reprod.* 1991; 6(9): 1265-1274.
61. Walensky LD, Snyder SH. Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. *J Cell Biol.* 1995; 130(4): 857-869.
62. Spungin B, Breitbart H. Ca mobilization and influx during sperm exocytosis. *J Cell Sci.* 1996; 109 (Pt 7): 1947-1955.
63. Aitken RJ, Buckingham DW, Fang HG. Analysis of the responses of human spermatozoa to A23187 employing a novel technique for assessing the acrosome reaction. *J Androl.* 1993; 14(2): 132-141.
64. De Jonge CJ. Diagnostic significance of the induced acrosome reaction. *Reprod Med Rev.* 1994; 3: 159-178.
65. De Jonge C. Attributes of fertile spermatozoa: an uptake. *J Androl.* 1999; 20(4): 463-473.
66. Benoff S, Jacob A, Hurley IR. Male infertility and environmental exposure to lead and cadmium. *Hum Reprod Update.* 2000; 6(2): 107-121.
67. Arabi M, Mohammadpour AA. Adverse effects of cadmium on bull spermatozoa. *Vet Res Commun.* 2006; 30(8): 943-951.
68. Feng HL, Hershlag A, Han YB, Zheng LJ. Localizations of intracellular Ca and Ca²⁺-ATPase in hamster spermatogenic cells and spermatozoa. *Microsc Res Tech.* 2006; 69(8): 618-623.
69. Whitaker M. Ca at fertilization and in early development. *Physiol Rev.* 2006; 86(1): 25-88.
70. Homa ST, Carroll J, Swann K. The role of Ca in mammalian oocyte maturation and egg activation. *Hum Reprod.* 1993; 8(8): 1274-1281.

An Overview of The Globozoospermia as A Multigenic Identified Syndrome

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Abstract

Acrosome plays an integral role during fertilization and its absence in individuals with globozoospermia leads to failure of *in vitro* fertilization (IVF) and oocyte activation post-intracytoplasmic sperm injection (ICSI). A variety of processes, organelles and structures are involved in acrosome biogenesis including, trans-golgi network (TGN), acroplaxome and cellular trafficking. This review aims to explain roles of related signals and molecules involved in this process and also describe how their absence in form of mutation, deletion and knockout model may lead to phenomenon referred to globozoospermia.

Keywords: Acrosome, Globozoospermia, Male Infertility

Citation: Modarres P, Tavalaei M, Ghaedi K, Nasr-Esfahani MH. An overview of the globozoospermia as a multigenic identified syndrome. *Int J Fertil Steril*. 2019; 12(4): 273-277. doi: 10.22074/ijfs.2019.5561.

Introduction

Fertilization is a multifactorial process for fusion of gametes to initiate development of a new individual. For successful fertilization to occur, each process needs to take place in a coordinated manner. One of the main steps of fertilization is acrosome reaction during which proteolytic contents of acrosome is released to facilitate zona binding and penetration to zona and the oocyte by sperm (1). Structural and functional anomalies of acrosome lead to inability of sperm to penetrate oocyte, thereby resulting in failed fertilization and infertility. Furthermore, different studies show that when barriers of fertilization are bypassed during intra-cytoplasmic insemination, in certain cases with acrosome anomalies, the ability of sperm to induce fertilization is still diminished due to inability of the oocyte to induce activation (2-4).

Total absence of acrosome was first reported by Schirren et al. (5) which manifested by round head spermatozoa appearance. This syndrome has termed "globozoospermia" with a prevalence of 0.1% among infertile male population and two subtypes: complete (type-I: 100% spermatozoa are round head) or partial (type-II: over 50% spermatozoa are round head). Further genetic pedigree analysis revealed genetic basis with possible autosomal recessive inheritance is responsible for incidence of this syndrome (6). Individuals with globozoospermia commonly show no mental and physical abnormalities, and generally have

normal karyotype with no micro-deletion in chromosome Y (7). However, sperm cells of the affected persons are acrosome-less, and incapable of penetrating in zona pellucida (ZP). Considering the importance of acrosome in fertilization and oocyte activation, this review aimed to describe the genetic and molecular aspects of globozoospermia.

Genetic aspects of globozoospermia

Literature survey shows different approaches were taken by various researchers to detect genetic and molecular bases of globozoospermia. These approaches include: i. Purposefully designed knockout mice for a variety of genes including: *Hrb*, *Zpbp1*, *Hsp90b1*, *Vps54*, *SAMP32* (*SPACA1*), ii. Knockout mice approach for different purpose which later exhibited globozoospermia manifestation. The target genes were as: *Csnk2a2*, *GOPC*, *Gba2*, *PICK1*, iii. Whole-genome scan analysis which were carried out using SNP-array approach on the genome of globozoospermia and the genes associated with this syndrome. These genes identifiers are: *SPATA16* and *DPY19L2*, and iv. Assessment of protein localization associate with acrosome biogenesis such as: *SPGL4*, *Calicin*.

Among the 13 genes involved in globozoospermia, they were mostly related to Golgi network, acrosome formation, sperm head shaping (anchorage of acrosome to nucleus) and zona binding. Only, four genes have been so far de-

Received: 5/May/2018, Accepted: 30/May/2018

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 4, Jan-Mar 2019, Pages: 273-277

tected in individuals presenting globozoospermia including *DPY19L2*, *SPATA16*, *PICK1* and *Calicin* (6-10). It is important to note that in addition to genetic defects, deregulation of proteins (up or down regulation) can also result in the onset of globozoospermia. To further elucidate the role of these 13 genes, below section provides the cellular and molecular mechanisms in acrosome biogenesis.

Acrosome biogenesis

Acrosome structure is divided into two segments, anterior and equatorial segments. The former segment contains enzymes that are released during acrosome reaction while the latter segment is predominantly involved in sperm-oocyte fusion. Biogenesis of acrosome begins during meiosis and continues through early stages of spermiogenesis which is divided into four steps including golgi, cap, acrosomal and maturation phases (Fig.1A) (11). In golgi phase, pro-acrosomal granules (PAGs) derived from endoplasmic reticulum (ER) are transported to golgi sacs through anterograde pathway. Subsequently, PAGs are transported toward sperm nucleus where they bind to an actin-keratin containing cytoskeletal plate termed “acroplaxome”. In cap phase, PAGs fuse with each other to form a structure known as “acrosomal cap”. In acrosomal phase, cap begins to spread over anterior part of nucleus to form an acrosome like structure. In maturation phase, following condensation and elongation of nucleus with the help of manchette, the equatorial segment of acrosome is shaped. At this stage, the acrosome is surrounded by two distinct membranes known as “inner” and “outer” acrosomal membranes. Inner acrosomal membrane locates in vicinity of nuclear membrane, tightly anchors the acrosome to the nuclear envelop through cytoskeletal components known as “perinuclear theca” (Fig.1B, C) (12).

Originally, acrosome was described as a modified lysosome while recent literatures suggest that in addition to PAGs forming from Golgi network, early endosome (EE) may also have a role in acrosomal biogenesis (Fig.1B). Hence it is agreed that cargos originated from Golgi apparatus are sorted to plasma membrane, subsequently are recruited back into cytoplasm and incorporate into developing pro-acrosomes (13). During acrosomal biogenesis, particular proteins are involved that their absence or defect may result in globozoospermia.

Globozoospermia and associated proteins

Csnk2a2

Casein kinase II α ' polypeptide (Csnk2a2) was the first introduced protein whose gene was associated with globozoospermia. This protein is a kind of serine-threonine kinase which relates to nuclear matrix. Multiple forms of acrosome imperfection like complete lack of acrosome, indented/detached acrosome from nucleus, and acrosomal remnants were recognized in spermatozoa of *Csnk2a2*-deficient mice. In other words, mice lacking the *Csnk2a2* gene demonstrated aberrant development in both nucleus and acrosome (14).

GBA2

β -Glucosidase 2 (GBA2) is a glycolipid hydrolase resident in ER and its relation to globozoospermia was first recognized in glycolipid storage disease due to deficiency of *Gba2* in male mice with reduced fecundity. Glucosylceramides are normally transferred from developing germ cells to Sertoli cells for subsequent breakdown. Loss of the GBA2 results in accumulation of glucosylceramide in Sertoli cells and disrupts transport of glycolipid from germ cells which in turn interrupts normal Sertoli-germ cell interactions. Therefore, this defect leads to formation of abnormal sperm (Fig.1A, D). Unlike in mice, mutational assessments for *GBA2* in 3 unrelated families, originating from Britain, Canada, and Germany, have been unfruitful to show an association with globozoospermia in man (15).

SPATA16

Spermatogenesis-associated 16 (SPATA16), also known as NYD-SP12, is a human testis specific protein and its ortholog encoding gene is expressed in mouse spermatocyte and spermatids. SPATA16 has a subcellular localization in Golgi apparatus and pro-acrosomal vesicles being transported to acrosome. Its function is sorting and modification of acrosomal enzymes in Golgi network (16). This protein also interacts with other proteins involved in acrosomal biogenesis including GOPC and Hrb (Fig.1B). *SPATA16* was the first gene which was shown to contribute to human globozoospermia with an autosomal dominant pattern of inheritance (6).

Hrb

Hrb, formerly known as human Rev-binding/interacting protein (hRIP), is the cofactor of HIV-1 Rev protein, involved in shuttling of proteins between nucleus and cytoplasm. Hrb interacts with proteins involved in nucleocytoplasmic trafficking (17). Based on these functions, *Hrb* mice knockout model revealed that, Hrb is involved in vesicle to vesicle docking, fusion of pro-acrosomal vesicles with acrosome and thereby acrosomal biogenesis (Fig.1B). Therefore, its absence was associated with globozoospermia (18). Further analysis of *Hrb*^{-/-} mice revealed a second role for Hrb in formation of acroplaxome plaque. Acroplaxome is encompassed by 3 proteins including: F-actin, Sak57 (an ortholog of keratin5) and myosin Va. In *Hrb* deficient mice, keratin 5 filament bundle in acroplaxome is missing and the strength of acrosome vesicle in binding to nucleus is reduced which its outcome is manifested as globozoospermia (19).

PICK1

Protein interacting with C kinase 1 (PICK1) was initially found in brain. It plays an important role in protein trafficking of neurons. Although the *PICK1*^{-/-} mice were produced to study the brain function but these mice were infertile. PICK1 like GOPC has a postsynaptic density 95, discs large, and zonula occludens-1 (PDZ) domain which

is involved in PAG trafficking (Fig.1B) (20). So far one mutation in this gene has been reported to be associated with globozoospermia (9).

GOPC

GOPC gene, encodes Golgi-associated PDZ and coiled-coil motif containing protein (GOPC). GOPC protein has 5 domains including: one PDZ domain, two coiled-coil motifs, and two conserved domains with unknown function (21). GOPC is involved in PAG transport from Golgi network to acrosome and its absence (*GOPC*^{-/-}) is associated with globozoospermia (Fig.1B). In addition to lack of acrosome, other deformities associated with this defect, are lack of post-acrosomal sheath or peri-nuclear theca (22) and coiled-coil tail (23).

ZBPB1

ZP binding protein 1 (ZBPB1) or Sp38 or Iam 38 and its paralog, ZBPB2, were described as acrosomal proteins in mice and human. *ZBPB1*- deficient male mice are sterile and present round-head spermatozoa due to disrupted acrosome biogenesis. *Zbpb1* is an intra-acrosomal protein and *Zbpb1*-deficient spermatids demonstrate defective protein matrix assembly and results in fragmentation of the abnormal acrosomes (Fig.1B) (24).

Considering candidate genes responsible for abnormal sperm head morphology, heterozygous mutation in ZBPB1 were described in patients with aforementioned condition, however direct involvement of ZBPB1 in the onset of such conditions remains to be clarified (25).

SPACA1

Sperm acrosome associated 1 (SPACA1) or SAMP32 (sperm acrosomal membrane-associated protein 32) is a testis-specific transmembrane protein involved in sperm-egg interaction. During elongation stage of developing spermatozoa, this protein is localized in inner acrosomal membrane (Fig.1B) (26) and no role has been envisaged in acrosome reaction. The role of this protein in globozoospermia was initially recognized when this protein was absent in *Gopc*- and *Zbpb1*-disrupted mouse line. However, later studies revealed that “disruption of *Gopc* caused a significant decrease in SPACA1 and ZBPB1” while “disruption of *Zbpb1* caused loss of SPACA1 whereas GOPC was unaffected” and “disruption of *Spaca1* did not affect the amounts of GOPC and ZBPB1 in the testis”. Thereby, suggesting that *Spaca1* is likely downstream of these two genes. *Spaca1* deficiency leads to failure of acrosome thinning, coinciding with instability/or loss of acroplaxome and nuclear plate (27) and unlike most of aforementioned proteins, it has no role in protein transport in golgi network or in acrosome formation.

Hsp90b1

Heat shock protein 90b1 (Hsp90b1), a member of heat shock protein 90 family, is a testis specific endoplasmic

chaperone involving in entire folding, activation and/or degradation of ER proteins (Fig.1B). It was shown that *Hsp90b1*- null sperm cells are round and not able to fertilize the oocyte. Therefore, absence of this protein showed a potential role in the incidence of globozoospermia (28). Recent study has hypothesized that phosphorylation of Hsp90b1 along with other chaperon proteins during sperm capacitation leads to the formation of ZP-recognized protein complexes and/or the translocation of these complexes to the surface of spermatozoa (29).

Vps54

Vps54 is a protein apparently involved in tethering of vesicles from endosomes to the trans-golgi sacs (13). This is an alternative pathway in acrosome biogenesis as mentioned earlier. The role of this protein in acrosomal biogenesis was found when wobbler mouse with *Vps54*(*L967Q*) mutation were found to cause sterility. The protein codified by the Vps54 gene, has an active role in vesicular retrograde trafficking and like Hrb gene affects proacrosomal vesicle coalesces with acrosome (Fig.1B) (30).

SPAG4L/4L-2

SPAG4L and its isoform, are testis specific proteins, belong to SUN domain proteins. These transmembrane proteins are located on inner nuclear membrane (INM). By interacting with their partner on outer nuclear membrane (ONM), known as KASH domain can anchor or create linkage to nucleo- and cytoskeleton complex (LINC complex) (Fig.1C) (31).

Different members of this anchoring system have been discovered but their role in acrosomal biogenesis remains to be determined. Among these proteins, absence of SPAG4L/4L-2 has been associated with globozoospermia. SPAG4L/4L-2 is localized on apical side of nuclear membrane of developing spermatid and it may have a function in docking of acrosome vesicle to nuclear membrane (31).

DPY19L2

DPY19L2, similar to SPAG4L/4L-2, is a transmembrane protein with 8-10 predicted domains in inner nuclear membrane. The expression of this protein is restricted to testis and like SPAG4 (or SUN5) is involved in anchorage of cytoskeleton to nuclear membrane (Fig.1C). Therefore, its absence leads to instability and dissociation of the layered structure of acroplaxome, the nuclear/acrosome bridging region. Thereby, its absence results in formation of round head spermatozoa (32). Ellnati et al. (10) revealed that *DPY19L2* gene has an inevitable relationship with globozoospermia. They have shown that *DPY19L2* is one of the main genes responsible for globozoospermia. In this regard, a wide spectrum of plausible mutations for this gene has been detected in globozoospermic individuals such as: deletion of the whole locus, nonsense, missense, splicing mutations, partial deletion in different regions of the gene encompassing exons 8, 9, 11, 15, 21 and intron 11 (10, 33-36).

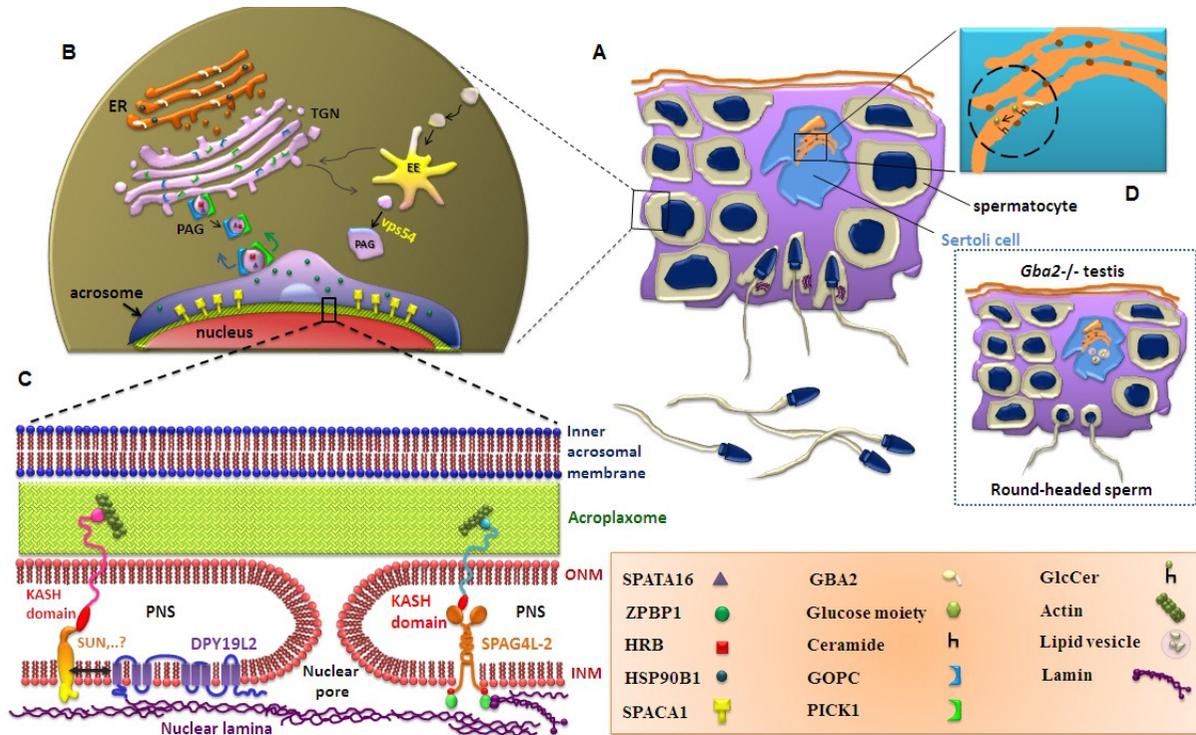


Fig.1: Schematic illustration of subcellular distribution and localization of several proteins involved in acrosome biogenesis in germ cells during spermiogenesis. **A.** Lack/disruption of each protein leads to impaired acrosome formation, and characterized as globozoospermia. **B.** SPATA16 and HRB are proteins which are transported in proacrosomal vesicle from Golgi to acrosome via PAG transporter proteins like GOPC and PICK1. Vps54 has a possible role in tethering of vesicles from early endosomes (EE) to the trans-Golgi network (TGN). SPACA1 is a transmembrane protein localized in inner acrosomal membrane of developing spermatozoa. Spaca1 deficiency leads to failure of acrosome thinning coincides with instability/loss of acroplaxome and nuclear plate. **C.** DPY19L2 and SPAG4L/4L-2 are transmembrane proteins located on inner nuclear membrane (INM) and are likely to be participating to form linkage to nucleo- and cytoskeleton complex (LINC) complex. PNS: perinuclear space, and **D.** GBA2 is a non-lysosomal glycosylceramidase (GlcCer) involved in catalysis of glycolipids, leading to releasing glucose moiety in ER lumen. Right inset: accumulation of lipid vesicles in testicular Sertoli cell of *Gba2*-null mice.

Calicin

Calicin is one of the subacrosomal cytoskeletal proteins involved in acrosomal biogenesis which its absence results in globozoospermia (8).

The proteomics of round-head spermatozoa:

Collectively, it is evident that numerous proteins are involved in acrosomal biogenesis and the absence of each protein may result in globozoospermia phenotype. One approach to distinguish proteins associated with globozoospermia is comparative proteomics between normozoospermia and globozoospermia. The results of this study have shown up/down regulation of several proteins in affected subjects. Spermatozoa acrosome membrane-associated protein 1 (SAMP1) and sperm protein associated with the nucleus on the X chromosome (SPANX) are among the proteins that their expression was shown to be down regulated (37). SAMP1 is a glycoprotein receptor residing in inner nuclear membrane and its absence results in mislocalization of the SUN1 (38). SPANX also acts as a nuclear envelope protein residing in post-acrosomal perinuclear theca and is expected to be associated with acrosome-nucleus binding and down regulation of this protein in globozoospermia may be underlying cause of the lack of acrosome (37).

Conclusion

Taken together, the results of this study suggest that mutation, deletion of genes products associate with Golgi apparatus, formation of acroplaxome or those associated with nucleo-cytoskeleton involved in attachment of acrosome with nucleus have a potential role in induction of globozoospermia.

Acknowledgements

We would like to express our gratitude to staff of Royan institute. There is no financial support and conflict of interest in this study.

Authors' Contributions

P.M; Search and collection of articles, interpretation, manuscript writing. M.T, K.G., M.H.N.-E.: Manuscript writing and final approval of manuscript. All authors read and approved the final manuscript.

References

- Ikawa M, Inoue N, Benham AM, Okabe M. Fertilization: A sperm's journey to and interaction with the oocyte. *J Clin Invest.* 2010; 120(4): 984-994.
- Nasr-Esfahani MH, Razavi S, Javdan Z, Tavalaee M. Artificial oocyte activation in severe teratozoospermia undergoing

- intracytoplasmic sperm injection. *Fertil Steril*. 2008; 90(6): 2231-2237.
3. Nasr-Esfahani MH, Deemeh MR, Tavalaei M. Artificial oocyte activation and intracytoplasmic sperm injection. *Fertil Steril*. 2010; 94(2): 520-526.
 4. Nasr-Esfahani MH, Tavalaei M, Deemeh MR, Arbabian M, Parrington J. Can assessment of total acrosin activity help predict failed or low fertilization rate ICSI for implementation of artificial oocyte activation? *Open Androl J*. 2010; 2(1): 19-26.
 5. Schirren CG, Holstein AF, Schirren C. Morphogenesis of round-headed spermatozoa of humans. *Andrologia*. 1971; 3(3): 117-125.
 6. Dam AH, Kosciński I, Kremer JA, Moutou C, Jaeger AS, Oudakker AR, et al. Homozygous mutation in SPATA16 is associated with male infertility in human globozoospermia. *Am J Hum Genet*. 2007; 81(4): 813-820.
 7. Dam AH, Feenstra I, Westphal JR, Ramos L, van Golde RJ, Kremer JA. Globozoospermia revisited. *Hum Reprod Update*. 2007; 13(1): 63-75.
 8. Escalier D. Failure of differentiation of the nuclear-perinuclear skeletal complex in the round-headed human spermatozoa. *Int J Dev Biol*. 1992; 34(2): 287-297.
 9. Liu G, Shi QW, Lu GX. A newly discovered mutation in PICK1 in a human with globozoospermia. *Asian J Androl*. 2010; 12(4): 556-560.
 10. Ellnati E, Kuentz P, Redin C, Jaber S, Vanden Meerschaut F, Makarian J, et al. Globozoospermia is mainly due to DPY19L2 deletion via non-allelic homologous recombination involving two recombination hotspots. *Hum Mol Genet*. 2012; 21(16): 3695-3702.
 11. Moreno RD, Alvarado CP. The mammalian acrosome as a secretory lysosome: new and old evidence. *Mol Reprod Dev*. 2006; 73(11): 1430-1434.
 12. Yoshinaga K, Toshimori K. Organization and modifications of sperm acrosomal molecules during spermatogenesis and epididymal maturation. *Microsc Res Tech*. 2003; 61(1): 39-45.
 13. Berruti G, Ripolone M, Ceriani M. USP8, a regulator of endosomal sorting, is involved in mouse acrosome biogenesis through interaction with the spermatid ESCRT-0 complex and microtubules. *Biol Reprod*. 2010; 82(5): 930-939.
 14. Xu X, Toselli PA, Russell LD, Seldin DC. Globozoospermia in mice lacking the casein kinase II alpha catalytic subunit. *Nat Genet*. 1992; 23(1): 118-121.
 15. Yildiz Y, Matern H, Thompson B, Allegood JC, Warren RL, Ramirez DM, et al. Mutation of beta-glucosidase 2 causes glycolipid storage disease and impaired male fertility. *J Clin Invest*. 2006; 116(11): 2985-2994.
 16. Xu M, Xiao J, Chen J, Li J, Yin L, Zhu H, et al. Identification and characterization of a novel human testis-specific Golgi protein, NYDSP12. *Mol Hum Reprod*. 2003; 9(1): 9-17.
 17. Doria M, Salcini AE, Colombo E, Parslow TG, Pelicci PG, Di Fiore PP. The eps15 homology (EH) domain-based interaction between eps15 and hrb connects the molecular machinery of endocytosis to that of nucleocytoplasmic transport. *J Cell Biol*. 1999; 147 (7): 1379-1384.
 18. Kang-Decker N, Mantchev GT, Juneja SC, McNiven MA, Van Deursen JM. Lack of acrosome formation in Hrb-deficient mice. *Science*. 2001; 294(5546): 1531-1533.
 19. Kierszenbaum AL, Tres LL, Rivkin E, Kang-Decker N, van Deursen JM. The acroplaxome is the docking site of Golgi-derived myosin Va/Rab27a/b-containing proacrosomal vesicles in wild-type and Hrb mutant mouse spermatids. *Biol Reprod*. 2004; 70(5): 1400-1410.
 20. Xiao N, Kam C, Shen C, Jin W, Wang J, Lee KM, et al. PICK1 deficiency causes male infertility in mice by disrupting acrosome formation. *J Clin Invest*. 2009; 119(4): 802-812.
 21. Yao R, Ito C, Natsume Y, Sugitani Y, Yamanaka H, Kuretake S, et al. Lack of acrosome formation in mice lacking a Golgi protein, GOPC. *Proc Natl Acad Sci*. 2002; 99(17): 11211-11216.
 22. Ito C, Suzuki-Toyota F, Maekawa M, Toyama Y, Yao R, Noda T, et al. Failure to assemble the peri-nuclear structures in GOPC deficient spermatids as found in round-headed spermatozoa. *Arch Histol Cytol*. 2004; 67(4): 349-360.
 23. Suzuki-Toyota F, Ito C, Toyama Y, Maekawa M, Yao R, Noda T, et al. The coiled tail of the round-headed spermatozoa appears during epididymal passage in GOPC-deficient mice. *Arch Histol Cytol*. 2004; 67(4): 361-371.
 24. Lin YN, Roy A, Yan W, Burns KH, Matzuk MM. Loss of zona pellucida binding proteins in the acrosomal matrix disrupts acrosome biogenesis and sperm morphogenesis. *Mol Cell Biol*. 2007; 27(19): 6794-6805.
 25. Yatsenko AN, O'Neil DS, Roy A, Arias-Mendoza PA, Chen R, Murthy LJ, et al. Association of mutations in the zona pellucida binding protein 1 (ZBP1) gene with abnormal sperm head morphology in infertile men. *Mol Hum Reprod*. 2012; 18(1): 14-21.
 26. Hao Z, Wolkowicz MJ, Shetty J, Klotz K, Bolling L, Sen B, et al. SAMP32, a testis-specific, isoantigenic sperm acrosomal membrane-associated protein. *Biol Reprod*. 2002; 66(3): 735-744.
 27. Fujihara Y, Satouh Y, Inoue N, Isotani A, Ikawa M, Okabe M. SPACA1-deficient male mice are infertile with abnormally shaped sperm heads reminiscent of globozoospermia. *Development*. 2012; 139(19): 3583-3589.
 28. Audouard C, Christians E. Hsp90β1 knockout targeted to male germline: a mouse model for globozoospermia. *Fertil Steril*. 2011; 95(4): 1475-1477.
 29. Bromfield EG, Nixon B. The function of chaperone proteins in the assemblage of protein complexes involved in gamete adhesion and fusion processes. *Reproduction*. 2013; 145(2): R31-R42.
 30. Paiardi C, Pasini ME, Gioria M, Berruti G. Failure of acrosome formation and globozoospermia in the wobbler mouse, a Vps54 spontaneous recessive mutant. *Spermatogenesis*. 2011; 1(1): 52-62.
 31. Frohnert C, Schweizer S, Hoyer-Fender S. SPAG4L/SPAG4L-2 are testis-specific SUN domain proteins restricted to the apical nuclear envelope of round spermatids facing the acrosome. *Mol Hum Reprod*. 2010; 17(4): 207-218.
 32. Pierre V, Martinez G, Coutton C, Delarochette J, Yassine S, Novella C, et al. Absence of Dpy19l2, a new inner nuclear membrane protein, causes globozoospermia in mice by preventing the anchoring of the acrosome to the nucleus. *Development*. 2012; 139(16): 2955-2965.
 33. Harbuz R, Zouari R, Pierre V, Ben Khelifa M, Kharouf M, Coutton C, et al. A recurrent deletion of DPY19L2 causes infertility in man by blocking sperm head elongation and acrosome formation. *Am J Hum Genet*. 2011; 88(3): 351-361.
 34. Kosciński I, Ellnati E, Fossard C, Redin C, Muller J, Velez de la Calle J, et al. DPY19L2 deletion as a major cause of globozoospermia. *Am J Hum Genet*. 2011; 88(3): 344-350.
 35. Coutton C, Zouari R, Abada F, Ben Khelifa M, Merdassi G, Triki C, et al. MLPA and sequence analysis of DPY19L2 reveals point mutations causing globozoospermia. *Hum Reprod*. 2012; 27(8): 2549-2558.
 36. Zhu F, Gong F, Lin G, Lu G. DPY19L2 gene mutations are a major cause of globozoospermia: identification of three novel point mutations. *Mol Hum Reprod*. 2013; 19(6): 395-404.
 37. Liao TT, Xiang Z, Zhu WB, Fan LQ. Proteome analysis of round-headed and normal spermatozoa by 2-D fluorescence difference gel electrophoresis and mass spectrometry. *Asian J Androl*. 2009; 11(6): 683-693.
 38. Gudise S, Figueroa RA, Lindberg R, Larsson V, Hallberg E. Samp1 is functionally associated with the LINC complex and A-type lamina networks. *J Cell Sci*. 2011; 124(Pt 12): 2077-2085.

Prevalence of Sexual Dysfunction among Infertile Women in Iran: A Systematic Review and Meta-analysis

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Abstract

Infertile women are at a higher risk of sexual dysfunction compared to fertile women. Infertility is a major source of stress, anxiety, and depression, which strongly affects sexual health. The aim of this study is to estimate the prevalence of female sexual dysfunction (FSD) among infertile Iranian women. We searched the main international databases (Web of Science, PubMed, Medline, and Scopus) and national databases (Scientific Information Database, Magiran, and IranMedex) from their inception until April, 2017. Due to heterogeneity between the studies, the extracted data were pooled using a random-effects model by Stata software. Out of 313 retrieved studies, we included 18 studies of 3419 infertile women in the meta-analysis. The pooled prevalence of FSD was 64.3% [95% confidence interval (CI): 53.3-75.3]. Our findings revealed that sexual desire (59.9%, 95% CI: 38.7-81.2) was the most prevalent disorder and vaginismus (19.2%, 95% CI: 11.3-27.2) was the least prevalent among infertile women. The results of our meta-analysis suggested that more than 64% of infertile Iranian women reported sexual dysfunction, which was meaningfully high. This study also showed that sexual desire was significantly more common than other sexual dysfunction dimensions and the prevalence of vaginismus was the least common.

Keywords: Female, Infertility, Iran, Prevalence

Citation: Omani-Samani R, Amini P, Navid B, Sepidarkish M, Maroufizadeh S, Almasi-Hashiani A. Prevalence of sexual dysfunction among infertile women in Iran: a systematic review and meta-analysis. *Int J Fertil Steril*. 2019; 12(4): 278-283. doi: 10.22074/ijfs.2019.5395.

Introduction

The estimated prevalence of infertility is approximately 9% worldwide (1). Infertility has negative effects on emotional health, quality of life, and a couple's sexual relationship (2, 3). Infertility has a greater impact of psychosexual behaviour in women than men (4). Sexual function is one of the most important components of quality of life and social health (5). The estimated prevalence of sexual problems in Iranian women is 31.5%, and for Iranian men, it is 18.8% (6). Many studies report that sexual dysfunction is more common among infertile women (7, 8).

Sexual dysfunction has several domains. The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) lists the types of sexual dysfunctions in females as female sexual interest/arousal disorder, female orgasmic disorder, and genito-pelvic pain/penetration disorder (9). The Female Sexual Function Index (FSFI) contains four domains: sexual arousal, orgasm, satisfaction, and pain (10).

There is an association between sexual dysfunction and infertility (11). Sexual dysfunction may cause dif-

ficulties in sexual function during attempts to conceive (12). In order to perform the diagnostic assessment and sexual dysfunction therapy in infertile women, it is necessary to specify the prevalence of these disorders. The prevalence of sexual dysfunction varies across populations and is affected by medical, psychological, socio-economic, cultural, and ethnic factors (13).

Many studies conducted in Iran to evaluate the prevalence of sexual dysfunction among infertile women have reported various findings (14-17). Thus, we conducted this meta-analysis to estimate the prevalence rate of sexual dysfunction in infertile Iranian women.

Materials and Methods

Search strategy

Royan Institute approved this systematic review and meta-analysis (code: 95000051). The authors followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist to perform this meta-analysis (18). The authors searched for the prevalence of female sexual dysfunction (FSD) in infertile Iranian women. We searched published literature in the

Received: 7/Sep/2017, Accepted: 7/May/2018

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 4, Jan-Mar 2019, Pages: 278-283

international (Web of Science, PubMed, Scopus) and national (Magiran, SID, and IranMedex) electronic databases from their inception until April 2017. Key words used for the search included “sexual problem”, “sexual disability”, “sexual dysfunction”, “sexual dysfunction, physiological”, “sexual problems”, “sexual pain”, “orgasm”, “lubrication”, “sexual excitement”, “sexual desire”, “dyspareunia”, “vaginismus”, “Iran”, “infertility”, “infertility, female”, “cross-sectional study”, “prevalence study” and “prevalence”. No time restriction was applied to the searches and we included both Farsi and English languages in the study. In addition to the mentioned databases, the grey literatures were searched using Google Scholar for the possibility of missed papers as recommended by Haddaway et al. (19). We also checked the reference lists of the included articles for additional potentially applicable papers.

Inclusion and exclusion criteria

Studies with the estimated prevalence rates of FSD, observational studies, studies in Farsi and English languages, and those without any restricted published date were included in this study. Excluded from this meta-analysis were interventional studies, repeated or duplicated studies, and studies with no relevant reported data.

Data extraction and quality assessment

In this meta-analysis, 2 authors (AAH and SM) separately extracted the required data from the included studies. Data extracted were: first Authors' name, year of publication, place of study, published year of study, mean age, infertility year, sample size, type of questionnaire, and the prevalence estimate of FSD and its dimensions. Then, 2 reviewers (AAH and MS) independently performed the quality assessment based on our modified STROBE checklist (20). The quality of the papers was low (22.22%), moderate (61.11%), and high (16.67%). This checklist contained sample size, sampling method, analysis, generalizability, quality of results reported, and study design.

Statistical analysis

The pooled prevalence was estimated by the “metan” command in Stata. Statistical heterogeneity between studies was checked by the Cochrane Q test and I² statistics. Because of low primary studies, for the Cochrane Q test, we considered a P<0.10 to be statistically significant. An value of 25% indicated low heterogeneity, 50% was moderate, and 75% indicated high heterogeneity (21).

The outcome measure of study was prevalence of sexual dysfunction in infertile women. In terms of the outstanding heterogeneity among the studies, we applied a random effect model to pool the primary prevalence rates. To explain the sources of between-study heterogeneity, meta-regression was performed for the year of the study, the sample size, and type of questionnaire. By running the

“metainf” command, we conducted sensitivity analyses by excluding each study from the analysis to examine the influence of each study on the pooled estimate. The Funnel plot, Begg's rank correlation, and Egger's weighted regression tests were used to assess publication bias (22, 23). The level of significance in these tests was less than 0.10 because of the statistical power. Finally, cumulative meta-analysis was performed to investigate whether the amount of prevalence changed noticeably over time (“metacum” command). All statistical analyses were performed using Stata version 14.0 (Stata Corp., College Station, TX, USA).

Results

Study selection

The details of the study selection method are shown in Figure 1. We identified a total of 313 relevant papers; after removal of the duplicates, 271 papers remained. After screening the titles and abstracts, we disqualified 228 papers, and resumed the full texts for 43 relevant papers. Next, we excluded all non-eligible studies, which left a total of 18 cross-sectional or case control studies based on the inclusion criteria for the meta-analysis.

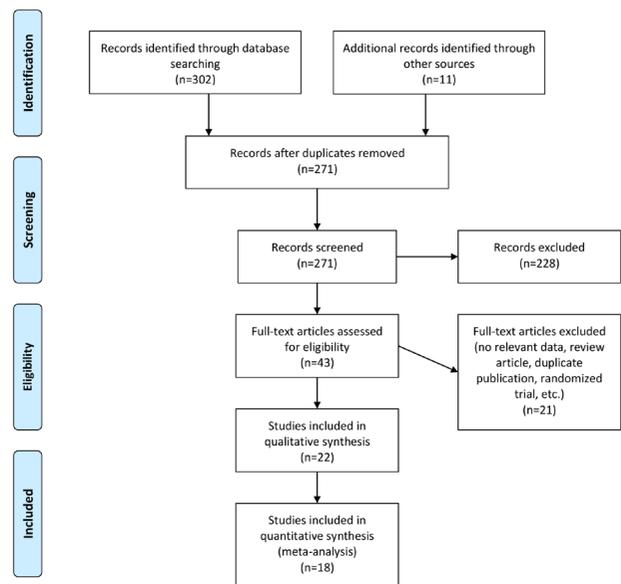


Fig.1: Flow diagram of the literature search for studies included in the meta-analysis.

Study characteristics

The majority (two-thirds) of the studies used the FSFI questionnaire to assess the prevalence of FSD. The lowest prevalence of FSD among infertile women was 46.6%, whereas the highest prevalence of FSD was 87.1%. These studies were published between 2001 and 2017 and had a diverse sample size that ranged from 30 to 604 cases, with a total of 3419 infertile women. Additional information about each primary study included in this analysis is shown in Table 1.

Table 1: Characteristics of the primary studies included in the meta-analysis

ID	Author	Year published	City	Sample size	Age (Y) (mean \pm SD)	Mean years of infertility (mean \pm SD)	Questionnaire	Sampling method	Quality assessment
1	Sargolzaee et al. (24)	2001	Mashhad	30	25.77 \pm 5.08	4.2 \pm 3.09	GSF	Random	Moderate
2	Besharat and Hoseinzadeh Bazargani (25)	2006	Tehran	45	28.8 \pm 4.68	NA	Golombok-Rust	Convenience	Low
3	Tayebi and Yassini Addakani (26)	2007	Yazd	300	27.93 \pm 4.8	5.42 \pm 3.2	NR	Volunteer	Moderate
4	Khademi et al. (27)	2008	Tehran	100	26.9 \pm 5	5.3 \pm 3.7	SFQ	Volunteer	Moderate
5	Fahami et al. (28)	2009	Isfahan	140	29 \pm 5.5	6.5 \pm 5.2	FSFI	Convenience	Moderate
6	Pakpour et al. (8)	2012	5 cities	604	30 \pm 7.8	NA	FSFI	Convenience	High
7	Aghamohammadian Sharbaf (29)	2014	Mashhad	200	28.8 \pm 6.2	NA	FSFI	Convenience	Moderate
8	Basirat et al. (30)	2014	Babol	208	27.85 \pm 5.7	NA	FSFI	NA	High
9	Davari Tanha et al. (11)	2014	Tehran	320	29.66	NA	FSFI	NA	Moderate
10	Hashemi et al. (31)	2014	Tehran	128	30.9 \pm 4.9	NA	FSFI	NA	Moderate
11	Jamali et al. (32)	2014	Jahrom	100	28.56 \pm 5.72	NA	FSFI	Random	High
12	Jamali et al. (33)	2014	Jahrom	502	30.95 \pm 6.80	NA	FSFI	Convenience	Moderate
13	Karamidehkordi and Roudsari (34)	2014	Mashhad	130	27 \pm 4.58	NA	FSFI	Convenience	Low
14	Alirezaee et al. (35)	2014	Mashhad	85	NA	NA	FSFI	Convenience	Low
15	Bakhtiari et al. (36)	2016	Babol	236	26.1 \pm 5.3	60.2 \pm 8.4 months	DSM	Convenience	Moderate
16	Mirblouk et al. (37)	2016	Guilan	147	31.66 \pm 6.8	NA	FSFI	NA	Moderate
17	Zare et al. (38)	2016	Mashhad	110	29.2 \pm 4.9	4.85 \pm 3.53	Golombok-Rust	Convenience	Moderate
18	Billar et al. (39)	2017	2 cities	34	42	NA	FSFI	Convenience	Low

GSF; Global Sexual Functioning Scale, SFQ; Sexual Function Questionnaire, FSFI; Female Sexual Function Index, NR; Not reported, and DSM; The diagnostic and statistical manual of mental disorders.

Evaluation of heterogeneity and meta-analysis

The results of Cochran's Q test and I² statistics displayed considerable heterogeneity among the primary studies included for FSD (Q=194.04, P=0.0001 and I²: 95.4%); thus, we used the random effects model for analysis. The pooled prevalence of FSD was 64.3% (95% CI: 53.3-75.3). As shown in Figure 2, the lowest prevalence of FSD was reported by Basirat et al. (30) in Babol, Northern Iran (46.6%, 95% CI: 36.7%-56.5%) and Jamali et al. (33) reported the highest prevalence in Jahrom, Southern Iran (87.1%, 95% CI: 83.9%-90.3%).

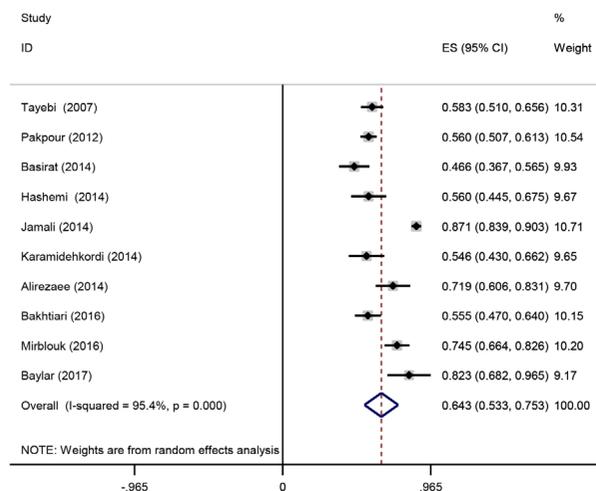


Fig.2: Forest plot that shows the prevalence of female sexual dysfunction (FSD) among infertile Iranian women.

The pooled estimated prevalence of different dimensions of sexual dysfunction that included sexual desire, sexual excitement, orgasm, dyspareunia and vaginismus is presented in Table 2. The results showed that the most prevalent sexual disorder was related to sexual desire (59.9%; 95% CI: 38.7-81.2) and the least prevalent was vaginismus (19.2%, 95% CI: 11.3-27.2).

Table 2: The pooled estimated prevalence of different dimensions of sexual dysfunction

Disorder	Number of included studies	Pooled estimated (%)	95% CI	I ² (%)
Sexual desire	8	59.9	38.7-81.2	99.2
Sexual excitement	4	52.3	29.6-75.0	96.9
Orgasm	7	53.8	27.9-79.7	99.4
Dyspareunia	6	52.9	29.4-76.4	98.8
Vaginismus	2	19.2	11.3-27.2	82.6

CI; Confidence interval and I²; I square.

Publication bias

We used Begg's test to assess for probable publication bias of FSD prevalence. The results showed no evidence of any publication bias (P=0.325).

Meta-regression

In order to assess the sources of heterogeneity, we in-

cluded 4 variables in a univariate meta-regression. The results suggested that the study sample size ($P=0.992$), date ($P=0.366$), type of questionnaire ($P=0.418$), and age ($P=0.070$) were not accountable for the heterogeneity in the FSD prevalence. Therefore, we used the random effect model because of the presence of heterogeneity between studies.

Sensitivity analysis and cumulative meta-analysis

In order to calculate the influence of each primary study, a sensitivity analysis was performed by removing each study from the analysis and calculating the point estimates. The results of the sensitivity analysis (Table 3) showed that after removal of the individual studies, the pooled prevalence of FSD ranged from 61.2%, after excluding Jamali et al. (33) to 66.2% after excluding Basirat et al. (30).

Table 3: Sensitivity analysis to estimate the pooled prevalence by removal of each individual study

Study omitted	Pooled prevalence	95% CI	
Basirat et al. (30)	0.662	0.550	0.774
Karamidehkordi and Roudsari (34)	0.653	0.537	0.769
Bakhtiari et al. (36)	0.653	0.536	0.770
Hashemi et al. (31)	0.652	0.535	0.768
Pakpour et al. (8)	0.653	0.535	0.770
Tayebi and Yassini Addakani (26)	0.650	0.530	0.769
Alirezaee et al. (35)	0.635	0.515	0.754
Mirblouk et al. (37)	0.631	0.509	0.754
Billar et al. (39)	0.625	0.508	0.742
Jamali et al. (33)	0.612	0.546	0.678

After sorting the studies based on publication year, the cumulative meta-analysis showed that the overall prevalence estimate was not constant over time; rather there was an increase after 2014 (Fig.3).

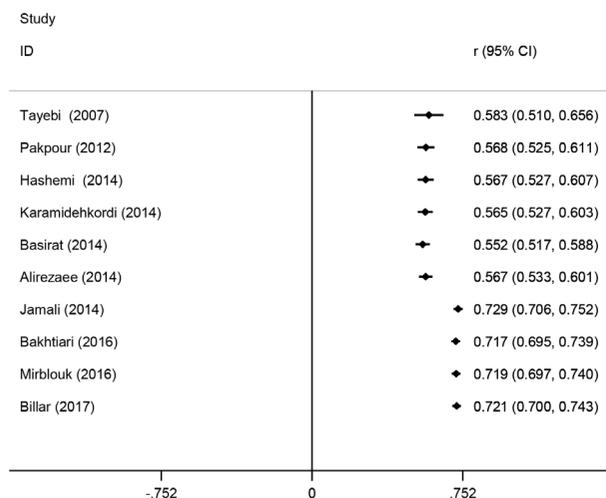


Fig.3: Cumulative meta-analysis of female sexual dysfunction (FSD) by sorting the studies based on publication time.

Discussion

The tendency of having sexual intercourse is strongly affected by pregnancy, which results in a low FSD score. It is well-known that infertile women are at a higher risk of sexual dysfunction compared to fertile women (33, 40, 41). Infertility is a major source of stress, anxiety and depression, which strongly affects sexual health. It has been shown that sexual dysfunction simultaneously compounds the disappointment of childlessness and the distress of medical treatment among infertile patients (42). However, sex is less defined as a loving act and considered more of a clinical tool among infertile couples (14). Our study has revealed that 64% of infertile women in Iran have sexual dysfunction. The studies were published with different sample sizes over a 17-year period. However, the current study showed that the diversity in the FSD prevalence was not affected by sample size, date, age, and type of questionnaires in Iran. This study also demonstrated that vaginismus was significantly less prevalent than other sexual dysfunction dimensions. Regarding the vast spectrum of vaginismus, women might not be aware of their disorder, which might lead to a low prevalence rate. Psychological variables are the most responsible factors for vaginismus (43).

Although the prevalence of sexual desire was higher than the other dimensions, dyspareunia, orgasm, and sexual excitement did not considerably differ in prevalence ratios. Based on the results of our meta-analysis, the prevalence of FSD among infertile women in Iran was noticeable. This might be due the adverse consequences of infertility such as personal and marital distress, depression, anxiety, reduction in self-esteem, and greater risk of psychological pressure that strongly contributes to sexual dysfunction in women (44). It has been demonstrated that both of the diagnosis of infertility and the treatments affect FSD (41). Some local and cultural aspects could reduce the amount of sexual functioning among Iranian infertile women such as lack of sexual knowledge and poor emotional relationship, the presence of economic problems, and pregnancy as the only point for sexual function.

Keskin et al. (45) found that 64.8% of women with primary infertility and 76.5% of those with secondary infertility had sexual dysfunction. Aggarwal et al. (4) reported that 63.67% of infertile women had FSD. Potential factors such as kidney failure, multiple sclerosis, heart disease and bladder problems, hormonal dysfunctions, and social and psychological problems might be responsible (8, 46). In comparison to the Middle East, the FSD prevalence rate is relatively higher in Iran (47). However, the difference is not considerable, which might be due to the similarities in culture and the same amount of development. However, the respondents were self-reporting in Iranian studies. According to cultural conditions, patients might not provide the exact responses to the questions and there might be biases in the prevalence rate.

There may be a two-way relationship between infertility and sexual dysfunction. Infertility changes sexual feelings and sexual dysfunction may result in infertility. Howev-

er, numerous potential factors cause the increase in FSD prevalence among infertile women and include involuntary childlessness, woman's age, husband's age, the lack of knowledge about marital issues, lack of training in the society, socio-economic status, infertility characteristics, the relationship with partner, duration of marriage, medical problems, depression, anxiety, loss of self-esteem, menopausal status, history of previous infertility treatment, income level, lower educational level, frequency of intercourse, and higher partner education (8, 45, 48-53).

Infertility affects the dimensions of sexual dysfunction (2, 37, 44, 53). In 2 different studies conducted by Keskin et al. (45) in Turkey and Pakpour et al. (8) in Iran, the researchers reported that the prevalence of sexual desire, orgasm, and satisfaction decreased among women with secondary infertility compared to those with primary infertility. Iris et al. (2) investigated the effects of infertility and infertility duration on female sexual function. They demonstrated that the mean score of all sexual functions such as desire, arousal, lubrication, orgasm, sexual satisfaction, and pain, as well as the total score decreased over time. The similarity of the prevalence ratios among the FSD dimensions in Iran might be due to the similarity in their risk factors. These potential factors could explain the difference in prevalence ratios across countries. Berger et al. (54) assessed the association between infertility and sexual dysfunction in men and women. They indicated that desire was strongly associated with problems in achieving pregnancy and infertility. These researchers introduced sexual dysfunction as a complex issue among couples with infertility and suggested that health policy makers should utilize appropriate medical therapy and psychosocial tools for infertile couples.

However, the power of statistical tools that has identified the heterogeneity in the studied meta-analysis differs according to the sample size of the studies as well as the number of included studies. The chi-square test is strongly affected by these limitations, such that a non-significant result must not be taken as evidence of lack of heterogeneity. On the other hand, the power of the chi-square test is high when many studies are included in a meta-analysis. The I^2 value depends on the magnitude of the prevalence ratios (55). In our meta-analysis, the result of chi-square test has been confirmed by the I^2 test, which addressed considerable heterogeneity among the reported prevalence ratios of the included studies. These studies were conducted in different regions of the country. The heterogeneity might be due to the diversities in the ethnic and cultural conditions, uneven development regions and disparity in the amount of knowledge, particularly about sexual performance.

Limitations in this study included the use of different questionnaires with different scoring methods to assess the prevalence of sexual dysfunction; therefore, we did not pool all of the scores in a continuous scale. In some studies, the scores of the questionnaires (in a continuous scale) was reported, whereas in other studies, the preva-

lence of FSD (in a categorized scale) was reported. There were different cut-offs for the questionnaires. For example, the point at which a woman was classified as having a sexual dysfunction or not might have been used in the studies. However, we ignored this issue and pooled the reported prevalence rate.

Conclusion

The results of current meta-analysis discovered that prevalence of FSD in infertile Iranian women was considerable. More than 64% of these women had sexual dysfunction. This study also showed that sexual desire was significantly more common than other sexual dysfunction dimensions and that the prevalence of vaginismus was less than the other dimensions.

Acknowledgements

The authors express their appreciation to Royan Institute for financial and scientific support. The authors declare that they have no conflicts of interest.

Authors' Contributions

A.A.-H, R.O.-S., M.S.; Contributed to the study conception and design. A.A.-H., M.S., S.M., P.A., B.N.; Performed the literature search, data extraction and quality assessment. A.A.-H., R.O.-S.; Performed data analysis and interpretation. All authors drafted and reviewed the manuscript and approved the final version of manuscript for submission.

References

1. Boivin J, Bunting L, Collins JA, Nygren KG. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod.* 2007; 22(6): 1506-1512.
2. Iris A, Aydogan Kirmizi D, Taner CE. Effects of infertility and infertility duration on female sexual functions. *Arch Gynecol Obstet.* 2013; 287(4): 809-812.
3. Monga M, Alexandrescu B, Katz SE, Stein M, Ganiats T. Impact of infertility on quality of life, marital adjustment, and sexual function. *Urology.* 2004; 63(1): 126-130.
4. Aggarwal RS, Mishra VV, Jasani AF. Incidence and prevalence of sexual dysfunction in infertile females. *Middle East Fertility Society Journal.* 2013; 18(3): 187-190.
5. Wischmann TH. Sexual disorders in infertile couples. *J Sex Med.* 2010; 7(5): 1868-1876.
6. Bakoyi F, Sh O, Nasiri F. Determine the frequency sexual dysfunction in married women and some related factors in Babol. *J Babol Univ Med Sci.* 2006; 9: 59-64.
7. Fakhri A, Pakpour AH, Burri A, Morshedi H, Zeidi IM. The female sexual function index: translation and validation of an Iranian version. *J Sex Med.* 2012; 9(2): 514-523.
8. Pakpour AH, Yekaninejad MS, Zeidi IM, Burri A. Prevalence and risk factors of the female sexual dysfunction in a sample of infertile Iranian women. *Arch Gynecol Obstet.* 2012; 286(6): 1589-1596.
9. IsHak WW, Tobia G. DSM-5 changes in diagnostic criteria of sexual dysfunctions. *Reprod Sys Sexual Disorders.* 2013; 2: 122.
10. Rosen R, Brown C, Heiman S, Leiblum S, Meston C, Shabsigh R, et al. The female sexual function index (FSFI): a multidimensional self-report instrument for the assessment of female sexual function. *J Sex Marital Ther.* 2000; 26(2): 191-208.
11. Davari Tanha F, Mohseni M, Ghajarzadeh M. Sexual function in women with primary and secondary infertility in comparison with controls. *Int J Impot Res.* 2014; 26(4): 132-134.
12. Khayata GM, Rizk DE, Hasan MY, Ghazal-Aswad S, Asaad MA.

- Factors influencing the quality of life of infertile women in United Arab Emirates. *Int J Gynaecol Obstet.* 2003; 80(2): 183-188.
13. Hosseini Tabaghdehi M, Keramat A, Khosravi A. Prevalence of female sexual dysfunction in Iran: a meta-analysis study. *International Journal of Health Studies.* 2016; 2(4): 10-13.
 14. Zare Z, Amirian M, Golmakani N, Mazlom R, Laal Ahangar M. Sexual dysfunction in infertile women. *Int J Reprod Biomed (Yazd).* 2016; 14(2): 89-94.
 15. Aggarwal RS, Mishra VV, Jasani AF. Incidence and prevalence of sexual dysfunction in infertile females. *Middle East Fertil Soc J.* 2013; 18(3):187-190.
 16. Diamond MP, Legro RS, Coutifaris C, Alvero R, Robinson RD, Casson PA, et al. Sexual function in infertile women with polycystic ovary syndrome and unexplained infertility. *Am J Obstet Gynecol.* 2017; 217(2): 191. e1-191. e19.
 17. Khademi A, Alleyassin A, Amini M, Ghaemi M. Evaluation of sexual dysfunction prevalence in infertile couples. *J Sex Med.* 2008; 5(6): 1402-1410
 18. Moher D, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Ann Intern Med.* 2009; 151(4): 264-269, W64.
 19. Haddaway NR, Collins AM, Coughlin D, Kirk S. The role of google scholar in evidence reviews and its applicability to grey literature searching. *PLoS One.* 2015; 10(9): e0138237.
 20. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP; STROBE Initiative. Strengthening the reporting of observational studies in epidemiology (STROBE) statement: guidelines for reporting observational studies. *BMJ.* 2007; 335(7624): 806-808.
 21. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med.* 2002; 21(11): 1539-1558.
 22. Begg CB, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. *Biometrics.* 1994; 50(4): 1088-1101.
 23. Egger M, Davey Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. *BMJ.* 1997; 315(7109): 629-634.
 24. Sargolzaee M, Moharreri F, Arshadi H, Javadi K, Karimi S, Fayyazi-bordbar M. Psychosexual and depression disorders in infertile female referring to Mashhad Infertility Treatment Center. *J Reprod Infertil.* 2001; 2(4): 46-52.
 25. Besharat MA, Hoseinzadeh Bazargani R. A comparative study of fertile and infertile women's mental health and sexual problems. *Iranian Journal of Psychiatry and Clinical Psychology.* 2006; 12(2): 146-153.
 26. Tayebi N, Yassini Addakani SM. The prevalence of sexual dysfunctions in infertile women. *Middle East Fertil Soc J.* 2007; 12(3): 184-187.
 27. Khademi A, Alleyassin A, Amini M, Ghaemi M. Evaluation of sexual dysfunction prevalence in infertile couples. *J Sex Med.* 2008; 5(6): 1402-1410.
 28. Fahami F, Najarian M, Kohan S. Comparison among some effects of sexual dysfunction in female spouses of fertile and infertile couples. *Iran J Nurs Midwifery Res.* 2009; 14(1): 25-28.
 29. Aghamohammadian Sharbaf HR, Zarezade Kheibari S, Horouf Ghanad M, HokmAbadi ME. The relationship between perfectionism and sexual function in infertile women. *Iranian Journal of Obstetrics, Gynecology and Infertility.* 2014; 17(97): 9-17.
 30. Basirat Z, Pasha H, Esmailzadeh S, Faramarzi M. Evaluation of the Female Sexual Dysfunction in a Sample of Iranian Infertile Women. *Br J Med Med Res.* 2014; 4(36): 5825-5838.
 31. Hashemi S, Ramezani Tehrani F, Noroozadeh M, Rostami dovom M, Azizi F. Infertility, the most adverse outcome among sexual function outcome affecting of Iranian women with polycystic ovarian syndrome. *Iranian Journal of Endocrinology and Metabolism.* 2014; 16(3): 197-204.
 32. Jamali S, Rasekh Jahromi A, Javadpour S. Sexual function in fertile and infertile women referring to the Jahrom Infertility in 2011. *Jundishapur J Chronic Dis Care.* 2014; 3(1): 11-20.
 33. Jamali S, Zarei H, Rasekh Jahromi A. The relationship between body mass index and sexual function in infertile women: A cross-sectional survey. *Iran J Reprod Med.* 2014; 12(3): 189-198.
 34. Karamidehkordi A, Roudsari RL. Body image and its relationship with sexual function and marital adjustment in infertile women. *Iran J Nurs Midwifery Res.* 2014; 19(7 Suppl 1): S51-S58.
 35. Alirezaee S, Ozgoli G, Alavi Majd H. Comparison of sexual self-efficacy and sexual function in fertile and infertile women referred to health centers in Mashhad in 1392. *Pajoohandeh.* 2014; 19(3): 131-136.
 36. Bakhtiari A, Basirat Z, Nasiri-Amiri F. Sexual dysfunction in women undergoing fertility treatment in iran: prevalence and associated risk factors. *J Reprod Infertil.* 2016; 17(1): 26-33.
 37. Mirblouk F, Asgharnia DM, Solimani R, Fakor F, Salamat F, Mansoori S. Comparison of sexual dysfunction in women with infertility and without infertility referred to Al-Zahra Hospital in 2013-2014. *Int J Reprod Biomed (Yazd).* 2016; 14(2): 117-124.
 38. Zare Z, Amirian M, Golmakani N, Mazlom R, Ahangar ML. Sexual dysfunction in infertile women. *Int J Reprod Biomed (Yazd).* 2016; 14(2): 89-94.
 39. Billar S, Jahanbakhshi Z, Jahangir A. Studying sexual malfunctioning, mental health, depression, anxiety, and stress in menopausal, unproductive, and addict women. *Pajoohesh Nameh Zanan.* 2017; 7(17): 1-15.
 40. Mendes de Leon CF, Dilillo V, Czajkowski S, Norten J, Schaefer J, Catellier D, et al. Psychosocial characteristics after acute myocardial infarction: the ENRICH pilot study. *Enhancing recovery in coronary heart disease. J Cardiopulm Rehabil.* 2001; 21(6): 353-362.
 41. Cwikel J, Gidron Y, Sheiner E. Psychological interactions with infertility among women. *Eur J Obstet Gynecol Reprod Biol.* 2004; 117(2): 126-131.
 42. Grill E, Schattman GL. Female sexual dysfunction and infertility. In: *Lipshultz LI, Pastuszak AW, Goldstein AT, Giraldi A, Perelman MA, editors. Management of sexual dysfunction in men and women.* New York: Springer; 2016; 337-342.
 43. Watts G, Nettle D. The role of anxiety in vaginismus: a case-control study. *J Sex Med.* 2010; 7(1 Pt 1): 143-148.
 44. Millheiser LS, Helmer AE, Quintero RB, Westphal LM, Milki AA, Lathi RB. Is infertility a risk factor for female sexual dysfunction? A case-control study. *Fertil Steril.* 2010; 94(6): 2022-2025.
 45. Keskin U, Coksuer H, Gungor S, Ercan CM, Karasahin KE, Baser I. Differences in prevalence of sexual dysfunction between primary and secondary infertile women. *Fertil Steril.* 2011; 96(5): 1213-1217.
 46. Jaafarpour M, Khani A, Khajavikhan J, Suhrabi Z. Female sexual dysfunction: prevalence and risk factors. *J Clin Diagn Res.* 2013; 7(12): 2877-2880.
 47. Shaeer O, Shaeer K, Shaeer E. The Global Online Sexuality Survey (GOSS): female sexual dysfunction among Internet users in the reproductive age group in the Middle East. *J Sex Med.* 2012; 9(2): 411-424.
 48. Ishak IH, Low WY, Othman S. Prevalence, risk factors, and predictors of female sexual dysfunction in a primary care setting: a survey finding. *J Sex Med.* 2010; 7(9): 3080-3087.
 49. West SL, Vinikoor LC, Zolnoun D. A systematic review of the literature on female sexual dysfunction prevalence and predictors. *Annu Rev Sex Res.* 2004; 15: 40-172.
 50. Schover LR, van der Kaaij M, van Dorst E, Creutzberg C, Huyghe E, Kiserud CE. Sexual dysfunction and infertility as late effects of cancer treatment. *EJC Suppl.* 2014; 12(1): 41-53.
 51. Turan V, Kopuz A, Ozcan A, Kocakaya B, Sahin C, Solmaz U. Sexual dysfunction in infertile Turkish females: prevalence and risk factors. *Eur J Obstet Gynecol Reprod Biol.* 2014; 182: 128-131.
 52. Piva I, Lo Monte G, Graziano A, Marci R. A literature review on the relationship between infertility and sexual dysfunction: Does fun end with baby making? *Eur J Contracept Reprod Health Care.* 2014; 19(4): 231-237.
 53. Oskay UY, Beji NK, Serdaroglu H. The issue of infertility and sexual function in Turkish women. *Sexuality and Disability.* 2010; 28(2): 71-79.
 54. Berger MH, Messori M, Pastuszak AW, Ramasamy R. Association Between Infertility and Sexual Dysfunction in Men and Women. *Sex Med Rev.* 2016; 4(4): 353-365.
 55. Higgins JP, Green S. *Cochrane handbook for systematic reviews of interventions.* The Cochrane Collaboration: John Wiley & Sons; 2011.

Association of Tumor Necrosis Factor- α ($TNF-\alpha$) -308G>A and -238G>A Polymorphisms with Recurrent Pregnancy Loss Risk: A Meta-Analysis

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Abstract

Background: Multiple studies have been carried out examining the association of tumor necrosis factor- α gene ($TNF-\alpha$) promoter region polymorphisms with recurrent pregnancy loss (RPL) risk. However, the results remain controversial and incomplete. Hence, we performed a meta-analysis to evaluate the association of the $TNF-\alpha$ -308G>A and -238G>A polymorphisms with RPL risk.

Materials and Methods: In this meta-analysis, a comprehensive search of PubMed, Web of Knowledge and EMBASE was performed to identify relevant studies published until December 1, 2017. The associations were assessed by odds ratio (OR) and its corresponding 95% confidence interval (CI).

Results: A total of 29 case-control studies, comprising 20 studies on $TNF-\alpha$ -308G>A (3,461 cases and 3,895 controls) and nine studies on $TNF-\alpha$ -238G>A (2,589 cases and 2,664 controls), were included in the meta-analysis. Overall, we found $TNF-\alpha$ -308G>A to be associated with an increase in RPL risk under the homozygote (OR=1.716, 95% CI: 1.210-2.433, P=0.002) and the recessive (OR=1.554, 95% CI: 1.100-2.196, P=0.012) models. $TNF-\alpha$ -238G>A was also significantly associated with increased risk of RPL under the allele model (OR=1.554, 95% CI: 1.100-2.196, P=0.012). Stratified analysis revealed a more significant association between the $TNF-\alpha$ -308G>A polymorphism and increased RPL risk in Asians under the homozygote (OR=2.190, 95% CI: 1.465-3.274, P<0.001), the dominant (OR=1.642, 95% CI: 1.269-2.125, P<0.001) and the recessive (OR=1.456, 95% CI: 1.039-2.040, P=0.029) models, but not in Caucasians. A non-significant association was, however, identified between $TNF-\alpha$ -238G>A and RPL risk based on ethnicity. Moreover, $TNF-\alpha$ -308G>A and -238G>A polymorphisms were significantly associated with increased risk of RPL in high quality studies and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) subgroups.

Conclusion: The present meta-analysis demonstrates that $TNF-\alpha$ -308G>A and -238G>A polymorphisms are associated with an increased risk of RPL.

Keywords: Meta-Analysis, Miscarriage, Polymorphism, Pregnancy Loss, Tumor Necrosis Factor- α

Citation: Aslebahar F, Neamatzadeh H, Meibodi B, Karimi-Zarchi M, Tabatabaei RS, Noori-Shadkam M, Mazaheri M, Dehghani-Mohammadabadi R. Association of tumor necrosis factor- α (TNF- α) -308G>A and -238G>A polymorphisms with recurrent pregnancy loss risk: a meta-analysis. *Int J Fertil Steril.* 2019; 12(4): 284-292. doi: 10.22074/ijfs.2019.5454.

Introduction

Recurrent pregnancy loss (RPL) is traditionally defined as the occurrence of three or more (≥ 3) consecutive pregnancy losses; however, the American Society of Reproductive Medicine (ASRM) has recently redefined RPL as two or more pregnancy losses (1, 2). It is estimated that up to 3% of fertile couples have been diagnosed with RPL (3). Moreover, RPL is accompanied by an increased risk of other pregnancy complications such as preterm birth or small for gestational age newborns (4). RPL remains

one of the most important issues in reproductive medicine and there are multiple barriers to the prevention, diagnosis and treatment of it (5).

Many studies have been undertaken to identify the underlying aetiology, however, the cause of miscarriage can be identified in only 50% of cases (1, 6). Maternal age and number of previous miscarriages are two independent risk factors for a further miscarriage (7). Moreover, the known causes of RPL include chromosomal and metabolic abnormalities, uterine anomalies and

Received: 30/Dec/2017, Accepted: 1/May/2018

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 4, Jan-Mar 2019, Pages: 284-292

immunologic factors (8). The parental carriers of balanced structural chromosomal rearrangements including balanced reciprocal and Robertsonian translocations are responsible for 2-4% of RPL cases (5-9). There is much evidence that tumor necrosis factor- α (TNF- α) also plays an important role in the implantation, placentation and pregnancy outcome (10).

TNF- α , a key pro-inflammatory cytokine, is secreted by macrophages and plays an important role in apoptotic cell death and initiating an immune response (10, 11). TNF- α is located on human chromosomes 6p21.3, spanning 2,762bp, and contains 4 exons (11, 12). There are several common single nucleotide polymorphisms (SNPs) in TNF- α which can regulate the transcription and production of TNF- α (12). To date, several promoter region SNPs in TNF- α have been reported, among which two, namely -308G>A (rs1800629) and -238G>A (rs361525), are most frequently studied in RPL (11-13). These polymorphisms have been shown to contribute to the susceptibility of several autoimmune conditions (9, 12, 13). Moreover, numerous studies have examined the association between TNF- α polymorphisms and risk of RPL; however, results have been controversial and inconclusive. These inconsistencies may be partly due to low sample sizes, false positive findings, publication bias, ethnic and geographical heterogeneity, and different characteristics among studies such as sources of controls. Meta-analysis is an important tool to obtain an unbiased estimate of the role of genetic variation in disease susceptibility (14). Thus, we conducted a meta-analysis to comprehensively evaluate the association of TNF- α -308G>A and -238G>A polymorphisms with susceptibility to RPL.

Materials and Methods

Search strategy

This meta-analysis was conducted and reported in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines. All studies published up to December 1, 2017 reporting the association of the TNF- α -308G>A and -238G>A polymorphisms with RPL were identified by searching the literature in databases including PubMed, EMBASE, ISI Web of Science, Google Scholar, China National Knowledge Infrastructure (CNKI), and Wanfang. The following combination of MeSH terms and keywords was used: (“tumor necrosis factor alpha” OR “TNF- α ” OR “cachexin” OR “cachectin”) AND (“-308G>A” OR “rs1800629” OR “-238G>A” OR “rs361525”) AND (“recurrent pregnancy loss” OR “pregnancy loss” OR “miscarriage” OR “RPL” OR “habitual abortion” OR “abortion” OR “unexplained recurrent spontaneous abortion”) AND (“gene” OR “allele” OR “genotype” OR “mutation” OR “variant” OR “variation” OR “polymorphism”). In addition, the reference lists of retrieved articles, reviews and previous meta-analyses were manually screened for additional studies. In the case where more than one article was published by the same author using the same case series, the study with the largest sample size was investi-

gated. Moreover, no restrictions were placed on language, and only published studies with full-text articles were included.

Inclusion and exclusion criteria

Studies fulfilling the following selection criteria were included in this meta-analysis: i. Has original and published data, ii. Uses case-control or cohort design, iii. Examines the associations of TNF- α -308G>A and -238G>A polymorphisms with RPL risk, and iv. Provides sufficient data for calculation of odds ratio (OR) with 95% confidence interval (CI). In addition, the following exclusion criteria were also used: i. Not relevant to TNF- α -308G>A and -238G>A polymorphisms and RPL, ii. The design is based on family or sibling pairs, iii. No usable data reported, iv. The study only involved a case population, v. Animal studies, vi. Duplicated publications, and vii. Abstracts, case-only articles, editorials, and reviews. If studies had partly overlapped subjects, only the one with the largest sample size was included.

Data extraction

Two investigators independently reviewed full manuscripts of eligible studies, and the relevant data were extracted into predesigned data collection forms. Any discrepancy was resolved by discussion or consensus by involving a third reviewer when required. The following data were collected from each study: first Authors' surname, year of publication, country of origin, ethnicity of the study population, genotyping method, source of control groups (population- or hospital-based controls), total number of cases and controls as well as numbers of cases and controls for each TNF- α SNP genotype, and deviation from Hardy-Weinberg Equilibrium (HWE) of the control group. Diverse ethnicities were categorized as Asian, African, Latinos and Caucasian.

Statistical analysis

The association of TNF- α -308G>A and -238G>A polymorphisms with RPL was estimated by ORs and their 95% CIs. Z-test was carried out to evaluate the statistical significance of pooled ORs ($P < 0.05$ was considered statistically significant). The pooled ORs for both polymorphisms were performed under the following five genetic models: allele model (B vs. A), homozygote model (BB vs. AA), heterozygote model (BA vs. AA), dominant model (BA+BB vs. AA) and recessive model (BB vs. BA+AA). The heterogeneity of studies was assessed by using Cochran's Q-test and the I^2 test. A significance level of < 0.10 was used to indicate heterogeneity among studies. Moreover, a high value of I^2 indicated a higher probability of the existence of heterogeneity ($I^2 = 0$ to 25%, no heterogeneity; $I^2 = 25$ to 50%, moderate heterogeneity; $I^2 = 50$ to 75%, large heterogeneity; and $I^2 = 75$ to 100%, extreme heterogeneity). When between-study heterogeneity was found a random-effects model was performed; otherwise, a fixed-effects model (Mantel-Haenszel method) was employed. HWE of genotype

distribution in the controls of included studies was conducted using an online program (<http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>), and $P < 0.05$ was considered as significant deviation from HWE. Stratified analyses were performed according to ethnicity, source of controls and study quality (HWE status). To validate the reliability and stability of the results, sensitivity analysis was performed with a single study in the meta-analysis being removed each time to reflect the influence of the individual data set on the pooled OR. The funnel plot was employed to examine publication bias. Egger's regression analysis was used for re-evaluation of publication bias. The significance of the intercept was determined by the t test suggested by Egger, with $P < 0.10$ considered as representative of statistically significant publication bias. Funnel plots and Egger's linear regression tests were used to provide a diagnosis of the potential publication bias. In the presence of bias, we utilized the Duval and Tweedie non-parametric "trim and fill" method to adjust results. The statistical analysis for this meta-analysis was performed by using the comprehensive meta-analysis (CMA) version 2.20 software (Biostat, USA) using two-sided P values.

Results

The search process and search outcomes are listed in Figure 1. One hundred and forty-six potential studies were collected by database and manual search. After screening titles and abstracts, 30 studies were considered duplicates and were excluded. Following the full-text review, 45 articles were excluded as they were reviews, case reports, not case-control designed, contained no data concerning RPL and two lacked any data concerning the polymorphisms of interest. Moreover, we also examined recent reviews and meta-analyses; no additional relevant study was found. Finally, 29 case-control studies in 20 publications with 5,050 RPL cases and 6,559 controls were included in the meta-analysis. There were 20 case-control studies (15-34) for the *TNF- α -308G>A* polymorphism including 3,461 RPL cases and 3,895 controls, and nine case-control studies (25-28, 30-32, 34) for the *TNF- α -238G>A* polymorphism including 2,589 RPL cases and 2,664 controls (Table 1). Data from studies published between 2001 and 2017 were pooled to the meta-analysis. Among the 29 case-control studies, there were eight studies of Caucasians, 16 studies of Asians, two studies of Latinos and two studies of Africans. Studies had been carried out in United Kingdom, Brazil, Argentina, Germany, Iran, Mexico, Tunisia, Italy, China, Bahrain, India, Korea, and Saudi Arabia. The *TNF- α* polymorphisms were genotyped by five methods including polymerase chain reaction-amplification refractory mutation system (PCR-ARMS), PCR-restriction fragment length polymorphism (PCR-RFLP), sequence-specific oligonucleotide (SSO), PCR-sequence-specific primer (PCR-SSP) and direct sequencing. The distribution of genotypes in the controls of five studies (20, 26, 27, 32, 33) deviated from HWE expectations ($P < 0.05$). The

detailed characteristics of each study and genotype distributions included in the meta-analysis are presented in Table 1.

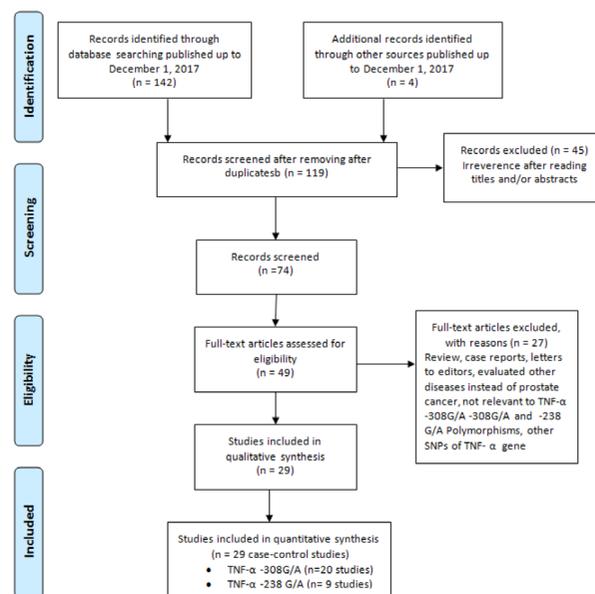


Fig.1: Flow chart of literature search and study selection.

Quantitative data synthesis

TNF- α -308G>A polymorphism

Table 2 lists the main results of the meta-analysis of *TNF- α -308G>A* polymorphism and RPL risk. When all the eligible studies were pooled into the meta-analysis, a significant association was found under the homozygote model (AA vs. GG: OR=1.716, 95% CI: 1.210-2.433, $P=0.002$, Fig.2A) and the recessive model (AA vs. AG+GG: OR=1.554, 95% CI: 1.100-2.196, $P=0.012$, Fig.2B). In addition, significant between-study heterogeneity was detected for all genetic models.

When stratified by ethnicity, a significant association with increased RPL risk was observed among Asians under the homozygote genetic model (AA vs. GG: OR=2.190, 95% CI: 1.465-3.274, $P \leq 0.001$), the dominant model (AA+AG vs. GG: OR=1.642, 95% CI: 1.269-2.125, $P \leq 0.001$) and the recessive model (AA vs. AG+GG: OR=1.901, 95% CI: 1.279-2.828, $P=0.002$), but not among Caucasian populations. Interestingly, stratified analysis according to genotyping technique revealed a significantly increased risk of RPL in those studies using PCR-RFLP under the homozygote genetic model (AA vs. GG: OR=1.828, 95% CI: 1.253-2.667, $P=0.002$), the dominant model (AA+AG vs. GG: OR=1.387, 95% CI: 1.016-1.892, $P=0.039$) and the recessive model (AA vs. AG+GG: OR=1.666, 95% CI: 1.147-2.421, $P=0.007$). Subgroup analysis of studies with high quality data did not show a significant association between this polymorphism and increased risk of RPL. Summary results of different comparisons are listed in Table 2.

Table 1: Main characteristics of studies included in this meta-analysis

First author (Y)	Country (Ethnicity)	Genotyping method	SOC	Case/Control	Cases			Controls			MAFs	HWE				
					Genotype	Allele	Allele	Genotype	Allele	Allele						
TNF-α-308G>A																
Babbage et al. (15)	UK (Caucasian)	PCR-ARMS	HB	43/73	GG	AG	AA	G	A	GG	AG	AA	G	A	0.137	0.106
Reid et al. (16)	UK (Caucasian)	PCR-RFLP	HB	17/43	9	6	2	24	10	29	13	1	71	15	0.174	0.744
Baxter et al. (17)	UK (Caucasian)	SSO	NR	76/138	51	25	(AG+AA)	-	-	94	44	(AG+AA)	-	-	NA	NA
Daher et al. (18)	Brazil (Caucasian)*	PCR-SSP	NR	48/108	36	12	(AG+AA)	-	-	89	19	(AG+AA)	-	-	NA	NA
Prigoshin et al. (19)	Argentina(Caucasian)	PCR-SSP	NR	41/54	35	6	(AG+AA)	-	-	49	5	(AG+AA)	-	-	NA	NA
Pietrowski et al. (20)	Germany(Caucasian)	PCR-RFLP	PB	168/222	133	33	2	299	37	167	41	14	375	69	0.155	≤ 0.001
Kamali-Sarvestani et al. (21)	Iran (Asian)	ASO-PCR	PB	131/143	117	14	0	248	14	122	21	0	265	21	0.073	0.343
Quintero-Ramos et al. (22)	Mexico (Latinos)	PCR-SSP	PB	122/214	113	8	1	234	10	182	30	2	394	34	0.079	0.543
Zammitti et al. (23)	Tunisia (African)	PCR-RFLP	PB	372/274	319	39	14	677	67	222	47	5	491	57	0.104	0.186
Palmirotta et al. (24)	Italy (Caucasian)	Sequencing	HB	100/100	87	13	0	187	13	76	21	3	173	27	0.135	0.313
Liu et al. (25)	China (Asian)	Sequencing	HB	132/152	110	22	0	242	22	138	13	1	289	15	0.049	0.276
Finan et al. (26)	Bahrain(Asian)	PCR-RFLP	PB	204/248	164	32	8	360	48	212	32	4	447	49	0.080	0.040
Kaur et al. (27)	India (Asian)	PCR-RFLP	PB	50/50	39	6	5	84	16	41	7	2	89	11	0.110	0.043
Gupta et al. (28)	India (Asian)	PCR-RFLP	PB	300/500	229	62	9	520	80	425	70	5	920	80	0.080	0.274
Bompeixe et al. (29)	Brazil (Caucasian)*	PCR-SSP	NR	61/75	45	16	(AG+AA)	-	-	59	16	(AG+AA)	-	-	NA	NA
Lee et al. (30)	Korea (Asian)	PCR-RFLP	PB	357/236	319	36	2	674	40	213	21	2	447	25	0.053	0.082
Alkhourji et al. (31)	Saudi (Asian)	PCR-SSP	NR	65/65	33	24	8	108	22	47	14	4	90	40	0.169	0.059
Liu et al. (32)	China (Asian)	PCR-RFLP	NR	284/284	144	105	35	393	175	205	61	18	471	35	0.170	≤ 0.001
Sudhir et al. (33)	India (Asian)	PCR-RFLP	HB	115/111	76	34	5	186	44	87	18	6	192	30	0.135	0.001
Ma et al. (34)	China (Asian)	PCR-RFLP	PB	775/805	683	86	6	1452	98	726	76	3	1528	82	0.050	0.506
TNF-α-238G>A																
Zammitti et al. (23)	Tunisia (African)	PCR-RFLP	PB	372/274	264	88	20	616	128	215	52	7	482	66	0.120	0.084
Palmirotta et al. (24)	Italy (Caucasian)	Sequencing	HB	100/100	84	16	0	184	16	94	6	0	194	6	0.030	0.757
Finan et al. (26)	Bahrain(Asian)	PCR-RFLP	PB	204/248	148	52	4	348	60	200	48	0	448	48	0.096	0.091
Liu et al. (25)	China (Asian)	Sequencing	HB	132/152	128	4	0	260	4	135	17	0	287	17	0.055	0.465
Gupta et al. (27)	India (Asian)	PCR-RFLP	PB	300/500	121	63	16	509	91	154	113	31	891	109	0.293	0.138
Lee et al. (30)	Korea (Asian)	PCR-RFLP	PB	357/236	330	26	1	686	28	228	8	0	464	8	0.016	0.791
Alkhourji et al. (31)	Saudi (Asian)	PCR-SSP	NR	65/65	55	7	3	117	13	57	8	0	122	8	0.061	0.597
Liu et al. (32)	China (Asian)	PCR-RFLP	NR	284/284	240	30	14	510	58	249	35	0	533	35	0.061	0.268
Ma et al. (34)	China (Asian)	PCR-RFLP	PB	775/805	732	41	2	1505	45	745	57	3	1547	63	0.039	0.097

*: Authors declared that the ancestry of the participants was European (Caucasians). ARMS-PCR, Amplification refractory mutation system-polymerase chain reaction, RFLP; Restriction fragment length polymorphism, SSO; Sequence-specific oligonucleotide, SSP; Sequence-specific primer, ASO-PCR; Allele-specific polymerase chain reaction; SOC; Source of controls, HB; Hospital based, NR; Not reported, MAFs; Minor allele frequencies, HWE; Hardy-weinberg equilibrium, and NA; Not applicable.

Table 2: Results of meta-analysis for the association of the *TNF- α -308G>A* polymorphism and risk of RPL

Subgroup	Genetic model	Type of model	Heterogeneity		Odds ratio (OR)			Publication bias		
			I ² (%)	P _H	O _R	95% CI	Z _{test}	P _{OR}	P _{Begg}	P _{Egger}
Overall	A vs. G	Random	87.66	≤0.001	1.151	0.805-1.646	0.769	0.442	0.964	0.296
	AA vs. GG	Fixed	31.51	0.117	1.782	1.270-2.500	3.342	0.001	0.198	0.038
	AG vs. GG	Random	52.75	0.009	0.699	0.411-1.190	-1.319	0.187	0.443	0.956
	AA+AG vs. GG	Random	66.08	≤0.001	1.235	0.981-1.554	1.797	0.072	1.000	0.470
	AA vs. AG+GG	Fixed	27.21	0.156	1.624	1.162-2.272	2.836	0.005	0.092	0.084
By ethnicity										
Caucasian	A vs. G	Random	63.68	0.041	0.859	0.492-1.498	-0.537	0.591	0.308	0.416
	AA vs. GG	Fixed	53.96	0.089	0.416	0.145-1.190	-1.66	0.102	0.734	0.562
	AG vs. GG	Random	85.78	≤0.001	0.540	0.042-7.007	-0.471	0.637	0.734	0.873
	AA+AG vs. GG	Fixed	26.01	0.230	0.990	0.759-1.291	-0.077	0.939	0.071	0.198
	AA vs. AG+GG	Fixed	50.44	0.109	0.407	0.143-1.158	-1.685	0.092	0.734	0.564
Asian	A vs. G	Random	90.54	≤0.001	1.543	0.880-2.706	1.514	0.130	0.536	0.335
	AA vs. GG	Fixed	0.00	0.654	2.190	1.465-3.274	3.822	≤0.001	0.265	0.071
	AG vs. GG	Fixed	0.00	0.532	0.885	0.557-1.314	-0.713	0.476	0.386	0.617
	AA+AG vs. GG	Fixed	47.93	0.052	1.642	1.269-2.125	3.771	≤0.001	0.754	0.224
	AA vs. AG+GG	Fixed	0.00	0.632	1.901	1.279-2.828	3.174	0.002	0.265	0.243
Genotyping technique										
PCR-RFLP	A vs. G	Random	90.14	≤0.001	1.516	0.917-2.507	1.622	0.105	1.000	0.708
	AA vs. GG	Fixed	46.06	0.062	1.828	1.253-2.667	3.130	0.002	0.602	0.325
	AG vs. GG	Random	55.32	0.022	0.760	0.390-1.482	-0.804	0.421	0.916	0.717
	AA+AG vs. GG	Random	67.69	0.002	1.387	1.016-1.892	2.061	0.039	0.754	0.434
	AA vs. AG+GG	Fixed	43.51	0.078	1.666	1.147-2.421	2.677	0.007	0.602	0.513
Studies quality (HWE)										
High quality studies	A vs. G	Random	77.52	≤0.001	0.974	0.620-1.528	-0.116	0.908	1.000	0.254
	AA vs. GG	Fixed	7.84	0.370	1.561	0.821-2.970	1.358	0.174	0.536	0.074
	AG vs. GG	Random	54.98	0.030	0.514	0.188-1.406	-1.296	0.195	0.265	0.418
	AA+AG vs. GG	Random	53.50	0.011	1.208	0.904-1.616	1.276	0.202	1.000	0.504
	AA vs. AG+GG	Fixed	9.35	0.358	1.584	0.836-2.999	1.411	0.158	0.265	0.046

RPL; Recurrent pregnancy loss, PCR-RFLP; Polymerase chain reaction-restriction fragment length polymorphism, CI; Confidence interval, and HWE; Hardy-weinberg equilibrium.

TNF- α -238G>A polymorphism

Table 3 lists the main results of the meta-analysis of *TNF- α -238G>A* polymorphism and RPL risk. When all the eligible studies were pooled into the meta-analysis, a significant association was found under the allele model (A vs. G: OR=1.456, 95% CI: 1.039-2.040, P=0.029, Fig.2C). Interestingly, when stratified by ethnicity, there was no significant association with an increased RPL risk in the Asian population. Stratified analysis according to genotyping technique revealed a significantly increased risk of RPL in those studies involving PCR-RFLP under the allele model (A vs. G: OR=1.418, 95% CI: 1.077-1.867, P=0.013).

Minor allele frequencies

The minor allele frequencies (MAFs) of the *TNF- α -308G>A* and *-238G>A* polymorphisms are presented in Table 1. The MAF for *TNF- α -238G>A* polymor-

phism was ranged between 3.0-29.3% in overall population. However, the allele and genotype distributions of *TNF- α -308G>A* polymorphism showed ethnic variation. The MAF in the Asian and Caucasian populations were 10.95% (4.9-17.0%) and 15.45% (13.5-17.4%) respectively, showing a lower frequency in Asians (Table 1).

Test of heterogeneity and sensitivity analyses

There was significant heterogeneity among these studies for the *TNF- α -308G>A* polymorphism under the allele model (A vs. G: P_H≤0.001), the heterozygote model (AG vs. GG: P_H=0.009) and the dominant model (AA+AG vs. GG: P_H≤0.001). Then, we assessed the source of heterogeneity by meta-regression analysis. Surprisingly, ethnicity, glaucoma types, genotyping methods and study quality did not contribute to substantial heterogeneity in the meta-analysis (Table 2).

Table 3: Results of meta-analysis for the association of the *TNF-α* -238G>A polymorphism and risk of RPL

Subgroup	Genetic model	Type of model	Heterogeneity		Odds ratio (OR)				Publication bias	
			I ² (%)	P _H	OR	95% CI	Z _{test}	P _{OR}	P _{Beggs}	P _{Eggers}
Overall	A vs. G	Random	74.99	≤0.001	1.456	1.039-2.040	2.181	0.029	0.348	0.801
	AA vs. GG	Random	59.35	0.022	2.134	0.792-5.751	1.498	0.134	1.000	0.088
	AG vs. GG	Random	68.43	0.001	1.051	0.746-1.482	0.284	0.776	0.754	0.820
	AA+AG vs. GG	Random	68.61	0.001	11.94	0.864-1.652	1.073	0.283	1.000	0.705
	AA vs. AG+GG	Fixed	48.85	0.068	1.374	0.865-2.184	1.345	0.179	0.763	0.084
By Ethnicity										
Asian	A vs. G	Random	73.09	0.001	1.269	0.872-1.848	1.245	0.213	1.000	0.711
	AA vs. GG	Random	58.47	0.034	2.420	0.640-9.154	1.302	0.193	0.707	0.039
	AG vs. GG	Random	64.64	0.009	0.906	0.625-1.316	-0.517	0.605	1.000	0.950
	AA+AG vs. GG	Random	67.71	0.005	1.056	0.733-1.523	0.293	0.769	0.763	0.919
	AA vs. AG+GG	Fixed	51.39	0.068	1.151	0.667-8.131	1.411	0.158	0.707	0.064
Genotyping technique										
PCR-RFLP	A vs. G	Random	64.12	0.016	1.418	1.077-1.867	2.491	0.013	0.060	0.630
	AA vs. GG	Random	62.66	0.020	1.920	0.679-5.428	1.231	0.218	0.707	0.165
	AG vs. GG	Random	65.63	0.012	1.070	0.773-1.481	0.409	0.683	0.259	0.341
	AA+AG vs. GG	Random	65.67	0.012	1.213	0.895-1.643	1.244	0.214	0.452	0.232
	AA vs. AG+GG	Fixed	52.34	0.062	1.318	0.825-2.107	1.156	0.248	0.452	0.164

RPL; Recurrent pregnancy loss, PCR-RFLP; Polymerase chain reaction-restriction fragment length polymorphism, and CI; Confidence interval.

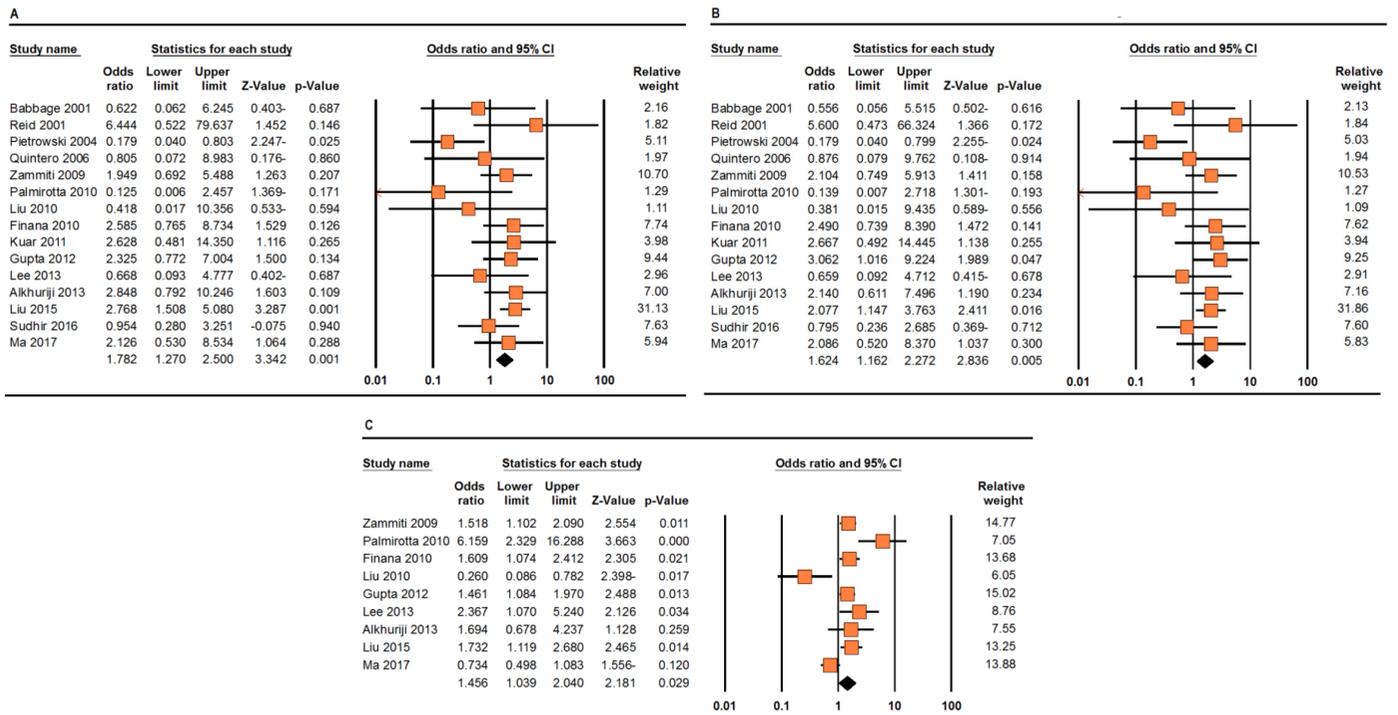


Fig.2: Forest plots for the association of the *TNF-α* -308G>A and -238G>A polymorphisms with Recurrent pregnancy loss (RPL) susceptibility. **A.** *TNF-α* -308G>A (homozygote model: AA vs. GG), **B.** *TNF-α* -308G>A (recessive model: AA vs. AG+GG), and **C.** *TNF-α* -238G>A (allele model: A vs. G).

Additionally, we performed sensitivity analysis to confirm the stability and reliability of our results by sequentially omitting individual eligible studies. When any single study was excluded, the corresponding ORs were not considerably changed, indicating the stability of the estimated OR. In addition, we excluded the studies in which genotype distribution in the controls deviated from HWE expectations, and in the homozygote and recessive models, the studies were found to affect the corresponding pooled ORs with heterogeneity removed under the recessive model (Table 2). The supplementary sensitivity analysis thus showed that the results of the present meta-analysis are reliable.

Publication bias

We used the funnel plot and Egger's linear regression to assess publication bias of the included studies. The shapes of the funnel plots indicated no obvious asymmetry (Fig.3). The results of Egger's test also showed no strong statistical evidence of publication bias (Table 2). However, Egger's test found evidence for the publication bias between *TNF- α -308G>A* polymorphism and RPL risk under the homozygote model (AA vs. GG: $P_{\text{Egger's}}=0.273$, $P_{\text{Egger's}}=0.046$, Fig.3B). Therefore, we used the Duval and Tweedie non-parametric "trim and fill" method to adjust for publication bias. However, the results with and without "trim and fill" did not lead to different conclusions, indicating that our results are statistically robust.

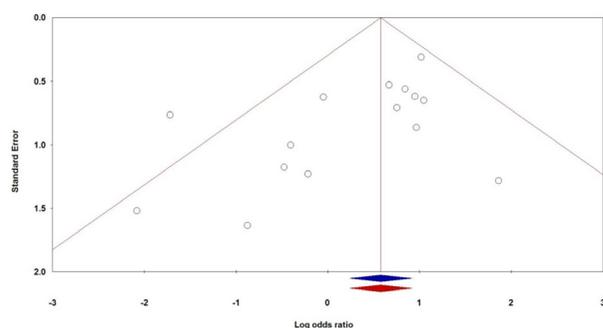


Fig.3: Begg's funnel plots of the *TNF- α -308G>A* polymorphism and RPL susceptibility under the homozygote model for publication bias test (blue without and red with trim and fill test).

Discussion

Although RPL is one of the most common public health issues, little is known regarding genetic its susceptibility factors (35). Genetic variants in *TNF- α* have been associated with the etiology of RPL (9). Several important polymorphisms in the promoter region of *TNF- α* have been identified, such as the *-308G>A* and *-238 G>A* variants (27-34). Hitherto, the associations between polymorphisms in the promoter region of *TNF- α* and the risk of RPL have remained inconclusive. Thus, we performed a CMA to evaluate the association of *TNF- α -308G>A* polymorphisms with risk of RPL. The meta-analysis found a significant association between the *TNF- α -308G>A* polymorphism and RPL under the homozygote and re-

cessive models. Moreover, to the best of our knowledge, this meta-analysis is the first to evaluate the association of *TNF- α -238G>A* polymorphism with RPL. The results of our meta-analysis revealed that the *TNF- α -238G>A* polymorphism was associated with the increased risk of RPL under the allele model. However, due to the limited number of available published studies on the *TNF- α -238G>A* polymorphism, further studies with larger sample sizes are needed to reach a more convincing conclusion.

The subgroup analyses revealed a significant association between *TNF- α -308G>A* polymorphism and RPL in Asian populations under the three genetic models. However, for Caucasians, the results indicated that this polymorphism is not associated with increased risk of RPL. The inconsistency of subgroup analysis with pooled estimates may be due to genetic diversity in different ethnicities. Furthermore, given that RPL is a multifactorial condition, beside genetic factors, endogenous and exogenous factors play a major role in RPL aetiology. Similarly, the included studies in this meta-analysis differed in their findings with regard to the association between *TNF- α -308G>A* polymorphism and risk of RPL. It is therefore possible that the difference between the studies may reflect the different ethnicities been investigated, because geographical regions may have different genetic and environmental factors that might affect the findings. Thus, this discrepancy might also be due to other factors such as maternal cigarette smoking, caffeine consumption, alcohol consumption, maternal age, number of previous miscarriages, diabetes mellitus, infective agents, endocrine factors, uterine anatomic abnormalities and antiphospholipid antibody syndrome (APS). Therefore, the relationship between this polymorphism and RPL might vary by ethnicity.

Recently, a meta-analysis by Li et al. (9) with 1,430 RPL cases and 1,727 healthy controls was performed to investigate the associations of *TNF- α* polymorphisms with RPL. Their results suggested that the *TNF- α -308G>A* polymorphism was associated with increased RPL risk. Our results are consistent with their meta-analysis. However, their meta-analysis generated contradictory results due to insufficient power because the number of studies was considerably smaller than that needed to achieve robust conclusions. In addition, due to small size, they could not rule out the possibility that publication bias was undetected. More recently, two new epidemiological studies (33, 34) have been performed to estimate the effect of the *TNF- α -308G>A* polymorphism on RPL risk in Asian populations. Moreover, we found that Li et al. had overlooked seven studies on the *TNF- α -308G>A* polymorphism and RPL risk in their meta-analysis. Hence, it may significantly affect their total results and RPL results. In the present meta-analysis, by including more nine case-control studies, we found that the *TNF- α -308G>A* polymorphism was associated with risk of RPL.

Between-study heterogeneity and publication bias are

important issues that cannot be ignored in a meta-analysis (36, 37). In addition, between-study heterogeneity might distort the conclusion of a meta-analysis (38). The study designs, source of controls subjects, ethnicity, genotyping method, sample size and other variables may contribute to the heterogeneity (37-40). Here, we detected moderate between-study heterogeneity across studies under the allele, heterozygote and the dominant models. We thus selected the random-effects model to summarize the ORs. We performed meta-regression analysis to find the source of between-study heterogeneity. However, after the subgroup analysis by ethnicity, heterogeneity still remained in Caucasians. In contrast, in the Asians, there were no heterogeneity, indicating that heterogeneity could be partly accounted for by the genetic distribution of different ethnicities between continents. We observed publication bias for the association between the *TNF-α* -308G>A polymorphism and RPL risk under the homozygote model. After subgroup analysis by ethnicity, the publication bias disappeared in both Caucasians and Asians.

This meta-analysis has a number of limitations that should be noted. First, we could not perform further subgroup analysis by ethnicity among Caucasians and other ethnicities because of the limited number of published studies. Secondly, we strictly followed the inclusion and exclusion criteria to reduce possible selection bias. However, the Egger's linear regression test showed little publication bias of overall analysis under the homozygote genetic model. Thirdly, the sources of literature searched were from a limited selection of electronic databases, and we failed to retrieve unpublished studies and articles written in other languages and also unpublished studies that might meet the inclusion criteria. Thus, potentially, publication bias might exist even though the funnel plots were found to be symmetrical. Fourthly, subgroup analyses regarding age, number of miscarriage, and other risk factors such as chronic disease were not conducted since the primary literature lacked sufficient data. Finally, the *TNF-α* -308G>A and -238G>A polymorphisms were not analyzed in combination with other polymorphisms of *TNF-α* and the effect of gene-gene interactions on RPL development was not undertaken. The relationship between polymorphisms of *TNF-α* gene and other genetic and environmental risk factors may be highly complicated, and extensive research is still required to ascertain how exactly the *TNF-α* polymorphisms affect the susceptibility of an individual to RPL.

Conclusion

This meta-analysis confirmed the association of the *TNF-α* -308G>A and -238G>A promoter region polymorphisms with increased risk of RPL. Moreover, our meta-analysis suggests that *TNF-α* -308G>A is more likely to be associated with the risk of RPL in Asians than Caucasians. Further large, well-designed case-control studies are needed to confirm these findings.

Acknowledgements

Authors would to thank Dr. Seyed Mahdi Kalantar for her assisting in imaging data collection. No specific funding was obtained to support this study. The authors declare no conflict of interest.

Authors' Contributions

F.A., B.M., H.N., M.K.-Z.; Contributed to the conception of the study. M.M.; Contributed to the critical review of the intellectual content. R.S.T., M.N.-S., R.D.-M.; Contributed to the data collection and analysis, interpretation of data. All authors contributed to the writing of the manuscript and final approval of the version to be published.

References

- Kamali M, Hantoushzadeh S, Borna S, Neamatzadeh H, Mazaheri M, Noori-Shadkam M, et al. Association between thrombophilic genes polymorphisms and recurrent pregnancy loss susceptibility in the Iranian population: a systematic review and meta-analysis. *Iran Biomed J.* 2018; 22(2): 78-89.
- Neamatzadeh H, Ramazani V, Kalantar SM, Ebrahimi M, Sheikhhah MH. Serum immune reactivity against β2-Glycoprotein-I and anti-neutrophil cytoplasmic auto-antibodies by ELI-P-Complex Screening Technology in recurrent miscarriage. *Minerva Ginecol.* 2016; 68(3): 243-249.
- Larsen EC, Christiansen OB, Kolte AM, Macklon N. New insights into mechanisms behind miscarriage. *BMC Med.* 2013; 11: 154.
- Ford HB, Schust DJ. Recurrent pregnancy loss: etiology, diagnosis, and therapy. *Rev Obstet Gynecol.* 2009; 2(2): 76-83.
- Elsokkary M, Elshourbagy M, Labib K, Mamdouh A, El-Shahawy Y, Nossair WS, et al. Assessment of hysteroscopic role in management of women with recurrent pregnancy loss. *J Matern Fetal Neonatal Med.* 2018; 31(11): 1494-1504.
- Colagar AH, Mosaieby E, Seyedhassani SM, Mohajerani M, Arasteh A, Kamalidehghan B, et al. T4216C mutation in NADH dehydrogenase I gene is associated with recurrent pregnancy loss. *Mitochondrial DNA.* 2013; 24(5): 610-612.
- Jeve YB, Davies W. Evidence-based management of recurrent miscarriages. *J Hum Reprod Sci.* 2014; 7(3): 159-169.
- Cao Y, Zhang Z, Zheng Y, Yuan W, Wang J, Liang H, et al. The association of idiopathic recurrent early pregnancy loss with polymorphisms in folic acid metabolism-related genes. *Genes Nutr.* 2014; 9(3): 402.
- Li HH, Xu XH, Tong J, Zhang KY, Zhang C, Chen ZJ. Association of *TNF-α* genetic polymorphisms with recurrent pregnancy loss risk: a systematic review and meta-analysis. *Reprod Biol Endocrinol.* 2016; 14: 6.
- Alijotas-Reig J, Esteve-Valverde E, Ferrer-Oliveras R, Llorba E, Gris JM. Tumor necrosis factor-α and pregnancy: focus on biologics. An updated and comprehensive review. *Clin Rev Allergy Immunol.* 2017; 53(1): 40-53.
- Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. *Front Immunol.* 2014; 5: 491.
- Ghamari E, Farnia P, Saif S, Marashian M, Ghanavi J, Farnia P, et al. Comparison of single nucleotide polymorphisms [SNP] at *TNF-α* promoter region with *TNF* receptor 2 (*TNFR2*) in susceptibility to pulmonary tuberculosis; using PCR-RFLP technique. *Am J Clin Exp Immunol.* 2016; 5(4): 55-61.
- Banday MZ, Balkhi HM, Hamid Z, Sameer AS, Chowdri NA, Haq E. Tumor necrosis factor-α (*TNF-α*)-308G/A promoter polymorphism in colorectal cancer in ethnic Kashmiri population-A case control study in a detailed perspective. *Meta Gene.* 2016; 9: 128-136.
- Sobhan MR, Mehdinejad M, Jamaladini MH, Mazaheri M, Zare-Sheneh M, Neamatzadeh H. Association between aspartic acid repeat polymorphism of the asporin gene and risk of knee osteoarthritis: A systematic review and meta-analysis. *Acta Orthop Traumatol Turc.* 2017; 51(5): 409-415.
- Babbage SJ, Arkwright PD, Vince GS, Perrey C, Pravica V, Quenby S, et al. Cytokine promoter gene polymorphisms and idiopathic

- recurrent pregnancy loss. *J Reprod Immunol.* 2001; 51(1): 21-27.
16. Reid JG, Simpson NA, Walker RG, Economidou O, Shillito J, Gooi HC, et al. The carriage of pro-inflammatory cytokine gene polymorphisms in recurrent pregnancy loss. *Am J Reprod Immunol.* 2001; 45(1): 35-40.
 17. Baxter N, Sumiya M, Cheng S, Erlich H, Regan L, Simon A, et al. Recurrent miscarriage and variant alleles of mannose binding lectin, tumour necrosis factor lymphotoxic alpha genes. *Clin Exp Immunol.* 2001; 126(3): 529-534.
 18. Daher S, Shulzhenko N, Morgun A, Mattar R, Rampim GF, Camano L, et al. Associations between cytokine gene polymorphisms and recurrent pregnancy loss. *J Reprod Immunol.* 2003; 58(1): 69-77.
 19. Prigoshin N, Tambutti M, Larriba J, Gogorza S, Testa R. Cytokine gene polymorphisms in recurrent pregnancy loss of unknown cause. *Am J Reprod Immunol.* 2004; 52(1): 36-41.
 20. Pietrowski D, Bettendorf H, Keck C, Burkle B, Unfried G, Rienecker EK, et al. Lack of association of TNF α gene polymorphisms and recurrent pregnancy loss in Caucasian women. *J Reprod Immunol.* 2004; 61(1): 51-58.
 21. Kamali-Sarvestani E, Zolghadri J, Ghareisi-Fard B, Sarvari J. Cytokine gene polymorphisms and susceptibility to recurrent pregnancy loss in Iranian women. *J Reprod Immunol.* 2005; 65(2): 171-178.
 22. Quintero-Ramos A, Valdez-Vélázquez LL, Hernández G, Baltazar LM, Padilla-Gutiérrez JR, Valle Y, et al. Assessment of five thrombophilic genetic polymorphisms among couples with habitual abortion. *Gac Med Mex.* 2006; 142(2): 95-98.
 23. Zammiti W, Mtraoui N, Finan RR, Almawi WY, Mahjoub T. Tumor necrosis factor alpha and lymphotoxin alpha haplotypes in idiopathic recurrent pregnancy loss. *Fertil Steril.* 2009; 91(5): 1903-1908.
 24. Palmirotta R, La Farina F, Ferroni P, Ludovici G, Nigro C, Savonaro A, et al. TNFA gene promoter polymorphisms and susceptibility to recurrent pregnancy loss in Italian women. *Reprod Sci.* 2010; 17(7): 659-666.
 25. Liu C, Wang J, Zhou S, Wang B, Ma X. Association between -238 but not -308 polymorphism of Tumor necrosis factor alpha (TNF- α)v and unexplained recurrent spontaneous abortion (URSA) in Chinese population. *Reprod Biol Endocrinol.* 2010; 8: 114.
 26. Finan RR, Al-Irhayim Z, Mustafa FE, Al-Zaman I, Mohammed FA, Al-Khateeb GM, et al. Tumor necrosis factor-alpha polymorphisms in women with idiopathic recurrent miscarriage. *J Reprod Immunol.* 2010; 84(2): 186-192.
 27. Kaur A, Kaur A. Recurrent pregnancy loss: TNF- α and IL-10 polymorphisms. *J Hum Reprod Sci.* 2011; 4(2): 91-94.
 28. Gupta R, Prakash S, Parveen F, Agrawal S. Association of CTLA-4 and TNF-alpha polymorphism with recurrent miscarriage among North Indian women. *Cytokine.* 2012; 60(2): 456-462.
 29. Bompeixe EP, Carvalho Santos PS, Vargas RG, von Linsingen R, Zeck SC, Wowk PF, et al. HLA class II polymorphisms and recurrent spontaneous abortion in a Southern Brazilian cohort. *Int J Immunogenet.* 2013; 40(3): 186-191.
 30. Lee BE, Jeon YJ, Shin JE, Kim JH, Choi DH, Jung YW, et al. Tumor necrosis factor-alpha gene polymorphisms in Korean patients with recurrent spontaneous abortion. *Reprod Sci.* 2013; 20(4): 408-417.
 31. Alkhuriji AF, Alhimaidei AR, Babay ZA, Wary AS. The relationship between cytokine gene polymorphism and unexplained recurrent spontaneous abortion in Saudi females. *Saudi Med J.* 2013; 34(5): 484-489.
 32. Liu RX, Wang Y, Wen LH. Relationship between cytokine gene polymorphisms and recurrent spontaneous abortion. *Int J Clin Exp Med.* 2015; 8(6): 9786-9792.
 33. Sudhir N, Badaruddoza, Beri A, Kaur A. Association of tumor necrosis factor-alpha 308G/A polymorphism with recurrent miscarriages in women. *J Hum Reprod Sci.* 2016; 9(2): 86-89.
 34. Ma J, Zhang X, He G, Yang C. Association between TNF, IL1B, IL6, IL10 and IFNG polymorphisms and recurrent miscarriage: a case control study. *Reprod Biol Endocrinol.* 2017; 15(1): 83.
 35. Govindaiah V, Naushad SM, Prabhakara K, Krishna PC, Radha Rama Devi A. Association of parental hyperhomocysteinemia and C677T Methylene tetrahydrofolate reductase (MTHFR) polymorphism with recurrent pregnancy loss. *Clin Biochem.* 2009; 42(4-5): 380-386.
 36. Kamali M, Hamadani S, Neamatzadeh H, Mazaheri M, Zare-Shehneh M, Modares-Gilani M, et al. Association of XRCC2 rs3218536 polymorphism with susceptibility of breast and ovarian cancer: a systematic review and meta-analysis. *Asian Pac J Cancer Prev.* 2017; 18(7): 1743-1749.
 37. Sadeghiyeh T, Hosseini-Bioui F, Mazaheri M, Zare-Shehneh M, Neamatzadeh H, Poursharif Z. Association between catechol-o-methyltransferase Val158Met (158G/A) polymorphism and suicide susceptibility: a meta-analysis. *J Res Health Sci.* 2017; 17(2): e00383.
 38. Mehdinejad M, Sobhan MR, Mazaheri M, Zare-Shehneh M, Neamatzadeh H, Kalantar SM. Genetic association between ERCC2, NBN, RAD51 gene variants and osteosarcoma risk: a systematic review and meta-analysis. *Asian Pac J Cancer Prev.* 2017; 18(5): 1315-1321.
 39. Namazi A, Abedinzadeh M, Nourbaksh P, Neamatzadeh H. Association between the XRCC3 Thr241Met polymorphism and risk of colorectal cancer: a meta-analysis of 5,193 cases and 6,645 controls. *Asian Pac J Cancer Prev.* 2015; 16(6): 2263-2268.
 40. Jafari-Nedooshan J, Forat-Yazdi M, Neamatzadeh H, Zare-Shehneh M, Kargar S, Seddighi N. Genetic association of XRCC1 gene rs1799782, rs25487 and rs25489 polymorphisms with risk of thyroid cancer: a systematic review and meta-analysis. *Asian Pac J Cancer Prev.* 2017; 18(1): 263-270.

Evaluation of Osteopontin Gene Expression in Endometrium of Diabetic Rat Models Treated with Metformin and Pioglitazone

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Abstract

Background: Osteopontin (*Opn*) is one of the co-factors involved in cell adhesion and invasion during the implantation process. Several reports have shown *Opn* expression changes in diabetic condition in several tissues. In addition, an increased incidence of spontaneous abortion is reported in diabetic women. We, therefore, designed a study to evaluate the effects of diabetes on *Opn* expression at implantation time after treatment with metformin and pioglitazone.

Materials and Methods: In this interventional and experimental study, 28 rats were randomly divided into four groups, namely control, diabetic, pioglitazone-treated diabetic rats and metformin-treated diabetic rats. Streptozotocin (STZ) and nicotinamide (NA) were used to induce type 2 diabetes (T2D). During the implantation window, the endometrium was removed and the expression of *Opn* was analysed by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Results: *Opn* expression was significantly higher (30.70 fold-changes) in the diabetic group in comparison with the control group (P=0.04). Furthermore, the expression of *Opn* was significantly lower in the diabetic group treated with pioglitazone when compared with the diabetic group (P=0.04).

Conclusion: According to the high *Opn* expression and the possibility of increased adhesion of endometrial epithelial cells, the invasion of blastocyst may be affected and thus reduced. As pioglitazone significantly reversed the upregulation of *Opn* in diabetic rats, it may be considered as a therapeutic compound for treating T2D.

Keywords: Diabetes, Endometrium, Implantation, Osteopontin

Citation: Hosseiny ZS, Nikpour P, Bakhtiary A, Mostafavi FS, Matinfar M, Jahani M, Aboutorabi R. Evaluation of osteopontin gene expression in endometrium of diabetic rat models treated with metformin and pioglitazone. *Int J Fertil Steril.* 2019; 12(4): 293-297. doi: 10.22074/ijfs.2019.5471.

Introduction

Globally, approximately 60 to 80 million couples are likely to be affected by infertility per year (1). About 15% of couples suffer from subfertility around the world (2). The rate of male and female infertility incidences have been estimated to be equal with each explaining approximately 35-40% of cases. The cause of infertility in the remaining proportion of cases (male or female) may be due to a combination of disorders in the two genders (20-30%) (3), through which, 15-30% of cases are diagnosed as unexplained infertility (4).

The first step to achieve a successful pregnancy is embryo implantation which needs an intact embryo, an endometrium and the synchronization between them (5). Failure in implantation is multifactorial and may be due to embryonic or maternal dysfunction during the dialogue window between them. Coagulation, immunological, en-

dothelial, endocrine and metabolic disorders are among the most common known causative factors of subfertility (6, 7).

Diabetes is a variety of metabolic diseases in which individuals are unable to produce or uptake adequate levels of insulin, resulting in high levels of blood glucose (8). Diabetes mellitus could deregulate a variety of cellular and molecular pathways (9).

A total of 425 million diabetic individuals have been reported worldwide in 2017 and it is estimated that this population will reach 629 million by 2045 (10). The incidence of type 2 diabetes (T2D) is also rising and is accompanied with age reduction in its onset, especially in women (11).

Diabetes can seriously affect the outcome of embryo implantation and pregnancy. It seems diabetes mellitus

Received: 5/Feb/2018, Accepted: 19/May/2018

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 4, Jan-Mar 2019, Pages: 293-297

impairs the molecular functions of the female reproductive system and thus causes improper implantation and/or fetal loss (12).

Some reports have shown that miscarriage, neonatal morbidity and mortality, and neonatal congenital malformations are observed in women who suffer from T2D (13, 14).

Administration of metformin to T2D patients for blood glucose level reduction is common. Metformin affects cell insulin resistance, descends gluconeogenesis by liver and increases blood glucose utilization, therefore leading to euglycemia (15).

Pioglitazone is a member of the thiazolidinediones (TZDs) family, which is used as an antidiabetic drug. It acts by binding to peroxisome proliferator-activated receptor gamma (PPAR- γ). This drug therefore improves glycemic control by increasing insulin sensitivity at cellular level (16).

The association of subfertility or infertility with diabetes, as a metabolic disease, has been previously evaluated (17). However, the effect of diabetes on gene expression at the transcript and protein levels have not been evaluated during the implantation window (14).

The embryo-maternal crosstalk during the implantation window involves several genes which ought to be expressed at the right time either in the blastocyst or the endometrium (18). Receptivity of endometrium is the key point for implantation of the blastocyst (18, 19). This receptivity is provided by a number of molecules which reach their peak values during the window of implantation (5). Some of these molecules include integrins, mucins, vascular endothelial growth factor (VEGF), and osteopontin (20). Integrin family members act as receptors for multiple ligands such as osteopontin, laminin and collagen.

In rats, Opn gene has 7 exons, and its location is on the 14p22 (21). Osteopontin (Opn) promotes cell adhesion and invasion through its Arg-Gly-Asp domain (5). Opn is shown to be present at a high level in the epithelial layer (during mid-secretory phase) in human, mouse and rabbit uterine (22, 23). Osteopontin has been also identified as a protein associated with metastatic cancers, as an extracellular matrix protein of bones and teeth, as a cytokine produced by activated lymphocytes and macrophages, and as a major constituent of the uterus and placenta during pregnancy (22).

Given the rise of T2D prevalence its effects on the female reproductive system, we quantified the expression of *Opn* in the endometrium of diabetic rat models to examine the association of *Opn* with T2D and evaluate the molecular effect of metformin and pioglitazone treatments on *Opn* expression.

Materials and Methods

Animal and maintenance

This interventional and experimental study on diabetic rat models was conducted at the Central Laboratory of Isfahan University of Medical Sciences in 2017. This work has the Ethical Committee code number IR.MUI.REC.1394.1.184.

Adult virgin female Wistar rats weighting 200-250 g were obtained from Pasteur Institute of Iran, aged 6-8 weeks, maintained in conventional wire mesh cages at room temperature $21 \pm 1^\circ\text{C}$ and humidity of 45-50% with light/dark cycle. Rats had access to standard dry pellets and water.

Induction of diabetes

Diabetes was induced in rats by injecting 60 mg/kg streptozotocin (STZ, Sigma-Aldrich Chemie, Germany) intraperitoneally. Fifteen minutes prior to STZ injection, 200-230 mg/kg nicotinamide (NA, Sigma-Aldrich Chemie, Germany) was injected intraperitoneally (24-26).

Blood samples were taken from the tail vein and glucose level values were measured using a glucometer (HemoCue Glucose 201+, Ångelholm, Sweden). Rats with blood glucose levels above 250 mg/dl were considered manifestly as diabetic (27).

Study design and tissue collection

The 28 rats were randomly categorized into four groups (n=7), namely control, diabetic, Pioglitazone-treated and metformin-treated diabetic rats.

The first group of rats was the control group and did not receive any substance. The second (diabetic) group did not receive any treatment except for STZ and NA. The third group received 20 mg/kg/day of pioglitazone for diabetes treatment (28), and the final group received 100 mg/kg/day of metformin (12). Treatments were administered by orogastric gavage and continued for 4 weeks (28).

Rats were maintained in diabetic condition for 3 weeks (one sexual cycle) and then underwent treatment with metformin or pioglitazone.

Treatments with the two drugs lasted 4 weeks and for the next step, each of the 3 female rats were mated with 1 male rat and vaginal plug was observed the following morning (first day of pregnancy). Animals were anesthetized and sacrificed on the 4th day of pregnancy, considered as the implantation day (6). The rat uteri were then removed, snap-frozen in liquid nitrogen and stored at -80°C for further analysis.

Real time polymerase chain reaction

RNA extraction

Total RNA was isolated from epithelial cells of endometrium using the RNX plus solution (Cinnagen, Iran) according to the manufacturer's instructions and as previously described. The purity and integrity of the extracted RNA were assessed by optical density measurements (260/280 nm ratios) and by visual observation of samples electrophoresed on agarose gels. For elimination of genomic DNA, RNA was treated with RNase-free DNase (Qiagen, Germany)

cDNA synthesis

Complementary DNA (cDNA) synthesis was carried out by using a cDNA synthesis Kit (Yektatajhez, Iran). Briefly, the synthesis mixture was prepared by adding 4 μl of 5 X first-

strand buffer, 1 µl of dNTPs, 0.5 µl of RNasin and 1 µl of M-MLV. Approximately 1 µg of RNA and random hexamer primers were finally added to the mixture in a 20 µl reaction.

Quantitative real-time polymerase chain reaction

Specific primers for the rat β-actin (as an internal control, Accession number: NM_031144) and osteopontin (NM_012881.2) genes were designed with Genrunner software version 3.05 (Hastings Software, Hastings, NY, USA). All designed primers were checked against the the rat genome using BLAST to make sure they are not complementary with other regions of genome.

The sequences of the designed primers are as follow:

SPPI-

F: 5'-AGGAGAAGGCGCATTACAG-3'
R: 5'-GCTTTCATTGGAGTTGCTTG -3'
with an amplicon size of 160 bp and

β-actin-

F: 5'-GCCTTCCTTCCTGGGTATG-3'
R: 5'-AGGAGCCAGGGCAGTAATC-3'
with an amplicon size of 165 bp.

PCR was carried out by using the specific primers along with the Maxima™ SYBR Green/ROX qPCR Master MIX (Fermentas, Lithuania) and run on an Applied Biosystems StepOnePlus instrument. The PCR cycling conditions were an initial denaturation step at 95°C for 10 minutes, followed by 40 amplification cycles of denaturation at 95°C for 10 seconds, annealing at 60°C and 58.8°C for β-actin and osteopontin genes respectively for 10 seconds, and extension at 72°C for 10 seconds. All samples were measured in duplicate. The 2^{-ΔΔCt} method was utilized to quantify the relative levels of gene expression.

Statistical analyses

Statistical analyses were performed using SPSS version 18.0 (SPSS Inc, Chicago, IL, USA). All data are expressed as mean ± standard error of mean (SEM) from at least in triplicate at two separate experiments. Differences between groups were analyzed using Analysis of Variance (ANOVA) with post hoc multiple comparisons. Statistical significance was defined as P<0.05.

Results

Figure 1 shows fasting plasma glucose concentrations in diabetic rat models (399.28 ± 84.61) and in those treated with metformin (103.28 ± 14.12) and pioglitazone (99.29 ± 6.70). There was a significant difference between the diabetic group and all other groups (P=0.0001).

The differential expression of the target gene was compared with the house keeping gene (β-actin) in all samples. As shown in Table 1 and Figure 2, the mean of *Opn* expression in the diabetic group (30.70 ± 11.65) was significantly different from the control group (1) (P=0.04) but no significant difference was observed between diabetic and metformin treated group (42.11 ±

19.07) (P=0.07). Therefore in diabetic group and diabetic treated with metformin group upregulation in expression of *Opn* gene were observed. Also, the diabetic treated with pioglitazone group (0.55 ± 0.22) showed no significant difference compared with the control group (1) (P=0.3).

Figure 3 shows the non-significant difference observed in *Opn* mRNA expression in metformin treated group (1.371 ± 0.621) compared to diabetic group (1) (P=0.62). On the other hand, there was a significant reduction in the expression of *Opn* in pioglitazone treated group (0.017 ± 0.007) compared with the diabetic group (1) (P=0.04).

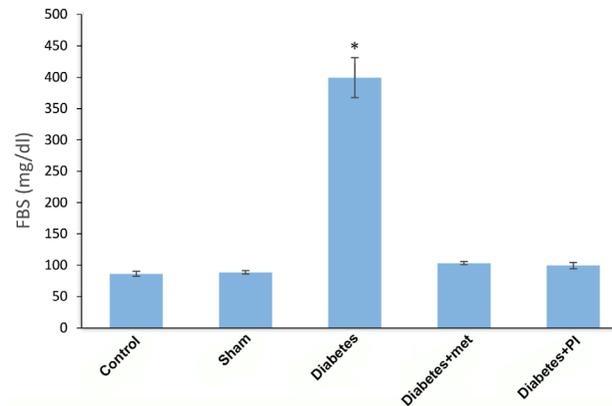


Fig.1: Fasting plasma glucose concentrations in diabetic rat models and in those treated with metformin and pioglitazone. *: Significant difference between the diabetic group and other groups (P=0.0001), FBS; Fasting blood sugar, met; Metformin, and PI; Pioglitazone.

Table 1: Mean level of *Opn* transcript expression in different groups

Groups	Specific (mean ± SEM)	Normalized (mean ± SEM)
Control	0.35	1
Sham	0.29 ± 0.12	0.83 ± 0.35
Diabetic	10.99 ± 4.17	30.70 ± 11.65
Pioglitazone treated	0.19 ± 0.08	0.55 ± 0.22
Metformin treated	15.08 ± 6.83	42.11 ± 19.07

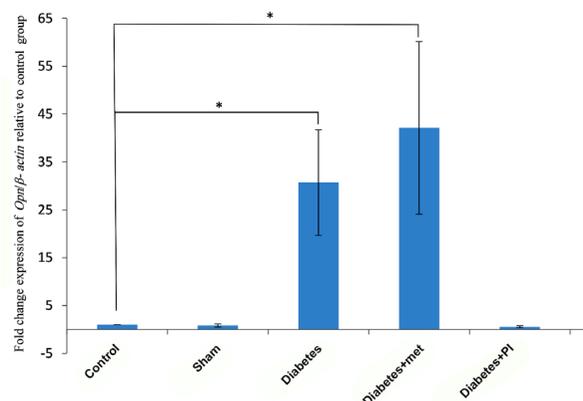


Fig.2: Fold change expression of *Opn* /β-actin gene relative to the control group based on real time polymerase chain reaction analysis in the rat endometrium. *Opn* transcript expression was significantly higher in the diabetic and diabetic+met groups in comparison with the control subjects (P=0.04). *: P<0.05, met; Metformin, and PI; Pioglitazone.

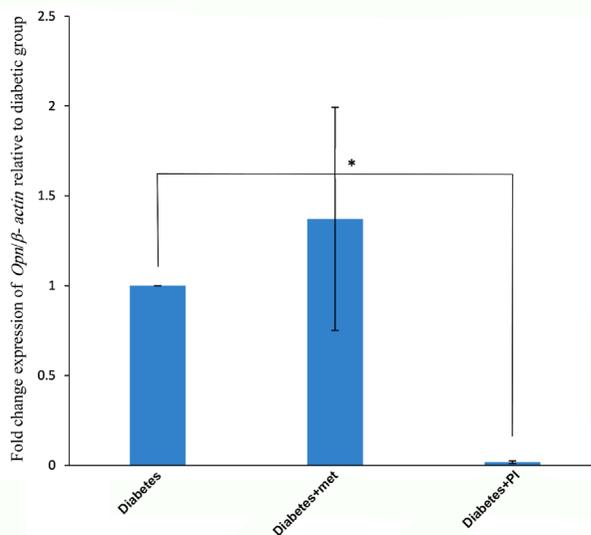


Fig.3: Fold change expression of *Opn*/β-actin gene relative to the diabetic group. *Opn* mRNA expression was significantly lower in the diabetic group treated with pioglitazone ($P=0.04$). *, $P<0.05$, met; Metformin, and PI; Pioglitazone.

Discussion

This study was designed to investigate the effects of diabetes on *Opn* gene expression at the implantation stage after being treated with metformin and pioglitazone. Osteopontin transcript expression was significantly higher in the diabetic group in comparison with the control group. Furthermore, evidence for the ability of pioglitazone to downregulate *Opn* expression was shown. Given the high *Opn* expression and the possibility of increased adhesion of endometrial epithelial cells, the invasion of blastocyst may be affected and thus reduced. As pioglitazone significantly reverted the *Opn* expression in diabetic rats, it may be considered as a therapeutic compound to act against this molecular perturbation.

Receptivity of endometrium, mature blastocyst and dialogue between them are essential for the multifactorial nature of embryo implantation. The duration of this dialogue window is different among mammals, but should be present for a limited time for embryo reception (5).

Failure in the onset of pregnancy is widely due to inappropriate endometrial receptivity (29). Several endometrial growth factors, cytokines and adhesion molecules such as osteopontin cooperate in molecular pathways which are necessary for pregnancy (30, 31).

Opn transcript level increases at the implantation sites from day 5 to 8 of pregnancy in the mouse uterus. Therefore, *Opn* expression is thought to be essential for hatching and adhering the trophoblast to the endometrium (5).

In addition, at the protein expression level, *Opn* and β3 integrin positive cells were significantly higher on the 5th day of pregnancy. The presence of these two proteins were proposed as suitable markers for predicting the fate of ongoing implantation by the authors (32). *Opn* expression is also reported during the peri-implantation period, which is under control of progesterone in rabbit (33).

Young et al. (34) showed that in the proliferative

phase of the menstrual cycle, *Opn* was not observed, however, its presence was observed during the secretory phase.

Opn expression has also been studied in normal endometrium during implantation in human (32), rat (23), mouse (35), sheep and pig (36). Here, we evaluated its expression in diabetic rats during the implantation window and observed significant overexpression when compared with the control group.

Diabetes mellitus in women could cause reduction of fertility, poor reproduction outcome and molecular abnormalities in ovary and endometrium (37). In diabetic mice models, the implantation outcome is shown to be lower than control mice (14).

Takemoto et al. (38) observed enhanced *Opn* expression levels in cultured rat aortic smooth muscle cells which were maintained in a medium with high glucose levels and suggested that it may be involved in the development of diabetic vascular complication.

Streptozotocin-induced diabetes mellitus in rats leads to the reduction of endometrial thickness while treating with pioglitazone and zinc improves the damages in the endometrium (25).

Up-regulation of *Opn* has been reported in renal tissue of diabetic rat models, which may implicate this molecule as a potential key pathophysiologic factor in diabetic nephropathy. Treatment with pioglitazone is thought to suppress *Opn* expression levels (39).

Consistently, we observed a significant reduction in the expression of *Opn* in the group treated with pioglitazone in comparison with the diabetic group. This indicates that pioglitazone has the ability to control the overexpression of *Opn* gene, which is probably in relation to the effect of pioglitazone on *PPAR-γ* (a regulator of gene expression). Further studies are nevertheless required to suggest administration of pioglitazone.

Another common drug for the treatment of diabetes is metformin, which causes an increase in intracellular magnesium concentration along with a lower blood glucose level in the uterus and ovary (12). Metformin seems to have positive effects on other organs such as the kidney where it significantly protects renal function in diabetic nephropathy (40). In this study, we observed no significant effect of metformin on *Opn* expression in the endometrium of diabetic rats.

Conclusion

We conclude that due to the high expression of *Opn* and the possibility of increased adhesion of endometrial cells to each other, the invasion of blastocyst into uterine epithelium is likely reduced. Also, pioglitazone significantly down-regulates the expression *Opn* back to its normal levels in the female diabetic rats.

Acknowledgements

The authors express their gratitude to Isfahan University of Medical Sciences for its financial support by project numbers 395210 and 194184. There is no conflict of interest in this study.

Authors' Contributions

R.A., P.N.; Were responsible for overall supervision from study design to statistical analysis and revision of the manuscript. Z.S.H., A.B., M.J.; Conducted molecular experiments and RT-qPCR analysis. F.S.M., M.M.; Were responsible for counseling diabetes and its complications and also editing and approving the final version of this manuscript. All authors read and approved the final manuscript.

References

1. Sudha G, Reddy KSN. Causes of female infertility: a cross-sectional study. *International Journal of Latest Research in Science and Technology*. 2013; 2(6): 119-123.
2. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. *Reprod Biol Endocrinol*. 2015; 13: 37.
3. Deyhoul N, Mohamaddoost T, Hosseini M. Infertility-related risk factors: a systematic review. *Int J Womens Health Reprod Sci*. 2017; 5(1): 24-29.
4. Quaas A, Dokras A. Diagnosis and treatment of unexplained infertility. *Rev Obstet Gynecol*. 2008; 1(2): 69-76.
5. Qi QR, Xie QZ, Liu XL, Zhou Y. Osteopontin is expressed in the mouse uterus during early pregnancy and promotes mouse blastocyst attachment and invasion in vitro. *PLoS One*. 2014; 9(8): e104955.
6. Aplin JD, Kimber SJ. Trophoblast-uterine interactions at implantation. *Reprod Biol Endocrinol*. 2004; 2: 48.
7. Tuckerman EM, Laird SM, Prakash A, Li TC. Expression of integrins in the endometrium of women with recurrent miscarriage. *Fertil Steril*. 2006; 86(3): 755-757.
8. Beck JK, Cogen FR. Outpatient management of pediatric type 1 diabetes. *J Pediatr Pharmacol Ther*. 2015; 20(5): 344-357.
9. Saeedi Borujeni MJ, Esfandiary E, Taheripak G, Codoñer-Franch P, Alonso-Iglesias E, Mirzaei H. Molecular aspects of diabetes mellitus: resistin, MicroRNA and exosome. *J Cell Biochem*. 2018; 119(2): 1257-1272.
10. Kim ES, Jeong JS, Han K, Kim MK, Lee SH, Park YM, et al. Impact of weight changes on the incidence of diabetes mellitus: a Korean nationwide cohort study. *Sci Rep*. 2018; 8(1): 3735.
11. Feig DS, Murphy K, Asztalos E, Tomlinson G, Sanchez J, Zinman B, et al. Metformin in women with type 2 diabetes in pregnancy (MiTy): a multi-center randomized controlled trial. *BMC Pregnancy Childbirth*. 2016; 16(1): 173.
12. Gales C, Zamfir C, Radulescu D, Stoica B, Nechifor M. Protective effect of magnesium and metformin on endometrium and ovary in experimental diabetes mellitus. *Magnes Res*. 2014; 27(2): 69-76.
13. Greene MF. Spontaneous abortions and major malformations in women with diabetes mellitus. *Semin Reprod Endocrinol*. 1999; 17(2): 127-136.
14. Wang TS, Gao F, Qi QR, Qin FN, Zuo RJ, Li ZL, et al. Dysregulated LIF-STAT3 pathway is responsible for impaired embryo implantation in a Streptozotocin-induced diabetic mouse model. *Biology Open*. 2015; 4(7): 893-902.
15. Ainuddin JA, Karim N, Zaheer S, Ali SS, Hasan AA. Metformin treatment in type 2 diabetes in pregnancy: an active controlled, parallel-group, randomized, open label study in patients with type 2 diabetes in pregnancy. *J Diabetes Res*. 2015; 2015: 325851.
16. Filipova EP, Uzunova KH, Vekov TY. Comparative analysis of therapeutic efficiency and costs (experience in Bulgaria) of oral anti-diabetic therapies based on glitazones and gliptins. *Diabetol Metab Syndr*. 2015; 7: 63.
17. Basmatzou T, Konstantinos Hatziveis M. Diabetes mellitus and influences on human fertility. *International Journal of Caring Sci-*

- ences. 2016; 9(1): 371-379.
18. Altmäe S, Martínez-Conejero JA, Salumets A, Simón C, Horcajadas JA, Stavreus-Evers A. Endometrial gene expression analysis at the time of embryo implantation in women with unexplained infertility. *Mol Hum Reprod*. 2010; 16(3): 178-187.
19. Boivin J, Bunting L, Collins JA, Nygren KG. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod*. 2007; 22(6): 1506-1512.
20. Liu JL, Zhao M, Peng Y, Fu YS. Identification of gene expression changes in rabbit uterus during embryo implantation. *Genomics*. 2016; 107(5): 216-221.
21. Homepage N. spp1 rat gene 27.06.2018. Available from: <https://www.ncbi.nlm.nih.gov/gene/25353>.
22. Johnson GA, Burghardt RC, Bazer FW. Osteopontin: a leading candidate adhesion molecule for implantation in pigs and sheep. *J Anim Sci Biotechnol*. 2014; 5(1): 56.
23. Kang YJ, Forbes K, Carver J, Aplin JD. The role of the osteopontin-integrin $\alpha v \beta 3$ interaction at implantation: functional analysis using three different in vitro models. *Hum Reprod*. 2014; 29(4): 739-749.
24. Rossetti L, DeFronzo RA, Gherzi R, Stein P, Andraghetti G, Falzetti G, et al. Effect of metformin treatment on insulin action in diabetic rats: in vivo and in vitro correlations. *Metabolism*. 1990; 39(4): 425-435.
25. Gales C, Zamfir C, Stoica B, Nechifor M. Zinc and pioglitazone effects on ovaries and endometrium in diabetes. *Farmacologia*. 2015; 63(1): 52-56.
26. Masiello P, Broca C, Gross R, Roye M, Manteghetti M, Hillaire-Buys D, et al. Experimental NIDDM: development of a new model in adult rats administered streptozotocin and nicotinamide. *Diabetes*. 1998; 47(2): 224-229.
27. Zabihi S, Wentzel P, Eriksson UJ. Altered uterine perfusion is involved in fetal outcome of diabetic rats. *Placenta*. 2008; 29(5): 413-421.
28. Eissa LA, Abdel-Rahman N, Eraky SM. Effects of omega-3 fatty acids and pioglitazone combination on insulin resistance through fibroblast growth factor 21 in type 2 diabetes mellitus. *Egyptian Journal of Basic and Applied Sciences*. 2015; 2(2): 75-86.
29. Macklon NS, Stouffer RL, Giudice LC, Fauser BC. The science behind 25 years of ovarian stimulation for in vitro fertilization. *Endocr Rev*. 2006; 27(2): 170-207.
30. Aghajanova L, Hamilton AE, Giudice LC. Uterine receptivity to human embryonic implantation: histology, biomarkers, and transcriptomics. *Semin Cell Dev Biol*. 2008; 19(2): 204-211.
31. Gong X, Tong Q, Chen Z, Zhang Y, Xu C, Jin Z. Microvascular density and vascular endothelial growth factor and osteopontin expression during the implantation window in a controlled ovarian hyperstimulation rat model. *Exp Ther Med*. 2015; 9(3): 773-779.
32. Liu N, Zhou C, Chen Y, Zhao J. The involvement of osteopontin and $\beta 3$ integrin in implantation and endometrial receptivity in an early mouse pregnancy model. *Eur J Obstet Gynecol Reprod Biol*. 2013; 170(1): 171-176.
33. Apparao KB, Illera MJ, Beyler SA, Olson GE, Osteen KG, Corjay MH, et al. Regulated expression of osteopontin in the peri-implantation rabbit uterus. *Biol Reprod*. 2003; 68(5): 1484-1490.
34. Young MF, Kerr JM, Termine JD, Wewer UM, Wang MG, McBride OW, et al. cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). *Genomics*. 1990; 7(4): 491-502.
35. von Wolff M, Strowitzki T, Becker V, Zepf C, Tabibzadeh S, Thaler CJ. Endometrial osteopontin, a ligand of $\beta 3$ -integrin, is maximally expressed around the time of the "implantation window". *Fertil Steril*. 2001; 76(4): 775-781.
36. Girotti M, Zingg HH. Gene expression profiling of rat uterus at different stages of parturition. *Endocrinology*. 2003; 144(6): 2254-2265.
37. Cardozo E, Pavone ME, Hirshfeld-Cytron JE. Metabolic syndrome and oocyte quality. *Trends Endocrinol Metab*. 2011; 22(3): 103-109.
38. Takemoto M, Yokote K, Yamazaki M, Ridall AL, Butler WT, Matsumoto T, et al. Enhanced expression of osteopontin by high glucose: involvement of osteopontin in diabetic macroangiopathy. *Ann N Y Acad Sci*. 2000; 902: 357-363.
39. Zhang SJ, Wang JP. Effect of pioglitazone on renal osteopontin expression in diabetic rats. *Journal of Chinese Pharmaceutical Sciences*. 2013; 48(6): 441-445.
40. Zhang S, Xu H, Yu X, Wu Y, Sui D. Metformin ameliorates diabetic nephropathy in a rat model of low-dose streptozotocin-induced diabetes. *Exp Ther Med*. 2017; 14(1): 383-390.

Detection of Partial AZFc Microdeletions in Azoospermic Infertile Men Is Not Informative of MicroTESE Outcome

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Abstract

Background: Microdeletions of the Yq chromosome are among the most frequent genetic etiological factor of male infertility which spans the azoospermia factor regions (AZFa, AZFb and AZFc). Microdeletions are mostly seen in the AZFc region and usually cover genes actively involved in spermatogenesis. Partial AZFc microdeletions may also occur with various spans, namely gr/gr, b2/b3 and b1/b3. It is known that the outcome of microtesticular sperm extraction (TESE), the surgical process for sperm retrieval from the testis in infertile azoospermic men, may be predicted based on the type of AZF microdeletion. We therefore aimed to evaluate the correlation between partial AZFc microdeletions and microTESE results.

Materials and Methods: In this cross-sectional study, 200 infertile azoospermic men referred to the Royan Institute were examined for the presence of partial AZFc microdeletions before undergoing microTESE. Partial AZFc microdeletions were detected by multiplex polymerase chain reaction (PCR) of seven different sequence-tagged site (STS) markers. The data were analyzed with the Chi-square test.

Results: Among the 90 patients (45%) with a positive microTESE outcome, 9 (10%) showed a partial microdeletion in AZFc region. Of the 110 (55%) patients with a negative microTESE outcome, 7 (6.3%) had an AZFc partial microdeletion. With respect to the span of the microdeletions, among the 200 patients, 11 (5.5%) were gr/gr and 5 (2.5%) were b2/b3. Statistical analysis showed no significant difference between the patients with and without partial AZFc microdeletions with respect to microTESE outcome.

Conclusion: Partial AZFc microdeletions is not a predictor of microTESE outcome in azoospermic men.

Keywords: Azoospermia, Infertility, Microsurgery, Sperm Retrieval, Y Chromosome

Citation: Miraghazadeh A, Sadighi Gilani MA, Reihani-Sabet F, Ghaehri A, Borjian Boroujeni P, Zamanian M. Detection of partial AZFc microdeletions in azoospermic infertile men is not informative of microTESE outcome. *Int J Fertil Steril.* 2019; 12(4): 298-302. doi: 10.22074/ijfs.2019.5397.

Introduction

Infertility, defined as the failure to conceive after one year of regular intercourse, is caused by male factors in 15% of cases (1, 2). Genetic factors play a role in at least 10% of male infertility cases, of which chromosomal disorders and Y chromosome microdeletions are the most prevalent (3). The azoospermia factor (AZF) region is located on the long arm of Yq and contains important genes involved in the process of spermatogenesis. Microdeletions in the AZF regions (a, b and c) usually lead to azoospermic/severe oligospermic male infertility (4).

AZFc is the most common Yq microdeletion (5) spanning about 3.5 Mbp of Yq and is divided into three smaller subcategories of partial microdeletions, namely gr/gr, b2/b3 and b1/b3 (6, 7). In the classic AZFc microdeletion (b2/b4), four copies of DAZ are removed leading to spermatogenic failure (5). The gr/gr deletion, the most frequent partial AZFc deletion, removes around half of the b2/b4 region and may be clinically presented by various phenotypes based on ethnic and geographic origin (8-10). The b2/b3 and b1/b3 partial deletions are rare and have been studied less often (11, 12).

Received: 11/Sep/2017, Accepted: 22/Apr/2018

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The chance of sperm retrieval during surgical or microsurgical procedures such as testicular sperm extraction (TESE), known as microTESE, may be predicted if there is a microdeletion in the AZF region (13). This chance is virtually zero in cases with AZFa and AZFb, however, in AZFc microdeletion carriers, this may increase to more than 50% (14). Since there was no previous report on the chance of sperm retrieval in men with AZFc partial microdeletions, we first determined the frequency of such deletions in a cohort of azoospermic/severe oligospermic men and then assessed its relationship with microTESE outcome.

Materials and Methods

This cross-sectional study was conducted during 2013-2014. A total of 200 infertile men with azoospermia/severe oligospermia, as candidates for microTESE surgery at the Royan Infertility Center, were included. The study was approved by the Ethics Committee of the Royan Institute (IR.ACECR.ROYAN.REC.1395.1) and written consent was obtained from the patients. The patients were checked for AZF full microdeletions and reported as normal based on the EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions (13). Infertile patients with obstructive azoospermia, varicocele, cryptorchidism, endocrine problems, history of chemotherapy or radiotherapy and abnormal karyotype were excluded from the study. MicroTESE candidates were then checked for the presence of AZFc partial microdeletions.

PAXgene Blood DNA kit (Qiagene, Germany) and

the salting out method were used for DNA extraction. Quality and concentration of extracted DNA from peripheral blood was checked by Nanodrop Spectrophotometer 2000 (Thermo Scientific, USA). AZFc partial deletions were analyzed using multiplex polymerase chain reaction (PCR) of seven sequence-tagged site (STS) markers as previously described (7). In brief, STS markers for each AZFc subregion (gr/gr, b2/b3 and b1/b3) were selected and specific primers with predetermined products size were designed (Table 1). Two multiplex PCR reactions (A and B) were prepared by mixing 0.5 μ l of 10 mM dNTP mix (Bioron Germany), 0.3 μ l of Taq DNA polymerase (Bioron, Germany), 0.55 μ l of 15 mM MgCl₂ and 0.25 μ l of 10 pM forward and reverse primers in a total volume of 25 μ l. For mix A, the PCR cycling conditions were 94°C for 4 minutes, 40 cycles of 94°C for 30 seconds, 64°C for 40 seconds and 72°C for 45 seconds and a final extension at 72°C for 10 minutes. For mix B, the annealing temperature was set at 41°C. Mix A contained primers for the 1191, 1291 and 1258 STS markers while mix B contained primers for STS markers 1161, 1197, 1206 and 1201. DNA from healthy male and female controls were used as internal positive and negative controls. The PCR products were separated on 3% agarose gels stained with Sybr green (ABM, Germany).

Statistical analyses were performed using SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA). Continuous variables were analyzed using the independent sample t test and categorical variables were analyzed using chi-square test. P<0.05 was considered statistically significant.

Table 1: The sequence-tagged site (STS) markers included in each multiplex polymerase chain reaction (PCR) mix. Each marker and its relationship to azoospermia factor c (AZFc) subregions, its related primer set and product size are shown

	Marker	b2/b4	b2/b3	gr/gr	b1/b3	Primer sequence (5'-3')	Product size (bp)
Mix A	SY1197	+	+	+	-	F: TCATTTGTGTCCTTCTCTTGGA R: CTAAGCCAGGAAGTTGCCAC	435
	SY1161	+	+	+	-	F: CGACACTTTTGGGAAGTTTCA R: TTGTGTCCAGTGGTGCTTA	330
	SY1201	+	+	+	+	F: CCGACTTCCACAATGGCT R: GGGAGAAAAGTTCTGCAACG	677
	SY1206	-	+	+	+	F: ATTGATCTCCTTGGTTCCCC R: GACATGTGTGGCCAATTGA	394
Mix B	SY1191	-	-	+	-	F: CCAGACGTTCTACCCTTTCG R: GAGCCGAGATCCAGTTACCA	385
	SY1291	-	+	-	-	F: TAAAAGGCAGAACTGCCAGG R: GGGAGAAAAGTTCTGCAACG	527
	SY1258	+	+	+	+	F: AACCCCACTCTAGCAAAAATATG R: TAGGTGACAGGGCAGGATTC	930

Table 2: The clinical characteristics of infertile men

Patients groups	Age (Y)	FSH (mIU/mL)	LH (mIU/mL)	Testosterone (mg/mL)
TESE positive n=90	39.19 ± 6.6	22.87 ± 17.05	11.4 ± 8.20	3.86 ± 4.95
TESE negative n=110	39.22 ± 6.22	24.71 ± 15.61	15.12 ± 10.66	3.59 ± 1.97

Values are mean ± SEM (P=0.974, P=0.431, P=0.006, P=0.627, respectively). FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, TESE; Testicular sperm extraction. Normal ranges: FSH: 2-10 mIU/mL, LH: 1.0-9.5 mIU/mL, Testosterone: >2.4 mg/mL.

Results

Demographic characteristics of the patients including age and hormonal profile are summarized in Table 2. Of the 200 infertile men, 90 cases had a successful micro-TESE. In assessing partial AZFc deletions, we identified 11 cases of gr/gr and 5 cases of b2/b3 microdeletions among all cases, however, no b1/b3 partial AZFc microdeletion was identified. In samples with a gr/gr deletion, a 527 bp PCR product representing the sY1291 STS marker was missing while those carrying b2/b3 deletions lacked a 385 bp product for the sY1191 STS marker (Fig.1). We detected partial AZFc deletions in 9 and 7 cases among successful and failed microTESE groups respectively (Table 2). There was no statistically significant difference in the frequency of AZFc partial deletions between successful and failed microTESE groups (Fig.2). For gr/gr deletion the frequencies were 8% and 3.6% for successful and failed groups respectively. Finally, for b2/b3 deletion, we detected a frequency of 2.2% in successful group for sperm retrieval versus 2.7% in failed group (Table 3).

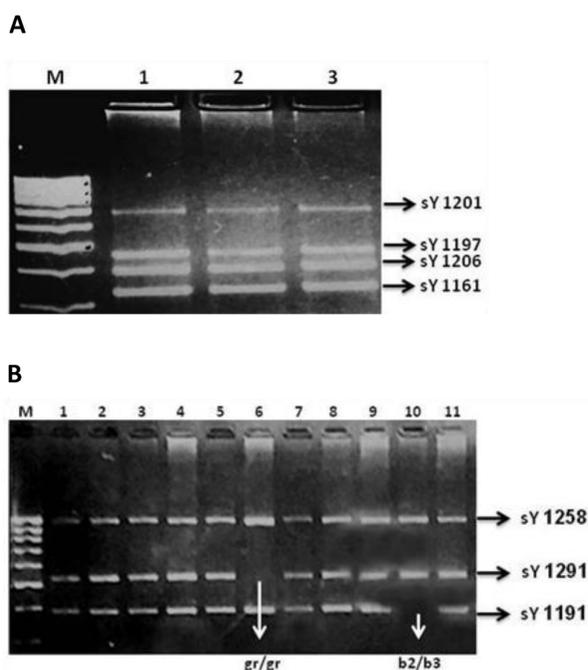


Fig.1: Results for multiplex polymerase chain reaction (PCR). **A.** Mix A, the band sizes match the relevant sequence-tagged site (STS) markers. No deletion was detected in three samples and **B.** Mix B, sample 6 is showing a missing band for sY1291 which is representative for a gr/gr deletion while sample 10 has a missing band for sY1191 which is indicative of a b2/b3 deletion in the azoospermia factor c (AZFc) region.

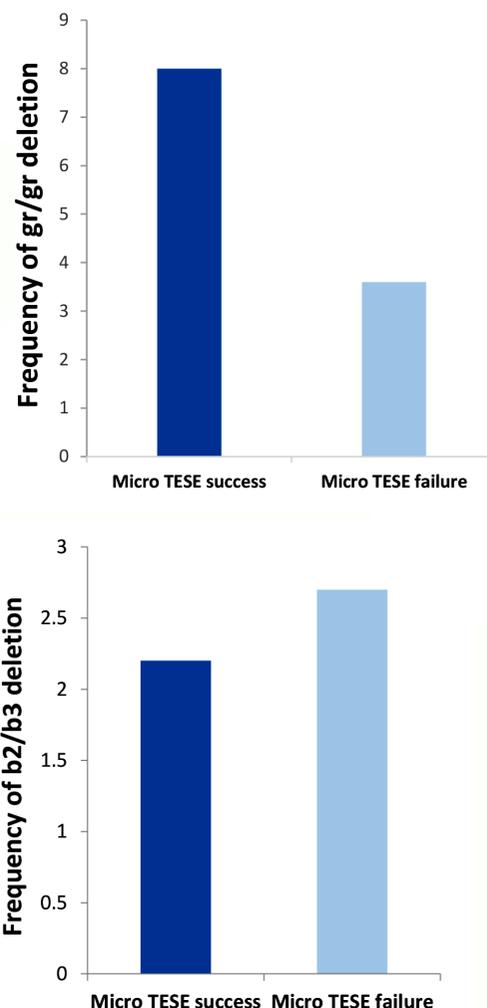


Fig.2: Partial azoospermia factor c (AZFc) microdeletions in infertile men with micro testicular sperm extraction (TESE) surgery. **A.** Percentage of gr/gr deletions. Comparison showed no significant difference (P=0.201) in those with successful micro TESE compared to those with failed surgery and **B.** Percentage of b2/b3 microdeletion. Also no significant difference was detected between those with and without sperm retrieval during micro TESE surgery (P=0.82).

Table 3: AZFc partial deletions in infertile men based on microTESE outcome. The number and percentage of AZFc partial deletions in each category are shown

Micro TESE result	n	AZFc partial deletion		
		gr/gr	b2/b3	Total
Successful	90	7 (8%)	2 (2.2%)	9 (10%)
Failed	110	4 (3.6%)	3 (2.7%)	7 (6.4%)
P value	-	0.201	0.820	0.346
Total	200	11 (5.5%)	5 (2.5%)	16 (8%)

AZF; Azoospermia factor and TESE; Testicular sperm extraction.

Discussion

Infertile men with azoospermia/severe oligospermia are usually selected for sperm retrieval surgeries such as microTESE for further assisted reproductive technology (ART) procedures. It is usually recommended to determine AZF microdeletions before surgery to predict the chance of microTESE success (13). For cases carrying AZFa and AZFb microdeletions, this chance is considered as virtually zero while for those with AZFc deletions it can reach up to 50% (15). In AZFc cases, there is also a chance for transmission of infertility to the male offspring (2).

Previous reports on the importance of AZFc partial deletions such as gr/gr and b2/b3 are controversial and seemingly due to ethnic heterogeneity and differences in genetic backgrounds (7-12). Ferlin et al. (7) reported the gr/gr deletion as a risk factor in infertile men of a Caucasian population. In a study on the Sri Lankan population, the gr/gr deletion frequency was reported as equal (4.2%) in both fertile and infertile groups and therefore found not to be associated with spermatogenesis (16). On the contrary, Eloualid et al. (11) and Choi et al. (17) reported contributory effects on spermatogenesis for b2/b3 and gr/gr deletions in other populations.

Giachini et al. (18, 19) examined infertile Italian men and detected a higher frequency for the gr/gr deletion in the oligo/azoospermic group but found no difference for the b2/b3 deletion. Furthermore, in a previous study on 100 Iranian infertile men, the gr/gr deletion was linked to spermatogenic failure (20). In contrast, Stahl et al. (10) emphasized that the gr/gr deletion is not an appropriate factor for predicting impaired spermatogenesis. Therefore, effects of partial AZFc microdeletions on spermatogenesis as well as the chance for sperm retrieval during microTESE has remained inconclusive. Given that most of these studies had relatively small sample sizes, we examined this association on a relatively larger sample set of 200 patients. The frequency of AZFc partial deletions for patients with successful and failed microTESE outcome were 10% and 6.4% respectively, further questioning the association, similar to some previous studies (10, 16, 21) who reported a lack of association between AZFc partial deletions and spermatogenic failure. No significant difference was also observed when gr/gr and b2/b3 deletions were considered separately. In present study, one major limitation is the relatively small sample size which could limit the statistical power and be the reason for the absence of significant relationship between micro TESE results and AZFc microdeletions. In addition to a bigger sample size, it is recommended to consider gene content in partial AZFc deletions in future studies.

Conclusion

We found no evidence for partial AZFc microdeletion influencing outcome of sperm extraction during microTESE. A larger association study may reveal the diagnostic value of such deletions in men who are candidates for microTESE.

Acknowledgements

This study was designed, financially supported and performed in Royan Institute for Reproductive Biomedicine. Authors, hereby, declare no conflicts of interest.

Authors' Contributions

A.M.; Performed the experiments, drafted the manuscript. M.A.S.G.; Contributed to the conception and helped in designing the study, performed the micro TESE. A.G.; Analyzed the data. F.R.-S., P.B.B; Helped in optimizing the experiments. M.Z.; Contributed substantially to the conception of study, designed the study and the experiments, provided critical revision of the manuscript. All authors read and approved the final manuscript.

References

- Friel A, Houghton JA, Maher M, Smith T, Noël S, Nolan A, et al. Molecular detection of Y chromosome microdeletions: an Irish study. *Int J Androl.* 2001; 24(1): 31-36.
- Foresta C, Moro E, Ferlin A. Y Chromosome microdeletions and alterations of spermatogenesis. *Endocr Rev.* 2001; 22(2): 226-239.
- Lin YN, Matzuk MM. Genetics of male fertility. In: Rosenwaks Z, Wassarman PM, editors. *Human fertility: methods and protocols.* 1st edition. New York: Springer; 2014; 25-37.
- Hamada AJ, Esteves SC, Agarwal A. A comprehensive review of genetics and genetic testing in azoospermia. *Clinics (Sao Paulo).* 2013; 68 Suppl 1: 39-60.
- Page DC. On low expectations exceeded; or, the genomic salvation of the Y chromosome. *Am J Hum Genet.* 2004; 74(3): 399-402.
- O'Flynn O'Brien KL, Varghese AC, Agarwal A. The genetic causes of male factor infertility: a review. *Fertil Steril.* 2010; 93(1): 1-12.
- Ferlin A, Tessari A, Ganz F, Marchina E, Barlati S, Garolla A, et al. Association of partial AZFc region deletions with spermatogenic impairment and male infertility. *J Med Genet.* 2005; 42(3): 209-213.
- Lynch M, Cram DS, Reilly A, O'Bryan MK, Baker HW, de Kretser DM, et al. The Y chromosome gr/gr subdeletion is associated with male infertility. *Mol Hum Reprod.* 2005; 11(7): 507-512.
- Stouffs K, Lissens W, Tournaye H, Haentjens P. What about gr/gr deletions and male infertility? Systematic review and meta-analysis. *Hum Reprod Update.* 2011; 17(2): 197-209.
- Stahl PJ, Mielnik A, Margreiter M, Marean MB, Schlegel PN, Paduch DA. Diagnosis of the gr/gr Y chromosome microdeletion does not help in the treatment of infertile American men. *J Urol.* 2011; 185(1): 233-237.
- Eloualid A, Rhaissi H, Reguig A, Bounaceur S, El Houate B, Abidi O, et al. Association of spermatogenic failure with the b2/b3 partial AZFc deletion. *PLoS One.* 2012; 7(4): e34902.
- Lu C, Zhang J, Li Y, Xia Y, Zhang F, Wu B, et al. The b2/b3 subdeletion shows higher risk of spermatogenic failure and higher frequency of complete AZFc deletion than the gr/gr subdeletion in a Chinese population. *Hum Mol Genet.* 2009; 18(6): 1122-1130.
- Krausz C, Hoefsloot L, Simoni M, Tüttelmann F; European Academy of Andrology; European Molecular Genetics Quality Network. EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions: state-of-the-art 2013. *Andrology.* 2014; 2(1): 5-19.
- Park SH, Lee HS, Choe JH, Lee JS, Seo JT. Success rate of microsurgical multiple testicular sperm extraction and sperm presence in the ejaculate in Korean men with Y chromosome microdeletions. *Korean J Urol.* 2013; 54(8): 536-540.
- Tsujimura A, Matsumiya K, Takao T, Miyagawa Y, Koga M, Takeyama M, et al. Clinical analysis of patients with azoospermia factor deletions by microdissection testicular sperm extraction. *Int J Androl.* 2004; 27(2): 76-81.
- Fernandes S, Paracchini S, Meyer LH, Florida G, Tyler-Smith C, Vogt PH. A large AZFc deletion removes DAZ3/DAZ4 and nearby genes from men in Y haplogroup N. *Am J Hum Genet.* 2004; 74(1): 180-187.
- Choi J, Song SH, Bak CW, Sung SR, Yoon TK, Lee DR, et al. Impaired spermatogenesis and gr/gr deletions related to Y Chromosome haplogroups in Korean men. *PLoS One.* 2012; 7(8): e43550.
- Giachini C, Nuti F, Marinari E, Forti G, Krausz C. Partial AZFc de-

- letions in infertile men with cryptorchidism. *Hum Reprod.* 2007; 22(9): 2398-2403.
19. Giachini C, Guarducci E, Longepied G, Degl'Innocenti S, Becherini L, Forti G, et al. The gr/gr deletion(s): a new genetic test in male infertility? *J Med Genet.* 2005; 42(6): 497-502.
 20. Rezaei Z, Motvali bashi M, Hojati Z, Mahmodi R. Molecular Study of Partial Deletions of AZFc Region of the Y Chromosome in Infertile Men. *Journal of Kerman University of Medical Sciences.* 2013; 20(5): 460-469.
 21. Hucklenbroich K, Gromoll J, Heinrich M, Hohoff C, Nieschlag E, Simoni M. Partial deletions in the AZFc region of the Y chromosome occur in men with impaired as well as normal spermatogenesis. *Hum Reprod.* 2005; 20(1): 191-197.
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Varicocelectomy May Improve Results for Sperm Retrieval and Pregnancy Rate in Non-Obstructive Azoospermic Men

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Abstract

Background: Assessing the net-results of microsurgical varicocelectomy in infertile men with non-obstructive azoospermic (NOA) and clinical varicocele in five years at Royan Institute.

Materials and Methods: This is a descriptive retrospective cohort study. A backward-looking review of patients treated for NOA and varicocele from march 2011 to march 2016 was performed. In addition, MDTESE results of 57 patients with NOA and clinical varicocele, with 537 NOA patients without varicocele were compared.

Results: Of 57 patients who underwent varicocelectomy, eight patients (14%) had sperm on sperm analysis post-operatively. One of the eight patients was single, and one of them had spontaneous pregnancy (1/7) 14%, and one had a child by microinjection (1/7) 14%. Out of these 8 patients, 6 had hypospermatogenesis pathology. Of 38 patients who underwent MDTESE, 14 patients (36%) had sperm on their testis tissues, but one of them had no egg fertilization. Therefore, the fertilization rate was (92%). Of the remaining 13 patients, 3 had live child birth (3/13) 23%. Sperm retrieval rate (SRR) in NOA men without clinical varicocele was lower from those who had varicocele and NOA (22 vs. 36%). Also live birth rate in NOA men with varicocelectomy was higher than NOA men without varicocele (23 vs. 11%).

Conclusion: Microsurgical varicocelectomy in NOA men may have positive effects on post-operative sperm in ejaculate and natural or assisted pregnancies, but it seems that the effect is more significant on MDTESE results and following successful microinjection. Meanwhile, SRR and live birth rate was higher in our patients compare to NOA men without clinical varicocele.

Keywords: Azoospermia, Testicular Sperm Retrieval, Varicocele

Citation: Sajadi H, Hosseini J, Farrahi F, Dadkhah F, Sepidarkish M, Sabbaghian M, Eftekhari-Yazdi P, Sadighi Gilani MA. Varicocelectomy may improve results for sperm retrieval and pregnancy rate in non-obstructive azoospermic men. *Int J Fertil Steril.* 2019; 12(4): 303-305. doi: 10.22074/ijfs.2019.5344.

Introduction

Once infertile men with non-obstructive azoospermic (NOA) had no other options than adopting a child or using sperms of a donor to father a child, however, nowadays they are provided with other alternatives, which are given to them by the introduction of sperm retrieval from their testis and then entering an Intracytoplasmic sperm injection (ICSI) cycle (1).

Varicocele, that is associated with a progressive decline in testicular function, occurs in about 15% of total male population, 35% of men with primary infertility and between 75 to 81% of men with secondary infertility (2).

As a treatment, varicocelectomy improves both sper-

matogenesis and the function of Leydig cells (2). Moreover, it has been broadly reported that the density and the motility of sperms has improved in OAT patients after varicocelectomy. Nevertheless, the value of varicocelectomy is still arguable in men with azoospermia (1).

In previous studies the effects of varicocelectomy in these patients were shown to be less significant (1) but in recent studies better results have been reported for varicocelectomy in NOA patients (3-5). Therefore, several questions occur regarding sperm in the ejaculate of NOA males who will undergo varicocelectomy procedure. These questions include: are these couples able to have natural or assisted pregnancies while avoiding the

Received: 30/Jun/2017, Accepted: 10/Jul/2018

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 4, Jan-Mar 2019, Pages: 303-305

need for TESE? Does varicocelelectomy in these groups of patients improve SRR with MDTESE? How are the MDTESE results of these groups of patients with NOA males without varicocelelectomy compared?

The present study aimed to assess the net-result of microsurgical varicocelelectomy in infertile men with NOA with clinical varicocele in the past five years at Royan institute.

Materials and Methods

This is a retrospective cohort study. A backward-looking review of patients treated for NOA and palpable varicocele in Royan institute from March 2011 to March 2016 was performed.

57 men with NOA and clinical varicocele in their physical examination have been reviewed. Known cases of obstructive azoospermia, non-palpable varicocele, female factor infertility and genetic abnormalities like klinefelter syndrome and Y-chromosome microdeletion were excluded from the study.

The cases of varicocele were identified by scrotal examinations performed by expert surgeons with the patients in standing position and during valsalva's manoeuvre. The disease was categorized in 3 grades: grade 1 if it was palpable just during the maneuver, grade 2 if it was palpable without the maneuver and grade 3 if it was visible.

All patient charts were also reviewed for age, infertility duration, postoperation complications, testis volume, follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), testicular sonographic findings, genetic abnormalities, testicular biopsy results, sperm in ejaculate, MDTESE, fertilization rate, pregnancy and delivery rate.

In order to stay away from retrieval of testicular sperm, all NOA patients who underwent microsurgical varicocelelectomy in Royan institute were inspected to find out if these patients had enough sperm in ejaculate postoperatively. Also, both assisted and unassisted pregnancy rates were evaluated using postoperative ejaculated sperm.

In addition, we have evaluated the MDTESE results in these patients and reviewed their fertilization, pregnancy and delivery rates.

Finally, we have compared the results of our 57 patients with NOA and clinical varicocele to 537 NOA patients without varicocele. All patients in our study had been treated in Royan institute.

Statistical analysis

For categorical and continuous variables data was reported in forms of proportions and mean \pm SD, respectively. Pearson chi-square tests and Student's t test were used to assess differences between baseline demographic and clinical characteristics. Since the sample size was small and data had several unbalanced and highly predictive risk factors (complete separation or quasi separation

problems), multiple logistic regression model was performed using firthlogit to examine possible association between the outcome of interest (sperm retrieval) and microdissection TESE. The presence of the problem mentioned above in logistic regression models can result in bias in odds ratio (OR) estimates away from 1. Firthlogit command did not use maximum log likelihood but penalized log likelihood instead to reduce bias. All data analysis was completed using stata version 14 (STATA Corp, College Station, TX).

Results

For the 57 patients who were enrolled in our study from March 2011 to March 2016, the mean duration of infertility was $4.29 \pm (3.97)$ years (range 1-12) and the mean testicular volume values were $17.36 \pm (6.39)$ cc (range 3.2-31).

40 patients treated for varicocelelectomy had karyotype analysis and azoospermia factor (AZF) microdeletion in their charts and their karyotypes were NL 46XY and no one had microdeletion.

Of the 57 patients, 8 (14.03%) acquire motile sperms in a postoperative sperm analysis. Of these 8 patients, 6 had hypospermatogenesis, 1 had maturation arrest and 1 had sertoli only syndrome (SOS) in histopathology.

One of the patients was single, and one of them had spontaneous pregnancy (1/7) 14%, and one had children through microinjection (1/7) 14%.

Microdissection TESE was applied to 38 (66.7) NOA patients, who had negative sperm postoperatively (Table 1). The mean interval between varicocelelectomy and microTESE was 13.7 months (range 3 to 17). Prior to operation varicocele grades were 1, 2, and 3 in 8 (21.05%), 16 (42.1%) and 14 (36.84%) of these patients, respectively. Of 38 patients who underwent MDTESE, 14 (36.8%) had sperm in their testis tissues. Of these 14 patients, 8 had maturation arrest, 3 had hypospermatogenesis and 3 had SOS in their biopsies. In addition, of these 14 patients one had no egg fertilization, therefore, the fertilization rate was 13 (92%).

A total of 530 patients with NOA without varicocele were selected as the control group. Characteristics of the control group are described in Table 1. The mean \pm SD patient age was $33.84 \pm (7.27)$ years for the cases and $34.10 \pm (6.35)$ years for the controls ($P=0.810$). The controls had a significantly higher FSH [$22.48 \pm (14.47)$] (mIU/mL) compared to the case group [$17.50 \pm (16.65)$] ($P=0.05$). Base on other laboratory parameters, no significant difference was observed between these two groups.

Sperm retrieval rate by MDTESE in the cases and the control groups were 14/38 (36.8%) and 119/530 (22.3%), respectively (OR=2.03, 95% CI: 1.01-4.05, $P=0.041$). The live birth rate in the cases and the control groups was 21.42% (3 of 14 cases) and 11.7% (14 of 119 controls), respectively (OR=2.21, 95% CI: 0.59-8.14, $P=0.219$).

Table 1: Characteristics of patients

Parameter	Cases	Controls	P value
Age (Y)	33.84 ± 7.27	34.10 ± 6.35	0.810
FSH (mIU/mL)	17.50 ± 16.65	22.48 ± 14.47	0.05
LH (mIU/mL)	8.44 ± 6.80	8.84 ± 7.18	0.743
Testosterone (ng/ml)	3.20 ± 2.38	3.48 ± 2.23	0.477
SRR	14(36.8)	119(22.3)	0.410
Live birth rate	3(21.42)	14(11.7)	0.219

Values given as mean ± SD, or n(%) unless otherwise indicated. FSH; Follicular stimulating hormone, LH; Luteinizing hormone, and SRR; Sperm retrieval rate.

Discussion

While the impact of varicocelectomy has been widely considered in oligoasthenoteratozoospermia (OAT) patients, this surgical procedure's benefit in patients with NOA is limited and still arguable.

Tulloch was the first who studied the importance of varicocelectomy for treatment of NOA in 1952 (6). After that, many studies investigated the effect of this surgical procedure on NOA patients.

From 57 patients who underwent varicocelectomy at Royan institute, eight patients (14%) had sperm on sperm analysis postoperatively and only one of the patients had spontaneous pregnancy. So in terms of postoperative sperm in ejaculate the effect of varicocelectomy in our patients was little, and this result was nearly the same as the findings of the study by Shlegele et al. (22%) (1). Other studies have reported variable results from 34 to 44% (3-5).

So far, studies report testicular histology as one of the most important predictor factor outcomes (7-9). Our data support this as the most histopathological predictor of postoperative sperm in the ejaculate was hypospermatogenesis: from 8 patients who had achieved motile sperm postoperatively, 6 had hypospermatogenesis.

Sperm retrieval rate by MDTES was (36/8%) in NOA men with varicocelectomy compared to (22%) in NOA cases without varicocele. Therefore, varicocelectomy in NOA men with varicocele may improve the chance of SRR compared to NOA men without varicocele.

Live birth rate/embryo was 21% in our patients in comparison with 11% in NOA without varicocele.

Conclusion

According to our current data, we suggest that microsurgical varicocelectomy in NOA patients may have positive effects on postoperative sperm in the ejaculate and spontaneous or assisted pregnancies, but it seems that this effect is more significant on MDTESE results when fol-

lowing successful microinjection. Meanwhile, SRR and live birth rate were higher in our patients compared to NOA males without clinical varicocele. The most histopathologic finding in microTESE-positive patients was maturation arrest, which shows the need of MDTESE for sperm retrieval in such patients.

Thus, varicocelectomy is a key factor in NOA patients to increase the likelihood of SRR in MDTESE. Nevertheless, studies with a larger population and a longer follow-up period are needed in order to prove MDTESE benefits in these patients.

Acknowledgements

We thank all individuals who provided help in this study, especially the Royan Institute staff. There is no financial support associated with this study. The authors declare no conflict of interest.

Authors' Contributions

H.S.; Participated in study design, data collection and evaluation, and interpretation of data. J.H., F.F., F.D.; Participated in microsurgical varicocelectomy, TESE, MDTESE. M.Se.; Participated in statistical analysis. M.Sa.; Data collection and participated in drafting and revising the manuscript. P.E.-Y.; Participated in embryological data gathering. M.A.S.G.; Participated in both study design and interpretation of data, and was responsible for overall supervision. All authors read and approved the final manuscript.

References

- Schlegel PN, Kaufman J. Role of varicocelectomy in men with non-obstructive azoospermia. *Fertil Steril.* 2004; 81(6): 1585-1588.
- Wein AJ, kavoussi LR, Partin AW, Peters CA. *Campbell Walsh urology.* 11th edition. Philadelphia, USA. Elsevier; 2015; 604.
- Youssef T, Abd-Elaal E, Gaballah G, Elhanbly S, Eldosoky E. Varicocelectomy in men with nonobstructive azoospermia: is it beneficial? *Int J Surg.* 2009; 7(4): 356-360.
- Zampieri N, Bosaro L, Costantini C, Zaffagnini S, Zampieri G. Relationship between testicular sperm extraction and varicocelectomy in patients with varicocele and nonobstructive azoospermia. *Urology.* 2013; 82(1): 74-77.
- Esteves SC, Miyaoka R, Roque M, Agarwal A. Outcome of varicocele repair in men with nonobstructive azoospermia: systematic review and meta-analysis. *Asian J Androl.* 2016; 18(2): 246-253.
- Tulloch WS. A consideration of sterility factors in the light of subsequent pregnancies. II. Sub fertility in the male. (Tr. Edinburgh Obst. Soc. Session 104). *Edinb Med J.* 1951-1952; 59: 29-34.
- Matthews GJ, Matthews ED, Goldstein M. Induction of spermatogenesis and achievement of pregnancy after microsurgical varicocelectomy in men with azoospermia and severe oligoasthenospermia. *Fertil Steril.* 1998; 70(1): 71-75.
- Kim ED, Leibman BB, Grinbat DM, Lipshultz LI. Varicocele repair improve semen parameters in azoospermic men with spermatogenic failure. *J Urol.* 1999; 162(3 Pt 1): 737-740.
- Kadioglu A, Tefkeli A, Cayan S, Kendirali E, Erdemie F, Tellaloglu S. Microvascular inguinal varicocele repair in azoospermic men. *Urology.* 2001; 57(2): 328-333.

Significant Correlation between High-Risk HPV DNA in Semen and Impairment of Sperm Quality in Infertile Men

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Abstract

Background: Human papillomavirus (HPV) is a DNA virus that causes sexually transmitted infections (STI). Recent reports suggest that HPV may affect sperm parameters and lead to male infertility. This study aims to evaluate the correlation between seminal high-risk HPV infection and impairment of sperm quality in infertile Iranian men.

Materials and Methods: In this case-control study, we collected fresh semen samples from 70 fertile men and 70 confirmed infertile men who referred to Yazd Infertility Centre in 2015. Semen analyses were performed according to the World Health Organization (WHO) guidelines. High-risk HPV DNA was detected by real-time polymerase chain reaction (PCR).

Results: A total of 140 subjects participated in the current study. Among 70 confirmed infertile males, only 8 (11.43%) cases tested positive for high-risk HPV and all fertile men were HPV-negative. This data revealed a significant association between high-risk HPV and male infertility ($P=0.03$). The percentage of normal sperm morphology and sperm motility rate significantly declined in men infected with HPV ($P<0.001$).

Conclusion: There was a significantly higher prevalence of high-risk HPV in infertile men than fertile men. HPV infection seemed to be a risk factor for male infertility. Additional, larger studies should be conducted to confirm the impact of HPV on male infertility.

Keywords: Human Papillomavirus, Male Infertility, Sperm Motility

Citation: Moghimi M, Zabihi-Mahmoodabadi S, Kheirkhah-Vakilabad A, Kargar Z. Significant correlation between high-risk HPV DNA in semen and impairment of sperm quality in infertile men. *Int J Fertil Steril.* 2019; 12(4): 306-309. doi: 10.22074/ijfs.2019.5421.

Introduction

Human papillomavirus (HPV) infection is caused by a DNA virus and it is considered the most common sexually transmitted disease (STD) worldwide (1). Currently, over 150 different HPV genotypes have been identified and classified as low-risk or high-risk according to oncogenic potential of the virus (2, 3). Low-risk HPVs mostly cause self-limited infections such as skin warts and are not usually associated with neoplasia (4). High-risk types of HPV are strongly linked to several cancers in both men and women (5). HPV is responsible for 630,000 new cancer cases per year worldwide and accounts for 0.8% of all cancers in men and 8.6% in women (6).

HPV may be found anywhere in the male reproductive tract such as external genitalia, epididymis, vas deferens, and urethra (7). Although HPV can infect the semen, its role as a direct cause of infertility is not clear (8).

Sexually active couples who cannot achieve pregnancy after one year are considered infertile. According to this

definition, approximately 15-20% of couples are infertile. Despite the available advanced diagnostic methods, approximately 20-35% of infertile men have unexplained infertility (9). Approximately 6-10% of male infertility is due to male genital tract infections.

Evidence suggests that sexually transmitted infections (STI) such as *Treponema pallidum*, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* can lead to reduced fertility or infertility. Sexually transmitted viral infections that include herpes simplex virus (HSV), cytomegalovirus (CMV), and human immunodeficiency virus (HIV) also alter semen parameters and fertility.

In addition, HPV has been recently considered as an infectious agent that affects fertility (10). Several studies have reported that HPV virions can bind to the head of sperm and directly reduce its motility (11). It has been suggested that HPV infections might be linked to unexplained infertility in men. In addition, a significant correlation between seminal HPV DNA infection and lower



total sperm count has also been reported (12).

The prevalence of sperm infection by HPV is reported to be 2-31% in the general male population and 10-35% in men with unexplained infertility (13). Some studies have indicated a significant relationship between seminal HPV infection and sperm quality. On the other hand, other studies have not found a significant association between abnormal sperm parameters and HPV infection in infertile men (9, 14). Therefore, the effect of HPV infection on impaired sperm quality and male infertility is debatable. A few studies have sought to determine the impact of HPV on fertility in Iran, with inconsistent results (14, 15). The current study aimed to determine the correlation between seminal high-risk HPV infection and impairment of sperm quality in infertile Iranian men in an attempt to increase information about the possible effect of HPV infection on alterations of male fertility.

Materials and Methods

We conducted this case-control study on 70 infertile male patients and 70 confirmed fertile males who referred to Yazd Infertility Center in 2015. The Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran approved this study (IR.SSU.MEDICINE.REC.1393.130) and all participants signed the study informed consent form. The major inclusion criterion of the case group was infertility after at least one year of unprotected sexual intercourse. The main exclusion criteria of the case group were as follows: chromosome abnormalities, azoospermia, undescended testis, and history of orchitis or varicocele. In addition, we excluded men whose spouses had histories of uterine and ovarian disorders. The control group included fertile men who had at least one child. The exclusion criterion of the control group was the presence of genital warts. The semen samples were collected and allowed to liquefy for 1 hour at 37°C. The spermogram was carried out for every specimen to determine semen parameters such as total sperm number, sperm motility, and morphology according to the World Health Organization (WHO) guidelines. Semen samples were immediately stored at -20°C until nucleic acid extraction and HPV detection.

DNA extraction and human papillomavirus detection

The semen samples were centrifuged at 2500 rpm for 10 minutes. The supernatants were removed, and the pellets were transferred to Eppendorf tubes. DNA extraction was performed with a RIBO-prep Extraction Kit (AmpliSens, Russia) according to the manufacturer's instructions. High-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 were detected using the AmpliSens® HPV HCR Screen-titre-FRT kit (AmpliSens, Russia) based on multiplex real-time polymerase chain reaction (PCR). The real-time PCR assays were performed using an ABI Step One Plus system (Applied

Biosystems, Foster City, CA, USA). The reaction mixture in a total volume of 25 µl contained 10 µl of extracted DNA and 15 µl of the master mix according to the kit instructions. The conditions for real-time PCR assay consisted of an initial denaturation phase at 95°C for 15 minutes followed by 60 cycles at 95°C for 5 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 15 seconds.

Statistical analysis

General descriptive data of fertile and infertile men and the values of sperm parameters were presented as mean ± SD. Data were analysed using Stata statistical software, version 14 (StataCorp LLC, College Station, TX, USA). Univariate and multivariate logistic regression analysis were used for comparison of participant characteristics and sperm parameters between infertile and fertile men. Penalized logistic regression model with data augmentation was performed to compare the frequency of high-risk HPV DNA between infertile and fertile men (16). We compared the sperm parameters in infertile men between the HPV-positive and HPV-negative group using the independent samples t test. $P < 0.05$ were considered statistically significant.

Results

A total of 140 men with an average age of 32.74 ± 5.21 years participated in the study. Table 1 summarizes the characteristics and sperm parameters for the fertile and infertile men. Data analysis according to logistic regression showed no statistically significant difference in fertility between smokers and nonsmokers ($P = 0.42$). In addition, the results in Table 1 showed no significant correlation between education, length of marriage, and male infertility ($P > 0.05$). Sperm parameters that included counts, total motility, progressive motility, and normal morphology rate in infertile men were significantly lower than the fertile group (Table 1, $P < 0.05$). Overall, the prevalence of high-risk HPV DNA infection in semen was 5.7%. Among 70 subjects in the case group, 8 (11.43%) men were HPV positive. None of the men in the control group had any HPV infection.

Penalized logistic regression analysis via data augmentation revealed a significant association between high-risk HPV infection and male infertility (Table 1, $P = 0.03$). The probability of fertility in high-risk HPV-positive men was 90% less than those not infected with HPV [odds ratio (OR): 0.1, 95% confidence interval (CI): 0.01-0.82]. In the case group (infertile men), sperm motility, progressive motility and percentage of normal sperm morphology of the HPV-positive subjects showed statistically significant decreases compared with HPV-negative cases (Table 2). Sperm concentration in the infertile men infected with HPV was lower than the HPV negative group, but this difference was not significant (Table 2, $P = 0.41$).

Table 1: Characteristics of participants and sperm parameters in the total study population

Characteristics/parameters	Fertile men n=70	Infertile men n=70	OR adjusted	95% CI	P value ^a
Mean age (Y ± SD)	33.61 ± 5.25	31.88 ± 5.18	1.6	1.23-2.36	0.99
Education n (%)					
Illiterate	2 (1.4)	1 (0.7)	0.37	0.27-3.12	0.61
Primary	6 (4.3)	4 (2.9)	0.59	0.47-1.78	0.46
Secondary	23 (16.4)	18 (12.9)	0.71	0.39-1.13	0.37
College	39 (27.8)	47 (33.6)	1	-	Reference
Married period (Y)	5.96 ± 4	5.73 ± 4.46	0.93	0.77-1.12	0.43
Smoking (%)	17 (24.3)	23 (32.9)	0.68	0.49-1.14	0.42
Sperm count (million/ml)	93.50 ± 37.95	59.64 ± 30.21	1.01	0.95-0.98	0.021
Sperm motility (%)	59.39 ± 8.86	31.21 ± 14.04	1.05	0.80-0.89	0.042
Sperm progressive motility (%)	37.54 ± 17.08	6.09 ± 5.20	1.06	0.64-0.82	0.016
Normal sperm morphology rate (%)	40.10 ± 9.48	14.26 ± 11.45	1.03	0.78-0.88	0.031
HPV DNA infection of semen	0	8 (11.43)	0.1	0.01-0.82	0.03 ^b

Data are presented as mean ± SD or n (%). HPV; Human papillomavirus, OR; Odds ratio, CI; Confidence interval, ^a; P value obtained by univariate and multivariate logistic regression analysis, and ^b; P value obtained by penalized logistic regression model via data augmentation.

Table 2: Comparison of sperm parameters between infertile men infected with HPV and the HPV-negative group

Sperm parameters	HPV positive	HPV negative	P value ^a
Sperm count (million/ml)	51.38 ± 29.29	60.71 ± 30.39	0.41
Sperm motility (%)	23.50 ± 13.50	32.21 ± 13.90	0.04
Sperm progressive motility (%)	0.63 ± 1.77	6.79 ± 5.08	<0.001
Normal sperm morphology rate (%)	7.13 ± 2.64	15.18 ± 11.83	<0.001

Sperm parameters are presented as mean ± SD. HPV; Human papillomavirus and ^a; P value obtained by independent samples t test.

Discussion

The effect of HPV on sperm quality and male infertility is controversial. Results of the current study have indicated that seminal high-risk HPV in infertile men was higher than in the fertile men, and it could impair sperm quality. These data suggested a possible role for HPV in male infertility. We found an HPV infection prevalence of 11.43% in semen of men who suffered from infertility. Previous studies have also reported a prevalence of HPV in infertile men that ranged from 10 to 30% (13). The few studies conducted on infertile men in Iran have estimated the HPV prevalence to be approximately 25 to 30% (15). The difference in prevalence might be due to the use of different diagnostic tests to detect HPV.

Some STI impair semen quality by inducing epididymitis, orchitis or urethritis (12, 17). The mechanism by which HPV affects sperm quality is still poorly understood. In the current study, HPV decreased significantly total sperm motility and progressive motility. Previous studies on sperm parameters in relation to HPV infection reported conflicting findings. A few studies have reported enhanced motility and progression in HPV-exposed sperm (18, 19). Bezold et al. (12) demonstrated that HPV infection in asymptomatic men was associated with low sperm quality and changes in progressive motility. Con-

sistent with our finding, the majority of previous studies observed reduced sperm motility in men infected with HPV (20-22). In contrast, other studies have shown no association between HPV infection and sperm quality parameters (17). A study conducted in Iran has also found a significant relationship between HPV and alteration in sperm motility (15). These contradictory results might be attributed to differences in sample size and sensitivity of HPV detection methods.

In the present study, the normal sperm morphology rate in HPV-positive cases significantly decreased compared with its rate in HPV-negative men. Several studies reported no difference in sperm morphology between HPV-infected and noninfected infertile men (17). The studies conducted in Iran also have not found a significant relationship between HPV and abnormal sperm morphology (12, 13). The relationship between HPV and its negative impact on sperm morphology remains poorly understood, but it could be due to binding of HPV to the spermatozoa head (23). Yang et al. (7) found that abnormal morphology of sperm clearly increased in HPV-infected individuals.

In this study, high-risk HPV infection did not significantly affect total sperm count in infertile men. Rintala et al. obtained similar results from infertile men infected with high-risk types of HPV (24). Unlike our result, Nasseri et al. (15) reported that HPV decreased sperm cell counts in infertile Iranian men. Several studies also reported a significant relationship between the lower total sperm count and HPV infection in semen (12, 14).

The limitation of our study included the absence of data on volume and pH of semen, which was not obtained in each semen analysis. However, most previous studies have not reported any connection between seminal HPV infection and alteration in these parameters (7, 17).

Conclusion

Taken together, the result of the present study indicated

that HPV could be a risk factor for male infertility. The prevalence of high-risk HPV in infertile men was significantly higher than fertile men. HPV decreased sperm motility and normal morphology rate. Further, larger studies would be required to confirm the impact of HPV on male infertility.

Acknowledgements

This research was financially supported by Shahid Sadoughi University of Medical Sciences. The authors would like to thank all participants of the study. The authors have no conflict of interest.

Authors' Contribution

M.M.; Participated in study design and data collection. S.Z.-M.; Contributed to all experimental work and the acquisition of data. A.Kh.-V., Z.K. Contributed to the statistical analysis and interpretation of data. All authors performed editing and approving the final version of this paper for submission, participated in the finalization of the manuscript, and approved the final draft.

References

- Dadashi M, Vaezjalali M, Fallah F, Goudarzi H, Nasiri MJ, Owlia P, et al. Epidemiology of human papillomavirus (HPV) infection among Iranian women identified with cervical infections: a systematic review and meta-analysis of national data. *IEM*. 2017; 3(2): 68-72.
- Elmi AA, Bansal D, Acharya A, Skariah S, Dargham SR, Abu-Raddad LJ, et al. Human papillomavirus (HPV) infection: molecular epidemiology, genotyping, seroprevalence and associated risk factors among Arab women in Qatar. *PLoS One*. 2017; 12(1): e0169197.
- Doosti M, Bakhshesh M, Zahir ST, Shayestehpour M, Karimi-Zarchi M. Lack of evidence for a relationship between high risk human papillomaviruses and breast cancer in Iranian patients. *Asian Pac J Cancer Prev*. 2016; 17(9): 4357-4361.
- Doosti M, Bakhshesh M, Hatami A, Taghipour-Zahir S, Moghimi M, Shayestehpour M. The relationship of low-risk (6, 11) and high-risk types (16, 18) of papilloma virus and human breast cancer in women. *Journal of Isfahan Medical School*. 2014; 32(279): 368-387.
- McBride AA. Oncogenic human papillomaviruses. *Philos Trans R Soc Lond B Biol Sci*. 2017; 372(1732). pii: 20160273.
- de Martel C, Plummer M, Vignat J, Franceschi S. Worldwide burden of cancer attributable to HPV by site, country and HPV type. *Int J Cancer*. 2017; 141(4): 664-670.
- Yang Y, Jia CW, Ma YM, Zhou LY, Wang SY. Correlation between HPV sperm infection and male infertility. *Asian J Androl*. 2013; 15(4): 529-532.
- Schuppe HC, Pilatz A, Hossain H, Diemer T, Wagenlehner F, Weidner W. Urogenital infection as a risk factor for male infertility. *Dtsch Arztebl Int*. 2017; 114(19): 339-346.
- Golob B, Poljak M, Verdenik I, Kolbezen Simoniti M, Vrtačnik Bokal E, Zorn B, et al. High HPV infection prevalence in men from infertile couples and lack of relationship between seminal HPV infection and sperm quality. *Biomed Res Int*. 2014; 2014: 956901.
- Pereira N, Kucharczyk KM, Estes JL, Gerber RS, Lekovich JP, Elias RT, et al. Human papillomavirus infection, infertility, and assisted reproductive outcomes. *J Pathog*. 2015; 2015: 578423.
- Foresta C, Patassini C, Bertoldo A, Menegazzo M, Francavilla F, Barzon L, et al. Mechanism of human papillomavirus binding to human spermatozoa and fertilizing ability of infected spermatozoa. *PLoS One*. 2011; 6(3): e15036.
- Bezold G, Politch JA, Kiviat NB, Kuypers JM, Wolff H, Anderson DJ. Prevalence of sexually transmissible pathogens in semen from asymptomatic male infertility patients with and without leukocytospermia. *Fertil Steril*. 2007; 87(5): 1087-1097.
- Foresta C, Noventa M, De Toni L, Gizzo S, Garolla A. HPV-DNA sperm infection and infertility: from a systematic literature review to a possible clinical management proposal. *Andrology*. 2015; 3(2): 163-173.
- Tafvizi F, Fathi MH, Tahmasebi Fard Z. Lack of association between HPV and sperm parameters as a risk factor in infertile men admitted to infertility clinic. *Pars Journal of Medical Sciences*. 2015; 13(3): 37-45.
- Nasseri S, Monavari SH, Keyvani H, Nikkhoo B, Vahabpour Roudsari R, Khazeni M. The prevalence of Human Papilloma Virus (HPV) infection in the oligospermic and azoospermic men. *Med J Islam Repub Iran*. 2015; 29: 272. eCollection 2015.
- Greenland S, Mansournia MA, Altman DG. Sparse data bias: a problem hiding in plain sight. *BMJ*. 2016; 352: i1981.
- Garolla A, Pizzol D, Foresta C. The role of human papillomavirus on sperm function. *Curr Opin Obstet Gynecol*. 2011; 23(4): 232-237.
- Connelly DA, Chan PJ, Patton WC, King A. Human sperm deoxyribonucleic acid fragmentation by specific types of papillomavirus. *Am J Obstet Gynecol*. 2001; 184(6): 1068-1070.
- Brossfield JE, Chan PJ, Patton WC, King A. Tenacity of exogenous human papillomavirus DNA in sperm washing. *J Assist Reprod Genet*. 1999; 16(6): 325-328.
- Garolla A, Lenzi A, Palu G, Pizzol D, Bertoldo A, De Toni L, et al. Human papillomavirus sperm infection and assisted reproduction: a dangerous hazard with a possible safe solution. *Hum Reprod*. 2012; 27(4): 967-973.
- Foresta C, Pizzol D, Moretti A, Barzon L, Palù G, Garolla A. Clinical and prognostic significance of human papillomavirus DNA in the sperm or exfoliated cells of infertile patients and subjects with risk factors. *Fertil Steril*. 2010; 94(5): 1723-1727.
- Gizzo S, Ferrari B, Noventa M, Ferrari E, Patrelli TS, Gangemi M, et al. Male and couple fertility impairment due to HPV-DNA sperm infection: update on molecular mechanism and clinical impact -systematic review. *Biomed Res Int*. 2014; 2014: 230263.
- Cai T, Wagenlehner FM, Mondaini N, D'Elia C, Meacci F, Migno S, et al. Effect of human papillomavirus and Chlamydia trachomatis co-infection on sperm quality in young heterosexual men with chronic prostatitis-related symptoms. *BJU Int*. 2014; 113(2): 281-287.
- Rintala MA, Grenman SE, Pollanen PP, Suominen JJ, Syrjanen SM. Detection of high-risk HPV DNA in semen and its association with the quality of semen. *Int J STD AIDS*. 2004; 15(11): 740-743.

Association between The Number of Retrieved Mature Oocytes and Insulin Resistance or Sensitivity in Infertile Women with Polycystic Ovary Syndrome

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Abstract

Background: The objective of this study was to describe the association between luteinizing hormone (LH)/follicle-stimulating hormone (FSH) ratio and demographic variables and maturation stage of oocytes in insulin-resistant and insulin-sensitive patients with polycystic ovary syndrome (PCOS) in comparison with control group.

Materials and Methods: In this case-control study, 60 patients with *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) indication were subdivided into 3 groups as follow: 20 subjects were assigned to control (fertile women with male infertility history) group, 20 subjects with PCOS were insulin resistant (IR) and 20 subjects with PCOS were insulin sensitive (IS). After puncture, retrieved oocytes were classified into metaphase II (MII) as mature and in metaphase I (MI) or germinal vesicle stage (GV) as immature. Regression analyses were used to explore the association between MII oocyte number and demographic and clinical variables.

Results: LH/FSH ratio was significantly higher in PCOS-IR women compared to controls but not significantly different from that of PCOS-IS group. PCOS-IR women had lower MII oocyte number compared with that of controls. According to multiple regression analysis, the number of previous assisted reproductive technology (ART) cycles was negatively associated with the number of MII oocytes.

Conclusion: Insulin resistance can be associated with reductions in MII oocyte number in patients with PCOS.

Keywords: Assisted Reproductive Technology, Insulin Resistance, Oocyte, Polycystic Ovary Syndrome

Citation: Hassani F, Oryan S, Eftekhari-Yazdi P, Bazrgar M, Moini A, Nasiri N, Ghaheeri A. Association between the number of retrieved mature oocytes and insulin resistance or sensitivity in infertile women with polycystic ovary syndrome. *Int J Fertil Steril.* 2019; 12(4): 310-315. doi: 10.22074/ijfs.2019.5422.

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women of reproductive age, which is mostly associated with hyperinsulinemia, hyperandrogenism and anovulatory infertility (1, 2). The prevalence of PCOS is 6-26% depending on differences in study populations background and the applied diagnostic criteria (3, 4).

Follicular arrest (FA) and dysregulation of paracrine ac-

tivity in follicles are noticeable ovarian signs in PCOS patients (5). Insulin plays an important role in regulating the response of human follicular cells to gonadotropins (6). Evaluation of the association between insulin resistance and ovarian hyperstimulation syndrome (OHSS) revealed that hyperinsulinemia may lead to disruption of ovarian steroidogenesis, which in turn increases the secretion of ovarian androgens by dysregulation of cytochrome P450c17 α activity (7, 8). High levels of insulin can occupy the insulin-like growth factor-1 (IGF-1) receptors

Received: 29/Sep/2017, Accepted: 5/May/2018

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 4, Jan-Mar 2019, Pages: 310-315

therefore simulating and disturbing their function and subsequently resulting in hyperandrogenism (9).

Recently, it has been observed that insulin can modulate steroidogenesis through its own receptor. Moreover, in case of insulin resistance, the steroidogenesis appears to be preserved likely by various mechanisms of regulation of receptors receptivity in different tissue (10). *In vitro* studies using cultured pituitary cells have demonstrated that insulin increases the luteinizing hormone (LH) secretion during the enhancement of pituitary responsiveness to gonadotropin releasing hormone (GnRH) (11, 12). Furthermore, DiVall et al. (13) observed that increased insulin receptor signaling in GnRH neurons of obese female mice, elevated GnRH pulsatile secretion and consequent LH secretion resulting in reproductive abnormality.

Excessive ovarian androgen production has also been implicated in the pathogenesis of PCOS. It has been postulated that hyperinsulinemia in case of insulin resistance, is associated with capacity of ovarian androgen production (4, 5). Severe insulin resistance causes a compensatory hyperinsulinemia, which stimulates ovarian androgen production in the presence of sufficient LH (14).

An *in vitro* study showed that insulin and IGF-I stimulate androgen production in incubated human stroma and theca cells. In some women with insulin-resistance-induced hyperandrogenism, an acute rise in circulating androgens may be induced by increases in glucose concentration. The effect of circulating androgen rise is dependent on the amount of insulin secreted in response to glucose enhancement. These data suggest that hyperinsulinemia may play a central role in the development of ovarian hyperandrogenism (14). According to a previous study, insulin can induce steroid secretion (i.e. insulin acts as a co-gonadotropin) (15). Increased LH serum level and enhanced ratio of LH/follicle-stimulating hormone (FSH) are seen in many of PCOS patients. Frequent coexistence of elevated LH and increased insulin concentrations leads to more severe manifestations of PCOS manifestations (15, 16).

Folliculogenesis and oocyte maturation are complex processes that require the action of both LH and FSH (17, 18). In PCOS patients high LH levels have been associated with significant decreases in oocyte maturation and fertilization rates, and impaired embryo quality (17, 19). Hyperinsulinemia may impair the competence of oocyte development. Subsequently, higher percentages of low-quality oocytes in PCOS may cause lower fertilization rates and decreased embryos quality that have been reported in PCOS patients compared to healthy women with assisted reproductive technology (ART) indication (18, 19).

In this study, we prospectively evaluated the association between different factors [age, body mass index (BMI), number of previous ART cycle, and LH/FSH ratio] and oocyte maturity in insulin-resistant and insulin-sensitive women with PCOS in comparison with control group.

Materials and Methods

Patient selection

In this case-control study, each of 40 patient's case seeking assisted reproduction at Royan Institute from April 2014 to January 2015 was analyzed. Written informed consent was obtained from all the participants. Ethics approval was obtained from the local Ethics committee of Royan Institute (no.EC/93/1138). PCOS patients were allocated to one of the two groups formed based on the level of fasting insulin (FI): insulin resistant (PCOS-IR; FI \geq 12 mg/dl) and insulin sensitive (PCOS-IS; FI $<$ 12 mg/dl). In control group, 20 women with regular menstrual cycle without known diseases (i.e. fertile women with male infertility history) were included. Exclusion criteria were impaired thyroid, renal or hepatic function, congenital adrenal hyperplasia (CAH), endometriosis, premature ovarian insufficiency (POI), functional hypothalamic amenorrhea (FHA), unexplained infertility (UI) and age $>$ 36 years.

Stimulation protocol

In order to controlled induce ovarian stimulation (COS), daily subcutaneous injection of recombinant human FSH (rFSH, Gonal F®; Serono Pharma, Switzerland) was started from the second day of the cycle. Starting dose of rFSH was adjusted individually depending on patients response measured by transvaginal ultrasonography, antral follicle count (AFC), levels of serum estradiol (E2) and AMH. A GnRH antagonist-cetrorelix (Cetrotide®, Merck Serono, Germany) was administered subcutaneously when at least two ovarian follicles reached 14 mm in diameter. The protocol consisted of daily subcutaneous injections of Cetrotide 0.25 mg, until the criteria for human chorionic gonadotropin (hCG) administration were met. For final oocyte maturation, when the dominant follicle reached \geq 18 mm in diameter with the following two follicles \geq 16 mm and E2 levels between 1000-4000 pg/mL, an intramuscular injection of 10.000 IU hCG (Pregnyl®, Organon, Holland) or subcutaneous injection of 250 μ g hCG (Ovitrelle®, Merck Serono, France) was given.

Oocytes retrieval

Oocyte Pick-up (OPU) was done using transvaginal ultrasound-guided follicle aspiration, 36 hours after hCG administration to collection tubes. Following OPU, cumulus-oocyte complexes were washed several times in fertilization medium (G-IVF®, Vitrolife, Sweden) to remove blood and cell debris, and incubated for two hours in fertilization medium (G-IVF®, Vitrolife, Sweden). Retrieved oocytes were classified into metaphase II (MII) stage as mature and metaphase I (MI) or germinal vesicle (GV) stage as immature. Oocyte denudation was performed using 80 IU of hyaluronidase (Sigma, USA) (20). Participants underwent intracytoplasmic sperm injection (ICSI). The spermatozoa were prepared using density gradient centrifugation (AllGrad®; LifeGlobal, USA) for

PCOS patients or standard swim-up method for control group. PCOS patients' quality of sperm were compatible with WHO criteria 2010; however, in the control group, male factor infertility existed with respect to sperm quality (i.e. oligo, asteno, teratozoospermia or combinations of these conditions) according to the WHO parameters. After sperm microinjection into the MII oocytes, fertilization was confirmed 16 to 17 hours after ICSI, by the presence of two pronuclei (2PN) and a second polar body. Zygotes were individually placed in 20 μ l fresh G-1TM medium (Vitrolife) supplemented with 10% recombinant human serum albumin (HAS-solutionTM, Vitrolife) under oil (OVOILTM, Vitrolife) for a 72 hour culture.

Embryological assessment

Based on our laboratory standards, embryos were graded at the pronuclear and cleavage stages. The quality of the embryos at cleavage stage were classified according to the following criteria: [excellent quality (≥ 4 cells or ≥ 8 cells and $< 10\%$ fragmentation), good quality (≥ 4 cells or ≥ 8 cells and $10\text{--}20\%$ fragmentation) and poor quality (< 4 cells or < 8 cells and $> 20\%$ fragmentation)] (21, 22). Decision on the number of transferred embryos was made based on 2013 ASRM embryo-transfer guidelines (23). Seventy two hours after ICSI, mainly a maximum of two embryos of excellent grade or good quality were transferred to uterine cavity by a Labotect catheter (Labotect, Germany).

Luteal support

On the day of oocyte retrieval, luteal phase support included Cyclogest[®] 200 mg (Actavis, UK) vaginal suppositories, twice daily (bid) for 14 days. Endometrial thickness was between 8 to 11 mm and showed a triple-line pattern as examined by vaginal ultrasonography on the day of hCG injection. Gestation was confirmed by pregnancy test 14 days after ET. Clinical pregnancy confirmed when a gestational sac with fetal cardiac activity was detectable after 7 weeks of gestation. Biochemical gestation was not taken into consideration at any stage of the study.

Statistical analysis

In this study, categorical variables are presented as number (%) and continuous variables as mean \pm SD or median (minimum-maximum; inter-quartile range) where appropriate. Statistical comparisons of means of the three study groups were performed using ANOVA or its non-parametric equivalent, Kruskal-Wallis test. Independent t-test was used to assess mean differences in FI between IR and IS- PCOS groups. Chi-square analysis was used for qualitative data. Univariate and backward multiple linear regression, including all variables, were used to evaluate the association between MII oocyte number and some demographic and clinical variables. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA). All statistical tests were 2-tailed and a $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics of participants are shown in Table 1. BMI was significantly higher in PCOS-IR group (29.97 ± 4.39) compared to control group (25.12 ± 4.21 , $P=0.023$), this difference was not significant between PCOS-IS (26.31 ± 8.03) and either of PCOS-IR and control groups. PCOS-IR women had significantly fewer number of MII oocytes (8.10 ± 3.61) compared to controls (11.57 ± 5.11 , $P=0.028$); but, the number of MII oocytes was not significantly different between PCOS-IS women (9.05 ± 3.37) and control subjects. There were no significant differences in MI, GV and dead oocytes between PCOS groups (IR and IS) and control group. Fasting insulin was significantly higher in PCOS-IR (19.30 ± 9.60 mg/dl) compared to PCOS-IS group (6.67 ± 2.89 mg/dl, $P=0.006$). Other criteria including age, previous ART history, number of retrieved oocytes, number of 2PN embryos, total number of embryo and success rate did not differ significantly among the three groups.

LH/FSH ratio was significantly higher in PCOS-IR women (1.67 ± 1.75) compared to controls (0.94 ± 0.68 , $P=0.047$) but not significantly different from that of PCOS-IS group (1.45 ± 0.94) (Fig.1). Regression analysis results are presented in Table 2. On the basis of the univariate analysis, LH/FSH in study groups (PCOS-IR, PCOS-IS and control) were significantly associated with MII oocyte number. After adjusting for other variables, based on the multiple linear regression model results, LH/FSH was no longer statistically significant; however, on average, the PCOS-IR group had 4.09 MII oocytes less than the control group and the PCOS-IS group had 3.21 MII oocytes less than the control group. Multiple linear regression model also identified that the number of previous ART cycles was negatively associated with MII oocyte number. As shown in Table 2, for each unit increase in previous ART cycle number, the expected number of MII oocyte decreases by 1.23. Other variables included in the univariate model and displayed in Table 2, were not significantly associated with MII oocyte number in the multiple model.

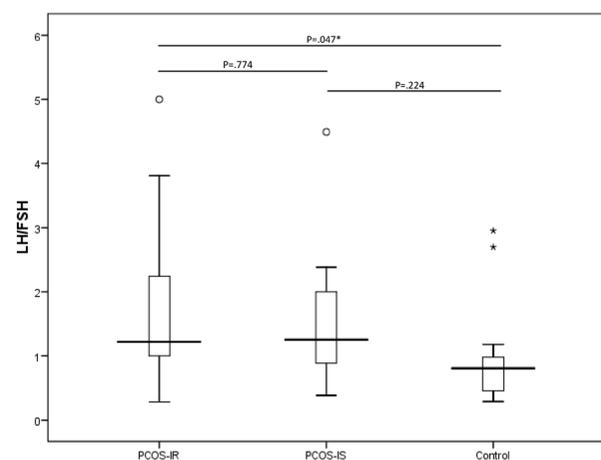


Fig.1: Boxplots of LH/FSH in PCOS-IR, PCOS-IS and control group. LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, PCOS; Polycystic ovary syndrome, IR; Insulin resistant, and IS; Insulin sensitive. Extreme values are indicated using asterisks and outliers are indicated using circles.

Table 1: Demographic and clinical characteristics of recruited patients

	PCOS-IR n=20	PCOS-IS n=20	Control n=20	P value
Age (Y)	30.04 ± 4.45	28.00 ± 4.00	29.05 ± 5.18	0.381
Previous ART				
Yes	7 (25.9)	7 (25.9)	13 (48.2)	0.108
No	15 (42.9)	12 (34.3)	8 (22.8)	
Fasting insulin (mg/dl)	19.30 ± 9.60	6.67 ± 2.89	-	0.006
BMI	29.97 ± 4.39 ^a	26.31 ± 8.03 ^{ab}	25.12 ± 4.21 ^b	0.023
Oocyte retrieved	11.90 ± 7.09	13.58 ± 11.26	12.90 ± 5.591	0.814
MII	8.01 ± 3.61 ^a	9.05 ± 3.37 ^{ab}	11.57 ± 5.11 ^b	0.028
MI	0 (0-3; 1)	0 (0-2; 0)	0 (0-1; 1)	0.806
GV	0 (0-2; 0)	0 (0-4; 1)	0 (0-3; 1)	0.468
Degenerated/dead	0 (0-1; 0)	0 (0-4; 1)	0 (0-2; 0.5)	0.125
Total embryo	7.15 ± 4.29	9.44 ± 7.34	7.95 ± 4.07	0.415
2PN embryos	6 (0-14; 4.75)	7 (1-13; 3.25)	9 (2-18; 4.5)	0.723
Fertilization (%)	73.28 ± 25.43	75.90 ± 25.89	76.01 ± 17.24	0.914

PCOS; Polycystic ovary syndrome, IR; Insulin resistant, IS; Insulin sensitive, ART; Assisted reproductive technology, BMI; Body mass index, MI; Metaphase I oocytes, MII; Metaphase II oocytes, GV; Germinal vesicle stage, 2PN; Two pronuclei. Continuous variables are presented as mean ± SD or median (minimum-maximum; inter-quartile range) when appropriate. Categorical variables are presented as number (%). Different letters indicate significantly different means (as evaluated by ANOVA and Tukey post-hoc test). A P<0.05 was considered significant.

Table 2: Regression analysis for factors associated with MII oocytes number

	Univariate analysis			Multiple analysis		
	B	SE	P value	B	SE	P value
Age	-0.01	0.13	0.910			
BMI	-0.14	0.09	0.143			
LH/FSH	-1.22	0.56	0.035			
No. of previous ART cycle	-0.71	0.61	0.251	-1.23	0.59	0.043
Group						
PCOS-IR vs. control	-3.47	1.30	0.010	-4.09	1.29	0.003
PCOS-IS vs. control	-2.52	1.33	0.064	-3.21	1.34	0.020

B; Unstandardized coefficient, SE; Standard error, BMI; Body mass index, LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, ART; Assisted reproductive technology, PCOS; Polycystic ovary syndrome, IS; Insulin sensitive, and IR; Insulin resistant.

Discussion

PCOS is a heterogeneous endocrinopathy; insulin resistance and elevated LH/FSH ratio play a potential role in the pathogenesis of the disorder (24). However, according to the 2003 rotterdam ESHRE/ASRM-sponsored PCOS Consensus workshop group, increased LH/FSH ratio and IR will not be considered the main criteria for the diagnosis of PCOS and more research is needed in this area (19). Banaszewska et al. (25) studied a rare subgroup of PCOS women and observed that increased LH levels and IR occurred simultaneously. In this study, in terms of biochemical factors, there was a significant increase in LH/FSH ratio in PCOS-IR compared to control group (P<0.05). We described the demographic characteristic of these patients and the outcome of IVF/ICSI treatment. According to our data, in the PCOS-IR group, mean number of MII was almost 4 units less than that of the control group. Moreover, in the PCOS-IS group, the number of mature oocytes was on average almost 3 units lower than that of the healthy women. As shown by Colton et al. (26),

continuation of meiosis and maturation of oocytes were defected in a diabetic mouse model. Also, it was reported that insulin resistance can impair normal fertilization or chromosomal abnormalities in affected oocytes (27). These findings explain aberrant relationships between oocytes and surrounding cumulus cells (26).

According to recent studies, insulin signals relay through multiple pathways, many of them are active in ovarian follicular cells especially oocytes. These pathways are able to interact with each other and also with gonadotropins. Thus, insulin has a direct regulatory effect on ovarian physiology (28). According to the results of Burghen et al. (29), insulin resistance was common in PCOS women even if the patients were obese. It was indicated that high levels of insulin can bind to not only insulin receptors but also ovarian IGF1 receptors, the latter stimulate steroidogenesis. In particular, this effect is mediated by aromatase activity (30). There is a synergism between insulin and gonadotropins (LH and FSH) that can intensify steroidogenesis. Poretsky and Kalin (30) have proposed that LH

can induce interstitial cells of ovary to differentiate into androgen-producing cells. Hyperinsulinemia possibly accelerates this process. This hyperandrogenic condition would result in follicular atresia. Tarlatzis et al. (31) found that elevated local androgens and loss of estrogen production lead to follicular growth arrest.

The strengths of this study were evaluation of the effects of increased LH/FSH level and insulin resistance on the oocyte maturation. This study has a prospective scheme and all patients underwent ICSI and received GnRH antagonist. Besides, the statistical calculations were accomplished by multivariable analysis. Finally, we observed lower number of MII oocytes in IR patients. The mechanisms underlying these results are unclear; although previous studies showed that ovarian folliculogenesis may be disrupted by high LH levels (32). Tarlatzis et al. (31) reported that elevated LH/FSH in human menopausal gonadotropin (HMG)-stimulated PCOS women may have a detrimental effect on the maturation of oocytes. However, according to Wisner et al. (33) higher numbers of mature oocytes were retrieved from PCOS women with higher LH/FSH ratio. Since according to the inclusion criteria of the study, only women who had indication for *in vitro* maturation (IVM) treatment were recruited, the results were inconsistent.

According to some studies, insulin resistance is not a disease but it can develop numerous metabolic alterations (34, 35). These metabolic disturbances can make lesions in cumulus-oocyte complexes (COCs) of murine. In fact, outbreak of insulin resistance may halt the progression of meiosis and postpone oocytes maturation (26). Although increased secretion of LH and insulin resistance is common characteristics of PCOS, in lean PCOS amplified growth hormone (GH) pulsatility in addition to LH hypersecretion induce theca cells to release androgens. In obese PCOS patients, elevated IGFs levels induced by insulin, stimulate granulosa cells to produce androgens. These processes enhance hyperandrogenemia and anovulation (36). Our data suggested that insulin resistance may diminish oocyte maturity.

According to previous studies, the success probability is essentially constant across the first three IVF attempts (37). In the other words, a maximum of three IVF treatment cycles lead to one child birth (38). Tan et al. (37) reported that there were no statistically significant differences in the pregnancy failure rates between those attempting a second course of treatment and those attempting the first course. In both groups of patients, the pregnancy failure rates were more or less the same in all age groups until the women were at least 35 years old (37, 39).

Based on the results of this study, for each unit increase in previous IVF/ICSI failure, the expected number of MII oocytes decreases by 1.23 in PCOS women. Moreover, to the best of our knowledge, the present study is the first report showing that the history of IVF/ICSI failure is associated with reduction of MII oocytes in PCOS-IR patients. Also, our results showed that retrieval of lower

numbers of mature oocytes from PCOS women might be in part related to insulin resistance. Our study suggested that treatment of insulin resistance should be considered in PCOS-IR patients who have a history of canceled IVF cycles. This can help to achieve greater numbers of mature oocytes.

Conclusion

Insulin resistance is a common metabolic abnormality in PCOS patients and PCOS-IR women had lower MII oocytes than control group. Collectively, histories of ART failure and insulin resistance are two important factors in predicting the number of mature oocytes in PCOS-IR patients.

Acknowledgements

We express our appreciation to the staff of the Embryology Laboratory at Royan Institute for their assistance. The authors wish to thank Dr. Ramin Rezaee for editing the manuscript. The authors received no financial support for research, authorship, and/or publication of this article. The authors declare no potential conflicts of interest in this study.

Authors' Contributions

F.H., S.O., P.E.-Y., M.B., A.M., N.N., A.G.; Contributed to manuscript drafting and revisiting. F.H., S.O., P.E.-Y., M.B., A.M.; Collected and collated data. F.H., P.E.-Y., N.N.; Assisted in the study design. A.G.; Did the statistical analysis. All authors read and approved the final version of the manuscript.

References

1. Shah D, Rasool S. Polycystic ovary syndrome and metabolic syndrome: the worrisome twosome? *Climacteric*. 2016; 19(1): 7-16.
2. Moghetti P, Tosi F, Bonin C, Di Sarra D, Fiers T, Kaufman JM, et al. Divergences in insulin resistance between the different phenotypes of the polycystic ovary syndrome. *J Clin Endocrinol Metab*. 2013; 98(4): E628-E637.
3. Tehrani FR, Simbar M, Tohidi M, Hosseiniapanah F, Azizi F. The prevalence of polycystic ovary syndrome in a community sample of Iranian population: Iranian PCOS prevalence study. *Reprod Biol Endocrinol*. 2011; 9: 39.
4. Yildiz BO, Bozdag G, Yapici Z, Esinler I, Yarali H. Prevalence, phenotype and cardiometabolic risk of polycystic ovary syndrome under different diagnostic criteria. *Hum Reprod*. 2012; 27(10): 3067-3073.
5. Xi W, Yang Y, Mao H, Zhao X, Liu M, Fu S. Circulating anti-müllerian hormone as predictor of ovarian response to clomiphene citrate in women with polycystic ovary syndrome. *J Ovarian Res*. 2016; 9: 3.
6. Stocco DM, Wang X, Jo Y, Manna PR. Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. *Mol Endocrinol*. 2005; 19(11): 2647-2659.
7. Hayes MG, Urbanek M, Ehrmann DA, Armstrong LL, Lee JY, Sisk R, et al. Genome-wide association of polycystic ovary syndrome implicates alterations in gonadotropin secretion in European ancestry populations. *Nat Commun*. 2015; 6: 7502.
8. Unlühizarci K, Keleştimur F, Bayram F, Sahin Y, Tutuş A. The effects of metformin on insulin resistance and ovarian steroidogenesis in women with polycystic ovary syndrome. *Clin Endocrinol (Oxf)*. 1999; 51(2): 231-236.
9. Homburg R, Pariente C, Lunenfeld B, Jacobs HS. The role of insulin-like growth factor-1 (IGF-1) and IGF binding protein-1 (IG-

- FBP-1) in the pathogenesis of polycystic ovary syndrome. *Hum Reprod.* 1992; 7(10): 1379-1383.
10. Barbieri RL. Update in female reproduction: a life-cycle approach. *J Clin Endocrinol Metab.* 2008; 93(7): 2439-2446.
 11. Adashi EY, Hsueh AJ, Yen SS. Insulin enhancement of luteinizing hormone and follicle-stimulating hormone release by cultured pituitary cells. *Endocrinology.* 1981; 108(4): 1441-1449.
 12. Allon MA, Leach RE, Dunbar J, Diamond MP. Effects of chronic hyperandrogenism and/or administered central nervous system insulin on ovarian manifestation and gonadotropin and steroid secretion. *Fertil Steril.* 2005; 83 Suppl 1: 1319-1326.
 13. DiVall SA, Herrera D, Sklar B, Wu S, Wondisford F, Radovick S, et al. Insulin receptor signaling in the GnRH neuron plays a role in the abnormal GnRH pulsatility of obese female mice. *PLoS One.* 2015; 10(3): e0119995.
 14. Barbieri RL, Smith S, Ryan KJ. The role of hyperinsulinemia in the pathogenesis of ovarian hyperandrogenism. *Fertil Steril.* 1988; 50(2): 197-212.
 15. Codner E, Iñiguez G, Villarreal C, Lopez P, Soto N, Sir-Petermann T, et al. Hormonal profile in women with polycystic ovarian syndrome with or without type 1 diabetes mellitus. *J Clin Endocrinol Metab.* 2007; 92(12): 4742-4746.
 16. Allahbadi GN, Merchant R. Polycystic ovary syndrome and impact on health. *Middle East Fertil Soc J.* 2011; 16(1): 19-37.
 17. Mermillod P, Oussaid B, Cognie Y. Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. *J Reprod Fertil Suppl.* 1999; 54: 449-60.
 18. Filicori M. The role of luteinizing hormone in folliculogenesis and ovulation induction. *Fertil Steril.* 1999; 71(3): 405-414.
 19. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertil Steril.* 2004; 81(1): 19-25.
 20. Wissing ML, Sonne SB, Westergaard D, Nguyen Kd, Belling K, Host T, et al. The transcriptome of corona radiata cells from individual MII oocytes that after ICSI developed to embryos selected for transfer: PCOS women compared to healthy women. *J Ovarian Res.* 2014; 7: 110.
 21. Eftekhari-Yazdi P, Valojerdi MR, Ashtiani SK, Eslaminejad MB, Karimian L. Effect of fragment removal on blastocyst formation and quality of human embryos. *Reprod Biomed Online.* 2006; 13(6): 823-832.
 22. Valojerdi MR, Karimian L, Yazdi PE, Gilani MA, Madani T, Baghestani AR. Efficacy of a human embryo transfer medium: a prospective, randomized clinical trial study. *J Assist Reprod Genet.* 2006; 23(5): 207-212.
 23. Practice Committee of American Society for Reproductive Medicine, Practice Committee of Society for Assisted Reproductive Technology. Criteria for number of embryos to transfer: a committee opinion. *Fertil Steril.* 2013; 99(1): 44-46.
 24. Moran C, Garcia-Hernandez E, Barahona E, Gonzalez S, Bermudez JA. Relationship between insulin resistance and gonadotropin dissociation in obese and nonobese women with polycystic ovary syndrome. *Fertil Steril.* 2003; 80(6): 1466-1472.
 25. Banaszewska B, Spaczyński RZ, Pelesz M, Pawelczyk L. Incidence of elevated LH/FSH ratio in polycystic ovary syndrome women with normo- and hyperinsulinemia. *Rocz Akad Med Białymst.* 2003; 48: 131-134.
 26. Colton SA, Pieper GM, Downs SM. Altered meiotic regulation in oocytes from diabetic mice. *Biol Reprod.* 2002; 67(1): 220-231.
 27. Diamond MP, Moley KH, Pellicer A, Vaughn WK, DeCherney AH. Effects of streptozotocin- and alloxan-induced diabetes mellitus on mouse follicular and early embryo development. *J Reprod Fertil.* 1989; 86(1): 1-10.
 28. Dupont J, Scaramuzzi RJ. Insulin signalling and glucose transport in the ovary and ovarian function during the ovarian cycle. *Biochem J.* 2016; 473(11): 1483-1501.
 29. Burghen GA, Givens JR, Kitabchi AE. Correlation of hyperandrogenism with hyperinsulinism in polycystic ovarian disease. *J Clin Endocrinol Metab.* 1980; 50(1): 113-116.
 30. Poretsky L, Kalin MF. The gonadotropic function of insulin. *Endocr Rev.* 1987; 8(2): 132-141.
 31. Tarlatzis BC, Grimbizis G, Pournaropoulos F, Bontis J, Lagos S, Spanos E, et al. The prognostic value of basal luteinizing hormone: follicle-stimulating hormone ratio in the treatment of patients with polycystic ovarian syndrome by assisted reproduction techniques. *Hum Reprod.* 1995; 10(10): 2545-2549.
 32. van der Spuy ZM, Dyer SJ. The pathogenesis of infertility and early pregnancy loss in polycystic ovary syndrome. *Best Pract Res Clin Obstet Gynaecol.* 2004; 18(5): 755-771.
 33. Wisner A, Shehata F, Holzer H, Hyman JH, Shalom-Paz E, Son WY, et al. Effect of high LH/FSH ratio on women with polycystic ovary syndrome undergoing in vitro maturation treatment. *J Reprod Med.* 2013; 58(5-6): 219-223.
 34. Reaven GM. The insulin resistance syndrome: definition and dietary approaches to treatment. *Annu Rev Nutr.* 2005; 25: 391-406.
 35. Diamanti-Kandaraki E, Dunaif A. Insulin resistance and the polycystic ovary syndrome revisited: an update on mechanisms and implications. *Endocr Rev.* 2012; 33(6): 981-1030.
 36. Morales AJ, Laughlin GA, Butzow T, Maheshwari H, Baumann G, Yen SS. Insulin, somatotrophic, and luteinizing hormone axes in lean and obese women with polycystic ovary syndrome: common and distinct features. *J Clin Endocrinol Metab.* 1996; 81(8): 2854-2864.
 37. Tan SL, Doyle P, Maconochie N, Edwards RG, Balen A, Bekir J, et al. Pregnancy and birth rates of live infants after in vitro fertilization in women with and without previous in vitro fertilization pregnancies: a study of eight thousand cycles at one center. *Am J Obstet Gynecol.* 1994; 170(1 Pt 1): 34-40.
 38. Olivius K, Friden B, Lundin K, Bergh C. Cumulative probability of live birth after three in vitro fertilization/intracytoplasmic sperm injection cycles. *Fertil Steril.* 2002; 77(3): 505-510.
 39. Schmittlein DC, Morrison DG. A live baby or your money back: the marketing of in vitro fertilization procedures. In: Carrell DP, Peterson CM, editors. *Reproductive endocrinology and infertility: integrating modern clinical and laboratory practice.* New York: Springer; 2010; 101-116.

Dietary Patterns and The Outcomes of Assisted Reproductive Techniques in Women with Primary Infertility: A Prospective Cohort Study

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Abstract

Background: Infertility is one of the most common challenges that women in reproductive age would encounter today. The maternal nutritional status could be a determinant of oocyte quality and embryonic growth. This study was conducted to assess the relationship between dietary patterns and reproductive outcomes in infertile women.

Materials and Methods: This prospective cohort study was conducted on 140 women with primary infertility who had referred to Isfahan Fertility and Infertility center, Isfahan, Iran. The average number of total oocytes and metaphase II oocytes, the fertilization rate, the ratio of good and bad quality embryo and biochemical and clinical pregnancy were considered as the outcomes of assisted reproductive techniques (ART). A 168-item food frequency questionnaire was used for estimating the dietary intakes during the last year. Factor analysis was used for identifying the dietary patterns and analysis of variance (ANOVA), analysis of covariance (ANCOVA), chi-square, and logistic regression analysis were used for assessing the relation between dietary patterns and ART's outcomes.

Results: Three major dietary patterns (the healthy, western and unhealthy diet) were identified. Women with high adherence to the "healthy diet" had a higher educational level and were employed. There was a significant increase in the average number of total oocytes (P -trend=0.009) and metaphase II oocytes (P -trend=0.006) in the third tertile of "healthy diet" compared to the first tertile. Also, women with high adherence to the second tertile of "unhealthy" diet compared to the first tertile, had a significantly lower chance of getting pregnant [odds ratio (OR): 0.14, 95% confidence interval (CI): 0.3-0.7].

Conclusion: Nutrition status could affect infertility treatment outcomes. Greater adherence to the healthy diet may enhance oocyte quality and quantity. Unhealthy diet could adversely affect the chance of getting pregnant.

Keywords: Assisted Reproductive Technique, Infertility, *In Vitro* Fertilization, Nutrition

Citation: Jahangirifar M, Taebi M, Nasr-Esfahani MH, Askari Gh. Dietary patterns and the outcomes of assisted reproductive techniques in women with primary infertility: a prospective cohort study. *Int J Fertil Steril.* 2019; 12(4): 316-323. doi: 10.22074/ijfs.2019.5373.

Introduction

Infertility is defined as the inability to conceive a clinical pregnancy after 12 months of unprotected intercourse (1). It is one of the most common challenges faced by women of reproductive age today (2). Infertility is estimated to affect between 8 to 12% of reproductive-aged couples worldwide (3) and the overall mean of infertility in Iran is 13.2% (4).

According to the Society for Assisted reproductive technology (ART), of 39,573 assisted reproductive cycles performed in the United States clinics among women younger than the age of 35 in 2014, only 37.1% of fresh nondonor ART cycles have resulted in live

births (5). So it can be concluded that there is more room for research for improving ART outcomes. One focus area for researchers could be the relation between intra-follicular environment and oocyte quality. Indeed, a viable pregnancy is highly related to the oocyte quality which is related to the follicular environment (6). In this regard, it has been shown that oocyte growth is sensitive to changes in the follicular environment especially nutrient changes. Variance in maternal nutrition can have a significant effect on the metabolic activity of oocytes, oocyte quality and the resultant embryo and its development (7). Therefore, preconception maternal nutritional status is shown to be an important determinant of embryonic and fetal growth (8). Despite

Received: 24/Jul/2017, Accepted: 3/Feb/2018

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 4, Jan-Mar 2019, Pages: 316-323

these conclusions, the effect of preconception interventions such as nutritional diet on fertility and pregnancy outcomes is unclear (9) and human studies in this field are few.

However, animal studies have shown that deficiencies or excesses in a range of macro- and micronutrients during pre-conception period can lead to impairments in fertility and fetal development and affect the long-term offspring health (10). Improper nutrition is a growing problem worldwide and it is estimated that up to 57.8% of the world's adult population (3.3 billion people) could be either overweight or obese by 2030 (11).

There are many studies that examine the impact of micro- and macronutrients on reproductive health and pregnancy outcomes (7). But recently, there is a new interest in how the overall diet can affect reproductive outcomes especially in infertile women. This approach is more realistic because it reflects the way in which food is consumed and takes into account complicated interactions between nutrients in the diet (12). Because of the association between nutrient intakes and dietary patterns, the effect of a single nutrient may be confounded by the effect of dietary patterns. Also the effect of multiple nutrients rather than a single nutrient could be big enough to detect (13). Human studies on whether or not dietary patterns can affect *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) outcomes are few and the relation between these two parameters is not clear. So there is a potential need for more research on the relation between dietary patterns and infertility treatment outcomes.

The aim of the present study was to assess the relation between dietary patterns and reproductive outcomes in women with primary infertility seeking ART.

Materials and Methods

The present study was a prospective cohort study that was performed at Isfahan Fertility and Infertility center, Isfahan, Iran. A simple sampling design was used. The following formula was used for calculating the sample size:

$$n = \frac{(Z_1 + Z_2)^2 \cdot 2P(1-P)}{d^2}$$

$$Z_1 = 1.96$$

$$Z_2 = 0.84$$

P=40% (an estimate of pregnancy rate in fresh nondonor IVF cycles in each tertile (14))

$$d = 0.6P$$

The sample size for each tertile was estimated to be 65 women plus a 10% sample dropping. So 217 participants were included in the study.

Between August 2015 and January 2016, 217 women with primary infertility aged between 20-45 years who were undergoing suppression protocol for IVF/ICSI,

were invited to participate in this study. The inclusion criteria were having female infertility (idiopathic or ovarian infertility), not having significant changes in diet during the last 3 months or having a specific diet, not consuming alcohol and not smoking, not having a history of diseases affecting metabolism including diabetes, galactosemia, maple syrup urine disease (MSUD), phenylketonuria (PKU), inflammatory bowel disease, celiac disease, chronic pancreatitis, nephrotic syndrome, acute and chronic kidney failure, dialysis, hypothyroidism and hyperthyroidism (15, 16) and not using drugs that affect the metabolism of macronutrients including antihyperglycemic and lipid-altering agents (metformin hydrochloride consumption considered as confounding variable). Exclusion criteria were deciding to hire a surrogate mother, having male factor infertility, and discontinuing cooperation in the study or ART process.

All participants filled out the 168-item food frequency questionnaire (FFQ) and the short form of international physical activity questionnaire (IPAQ). Also their demographic characteristics were recorded. Body mass index (BMI) was calculated as weight in kg divided by the square of height in meters (kg/m²). The waist circumference was measured with a non-stretch tape to the nearest 0.1 cm between the lowest rib margin and the iliac crest with minimal clothing (17).

In the laboratory, the researcher recorded the total number of retrieved oocytes and the number of Metaphase II oocytes. Then IVF/ICSI procedure was conducted based on the standard protocols using G-V series media form VitroLife (18). Fertilization rate was defined as the ratio of zygotes with two pronuclei observed 16-18 hours after insemination divided by the number of inseminated oocytes (19). Embryos were scored using a four-point scale on day 3. One point was assigned to all cleaved embryos, and an additional point was added for each of the following features: the absence of fragmentation (or fragmentation involving 25% of embryonic surface), the absence of irregularities in blastomere size or shape, and 8-cell stage on day 3 (20). Therefore, embryo score ranged from 2 to 4. Embryos with a score of 2 were considered as a bad quality embryo, and a score of 4 indicated good quality embryo. Their rates were calculated by dividing the number of the bad or good embryos by the total number of embryos. Biochemical pregnancy was confirmed by β HCG test, 12 days after embryo transfer (21). Clinical pregnancy was defined as the presence of one or more gestational sacs during transvaginal scan 3 weeks after embryo transfer (22).

Questionnaires

The 168-item FFQ was validated in a previous study (23) for estimating dietary intakes and their frequency of consumption for each food during the last year. The amount of each food item was converted into gram using household scale guide and calculated for one day. Food items were categorized into food groups according to the

similarity of their nutrient composition and logged into SPSS software. IPAQ was used for assessing daily physical activity as a confounder. The physical activity was computed by weighting each type of activity by its energy requirements defined in MET-Min/Day. Then it was converted to MET-h/week and reported. This questionnaire has been validated in previous studies (24).

Ovulation induction

In this study, suppression protocol was used. On the second day of the last menstrual period, when no ovarian cyst was observed in a transvaginal ultrasound scan, ovarian stimulation was commenced with Gonal-F (Serono, Switzerland) in combination with Menogon (Ferring, Germany). Serial ultrasound scans were carried out and when the dominant follicle reached the size of 13-14 mm, gonadotropin-releasing hormone (GnRH) antagonist was administered daily. Ovulation was triggered with 10,000 IU hCG, when the size of dominant follicles reached 17-18 mm. After 36 hours, transvaginal oocyte retrieval was carried out.

Ethical considerations

All participants were informed of the details of the study and were allowed to leave the study at any time. Informed consent was obtained from all the women who agreed to participate in the study. The study was approved by the Ethics Committee of the Isfahan University of Medical Sciences.

Statistical analysis

To identify major dietary patterns, factor analysis was used with oblique transformation. Factors were retained using the Scree test, if their eigenvalues were >1.5 . Three factors were selected as major dietary patterns based on the Scree test and eigenvalues. Dietary patterns were labeled based on the previous knowledge about nutrition and according to the food groups with highest factor loading (25). All the participants were in all the dietary patterns and, based on their personal score, were divided into tertiles; meaning that, those who had the lowest and highest intake of foods in each dietary pattern were placed respectively in the first and third tertiles. Significant differences in general characteristics across tertiles were assessed by ANOVA for continuous variables. Chi-square test was used for assessing the distribution of categorical variables across tertiles. ANOVA and ANCOVA analysis were used to determine the association of dietary patterns with fertility markers including the average number of total oocytes, the average number of metaphase II oocyte and the ratio of good and bad quality embryo. All models were adjusted for age, marriage age, BMI, waist circumference, supplement consumption, metformin consumption and physical activity. Logistic regression analysis was used (with covariates as above) to calculate adjusted odds ratios (OR) and 95% confidence interval (CI) for assessing the association of dietary patterns with biochemical and clinical pregnancy.

To perform all the statistical analyses, SPSS software version 20.0 (Armonk, NY: IBM Corp) was used. $P < 0.05$ was considered significant.

Results

A total of 217 infertile women participated in this study. Because of discontinuing the treatment due to ovarian hyperstimulation syndrome, unwillingness to continue the study, and diagnosing male factor infertility in the next steps of research, 77 cases were excluded from the study.

Three major dietary patterns were identified among 140 women. The first dietary pattern was named the "healthy diet" and included high consumption of fruits, nuts, vegetables, red and white meat, dairy, green olive, cream, and legume. The second dietary pattern was labeled as the "western diet" and was comprised of high consumption of sweet drinks, sweets, caffeinated drinks, potatoes, fast foods, whole grains, refined grains, liquid oils, and salt. The third dietary pattern was called the "unhealthy diet" and contained high consumption of mayonnaise, butter, egg, junk foods and solid oils (Table 1). Demographic characteristics of the participants according to the tertiles of dietary patterns are presented in Table 2. The results showed that women with high adherence to the "healthy diet" had a higher educational level and were employed.

Table 1: Factor loading for food groups of the three dietary patterns identified from food frequency questionnaire (FFQ) in 140 infertile women

Food groups	Healthy	Western	Unhealthy
	dietary pattern		
Fruits	0.750	-	-
Nuts	0.672	-	-
Vegetables	0.597	-	-
Meat	0.535	-	-
Dairy	0.418	-	-
Green olive	0.443	-	-
Cream	0.272	-	-
Legume	0.142	-	-
Sweet drinks	-	0.782	-
Sweets	-	0.519	-
Caffeinated drinks	-	0.480	-
Potato	-	0.416	-
Fast foods	-	0.344	-
Whole grain	-	-0.334	-
Refined grain	-	0.303	-
Liquid oil	-	0.298	-
Salt	-	0.237	-
Mayonnaise sauce	-	-	0.777
Butter	-	-	0.738
Egg	-	-	0.509
Junk foods	-	-	0.320
Solid oil	-	-	0.279
Variance explained (%)	12.679	8.892	7.133

Table 2: Demographic characteristics by tertiles of the dietary patterns

Characteristic	n=140	Tertile of healthy pattern			Tertile of western pattern			Tertile of unhealthy pattern			P value		
		T ₁ , n=46	T ₂ , n=47	T ₃ , n=47	T ₁ , n=46	T ₂ , n=47	T ₃ , n=47	T ₁ , n=46	T ₂ , n=47	T ₃ , n=47			
Age (Y), mean ± SD	32.4 ± 5.2	32.4 ± 5.6	33.2 ± 5.2	31.6 ± 4.7	0.294	31.3 ± 5	33.6 ± 5	32.3 ± 5.4	0.106	32.7 ± 4.7	33 ± 5.3	31.5 ± 5.6	0.324
BMI (kg/m ²), mean ± SD	28.1 ± 4.9	28.6 ± 5.5	28.3 ± 4.4	27.2 ± 4.6	0.338	28.2 ± 5.2	28.1 ± 4.5	27.9 ± 4.9	0.969	28.2 ± 5	28.2 ± 4	27.7 ± 5.6	0.875
Waist circumference (cm), mean ± SD	83.4 ± 10.4	84.3 ± 11.7	83.6 ± 9.2	82.3 ± 10.1	0.642	84 ± 10	83.5 ± 10	82.7 ± 11.3	0.842	84.1 ± 10.1	82.5 ± 7.9	83.6 ± 12.8	0.747
Education, count (%)					0.003				0.357				0.864
Under diploma	37 (26.4)	21 (45.7)	11 (23.4)	5 (10.6)	-	14 (30.4)	9 (19.1)	14 (29.8)	-	10 (21.7)	15 (31.9)	12 (25.5)	-
Diploma	45 (32.1)	13 (28.3)	16 (34)	16 (34)	-	11 (23.9)	20 (42.6)	14 (29.8)	-	16 (34.8)	14 (29.8)	15 (31.9)	-
Academic	58 (41.4)	12 (26.1)	20 (42.6)	26 (55.3)	-	21 (45.7)	18 (38.3)	19 (40.4)	-	20 (43.5)	18 (38.3)	20 (42.6)	-
Employment status, count (%)					0.033				0.207				0.423
Housewife	110 (78.6)	42 (91.3)	35 (74.5)	33 (70.2)	-	39 (84.8)	38 (80.9)	33 (70.2)	-	37 (80.4)	34 (72.3)	39 (83)	-
Employed	30 (21.4)	4 (8.7)	12 (25.5)	14 (29.8)	-	7 (15.2)	9 (19.1)	14 (29.8)	-	9 (19.6)	13 (27.7)	8 (17)	-
The cause of infertility, count (%)					0.523				0.111				0.482
Ovarian	105 (75)	33 (71.7)	38 (80.9)	34 (72.3)	-	39 (84.8)	35 (74.5)	31 (66)	-	34 (73.9)	33 (70.2)	38 (80.9)	-
Idiopathic	35 (25)	13 (28.3)	9 (19.1)	13 (27.7)	-	7 (15.2)	12 (25.5)	16 (34)	-	12 (26.1)	14 (29.8)	9 (19.1)	-

*; P value from One-way analysis of variance for continuous quantitative variables and from Chi-square test for categorical variables.

Table 3: Comparison of the assisted reproductive technology outcomes by tertiles of the dietary patterns in infertile women

Variables	Healthy pattern			Western pattern			Unhealthy pattern			P value		
	T ₁	T ₂	T ₃	T ₁	T ₂	T ₃	T ₁	T ₂	T ₃			
The average number of total oocyte												
Model ₁ ^a (mean ± SD)	7.1 ± 6.1	10.6 ± 8.6	12.2 ± 9	0.009	12 ± 9.3	8.7 ± 6.7	9.2 ± 8.3	0.119	9.8 ± 7.9	9.8 ± 8.8	10.4 ± 8.1	0.929
Model ₂ ^b (mean ± SE)	7.4 ± 1.2	11.1 ± 1.2	11.4 ± 1.3	0.053	12.1 ± 1.2	9.5 ± 1.1	8.4 ± 1.2	0.088	9.8 ± 1.2	10.5 ± 1.2	9.6 ± 1.2	0.837
The average number of metaphase II oocyte												
Model ₁ (mean ± SD)	6.1 ± 5.3	8.6 ± 6.8	10.6 ± 7.9	0.006	10 ± 7.4	7.5 ± 6.3	7.9 ± 7.1	0.184	8.6 ± 7	8.2 ± 7.6	8.6 ± 6.4	0.956
Model ₂ (mean ± SE)	6.2 ± 1	8.9 ± 1	10.2 ± 1.1	0.034	9.9 ± 1	8.1 ± 1	7.4 ± 1	0.225	8.6 ± 1	8.8 ± 1	7.9 ± 1	0.824
The fertilization rate												
Model ₁ (mean ± SD)	0.61 ± 0.4	0.7 ± 0.3	0.7 ± 0.2	0.168	0.7 ± 0.3	0.7 ± 0.3	0.7 ± 0.3	0.776	0.7 ± 0.3	0.7 ± 0.3	0.6 ± 0.3	0.649
Model ₂ (mean ± SE)	0.6 ± 0.05	0.7 ± 0.05	0.7 ± 0.05	0.310	0.7 ± 0.05	0.7 ± 0.05	0.7 ± 0.05	0.545	0.7 ± 0.05	0.7 ± 0.05	0.6 ± 0.05	0.536
The ratio of good quality embryo												
Model ₁ (mean ± SD)	0.2 ± 0.3	0.2 ± 0.2	0.2 ± 0.3	0.705	0.2 ± 0.3	0.2 ± 0.3	0.2 ± 0.3	0.870	0.2 ± 0.3	0.2 ± 0.3	0.2 ± 0.3	0.433
Model ₂ (mean ± SE)	0.2 ± 0.05	0.2 ± 0.04	0.2 ± 0.05	0.874	0.2 ± 0.04	0.2 ± 0.04	0.2 ± 0.04	0.656	0.2 ± 0.04	0.2 ± 0.04	0.2 ± 0.04	0.352
The ratio of bad quality embryo												
Model ₁ (mean ± SD)	0.2 ± 0.3	0.4 ± 0.3	0.3 ± 0.3	0.159	0.3 ± 0.3	0.3 ± 0.3	0.3 ± 0.3	0.559	0.3 ± 0.3	0.3 ± 0.3	0.3 ± 0.3	0.733
Model ₂ (mean ± SE)	0.3 ± 0.05	0.4 ± 0.05	0.3 ± 0.05	0.425	0.3 ± 0.05	0.3 ± 0.05	0.4 ± 0.05	0.653	0.3 ± 0.05	0.3 ± 0.05	0.3 ± 0.05	0.923
The biochemical pregnancy [OR (IC)] ^d												
Model ₁ (mean ± SD)	1	1.5 (0.4-5.3)	1.1 (0.3-4.2)	0.816	1	1.4 (0.3-5.5)	1.4 (0.4-5.1)	0.858	1	0.14 (0.3-0.7)	0.72 (0.2-2.6)	0.036
Model ₂ (mean ± SE)	1	1.3 (0.3-5.8)	1.3 (0.3-6.7)	0.914	1	1.8 (0.3-9.4)	1.1 (0.2-5.9)	0.749	1	0.09 (0.01-0.6)	0.9 (0.1-5.9)	0.022
The clinical pregnancy[OR (IC)]												
Model ₁ (mean ± SD)	1	1.5(0.4-5.3)	1.1(0.3-4.2)	0.816	1	1.4(0.3-5.5)	1.4(0.4-5.1)	0.858	1	0.14 (0.3-0.7)	0.72(0.2-2.6)	0.036
Model ₂ (mean ± SE)	1	1.3(0.3-5.8)	1.3(0.3-6.7)	0.914	1	1.8(0.3-9.4)	1.1(0.2-5.9)	0.749	1	0.09 (0.01-0.6)	0.9(0.1-5.9)	0.022

Model₁^a; Crude, Model₁^b; Adjusted for age, marriage age, BMI, waist circumference, physical activity, total energy intake, supplement consumption, duration of metformin consumption, ^c; P trends from ANOVA analysis for model₁, and from ANCOVA analysis for model₂ in quantitative variables and p trends from logistic regression analysis for qualitative variables, and ^d; OR (CI): Odds ratio and 95% interval confidence calculated by logistic regression analysis.

There was a significant increase in the average number of total oocytes (P-trend=0.009) and metaphase II oocytes (P-trend=0.006) in the third tertile of “healthy diet” compared to the first tertile. After adjusting for confounding variables, these relations remained significant for the average number of total oocytes (P-trend=0.053) and metaphase II oocytes (P-trend=0.034). But the “western diet” and the “unhealthy diet” were not associated with the average number of total and metaphase II oocytes. Women with high adherence to the second tertile of “unhealthy diet” compared to the first tertile, had a significantly lower chance of getting pregnant (chemical and clinical pregnancy) (model₁: OR: 0.14, 95% CI: 0.3-0.7, model₂: OR: 0.09, 95% CI: 0.01-0.6). None of these dietary patterns were associated with the fertilization rate and the ratio of good and bad quality embryo (Table 3).

Discussion

The present study assessed the relation between dietary patterns and outcome of ART in infertile women undergoing IVF/ICSI procedure. The first dietary pattern included high consumption of fruits, nuts, vegetables, red and white meat, dairy, green olive, cream, and legume. Intake of fruits, nuts, and vegetables was outstanding. So this dietary pattern was called as the “healthy diet” (26). The second dietary pattern comprised of high consumption of sweet drinks, sweets, caffeinated drinks, potatoes, oil, fast food, refined grains, whole grains and salt. The highest factor loadings belonged to sweet drinks, sweets, potato, refined grains, and salt, so it was labeled as the “western diet” (27). The third dietary pattern was rich in unhealthy food groups such as mayonnaise sauce, butter, junk foods and solid oils and was named the “unhealthy diet”.

Based on the results, women with the highest “healthy diet” score had a higher educational level and were employed. So higher education and being employed were predictors of a healthy diet. This relation has been seen in previous studies too (28).

Present findings showed that dietary patterns were related to some ART outcomes. The average number of total and metaphase II oocytes was increased significantly in women with high adherence to the “healthy diet”. These relations were significant even after taking confounding variables into account.

A randomized clinical trial showed that having a healthy diet rich in fruits and vegetable, like dietary approach for stopping hypertension (DASH), can have a positive effect on total antioxidant capacity (TAC). In this study, 60 women with polycystic ovary syndrome (PCOS) were equally randomized into DASH diet (being rich in fruits, vegetables, whole grains and low-fat dairy products, as well as low in saturated fats, cholesterol, refined grains and sweets) and control groups. After a 12-week follow-up, the DASH diet group had an increase in TAC (29). In another study it was shown that the DASH diet is associated with an increase in plasma TAC in obese patients with PCOS (30).

In this study, the “healthy diet” was richer in vegetable, fruits, and nuts. These food groups contained a high level of antioxidants (31). Antioxidants in fruits and vegetables may significantly contribute to an antioxidant capacity increase in plasma (32). The TAC levels may have a positive effect on the percentage of metaphase II stage oocyte and oocyte quality (33).

No relation was observed between fertilization rate and all three dietary patterns. Despite the fact that male factor infertility had been excluded in this study, environmental factors such as nutrition and job might affect the sperm quality and fertilization.

In the present study, the chance of getting pregnant was decreased significantly in women who had more adherence to the “unhealthy diet”. This diet contained foods high in saturated fatty acids like mayonnaise sauce, butter, and solid oils. A previous study reported that dietary fatty acid content reflects in the fatty acid profile of follicular fluid (34). Beneficial effects have been considered for unsaturated fatty acids and deleterious effects for saturated fatty acids in elevated concentrations (35). Haggarty et al. (36) showed that human embryos which were developed beyond the four-cell stage had a higher ratio of unsaturated compared to saturated fatty acids. Also Luzzo et al. (37) showed that in mice with a high-fat diet the percentage of embryos developing to a blastocyst stage was significantly lower than the control group (~50% vs. 65%). So there was a lower chance to go on to implant and establish pregnancy similar to the control groups.

No relation was found between the healthy and western diets and the chance for getting pregnant.

A prospective cohort study in the Netherlands on 161 subfertile couples undergoing IVF/ICSI treatment showed that the Mediterranean diet, as a healthy diet, has a 40% increased probability of achieving pregnancy after IVF/ICSI treatments (38). Also findings of a case-control study conducted on 485 women with difficulty getting pregnant and 1,669 age-matched controls who had at least one child showed that there was a lower risk of difficulty getting pregnant in the highest quartile of adherence to the Mediterranean-type pattern compared to the lowest quartile. But having western dietary pattern did not have a statistically significant relation with consulting a physician because of difficulty in getting pregnant (39).

Some limitations in the present study might cause these null findings. Although male factor infertility was excluded, environmental factors like nutrition were uncontrollable and could affect fertilization and establishment of pregnancy. Also we could not distinguish chromosomally abnormal embryos from normal. These abnormal embryos might fail to implant and be less affected by the diet (40). So chromosomal abnormalities in blastocysts could nullify the results. Finally, despite controlling various confounders in the present analysis, residual confounders, which were unknown, could not be excluded.

One strength of the present study was its prospective

cohort design so multiple assisted reproductive outcomes could have been evaluated. Also, the current study is the first follow-up study that was performed in countries undergoing nutrition transition. For assessing dietary intakes, a validated FFQ which reflects Iranian food consumption was used and filled out by an experienced interviewer. Also, many confounder variables such as age, marriage age, BMI, waist circumference, physical activity, total energy intake, supplement consumption and duration of metformin consumption were adjusted.

Since the number of questions was too much, it is probable that some of the participants had not answered the food frequency questionnaire accurately and this was one of the limitations of the present study. Although a validated FFQ was used and filled out by an experienced interviewer, measurement bias was unavoidable and might underestimate or overestimate dietary intakes.

Results of the present study suggested that the average number of total and metaphase II oocytes were significantly higher in women with high adherence to “healthy diet” than the others, with low adherence. Also having “unhealthy diet” decreased the chance of getting pregnant after IVF or ICSI procedure.

Conclusion

The present study showed that having a “healthy diet” had a positive effect and could cause an increase in the average number of total and metaphase II oocytes and an “unhealthy diet” could decrease the chance of getting pregnant. The fertilization rate and the ratio of good and bad quality embryo were not affected by any of the dietary patterns. The results showed that nutrition status could affect infertility treatment outcomes. So nutritional interventions before attempting the infertility treatments could improve outcomes, reduce costs, and increase the mental and fertility health in couples. Considering the small number of conducted studies, it is suggested to perform more investigations on this issue to elucidate this relation and evaluate nutritional effects on reproductive health especially in infertile women.

Acknowledgements

The researchers express their gratitude to the Vice-Chancellor for Research of Isfahan University of Medical Sciences and Isfahan Fertility and Infertility Center. We would like to thank them for their cooperation as well as all the participants who made this study possible. This study was funded and supported by Isfahan University of Medical Sciences, Isfahan, Iran (Grant No.: 394475, Date: 8/25/2015). There is no conflict of interest in this study.

Authors' Contributions

M.J.; Participated in study design, collection, evaluation, and interpretation of data, statistical analysis, drafting the manuscript. M.T.; Contributed to study design, evaluation and interpretation of data, revising and editing

the manuscript. M.H.N.-H.; Conducted all experimental work, evaluation of data, revising and editing the manuscript. Gh.A.; Contributed to study design, evaluation and interpretation of data, and statistical analysis. All authors read and approved the final manuscript.

References

- Hershberger PE, Stevenson EL. Advancing the care of individuals and couples at risk for and diagnosed with infertility. *J Obstet Gynecol neonatal Nurs.* 2016; 45(1): 98-99.
- Rizk B, Olsen ME. *Challenging office cases in obstetrics and gynecology.* Johnson City: Jaypee Brothers Medical Publishers Ltd; 2016; 65.
- Ombelet W, Cooke I, Dyer S, Serour G, Devroey P. Infertility and the provision of infertility medical services in developing countries. *Hum Reprod Update.* 2008; 14(6): 605-621.
- Dirkavand-Moghaddam A, Delpisheh A, Sayehmiri K. the prevalence of infertility in iran, a systematic review. *Iran J Obstet Gynecol Infertil.* 2014; 16(81): 1-7.
- Centers for Disease Control and Prevention, American Society for Reproductive Medicine, Society for Assisted Reproductive Technology. 2014 Assisted Reproductive Technology: National Summary Report. 2016.
- Meldrum DR. Introduction: nongenetic markers of oocyte and embryo competence. *Fertil Steril.* 2015; 103(2): 301-302.
- Gu L, Liu H, Gu X, Boots C, Moley KH, Wang Q. Metabolic control of oocyte development: linking maternal nutrition and reproductive outcomes. *Cell Mol Life Sci.* 2014; 72(2): 251-271.
- van Driel LMJW, Zwolle LJH, de Vries JHM, Boxmeer JC, Lindemans J, Steegers EAP, et al. The preconception nutritional status of women undergoing fertility treatment: use of a one-year post-delivery assessment. e-SPEN, the European e-Journal of Clinical Nutrition and Metabolism. 2010; 5(6): e284-e291.
- Temel S, van Voorst SF, Jack BW, Denктаş S, Steegers EA. Evidence-based preconceptional lifestyle interventions. *Epidemiol Rev.* 2014; 36: 19-30.
- Sinclair KD, Watkins AJ. Parental diet, pregnancy outcomes and offspring health: Metabolic determinants in developing oocytes and embryos. *Reprod Fertil Dev.* 2014; 26(1): 99-114.
- Kelly T, Yang W, Chen C-S, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. *Int J Obes (Lond).* 2008; 32(9): 1431-1437.
- Cucó G, Fernández-Ballart J, Sala J, Viladrich C, Iranzo R, Vila J, et al. Dietary patterns and associated lifestyles in preconception, pregnancy and postpartum. *Eur J Clin Nutr.* 2006; 60(3): 364-371.
- Hu FB. Dietary pattern analysis: a new direction in nutritional epidemiology. *Curr Opin Lipidol.* 2002; 13(1): 3-9.
- Dumesic DA, Meldrum DR, Katz-Jaffe MG, Krisher RL, Schoolcraft WB. Oocyte environment : follicular fluid and cumulus cells are critical for oocyte health. *Fertil Steril.* 2015; 103(2): 303-316.
- Barazzoni R, Deutz NEP, Biolo G, Bischoff S, Boirie Y, Cederholm T, et al. Carbohydrates and insulin resistance in clinical nutrition: recommendations from the ESPEN expert group. *Clin Nutr.* 2016; 36(2): 355-363.
- Mescka CP, Wayhs CA, Vanzin CS, Biancini GB, Guerreiro G, Manfredini V, et al. Protein and lipid damage in maple syrup urine disease patients: L-carnitine effect. *Int J Dev Neurosci.* 2013; 31(1): 21-24.
- Taebe M, Sadat Z, Saberi F, Kalahroudi MA. Early pregnancy waist-to-hip ratio and risk of preeclampsia: a prospective cohort study. *Hypertens Res.* 2015; 38(1): 80-83.
- Simon L, Castillo J, Oliva R, Lewis SE. Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes. *Reprod Biomed Online.* 2011; 23(6): 724-734.
- Opøien HK, Fedorcsak P, Omland AK, Abyholm T, Bjercke S, Ertzeid G, et al. In vitro fertilization is a successful treatment in endometriosis-associated infertility. *Fertil Steril.* 2012; 97(4): 912-918.
- Nasr-Esfahani MH, Tavalae M, Deemeh MR, Arbabian M, Par-rington J. Can assessment of total acrosin activity help predict failed or low fertilization rate ICSI for implementation of artificial oocyte activation? *Open Androl J.* 2010; 2(1): 19-26.
- Van Vaerenbergh I, Fatemi HM, Blockeel C, Van Lommel L, In'T Veld P, Schuit F, et al. Progesterone rise on HCG day in GnRH antagonist/rFSH stimulated cycles affects endometrial gene expression. *Reprod Biomed Online.* 2011; 22(3): 263-271.

22. Cobo A, Meseguer M, Remohí J, Pellicer A. Use of cryo-banked oocytes in an ovum donation programme: A prospective, randomized, controlled, clinical trial. *Hum Reprod.* 2010; 25(9): 2239-2246.
23. Asghari G, Rezazadeh A, Hosseini-Esfahani F, Mehrabi Y, Mirmiran P, Azizi F. Reliability, comparative validity and stability of dietary patterns derived from an FFQ in the Tehran lipid and glucose study. *Br J Nutr.* 2012; 108(6): 1109-1117.
24. Baghiani Moghaddam MH, Bakhtari Aghdam F, Asghari Jafarabadi M, Allahverdipour H, Dabagh Nikookheslat S, Safarpour S. The Iranian version of International Physical Activity Questionnaire (IPAQ) in Iran: Content and construct validity, factor structure, internal consistency and stability. *World Applied Sciences Journal.* 2012; 18(8): 1073-1080.
25. Ocké MC. Evaluation of methodologies for assessing the overall diet: dietary quality scores and dietary pattern analysis. *Proc Nutr Soc.* 2013; 72(2): 191-199.
26. Newby PK, Tucker KL. Empirically derived eating patterns using factor or cluster analysis: a review. *Nutr Rev.* 2004; 62(5): 177-203.
27. Halton TL, Willett WC, Liu S, Manson JE, Stampfer MJ, Hu FB. Potato and french fry consumption and risk of type 2 diabetes in women. *Am J Clin Nutr.* 2006; 83(2): 284-290.
28. Correa Leite ML, Nicolosi A, Cristina S, Hauser WA, Pugliese P, Nappi G. Dietary and nutritional patterns in an elderly rural population in Northern and Southern Italy: (I). A cluster analysis of food consumption. *Eur J Clin Nutr.* 2003; 57(12): 1514-1521.
29. Azadi-Yazdi M, Karimi-Zarchi M, Salehi-Abargouei A, Fallahzadeh H, Nadjarzadeh A. Effects of Dietary Approach to Stop Hypertension diet on androgens, antioxidant status and body composition in overweight and obese women with polycystic ovary syndrome: a randomised controlled trial. *J Hum Nutr Diet.* 2017; 30(3): 275-283.
30. Asemi Z, Samimi M, Tabassi Z, Shakeri H, Sabihi SS, Esmailzadeh A. Effects of DASH diet on lipid profiles and biomarkers of oxidative stress in overweight and obese women with polycystic ovary syndrome: a randomized clinical trial. *Nutrition.* 2014; 30(11-12): 1287-1293.
31. Alasalvar C, Bolling BW. Review of nut phytochemicals, fat-soluble bioactives, antioxidant components and health effects. *Br J Nutr.* 2015; 113 Suppl 2: S68-S78.
32. Harasym J, Oledzki R. Effect of fruit and vegetable antioxidants on total antioxidant capacity of blood plasma. *Nutrition.* 2014; 30(5): 511-517.
33. Kazemi A, Ramezan-zadeh F, Nasr-Esfahani MH, Saboor-Yaraghi AA, Ahmadi M. Does dietary fat intake influence oocyte competence and embryo quality by inducing oxidative stress in follicular fluid? *Iran J Reprod Med.* 2013; 11(12): 1005-1012.
34. Wonnacott KE, Kwong WY, Hughes J, Salter AM, Lea RG, Garnsworthy PC, et al. Dietary omega-3 and -6 polyunsaturated fatty acids affect the composition and development of sheep granulosa cells, oocytes and embryos. *Reproduction.* 2010; 139(1): 57-69.
35. Leroy JL, Sturmey RG, Van Hoeck V, De Bie J, McKeegan PJ, Bols PE. Dietary fat supplementation and the consequences for oocyte and embryo quality: Hype or significant benefit for dairy cow reproduction? *Reprod Domest Anim.* 2014; 49(3): 353-361.
36. Haggarty P, Wood M, Ferguson E, Hoad G, Srikantharajah A, Milne E, et al. Fatty acid metabolism in human preimplantation embryos. *Hum Reprod.* 2006; 21(3): 766-773.
37. Luzzo KM, Wang Q, Purcell SH, Chi M, Jimenez PT, Grindler N, et al. High fat diet induced developmental defects in the mouse: oocyte meiotic aneuploidy and fetal growth retardation/brain defects. *PLoS One.* 2012; 7(11): e49217.
38. Vujkovic M, de Vries JH, Lindemans J, Macklon NS, van der Spek PJ, Steegers EA, et al. The preconception Mediterranean dietary pattern in couples undergoing in vitro fertilization/intracytoplasmic sperm injection treatment increases the chance of pregnancy. *Fertil Steril.* 2010; 94(6): 2096-2101.
39. Toledo E, Lopez-del Burgo C, Ruiz-Zambrana A, Donazar M, Navarro-Blasco I, Martínez-González MA, et al. Dietary patterns and difficulty conceiving: a nested case-control study. *Fertil Steril.* 2011; 96(5): 1149-1153.
40. Gaskins AJ, Rich-Edwards JW, Hauser R, Williams PL, Gillman MW, Penzias A, et al. Prepregnancy dietary patterns and risk of pregnancy loss. *Am J Clin Nutr.* 2014; 100(4): 1166-1172.

Satisfaction with Information Provided to Infertile Patients Who Undergo Assisted Reproductive Treatment

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Abstract

Background: Potentially modifiable factors, such as the appropriate informing process given to infertile patients, can affect their infertility knowledge and information. This study aims to assess infertility information provided to Iranians who undergo assisted reproductive treatment.

Materials and Methods: In this cross-sectional study, participants recruited were a convenience sample of all infertile patients who received assisted reproductive treatments from Royan Institute, Tehran, Iran. Inclusion criteria consisted of: patient's first time visit, no previous infertility treatment failures, and referral to the centre between January and March 2015. A 20-item tool designed by researchers measured patient satisfaction with the infertility informing process. This tool included cause of infertility, type of recommended treatment, diagnostic procedures, approximate treatment duration, success rate of the treatment, approximate cost of treatment, and non-therapeutic factors in treatment success.

Results: A total of 235 infertile patients were invited to participate in the study, from which 200 (100 men and 100 women) participants completely responded to the questionnaire with a response rate of approximately 85%. The mean age of participants was 30.93 ± 5.56 years. In terms of satisfaction with information provided about the cause of infertility, male responders reported the lowest mean score of 3.59 ± 1.05 compared to female responders (3.82 ± 0.85 , $P=0.078$). Infertile women had a greater mean score of 3.85 ± 0.78 than infertile men (3.58 ± 1.29) in satisfaction with information provided about the type of recommended treatment ($P=0.037$). There was a statistically significant difference between males (3.26 ± 1.04) and females (3.58 ± 0.93) in satisfaction with approximate treatment duration ($P=0.031$).

Conclusion: According to the results, most infertile patients were satisfied with the informing process related to the cause of infertility and recommended therapies. Information about infertility should be provided more systematically to all treated patients by medical staff, especially in terms of success rate of treatment and financial cost of therapy.

Keywords: Awareness, Health Promotion, Infertility, Information, Satisfaction

Citation: Ezabadi Z, Mollaahmadi F, Sazvar S, Vesali S, Omani-Samani R. Satisfaction with information provided to infertile patients who undergo assisted reproductive treatment. *Int J Fertil Steril.* 2019; 12(4): 324-328 doi:10.22074/ijfs.2019.5314.

Introduction

Infertility is defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (1). Infertility is a biomedical health problem and a reproductive system disease or dysfunction (2). Some potentially modifiable factors, such as provision of appropriate information to infertile patients, can affect infertility knowledge and information seeking behaviour among people who undergo assisted reproductive treatment.

Health-seeking behaviour among couples with infertility is directly related to their understanding of reproductive biology and their beliefs about infertility. Those with better knowledge of fertility health issues may show improved use of health care resources with a consequent reduction in infertility (3, 4).

Infertility awareness is considered a critical first step towards fertility preservation or infertility care by lifestyle modifications or changes (1, 5). Because fertility knowledge is associated with education, recommended health

Received: 30/May/2017, Accepted: 25/Feb/2018

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 4, Jan-Mar 2019, Pages: 324-328

promotion strategies should begin with educational interventions (2). Fertility health promotion represents that knowledge is a key factor associated with fertility self-care and the initiation of treatment. Focusing on education about fertility issues is needed to prevent fear and unnecessary delay in seeking help when faced with problems in conception (5).

Providing relevant information to patients, respecting their wishes, and considering their capacity to make treatment decisions is crucial for high-quality and patient-centred fertility care (2). Satisfaction with care can originate from adequate patient education, which enables patients to give greater understanding of and participation in medical decision-making, and often results in better health outcomes (6). There is an enormous disparity in the literature about the perspective of the infertile clients' satisfaction with infertility information provided by medical staff. Relatively little is known about satisfaction with information provided to infertile patients who receive infertility treatment. This study is the first in Iran to examine satisfaction with information provided to infertile patients who undergo assisted reproduction treatment.

Materials and Methods

This descriptive cross-sectional study was the first phase of a large survey on women and men who undergo infertility treatment in the largest referral fertility clinic in Iran, Royan Institute, where people are examined from all socio-economic and ethnic backgrounds. Participants recruited were a convenience sample of all infertile patients who received first-time assisted reproductive treatments, and who did not have any previous infertility treatment failures. Patients were seen at Royan Institute between January and March, 2015.

In this questionnaire-based study, the researchers developed a tool that was validated on the basis of a literature review. The questionnaire included questions about satisfaction with information about cause of infertility (3 questions); type of recommended treatment (3 questions); diagnostic procedures (3 questions); approximate treatment duration (3 questions); success rate of the treatment (3 questions); approximate financial cost of treatment (3 questions); and non-therapeutic factors in treatment success such as diet, exercise, taking supplements, and cigarette smoking (2 questions) to measure satisfaction with infertile patients' self-perception of the informing process.

Demographic and clinical information of the participants were gathered from their records in the fertility centre. Question types included yes/no, a 5-point Likert scale that ranged from 1 to 5 (dissatisfied, low satisfaction, neither satisfied nor dissatisfied, satisfied, very satisfied), and choice of one option. The questionnaire was also designed for the Iranian context and validated by a group of 18 gynaecologists, embryologists, meth-

odologists, and nurses for content, ease of understanding, and acceptability. Face validity was performed by a Persian literature expert and the wording of the questions was adapted to the context and perspectives of the participants. One interviewer who was aware of the main objective of the present study was responsible for distribution and collection of the questionnaires among participants.

The Ethics Committee of Royan Institute approved this study (EC/92/106). Aims of the study were clearly explained for all participants prior to the investigation. Voluntarily completion of the questionnaire was considered as consent. Eligible individuals were assured that their confidentiality and anonymity, and that their decision to participate in or withdraw from the study would not impact their current or future relationship with the clinic. Participants were also assured that their level of satisfaction did not affect provision of care services.

Statistical analyses were carried out using the Statistical Package for Social Science (SPSS, version 20.0 for Windows; SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean \pm SD (standard deviation) and categorical variables as numbers (percentages). Responses with the 5-point Likert scale (range: 1 to 5) were compared by the independent samples t test because it is robust when one might encounter ordinal scaled data. The statistical issue was demonstrated by Heeren and D'Agostino (7) in 1987 as previously explained. $P < 0.05$ was considered statistically significant.

Results

In this study, 235 infertile patients were invited to participate. In total, 200 participants (100 men and 100 women) responded to the questionnaire completely and returned completed questionnaires, which yielded a response rate of about 85%. The mean age of participants was 30.93 ± 5.56 years. Of participants, 67 (33.5%) patients were diagnosed with male infertility and 31 (15.5%) had female infertility. Recommended therapies to the patients included the following: 36 (18%) *in vitro* fertilization (IVF), 52 (26%) microinjection, and 71 (35.5%) intrauterine injection (IUI). Among diagnostic procedures used for the participants, 95 (47.5%) patients were diagnosed by ultrasound and 114 (57%) by blood and urine tests. Table 1 lists the demographic characteristics of the infertile study participants.

A total mean score of satisfaction with the informing process for each area in Table 2. As shown, the highest mean scores of satisfaction with the informing process to patients were related to cause of infertility (3.71 ± 0.96) and recommended therapies (3.72 ± 0.91). The lowest mean score satisfaction with informing process to the patients was 3.31 ± 1.10 for the approximate financial cost of treatment.

Table 1: Demographic and clinical characteristics of the study participants (n=200)

Demographic and clinical variables	n (%)
Sex	
Male	100 (50)
Female	100 (50)
Cause of infertility	
Male	67 (33.5)
Female	31 (15.5)
Both	54 (27)
Unknown	36 (18)
No answer	12 (6)
Recommended therapies	
<i>In vitro</i> fertilization (IVF)	36 (18)
Micro injection	52 (26)
Intra uterine injection (IUI)	71 (35.5)
Other	5 (2.5)
No answer	22 (11)
Diagnostic procedures	
Hysteroscopy	14 (7)
Ultrasound	95 (47.5)
Blood and urine	114 (57)
Laparoscopy	7 (3.5)
Pap smear	41 (20.5)
Genetic counseling	10 (5)
Hysterosonography	4 (2)
Hystrosalpangiography	39 (19.5)
Sperm motility	73 (36.5)
No answer	30 (15)

In terms of satisfaction with information provided about cause of infertility, male responders reported the lowest mean score (3.59 ± 1.05) compared to female responders (3.82 ± 0.85); there was no statistically significant difference between men and women ($P=0.078$, Fig.1). Infertile women had a statistically greater mean score (3.85 ± 0.78) than infertile men (3.58 ± 1.29) in satisfaction with information provided about type of recommended treat-

ment ($P=0.037$). A statistically significant difference was observed between males (3.26 ± 1.04) and females (3.58 ± 0.93) in satisfaction with approximate treatment duration ($P=0.031$). More than half of the responders obtained their infertility treatment information (causes, therapies, diagnostic procedures, cost, duration, and success rate) from physicians instead of other medical staff ($P<0.001$, Table 3).

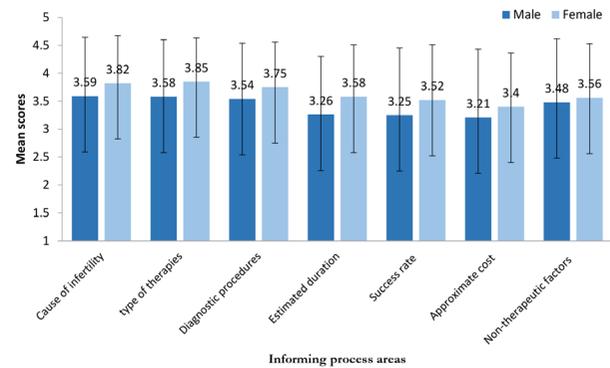


Fig.1: Patients' satisfaction with the informing process areas (n=200 participants).

Table 2: Description of infertile patients' satisfaction with the informing process

Areas	Mean	Standard deviation	Minimum	Maximum
Cause of infertility	3.71	0.96	1	5
Recommended therapies	3.72	0.91	1	5
Diagnostic procedures	3.64	0.91	1	5
Estimated treatment duration	3.42	0.99	1	5
Success rate of the treatment	3.39	1.11	1	5
Approximate cost of treatment	3.31	1.1	1	5
Non-therapeutic factors in treatment success	3.52	1.5	1	5

Table 3: Frequency of information on infertility treatment obtained from medical staff

Areas	Medical staff				
	Physician n (%)	Nurse n (%)	Midwife n (%)	Reception n (%)	Other n (%)
Cause of infertility	127 (67.2)	9 (4.2)	17 (9.0)	13 (5.8)	23 (12.2)
Recommended therapies	117 (65.0)	9 (5.0)	16 (8.9)	11 (5.6)	27 (15.0)
Diagnostic procedures	126 (71.6)	10 (5.7)	6 (3.4)	15 (8.5)	16 (9.1)
Estimated treatment duration	103 (60.6)	17 (10.0)	11 (6.5)	12 (7.1)	24 (14.1)
Treatment Success rate	92 (58.2)	20 (12.7)	18 (11.4)	6 (3.8)	19 (12.0)
Approximate cost of treatment	72 (47.7)	12 (7.9)	6 (4.0)	25 (16.6)	33 (21.9)
Non-therapeutic factors in treatment success	94 (58.8)	12 (7.5)	7 (4.4)	18 (11.3)	27 (16.9)

Discussion

To the best of our knowledge, this was the first national survey of infertile clients that pertained to satisfaction with information provision in infertility care. Determining the extent of the patients' satisfaction with information about infertility and its treatment would be beneficial for planning education programs related to the prevention of failures in infertility treatment or withdrawal. The findings of this study have provided useful insights into potentially modifiable factors that influence infertile patient's co-operation with medical staff in the infertility clinics and compliance with assisted reproductive treatments.

From this study, it was apparent that most infertile patients who participated were more satisfied with the informing process related to the cause of infertility and recommended therapies. In contrast, the vast majority of participants were less satisfied with the information provided for approximate financial cost of their treatment. Overall satisfaction with this infertility care centre was usually high in the survey, but provided no reliable measure for the quality of care (8-10). There were considerable knowledge gaps, particularly in relation to the impact of infertility treatment failure and infertility treatment history in other fertility care clinics.

Of note, those who had a history of infertility treatment were more aware of infertility-related information such as causes, therapeutic procedures, and financial cost. Hence, over-reporting of satisfaction with information provided to the patients was unavoidable. In this study, we attempted to recruit all infertile patients who received assisted reproductive treatments for first time and did not have any previous infertility treatment failures.

Problems exist with the absence of data registration from all Iranian infertility care clinics. However, objective data collection on satisfaction with information provision in infertility care is difficult. This study was in line with most studies that relied on interviews. Possibly, the answers of the respondents were to some extent biased by incorrect recall and self-interest (10, 11). Another limitation of this study was the obvious gender bias towards women in our sample; all women studied acquired higher scores of satisfaction with information provided in all 7 domains compared to the male respondents. It was likely that most infertile women have used the Internet for information on fertility-related problems. Men would be less likely to seek services for infertility than women, and many men from infertile couples do not undergo a male examination (12). Selection bias might occur against people who have a low income or a migration background, which is a common finding in interview studies on assisted reproductive treatment (8, 10).

To the best of our knowledge, we did not find any research that measured satisfaction with infertility treatment information available to infertile people in the literature. Rauprich et al. (10) investigated the views of patients (n=1590) and experts, including physicians (n=230) and psychosocial counsellors (n=66), in Germany on infor-

mation provision and decision-making in assisted reproduction treatment. Most participants had positive views for information on the chances for treatment success and physical risks of fertility treatment than for information on the risks and burden of multiple pregnancies, and on the emotional risks and burden associated with infertility treatment.

In the present study, both men and women participants were more satisfied with information provided about type of recommended treatment. The objective of another study was to assess patients' satisfaction with the investigation and initial management of infertility in 1366 women who attended outpatient clinics at 12 hospitals throughout Scotland. Overall, 87% of respondents were satisfied or very satisfied with their care, but there were a number of deficiencies identified.

A total of 86% felt they had not been given enough assistance with the emotional aspects of infertility, whereas 47% felt they were not given a clear plan for the future and 23% of those who had been given drug treatments reported receiving little or no information about the treatment or possible side-effects (11). In the present study, more than half of responders received their infertility information from physicians instead of other medical staff. A qualitative study with 6 group discussions on fertility knowledge and information-seeking behaviour among people of reproductive age revealed that most women and men who intended to have children in the future agreed that primary health care providers, such as general practitioners (GPs), were well placed to provide information regarding fertility and pregnancy health (13).

Despite the remaining limitations and risks of bias, the present methodical strategies have provided sufficient validity for the principal results of the study. The findings are limited to the particular context of fertility care in Iran, and are not transferable or generalizable elsewhere.

Conclusion

Information about infertility should be provided more systematically to all treated patients by medical staff, especially for success rate of treatment and financial cost of therapy. However, most infertile patient participants were more satisfied with the informing process related to the cause of infertility and recommended therapies. Therefore, the information should be clarified for all infertile patients prior to the onset of any therapeutic procedures.

Acknowledgements

The authors express their appreciation to all of the infertile patients who participated in this study. This project was funded by Royan Institute. There was no conflict of interest among authors.

Authors' Contributions

Z.E., R.O.-S.; Were the project leaders and responsible for the study conception and design. F.M.; Was involved

in acquisition of data. S.S.; Contributed significantly to the analysis. S.V.; Was responsible for interpretation of the data, and drafted and critically revised the manuscript. All of the authors provided their final approval for the completed manuscript.

References

1. Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, et al. The International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary on ART Terminology, 2009. *Hum Reprod.* 2009; 24(11): 2683-2687.
 2. Sabarre KA, Khan Z, Whitten AN, Remes O, Phillips KP. A qualitative study of Ottawa university students' awareness, knowledge and perceptions of infertility, infertility risk factors and assisted reproductive technologies (ART). *Reprod Health.* 2013; 10: 41.
 3. Bunting L, Boivin J. Decision-making about seeking medical advice in an internet sample of women trying to get pregnant. *Hum Reprod.* 2007; 22(6): 1662-1668.
 4. Thomson AA, Brown M, Zhang S, Stern E, Hahn PM, Reid RL. Evaluating acquisition of knowledge about infertility using a whiteboard video. *J Obstet Gynaecol Can.* 2016; 38(7): 646-650.
 5. Tough S, Tofflemire K, Benzie K, Fraser-Lee N, Newburn-Cook C. Factors influencing childbearing decisions and knowledge of perinatal risks among Canadian men and women. *Matern Child Health J.* 2007; 11(2): 189-198.
 6. Bennett LR, Wiweko B, Bell L, Shafira N, Pangestu M, Adayana IB, et al. Reproductive knowledge and patient education needs among Indonesian women infertility patients attending three fertility clinics. *Patient Educ Couns.* 2015; 98(3): 364-369.
 7. Heeren T, D'Agostino R. Robustness of the two independent samples t-test when applied to ordinal scaled data. *Stat Med.* 1987; 6(1): 79-90.
 8. Mourad SM, Hermens RP, Cox-Witbraad T, Groel RP, Nelen WL, Kremer JA. Information provision in fertility care: a call for improvement. *Hum Reprod.* 2009; 24(6): 1420-1426.
 9. van Empel IW, Nelen WL, Tepe ET, van Laarhoven EA, Verhaak CM, Kremer JA. Weaknesses, strengths and needs in fertility care according to patients. *Hum Reprod.* 2010; 25(1): 142-149.
 10. Rauprich O, Berns E, Vollmann J. Information provision and decision-making in assisted reproduction treatment: results from a survey in Germany. *Hum Reprod.* 2011; 26(9): 2382-2391.
 11. Souter VL, Penney G, Hopton JL, Templeton AA. Patient satisfaction with the management of infertility. *Hum Reprod.* 1998; 13(7): 1831-1836.
 12. Anderson JE, Farr SL, Jamieson DJ, Warner L, Macaluso M. Infertility services reported by men in the United States: national survey data. *Fertil Steril.* 2009; 91(6): 2466-2470.
 13. Hammarberg K, Zosel R, Comoy C, Robertson S, Holden C, Deeks M, et al. Fertility-related knowledge and information-seeking behaviour among people of reproductive age: a qualitative study. *Hum Fertil (Camb).* 2017; 20(2): 88-95.
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Correlation of Maternal Stress Because of Positive Aneuploidy Screening Serum Analytes and Uterine Arteries' Doppler Ultrasound Index: A Prospective Cohort Study

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Abstract

Background: Antenatal anxiety or maternal stress is a prevalent chronic mental disorder in pregnant women. We have assessed the effect of maternal stress from positive aneuploidy screening results on the changes in uterine artery blood flow.

Materials and Methods: We performed a prospective cohort (one sample) pilot study at a hospital in Tehran, Iran. A total of 60 pregnant women who were candidates for amniocentesis due to abnormal sequential screening test results entered the study. We conducted 2 standard psychological tests, the Spielberger's State-Trait Anxiety Inventory and the Beck Anxiety Inventory, to determine anxiety levels in the participants before amniocentesis and two weeks after amniocentesis. The uterine artery resistance index was also measured before and two weeks after amniocentesis. The level of maternal stress was compared with the uterine artery resistance index.

Results: Patients had a mean State Trait Anxiety Inventory score before amniocentesis of greater than 40, which meant that the mothers experienced high anxiety. There were no correlations between both inventories' anxiety scores and uterine artery blood flow before amniocentesis. However, two weeks after amniocentesis, we observed significant negative correlations between the State Anxiety ($P=0.0041$) and Trait Anxiety ($P=0.010$) Inventory scores and the uterine artery resistance indexes. Also, there was an association between the decreased right uterine artery resistance index and State Anxiety scores ($P=0.036$). There were significant correlations between State and Trait Anxiety scores and second trimester analytes of β -human chorionic gonadotropin (β -hCG, $P<0.001$), α -fetoprotein ($P<0.001$), and unconjugated estriol ($P=0.048$).

Conclusion: Maternal anxiety because of positive aneuploidy screening serum analytes and amniocentesis can affect perinatal outcomes via mood-based alterations in blood flow of the uterine arteries and the screening markers β -hCG, unconjugated estriol, and α -fetoprotein.

Keywords: Amniocentesis, Doppler, Prenatal Screening, Stress Disorders, Uterine Artery

Citation: Shirazi M, Pooransari P, Rahimi Sharbaf F, Niromanesh Sh, Sahebdel B, Shariat M, Pahlavan Z, Shirazi M, Ahmadian M. Correlation of maternal stress because of positive aneuploidy screening serum analytes and uterine arteries' doppler ultrasound index: a prospective cohort study. *Int J Fertil Steril.* 2019; 12(4): 329-334. doi: 10.22074/ijfs.2019.5359.

Introduction

Antenatal anxiety or maternal stress is a frequent complication in pregnant women (1). The prevalence of depression during pregnancy is 7-18% and anxiety disorders during pregnancy is 8.5% (2, 3). Mothers face tremendous anxiety because of pregnancy complications, labour pain, prenatal invasive procedures, the childbirth process, and concerns about the health and future of their infants (4).

Antenatal psychological disorders affect neonatal outcome and may increase the risk of spontaneous preterm de-

livery, low birth weight, caesarean section and instrumental delivery, as well as neonatal intensive care unit (NICU) admission (5, 6). Blood flow changes in the maternal foetal blood circulation, sympathetic arousal, and hypothalamic-pituitary-adrenal axis activation are possible mechanisms behind adverse pregnancy outcomes (7, 8). Although determining the relationship between anxiety status and changes in maternal-foetal blood circulation is not easy (9), Doppler ultrasound measurements have demonstrated a correlation between uterine-umbilical blood circulation and maternal psychological disorders (10).

Received: 23/Jul/2017, Accepted: 8/Apr/2018

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 4, Jan-Mar 2019, Pages: 329-334

The sequential screening test is an aneuploidy screening test that measures β -human chorionic gonadotropin (β -hCG), pregnancy associated plasma protein A, α -fetoprotein, unconjugated estriol, and inhibin A (11-13). And is performed during the first trimester (10 weeks and 3 days until 13 weeks and 6 days) and second trimester (15-18 weeks). Several investigations have found a correlation between maternal anxiety and changes in these serum analytes (14-16). A few studies suggested an association between uterine blood circulation and maternal serum markers; pregnant women with increased β -hCG had abnormal Doppler ultrasound findings in uterine arteries from the 22nd to 24th pregnancy weeks (17).

Amniocentesis is an invasive diagnostic technique that rules out foetal aneuploidy. Studies have revealed that pregnant women experience high levels of anxiety as a result of positive aneuploidy screening tests, the amniocentesis procedure, uncertainty, tension, and foetal injury (18-20). A study has demonstrated high levels of long-lasting anxiety related to prenatal diagnostic procedures that include positive biochemical screening or ultrasound chromosomal abnormality markers. Some measures are suggested to decrease anxiety, and include promotion of the transparency of delivered information, as well as psychological and emotional interventions for both the medical team and high risk pregnant women (21).

This study investigated the correlation between anxiety scores related to positive aneuploidy screening serum analytes and uterine artery resistance index. To the best of our knowledge, few studies have focused on this subject. We assessed the correlation between screening serum analytes and psychological anxiety scales as the secondary outcome.

Materials and Methods

This prospective cohort (one sample) pilot study was performed at Moheb Yas Hospital in Tehran in 2015. The study population consisted of 100 pregnant women who were candidates for amniocentesis because of positive sequential screening test results for aneuploidy (as exposure).

The inclusion criteria were: 20-37 years old, gestational age 15-20 weeks, 19-25 kg/m² body mass index, high risk for sequential markers, and maternal mental health.

The exclusion criteria were history of psychological, psychotic, or neurocognitive disorders; smoking or drug abuse; underlying diseases such as hypertension, diabetes, recent experience of stressful events; and positive aneuploidy culture results after amniocentesis.

All participants signed an informed consent and accepted well-timed attending for receiving prenatal care. We used 2 standard psychological tests, the Spielberger's State-Trait Anxiety Inventory (an index of obvious and hidden anxieties) and Beck's Anxiety Inventory before amniocentesis and two weeks later to determine the participants' anxiety level as the outcome.

All questionnaires were standard and their validities

in the Iranian population were identified to be more than 91%. The State-Trait Anxiety Inventory is a 40-item self-report inventory in which scores more than 43 suggest high anxiety and scores that range from 15 to 20 indicate no anxiety. Beck's Anxiety Inventory evaluates the responses to 21 multiple-choice, self-report questions to measure the severity of anxiety; a score of 16 or higher is considered moderate and severe anxiety (22, 23).

A single observer measured the uterine artery resistance index before amniocentesis using a Doppler ultrasound (ACUSON Sequoia 512TM, Siemens Healthcare GmbH, USA) with a convex multi-frequency transducer (3.0 to 5.0 MHz). Patients were placed in the trans-abdominal position. After taking the images, the Doppler measurement was performed in location of the uterine artery that originates from the iliac artery just lateral to the cervix at the endocervix. We took into consideration an insonation angle of less than 30 degrees and a Doppler gate of 2 mm.

The same perinatologist performed another Doppler ultrasound two weeks after amniocentesis, after the culture results were available. The anxiety scores of all participants were recorded. Data related to serum analytes that included β -hCG and pregnancy associated plasma protein A in the first trimester, and α -fetoprotein, β -hCG, unconjugated estriol, and inhibin A in the second trimester were also recorded in the checklists (code and master sheets). Finally, we compared the effect of maternal stress based on high risk aneuploidy with the uterine artery resistance indexes before and two weeks after amniocentesis. The association between serum analytes and participants' psychological status was also evaluated.

Data analysis

The Statistical Package for Social Sciences (SPSS) software version 19 (Chicago, IL, USA) was used for statistical analysis. Questionnaires' anxiety scores and variables related screening marker analytes and uterine artery resistance index were presented as mean standard deviation. In cases where the variables were normally distributed, we applied the paired t test and linear regression analysis with determination of beta coefficient and 95% confidence interval, to analyse the correlation between variables. This study had an 80% power. $P < 0.05$ was considered significant.

This study was an experimental pilot study; therefore, we could not calculate the sample size. A total of 60 subjects entered the study from April to September 2015 based on the inclusion criteria and capability of the study design.

Ethical considerations

The collected data was considered confidential and no extra costs were imposed on the participants. The participants received assurances about their right to withdraw from the study at any time. The Institutional Review Board of Tehran University of Medical Sciences, by taking into consideration the tenets of the Declaration of Helsinki, approved this study.

Results

From among 100 pregnant women who met the inclusion criteria, 60 women entered the study and 40 women were excluded because of lack of cooperation or positive aneuploidy culture results. No participant required any medical treatment and all just received routine prenatal care. Participant had a mean age of 32 ± 3.52 years and their mean gestational age at the time of amniocentesis was 16 ± 0.56 weeks.

Pearson correlation test of the results showed a significant correlation between Beck's Anxiety Inventory score with the State and Trait Anxiety Inventory scores ($P < 0.0001$, $r = 0.570$ and $r = 0.533$). There was a significant association between the State and Trait Anxiety Inventory scores ($P < 0.0001$, $r = 0.809$)

The mean State Trait Anxiety Inventory scores before amniocentesis were higher than 40 (43.45 ± 7.86 and 42.23 ± 7.98 , respectively), which indicated that mothers experienced high levels of anxiety. Although these mean values decreased after amniocentesis, there were no significant differences before and 2 weeks after amniocentesis (Table 1).

Table 1: Descriptive statistics of independent and dependent variables

Variables	Mean \pm SD	Min	Max
Before amniocentesis			
Beck's Anxiety Inventory	10.01 ± 8.99	1.00	43.00
Trait Anxiety Inventory	42.23 ± 7.98	26	66
State Anxiety Inventory	43.45 ± 7.86	30	66
Two weeks after amniocentesis			
Beck's Anxiety Inventory	9.23 ± 7.44	2.00	40
Trait Anxiety Inventory	41.18 ± 8.44	25	62
State Anxiety Inventory	33.38 ± 7.66	24	52
Before amniocentesis			
Left artery resistance index	0.64 ± 0.15	0.33	0.93
Right artery resistance index	0.69 ± 0.15	0.34	0.92
Two weeks after amniocentesis			
Left artery resistance index	0.65 ± 0.17	0.27	1.02
Right artery resistance index	0.66 ± 0.18	0.16	0.94
Index changes			
Left artery resistance index change	0.01 ± 0.21	-60.59	0.57
Right artery resistance index change	-0.02 ± 0.22	-0.48	0.44
First trimester serum analytes			
Pregnancy associated plasma protein A	0.90 ± 0.49	0.03	1.80
β -human chorionic gonadotropin (β -hCG)	1.81 ± 1.30	0.01	4.6
Second trimester serum analytes			
β -hCG	2.14 ± 1.32	0.20	6.27
Inhib A	1.66 ± 0.80	0.34	3.56
α -fetoprotein	1.35 ± 1.50	0.23	7.28
Unconjugated estriol	0.67 ± 0.26	0.34	1.31

Min; Minimum and Max; Maximum.

Before amniocentesis, the left artery mean resistance index was 0.64 ± 0.15 and the right artery mean resistance index was 0.69 ± 0.15 . At 2 weeks after amniocen-

tesis, the mean resistance index for the left artery was 0.65 ± 0.17 and it was 0.66 ± 0.18 for the right artery. Table 2 shows that no significant association existed before amniocentesis between Beck's Anxiety Inventory scores and the left uterine artery resistance index ($P = 0.394$, $\beta = 0.141$, 95% CI: -0.003 to 0.008) as well as the right uterine artery resistance index ($P = 0.897$, $\beta = 0.021$, 95% CI: -0.005 to 0.006). At 2 weeks after amniocentesis, there was also no significant association between Beck's Anxiety Inventory scores and the left uterine artery resistance index, ($P = 0.159$, $\beta = -0.227$, 95% CI: -0.010 to 0.002) and right uterine artery resistance index ($P = 0.599$, $\beta = 0.087$, 95% CI: -0.023 to 0.039).

There was no significant relationship between Beck's Anxiety Inventory score and change in the uterine artery resistance indexes.

Table 2: Association between uterine artery resistance index and Beck's Anxiety Inventory scores

Variables	P value	Beta	95% confidence interval	
			Lower bound	Upper bound
Before amniocentesis				
Left uterine artery resistance index	0.394	0.141	-0.003	0.008
Right uterine artery resistance index	0.897	0.021	-0.005	0.006
Two weeks after amniocentesis				
Left uterine artery resistance index	0.159	-0.227	-0.010	0.002
Right uterine artery resistance index	0.599	0.087	-0.023	0.039

Dependent variable in each model: uterine artery resistance.

We did not observe any association between the State-Trait Anxiety Inventory scores and blood flow of the uterine arteries, before amniocentesis. However, 2 weeks after amniocentesis, there was a significant association between the State Anxiety Inventory score and the uterine artery resistance index ($P = 0.041$, $\beta = 3.05$, 95% CI: -2.548 to 8.072) and for the Trait Anxiety Inventory score ($P = 0.010$, $\beta = -23.82$, 95% CI: -25.301 to -10.402). A significant relationship was observed between the State Anxiety Inventory scores and a decrease in the right uterine artery resistance index 2 weeks after amniocentesis ($P = 0.036$, $\beta = -0.274$, 95% CI: -0.015 to -0.001, Tables 3-5).

We found some associations between psychological scores and second trimester analytes. There was a significantly positive correlation between Beck's Anxiety Inventory scores and the α -fetoprotein level ($P < 0.001$, $\beta = 5.38$). β -hCG levels were significantly higher in pregnant women with higher Trait Anxiety Inventory scores ($P < 0.001$, $\beta = 3.94$). Unconjugated estriol levels were significantly higher in pregnant women with higher State Anxiety Inventory scores ($P = 0.048$, $\beta = 13.08$).

Table 3: Associations between uterine artery resistance index and Trait Anxiety Inventory

Variables	P value	Beta	95% confidence interval	
			Lower bound	Upper bound
Before amniocentesis				
Left uterine artery resistance index	0.940	-0.020	-0.011	0.10
Right uterine artery resistance index	0.930	-0.023	-0.010	0.009
After amniocentesis				
Left uterine artery resistance index	0.127	0.393	-0.002	0.019
Right uterine artery resistance index	0.010	-23.82	-25.301	-10.402

Dependent variable in each model: uterine artery resistance.

Table 4: Associations between uterine artery resistance index and State Anxiety Inventory

Variables	P value	Beta	95% confidence interval for B	
			Lower bound	Upper bound
Before amniocentesis				
Left uterine artery resistance index	0.945	-0.018	-0.010	0.10
Right uterine artery resistance index	0.603	0.132	-0.035	0.059
After amniocentesis				
Left uterine artery resistance index	0.754	-0.078	-0.012	0.009
Right uterine artery resistance index	0.041	3.05	-2.548	8.072

Dependent variable in each model: uterine artery resistance.

Table 5: Associations between alterations of uterine artery resistance indexes before and 2 weeks after amniocentesis to Beck's Anxiety Inventory and the State-Trait Anxiety Inventory

Dependent variables	P value	Beta	95% confidence interval for B	
			Lower bound	Upper bound
Beck's Anxiety Inventory				
Left artery resistance index change	0.077	-0.287	-0.014	0.001
Right artery resistance index change	0.915	0.017	-0.007	0.008
Trait Anxiety Inventory				
Left artery resistance index change	0.197	0.332	-0.005	0.022
Right artery resistance index change	0.208	-0.310	-0.023	0.005
State Anxiety Inventory				
Left artery resistance index change	0.841	-0.201	-0.015	0.012
Right artery resistance index change	0.036	-0.274	-0.015	-0.001

Dependent variable in each model: uterine artery resistance.

Discussion

During the antenatal period mothers face substantial anxiety, which may affect the prevalence of adverse obstetric outcomes. Some studies have found a correlation between psychological factors (depression, anxiety, and chronic stress) and preterm birth or low birth weight (4, 24, 25).

Since investigations have shown a higher prevalence of antenatal depression and anxiety disorders in pregnant Iranian women compared to the world average (26, 27), we assessed the role of psychological complications based on positive aneuploidy screening results in changes in uterine artery blood flow. Assessment of uterine artery blood flow by Doppler velocimetry is a valuable clinical tool to predict some adverse pregnancy outcomes (28, 29).

Studies have shown that informing pregnant women about the abnormal results of screening tests such as Down syndrome, or being aware of an invasive procedure like amniocentesis and waiting for its outcome causes tremendous distress and anxiety for these women.

The mean of the State-Trait Anxiety Inventory scores were more than 40 in our participants before amniocentesis, which showed a high anxiety level. In accordance with our results, Hoskovec and colleagues reported a significantly elevated mean state anxiety score in women with abnormal maternal serum screening tests. The score was significantly affected by factors like perceived risk and decision to undergo amniocentesis (30). Ng et al. (21), reported a mean trait anxiety score of 41.56 ± 6.35 in pregnant women before they underwent amniocentesis. In a prospective case-control study, Calışkan et al. (31) found that the maternal stress level based on Spielberger's State-Trait Anxiety Inventory in 60 pregnant women who underwent amniocentesis (48.9 ± 11.8) was significantly higher than normal pregnant women (33.5 ± 6.5).

There was a significant relationship between the State-Trait Anxiety Inventory scores and a decrease in the right uterine artery resistance index 2 weeks after amniocentesis. This finding might show that a 2 week period could provide an opportunity for mothers to cope with their anxiety. Possibly, participants were relieved after receiving the negative amniocentesis results, which reduced their concerns about their foetus' health status. In addition, high levels of anxiety could be associated with the amniocentesis process, which disappeared after 2 weeks. Possibly, the significant decrease in uterine artery resistance index could have been attributed to a decline in anxiety level.

Beck's Anxiety Inventory score can be repeated after one week. We have administered the test after two weeks; however, we did not find significant changes despite the reduction in the State Anxiety score. The Trait Anxiety score is due to more chronic anxiety feelings, which we did not expect any changes in 2 weeks as was shown in our participants.

Several investigations have shown correlations between psychological scales and uterine artery blood flow indexes through alteration of the stress hormones corticotropin releasing hormone and cortisol (32, 33). Stress can also decrease the level of pregnancy supporting hormones like progesterone that increase prostaglandin production and result in increased uterine contractility, blood vessel resistance, and decreased uterine artery blood flow (25). Monk et al. (34) have reported that in 101 second trimester pregnant women who had a history of mental illness, there were no significant associations found between different measures of maternal depression (Beck Depression Inventory, Hamilton Rating Scale for Depression-17) or anxiety (Hamilton Rating Scale for Anxiety) and uterine artery blood flow indexes. Mendelson et al. (8) detected a linear association between decreased uterine artery resistance indexes and advancing gestational age during the second half of pregnancy; however, their cross-sectional analyses did not reveal any significant correlations between individual psychological scores and uterine artery resistance indexes.

The reason that we observed a relationship between scores and decline in the resistance index in the right uterine artery, but not the left, might relate to other factors like placental site. Although we did not consider the placental location in the present study, other investigations pointed to the effects of placental site on uterine artery flow and resistance (35, 36).

Our findings showed a significantly positive correlation between Beck's Anxiety Inventory scores and α -fetoprotein levels. There was also a significant correlation between the unconjugated estriol levels and state scores. Altered α -fetoprotein and unconjugated estriol levels in depressed and anxious pregnant women might be due to alterations in the placental function and foeto-placental blood circulation (37-39).

Our results also showed a positive correlation between the trait anxiety scores and the second trimester β -hCG levels. On the other hand it seemed that pregnant women with high trait anxiety scores had abnormal patterns of uterine blood flow. Altered resistance index values, defective trophoblastic invasion, and transiently altered hormonal concentrations which might affect analyte transport might play a role in such abnormalities (31). Similar to our results, several studies have revealed interrelationships between high levels of β -hCG, nausea and vomiting during pregnancy, and psychological disturbances such as psychological stress (40). Oancea and colleagues found a significant correlation between high β -hCG levels in the second trimester and some maternal complications in 120 pregnant Caucasian women. They found that β -hCG had the highest predictive power for prenatal outcomes in the second trimester of pregnancy complicated by preeclampsia. This finding was also confirmed by Lee and Saha (40). To our knowledge, this study was among the few studies that focused on the association between psychological complications related to positive an euploidy

screening serum analytes and changes in uterine artery blood flow. Such correlations could show the importance of stress management interventions to improve pregnancy outcome.

This study, like other pilot studies, had some methodological limitations. We did not consider other related factors like parity and gestational age, which have been shown to affect uterine artery resistance index in other studies (7). A larger sample size and well-designed study would be required to evaluate the differences.

Conclusion

Maternal anxiety because of positive aneuploidy screening results and amniocentesis can affect perinatal outcomes via mood-based alterations in uterine artery blood flow resistance and screening markers like β -hCG, unconjugated estriol, and α -fetoprotein. We think that increasing pregnant women's knowledge about the probability of false-positive results of screening tests, administration of proper psychological interventions, and psychological counselling and training may significantly decrease the influence of anxiety and improve pregnancy outcomes. Further studies are required on this subject with greater numbers of participants.

Acknowledgements

This research has been financially supported by Tehran University of Medical Sciences (grant no: 36165). The authors would like to thank Seyed Muhammed Hussein Mousavi Nasab for his cooperation in text editing. There is no conflict of interest in this study.

Authors' Contributions

Mahb.S., B.S.; Carried out the design, coordinated the study, and participated in most of the experiments. S.N., F.R.S., Mam.S.; Coordinated and carried out all the experiments, performed data analysis, and participated in manuscript preparation. Z.P, P.P., Mahmo.S., M.A.; Provided assistance for all experiments and prepared the manuscript. All authors have read and approved the content of the manuscript.

References

1. Bisetegn TA, Mihretie G, Muche T. Prevalence and predictors of depression among pregnant women in Debretabor Town, Northwest Ethiopia. *PLoS One*. 2016; 11(9): e0161108.
2. Loomans EM, van Dijk AE, Vrijkotte TG, van Eijsden M, Stronks K, Gemke RJ, et al. Psychosocial stress during pregnancy is related to adverse birth outcomes: results from a large multi-ethnic community-based birth cohort. *Eur J Public Health*. 2013; 23(3): 485-491.
3. Non AL, Binder AM, Kubzansky LD, Michels KB. Genome-wide DNA methylation in neonates exposed to maternal depression, anxiety, or SSRI medication during pregnancy. *Epigenetics*. 2014; 9(7): 964-972.
4. Madhavanprabhakaran GK, D'Souza MS, Nairy KS. Prevalence of pregnancy anxiety and associated factors. *International Journal of Africa Nursing Sciences*. 2015; 3: 1-7.
5. Andersson L, Sundström-Poromaa I, Wulff M, Åström M, Bixo M. Neonatal outcome following maternal antenatal depression and

- anxiety: a population-based study. *Am J Epidemiol.* 2004; 159(9): 872-881.
6. Shirazi M, Azadi F, Shariat M, Niromanesh Sh, Shirazi M. Effectiveness of stress management training on stress reduction in pregnant women. *Tehran University Medical Journal.* 2016; 74(7): 493-499
 7. László KD, Liu XQ, Svensson T, Wikström AK, Li J, Olsen J, et al. Psychosocial stress related to the loss of a close relative the year before or during pregnancy and risk of preeclampsia. *Hypertension.* 2013; 62(1): 183-189.
 8. Mendelson T, DiPietro JA, Costigan KA, Chen P, Henderson JL. Associations of maternal psychological factors with umbilical and uterine blood flow. *J Psychosom Obstet Gynaecol.* 2011; 32(1): 3-9.
 9. Vythilingum B, Geerts L, Fincham D, Roos A, Faure S, Jonkers J, et al. Association between antenatal distress and uterine artery pulsatility index. *Arch Womens Ment Health.* 2010; 13(4): 359-364.
 10. Fu J, Yang R, Ma X, Xia H. Association between maternal psychological status and fetal hemodynamic circulation in late pregnancy. *Chin Med J (Engl).* 2014; 127(13): 2475-2478.
 11. Mousavi S, Hantoushadeh S, Sheikh M, Shariat M. Maternal anxiety and the second-trimester prenatal screening: a prospective cohort study. *Fetal Diagn Ther.* 2015; 38(4): 269-275.
 12. Baer RJ, Flessel MC, Jelliffe-Pawlowski LL, Goldman S, Hudgins L, Hull AD, et al. Detection rates for aneuploidy by first-trimester and sequential screening. *Obstet Gynecol.* 2015; 126(4): 753-759.
 13. reathnach FM, Malone FD, Lambert-Messerlian G, Cuckle HS, Porter TF, Nyberg DA, et al. First- and second-trimester screening: detection of aneuploidies other than down syndrome. *Obstet Gynecol.* 2007; 110(3): 651-657.
 14. Patrick J, Challis J, Natale R, Richardson B. Circadian rhythms in maternal plasma cortisol, estrone, estradiol, and estriol at 34 to 35 weeks' gestation. *Am J Obstet Gynecol.* 1979; 135(6): 791-798.
 15. Chou JY, Mano T, Feldman M. Inhibition of synthesis of alpha-fetoprotein by glucocorticoids in cultured hepatoma cells. *J Cell Biol.* 1982; 93(2): 314-317.
 16. Mylonas I, Jeschke U, Winkler L, Makovitzky J, Richter DU, Briese V, et al. Immunohistochemical expression of inhibin-alpha in human endometrium and the in vitro secretion of inhibin, estradiol and cortisol in cultured human endometrial glandular cells. *Arch Gynecol Obstet.* 2003; 268(3): 142-150.
 17. Gagnon A, Wilson RD; Society of Obstetricians and Gynaecologists of Canada Genetics Committee. *J Obstet Gynaecol Can.* 2008; 30(10): 918-932.
 18. Brajenović-Milić B, Martinac Dorčić T, Kuljanić K, Petrović O. Stress and anxiety in relation to amniocentesis: do women who perceive their partners to be more involved in pregnancy feel less stressed and anxious? *Croat Med J.* 2010; 51(2): 137-143.
 19. Kukulcu K, Buldukoglu K, Keser I, Keser I, Simşek M, Mendilicioğlu I, et al. Psychological effects of amniocentesis on women and their spouses: importance of the testing period and genetic counseling. *J Psychosom Obstet Gynaecol.* 2006; 27(1): 9-15.
 20. Kowalcek I, Muhloff A, Bachmann S, Gembruch M. Depressive reactions and stress related to prenatal medicine procedures. *Ultrasound Obstet Gynecol.* 2002; 19(1): 18-23.
 21. Ng CCM, Lai F M, Yeo GS. Assessment of maternal anxiety levels before and after amniocentesis. *Singapore Med J.* 2004; 45(8): 370-374.
 22. Mahram B. Standardization of Spielberger's test anxiety inventory in Mashhad. Presented for M.Sc., Tehran. Allameh Tabataba'ei University. 1994.
 23. Beck AT, Epstein N, Brown G, Steer RA. An inventory for measuring clinical anxiety: Psychometric properties. *J Consult Clin Psychol.* 1988; 56(6): 893-897.
 24. Choi SK, Park YG, Park IY, Ko HS, Shin JC. Impact of antenatal depression on perinatal outcomes and postpartum depression in Korean women. *J Res Med Sci.* 2014; 19(9): 807-812.
 25. Allison SJ, Stafford J, Anumba DO. The effect of stress and anxiety associated with maternal prenatal diagnosis on foeto-maternal attachment. *BMC Womens Health.* 2011; 11: 33.
 26. Karamoozian M, Askarizadeh G, Sanati K, Aminzadeh M. Stress management on pregnancy women. *J Nov Appl Sci.* 2015; 4(10): 1035-1042.
 27. Mangoli M, Ramezani T, Alizadeh SM. Screening mental disorders in pregnancy. *Iran J Psychiatry Clin Psychol.* 2003; 8(4): 45-55.
 28. Giordano R, Cacciatore A, Romano M, La Rosa B, Fonti I, Vigna R. Uterine artery Doppler flow studies in obstetric practice. *J Prenat Med.* 2010; 4(4): 59-62.
 29. Barati M, Shahbazian N, Ahmadi L, Masihi S. Diagnostic evaluation of uterine artery Doppler sonography for the prediction of adverse pregnancy outcomes. *J Res Med Sci.* 2014; 19(6): 515-519.
 30. Hoskovec J, Mastrobattista JM, Johnston D, Kerrigan A, Robbins-Furman P, Wicklund CA. Anxiety and prenatal testing: do women with soft ultrasound findings have increased anxiety compared to women with other indications for testing? *Prenat Diagn.* 2008; 28(2): 135-140.
 31. Çalışkan E, Ozkan S, Cakiroğlu Y, Yalçınkaya O, Polat A, Corakçı A. The effects of maternal anxiety prior to amniocentesis on uterine and fetal umbilical blood flow. *J Turk Ger Gynecol Assoc.* 2009; 10(3): 162-167.
 32. Florio P, Severi FM, Fiore G, Micheli L, Bocchi C, Nencini C, et al. Impaired uterine artery blood flow at mid gestation and low levels of maternal plasma corticotropin-releasing factor. *J Soc Gynecol Investig.* 2003; 10(5): 294-297.
 33. Bale TL, Baram TZ, Brown AS, Goldstein JM, Insel TR, McCarthy MM et al. Early life programming and neurodevelopmental disorders. *Biol Psychiatry.* 2010; 68(4): 314-319.
 34. Monk C, Newport DJ, Korotkin JH, Long Q, Knight B, Stowe ZN. Uterine blood flow in a psychiatric population: impact of maternal depression, anxiety, and psychotropic medication. *Biol Psychiatry.* 2012; 72(6): 483-490.
 35. Gupta V, Agarwal N. Correlation between placental location and uterine artery flow waveforms in uncomplicated pregnancies. *JIMSA.* 2014; 27(1): 17-18.
 36. Oloyede OA, Iketubosin F. Uterine artery Doppler study in second trimester of pregnancy. *Pan Afr Med J.* 2013; 15: 87.
 37. O'Donnell KJ, Bugge Jensen A, Freeman L, Khalife N, O'Connor T, Glover V. Maternal prenatal anxiety and downregulation of placental 11 β -HSD2. *Psychoneuroendocrinology.* 2012; 37(6): 818-826.
 38. Kane HS, Dunkel Schetter C, Glynn LM, Hobel CJ, Sandman CA. Pregnancy anxiety and prenatal cortisol trajectories. *Biol Psychol.* 2014; 100: 13-19.
 39. Gitlin D, Boesman M. 64 serum α -fetoprotein synthesis in the human and rat fetus and its inhibition in the rat. *Pediatr Res.* 1967; 1: 216-217.
 40. Lee NM, Saha S. Nausea and vomiting of pregnancy. *Gastroenterol Clin North Am.* 2011; 40(2): 309-334.

Expression of Ghrelin and Its Receptor mRNA in Bovine Oocyte and Cumulus Cells

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Abstract

Energy balance is regulated by ghrelin which is a neuroendocrine modulator. Ghrelin is expressed in reproductive organs. However, the role of ghrelin during *in vitro* maturation (IVM) and bovine preimplantational development is limited. The purpose of this study was to measure the expression of ghrelin (*GHRL*) and its receptor growth hormone secretagogue receptor 1A (*GHS-R1A*) mRNA, and determine cumulus oocyte complex (COC) viability after IVM with 0, 20, 40 and 60 pM of ghrelin. Also, pronuclear formation was recorded after *in vitro* fertilization (IVF). *GHRL* and *GHS-R1A* mRNA expression in oocyte and cumulus cells (CCs) was assessed using reverse transcription-polymerase chain reaction (PCR). Oocyte and CC viability were analyzed with the fluorescein diacetate fluorochrome-trypan blue technique. Pronuclear formation was determined 18 hours after IVF with Hoechst 33342. The results demonstrated that ghrelin mRNA is present in oocyte and CCs before and after 24 hours IVM with all treatments. Ghrelin receptor, *GHS-R1A*, was only detected in oocytes and CCs after 24 hours IVM with 20, 40 and 60 pM of ghrelin. Oocyte viability was not significantly different ($P=0.77$) among treatments. However, CC viability was significantly lower ($P=0.04$) when COCs were matured with ghrelin (77.65, 72.10, 66.32 and 46.86% for 0, 20, 40, and 60 pM of ghrelin, respectively). The chance of two pronuclei forming were higher ($P=0.03$) when ghrelin was not be added to the IVM medium. We found that ghrelin negatively impacts CC viability and pronuclear formation.

Keywords: Ghrelin, *GHS-R1A*, *In Vitro* Oocyte Maturation, mRNA Expression

Citation: Sirini MA, Anchordoquy JP, Quintana S, Furnus C, Relling AE, Anchordoquy JM. Expression of ghrelin and its receptor mRNA in bovine oocyte and cumulus cells. *Int J Fertil Steril.* 2019; 12(4): 335-338. doi: 10.22074/ijfs.2019.5393.

Nutrition has a strong influence on female bovine reproductive performance. In recent years, there has been a growing interest in investigating the relationship between nutrition and reproduction. In dairy cows, high milk yield leads to negative energy balance (NEB) which has adverse effects for fertility (1, 2). It has been suggested that metabolic hormones such as leptin and ghrelin might be signals that link fertility and energy status (3). Ghrelin is a neuroendocrine regulator of energy balance and food intake. Indeed, ghrelin plasma concentrations in cattle increase during fasting or NEB (4).

Previous studies have indicated that ghrelin regulates several reproductive functions (3, 4). Two subtypes of ghrelin receptors (*GHS-R*) have been identified, but only *GHS-R* type 1A (*GHS-R1A*) is functionally active (5). Recent investigations have localized ghrelin and *GHS-R1A* mRNA and protein expression to most reproductive tissues of dairy cattle (6). However, the expression of ghrelin and

its receptor in the bovine cumulus oocyte complex (COC) has not been yet described. Furthermore, the knowledge of ghrelin's role in oocyte maturation and preimplantational development is very limited (4, 7-9). Therefore, the purpose of this study was to investigate ghrelin (*GHRL*) and *GHS-R1A* mRNA expression in bovine oocyte and cumulus cells (CCs) after *in vitro* maturation (IVM) with different ghrelin concentrations, and evaluate the effect of ghrelin on oocyte and CC viability and pronuclear formation.

To perform this experimental research, bovine ovaries were obtained from an abattoir and transported to the laboratory in sterile NaCl solution (9 g/L) including the antibiotics streptomycin (100 mg/L) and penicillin (59 mg/L) at 37°C within 3 hours after slaughter. Ovaries were pooled, regardless of the estrous cycle stage of the donor. The COCs were aspirated from 3 to 8 mm follicles, using an 18-G needle connected to a sterile syringe. COCs with evenly granulated cytoplasm were selected

Received: 6/Sep/2017, Accepted: 10/Feb/2018

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International Journal of Fertility and Sterility
Vol 12, No 4, Jan-Mar 2019, Pages: 335-338

under a low power (20-30 X) stereomicroscope (Nikon, Japan), and washed twice in TCM-199 buffered with 15 mM HEPES and IVM medium. Groups of 10 COCs were transferred into 50 μ L of IVM medium under mineral oil (Squibb, USA). Incubation was performed at 39°C in an atmosphere of 5% CO₂ in air with saturated humidity for 24 hours. COCs were matured in IVM medium supplemented with 0, 20, 40, and 60 pM acylated ghrelin. The total number of matured COC was 1152. This total was divided on 200 COC for polymerase chain reaction (PCR) analysis, 480 for viability assay and 472 for pronuclear formation rates after *in vitro* fertilization (IVF).

After IVM, COCs were pipetted several times with a narrow-bore pipette in TCM-199 buffered with HEPES, and washed three times in calcium- and magnesium-free phosphate buffer solution (PBS) containing 1 mg/mL polyvinylpyrrolidone (PVP). Total RNA was isolated from CCs and oocytes with TRIzol (Invitrogen, CA) according to the manufacturer's instructions. Samples were then treated with a RNase-Free DNase kit (Qiagen, Germany). The RNA content of each sample was calculated through 260 nm absorbance. RNA quality was evaluated by the ratio of absorbance at 260 and 280 nm with a NanoVue spectrophotometer (NanoVue™-NV-General Electrics Healthcare Limited, UK). Complementary DNA (cDNA) was synthesized using a reaction mixture containing 1.5 μ g of total RNA, random hexamers and the M-MLV reverse transcriptase (Invitrogen-Life Technologies, USA), following the procedure suggested by the manufacturer. Polymerase chain reaction (PCR) was subsequently performed on the cDNA from oocytes and CCs. The reaction was performed at a final volume of 25 μ L containing 4 μ L cDNA, 0.85 pmol/mL of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate, PCR buffer 1X (50 mmol/L KCl and 10 mmol/L TrisHCl, pH=8.3) and 0.1% Triton X-100, 1.2 mmol/L MgCl₂, and 1.5 units of Taq DNA polymerase (Invitrogen, CA). The cDNA amplification reactions for (*GHRL*) and *GHS-R1A* were carried out with an initial denaturing step of 92°C for 3 minutes, followed by 35 cycles of 30 seconds at 92°C, 40 seconds at 60°C, and 40 seconds at 72°C, with a final elongation step of 72°C for 5 minutes. PCR products were verified on 2% agarose gel, stained with ethidium bromide, and visualized using a transilluminator with an UV filter. For the negative control, reverse transcription polymerase chain reaction (RT-PCR) procedures were carried out in the same manner, except that M-MLV reverse transcriptase was omitted during reverse transcription. The PCR reactions were performed in duplicates. Primers for each gene of interest were designed using Primer Premier Software (PREMIER Biosoft International, USA, Table 1), to avoid possible genomic DNA amplification, primers were designed to span exon-exon junctions. A total of 200 COCs were matured in two replicates (40 COCs per treatment). A time zero (T0, COC before IVM) treatment was used as the control group.

At the end of IVM, oocyte and CC viability were evaluated as follows. Oocytes were stripped of surrounding CCs by repeated pipetting in PBS containing 1 mg/mL PVP. Oocytes and CCs were incubated separately in the dark in 2.5 μ g/L fluorescein diacetate fluorochrome and 2.5 g/L trypan blue in PBS medium for 10 minutes at 37°C. Then, they were washed three times in PBS. The CCs were centrifuge at 200 x g for 5 minutes. The pellet was resuspended in 50 μ L of PBS. Oocytes and CC samples were transferred onto slides, which were immediately covered with cover slips and observed under a fluorescent microscope Olympus BX40 (Olympus, Japan) equipped with a 330-490 nm excitation filter and 420-520 nm emission filter. Live cells were visible with green fluorescence, whereas dead ones showed a characteristic blue staining under white light (Fig.1). A total of 480 COCs were matured in three replicates for this purpose.

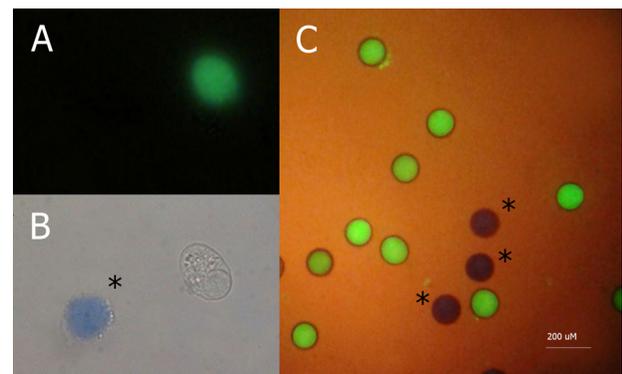


Fig.1: Oocyte and cumulus cells viability evaluated through fluorescein diacetate/trypan blue assay. The cell population was classified using the combined microscopic images obtained through light and fluorescence microscopy images. **A.** Alive cumulus cell (green) in the fluorescent field ($\times 1,000$ magnification), **B.** Dead cumulus cells (*) show a characteristic blue staining under white light ($\times 1,000$ magnification), and **C.** Alive (green) and dead (blue,*) bovine oocytes in a combined light and fluorescence field ($\times 40$ magnification).

The effect of different concentrations of ghrelin in the IVM medium on pronuclear formation was assessed after IVF (10). Expanded COCs were incubated in 50 μ L of Fert-TALP under mineral oil. Frozen semen from A bull of the same strain was used in all experiments. Motile spermatozoa were separated by a discontinuous Percoll gradient. The final sperm concentration in the IVF medium was 2×10^6 spermatozoa/mL. Incubation was performed at 39°C and 5% CO₂ in air with a saturated humidity for 18 hours. After IVF, presumptive zygotes were incubated in 0.1% (w/v) hyaluronidase in PBS solution for 5 minutes at 37°C and then oocytes were denuded by gentle pipetting. The presumptive zygotes were incubated in 5 mg/L Hoechst 33342 in PBS for 30 minutes at 37°C. Thereafter, they were examined under a fluorescent Olympus BX40 microscope (with a 365 nm excitation filter and a 400 nm emission filter) at $\times 200$ and $\times 400$ magnification to reveal the presence of pronuclei. A total number of 472 COCs were matured in three replicates for this purpose.

Table 1: Sequences of the primers for ghrelin (*GHRL*), growth hormone secretagogue receptor 1A (*GHS-R1A*) and the sizes of the reverse transcription polymerase chain reaction (RT-PCR) products

Gene	Primer sequence (5'-3')	Temperature annealing (°C)	Amplicon size (pb)
<i>GHS-R1A</i>	F: ACAGACCGTGAAGATGCT	60	164
	R: GGTAGAAGAGGACGAAAGA	60	164
<i>GHRL</i>	F: CTGAAGAAA CCCTGGCTAAC	57	107
	R: CGTGGTCTCGGAAGTGTC	57	107

Table 2: Fertilization status of putative zygotes produced *in vitro* with various ghrelin concentrations in IVM medium

Treatments	Number of oocytes	n (%) 1 PN	n (%) 2 PN	n (%) >2 PN	n (%) penetrated
0 pM ghrelin	115	33 ^a (28.6)	69 ^a (60.0)	1 ^a (0.8)	103 ^a (89.5)
20 pM ghrelin	116	59 ^b (50.8)	48 ^b (41.3)	0 ^a (0)	107 ^a (92.2)
40 pM ghrelin	122	64 ^b (52.4)	45 ^b (36.8)	1 ^a (0.8)	110 ^a (90.1)
60 pM ghrelin	119	69 ^b (57.9)	46 ^b (38.6)	2 ^a (1.6)	109 ^a (91.5)

IVM; *In vitro* maturation, PN; Pronucleus, ^{a, b}; Within a column, values without a common superscript are significantly different ($P < 0.05$), and COCs; Cumulus oocyte complex. Pronuclear rate was recorded 18 hours after insemination (472 COCs were matured and fertilized in three replicates). The presumptive zygotes were incubated in Hoechst 33342 and then examined under a fluorescent microscope at $\times 200$ and $\times 400$ magnification.

We used completely randomized block designs. Statistical models included the fixed effect of treatment (0 vs. 20 vs. 40 vs. 60 pM ghrelin) and the random effects of block (day of COCs collection, $n=3$). Oocyte and CC viability and rate of pronuclei presence were analyzed with logistic regression using the GENMOD procedure (SAS Institute, NC). Data for oocyte and CC viability and rate of pronuclei presence were expressed as a percentage. The level of significance was $P \leq 0.05$.

Using total RNA prepared from bovine oocytes and CCs and the specific primers for *GHRL*, RT-PCR showed a band of the expected size (107 bp) in agarose gel electrophoresis for all treatments (Fig.2). Thus, it seems clear that ghrelin mRNA is present in oocytes and CCs before and after 24 hours of IVM with 0, 20, 40 and 60 pM of ghrelin. On the other hand, the presence of *GHS-R1A* was only detected in oocytes and CCs after 24 hours of IVM with 20, 40 and 60 pM of ghrelin. The possibility of contaminating genomic ghrelin and *GHS-R1A* sequence amplification was excluded since the band of the expected size was only detected in the presence of reverse transcriptase.

Oocyte viability was not significantly different ($P=0.77$) among COCs treated with 0, 20, 40, or 60 pM of ghrelin during IVM (89.0, 87.1, 88.0 and 89.1%, respectively). However, CC viability was significantly lower ($P=0.04$) in COCs matured with ghrelin (72.10, 66.32 and 46.86% for 20, 40, and 60 pM of ghrelin, respectively) than in COCs matured with 0 pM of ghrelin (77.65%). No differences were found between 20 and 40 pM of ghrelin. The lowest CC viability rate was observed with 60 pM of ghrelin ($P=0.04$).

The incidence of polyspermy (>2 pronuclei) and the percentage of mature oocytes penetrated by spermatozoa did not differ among treatments ($P=0.96$). However, the chance of two pronuclei forming (normal fertilization)

were higher when ghrelin was not added to IVM medium ($P=0.03$, Table 2).

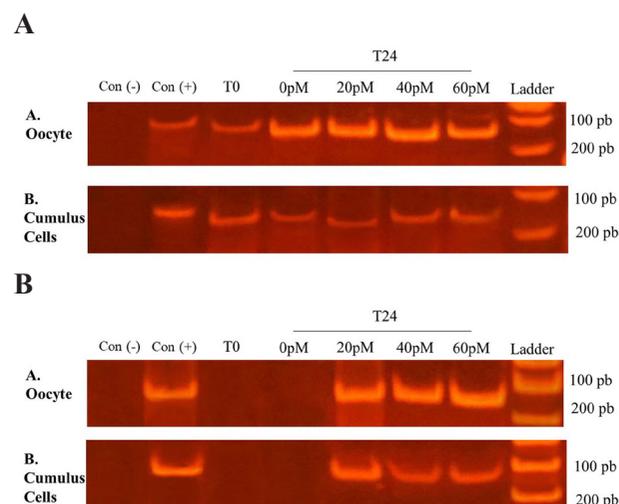


Fig.2: Agarose gel (2%) electrophoresis of polymerase chain reaction (PCR) products of *GHRL* and *GHS-R1A* cDNA. **A.** Agarose gel electrophoresis of PCR products of *GHRL* cDNA and **B.** Agarose gel electrophoresis of PCR products of *GHS-R1A* cDNA. COCs were matured 24 hours (T24) in IVM medium supplemented with 0, 20, 40, and 60 pM of ghrelin. A time zero (T0, COC before IVM) treatment was used. For the negative control [Con (-)], RT-PCR procedures were carried out in the same manner, except that M-MLV reverse transcriptase was omitted during reverse transcription. Hypothalamus tissue was used as a known positive control sample [Con (+)].

To our knowledge, this is the first study to report the expression of ghrelin and its receptor *GHS-R1A* in bovine oocytes and CCs. Our results indicate that ghrelin mRNA expression can be detected in oocytes and CCs both before and after IVM regardless of ghrelin presence during the IVM process. These findings support the idea that ghrelin may have an autocrine and/or paracrine effect within the follicular microenvironment. On the other hand, *GHS-R1A* mRNA expression was only detected when ghrelin was added to the IVM media, suggesting that the presence of ghrelin in the environment surround-

ing COCs may stimulate the expression of its functional receptor in both bovine oocytes and CCs. It has been demonstrated that ghrelin increases GHS-R mRNA levels in rat neurons (11). The mRNA expression of GHS-R1A is regulated by endogenous agonists, hormones and transcriptional factors (TFs) (12, 13). One of these factors, the pituitary-specific transcription factor (POU1F1) increases the expression of GHS-R1A and is present in oocytes and preimplantational embryos (13-15). García et al. (16) demonstrated that ghrelin induces the activation of Pit-1 (POU1F1) in anterior pituitary cells of infants. Although, in this study we did not examine the expression of Pit-1 in bovine COCs, this TF could have increased expression in the presence of ghrelin during IVM.

Cumulus cells play a key role in the acquisition of nuclear and cytoplasmic oocyte maturation (17). Furthermore, CCs protect the oocyte against oxidative stress and apoptosis (18). Likewise, CC damage leads to both lower fertilization and blastocyst formation rates (19, 20). In the present study, bovine COCs matured with different ghrelin concentrations resulted in a reduction of CC viability. Also, normal fertilization (formation of two pronuclei) was affected when oocytes were matured *in vitro* in the presence of ghrelin. Even though, the information about the effect of ghrelin on oocyte maturation and early embryo development is scarce and contradictory. However, our findings about the negative effect of ghrelin are in agreement with several publications (4, 8, 9).

Acknowledgements

This work was financially supported by grant from PIP 022-CONICET-MINCYT, Argentina. We are grateful to CIALE for the provision of bovine frozen semen, and the staff of Slaughterhouse Gorina S.A. for providing the bovine ovaries. Appreciation also due to A. Di Maggio for manuscript correction. We report no conflict of interests in this study.

Authors' Contributions

M.A.S., J.P.A.; Designed and performed the experiments, analyzed data. S.Q.; Performed molecular experiments. C.F., A.E.R.; Designed experiments and supervised the research. J.M.A.; Designed experiments and wrote the manuscript. All authors read and approved the final manuscript.

References

- Butler WR. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. *Livestock Prod Sci.* 2003; 83 (2-3): 211-218.

- van Kneysel AT, van den Brand H, Dijkstra J, Tamminga S, Kemp B. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. *Reprod Nutr Dev.* 2005; 45(6) :665-688.
- Evans JJ, Anderson GM. Balancing ovulation and anovulation: integration of the reproductive and energy balance axes by neuropeptides. *Hum Reprod Update.* 2012; 18(3): 313-332.
- Dovolou E, Messinis IE, Perikosta E, Dafopoulos K, Gutierrez-Adan A, Amiridis GS. Ghrelin accelerates *in vitro* maturation of bovine oocytes. *Reprod Domest Anim.* 2014; 49(4): 665-672.
- McKee KK, Palyha OC, Feighner SD, Hreniuk DL, Tan CP, Phillips MS, et al. Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors. *Mol Endocrinol.* 1997; 11(4): 415-423.
- Deaver SE, Hoyer PB, Dial SM, Field ME, Collier RJ, Rhoads ML. Localization of ghrelin and its receptor in the reproductive tract of Holstein heifers. *J Dairy Sci.* 2013; 96(1): 150-157.
- Popelkova M, Sirotkin AV, Bezakova A, Makarevic AV, Pivko J, Kacmarik J, et al. Effect of IGF-I, leptin, ghrelin and MAPK-ERK on the nuclear maturation of bovine oocytes. *Bull Vet Inst Pulawy.* 2006; 50(2): 179-181.
- Du C, Xilingaowa, Cao G, Wang C, Li H, Zhao Y, et al. Expression of the orexigenic peptide ghrelin in the sheep ovary. *Domest Anim Endocrinol.* 2009; 36 (2): 89-98.
- Wang Z, Lin P, Yu S. Effects of ghrelin on developmental competence and gene expression of *in vitro* fertilized ovine embryos. *Theriogenology.* 2013; 79(4): 695-701.
- Anchordoquy JP, Anchordoquy JM, Sirini MA, Mattioli G, Picco SJ, Furnus CC. Effect of different manganese concentrations during *in vitro* maturation of bovine oocytes on DNA integrity of cumulus cells and subsequent embryo development. *Reprod Domest Anim.* 2013; 48(6): 905-911.
- Nogueiras R, Tovar S, Mitchell SE, Rayner DV, Archer ZA, Dieguez C, et al. Regulation of growth hormone secretagogue receptor gene expression in the arcuate nuclei of the rat by leptin and ghrelin. *Diabetes.* 2004; 53(10): 2552-2558.
- Petersenn S, Rasch AC, Penschorn M, Beil FU, Schulte HM. Genomic structure and transcriptional regulation of the human growth hormone secretagogue receptor. *Endocrinol.* 2001; 142 (6): 2649-2659.
- Yin Y, Li Y, Zhang W. The growth hormone secretagogue receptor: its intracellular signaling and regulation. *Int J Mol Sci.* 2014; 15(3): 4837-4855.
- Joudrey EM, Lechniak D, Petrik J, King WA. Expression of growth hormone and its transcription factor, Pit-1, in early bovine development. *Mol Reprod Dev.* 2003; 64(3): 275-283.
- Laporta J, Driver A, Khatib H. Short communication: expression and alternative splicing of POU1F1 pathway genes in preimplantation bovine embryos. *J Dairy Sci.* 2011; 94(8): 4220-4223.
- García A, Alvarez CV, Smith RG, Diéguez C. Regulation of Pit-1 expression by ghrelin and GHRP-6 through the GH secretagogue receptor. *Mol Endocrinol.* 2001; 15(9): 1484-1495.
- Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev.* 2002; 61 (3): 414-424.
- Tatemoto H, Sakurai N, Muto N. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during *in vitro* maturation: role of cumulus cells. *Biol Reprod.* 2000; 63(3): 805-810.
- Høst E, Gabrielsen A, Lindenberg S, Smidt-Jensen S. Apoptosis in human cumulus cells in relation to zona pellucida thickness variation, maturation stage, and cleavage of the corresponding oocyte after intracytoplasmic sperm injection. *Fertil Steril.* 2002; 77 (3): 511-515.
- Seino T, Saito H, Kaneko T, Takahashi T, Kawachiya S, Kurachi H. Eight-hydroxy-2'-deoxyguanosine in granulosa cells is correlated with the quality of oocytes and embryos in an *in vitro* fertilization-embryo transfer program. *Fertil Steril.* 2002; 77(6): 1184-1190.

COMFFETI, Combined Fresh and Frozen Embryo Transfers per Individual: A New Index of Quality Control for The Performance of Embryologic Labs in The Emerging Era of Segmentation of Cycle and Freeze-All Strategy

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Abstract

The efficacy of *in vitro* fertilization (IVF) for treating human infertility has only one final efficacy index and that is the achievement of a delivery. However, with the evolution of the freeze-all strategy, a new problem is arising for evaluating the performance of an embryological team. The aim of the study was to present a new representative index, combining fresh and frozen embryo transfer success rates. In this opinion article, apart from the effectiveness of managing fresh gametes and embryos, we wish to evaluate the efficacy of the processes of both freezing and thawing of the produced embryos. The reporting of pregnancy rates of an IVF unit in the past was primarily laying in the fresh embryo transfer (ET) pregnancy rates. Now with the most frequent utilization of freeze-all strategy, it does not seem logical to report only on poor prognosis patients as all the good cases are postponed for thawed cycles. Ongoing implementation of the freeze-all strategy has indicated the need to establish a new representative index that may combine the success of both fresh and frozen cycles performed in the same woman; an index that may not be biased by the policy of an IVF center towards or against the freeze-all strategy. This newly proposed index, which is referred to as COMFFETI (Combined Fresh & Frozen Embryo Transfers per Individual), describes the optimal way to report final reproductive outcomes in the present opinion article.

Keywords: Embryo, Fresh, Frozen, Infertility

Citation: Papanikolaou EG, Timotheou E, Tatsi P, Janssens H, Grynberg M, Athanasiadis A, Zafeirati C, Najdecki R, Petousis S. COMFFETI, combined fresh and frozen embryo transfers per individual: a new index of quality control for the performance of embryologic labs in the emerging era of segmentation of cycle and freeze-all strategy. *Int J Fertil Steril.* 2019; 12(4): 339-342. doi: 10.22074/ijfs.2019.5424.

Currently, the efficacy of *in vitro* fertilization (IVF) for treating human infertility has only one final efficacy index, which is reaching a successful delivery. However, there are so many confounding factors that might alter the achievement of a pregnancy. These factors are either presently unmanageable by medicine, such as the genetic quality of the produced eggs, sperms and the endometrial receptivity capacity of each individual, or manageable by current technology, such as gonadotrophin capacity, equipment capacity, culture media capacity, air quality, scientist's expertise and so on (1, 2). In the last category the embryological team, who use the abovementioned techniques and equipment on a daily basis, play the central role for an optimal outcome (3).

Although, there are certain laboratory parameters that can be utilized to monitor the efficacy of a laboratory team, such as fertilization rate, degeneration rate, cleavage rate, blastulation rate, proportion of embryos for freezing, unfortunately the embryological staff are eventually solely judged by the pregnancy rates that their lab is achieving. This is unfortunate, because many confounders that intercede after their last involvement, such as the type of embryo transfer catheter, the capacity of the medical transferee, the quality of the endometrium, or the quality of the luteal support, are factors that can be critical for the efficiency of their work (4, 5). Moreover, with the increased number of freezing and sequential thawing steps, the vitrification technique is also considered as a

Received: 18/Oct/2017, Accepted: 16/May/2018

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Royan Institute
International Journal of Fertility and Sterility
Vol 12, No 4, Jan-Mar 2019, Pages: 339-342

new confounder in the evaluation of the embryologists' performance.

With the evolution of the Freeze-all strategy, a new problem is arising for evaluating the performance of an embryological team. Apart from the efficacy of the management of fresh gametes and embryos, we shall also take into consideration the efficacy of both freezing and thawing embryos. These are two extra procedures, which are both dependent on the expertise of the person who is performing these steps, therefore might potentially be at risk for mistakes. Moreover, the cryopreservation of all high responders and presumably good prognosis patients complicate the situation even more, as only poor or average responders are allowed to proceed with embryo transfer (ET), putting the probability of pregnancy with fresh embryo at risk in these poor prognosis patients.

Previously, the reports on pregnancy rates of an IVF unit were primarily based on the cases with fresh ET. Now with the frequent utilization of the freeze-all strategy there is a risks of reporting only poor prognosis patients, as all the good cases are postponed for thawed cycles.

The rationale for the freeze-all strategy and eventually segmentation of the IVF cycle has developed over the recent years and is based on two pathophysiological facts. The main one is the endometrial receptivity, which is definitely violated during ovarian stimulation due to the supra-physiological levels of the steroid hormones. Devroey et al. (6) have clearly demonstrated with endometrial biopsies in both agonist and antagonist protocols, that the early luteal endometrial histology is always advanced. Moreover, when this advancement lasts for more than 3 days, the endometrium may be considered as out-of-phase, which in turn significantly reduces the possibility of pregnancy. The second reason is the com-

plete eradication of severe ovarian hyperstimulation syndrome (OHSS) when fresh ET is withheld. Then, starting the next month, ET can be performed in a more naturally prepared endometrium with the absence of any risks for OHSS (7).

Ongoing implementation of the freeze-all strategy has indicated the need to establish a new representative index that may combine the success of both fresh and frozen cycles administered in the same woman, an index that may not be biased by the policy of an IVF center in favor of or against the freeze-all strategy (8, 9). This new index proposed as COMFFETI (Combined Fresh & Frozen Embryo Transfers per Individual) is described in the present opinion article.

The proposed COMFFETI index could be defined as a binomial variable [yes (1) or no (0)] reflecting the achievement of a pregnancy or not per individual (couple) at the end of each stimulated cycle; including fresh ET plus the thawed ET obtained by a single multifollicular ovarian stimulation cycle. This is a radically different index from the widely used index of pregnancy/delivery rate per transfer.

The basic difference lies in the fact that by the old way of reporting clinical or ongoing pregnancy per fresh ET reflects the potential of pregnancy achievement following this specific fresh ET (8), without considering the additional potential of success of surplus embryos obtained after the same ovarian stimulation. Therefore, an individual that may finally achieve a pregnancy at her third frozen ET (1 fresh and two frozen cycles failed) is considered as having a 0% pregnancy rate in her first attempt, while if the COMFFETI index would have been applied, this woman would have a 100% COMFFETI index, as she would have achieved a pregnancy with embryos still from the initial ovarian stimulation, even at her third frozen embryo transfer.

Table 1: Hypothetical outcomes for hypothetical couples and potential reports in clinic A, which is fresh-cycle friendly

Patient	Fresh COCs	Produced embryos	Destiny	ET (n)	Cryo (n)	Fresh outcome	1 st frozen	2 nd frozen	DR/ET	COMFFETI
1	2	1D2	Fresh ET	1D2	0	Neg	-	-		
2	9	6D3	Fresh ET	2D3	4D3	Delivery	-	-		
3	17	6D5	Fresh ET	2D5	4D5	Delivery	-	-		
4	12	5D5	Fresh ET	1D5	4D5	Neg	Neg	Neg		
5	15	4D5	Fresh ET	1D5	3D5	Neg	Neg	Delivery		
6	9	2D3	Fresh ET	2D3	2D3	Neg	-	-		
7	11	6D3	Fresh ET	2D3	4D3	Neg	Neg	Neg		
8	5	2D3	Fresh ET	2D3	0	Neg	-	-		
9	10	5D5	Fresh ET	2D5	3D5	Delivery	-	-		
10	10	6D3	Fresh ET	2D3	4D3	Neg	Neg	Neg		
Overall									30% (3/10)	40% (4/10)

COCs; Cumulus oocytes, ET; Embryo transfer, Neg; Negative, and DR/ET; Delivery rate per fresh embryo transfer.

Table 2: Hypothetical outcomes for the same hypothetical couples and potential reports in clinic B, which is freeze-ALL-friendly

Patient	Fresh COCs	Produced embryos	Destiny	ET (n)	Cryo (n)	Fresh outcome	1 st frozen	2 nd frozen	DR/ET	COMFFETI
1	2	1D2	Fresh ET	1D2	0	Neg	-	-		
2	9	6D3	Fresh ET	2D3	4D3	Delivery	-	-		
3	17	6D5	FRALL	0	6D5	No ET	Delivery	-		
4	12	5D5	Fresh ET	1D5	4D5	Neg	Neg	Neg		
5	15	4D5	FRALL	0	4D5	No ET	Neg	Delivery		
6	9	2D3	FRALL	0	2D3	No ET	Delivery	-		
7	11	6D3	Fresh ET	2D3	4D3	Neg	Neg	Neg		
8	5	2D3	Fresh ET	2D3	0	Neg	-	-		
9	10	5D5	FRALL	0	5D5	No ET	Delivery	-		
10	10	6D3	FRALL	0	6D3	No ET	Delivery	-		
Overall									20% (1/5)	60% (6/10)

COCs; Cumulus oocytes, FRALL; Freeze all, ET; Embryo transfer, Neg; Negative, and DR/ET; Delivery rate per fresh embryo transfer.

The value of this new index could be demonstrated in case we examine two theoretical examples of different IVF centers, of which the first is in favor of fresh ET, while the second is practicing the freeze-all technique. Table 1 presents the final outcomes of 10 patients undergoing IVF treatment in an IVF center performing fresh ET. In case there were three deliveries achieved by the first ET with fresh embryos, the traditional index would have been 30%. Table 2 presents the same 10 patients undergoing IVF treatment and their final outcomes in a center taking the freeze-all approach. In case an IVF Unit has as mainstream policy to freeze embryos, the traditional delivery index would be only 20%. For instance case number 5 (due to high response) and case number 10 (due to high follicular progesterone) withheld the fresh transfer and took the freeze-all approach. Therefore, a superficial outcome of the two centers would indicate that centers favoring the fresh ET policy are more successful in achieving clinical pregnancies/deliveries.

Our own data support what was mentioned in the examples above about the COMFFETI index. Based on the latest 50 cases of fresh embryo transfer cycles, the live birth rate has been 46% for the first embryo transfer, while the COMFFETI index has been 74% after two embryo transfers. Relatively, for the last 50 cases of the freeze-all strategy, the live birth rate was 58% for the first embryo transfer per case while the COMFFETI index was 82%. These results indicate the importance of using an index, which reflects the cumulative results of consecutive embryo transfers, especially in the freeze-all-friendly centers.

However, the consideration of pregnancies achieved “at the end of the day” according to COMFFETI index would radically change the situation. COMFFETI index would only have a slight increase from 20 to 30% in the first center, favoring fresh ETs, while in the second example, both cases 5 and 10 might have achieved a delivery with frozen embryos, and thus COMFFETI pregnancy rate could rise up to 60%, representing a totally different clinical outcome with regards to traditional index.

The additional great advantage that COMFFETI pregnancy rate provides is that it incorporates the implantation potential of all embryos produced from a single stimulated cycle. On the other hand, a drawback might be that COMFFETI rate is significantly related to the efficacy of cryopreservation techniques and the assumed increased success rates reported from the frozen cycles (10). Therefore, a complete freeze-all-friendly center may not actually achieve a pregnancy from the very first fresh embryo transfer with the traditional pregnancy rates being even 0%. Furthermore, one of the shortcomings of COMFFETI index is that you cannot include all the couples’ result into assessment, since some couples may take a long time to transfer all their vitrified embryos for ET. However, given the fact that especially when blastocysts are cryopreserved and subsequently all of them are transferred in consecutive natural cycles with assumed receptive endometria, an increased absolute number of clinical pregnancies might actually be achieved by this strategy (11).

The basic endpoint of a successful IVF treatment cycle is giving a healthy baby to the mother (12). The interval required for such a purpose may not be the primary concern of a rationale patient. The main concern that preoccupies every subfertile woman or man is the probability of having a healthy baby after a stimulated cycle. It is mentally much more encouraging to tell the woman or the couple that there is a 60% cumulative chance of a successful pregnancy by transferring all the embryos obtained through a single ovarian stimulation, rather than a 20% chance from the first fresh ET.

The COMFFETI index in reproductive medical practice may be used to give the infertile patients a more subjective view about the realistic possibilities to have a successful IVF cycle from the beginning of the treatment. Moreover, reporting to organizations like European Society of Human Reproduction and Embryology (ESHRE) and Center for Disease Control would become increasingly objective by using the above index as a higher number of centers are moving towards the freeze-all policy,

and therefore only poor cases or modest responders would be selected for fresh embryo transfers (13, 14).

Acknowledgements

There are no sources of funding for the research to be reported. None of the authors have any competing interests in the manuscript.

Authors' Contributions

E.G.P., E.T., P.T., S.P.; Had the conception of the basic idea and wrote the initial draft of the manuscript. E.G.P., R.N.; Contributed substantially to the conception of the study, or the analysis and interpretation. H.J., M.G., A.A., C.Z.; Provided critical revision of the article. All authors performed editing and approving the final version of this manuscript for submission, also participated in the finalization of the manuscript and approved the final draft.

References

1. Bourgain C, Devroey P. The endometrium in stimulated cycles for IVF. *Hum Reprod Update*. 2003; 9(6): 515-522.
2. Diedrich K, Fauser BC, Devroey P, Griesinger G; Evian Annual Reproduction (EVAR) Workshop Group. The role of the endometrium and embryo in human implantation. *Hum Reprod Update*. 2007; 13(4): 365-377.
3. Kent-First M. The critical and expanding role of genetics in assisted reproduction. *Prenat Diagn*. 2000; 20(7): 536-551.
4. Schoolcraft WB. Importance of embryo transfer technique in maximizing assisted reproductive outcomes. *Fertil Steril*. 2016; 105(4): 855-860.
5. Angelini A, Brusco GF, Barnocchi N, El-Danasouri I, Pacchiarotti A, Selman HA. Impact of physician performing embryo transfer on pregnancy rates in an assisted reproductive program. *J Assist Reprod Genet*. 2006; 23(7-8): 329-332.
6. Devroey P, Polyzos NP, Blockeel C. An OHSS-Free Clinic by segmentation of IVF treatment. *Hum Reprod*. 2011; 26(10): 2593-2597.
7. Papanikolaou EG, Humaidan P, Polyzos NP, Tarlatzis B. Identification of the high-risk patient for ovarian hyperstimulation syndrome. *Semin Reprod Med*. 2010; 28(6): 458-462.
8. Borges E Jr, Braga DP, Setti AS, Vingris LS, Figueira RC, Iaconelli A Jr. Strategies for the management of OHSS: results from freezing-all cycles. *JBRA Assist Reprod*. 2016; 20(1): 8-12.
9. De Vos A, Van Landuyt L, Santos-Ribeiro S, Camus M, Van de Velde H, Tournaye H, Verheyen G. Cumulative live birth rates after fresh and vitrified cleavage-stage versus blastocyst-stage embryo transfer in the first treatment cycle. *Hum Reprod*. 2016; 31(11): 2442-2449.
10. Rienzi L, Gracia C, Maggiulli R, LaBarbera AR, Kaser DJ, Ubaldi FM, Vanderpoel S, Racowsky C. Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Hum Reprod Update*. 2017; 23(2): 139-155.
11. Casper RF, Yanushpolsky EH. Optimal endometrial preparation for frozen embryo transfer cycles: window of implantation and progesterone support. *Fertil Steril*. 2016; 105: 867-872.
12. Jongbloed-Pereboom M, Middelburg KJ, Heineman MJ, Bos AF, Haadsma ML, Hadders-Algra M. The impact of IVF/ICSI on parental well-being and anxiety 1 year after childbirth. *Hum Reprod*. 2012; 27(8): 2389-2395.
13. European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE), Calhaz-Jorge C, de Geyter C, Kupka MS, de Mouzon J, Erb K, et al. Assisted reproductive technology in Europe, 2012: results generated from European registers by ESHRE. *Hum Reprod*. 2016; 31(8): 1638-1652.
14. Hornstein MD. State of the ART: assisted reproductive technologies in the United States. *Reprod Sci*. 2016; 23(12): 1630-1633.

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

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

1. Types of articles

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

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

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

C. Systematic Reviews

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

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

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

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

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

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

I. Debate.

2. Submission Process

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

A. Author contributions statements

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

B. Cover letter

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

C. Manuscript preparation

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text pages should have consecutive line numbers in the left margin beginning with title page and continuing through the last page of the written text. Each abbreviation must be defined in the abstract and text when they are mentioned for the first time. Avoid using abbreviation in title. Please use the international and standard abbreviations and symbols.

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

1. Hardy-Weinberg Equilibrium (HWE) calculations must be carried out and reported along with the P-values if applicable [see Namipashaki et al. 2015 (Cell J, Vol 17, N 2, Pages: 187-192) for a discussion].
2. Linkage disequilibrium (LD) structure between SNPs (if multiple SNPs are reported) must be presented.
3. Appropriate multiple testing correction (if multiple independent SNPs are reported) must be included.

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

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

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

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

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

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

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

The following components should be identified in the abstract:

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

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Tables and figures: Tables should have a short descriptive heading above them and also any footnotes. Figure's legend should contain a

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

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

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

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

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

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Abstract book:

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Thesis:

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

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

Conferences:

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

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Surname(s) and first letter of name & middle name(s) of author(s). Manuscript title. Journal title (abbr). Publication date (year);

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

Book:

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

Law:

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

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2. Text of manuscript and References prepared as stated in the "guide for authors" section.
3. Tables should be in a separate page. Figures must be sent in color and also in GIF or JPEG format with 300 dpi resolutions.
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