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Review Article

Female Reproductive Health in SARS-CoV-2 Pandemic Era

Reihaneh Nateghi, Ph.D.[1#], Shahriar Ghashghaei, M.D.[2#], Bahare Shokoohian, Ph.D.[1], Maryam Hezavehei, Ph.D.[1], Mahkameh Abbaszadeh, M.D.[2], Bita Ebrahimi, Ph.D.[1], Abolhossein Shahverdi, Ph.D.[1], Mehri Mashayekhi, M.D.[3], Anastasia Shpichka, Ph.D.[4], Peter Timashev, Ph.D.[4], Mohammad Hossein Nasr-Esfahani Ph.D.[5*], Massoud Vosough M.D., Ph.D.[2*]

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic struck global health systems with overgrowing demands in many fields of health care; yet, reproductive care, particularly pregnancy care remains a special focus of interest. Pregnancy is a major physiologic change that alters temporarily normal function of many organs, and specifically the immune system. Therefore, pregnant women are more susceptible to respiratory pathogens compared to the others. The current pandemic may have serious consequences on pregnancy whether directly or indirectly. In the present review, direct and indirect possible adverse effects of SARS-CoV-2 infection on female reproductive system by focusing on pregnancy and delivery has been discussed in details. In addition, the pregnancy consequences and whether maternal infection can affect infants were deliberated. The adverse impact of luck down and related psychological complications and obesity on pregnant women were discussed as well. Finally, the effects of SARS-CoV-2 vaccination on maternal health and pregnancy outcome was analyzed.

Keywords: COVID-19 Pandemic, Female Infertility, Female Reproductive Health, Fetal Development, SARS-CoV-2

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Introduction

Health systems are challenged by overwhelming requests created by SARS-CoV-2 pandemic. Yet, reproductive medicine including pregnancy care remains as an essential part of health services requiring special attention (1). Pregnancy makes changes on the immunity status and might make pregnant women more susceptible to respiratory pathogens and pneumonia (2). Pregnancy results in physiological adaptions such as airway edema, diaphragmatic elevation, more oxygen consumption, and pregnancy-related immune alterations (3). Moreover, swelling of upper respiratory tract because of high levels of estrogen and progesterone in addition to limited lung expansion capacity lead to the vulnerability of the pregnant woman to the respiratory pathogens (2, 3). Different processes in female reproductive system, including folliculogenesis, steroidogenesis, oocyte maturation are regulated by renin-angiotension aldosterone system (RAAS) that comprises the classic components of angiotensin converting enzyme (ACE), angiotensin 2 (Ang2) and angiotensin II type 1 receptor (AT1R) axis along with new discovered components i.e. Ang [1-7] and Mas. Angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) play the key role as entry receptors for SARS-CoV-2. Expression of ACE2 and TMPRSS2 was not only detected in epithelial cells and stromal cells of endometrium throughout the whole menstrual cycle (4); but also, the presence of these receptors were identified during first, second and third trimester of pregnancy (5). Moreover, during embryogenesis, ACE2 was identified in inner cell mass and trophoblast while TMPRSS2 was only seen in trophoblast. On contrast, none had significant expression in oocytes and cleavage embryos. Therefore, at each stage, certain cells are susceptible to infection by SARS-CoV-2. This paper focused on direct and indirect possible adverse effects of SARS-CoV-2 infection on the female reproductive health systems, the pregnancy consequences and whether maternal infection affects infants. Finally, the effects of SARS-CoV-2 vaccination on maternal health and pregnancy outcome was discussed.

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SARS-CoV-2 and female reproductive health system

Studies suggested that SARS-CoV-2 might cause dysfunction in the female reproductive system, directly or indirectly. The direct adverse effects are related to cytopathic impact of virus colonization and the indirect effects are associated with exacerbation caused by RAAS, inflammatory reactions, psychological disorders, and obesity.

Tissue distribution of ACE2 in the female reproductive system

The expression of ACE2 in human ovaries and endometrium has been reported. Throughout menstrual cycle, expression of ACE2 in endometrium changes based on the phase of cycle. In proliferative phase, expression of ACE2 is predominant in epithelial cells while in secretory phase, significant expression of this receptor is evident in both epithelial and stromal cells (6).

Data regarding the expression of ACE2 in oocytes and embryos are controversial. Previous publications indicated that high levels of ACE2 is expressed in the germ cells and early embryos (7) while some recent data reported the opposite. Recently, Stanley and colleagues revealed that co-expression of ACE2 and TMPRSS2 increased during oocyte maturity, therefore, primordial follicles have less susceptibility to the infection compared to the more matured follicles. Regardless, the study suggests that possibility of transient effects is low. In addition, ACE2 expression in human cumulus cells was reported, though TMPRSS2 expression was very low in the cumulus cells. Therefore, it seems that there is a low risk for infection in these type of cells (8). In contrast to the previous findings, Reis et al. (9) found that there is a slight possibility of presence of ACE2 and TMPRSS2 in oocytes. Furthermore, ACE2 was detected in follicular fluid (FF).

Although there were ACE2 receptors in the female reproductive tract, but there is no strong evidence for the virus colonization through ACE2 receptors in the female reproductive system so far.

Renin-angiotensin aldosterone system in COVID-19

There is a substantial correlation between RAAS components and gonadotropins; meaning that gonadotropins can increase RAAS components' expression (9) and vice versa (10-12) in addition that both can influence function of ovary (11-13).

High levels of gonadotropins' induces the expression of Ang (II) in FF (9). ACE2 uses Ang II as its key substrate to produce angiotensin [1-7], exerting vasodilatory activity via the mas receptor (MasR). Ang [1-7] and MasR, in the theca-interstitial cells, could raise the level of ovarian steroidogenesis and regulate the ovary physiologic functions such as follicular development, steroidogenesis, oocyte maturation, ovulation (10). Recently, the ability of ACE2/Ang [1-7]/MasR axis has been proved in enhancement of meiotic resumption and it is well-known that meiotic resumption can be adjusted by luteinizing hormone (12). In addition, regulation of ACE2 expression by gonadotropins, and its contribution in follicular development have been already mentioned (13). Reis and colleagues showed presence of ACE2 and active Ang [1-7]-MasR-ACE2 axis in the human ovarian follicles (9). The gonadotropin-dependent expression of ACE2 in human ovaries has widely covered in the literature, although ACE2 receptors in male reproductive system were more notable than female reproductive system (11, 14).

Due to correlation between female gonadotropins and ACE2 expression- as a part of RAAS system and key entry point for the SARS-CoV-2 -, there is a reasonable possibility of infection exacerbation in female reproductive system. Figure 1 illustrates different etiological pathways in pathogenesis of COVID-19 related female fertility complications.

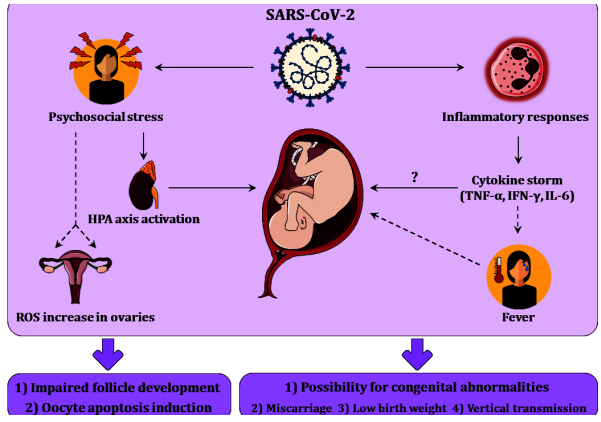

Fig. 1: These figure represents different etiological factors affecting female reproductive health system. Inflammatory reactions and psychosocial stress can cause many complications in pregnant mothers.

SARS-CoV-2 severe inflammatory response and female reproduction

Cytokine-storm is another serious consequence of SARS-CoV-2 infection. The plasma concentrations of different interleukins (IL) and tumor necrosis factor α (TNF-α) raised during SARS-CoV-2 infection which could lead to morbidity or even mortality due to multiple organ failure (15). The toxic effect of TNF on developmental competency was already shown. It was suggested that increased level of TNF-α in the maternal blood might be noxious for early embryo growth (16).

Other study reported that patients with SARS-CoV-2 had higher levels of inflammatory cytokines [TNF-α, interferon-γ (IFN-γ), IL-2, and IL-6] than control individuals (16-18). High levels of IL-6 were associated with the clinical intensity of SARS-CoV-2; thus, IL-6 level could be used as a biomarker in acute phase to determine the severity of infection (19), an independent predictor of mortality (20) and a hallmark for efficacy of possible treatments (21, 22).

SARS-CoV-2 and psychological factors in female reproduction

Previous studies have shown that viral diseases such as severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and H1N1 could initiate serious panic in societies like depression, anxiety, fear, and post-traumatic stress disorder (23, 24). Recent study showed that SARS-CoV-2 pandemic not only causes medical concerns, but also initiates different psychological complications. Frequency of anxiety, stress, and depression were reported to be around 31.9%, 29.6%, and 33.7% respectively in this pandemic (25).

Association between stress and reproductive function impairment in infertile women is acknowledged (26). This correlation could be identified by activating the hypothalamic-pituitary-adrenal (HPA) axis, body-stress response and dysregulation in hormones (27). Stress could increase reactive oxygen species (ROS) and oxidative stress in the ovaries, which lead to restricted development of follicles and apoptosis induction in oocytes. Consequently, impairments in female reproduction with adverse impacts on oocyte quality would be expected (28).

On the other hand, the growth of embryo might be affected by panic disorder during early pregnancy, and adverse outcomes in the maternal and fetal health would be expected (29).

SARS-CoV-2 and obesity in female reproduction

Worldwide, people are gaining extra weight during pandemic due to lockdown and limited physical activity, leading to increased obesity rate. The detrimental adverse effect of obesity on fertility and pregnancy has long been detected. Obesity results in hyperinsulinemia and impairment in hypothalamic-pituitary-gonadal (HPG) axis affects ovaries and endometrium. Eventually, obesity results in decline in pregnancy rate, rise in miscarriage and pregnancy complications as well as reduction in rate of still birth (30). Also, obesity is associated with increased risk of poly cystic ovary syndrome (PCOS) which causes anovulation and follicular atresia through ROS (31). Among pregnant women who were hospitalized, obesity was observed in more than a third of them (32). This could give rise to many complications including hypertension, preeclampsia and gestational diabetes in mother. In neonate, heart and neural defects, preterm birth and stillbirth are great risks (33).

SARS-CoV-2 infection and adverse outcomes in pregnancy

Mixed data regarding effects of SARS-CoV-2 on health of mother and infant/neonate exist, including serious effect on delivery, delivery outcome and vertical transmission.

Miscarriage and preterm delivery

In contribution to health of infant and neonates, miscarriage appears to not be a concern in infected patients as no significant risk was observed in this population (34, 35). Also, maternal infection may have no effect on infant growth (35-37). Despite this, in case of preterm delivery, some studies indicated higher risk in symptomatic mothers comparing to non-symptomatic/non-infected mothers (32, 34, 38) while others suggested no correlation (35, 37). Yet, based on the fact that the studies supporting higher pre-term delivery in symptomatic patients have a much higher sample size, we author believe SARS-CoV-2 infection increases the risk of pre-term delivery.

In contribution to maternal health, the adverse effects of SARS-CoV-2 before, during and after delivery has been demonstrated in literature. These effects include admission to intensive care unit (ICU), undergoing cesarean and operative vaginal birth and post-partum hemorrhage mainly observed in symptomatic patients along with many other complications (32, 37, 39).

The third trimester of pregnancy was the focal point of most studies on SARS-CoV-2 (34, 35, 40). The complication rate in first and second trimester mothers were similar to non-infected ones (34).

Vertical transmission of SARS-CoV-2

The vertical transmission could happen via three major routes: i. Placental blood during the course of pregnancy, ii. The birth canal in the course of labor, and iii. During the breastfeeding (41).

Though no sufficient data exist to drive a firm conclusion regarding vertical transmission, based on recent data, the vertical transmission can be deemed to be rare as many studies discussed its possibility (34, 35, 42).

In spite of controversial data, the presence of SARS-CoV-2 in placenta has yet to be determined based on further studies (35, 43-45); Though the vertical transmission through placenta has been ruled out based on the observations of Flannery et al. (42) that confirmed the cord blood to contain immunoglobulin G (IgG) without detection of IgM or IgA. The results were verified by other authors (34). Some studies even took a step further to introduce the placenta as a barrier against infection of infants (35, 46). Considering breastfeeding as a vertical transmission mechanism, the same fact applies here (47).

To emphasize on the term “rare”, it is valuable to mention that a few number of cases have been reported “intrauterine transmission”, (48, 49) “placental transmission”, (50, 51) and vertical transmission without mechanistic explanation (52, 53).

Maternal infection and autism disorder

It is noteworthy to mention that women who had an infection during the second trimester of pregnancy accompanied by a fever are more likely to have children with autism disorder (54). Another study showed that higher levels of IFN-γ, IL-4, and IL-5 were significantly associated with increased risk of autism disorder (55). Thus, it appears that increase in cytokines, particularly

IL-6 and IFN-γ during pregnancy may increase the risk of autism disorder.

Effects of SARS-CoV-2 vaccination on maternal health and pregnancy outcomes

The only data available regarding effects of vaccination on outcome of pregnancy, are from population received Pfizer-BioNTech and Moderna messenger ribonucleic acid (mRNA) based vaccines. More than 28,000 women received these types of vaccines during pregnancy. The reactions one day after vaccination was similar in pregnant and non-pregnant women. Of this population, pregnancy outcome in 827 who completed pregnancy was assessed. One-hundred four (12.6%) had spontaneous abortion which 96 (93.2%) occurred before 13 weeks of gestational age. Out of 712 live births, 700 (98.3%) were vaccinated during the third trimester. After spontaneous abortion, pre-term death was the second most common adverse effect with 9.4% incidence (56).

Conclusion

The expression of ACE2 and TMPRSS2 in female reproductive system during menstrual cycle and pregnancy (in all three trimesters) has been proven; yet, the mentioned fact does not necessarily mean that infection with SARS-CoV-2 leads to direct effect on female fertility. We believe that the direct effects of SARS-CoV-2 infection are mainly on maternal health before, during and after delivery period causing increased risk of admitting to ICU, caesarian and post-partum hemorrhage among many other complications. Except the risk of pre-term delivery in symptomatic mothers, no other significant risk is threatening the health of infant/neonate. If any risk exists, it is considered to be rare. Furthermore, vertical transmission from mother to infant/neonate is rare indicating that adverse effects of SARS-CoV-2 on health of infant/neonate is not the consequence of infection in them, rather the consequence of infection in mother and maternal clinical complications.

Nonetheless, the effects of SARS-CoV-2 on female fertility are mainly indirect. The indirect effects are regulated through specific mechanisms, i.e., cytokine storm, psychological disorder and obesity. These mechanisms may lead to increase the risk of pregnancy complications and eventually female infertility.

Safety of SARS-CoV-2 mRNA-based vaccines in pregnant women are not completely verified as pre-term delivery was reported, – although the rate was similar to before pandemic.

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Authors' Contributions

R.N., S.Gh.; Drafted the manuscript. M.H., M.A., B.E., Ab.Sh., M.M.; Contributed in acquisition of data and analysis. An.Sh., P.T.; Critically reviewed the manuscript. M.H.N.-E., M.V.; Involved in conception, design and final approval of the manuscript. All authors read and approved the final manuscript.

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Review Article

Reporting The Effects of Exposure to Monosodium Glutamate on The Regulatory Peptides of The Hypothalamic-Pituitary-Gonadal Axis

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Abstract

Monosodium glutamate (MSG) is a flavour enhancer that is used as a food additive (E621) in many parts of the world, especially in East Asian countries. However, in recent studies, it has been used as a neurotoxin because MSG is reported to cause neural degeneration in the hypothalamic arcuate of neonatal animals. The results of several studies show the negative effects of MSG injections on different parts of the hypothalamic-pituitary-gonadal (HPG) axis, in addition to its ability to inhibit secretion many reproductive neuropeptides, neurotrophic factors, and hormones, all of which play vital roles in the regulation of reproductive function. Oral administration or injection of large quantities of MSG into newborn animals results in a decrease in or overabundance of the production of many regulatory peptides of the male and female reproductive systems. In this review, we summarize the results of the most important studies that have examined the effect of oral consumption or injection of MSG on regulatory peptides of the HPG axis.

Keywords: Hormones, Neuropeptides, Neurotrophic Factors, Reproduction, Sodium Glutamate

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Introduction

Monosodium glutamate (MSG) is a common food additive for many foods from different countries, especially in East Asia. It has a special taste, umami, and is well-known in many countries by the name "savory" or "China salt". MSG is added as a flavour developing agent (E621) in the form of hydrolysed protein or as purified monosodium salt (1).

The growing use of MSG in processed foods is well-documented. It has been reported that more MSG is used in Europe (0.3-0.5 g/day) compared to Asia (1.2-1.7 g/day) (2), and the daily acceptable intake of MSG is proposed to be 30 mg/kg/body weight (BW)/day (3). Despite reports that MSG is safe for consumption (4, 5), the results of several studies show its potential toxicity. Excessive MSG consumption may exacerbate asthma (6) and migraine headaches are related to glutamate (7). Furthermore, the reported correlation between subcutaneous injections of MSG and increases in mRNA expression of interleukin-6 (IL-6), tumour necrosis factor-alpha (TNFα), and resistin, in addition to peroxisome proliferator-activated receptors alpha and gamma (PPARα and γ) and liver transaminases confirms its negative inflammatory and metabolic effects (8, 9). The neurotoxic effects of MSG include destruction of cells in the hypothalamic arcuate nucleus and surrounding areas, which might lead to obesity (10).

There are many indications of the toxic effects of MSG on the male and female reproductive systems. The presence of hypogonadism in MSG-injected mice was corrected by injections of physiological concentrations of oestradiol (11). Moreover, the plasma concentration of inhibin B decreased significantly in MSG-treated male rats (12). Oral gavage of MSG to pregnant mice penetrated the placental barrier and reached foetal tissues. The level of MSG in the foetal brain was twice as high as the maternal brain, and this increase in MSG concentration in the brain reflected negatively on motor tests performed on the newborn mice (13). Moreover, nutrition supplemented with MSG for 40 days caused cytoplasmic vacuolations, swollen mitochondria, and shrunken nuclei in spermatogenic, Sertoli, and Leydig cells, in addition to defects in the tubular basement membrane, damaged germ cells and seminiferous tubules, decreased diameter and height of the lining of the epithelium, and disorders in spermatogenic cells in male albino rats (14). On the other hand, in female virgin rats, it was observed that oral gavage for 30 to 40 days caused significant increases in the duration of the diestrus phase; diestrus index; numbers of primary and primordial follicles; size of the Graafian follicle; and a decrease in the duration of the proestrus, estrus, and metestrus phases and size of the corpus luteum (15).

The mechanism of MSG-induced damage, regardless of the organ or cell type, is explained by the induction of oxidative stress (16). In this phenomenon, the levels of reactive oxygen species increase within the cell, which

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leads to damages to cell proteins, lipids, polysaccharides, and nucleic acids, and results in deficiencies in many cellular activities (17). This increase in oxidative stress in MSG-treated animals was inferred by an increase in enzymatic activity of superoxide dismutase (SOD), glutathione-s-transferase (GST), and catalase in the livers of the treated rats (18). MSG induces apoptosis via lipid peroxidation (LPO) in the arcuate nucleus, hypothalamus, and other circumventricular organs (19). In addition, this oxidative stress drives cells to apoptosis in the rat thymocytes by reducing Bcl-2 expression (20); however, this is accompanied by an increase in intracellular calcium, which triggers a cascade of enzymatic activities and subsequent apoptosis (21) in addition to activation of calcium-dependent protease, calpain and apoptosis-inducing factor (AIF) (22). Remarkably, this toxicity can be reversed after the use of antioxidants (14).

Numerous reports of conflicting results exist about the toxic effects and safety of MSG on the reproductive system, the nervous system, and regulating glands. Therefore, it is important to shed light on the effect of MSG on the production of sex hormones and peptides that regulate the HPG axis to determine its effect on reproductive function. This review article examines studies that assessed the effects of various routes of administration (oral, subcutaneous, intraperitoneal) of MSG on the production of regulatory peptides of the hypothalamic-pituitary-gonadal (HPG) axis.

Monosodium glutamate and the hypothalamic-pituitary-gonadal axis regulatory peptides

Monosodium glutamate and reproductive neuropeptides

Kisspeptin is a critical neuropeptide in mammalian reproductive function because it controls the secretion of gonadotropin-releasing hormone (GnRH) (23). The results of a study indicated that MSG plays an indirect catalytic role for luteinizing hormone (LH) secretion. On the one hand, MSG has a positive role in stimulating kisspeptin neurons (24). On the other hand, the results of recent studies have shown involvement of neurokinin B in regulating the pituitary-gonadal axis (25). Immunotechnology techniques revealed less immunoreactivity for both somata and fibers of neurokinin B-producing neurons within the arcuate nucleus in the neonatal MSG-injected animals compared to control animals (26). Neuropeptide Y (NPY) plays an important role in reproduction because it affects kisspeptin/neurokinin B/dynorphin secreting neurons and the GnRH pathway (27). In general, numerous study results show decreases in hypothalamic NPY levels in MSG-injected animals (28). In other words, levels of NPY in the mediobasal and mediodorsal hypothalamus reduced significantly after neonatal rats were injected with MSG; however, at the same time, its Y1 and Y5 NPY receptors up-regulated (29).

In contrast, expression levels of NPY increased significantly in both the hypothalamus and the pituitary gland in the MSG-treated rats (30). Another study mentioned that treatment of mice with MSG arrested expression of NPY mRNA in the arcuate nucleus and reduced pro-opiomelanocortin (POMC) mRNA in the hypothalamus (31). POMC is a precursor polypeptide that expresses within the hypothalamus, pituitary glands, and brainstem. It is cleaved to various important neuroendocrine peptide derivatives like melanocyte-stimulating hormones (MSHs), adrenocorticotropic hormone (ACTH), and others (32). At the same time, an increase in its production has a negative effect on reproduction; studies have indicated that neonatal MSG-injected rats evoked an increase in POMC expression accompanied by an increase in ACTH expression in the pituitary gland, thus, the appearance of a state of stress that has a negative impact on reproduction (33). Subcutaneous injection of MSG (4 g/kg bw) in rats depleted hypothalamic pro-opiomelanocorticotropin-derived peptides (34).

Galanin, which is produced with its receptors GALR1, GALR2, and GALR3 in the hypothalamus, pituitary and different parts of the male and female reproductive systems and galanin-like peptide, which is a hypothalamic neuropeptide that binds to galanin receptors, are two peptides that play an important role in regulation of metabolism and reproductive function (35). Galanin-like immunoreactivity in the neonatal rats that received subcutaneous injections of MSG (4 g/kg bw) had significant reductions in the median eminence (ME), medial basal hypothalamus, and septal and preoptic regions (36). In addition, immunoreactivity of galanin was completely lost in the arcuate nucleus neurons of female rats that received subcutaneous injections (4 mg/g bw) of MSG (37). Vasoactive intestinal polypeptide (VIP) is a member of a family of neuropeptides and endocrine peptides that have an important role in the control of testosterone levels and testes aging (38), and in the female reproductive system (39). Although subcutaneous treatment of neonatal male Wistar rats with MSG caused an increase of major axes and somatic area of VIP neurons in the suprachiasmatic nucleus (SCN), there was a significant decrease in the VIP-immunoreactive neuronal density (40). This VIP-immunoreactive in SCN increased in another study (41). Agouti-related protein (AgRP) is a neuropeptide produced by the AgRP/NPY neurons of the arcuate nucleus in the hypothalamus and it plays a critical role in female puberty and reproduction via an effect on leptin secretion (42). Neonatal MSG injection nearly erased all AgRP immunoreactivity in the hypothalamus (43). Likewise, disappearance of AgRP immunoreactivity in the arcuate nucleus in mice injected with MSG was reported (Table 1) (44).

Table 1: Effect of MSG on reproductive neuropeptides

Reproductive neuropeptide	MSG effect	References
VIP	+	(41)
	-	(40)
Neurokinin B	-	(26)
Kisspeptin	+	(24)
POMC	+	(33)
	-	(34)
NPY	+	(30)
	-	(28, 29, 31)
Galanin	-	(37)
Galanin-like peptide	-	(36)
AgRP	-	(43, 44)

MSG; Monosodium glutamate, VIP; Vasoactive intestinal polypeptide, POMC; Pro-opiomelanocortin, NPY; Neuropeptide Y, AgRP; Agouti-related protein, +; Positive effect of MSG on neuropeptides, and -; Negative effect of MSG on neuropeptides.

Monosodium glutamate and reproductive neurotrophic factors

Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor thought to be involved in reproductive function because of its widespread expression with receptors located in different parts of the male and female reproductive systems. The results of many studies have indicated its importance in follicles, oocytes, and normal function of spermatozoa (45, 46). A significant down-regulation in the hypothalamic levels of BDNF was observed in induced obesity in male mice that received (3 g/kg bw) subcutaneous MSG injections (47). Nerve growth factor (NGF) is a neuropeptide and neurotrophic factor. In addition to its significance in the central and peripheral nervous systems, it has a role in the reproduction system (48). When adult rats were treated with MSG, the expression levels of *Ngf* increased significantly in both the hypothalamus and pituitary, yet remained unchanged in the adrenal gland (30). The immunoreactivity of low-affinity P75 neurotrophin receptor (p75NR), which is present in the reproductive tract of male rabbits, was reported during sexual maturation (49) p75NR reduced dramatically in the SCN of neonatal rats that received (2 mg/g bw) subcutaneous MSG injections (50).

Monosodium glutamate and reproductive hormones

MSG causes an imbalance in the secretion of many sex hormones by increasing the secretion of some of these hormones or by decreasing the secretion of others (Table 2).

Gonadotropin-releasing hormone

GnRH is produced by the hypothalamus, and it has a controlling role on the pituitary gland to secrete the hormones that regulate gonads. Therefore, disorders in the production of this hormone make the production of gametes almost impossible. The mean serum level of GnRH was significantly lower in rats that received oral or subcutaneous MSG compared to the control group of male albino rats (51).

Table 2: Effect of MSG on reproductive hormones

Reproductive hormone	MSG effect	References
GnRH	-	(51)
FSH/LH	+	(15)
	-	(52, 53)
Leptin	+	(54-56)
Oxytocin	+	(15, 57)
Progesterone	+	(58)
	-	(59)
Oestrogen	+	(15, 60)
Testosterone	-	(51, 53, 56, 59, 61-65)

MSG; Monosodium glutamate, GnRH; Gonadotropin-releasing hormone, FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, +; Sexual hormones positively affected by MSG exposure, and -; Sexual hormones negatively affected by MSG exposure.

In another study, there was considerably less perikarya immunoreactivity in the growth hormone-releasing hormone or LH-releasing hormone (LHRH) neurons of the arcuate nucleus in mice injected with MSG compared to control animals. This was also confirmed by a decrease in size of the anterior lobe of the hypophysis (66).

Follicle-stimulating hormone/luteinizing hormone

There are many reports that highlighted the negative role of MSG on the production and secretion of follicle-stimulating hormone (FSH) and LH; these hormones are critical for the maturation of reproductive organs and the production of male and female gametes. For example, studies have shown that animals injected with MSG have decreased plasma levels of FSH/LH (52, 53). Other studies indicated that the injection or gavage, respectively, of female Sprague Dawley rats with MSG had a negative effect on LH, but not FSH, producing cells in the anterior lobe of the pituitary gland (67, 68). Unlike previous studies that indicated a decrease in the concentration of FSH/LH hormones after oral gavage with MSG, a recent study revealed an increase in anterior pituitary LH and FSH secretion and accompanied by boost secretion of FSHRH and LHRH by paraventricular and supraoptic hypothalamic nuclei, which negatively impacted the reproductive system (15).

Oestrogen/progesterone

Several studies have reported a negative role of MSG on the production of oestrogens, which negatively affects mammalian metabolic and reproductive function. For example, MSG caused an obvious increase in oestradiol production by follicles (15). Adult female Sprague Dawley rats that received oral MSG treatment had a statistical increase in serum oestrogen levels. This effect decreased after administration of Diltiazem, which prevents the toxic effects of MSG (60). In terms of progesterone, which is considered vital for a stable pregnancy, there are contradictory reports about the effect of MSG on progesterone levels. For example, the results of a study indicated that serum progesterone levels in female pups that received subcutaneous injections of MSG were lower

than in the control females (59). The results of other studies indicated that MSG caused an increase in animal hormone production (58). However, in a recent study, the results indicated that there was no considerable effect of MSG on both oestrogen and progesterone production (69).

Testosterone

Mice with hyperleptinemia from injections of MSG had inhibited secretion of testosterone both *in vivo* and *in vitro* (56). In other studies, subcutaneous injections (4 mg/g bw) of MSG not only reduced the concentration of testosterone in the serum of the pups, but also reduced some of its derivatives such as dihydrotestosterone (59). Oral or subcutaneous administration of MSG to male albino rats considerably reduced serum concentrations of both testosterone and total cholesterol (51). In another study, the consumption of high amounts of MSG by male rats led to a notable decrease in the plasma testosterone levels, which led to partial infertility (53, 65). Moreover, there was an increase in corticosterone and a decrease in testosterone, which causes feminization in the MSG-treated male mice (64). Injections of MSG in neonatal male mice led to disorders in the sexual steroids in general, including testosterone (63). Purified Leydig cells from MSG-injected rats showed *in vitro* concentrations of 17-hydroxyprogesterone, delta-(4)-androstenedione, and testosterone that were significantly lower than control cells (62). Probably the negative effect of MSG was a result of the indirect suppression of hepatic enzymes that were vital to the production of testosterone and other sex steroids like cytochrome P450 2A2 (CYP2A2) and cytochrome P450 3A2 (CYP3A2) (61). However, some studies reported that MSG had no significant effect on serum levels of growth hormone (GH), LH, FSH, cortisol, prolactin, oestradiol, or testosterone (67).

Oxytocin

When virgin female Charles Foster rats were gavaged with MSG, the force of the uterine contractions increased significantly, which might be due to increased uterus sensitivity to oxytocin (15). In another study, there were increased oxytocin levels in the SCN, arcuate nucleus, and ME, and a decrease in oxytocin level in the paraventricular nucleus in rats that received intraperitoneal injections (4 mg/g bw) of MSG (57).

Leptin

Adult Siberian hamsters that received subcutaneous injections of MSG in the neonatal stage had higher serum leptin concentrations compared to the control counterparts (55). The same result was obtained in MSG-treated mice (54, 56).

Conclusion

Most MSG studies were conducted on neonatal animals due to incomplete formation of their blood-brain barriers and MSG cannot cross this barrier. However, MSG is toxic when consumed in large quantities. Subcutaneous or intraperitoneal injections or overconsumption of MSG in adult animals leads to obesity, accompanied by an imbalance in the production of regulatory peptides of the HPG axis, which includes neuropeptides, neurotrophic factors, and hormones that can lead to sexual dysfunction and possibly sterility.

In future studies, we propose to investigate the effect of MSG on the secretion of other hormones and regulating neuropeptides, which have not been studied, with emphasis on oral treatment due to its clinical importance because of MSG consumption as a food flavouring.

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Authors' Contributions

M.H.; Contributed to the design, implementation of the research, and writing the aspect related to the regulatory peptides of HPG in the manuscript. R.E.; Contributed to information regarding monosodium glutamate, its uses in the food industry, and some of its harmful effects. H.Kh.; Provided scientific and linguistic supervision of the manuscript, the revision process, and improved the analysis and structure of the manuscript. All authors read and approved the final manuscript.

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Original Article

Functional and Flow Cytometric Analysis of Buffalo Cryopreserved Spermatozoa: Comparison of Different Breeds and Incubation Times

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Abstract

Background: The purpose of this research was to compare the functional parameters of frozen-thawed Iranian Azari buffalo spermatozoa with imported semen samples of Italian Mediterranean buffalo (IMB) after the thawing process and 4 hours of incubation.

Materials and Methods: In this experimental study, a total of twenty-four ejaculates from four Iranian Azari buffalo bulls were collected. Semen samples were diluted in AndroMed extender at a concentration of 50×10^6 spermatozoa/ml. The diluted samples were filled in 0.5 ml straws and were frozen in a programmable freezer. For imported semen samples, twenty-four samples of four IMB were used, which were diluted in AndroMed extender and frozen by the same procedure. Frozen-thawed sperm motion patterns, mitochondrial activity, membrane integrity, DNA integrity, reactive oxygen species (ROS), and apoptosis status were evaluated immediately after thawing and 4 hours of incubation.

Results: Post-thawed sperm motility, progressive motility (PM), mitochondrial activity, membrane integrity were significantly higher in imported semen samples in compare with Iranian Azari buffalo. After 4 hours of incubation, sperm velocity patterns were superior in Iranian Azari semen samples. Moreover, the percentage of sperm cells with un-damaged DNA was higher in Iranian semen samples compared to imported samples at the time 0 of incubation. Following 4 hours of incubation, a significant increase in intracellular ROS level leads to reduced membrane integrity, mitochondrial activity, and DNA integrity in both buffalo breeds. At time 4, Iranian samples showed significantly lower apoptosis and higher dead spermatozoa compared to imported semen samples.

Conclusion: Our study showed that the post-thawed quality of Iranian Azari buffalo semen was comparable with imported samples after 4 hours of incubation. Further investigations are recommended to assess the *in vitro* and *in vivo* fertility rate of both buffalo breeds.

Keywords: Buffalo, Flow Cytometric Analysis, Iranian Azari Buffalo Breed, Italian Mediterranean Buffalo Breed, Sperm Cryopreservation

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Introduction

The current population of the buffalo is estimated to be around 200 million worldwide. Approximately 204,000 head of buffalo population is bred in Iran, where it provides about 2.8% and 2.5% of Iran's total milk and meat production, respectively (1). Some characteristics of the buffalo, including the production of high-quality milk, high adaptability to harsh climate conditions, high resistance to diseases, ability to consume the low-quality forage, as well as long productive life, made this animal valuable livestock (2, 3). However, a little consideration has been paid for the buffalo's breeding programs in Iran regarding improving their milk and meat production.

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Genetic improvement programs of buffalo received considerable attention in Italy (4). The Italian Mediterranean buffalo (IMB) is considered the best buffalo in the world, which has the highest high-fat milk production (average of 8.72 kg for Italian compared to 5.71 for Iranian Azari buffaloes during lactation period) (1, 5, 6). Moreover, it is the only breed of buffalo that has gone through a breeding selection program. The progeny test has also been accomplished to select high genetic value (high milk and meat production) bulls that are transported into semen collection centers for sperm freezing and artificial insemination (AI) (7).

Thanks to the progress of AI, it has been made possible to rapid improvement of genetic material through the propagation of desired genes from high genetic merit animals (8). The benefits of AI procedure have also been doubled by the successful freezing of the semen samples without comprising sperm quality and reducing fertilizing capability (9). Although, the fertility rate of post-thawed buffalo sperm under the field condition is poor (30% frozen-thawed vs. 60% fresh), and farmers are reluctant to breed buffalos by using AI procedure (9, 10).

Low fertility rate following AI procedure is another challenge. It may be due to sperm susceptibility to cryopreservation associated damages as well as female factors such as variable estrus length and estrus detection difficulties (11). Therefore, insemination timing and frozen-thawed spermatozoa quality play a prominent role in achieving desired results. In most previous studies, sperm quality was evaluated immediately after thawing (12-14). It seems that the post-thawed spermatozoa incubation for longer periods can broaden our understanding of the spermatozoa fertilizing capability. Several *in vitro* assessments have been developed for predicting the fertility potential of cryopreserved bull semen in AI procedure. Conventional semen assessments fail to detect some functional sperm impairments which are responsible for low fertility rate following AI procedure (15). Here, we aimed to evaluate motion characteristics, mitochondrial activity, membrane integrity, reactive oxygen species (ROS), DNA integrity, and apoptosis status of Iranian Azari and IMB semen samples during 4 hours of incubation.

Materials and Methods

This study was approved by Institutional Ethics Committee of Royan Institute (IR.ACECR.ROYAN.REC. 1395.143).

Semen collection and cryopreservation

The semen collection of Iranian Azari buffaloes was performed in Buffalo Breeding Center, Urmia, Iran. Twenty-four ejaculates from four mature buffalo bulls (*Bubalus bubalis*), were collected. The semen samples with the volume of 2-6 ml, progressive motility (PM) 70%, and the concentration of more than 1×10^9 spermatozoa/ml were enrolled in the study (12). The sperm concentration was determined by a digital photometer (IMV, France) and were diluted in AndroMed extender at a concentration of 50×10^6 spermatozoa/ml according to the manufacturer's instructions (Mintube, Germany) (16). The diluted samples were cooled to 4°C in 2 hours, and were left to equilibrate at 4°C for 2 hours. Then, samples were filled in 0.5 ml straws and were frozen in a cell freezer according to digit-cool (IMV Technologies, France) standard curve for bull semen (- 5°C/minutes from+4°C to -10°C; -40°C/minutes from -10°C to -100°C and -20°C/minutes from -100°C to 140°C). Also, twenty-four samples of four IMB were used, which were diluted in AndroMed extender and frozen by the same procedure. The frozen samples were thawed at 37°C for 30 seconds. Half of the samples were immediately analyzed after thawing (time 0), while the remainder was incubated at 37°C for 4 hours in a 5% CO_2 and analyzed after incubation (time 4).

All assessments were performed at the Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute, ACECR, Tehran, Iran.

Motion parameters

The motion parameters of Iranian Azari and imported frozen-thawed buffalo sperm with ~ 50×10^6 spermatozoa/ml concentration were analyzed by a computer-aided sperm analyzer (CASA, Sperm Class Analyzer, version 5, Microptic, Spain). The CASA system was adjusted for bull semen, according to Topraggaleh et al. (12). A 10 µl aliquot of semen sample was placed on a pre-warmed SpermTrack ® chamber (Proiser, Spain) and sperm motion characteristics, including total motility (TM, %), PM (%), curvilinear velocity (VCL, µm/seconds), straight line velocity (VSL, µm/seconds), average path velocity (VAP, µm/seconds), straightness (STR, %), linearity (LIN, %), amplitude of lateral head displacement (ALH, µm/seconds), wobble (WOB, %), and beat cross frequency (BCF, Hz) were analyzed in 5 randomly-selected microscopic fields with approximately 500 spermatozoa.

Membrane integrity

The sperm functional membrane integrity was assessed by hypo-osmotic swelling (HOS) test. Briefly, 50 µl of frozen-thawed samples were diluted in 500 µl of HOS solution [1.351 g fructose (1.05321, Merck, Germany) and 0.735 g sodium citrate dehydrate (W302600, Sigma-Aldrich, USA) were dissolved in 100 ml of distilled water; 190 mOsm/kg] and incubated for 45 minutes at 37°C in a 5% CO_2 (17). Afterward, 10 µl of suspension was placed on a glass slide and mounted with a coverslip. A total of 200 sperm cells were analyzed under a phase-contrast microscope (Olympus BX20) at a magnification of 400×. Sperm cells with coiled or swollen tail were considered as the functional plasma membrane.

Flow cytometry analysis

Using the FACS Calibur flow cytometer (BD Immuno-

cytometry Systems, USA), mitochondrial activity, DNA fragmentation, intracellular ROS, and apoptosis were analyzed. Imaging was made under excitation of an argon laser at 488 nm. To exclude debris and aggregates, the sperm cell population was gated using 90° and forward-angle light scatter. The green fluorescence (intact DNA and low mitochondrial activity) was measured with FL1 detector (530 nm), while the red fluorescence [damaged DNA, propidium iodide (PI)] was measured with FL3 detector (620 nm). The fluorescence of multimeric form of JC-1 (high mitochondrial activity) and Dihydroethidium (DHE) were determined with FL2 detector (585 nm). A minimum of 10.000 sperm cells was assessed in each sample at the flow rate of 100 cells/s and analyzed by Flowing Software version 2.5.1 (Cell Imaging Core, Finland).

Mitochondrial activity

The sperm mitochondrial activity was investigated using, JC-1 dye (T4069, Sigma-Aldrich, USA). Briefly, post-thawed semen specimens were centrifuged at 500 g for 5 minutes. The supernatant was removed, and cell pellets were resuspended in phosphate-buffered saline (PBS) at a final concentration of 1×10^6 cells/ml. Then, 1 µL of JC-1 stock solution [200 µM dissolved in DMSO (D2650, Sigma-Aldrich, USA)] was added into 1 ml of cell suspension, incubated at 37°C for 40 minutes in a dark place, and cells were finally subjected to flow cytometry (11).

DNA integrity

Sperm DNA damage was measured according to the sperm chromatin structure assay protocol. Post-thawed semen samples were centrifuged at 500 g for 5 minutes, supernatants were discarded, and remaining cells were diluted with Tris Null EDTA buffer (150 mM NaCl, 1 mM EDTA, and 10 mM Tris at pH=7.2) at a final concentration of 5×10^6 cells / ml. Then, 400 µl of acidic solution (0.15 M NaCl and 0.08 M HCl in 0.1% v/v Triton X-100) was added to 200 µl of diluted samples. After 30 seconds incubation, 1.2 ml of acridine orange (AO) solution [6 µg/ml AO (A8097, Sigma-Aldrich, USA), 0.1 M citric acid, 1 mM EDTA, 0.2 M Na_2HPO_4, and 0.15 M NaCl at pH=6.0] was added. Finally, cells were subjected to flow cytometry after 30 minutes incubation (18).

Reactive oxygen species

Intracellular ROS was determined by DHE. In brief, post-thawed semen samples were resuspended with PBS at a concentration of 1×10^6 cells/ml. An aliquot of 10 µl of DHE stock solution (1.25 mM, D 7008, Sigma-Aldrich, USA) was added into 1 ml of diluted semen samples, incubated at 25°C for 20 minutes, and subjected to flow cytometry (19).

Apoptosis status

Apoptosis status of frozen-thawed spermatozoa was determined by the double-stained method with Annexin V-fluorescein isothiocyanate (FITC) and PI according to the manufacturer's directions (IQP, Groningen, Netherlands). Sperm samples were washed in calcium buffer, and concentration was adjusted to 1×10^6 cells /ml. An aliquot of 10 µl of Annexin V-FITC (0.01 mg/ml) was added to 100 µl of sperm samples and incubated for 20 minutes on ice. Then, 10 µl of PI (1 µg/ml) was mixed with sperm suspension and incubated for 10 min on ice prior to evaluation with flow cytometry. Following analysis, sperm cells were classified into three categories: i. Annexin V and PI negative considered as viable non-apoptotic cells, ii. Annexin-V positive, but PI negative marked as apoptotic cells, and iii. Positive for both Annexin-V and PI as well as negative for Annexin-V but positive for PI were regarded as dead cells (20).

Statistical analysis

In statistical evaluation, the variance in repeated measurements was evaluated by the procedure GLM repeated measurement. Bull and origin were taken as factors. The result of different times, T0 and T4 (4 hours), were compared. A sample dependent t-test was used for two-group comparisons, and the Bonferroni test was used for multiple comparisons. All parameters were analyzed using the SPSS/PC software package (IBM SPSS Statistics Inc. version 25.0, Chicago, IL). The Statistical significance was set at $P<0.05$.

Results

Motion characteristics

Post-thawed Italian Mediterranean and Iranian Azari buffalo semen motion characteristics are displayed in Table 1. In both buffalo breeds, post-thawed sperm motion parameters, including TM, PM, VAP, VSL VCL, ALH, and BCF were significantly decreased following 4 hours of incubation. At the time 0, sperm characteristics, including TM, PM, VSL, STR, and ALH were significantly higher in Italian buffalo semen compared to Iranian samples. However, after 4 hours of incubation, statistical significant differences were not seen in TM, PM, VCL, VSL, and VAP between imported and Iranian buffalo semen samples. Iranian Azari buffalo semen showed significantly higher LIN, STR, and WOB compared to imported samples after 4 hours of incubation.

Membrane integrity, mitochondrial activity, DNA integrity, and ROS

As shown in Table 2, post-thawed membrane integrity and mitochondrial activity were significantly decreased during 4 hours of incubation in both of the buffalo breeds. However, the percentage of cells with intracellular ROS and damaged DNA were significantly increased following incubation in both the Italian Mediterranean and Iranian Azari buffalo semen samples. Italian buffalo semen samples showed significantly higher membrane integrity and mitochondrial activity compared to Iranian buffalo samples immediately after thawing. However, the percentage of sperm cells with fragmented DNA was significantly lower in Iranian Azari samples compared to imported straws at time 0 of incubation (4.60 ± 0.16 vs. 5.58 ± 0.20). No statistically significant differences were observed in all parameters among the Italian Mediterranean and Iranian Azari buffalo straws after 4 hours of incubation.

Table 1: Sperm motion characteristics between native and imported semen samples during 0 and 4 hours of incubations

Variables	Iranian Azari		Italian Mediterranean	
	0 hour	4 hours	0 hour	4 hours
TM (%)	56.77 ± 2.02^{aB}	33.69 ± 1.87^{b}	66.27 ± 1.78^{aA}	38.51 ± 2.43^{b}
PM (%)	41.19 ± 2.35^{aB}	19.44 ± 1.41^{b}	48.54 ± 2.57^{aA}	20.85 ± 1.77^{b}
VCL (µm/s)	69.55 ± 3.71^{a}	38.31 ± 2.40^{b}	78.39 ± 2.69^{a}	41.70 ± 3.10^{b}
VSL (µm/s)	37.24 ± 2.13^{aB}	21.26 ± 1.57^{b}	44.95 ± 2.44^{aA}	19.60 ± 2.46^{b}
VAP (µm/s)	52.51 ± 3.17^{a}	29.63 ± 2.47^{b}	59.70 ± 2.79^{a}	29.59 ± 3.59^{b}
LIN (%)	53.28 ± 0.72	54.42 ± 1.40^{A}	56.85 ± 2.12^{a}	42.20 ± 3.54^{bB}
STR (%)	71.26 ± 0.50^{B}	72.98 ± 1.16^{A}	74.70 ± 1.34^{aA}	63.63 ± 1.68^{bB}
WOB (%)	74.75 ± 0.89	74.81 ± 1.93^{A}	75.52 ± 1.62^{a}	64.29 ± 4.42^{bB}
ALH (µm)	2.60 ± 0.07^{aB}	1.72 ± 0.02^{bA}	2.98 ± 0.08^{aA}	1.93 ± 0.05^{bB}
BCF (Hz)	7.89 ± 0.16^{a}	7.47 ± 0.11^{b}	7.95 ± 0.17^{a}	7.22 ± 0.25^{b}

Data are presented as the mean ± SE. Small letters (a, b) in same raw indicate significant differences (P<0.05) between the 0 and 4 hours of incubation times, capital letters (A and B) in same raw indicate significant differences (P<0.05) between native and imported semen samples in the same time. TM; Total motility, PM; Progressive motility, VCL; Curvilinear velocity, VSL; Straight line velocity, VAP; Average path velocity, LIN; Linearity, STR; Straightness, WOB; Wobble, ALH; Lateral head displacement, and BCF; Beat cross frequency.

Table 2: Sperm membrane integrity, mitochondrial activity, DNA integrity and ROS between native and imported semen samples during 0 and 4 hours of incubations

Variables	Iranian Azari		Italian Mediterranean	
	0 hour	4 hours	0 hour	4 hours
Membrane integrity (%)	66.56 ± 2.26^{aB}	43.64 ± 2.50^{b}	73.96 ± 1.72^{aA}	47.76 ± 1.88^{b}
Mitochondrial activity (%)	37.15 ± 1.90^{aB}	19.38 ± 0.77^{b}	42.63 ± 1.83^{aA}	20.29 ± 0.92^{b}
DNA fragmentation (%)	4.60 ± 0.16^{bB}	6.73 ± 0.32^{a}	5.58 ± 0.20^{bA}	7.33 ± 0.39^{a}
ROS (%)	49.72 ± 1.66^{b}	63.68 ± 2.62^{a}	49.65 ± 2.10^{b}	65.50 ± 2.38^{a}

Data are presented as the mean ± SE. Small letters (a, b) in same raw indicate significant differences (P<0.05) between the 0 and 4 hours of incubation times, capital letters (A and B) in same raw indicate significant differences (P<0.05) between native and imported semen samples in the same time. ROS; Reactive oxygen species motility.

Table 3: Percent of live, early apoptosis, late apoptosis and necrosis between native and imported semen samples during 0 and 4 hours of incubations

Variables	Iranian Azari		Italian Mediterranean	
	0 hour	4 hours	0 hour	4 hours
Membrane integrity (%)	59.79 ± 2.09^{a}	24.98 ± 1.34^{b}	55.94 ± 1.78^{a}	26.38 ± 1.52^{b}
Mitochondrial activity (%)	10.30 ± 0.59^{b}	12.27 ± 0.60^{aB}	9.77 ± 0.67^{b}	16.32 ± 0.70^{aA}
DNA fragmentation (%)	29.49 ± 2.22^{b}	62.74 ± 1.56^{aA}	34.28 ± 1.83^{b}	57.28 ± 1.84^{aB}

Data are presented as the mean ± SE. Small letters (a, b) in same raw indicate significant differences (P<0.05) between the 0 and 4 hours of incubation times, capital letters (A and B) in same raw indicate significant differences (P<0.05) between native and imported semen samples in the same time.

Apoptosis status

The apoptosis status of frozen-thawed Italian Mediterranean and Iranian Azari buffalo semen is presented in Table 3. Following the incubation of semen samples, the percentage of live cells significantly decreased. Nevertheless, the percentage of dead spermatozoa, and also apoptotic spermatozoa were significantly increased during 4 hours of incubation in both buffalo semen samples. There were no significant differences in apoptosis status at time 0 between buffalo samples. However, at time 4, Iranian Azari samples showed significantly lower apoptosis and higher dead spermatozoa compared to Italian Mediterranean semen samples.

Discussion

In order to increase the milk production potential of Iranian buffalo, the Animal Breeding Center of Iran (ABCI, Karaj, Iran) imported semen samples of high genetic merit IMB bulls. However, follow-up of the inseminated samples, rate of fertilization as well as *in vitro* assessment of frozen-thawed imported samples were not precisely evaluated. For the first time, we compared *in vitro* characteristics of frozen-thawed sperm between Italian Mediterranean and native Iranian Azari buffalos.

The present study's data showed that sperm characteristics, including motion parameters, membrane integrity, and mitochondrial activity, were significantly higher in Italian spermatozoa compared to the Iranian Azari group. As expected, the quality of frozen-thawed spermatozoa could be influenced by the age, feeding, and housing conditions as well as environmental factors, including humidity, temperature, and day length (21, 22). Moreover, semen processing, including dilution, type of extender, equilibration, freezing, and thawing, influences the qual-

ity of frozen-thawed buffalo spermatozoa (13, 23, 24). A growing body of literature has shown that the quality of post-thawed buffalo spermatozoa has been diminished by increasing the temperature in tropical and subtropical countries during the years (21, 25, 26). Although we tried to minimize the extrinsic factors (such as feeding and housing condition, semen processing) of two study groups, the average temperature of the Iranian buffalo breeding place was higher than Italian ones during the semen collection period (February-April). Thus, the lower post-thawed quality of Iranian buffalo spermatozoa could be attributed to genetic differences between the two studied breeds as well as environmental conditions, higher temperature and lower weather humidity.

Another finding of this study was that incubation of post-thawed semen samples for 4 hours significantly decreased motility, velocity patterns, membrane integrity, and mitochondrial activity as well as increased intracellular ROS and DNA fragmentation in both of imported and native semen samples. These findings are in accordance with the results of Rastegarnia et al. (24), where incubation of post-thawed buffalo spermatozoa for 4 hours significantly decreased the sperm quality in soybean lecithin and egg yolk based extenders. In the nature, semen is deposited in the vagina near the external os of the cervix. Sperm cells may be transported to the site of fertilization in two phases. Rapid phase in which a lower quantity of sperm cells is transported to the ampulla region within a few minutes. And slow phase in which a large number of sperm cells move toward the oviduct over the 4-8 hours. This time of sperm transport to the site of fertilization is decreased to 30 minutes following AI procedure due to sperm deposition in the uterus' horns. Although assessments of sperm parameters immediately after thawing indicate sperm quality to some extent, *in vitro* incubation of thawed semen samples for longer periods of time broaden our understanding of spermatozoa's fertilizing capability. A large amount of the ROS is generated by the electron transport chain of mitochondria during the spermatozoa incubation (27). A high level of ROS, along with insufficient antioxidant defenses, leads to oxidative stress in spermatozoa (28). Excess production of ROS induces structural and biochemical alteration, including depletion of ATP, DNA fragmentation, and lipid peroxidation in spermatozoa (29). Therefore, decreased sperm motility, membrane integrity, mitochondrial activity, and DNA integrity are likely to be related to increased intracellular ROS levels during incubation.

Frozen in different semen collection condition was one of the major limitations of this study. Although we tried to minimize the differences between the semen processing and freezing of the both buffalo bulls, intrinsic factors like the genetics of the bulls could also affect the quality of frozen-thawed spermatozoa. Another limitation of this study was that, the comparison of the samples was performed only by analyzing sperm *in vitro* characteristics. Pregnancy rate, as well as delivery of live offspring following insemination of both semen samples, was not evaluated in this study. The main reason behind this problem is that the farming system of buffalo in Iran is based on small holders (99%) with an average herd size of five animals (1). Since management and environmental factors could differ between the buffalo breeders, comparison of pregnancy and delivery rate following insemination of Iranian Azari and IMB samples could be challenging. Therefore, in vivo studies on a larger buffalo population are required to investigate the pregnancy rate following insemination of these two buffalo breeds.

Conclusion

This study has shown that post-thawed sperm characteristics, including motility, PM, membrane integrity, and mitochondrial activity were significantly higher in Italian Mediterranean semen samples compared to Iranian Azari buffalo semen immediately after thawing. While after 4 hours of incubation, sperm velocity patterns were superior in Iranian Azari semen samples. Moreover, the percentage of sperm with intact DNA was higher in Iranian semen samples than imported samples at the time 0 of incubation. At time 4, Iranian samples showed significantly lower apoptosis and higher dead spermatozoa compared to imported semen samples. Our study indicated that the Iranian Azari buffalo semen were comparable to imported samples after 4 hours of incubation. One of the major limitations of this study was that the comparison of the Italian Mediterranean and Iranian Azari buffalo semen samples was performed only by analyzing sperm *in vitro* characteristics. Further studies are required to evaluate the *in vivo* and *in vitro* fertility rate of both buffalo breeds.

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Authors' Contributions

T.R.T., M.N.B., Y.K., A.Sh.; Participated in study design, data collection and evaluation, drafting and statistical analysis. A.K.-A.; Performed sample collection and semen freezing. A.M.A., P.R., M.Sh.; Conducted semen evaluation and flow cytometry analysis. A.F.B., P.I.; Contributed extensively in the data interpretation. M.Gu., M.Ga.; Performed statistical analysis. All authors performed editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript and approved the final draft.

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Original Article

Effects of Dietary Pomegranate Peel on Antioxidant Gene Expression and DJ-1 Protein Abundance in Ram Testes

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Abstract

Background: Pomegranate is an ancient fruit containing Punicalagin, which has known as an effective antioxidant. Pomegranate peel was recognized as a phenol and tannin source, and pomegranate seed contains unique fatty acid (Punicic acid). Limited information exists about the influences of pomegranate peel and seed on antioxidant enzymes and proteins in the male reproduction system. This study was performed to determine the pomegranate peel and seed effects on the expression of antioxidant genes and DJ-1 protein in ram's testis.

Materials and Methods: In this experimental study, twenty-one mature Iranian rams were randomly divided into three groups (n=7 in each group), and fed experimental diets consisted of a control diet (C), a diet containing dry pomegranate seed pulp (S), and a diet containing pomegranate peel (P) for 80 days. All rams were offered isoenergetic and isonitrogenous rations. Testicular tissue samples were collected, and expression of *Gpx1*, *Gpx4*, *Prdx4*, *Prdx5*, and *Sod2* genes was quantified by real-time polymerase chain reaction (RT-PCR). In addition, western blotting was used to evaluate DJ-1 expression at the protein level.

Results: *Gpx1* and *Sod2* mRNA levels in the peel group were significantly ($P<0.05$) higher than control. *Prdx5* mRNA level was increased ($P<0.05$) in the seeds group than in the control group. *Gpx4* and *Prdx4* expression were statistically not affected significantly by the experimental diet. Data analysis showed a significant ($P<0.05$) increase (1.5-fold) in the expression level of DJ-1 in peel groups than in control.

Conclusion: The expression of antioxidant genes and DJ-1 protein in ram testes are more influenced by pomegranate peel than seed.

Keywords: Antioxidant Genes, Pomegranate, Ram Testes

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Introduction

Infertility is a critical problem in couples' lives who are demanding a child. At present, near 7% of couples in the world are infertile, and half of these cases are related to male factors (1). In recent decades, extensive research and numerous publications have demonstrated pathological levels of reactive oxygen species (ROS) and oxidative stress (OS) following the weak potential of defense in the seminal plasma leading to spermatozoa damage and male infertility (2). In addition to the harmful effects of OS on sperm classical parameters like motility, morphology, and DNA, a high level of ROS can impair spermatogenesis and sperm maturation by the alternation of *H19* gene methylation (3).

Antioxidants enzymes are the only mechanism that protects sperm against the damaging effects of OS. Superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) are well-known enzymatic antioxidants in the seminal plasma and sperm, encoded by *NRF2* (4), *SOD*, *CAT*, glutathione S-transferase (*GST*), and glutathione peroxidase (*GPX*) genes. Alteration in these genes may lead to male infertility, and it seems that the improved activity of

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these genes may improve male fertility (5).

Antioxidant therapy and oral antioxidants are the most frequent suggestions to control male infertility induced by OS. In an ideal world, oral antioxidants in high concentration could improve spermatogenesis in the reproductive tract. Besides, they should increase the capacity of the seminal plasma clearance and leads to the reduction of ROS level in semen [reviewed by (6)]. In addition to synthetic antioxidants, herbal antioxidants such as Saffron (*Crocus sativus*) and Pomegranate (*Punica granatum*) positively affected sperm parameters such as motility and morphology (7, 8). Pomegranate from the *Punicacea* family is an ancient and aboriginal fruit of Iran known as a great antioxidant and used to treat several diseases such as dysentery or respiratory pathologies. Pomegranate has numerous polyphenols, including anthocyanins, minor flavonoids, and punicalagin in the peel, seed, and juice (9). The limited evidence available regarding the role of each component of this fruit (peel or seed) and its possible mechanisms related to successful therapy for male infertility. Some reports have shown that daily consumption of pomegranate juice increases the number of spermatogonia cells, sperm motility and decreases sperm lipid peroxidation in male rats (10). Moreover, using pomegranate juice could increase the expression level of *Sod*, *Gpx*, *Gst*, and *Gsh* antioxidant enzymes in male rat testis (11). On the other hand, dietary supplementation of pomegranate seed for cloned male goats could decrease OS and improve sperm motility, viability, and following sperm cryopreservation in this species (12).

Nevertheless, a higher proportion of presented data in papers is based on pomegranate juice consumption in animal models; peel or seed's effect and antioxidant effects on testis are still unclear. Furthermore, to our best knowledge, whether the inclusion of pomegranate peel (the source of polyphenols and tannins) or pomegranate seed (the source of unique fatty acid [FA]; Punicic acid) in the diet affects gene expression in testes has not been addressed.

With this background, the present research was performed to study the antioxidant effects of pomegranate peel, and seed on the expression of *Gpx1*, *Gpx4*, *Prdx4*, *Prdx5*, and *Sod2* genes and DJ-1 protein in testis following feeding rams with a daily diet contained pomegranate peel or pomegranate seed.

Materials and Methods

Preparation of pomegranate

In this experimental study, the pomegranate peels were freshly provided by the Sunich (Saveh, Iran). Pomegranate peels silage was prepared by mixing 95% pomegranate peels, 3% wheat straw, and 2% urea. Dried pomegranate seeds were bought from a local factory (Narni, Neyriz, Iran). Total tannins and phenolic compounds were measured by methods which are defined by Makkar (13) at Animal Science Research Institute of Iran, Karaj, Iran. The total phenol content and total tannin content of pomegranate peel were 3.09 and 1.81 percent, respectively. FA profiles in peels and seeds were determined at the Institute of Medicinal Plants, ACECR, Karaj, Iran by Gas Chromatography/Mass Spectrometry (GC/MS) (Agilent GC 6890 system, Agilent Technologies Co., Hewlett Packard, Wilmington, DE, USA) (Figs.S1, S2, Table S1, See Supplementary Online Information at www.ijfs.ir).

Animals and experimental design

Following approval of study protocol by the Ethics committee of Royan Institute (IR.ACECR.ROYAN.REC.1395.143); twenty-one Iranian fat-tailed rams (8 months of age; 27.03 ± 3.5 kg body weight) were housed in individual pens under a protective condition in the Animal Research Station, College of Agriculture, Shiraz University, Shiraz, Iran.

Rams were randomly divided into three groups (n=7 in each group); group I: control (basal diet without supplements), group II: pomegranate peel group (a diet containing 27% pomegranate peels silage), group III: pomegranate seed group (a diet containing 31% pomegranate seeds). All rations were isoenergetic and isonitrogenous. Animals received the diet as a total mixed ration (TMR) (according to National Research Council requirements of sheep and goats [NRC, 2007]), twice daily at 08:00 and 17:00 hours for 80 days (Table S2, See Supplementary Online Information at www.ijfs.ir). The first twenty days of the experiment was the adaptation period.

Sample collection, RNA isolation, and real-time quantitative reverse transcription polymerase chain reaction

At the end of the experimental period, all rams were sacrificed. Testis was collected, snap-frozen in liquid nitrogen (-196°C), and stored at - 80°C till further processing. Total RNA was isolated from testis tissues using TRIzol reagent (Invitrogen, USA) and dissolved in RNase-free water. The quantity and quality of extracted RNA were checked by a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., USA) and gel electrophoresis. Only samples with the A260/A280 ratio between 1.8 and 2 were used in this study. Two micrograms of total RNA from each sample were reverse transcribed into complementary DNA (cDNA) with random Hexamer primers using primeScriptTM1st strand cDNA synthesis kit (Takara, USA). The cDNA synthesized was kept at -20°C until needed. The transcript of selected genes was detected by real time-PCR step one plus Applied BioSystems and using SYBR Green qPCR Master Mix (Takara, USA). The optimal conditions of each reaction were as follows: pre- denaturation at 95°C for 10 minutes, denaturation at 95°C for 10 seconds (40 cycles), annealing at 60°C for 20 seconds, and extension at 72°C for the 20 seconds. Fold changes of expression were calculated using the $2^{-\Delta\Delta ct}$ method. Relative expression levels of target genes were normalized by *GAPDH* as the housekeeping gene (Table 1).

Table 1: Sequences of primers for the real-time polymerase chain reaction experiments

Gene	Primer sequence (5'-3')	Size (bp)
Sod	F: CTGCAAGGAACAACAGGTCT R: TTGGTGTACTTGGTGTAAGGC	190
Gpx1	F: GGACTACACCCAGATGAATGACC R: CGTTCTTGGCGTTTTCCTGATG	107
Gpx4	F: CGCAATGAGGCAAGACTGACG R: CGCATTACTCCCTGGCTCCTG	131
Prdx4	F: AAGGACTATGGCGTATATCTGGAA R: GGGCAGACTTCTCCGTGTTT	182
Prdx5	F: GGGAAGGAGACAGATTTGTTAC R: CACATTCAGGGATTTGACGAT	114
Gapdh	F: GGAGAAACCTGCCAAGTATG R: TGAGTGTCGCTGTTGAAGTC	126

Evaluation of DJ-1 protein level

Protein was extracted from frozen testis tissue samples using the TRIzol extraction method. Total protein concentration was measured using the Bradford reagent with human serum albumin (HSA) as the standard protein. An equal amount (30 µg) of total protein from each sample was separated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 110 v for 3 hours and were then transferred onto PVDF membranes (Bio-Rad, USA) at 12 v for 16 hours. The membranes were blocked with 2% non-fat dry milk in TBST solution (20 mM Tris-HCL pH=7.4, 15 mM NaCl, and 0.1 TWEEN-20) for 1 hour at room temperature under agitation. The blots were incubated with primary antibody (Anti-PARK7/DJ1, 1:10000, Abcam, USA) and Goat anti-Mouse IgG (H+L) Poly-HRP Secondary Antibody, HRP (1:60000, Thermofisher, USA) for 1h at room temperature.

Protein visualization was carried out using the enhanced chemiluminescence (ECL) detection system (Amersham, ECL Healthcare Life Sciences, Little Chalfont, UK). The intensities of protein bands on the scanned X-ray films were quantified using the ImageJ software version 1.50i (US National Institutes of Health, Bethesda, USA). The changes in the DJ-1 level were normalized against β-actin as a housekeeping protein.

Statistical analysis

Data were expressed as mean ± SEM. The normal distribution of data was confirmed by Kolmogorov-Smirnov's test. Various parameters were compared to each other using One-way ANOVA. Multiple comparisons were performed using Post-hoc: Tukey range's test. Statistical analysis was performed with the SPSS 20 software for Windows (SPSS Inc., Chicago, IL, USA). P<0.05 was considered statistically significant. The authors confirm that the data supporting the findings of this study are available within the article.

Results

Gene expression level of *Sod2*, *Gpx1*, *Gpx4*, *Prdx4*, and *Prdx5*

According to statistical analysis, the pomegranate peel group showed a significant increase in the expression level of *Sod2* (1.25 ± 0.06, P=0.01) and *Gpx1* (1.21 ± 0.06, P=0.02) in testes compared to the control group (Figs.1, 2). Contrary to *Sod2* and *Gpx1*, *Prdx5* level was significantly higher in the fed group with seed than the control group (1.17 ± 0.06, P=0.02, Fig.3). Nonetheless, mRNA abundance of *Gpx4* and *Prdx4* were not significantly affected by pomegranate peel or pomegranate seed inclusion in the diet compared to the control group (Figs.S3, S4, See Supplementary Online Information at www.ijfs.ir). Moreover, the comparison of pomegranate peel and seed results did not show any notable difference between these two groups.

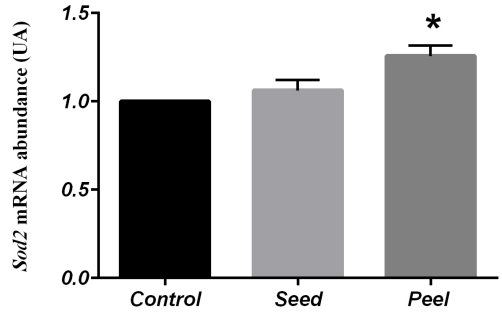

Fig.1: Comparison of *Sod2* gene expression in control and fed groups with pomegranate seed and pomegranate peel using One Way-ANOVA (Post-hoc test: Tukey's range test). Data represented as mean values ± SEM. *; Indicates significant difference among the evaluated groups (P<0.05).

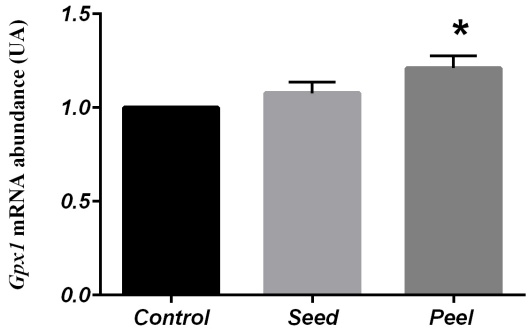

Fig.2: Comparison of *Gpx1* gene expression in control and fed groups with pomegranate seed and pomegranate peel using One Way-ANOVA (Post-hoc test: Tukey's range test). Data represented as mean values ± SEM. *; Indicates significant difference among the evaluated groups (P<0.05).

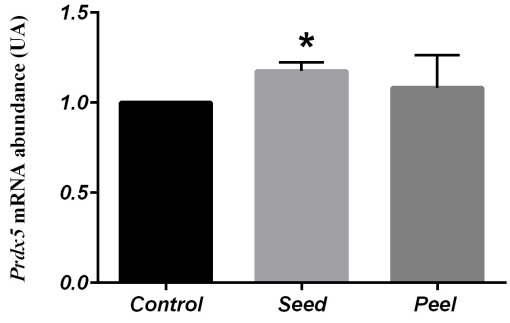

Fig.3: Comparison of *Prdx5* gene expression in control and fed groups with pomegranate seed and pomegranate peel using One Way-ANOVA (Post-hoc test: Tukey's range test). Data represented as mean values ± SEM. *; Indicates significant difference among the evaluated groups (P<0.05).

Level of DJ-1 protein

DJ-1 protein was elevated by pomegranate peel following the western blotting procedure (1.52 ± 0.22, P=0.04). However, the protein levels were unaltered by pomegranate seed (Figs.4, 5).

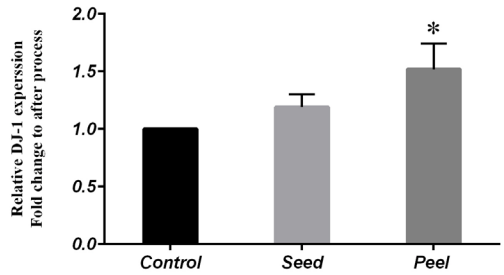

Fig.4: Comparison of relative DJ-1 expression in control and fed groups with pomegranate seed and pomegranate peel using One Way-ANOVA (Post-hoc test: Tukey's range test). Data represented as mean values ± SEM. *; Indicates significant difference among the evaluated groups (P<0.05).

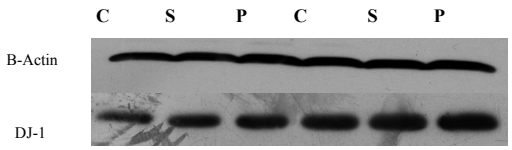

Fig.5: Western blot analysis of DJ-1 protein in control (C) and fed group with pomegranate seed (S) and peel (P).

Discussion

This study provides compelling evidence on the effective use of pomegranate peel on the relative expression of various antioxidant genes in rams' testes. OS is one of the critical factors of male infertility that can disrupt testicular function and affect spermatogenesis. Antioxidants are the first line of protection in front of this unfavorable incident that can save testis and sperm against excessive ROS damage. Nowadays, consuming natural antioxidants, especially pomegranate products, as an aborigine group in the Middle East is one of the researchers' interests in improving fertility in males. To better understand the pomegranate antioxidant mechanism on the testis, in the present study, pomegranate seed and peel effects were separately determined on antioxidant gene expression in Iranian fat-tailed rams testis, which fed pomegranate by-products.

Although the expression of *Sod2*, *Gpx1*, *PrdX5* antioxidant genes, and DJ-1 protein had affected by adding seed and peel of pomegranate to the regular feeding plan of rams, the effect of peel was more notable about these genes' expressions. Pomegranate has a high amount of polyphenol component of ellagitannins, especially punicalagin, as the most abundant soluble component of the peel with more than 50 percent potent antioxidant activity in pomegranate juice (14). As a putative mechanism, it was suggested that this polyphenol might increase the expression of *Sod* and *Gpx* antioxidant genes in testis, which has been reported in other species. Similarly, punicalagin and peel extract could reduce OS damage because of enhancement of the enzymatic capacity of SOD and GSH and decrease of lipid peroxidation in mouse (9) and rat testis (15), respectively. In this regard, Kang et al. showed the pomegranate powder through antioxidant inhibitory effects on melanin synthesis and tyrosinase activity, and the increase in Gpx1 may lead to prevention of melanogenesis in B16F10 melanoma cells via inactivation of the p38 signaling pathway (16).

In agreement with the mRNA behavior of *Sod* and *Gpx*, pomegranate peel significantly increased DJ-1 protein 1.5-fold compared to control. DJ-1 can protect cells and tissues against OS by enhancing the expression of several antioxidants such as SOD. In addition, this protein stabilizes Nuclear factor erythroid-2 related factor 2 (NRF2), a leading regulator for antioxidant proteins and detoxifying enzymes (17). Interestingly, pomegranate peel effectively increases DJ-1 protein level and antioxidant capacity in testis, which has not been reported before in previous studies. Therefore, the potential functions of peel were confirmed by western blot analysis and it could be proposed as a possible mechanism for the treatment of male infertility induced by OS and deficiency of DJ-1 function.

While this study revealed a high antioxidant potential for pomegranate peel according to a higher level of *Sod*, *Gpx*, and DJ-1, the seed had a more significant impact on *Prdx5* expression. *Prdx* family has a vital role in the safekeeping of cells against OS, especially hydrogen peroxide, and *Prdx4* and *Prdx5* have been found in spermatogonia (18). However, since there is no study regarding the effects of pomegranate's by-products on *Prdx* family, it is not clear how pomegranate seed can increase the expression level of *Prdx5*. The unique profiles of FAs and the presence of Punicic acid in pomegranate seed may be a reason for such response in testis which warrants further studies.

Conclusion

Even though both seed and peel of pomegranate are noteworthy in the stimulation of antioxidant capacity in testis, peel showed a higher impact on antioxidant genes such as *Sod2* and *Gpx1* as well as DJ-1 protein while seed only affected *Prdx5*. These findings will shed light and pave the way to acknowledging the importance of pomegranate by-products, especially pomegranate peel, as a natural antioxidant in the male antioxidant system. Besides clinical relevance, such research may result in considerable improvement in male infertility treatments.

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Authors' Contributions

M.N., L.R.Gh.; Investigation, data analyses, writing, original draft preparation, and visualization. A.Sh.; Supervision, writing, review and editing, and funding acquisition. Sh.M.; Methodology of protein evolution, validation, writing, review and editing. S.M.Gh.; Supervision, conceptualization, project administration, validation, writing, review and editing, and visualization. A.R.A.; Conceptualization, project administration, validation, writing, review and editing, and visualization. All authors read and approved the final manuscript.

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Original Article

A Prospective Randomised Comparative Clinical Trial Study of Luteal PhaseLetrozole versus Ganirelix Acetate Administration to Prevent Severity of Early Onset OHSS in ARTs

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Abstract

Background: Ovarian hyperstimulation syndrome (OHSS) is the most notable complication in ovulation induction for assisted reproductive techniques (ARTs) like *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Hence, we decided to evaluate the effect of the aromatase inhibitor, letrozole, versus gonadotrophin-releasing hormone (GnRH)-antagonist (ganirelix acetate) on prevention of severity of OHSS and reduction in serum estradiol (E2) levels when administered during the luteal phase after oocyte retrieval in IVF/ICSI cycles.

Materials and Methods: In this prospective single-centred, randomized, parallel-arm study, 122 patients were randomized to receive oral letrozole (n=61, 2.5 mg twice daily) or ganirelix acetate (n=61, 0.25 mg subcutaneously daily) from the day of egg retrieval for the next 7 days. Incidence and severity of early OHSS were the primary endpoints assessed by the signs, symptoms, and laboratory findings of OHSS (e.g., serum E2 levels). The secondary endpoints were patient satisfaction and the additional cost of therapy to prevent the severity of OHSS.

Results: Letrozole group had lower incidence of OHSS (13.1%) compared to 19.6% in ganirelix acetate group (P=0.32). Serum E2 levels on post-pick up days 5 and 7 were significantly lower in the letrozole group when compared to the ganirelix acetate group (P=0.001). The majority of the patients in both groups had no major complications. No significant difference was found between the study groups with respect to the incidence of OHSS (P=0.33). The additional cost per IVF cycle for prevention of severity of early-onset OHSS in the letrozole group was 5.32 USD compared to 267.26 USD in the ganirelix acetate group, which was almost fifty times costlier.

Conclusion: Letrozole and ganirelix acetate have the same efficiency for the overall prevention of OHSS, whereas letrozole was more effective in preventing moderate OHSS. Letrozole had better patient satisfaction and is cheaper compared to GnRH antagonists (Registration number: CTRI/2020/10/028674).

Keywords: Ganirelix Acetate, Gonadotrophin-Releasing Hormone Antagonist, *In Vitro* Fertilization, Letrozole, Ovarian Hyperstimulation Syndrome

Citation: **Choudhary RA, Vora PH, Darade KK, Pandey S, Ganla KN. A prospective randomised comparative clinical trial study of luteal phase letrozole versus ganirelix acetate administration to prevent severity of early onset OHSS in ARTs. Int J Fertil Steril. 2021; 15(4): 263-268. doi: 10.22074/IJFS.2021.139562.1042.**
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Introduction

In the latter part of the 20th century, infertility treatment revolved around achieving controlled ovarian hyperstimulation along with *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (1-4). Ovarian hyperstimulation syndrome (OHSS) is the most notable complication of ovulation induction in assisted reproductive technique (ART) (5). The contributing mechanism of OHSS is attributable to human chorionic gonadotropin (hCG) exposure either following ovulation trigger during IVF/ICSI (early OHSS) or by pregnancy achieved from embryo transfer (late OHSS) (6). The pathobiological basis for the prolonged luteotropic effect of hCG is a longer half-life of hCG (t1/2=24 to 36 hours) as compared to the luteinizing hormone (LH, t ½=20 minutes) (7). This is given to promote final follicular maturation prior to oocyte retrieval. OHSS is characterized by cystic enlargement of the ovaries and a fluid shift from the intravascular to the third space due to increased capillary permeability and ovarian neoangiogenesis.

The incidence of OHSS, as reported in the literature, varies from 3.1 to 6% for IVF cycles and increases proportionately depending on the risk profile of the patient (8, 9). Increased serum estradiol (E2) levels is an established risk factor for OHSS and hypercoagulability lead-

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ing to further complications (10, 11). Depending on the clinical manifestation and laboratory findings, OHSS is classified as mild, moderate, and severe (12). Additionally, based on the timing of occurrence, OHSS is classified as early OHSS that occurs within 9 days of hCG trigger; and late OHSS is seen after 10 days of administering hCG (13).

Primary treatment strategies to prevent OHSS include appropriate modification of ovulation induction protocol after identifying appurtenant patient risk factors (14). Further, the use of gonadotrophin-releasing hormone (GnRH) antagonists after oocyte retrieval in the luteal phase has been promoted for reducing the severity of OHSS and has become increasingly popular (15). Recently, the use of letrozole, an oral aromatase inhibitor, during the luteal phase has been hypothesized as one of the strategies to prevent OHSS as it significantly reduces the E2 levels in the blood (16). Nonetheless, because of its shortened half-life and its effects on reproductive physiology, researchers are showing more interest in this drug. Moreover, randomized clinical trials have also claimed the efficacy of letrozole in reducing serum E2 levels (17). Existing studies on letrozole have confirmed its effectiveness by comparing it with either a placebo or an active comparator such as aspirin or Cetrorelix (16). In Wang et al. (16) study, a head-to-head comparison of letrozole to Cetrorelix (GnRH antagonist) showed no difference in the incidence of moderate to severe OHSS, hospitalization days, or duration of the luteal phase. Considering this, it is prudent to know the outcomes with letrozole and ganirelix acetate in the context of decreasing OHSS after ovulation trigger with an inducing agent, such as hCG or dual trigger during ART. Thus, we conducted a prospective comparative study to evaluate the effect of letrozole versus ganirelix acetate on serum E2 levels when administered during the luteal phase after oocyte retrieval in IVF/ICSI cycles for prevention and decreasing the severity of OHSS.

Materials and Methods

Study design

This prospective single-centred, randomized, parallel-arm study was conducted at a private reproductive medicine clinic for a period of one year from 16 October 2019. Ethics committee approval was obtained from Independent Ethics Committee after submitting the study related documents [Ethic committee Reg No: ECR/1.679/Maruthi/lnd/ KA (2O18 -Letter dated 16-Aug-2019)], and the study was registered in the Clinical Trials Registry-India [CTRI Registration No CTRI/2020/10/028674]. The study was conducted by adhering to all established norms of Good Clinical Practice (GCP) guidelines and Ethical principles laid down in the Indian Council for Medical Research guidelines for biomedical research on human participants, 2018.

Study subjects

After obtaining written informed consent from 144 women, who are seeking ART, aged 20-30 years, with body mass index (BMI) between 18 to 29 kg/m^2, basal levels of E2≤50 pg/mL on day 1 of stimulation, anti-Müllerian hormone (AMH) >5 ng/ml, and antral follicle count (AFC) of >20 confirmed via ultrasound; and oocyte retrieval >25, serum E2 level >2500 pg/mL on the day of trigger and evidence of OHSS defined by documented clinical findings coupled with ultrasonographic evidence of ascites, or ovary diameter 10 cm on one or both sides, or puncture follicle number more than 30 (follicular diameter 14 mm on oocyte retrieval day) were recruited for the study. All women with serum E2 levels <2500 pg/mL on day of trigger and those who could not receive dual triggers (as outlined in the study subject section), coasting (withholding gonadotropin stimulation during controlled ovarian stimulation resulting in atresia of small follicles, or other preventive measures for managing OHSS), contraindications to letrozole or GnRH antagonist (ganirelix acetate), including severe liver and renal dysfunction were exempted.

Study procedures

Screened couples were counseled regarding the risk and symptoms of OHSS before starting the enrolment. Participants could enter the study only once. Controlled ovarian stimulation was achieved using recombinant follicle-stimulating hormone (rFSH) 225 IU and GnRH antagonist 0.25 mg added from day 6 of their stimulation (fixed antagonist protocol). A dual trigger was given in the form of hCG 2000 IU and triptorelin acetate 0.2 mg. All embryos were cryopreserved on day 3 for transfer in future cycles (freeze all protocol).

Intervention

Following administration of dual trigger, a total of 122 patients were randomized into letrozole and ganirelix acetate groups of 61 each. Letrozole group received 2.5 mg of oral letrozole (Letoval, Sun Pharma Laboratories Limited) twice daily. The ganirelix acetate group received ganirelix acetate 0.25 mg (Orgalutran, Merck Sharp, and Dohme) subcutaneously daily, from the day of egg retrieval for the next seven days. The computer randomization technique was used for randomization.

Endpoints

The primary outcome was the incidence and severity of early OHSS measured by the symptoms, signs, and laboratory findings suggestive of OHSS, serum E2, and serum progesterone on the day of hCG/trigger administration, days 5 and 7 after ovum pick-up, and days for menses after oocyte retrieval. The secondary measurements were the patient satisfaction and additional cost of therapy for the prevention of OHSS. All the study participants received dopamine agonist (Cabergoline) 0.5 mg once daily from the day of trigger for the next 8 days as a conservative therapy for OHSS. The Short Assessment of Patient Satisfaction (SAPS) questionnaire was used to assess patient satisfaction (18).

Criteria for the diagnosis and grading of ovarian hyperstimulation syndrome

- Mild OHSS: Abdominal bloating, mild abdominal pain, ovarian size usually < 8 cm
- Moderate OHSS: Moderate abdominal pain, nausea ± vomiting, ultrasound evidence of ascites, and ovarian size usually 8-12 cm
- Severe OHSS: Clinical ascites (± hydrothorax), oliguria (<300 ml/day or <30 ml/hour), haematocrit >0.45, hyponatraemia (sodium <135 mmol/L), hypo-osmolality (osmolality <282 mOsm/kg), hyperkalaemia (potassium >5 mmol/L), hypoproteinaemia (serum albumin <35 g/L), and ovarian size usually >12 cm
- Critical OHSS: Tense ascites/large hydrothorax, haematocrit > 0.55, white cell count > 25 000/ml, oliguria/anuria, thromboembolism, and acute respiratory distress syndrome

Statistical analysis

A minimum sample of 60 patients was required for each arm to detect a significant difference by considering a two-sided t test at a significance level of 95% and power of 90%. Statistical analysis was performed using SPSS 21 (SPSS, Chicago, IL, USA). Categorical variables were represented in terms of percentages. The continuous variables with normal distribution were presented as mean ± standard deviation and compared using paired t test, whereas Chi-square test was employed for dichotomous data. Mann-Whitney U test was performed for variables without normal distribution. A P<0.05 was considered statistically significant at a 95% confidence interval.

Results

A total of 122 subjects were included in the study (letrozole group, n=61 and ganirelix acetate group, n=61). All the details are clearly depicted in the CONSORT diagram (Fig.1). Anovulation was the most commonly encountered cause for infertility, followed by polycystic ovary syndrome (PCOS) and male factor infertility. Baseline characteristics of the study population are presented in Table 1.

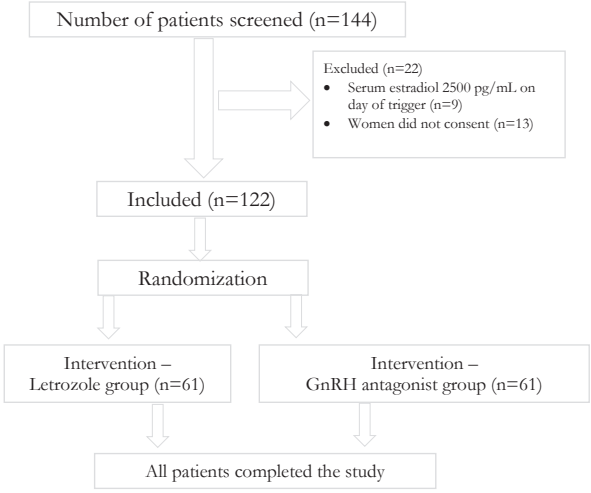

Fig.1: Consort diagram.

Table 1: Baseline characteristics of the study population

Parameters	Letrozole (n=61)	Ganirelix acetate (n=61)	P value*
Age (Y)	26.4 ± 2.4	26.8 ± 2.4	0.33^{NS}
BMI (kg/m^2)	22.3 ± 5.2	21.8 ± 5.9	0.65^{NS}
Duration of infertility (Y)	4.3 ± 1.3	4.4 ± 1.2	0.78^{NS}
Antral follicular count	26 ± 1.3	25 ± 1.8	> 0.05^{NS}
Anti-mullerian hormone (ng/ml)	6.1 ± 1.3	5.9 ± 1.4	> 0.05^{NS}
Follicle stimulating hormone (IU/L)	3.1 ± 1.1	3.2 ± 1.4	0.68^{NS}
Luteinizing hormone (IU/L)	3.5 ± 1.3	3.4 ± 1.1	0.68^{NS}
Oocytes retrieved	33.2 ± 15.4	34.9 ± 10	0.47^{NS}
Metaphase II oocytes	25.7 ± 11.5	27.8 ± 8.5	0.23^{NS}
Immature oocyte	3.2 ± 3.6	5.9 ± 10.7	0.06^{NS}
Germinal vesicle	4.3 ± 3.7	5.6 ± 3.1	0.05^{S}
Fertilised oocytes	24.9 ± 10.9	26.9 ± 8.4	0.26^{NS}
Embryos cryopreserved (D3)	24.2 ± 10.9	19.2 ± 14.4	0.03^{S}

Data are presented as mean ± standard deviation. *; Independent t test, BMI; Body mass index, NS; non-significant, and S; Significant.

The letrozole group (6.2 ± 4.18 days) had early menses after oocyte retrieval as compared to the ganirelix acetate group (10.6 ± 1.3 days), which was statistically significant (P=0.001). On the trigger day, serum E2 levels were significantly different between letrozole and ganirelix acetate groups [844 pg/mL, P=0.04, 95% confidence interval (CI): 45.96 to 1642.04]. In addition, mean serum E2 levels were significantly lower in the letrozole group compared to the ganirelix acetate group on post-pick up days 5 and 7 (Fig.2). Figure 3 represents mean serum progesterone levels between the study groups on the day of trigger and post-pick up days 5 and 7.

Statistically, no significant difference was found between the study groups with respect to the incidence of OHSS (P=0.33, Fig.4). According to the SAPS assessment, patients were 'very satisfied' with the route of administration and comfort of taking the treatment in the letrozole group compared to 'not so satisfied' in the ganirelix acetate group (20 vs. 14 and 20 vs. 12, respectively). A significant difference was observed between the study groups regarding patient satisfaction scores (Fig.5).

The additional cost per IVF/ICSI cycle for prevention of severity of early-onset OHSS in the letrozole group was 5.32 USD as compared to 267.26 USD in the ganirelix acetate group, which was almost fifty times costlier.

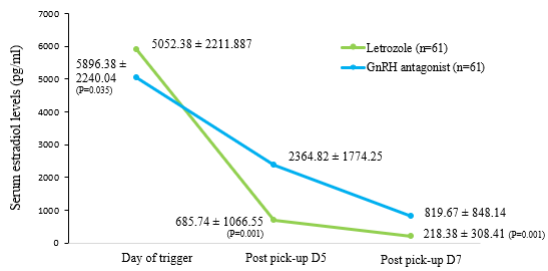

Fig.2: Mean serum estradiol levels between the study groups. D; Day and GnRH; Gonadotrophin-releasing hormone.

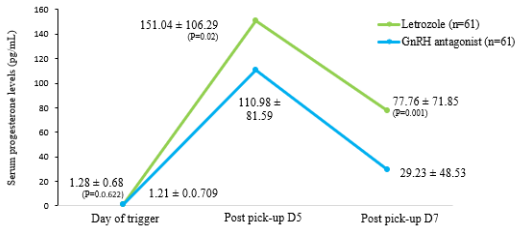

Fig.3: Mean serum progesterone levels between the study groups. D; Day and GnRH; Gonadotrophin-releasing hormone.

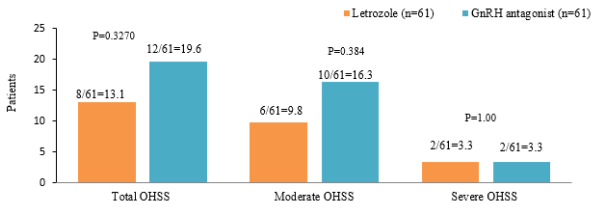

Fig.4: Distribution of OHSS between the study groups. OHSS; Ovarian hyperstimulation syndrome and GnRH; Gonadotrophin-releasing hormone.

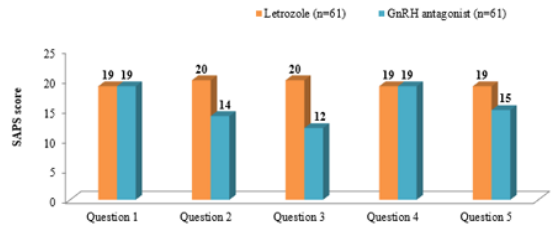

Fig.5: SAPS outcome score between the study groups. Q1; How satisfied are you with the explanations the doctor has given you about the your treatment?, Q2; Are you satisfied with the route of drugs (oral vs. injectable) being given to you?, Q3; Are you satisfied with the ease of taking these drugs (oral vs. injectable) being given to you?, Q4; Are you satisfied with the care you received in the clinic?, Q5; Are you satisfied with the overall treatment?, Scores: 1 to 5; Very dissatisfied, 5 to 10; Dissatisfied, 11 to 15; Satisfied, and 15 to 20; Very satisfied.

Discussion

Currently, the management of patients undergoing IVF/ICSI is centred on minimizing OHSS related complications while maintaining pregnancy efficacy outcomes. In the current study, mean serum E2 levels were significantly lower in the letrozole group compared to the ganirelix acetate group on post-pick up days 5 and 7. However, the majority of the patients in both groups had no significant complications.

In the current study, patients undergoing IVF/ICSI were treated during the luteal phase with either oral letrozole (aromatase inhibitors) or ganirelix acetate injection. In general, patients undergoing IVF have a drastic increase in their endogenous E2 levels starting with <60 pg/mL to >2500 pg/mL, or much higher if they develop OHSS. A high estrogenic milieu is a reasonable justification for forming a hypercoagulable state and puts patients at risk for venous or arterial thrombosis (18). Administration of letrozole during the luteal phase could be a new approach to reduce the risk of thrombosis associated with OHSS, as shown in the previous studies (19). Letrozole is an effective and extremely specific aromatase inhibitor. Letrozole hinders the aromatase enzyme by actively binding to the subunit cytochrome P450 of the enzyme resulting in an obstruction of androgen conversion into estrogen and reduces the risk of OHSS (20-22).

Published studies have proposed the use of letrozole would effectively reduce the E2 levels in patients with high risk for OHSS (16, 19). Similar findings were observed in the current study as well. As expected, letrozole has caused a more significant reduction in serum E2 levels in patients at high risk for OHSS in comparison to ganirelix acetate on day 7 post oocyte trigger, and a significant trend continued up to day 9. A similar trend was observed in Wang et al. (16) study carried out in 139 infertile women undergoing ART with high risk for OHSS. In that study, a significant decrease in the level of E2 was observed on the 4th, 7th, and 10th days after hCG administration for letrozole (5 mg) compared to support therapy. In a similar study by Chen et al. (17) carried out in women with polycystic ovary syndrome (n=181), the letrozole-treated group had significantly lowered E2 levels on the day of retrieval as compared to the non-letrozole group (1001.60 vs. 1690.65 pg/ml). As compared to the study by Chen et al. (17), the BMI was the same in our study. However, there were differences in the AFC (~25 vs. 18) and AMH (~6 vs. 5 ng/mL, respectively) in our study. Ovarian response was also higher in our study as compared to the Chen et al. (17) study: the number of oocytes retrieved (33 vs. 18), the number of fertilized oocytes (24 vs. 13), and the number of embryos cryopreserved (19 vs. 6, respectively). The potential mechanism behind reducing the E2 levels with letrozole treatment could be attributed to either corpus luteum mediated hypothesis as a result of the luteolytic effect or vascular endothelial growth facto (VEGF) mediating downstream pathways or steroidogenic pathway as demonstrated by previous studies (16, 19, 20, 23, 24). This may limit the undesirable negative effect of accumulative E2 concentrations and prevent complications due to hypercoagulability and OHSS in these women (25-27).

Recently, letrozole was recommended as a preventive treatment in women at high risk for OHSS (28). Corresponding to this, in the current study, the incidence of OHSS was numerically less in the letrozole group than the ganirelix group, although statistically non-significant (13.1 vs. 19.6%). Besides, letrozole was better than GnRH antagonist in preventing moderate OHSS (9.8 vs. 16.3%), though non-significant. Although Chen et al. (17), in their study demonstrated a decrease in the incidence rate of OHSS in the letrozole-treated group compared to the non-letrozole group (2.56 vs. 7.77%), the differences were not significant, possibly because of the small study sample size. Our findings comply with Mai et al.'s (29) findings, who compared the effectiveness of letrozole with aspirin in preventing early OHSS (in the luteal phase). This study has demonstrated less incidence of OHSS in patients receiving letrozole compared to aspirin, which could be credited to luteolysis rather than the VEFG effects.

On the other hand, with progesterone, we observed an

initial rise to 5 days, after which the levels witnessed a sharp decline at the end of day 7. Similar patterns of a peak at day 5 followed by a fall by day 7 were observed in the two studies reported by Lainas et al. (15, 30). This distinct pattern of a rise in progesterone is because of the luteotropic effect of hCG, which mimics LH but with a longer half-life of HCG up to 36 hours (7). Given the hormonal findings, it is reasonable to conclude that the aromatase enzyme inhibition by letrozole causes a significantly greater decrease in serum E2 compared to blocking the hypothalamic-pituitary-gonadal pathway by ganirelix. The smaller but significantly higher levels of progesterone require further validation.

In this study, no complications were observed in most participants; however, moderate OHSS was observed in a limited number of patients in the letrozole group compared to the ganirelix group. Additionally, participants in the letrozole group had early menses after oocyte retrieval compared to the ganirelix acetate group, and the difference noticed was -4.41 days.

Considering the results of the study, it is imperative to discuss the pathophysiological mechanisms for the beneficial effects observed in our study. Two main hypotheses are proposed for the development of OHSS: first, estrogen-mediated- as studies have shown that patients with high E2 levels of >2500 pg/mL are at increased risk of OHSS (10); however, there have been conflicting reports regarding the estrogen hypothesis. Second, corpus luteum mediated hypothesis due to the luteotropic effect, with VEGF mediating downstream pathways (16, 19). Letrozole increases local androgen levels and thereby influences the granulosa lutein cells to decrease VEGF and E2 levels (19, 28). Patients receiving letrozole have a shorter luteal phase and lower VEGF levels, indicating the corpus luteum pathway is a more plausible hypothesis. However, as research in this field is at a nascent stage, further studies are required in this regard.

Lastly, our study showed that patients prefer oral therapy due to ease of administration and the lower cost associated with the intervention. The cost of treatment with letrozole is significantly less than ganirelix (5.32 USD vs. 267.26 USD). Therefore, in a resource-constrained setting and a developing country like India, more patients would be able to afford the intervention and benefit from this therapy, making this approach patient-friendly in selected cases.

Although to the best of our knowledge, we are the first to report a head-to-head comparison between letrozole and ganirelix in mitigating OHSS symptoms when these agents are administered post-oocyte retrieval, our study has few limitations. Firstly, this was a single-centred unblinded trial design; hence it will not give a comprehensive view of the entire population. Secondly, we did not stratify patients with higher baseline E2 levels. This is a known risk factor for developing OHSS and could have confounded our results. Thirdly, other outcomes such as VEGF levels would have been worth exploring to understand the outcomes truly.

Conclusion

Letrozole and ganirelix acetate have the same efficiency for the overall prevention of OHSS, among which letrozole was more effective in preventing moderate OHSS. Letrozole had better patient satisfaction and was cheaper compared to GnRH antagonists. In the future, rigorous randomized trials are required to evaluate the effect of letrozole and its endocrine impact on the development of OHSS.

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Authors' Contributions

R.A.C.; Supervised the study, performed acquisition, analysis, and interpretation of data. R.A.C., P.H.V., K.K.D., S.P., K.N.G.; Design the study, manuscript preparation, editing, and critical review. All authors read and approved the final version of the manuscript.

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Original Article

Risk Factors Associated with Recurrent Pregnancy Loss and Outcome of Pre-Implantation Genetic Screening of Affected Couples

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Abstract

Background: Recurrent pregnancy loss (RPL) is a multifactorial disorder which affects up to 5% of couples around the world. Several factors are considered to be involved in RPL; but, the etiology remains unexplained in 35-60% of cases. The aim of this study was to assess the frequency of risk factors associated with RPL in a group of our clinic clients, and their pre-implantation genetic screening (PGS) outcome.

Materials and Methods: We designed a retrospective descriptive study among, 602 Iranian couples referred to the Royan Reproductive Clinic (Tehran-Iran) from 2006 to 2018. Their karyotyping test and PGS outcomes were analyzed. PGS had been applied by array comparative genomic hybridization (array-CGH) on embryos from these patients. Also, karyotyping test had been performed using standard cytogenetic techniques.

Results: G-banding analysis revealed a frequency of 15.61% chromosomal abnormalities in RPL couples. Also, the reciprocal translocations were more frequent (33/1204 cases) compared to the other structural abnormalities. Pregnancy rate per embryo transferred were 50% with array-CGH approach.

Conclusion: Our findings could confirm a positive correlation between chromosomal abnormalities and RPL rate. Applying PGS for the RPL couples, leads to improvement of pregnancy success rate.

Keywords: Array-CGH, Chromosomal Abnormalities, Recurrent Pregnancy Loss

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Introduction

Recurrent pregnancy loss (RPL) is an important and common phenomenon in the reproductive system, which affects 2-5% of couples (1). According to the American Society for Reproductive Medicine (ASRM), RPL is defined as two or more consecutive pregnancy losses before 20 weeks while, minimum of three failed pregnancy (<20 weeks gestation) is determined by European Society of Human Reproduction and Embryology (ESHRE) and the Royal College of Obstetricians and Gynecologists (RCOG) (2). RPL occurrence , a highly heterogeneous condition , was attributed to several causes including endocrine dysfunction, auto immune disorders, thrombophilia, genetic abnormalities, infectious diseases, uterine anomalies, sperm DNA fragmentation and epigenetics (3, 4). However, the reason for half of RPL cases is still unclear (1).

Genetic factors such as chromosomal rearrangements and gene mutations are responsible for 2-5% of the defined causes of RPL (4). Chromosomal balanced structural rearrangements, mainly reciprocal and Robertsonian translocations , were identified more common in couples with recurrent spontaneous abortions (5, 6). Also embryo chromosomal abnormalities such as, aneuploidy and polyploidy, were observed in 50-80% of aborted tissues, which are the most important reason for first-trimester spontaneous pregnancy loss (7). Nowadays, pre-implantation genetic screening (PGS) is performed to improve the *in vitro* fertilization (IVF) success rate (8) by embryo chromosomal abnormalities detection.

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Before advent of array comparative genomic hybridization (array-CGH), fluorescence in situ hybridization (FISH) technique was performed as screening approach for over two decades. Recently, due to FISH limitations, it has been recommended that this technique should be replaced by developed screening methods such as next generation sequencing (NGS) and array-CGH (9). Unlike FISH, array-CGH could analyze all 24 chromosomes and shows high accuracy for aneuploidy detection (10). Recent advances in NGS technology, enable to use this technique for chromosomal screening in preimplantation embryos (11). Moreover, using this technique is challenging due to detecting large insertions and deletions (indels) (>1 kb) and complex structural variations (12).

The present study is to find out the relation between chromosomal abnormality and RPL among patients referred to the Royan Reproductive Clinic (Tehran-Iran) from 2006 to 2018.

Materials and Methods

Patients

This retrospective descriptive study includes a total of 1204 individuals (602 couples) with RPL history (more than two consecutive pregnancy losses before 20 weeks of gestation) referring to the Royan Reproductive Clinic, Tehran, Iran, during the period of 2006 to 2018. Informed consent was obtained from all patients according to the Royan Institute Ethical Committee Guidelines. The study was performed in accordance with the Declaration of Helsinki and was approved by Institutional Review Board and Ethics Committees (Royan Institute: IR.ACECR.ROYAN.REC.1397.213, Zanjan University of Medical Sciences: ZUMS.REC.1396.182). Also, the most common RPL causes, such as hysterosalpingography, immunological tests, semen analysis, clotting assay, and blood tests for diabetes mellitus, hypothyroidism and infectious cause history were investigated for each couple.

In this study, severe intrauterine adhesions and Mullerian tract anomalies described as anatomical abnormalities in female reproduction system. Also, we categorized diabetes type II, polycystic ovarian syndrome, hypothyroidism, endometriosis and hyperprolactinemia as endocrine problems Thrombophilic genetic factors such as homozygous mutations in each of the *MTHFR* (C677T), Factor V Leiden (1691G > A), *PAI-1* (4G/4G) and prothrombin (G20210A) genes were reported. According to ESHRE guideline (3), thrombophilia-related mutations were evaluated for the patients who had additional risk factors for thrombophilia or had a family history. Also, sperm DNA fragmentation index (DFI) and high DNA stainability (HDS) were assessed by the sperm chromatin structure assay (SCSA). For the determination of sperm DNA damage, we considered DFI >25% or HDS >15%.

Cytogenetic analysis

Karyotyping from peripheral blood lymphocytes was performed for both male and female partners, according to standard cytogenetic techniques (12). Briefly, at least 25 metaphase cells were analyzed for each patient while every suspected mosaic cases received extensive work-up, additional cells were examined to exclude 10% mosaicism at a 95% confidence level. Polymorphic rearrangements including heterochromatin variants were considered normal karyotypes. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature criteria (ISCN) (13).

Pre-implantation genetic screening

Using array-CGH, PGS was performed to identify embryos chromosomal aneuploidy during assisted reproductive technology (ART) treatment. Following the long protocol ovarian stimulation (14), the mature oocytes were fertilized by intracytoplasmic sperm injection (ICSI) and cycles testing of blastomeres was performed in 3-day embryos.

Using array-CGH, single-cell numerical chromosomal abnormalities were verified that those cells removed on day 3 to 5 in early embryo stages. In this aim, the 24 sure ® Microarray Pack version 3.0 (Illumina®; cat. #: PR-10-408702-PK, USA) was applied.

The array slids was scanned in InnoScan 900 microarray scanner (INNOPSYS Inc., Carbonne, France) and, Data were analyzed using the BlueFuse Multi v3.1 software program (Illumina). Depending on the platform used, BlueFuse Multi software (BlueGnome Ltd, now Illumina) calculates median log 2 ratio for all the chromosomes, as the index of aneuploidy.

Results

Five hundred forty eight couples out of 602, (91.02%) had a first trimester abortion experience (<13 weeks). Also, the percentage of couples with ≥3 abortion was 78.24% (the average abortion was 3.5 ± 1.6). Karyotype analysis showed 8.13% (98/1204, 73 females and 25 males) chromosomal abnormalities in RPL patients. The reciprocal translocations were more frequent structural abnormality (2.74%) in 602 studied couples. The frequency and types of chromosomal abnormalities are shown in Tables 1 and 2.

Table 1: The frequency and types of chromosomal abnormality in 602 couples (1204 cases)

Type	Number	Frequency in 1204 cases (%)
Mosaicism	35	2.90
Translocation	40	3.32
Robertsonian	7	0.58
Reciprocal	33	2.74
Inversion	22	1.82
Super male	1	0.08
Total	99	8.22

Gynecologic structural abnormalities were identified in 16.77% (101/602) of the patients. Endocrine disorder and thrombotic complications were observed in 26.07% (157/602) and 4.15% (25/602) of the females, respectively, while, sperm DNA damage were detected in 14.95% of couples subjected to RPL (Table 3).

Pre-implantation genetic screening analysis

In this study, only 83 couples (83/602) were undertaken PGS with array-CGH platform. Only the last cycle of PGS was considered for each couple.

Table 2: Structural chromosome abnormalities of the carrier couples with recurrent pregnancy loss (RPL)

Structural chromosome abnormalities	Female	Male
Reciprocal translocation	46,XX,t(11;22)(q23;q11.2)	46,XY,t(1;2)(p36.2;q37.2)
	46, XX t(10;15)(q21;q21)	46,XY,t(16;6)(p12;q26)
	46,XX,t(16;6)(p12;q26)	46,XY,t(6;12)(q15;q15)
	46,XX,t(1;3)(q32;q13.2)	46,XY,t(1;13)(q43;q14)
	46,XX,t(5;16)(p15.1;q12.1)	46,XY,t(1;14)(q43;q25)
	46XX,t(1;13)(q21;q12.3)	46,XY,t(7;10)(q21.3;q26.2)
	46,XX,t(13;11)	46,XY,t(18;20)(q12.2;q13.1)
	46,XX,t(11;22)(q23;q11.2)	46,XY,t(1;3)(p35.1;p26)
	46,XX,t(4;12)(q35;q22)	46,XY,t(10;19)(q22;q13)
	46,XX,t(4;7)(q35;q31.2)	46,XY,t(1;7)(q21;q36)
	46,XX,t(2;18)(p24:q2.2)	46,XY,t(13q:16q)
	46,XX,t(6;18)(q25.1;q21.1)	46,XY,t(4;10)(q22;q21)
	46,XX,t(4;7)(q27;p14.1)	46,XY,t(4;8)(q33;q23)
	46,XX,t(3;20)(q13.3;p12)	46,XY,t(4;6)(q26;p24)
	46,XX,t(1;11)(p32.9;p14.3)	46,XY,t(1;15)(p36.1;p11.2)
	46,XX,t(2,3)(q12;q27)	46,XY,t(6;11)(q13;q25)
		46,XY,t(4;5)(p14;q15)
Robertsonian translocation	45,XX,t(13;14)(q10;q10)	46,XY,t(13q:16q)
	45,XX,der(14;15)(q10;q10)	45,XY,t(13,14)
	45,XX,der(14;21)(q10;q10)	
Inversion	46, XX, inv (5)(p13q13)	46,XY, inv(9)(p13q21)
	46,XX,inv(9)(p11q12)	46,XY,per inv(9)(p11q12)
	46,XX,inv(4)(q10q12)	46,XY,inv(11)(p15q13)
	46,XX,per inv(8)(p23.1q22.1)	

Table 3: Frequency of factors associated with recurrent pregnancy loss (RPL) in 602 couples

Type	Number	Frequency (%)
Couples with chromosomal abnormality	94	15.61
Anatomical abnormalities in female reproduction system	101	16.77
Uterine adhesions	86	14.28
Mullerian tract anomalies	15	2.49
Endocrine disorder in female	157	26.07
Diabetes type II	20	3.32
Polycystic ovary syndrome	43	7.14
Hypothyroidism	97	16.11
Endometriosis	7	1.16
Hyperprolactinemia	3	0.49
Thrombotic	25	4.15
Males with sperm DNA damages	90	14.95

Based on the PGS-array-CGH results, of 13 abnormal karyotype couples, 20.68% (12/58) of analyzed embryo were normal and all of them were transferred in 9 cycles. Finally, 33.33% (3/9) led to pregnancy and ended to live births. In the 70 normal karyotype couples, 70 cycles PGS-array-CGH were performed, and 29.92% (85/284) of embryos were normal. In 72.85% (51/70) of cycles, embryo transfers (ETs) were carried out and 52.94% (27/51) of ETs lead to successful pregnancy. Noticeably, 70.37% of pregnancies was led to live births (Fig.1, Table S1B, See Supplementary Online Information in www.ijfs.ir). The frequency of chromosomal abnormalities in PGS-array-CGH embryos is shown in Figure 2.

Totally, 46 abnormal embryos were developed from abnormal karyotype couples; which among these, 16 embryos (16/46-34.78%) showed a chaotic chromosomal complement. Abnormality in chromosomes 17 and 11 was not observed in the embryos. Also, 199 abnormal embryos were obtained from normal-karyotype couples; the high rate of chaotic embryos is significant (45/199-22.61%). Also, the lowest frequencies were related to abnormality in chromosomes 17 (4/199-2.01%) and 11 (6/199-3.01%).

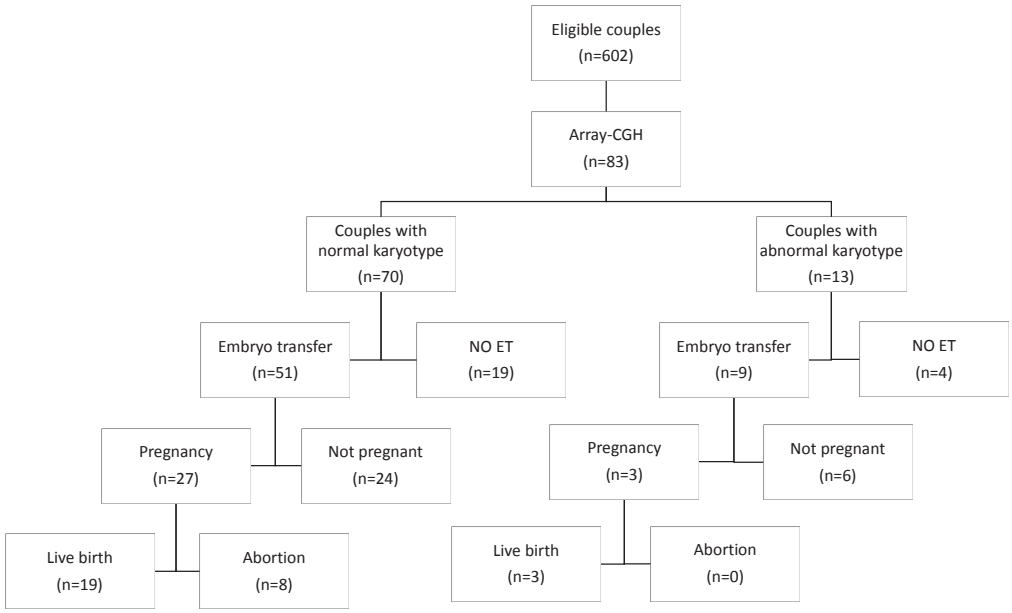
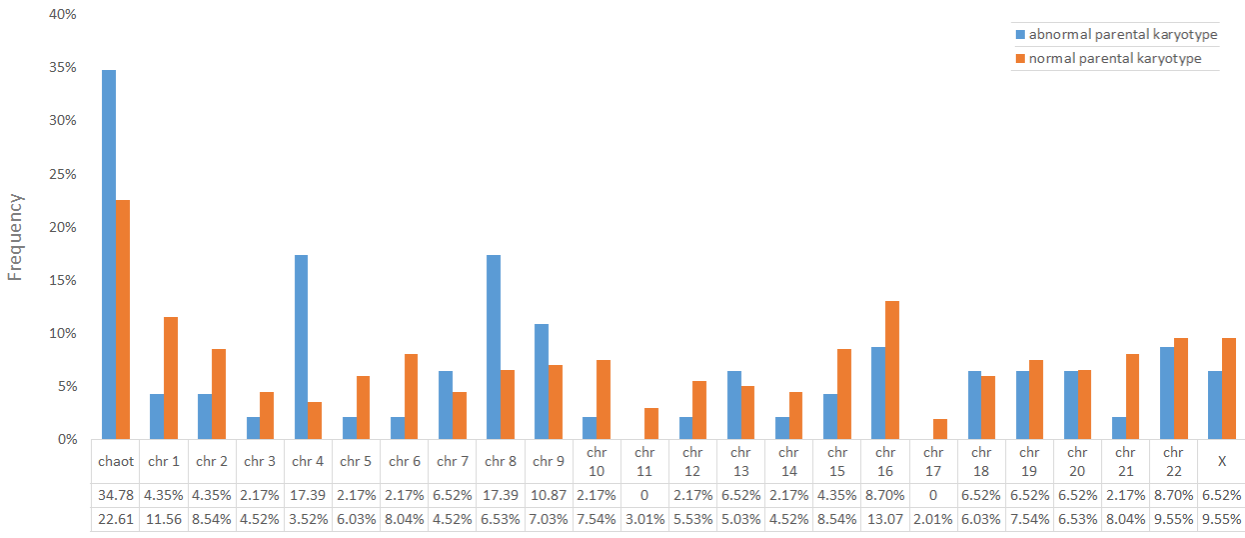


Fig.1: Flowchart of eligible subjects and their outcomes.



	chaot	chr 1	chr 2	chr 3	chr 4	chr 5	chr 6	chr 7	chr 8	chr 9	chr 10	chr 11	chr 12	chr 13	chr 14	chr 15	chr 16	chr 17	chr 18	chr 19	chr 20	chr 21	chr 22	X
abnormal parental karyotype	34.78	4.35%	4.35%	2.17%	17.39	2.17%	2.17%	6.52%	17.39	10.87	2.17%	0	2.17%	6.52%	2.17%	4.35%	8.70%	0	6.52%	6.52%	6.52%	2.17%	8.70%	6.52%
normal parental karyotype	22.61	11.56	8.54%	4.52%	3.52%	6.03%	8.04%	4.52%	6.53%	7.03%	7.54%	3.01%	5.53%	5.03%	4.52%	8.54%	13.07	2.01%	6.03%	7.54%	6.53%	8.04%	9.55%	9.55%

Fig.2: PGS-array-CGH and embryos chromosomal abnormalities frequency. PGS; Pre-implantation genetic screening and Array-CGH; Array comparative genomic hybridization.

Discussion

RPL is a multifactorial problem. Several studies were conducted to examine the prevalence of RPL risk factors (115-17). In this study, we evaluated five RPL associated factors, including chromosomal abnormality, anatomical character, endocrine, thrombotic defects and sperm DNA damages. Here, we observed high frequency of Endocrine disorder. Also, hypothyroidism was identified as the most common endocrine disorder, consistent with some previous reports (17, 18). The incidence of chromosomal abnormalities was 15.61%, which is inconsistent with previous studies. This different frequency was observed probably because of the variety in sample size and nationality (19, 20). Here, we observed translocation as a most common abnormality that is consistent with other investigations (21, 22). Noteworthy, the chromosome 9 inversion was the most frequent structural chromosomal abnormality in the present study of, it is associated with reproductive complications as described previously (23, 24). Although, Merrion and Maisenbacher (25) denied this association.

PGS technology has improved the IVF success rate by improving embryo selection for transfer and subsequently, reducing pregnancy loss. Recent molecular cytogenetics development, such as FISH and array-CGH, have provided a rapid embryonic chromosomes screening tool at the preimplantation stage (26). Because of some limitations, only small numbers of our participants could benefit of PGS service.

Chromosome 16 disruption was observed more than other chromosomal abnormality in embryos of the normal karyotype parents. It is consistent with previous studies (21, 27).

Conclusion

Clinical examination of a large proportion of Iranian couples with RPL history, indicated that hypothyroidism, anatomic factors and chromosomal anomalies are the major risk factors for RPL phenotype. Therefore, assessment of the mentioned factors would be useful for early diagnosis of RPL patients. Furthermore, identification of genetic causes of RPL could be considered to predict the risk of next pregnancy loss and would assist physicians for precise patient management in the clinic. Based on this retrospective study, it seems PGS platforms might provide a better chance for RPL couples.

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Authors' Contributions

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

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Original Article

The Clinical Outcome of Laparoscopic Surgery for Endometriosis on Pain, Ovarian Reserve, and Cancer Antigen 125 (CA-125): A Cohort Study

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Abstract

Background: Endometriosis is an important cause of chronic pain and infertility. Surgery is considered the gold standard for diagnosis and treatment. In this study, we aim to describe the clinical outcomes of women who undergo laparoscopic surgery for endometriosis.

Materials and Methods: In this cohort study, a total of 174 women who referred to Farmaniyeh Hospital, Tehran, Iran from August 2015 to December 2017 with surgical diagnoses of endometriosis stages III and IV enrolled. The participants' demographic, gynaecological, and clinical characteristics were recorded and they were asked to use a numeric rating scale (NRS) to record their severity of pain before and three months after surgery. Blood samples were also taken from the patients before and three months after surgery for measurement of serum levels of anti-Müllerian hormone (AMH) and cancer antigen 125 (CA-125). Data were analysed using SPSS version 21.

Results: The patients had a mean age of 34.86 ± 6.47 years, 60.9% were married, and 49.4% were housewives. The primary indication for surgery was pain (68.4%), followed by both pain and infertility in the remainder of patients. Types of endometriotic lesions included endometrioma (19%), deep infiltrating endometriosis (DIE, 3.4%), and both endometrioma and DIE (77.6%). There was a reduction in pain from 6.79 ± 2.19 before surgery to 1.48 ± 1.68 after surgery; serum AMH levels reduced from 2.80 ± 1.86 ng/mL to 1.76 ± 1.40 ng/mL and CA-125 reduced from 257.06 ± 220.25 U/mL to 23.27 ± 23.25 U/mL (all $P<0.001$). Of the 21.2% who experienced recurrence, 13.5% underwent additional surgery. The total additional surgery rate was 2.8%. Of the 55 patients with infertility, 78.1% became pregnant after surgery, 54.5% of which was spontaneous.

Conclusion: Surgical treatment of endometriosis had a favourable effect on the patients' pain and inflammation and resolved the patients' infertility with a minimal need for additional surgery.

Keywords: Anti-Mullerian Hormone, CA-125 Antigen, Endometriosis, Pain, Patient Outcome Assessment

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Introduction

Endometriosis is a benign gynaecologic disorder mainly observed in reproductive age women that has a global prevalence of 5-15% (1). The most common symptoms of endometriosis include chronic pelvic pain, dysmenorrhea, menorrhagia, dyspareunia, gastrointestinal (GI) complaints, and urinary symptoms (2); some cases may remain asymptomatic or may have mild symptoms, whereas others may only present with disease complications such as chronic pelvic pain and infertility (3). The disease severity of endometriosis varies based on the lesion site and penetration of the endometriotic lesions into the peritoneum; deep infiltrating endometriosis (DIE) has the worst prognosis and most severe symptoms (4).

Several hypotheses have been proposed for the pathogenesis of endometriosis; however, the majority of patients' symptoms appear to be associated with inflammation and proliferation of endometriotic lesions (5) that result from the secretion of cytokines and growth factors such as tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), and cancer antigen 125 (CA-125, used for diagnosis of endometriosis) (6). Anti-Müllerian hormone (AMH) is a member of the TGF-β superfamily and considered a valuable serum marker for a general

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measurement of ovarian reserve and also in women with endometriosis (7).

The clinical symptoms of endometriosis are not specific and serum/peripheral biomarkers can only predict endometriosis (8); therefore, direct observation of lesions during surgery and histologic confirmation of the specimens are considered the gold standard for diagnosis and treatment (9). A six-year follow-up of 1315 patients confirmed that laparoscopic treatment of endometriosis resulted in significant reductions in pain and resolution of infertility, which resulted in pregnancy after surgery (spontaneous or assisted) (10). Due to the need for ongoing reporting and more detailed follow-up after surgery for endometriosis-associated pain, in this study we aimed to describe the clinical characteristics of women who underwent laparoscopic surgery for treatment of endometriosis as well as the effect of surgery on patients' pain and serum levels of AMH and CA-125.

Materials and Methods

Study design

In this cohort study, all women who referred to Farmaniyeh Hospital in Tehran, from August 2015 to December 2017 for surgical treatment of endometriosis enrolled in this study. Diagnosis of endometriosis was based on the clinical symptoms of endometriosis and the results of imaging tests (ultrasound and magnetic resonance imaging [MRI]) in patients with severe pain or both pain and infertility who were indicated for surgical treatment. Those with surgically confirmed endometriosis stages III or IV were included in this study by the census method, after they received explanations about the study objectives and read and sign the written informed consent form. The Ethics Committee of Farmaniyeh Hospital approved the study protocol (FH-02-005). This study was conducted in accordance with the principles of the Declaration of Helsinki and its subsequent amendments.

The surgeries for all enrolled patients were performed by one surgical team of two gynaecologic laparoscopic surgeon.

For this purpose, after induction of general anaesthesia by the anaesthesiologist, the trocars were inserted in their place and the abdomen and pelvic cavity were explored. After visualization of the endometrioma, the cyst wall was excised and the ovarian adhesions were released to mobilize the ovaries, and the DIE lesions were totally resected.

The researcher used a study checklist to record the participants' demographic characteristics (age, height, weight, body mass index [BMI], marital status, educational level, and occupational status), gynaecological characteristics (age at menarche and menopause, menorrhagia, metrorrhagia, dysmenorrhea, dyspareunia, regular or irregular menstruation, history of endometriosis surgery, history of infertility before surgery, and indication for surgery), and endometriotic-related characteristics (type of endometriotic lesion, presence of endometrioma, its type and side, and the anatomical site of the DIE). The information was collected from the hospital medical records and completed by conferring with the patients (history taking). Cases of recurrence, reoperation, and pregnancy after surgery with or without assisted reproductive technique (ART) were recorded. Follow-up information was collected during post-surgical follow-up visits or by phone contact with patients who did not return for their follow-up visits. Recurrence was defined as recurrence of endometrioma or pain. Cases with recurrence were treated by medical therapy and surgery, if required.

The participants were asked to record their pain severity before and three months after surgery on a numeric rating scale (NRS), which was scored from 0 to 10 where 0 indicated no pain and 10 indicated the worst pain. Two blood samples were taken from the patients, one before surgery and three months after surgery. The samples were sent to the laboratory for measurement of serum levels of AMH and CA-125 antigen. The tumour markers were measured using an Enzyme linked Fluorescent Assay (ELFA) technique (TOSOH Co.) and CA-125 levels <35 were considered normal. AMH was measured using ELISA kits (Beckman Coulter Co., USA). AMH levels of 4.0-6.8 indicated optimal fertility, 2.2-4.0 indicated satisfactory fertility, 0.3-2.2 indicated low fertility, and <0.3 indicated very low fertility.

Cases who were not confirmed as having endometriosis by surgical inspection were excluded from the study and not included in the statistical analysis.

Statistical analysis

The results were described by frequency (%) for categorical variables and by mean ± standard deviation (SD) for quantitative variables. The results of the Kolmogorov-Smirnov test showed normal distribution of the data; therefore, we used the paired t test to compare numeric variables before and after surgery. The chi square test was used to compare frequencies between the groups. For statistical analysis, we used the IBM SPSS Statistics for Windows version 21.0 (IBM Corp., Armonk, NY, USA.) statistical software. $P<0.05$ were considered statistically significant.

Results

The 174 women who completed the study had a mean age of 34.86 ± 6.47 (18-49) years. Most (60.9%) were married, about half (49.4%) were unemployed/housewives, and 64.3% had an academic education. Table 1 lists the demographic and gynaecologic characteristics of the participants. The indication for surgery was pain in the majority (68.4%) of patients and both pain and infertility in the rest.

Types of endometriotic lesions included: endometrioma (19%, n=33), DIE (3.4%, n=6), and

both endometrioma and DIE (77.6%, n=135); 77.4% of cases with endometrioma were bilateral (n=130) and 22.6% (n=38%) were unilateral. The frequency of endometrioma located on the left side (57.9%) was significantly higher than the right side (42.1%, P=0.003), but the frequency of DIE on the left or right sides did not have any significant difference (47.6 vs. 52.4%, respectively, P=0.371). As shown, the most common site of DIE was the ovarian fossa and the least common site was the vaginal vault (1.19%).

Table 1: Demographic and gynaecologic characteristics of the study population

Variable	n	Mean ± SD or %
Age (Y)	174	34.86 ± 6.47
Weight (kg)	173	68.12 ± 13.88
Height (m)	173	164.90 ± 5.53
BMI (kg/m^2)	173	24.95 ± 4.40
Menarche age (Y)	133	12.15 ± 1.48
Menopause age (Y)	7	45.71 ± 1.25
Menorrhagia	44	25.3
Metrorrhagia	36	20.7
Dysmenorrhea	164	95.9
Dyspareunia	101	58.0
Irregular menstruation	42	24.1
Marital status		
Single	68	39.1
Married	106	60.9
Educational level		
Illiterate	16	9.2
High school graduate	34	19.5
B.Sc.	75	43.1
M.Sc.	22	12.6
Ph.D.	15	8.6
Not reported	12	6.9
Occupational status		
Housekeeper/unemployed	86	49.4
Employed	85	48.9
Previous surgery for endometriosis	24	13.8
Indication for surgery		
Pain and infertility	55	31.6
Pain	119	68.4

SD; Standard deviation and BMI; Body mass index.

Participants reported a reduction in pain from 6.79 ± 2.19 before surgery to 1.48 ± 1.68 after surgery; in addition, serum levels of AMH reduced from 2.80 ± 1.86 ng/mL before surgery to 1.76 ± 1.40 ng/mL after surgery and that of CA-125 from 220.25 ± 257.06 U/mL before surgery to 23.27 ± 23.25 U/mL after surgery (all P<0.001, Table 2). Figure 1 shows the trend of changes in pain, AMH, and CA-125. Postoperative follow-up showed recurrence in 21.2% of patients and 13.5% of these patients underwent additional surgery. The total reoperation rate was 2.8%. Of 55 patients who had a positive history of infertility, 43 (78.1%) became pregnant after surgery, 54.5% of these were spontaneous and without ART, and 23.6% with the use of ART.

Table 2: The post-surgical outcome of the study participants

Post-surgical outcome	n	Mean ± SD or (%)	P value
Pain score before surgery	173	6.79 ± 2.19	<0.001
Pain score after surgery	173	1.48 ± 1.68	
AMH before surgery (ng/mL)	142	2.80 ± 1.86	<0.001
AMH after surgery (ng/mL)	142	1.76 ± 1.40	
CA-125 before surgery (U/mL)	133	220.25 ± 257.06	<0.001
CA-125 after surgery (U/mL)	133	23.27 ± 23.25	
Recurrence	37	21.2	–
Reoperation (% of relapsed cases)	5	13.5	–
Reoperation (% of total cases)	5	2.8	
History of infertility before surgery	55	31.6	–
Pregnancy after surgery			
Without ART (% of infertile cases)	30	54.5	–
With ART (% of infertile cases)	13	23.6	–

SD; Standard deviation, AMH; Anti-Müllerian hormone, CA-125; Cancer antigen 125, and ART; Assisted reproductive technique.

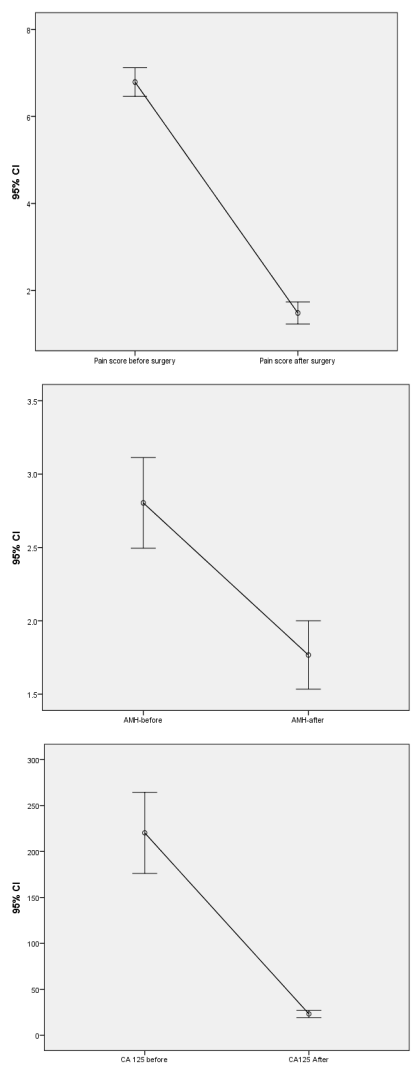

Fig.1: The changes in mean scores of pain, and serum levels of anti-Müllerian hormone (AMH) and cancer antigen 125 (CA-125) post-surgery compared to pre-surgery. CI; Confidence interval.

Discussion

In this study, we described the characteristics of 174 women with endometriosis, indicated for surgical treatment, and reported the follow-up results of these patients. The age range of 18 to 49 years (mean age: 34.86 years) and menopause in only seven of our patients confirmed the main occurrence of endometriosis in women of reproductive age and its rare occurrence after menopause (1). The mean age of menarche in the present study (12.15 years) also confirmed the results of a meta-analysis of 47 Iranian studies (11).

Endometrioma and DIE were present in 96.6% and 81% of patients, respectively, alone or in combination. The high frequency of coexistence of endometrioma and DIE refers to their association, as reported previously (12). Furthermore, as our patients were surgical candidates, the high frequency of endometrioma and DIE confirmed the association of these two with increased disease severity, as suggested previously (13). Most cases of endometrioma were bilateral (77.4%) and the frequency of left-sided endometrioma was higher. A higher frequency of endometriosis on the left side is anticipated to result from the anatomical differences of abdomen (presence of diaphragm on the right side) and hemipelvis (presence of sigmoid colon on the left side) (14). Right and left ovarian fossa were also the most common sites of DIE in our study, which was in line with evidence that suggested an association of ovarian involvement with more severe DIE (15).

The primary endometriosis symptoms in our patients included dysmenorrhea (95.9%) and dyspareunia (58%), while menstruation-related problems such as menorrhagia, metrorrhagia, and irregular menstruation had a frequency of 20-25%. Different frequencies have been reported in women with endometriosis (16, 17). However, of note is the significance of different forms of pain (dysmenorrhea and dyspareunia) in women with endometriosis in our study that referred to the importance of endometriosis-associated pain, which was in line with the results of previous studies that referred to the important role of pain and the impact of endometriosis on a woman's life (18). Pain was the indication of surgery in all patients (with or without infertility) and assessing the pain severity in our patients showed a high severity of pain in women before surgery (mean: 6.79), which confirmed the significance of pain in these patients. After surgery, a significant decrease was observed in patients' pain. In the study by Alborzi et al. (10), a six-year follow-up of patients showed reduced pain scores from 8.23 to 4.46 in 93.07% of patients. The differences in the scores could be due to the different evaluations of the patients from pain, as the assessment tool is a self-report tool and due to the different surgical details used because of the different sites and penetrations of endometriotic lesions. A meta-analysis of 1847 patients (23 studies) also showed significant reductions in pain after endometriosis surgery with a decrease of approximately 4.5-5.2 units in the pain scores, which differed based on the duration of the follow-up period (19). These results also confirmed the present study findings and suggested that the endometriotic lesions caused inflammation and activated the central nervous system (CNS). Therefore, excision of these lesions resolved these problems and the resulting pain (20). However, it has been reported that some cases may recur over time (21). In our study, we observed recurrence in 21.2% cases and a total of 2.8% required additional surgery. These results were in line with that reported by Asadzadeh and colleagues on an Iranian population where 28.6% of cases recurred after surgery (22). The different surgical details and the difference in the frequency of lesions' sites might affect the recurrence rate.

Another important aspect of endometriosis is infertility. In the present study, the majority of our patients were married and infertility was observed in more than half of the married cases (51.8%) and about one-third of all cases (31.6%), which referred to the significance of infertility in women with endometriosis. The follow-up in our study showed that 78.1% of infertile women became pregnant after surgery, 54.5% were spontaneous and 23.6% after ART. In a study by Alborzi et al. (10), 58.1% of infertile women became pregnant during the follow-up period, which was lower than the present study results. Furthermore, the frequency of infertility was also lower in their study (about 15%) compared to the current study. Infertility is one of the indications of surgical treatment for endometriosis and one of the main goals of the surgical treatment (23). Therefore, it is necessary to pay more attention to this issue. Depletion of ovarian reserve is reported to be one of the causes of endometriosis-related infertility (24). Hence, AMH, levels are commonly measured for assessment of ovarian reserve and it is also suggested to be measured in women with endometriosis (25). In the present study, although a statistically significant reduction was observed in serum levels of AMH, both pre- and post-surgical values were within the normal range. These results were in line with a report by Streuli and colleagues, which suggested that endometriosis did not result in decreased serum AMH levels and low AMH levels were only observed in patients with surgical histories of endometriosis (26). Others have also reported a decline in serum AMH levels after surgery (27) that was associated with bilaterality and disease severity (28), which confirmed the results of the present study.

As a reliable marker for diagnosis of endometriosis, CA-125 was also measured in the present study and the results showed that the significantly high mean pre-surgical level of CA-125 reduced after surgery. These results confirmed the previous study results, considering the high level of CA-125 in peritoneal fluid and sera of patients with endometriosis (8) and significant reduction (25.8%) in CA-125 levels after surgery (29). This high molecular weight glycoprotein, produced in the epithelium, can be a good marker for diagnosis and follow-up of endometriosis. One of the limitations of the present study was the small

sample size and nonrandomized patient enrolment. Furthermore, the pain assessment tool is a self-report tool and is exposed to subjective bias. Because of the multifactorial nature of endometriosis, the effect of confounders on the study results cannot be rejected; however, we evaluated a wide range of variables to overcome this issue.

Conclusion

The results of the present study showed that surgical treatment of endometriosis could have a favourable effect on patients' pain and infertility with a minimal rate of reoperation and acceptable recurrence rate. However, the role of AMH in endometriosis and the serum level of CA-125 after surgery should be studied in future research.

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Authors' Contributions

F.S., E.A., B.N.; Study concept and design. A.K., Sh.N.A.; Analysis and interpretation of data. F.S., B.N.; Drafting of the manuscript. F.S., A.K., Sh.N.A.; Critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Original Article

The Effects of Serum and Follicular Fluid Vitamin D Levels on Assisted Reproductive Techniques: A Prospective Cohort Study

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Abstract

Background: Based on studies on animal models, vitamin D plays an essential role in reproduction by controlling Ca and Mg levels. Despite these findings, the effects of vitamin D deficiency and supplementation on the outcome of assisted reproductive techniques (ART) remain controversial. Therefore, the aim of the present study was to assess the relationship between serum and follicular fluid 25-OH vitamin D levels on reproductive outcomes of infertile women.

Materials and Methods: This prospective cohort study included 150 infertile women who underwent *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). The participants were allocated to one of the three groups according to their serum and follicular fluid 25-OH vitamin D concentrations (less than 10 ng/ml, between 10 and 30 ng/ml and more than 30 ng/ml), and fertilization, cleavage and biochemical and clinical pregnancy rates were compared among the groups. Data was analyzed by SPSS software and using Chi-square and Spearman correlation coefficient.

Results: Serum and follicular fluid vitamin D levels significantly correlated with biochemical (P=0.008), (P=0.003) and clinical pregnancy (P=0.017), (P=0.001) rates respectively . However, the quality of embryos (P=0.125), (P=0.106) and fertilization rate (P=0.082), (P=0.059) were not associated with the level of serum and follicular fluid vitamin D.

Conclusion: This study found that women with higher levels of vitamin D in their serum and follicular fluid are significantly more likely to achieve pregnancy but without affecting the quality of embryo and fertility rate.

Keywords: Assisted Reproductive Techniques, Follicular Fluid, Infertility, Serum, Vitamin D

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Introduction

Infertility is a widespread problem, which affects many humans in the world. Today approximately 20% of couples are facing this problem. While more than half of them are seeking treatment options, approximately one-quarter of them accept child absenteeism (1). Generally, infertility is defined as the inability of couples to get pregnant after one year of regular unprotected sexual intercourse (2). It could be the result of a disease, stressful lifestyle, consumption of unhealthy foods and chemical medicines and exposure to industrial and environmental pollutants or other reasons (3). The most effective and costeffective way to prevent fertility problems or to treat these issues is nutritional modifications. Different food supplementations have significant roles in both prevention and treatment of infertility by their impact on the female and male reproductive systems. For instance, a deficiency in some vitamins and minerals can lead to infertility, and in fact, there have been recent reports suggesting a role for vitamin D in infertility (4).

The role of vitamin D in biological processes such as cell growth, metabolism modification, especially insulin function, autoimmune system and cardiovascular health is well known (5). This vitamin plays its role by interacting with vitamin D receptors (VDR) on various organs in the body (6). The presence of VDR in reproductive tissues such as testis, placenta, uterus and ovary has lead to the possibility that this vitamin is involved in reproductive processes as well (7). Disorders such as reduced fertility, diminished mating success, increased pregnancy complications, gonadal insufficiency, hypogonadism, uterine hypoplasia, impaired folliculogenesis (8) and infertility

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caused by vitamin D deficiency have been reported in animal models and human (9). There are several studies, which support the role of this vitamin in calcium transport through the placenta (10), placental steroidogenesis (11) and decidualization of endometrium (12). In addition, its function as a regulator of key target genes, which are related to implantation and establishment of the fetoplacental unit (13) has been identified.

It is believed that the vitamin D level in follicular fluid can be associated with its level in the body resources (14). There are contradictory results regarding the impact of this vitamin on the number of oocytes (8, 14) and embryo quality (15, 16) in assisted reproduction technique (ART). Follicular fluid, derived from both the follicular cell secretions and plasma (17), can be an important indicator of vitamin D levels, as it has been shown that serum vitamin D levels are related to the amount of this vitamin in the follicular fluid (15).

The presence of follicular fluid in many species shows its potential role in ovarian physiology, steroidogenesis (18), follicular growth and ovulation, oocyte maturation (19), and their transmission to fallopian tube (17). As follicular fluid provides a suitable environment for optimal growth of oocytes, which can have a direct effect on fertility (20), this study aimed to investigate the effects of serum and follicular levels of vitamin D on fertility and ART outcomes.

Materials and Methods

A prospective cohort study was performed on 150 women aged 18-40 years old with primary infertility, who had undergone assisted reproductive treatments [intracytoplasmic sperm injection + *in vitro* fertilization (ICSI+IVF)] at Isfahan Fertility and Infertility Center, Isfahan, Iran, from April to September 2015. A simple sampling design was used. Women who met the inclusion criteria were included in the study.

The inclusion criteria for this study consisted of female infertility, lack of endocrine disorders such as Cushing's syndrome, Hyper or Hypothyroidism, hyperprolactinemia (8), body mass index (BMI) 18-29 kg/m^2 (20), lack of congenital uterine anomalies and endometriosis (20), and not consuming drugs affecting vitamin D metabolism (21). The following formula was used for calculating the sample size:

$n = z^2 . s^2 / d^2$
$z = 1. 96$
s = an estimate of the standard deviation of vitamin D level
d= accuracy that considered 0.16.

After receiving the standard long gonadotropin-releasing hormone (GnRH)-a protocol by all the subjects, Buserelin Acetate 0.5 mg/day was injected intramuscularly on day 20-21 of menstrual cycle and 0.25 mg/day after mensuration until ovum pick up day. Then a subcutaneous injection of 75 IU/day recombinant follicle-stimulating hormone (FSH) was administered for ovarian stimulation. When at least two follicles reached 18-20 mm, human chorionic gonadotropin (HCG) 10,000 IU was administered through an intramuscular injection. After 34-36 hours, ovum was picked up. All the participants were followed by sequential vaginal ultrasound.

On the same day of ovum pick up, follicular fluid and serum samples were collected to determine the level of 25-OH vitamin D (HPLC system, euro immune kit, Germany). Vitamin D levels were defined as sufficient (30-100 ng/ml), insufficient (10-30 ng/ml), or deficient (<10 ng/ml). Fertilization was performed in the lab by an expert embryologist and the success rate of fertilization and embryo quality were investigated according to the number of blastomeres and fragmentation rate. Good-quality embryos (7 or more blastomeres and >20% fragmentation rate) were transferred to uterine 3 days after fertilization. Pregnancy was detected by serum β-hCG analysis (Electrochemiluminescence method, Roche, Germany) two weeks after embryo transfer, and a transvaginal ultrasound scan was employed at 3-4 weeks later to detect the intrauterine gestational sac. Itn this study, after ovum pick ups in all the women, luteal phase was supported with 400 mg suppository vaginal progesterone (cyclogest) twice per day for 10-12 weeks after pregnancy (Fig.1).

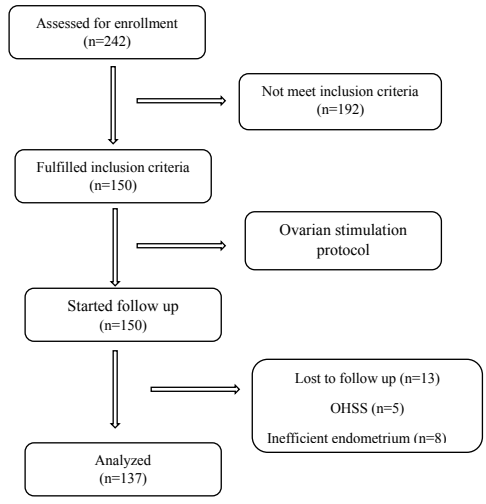

Fig.1: Study flowchart. OHSS; Ovarian hyperstimulation syndrome.

Ethical considerations

This study was approved by Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran (IR.MUI.REC.1394.3.147). A written informed consent was taken from each of the participants of this research.

Data analysis

Data were analyzed by SPSS software (version 16, SPSS Inc., Chicago, Ill., USA) and Chi-square and Spearman correlation coefficient. A value of $P \leq 0.05$ was considered statistically significant.

Results

This study included 150 infertile women. Thirteen

of them were excluded from the study due to ovarian hyperstimulation syndrome (OHSS) or because their endometrium was not ready for emberyo transfer. Ultimately, data analysis was performed on 137 patients. The patients' demographic characteristics are summarized in Table 1. The age of the females ranged from 21-40 with an average age of 30.30 ± 4.75 years. The mean of vitamin D level of follicular fluid and the serum was 26.99 ± 24.32 ng/ml and 26.37 ± 24.36 ng/ml, respectively. Also, there was a significant difference between the levels of vitamin D in serum and follicular fluid (r=0.711, P<0.001).

The results show that 45.8% of the subjects with positive biochemical pregnancy had serum vitamin D levels above 30 ng/ml, while 80.5% of the women with negative pregnancy rate were in the group with vitamin D levels less than 10 ng/ml. The statistical analysis indicated that there was a positive association between serum vitamin D levels and the incidence of biochemical pregnancy (P=0.008) and clinical pregnancy (P=0.017, Table 2).

In addition, in 44.4% of the females who had a positive biochemical pregnancy, the vitamin D level in follicular fluid was higher than 30 ng/ml, while in 84.6% of the women who had less than 10ng/ml vitamin D in their follicular fluid, a successful pregnancy did not occur. Statistically, a positive association was found between follicular fluid vitamin D levels and pregnancy (biochemical pregnancy, P=0.003, chemical pregnancy, P=0.001) (Table 2).

Table 1: Baseline characteristics of study population (n=137)

Characteristics	Mean ± SD or n (%)
Woman age (Y)	30.30 ± 4.75
Education level	
Graduate	69 (50.3)
High school	42 (30.6)
Middle school	15 (10.9)
Elementary	11 (8.2)
Occupation	
Housewife	104 (75.9)
Employed	33 (24.1)
Location	
Urban	128 (93.4)
Rural	9 (6.6)
Duration of infertility (Y)	6.16 ± 4.49
BMI (Kg/m^2)	24.70 ± 2.87
Follicular fluid vitamin D (ng/ml)	26.99 ± 24.32
Serum level of vitamin D (ng/ml)	26.37 ± 24.36

BMI; Body mass index.

Table 3 shows that there is no association between the serum level of vitamin D and the quality of embryo (r=0.126, P=0.125) or fertilization rate (r=0.019, P=0.082). Similarly, no associations were observed between the follicular fluid level of vitamin D and embryo quality (r=0.133, P=0.106) or fertilization rate (r=0.154, P=0.059).

Table 2: Serum and follicular fluid vitamin D levels and biochemical and clinical pregnancy rate

	Follicular fluid vitamin D (ng/ml)			P value*	X^2	Serum vitamin D (ng/ml)			P value*	X^2
	10>	10-30	30≤			10>	10-30	30≤		
Biochemical pregnancy										
Positive	6 (15.4)	14 (31.8)	24 (44.4)	0.003	8.661	8 (19.5)	14 (29.2)	22 (45.8)	0.008	7.094
Negative	33 (84.6)	30 (68.2)	30 (55.6)			33 (80.5)	34 (70.8)	26 (54.2)		
Clinical pregnancy										
Positive	3 (7.7)	13 (29.5)	22 (40.7)	0.001	11.938	6 (14.6)	14 (29.2)	18 (37.5)	0.017	5.653
Negative	36 (92.3)	31 (70.5)	32 (59.3)			35 (85.4)	34 (70.8)	30 (62.5)		

Data are presented as n (%). *; P≤0.05 was considered significant.

Table 3: Follicular fluid vitamin D levels and embryo quality and fertility rate

	10>	10-30	30≤	P value*
Follicular fluid vitamin D (ng/ml)				
Embryo quality	59.01 ± 28.72	66.45 ± 32.22	68.33 ± 28.23	0.106
Fertilization rate	55.12 ± 30.63	57.96 ± 30.94	66.62 ± 23.87	0.059
Serum vitamin D (ng/ml)				
Embryo quality	60.79 ± 30.29	64.97 ± 29.16	68.72 ± 30.23	0.125
Fertilization rate	60.65 ± 30.24	57.98 ± 29.30	62.40 ± 26.95	0.082

Data are presented as mean ± SD. *; P≤0.05 was considered significant.

Discussion

In this study, we sought to elucidate one of the most controversial issues in fertility, which is whether vitamin D affects assisted reproduction outcomes. Over the past decades there has been extensive investigation on the physiological roles of 25-OH vitamin D on ART outcomes, but the results of previous studies are very heterogeneous (22). This heretogenousity can be due to various factors affecting vitamine D levels including diet and the degree of exposure with sunlight (23). Nonetheless, independently of these factors, animal experimental studies have shown that vitamin D deficiency may affect fertility through Ca dependent/independent hemostasis (24). However, most reproductive consequnences of viatmin D deficiency are corrected. Consequently, this prospective cohort study was performed to evaluate the association of vitamin D levels in follicular fluid and serum on both biochemical and clinical pregnancy outcomes. We also evaluated the association between these two parameters with embryo quality and fertilization rates among participants.

It is noteworthy that the relationship between the level of 25-OH vitamin D in follicular fluid is a reflective of stores of vitamin D in the body (8, 14). Moreover, serum and follicular fluid 25(OH)D are directly related to each other (25).

Our results showed a significant association between the serum and follicular fluid vitamin D levels and pregnancy rate, which is in agreement with several previous published work (4, 8, 16, 26, 27), however, it is in contrast to other studies, which have reported no association (14, 25, 28-30) or inverse relation (15).

We also concluded that Vitamin D status is not associate with embryo quality and fertilization rates, despite the fact that association ofthe fertilization rate was close to be significant. These results are in accordance with Rudick et al. (16) and Aleyasin et al. (31). While Anifandis et al. (15) found that higher levels of this vitamin have a negative impact on embryo quality and therefore on IVF outcome.

Rudick et al. (16) showed an association between vitamin D and IVF success rate among non-Hispanic whites but not in Asians. They concluded that there was a statistically significant impact of race on the relationship between these two parameters. Our current finding are in contrast with previous results of investigations in Iran (14, 29) that suggested no relationship between vitamin D levels and the outcomes of ART. Definitely, the most surprising results were observed by the Anifandis et al. (15), as they reported an excess level of vitamin D in combination with a decreased level of follicular fluid glucose have an adverse effect on ART outcomes of infertile Greek women.

Considering the substantial discrepancies with published works, these results add to the literature the potential role of vitamin D on pregnancy rate among infertile couples undergoing infertility treatments. The possible mechanism can be explained as follows: firstly, vitamin D has been diagnosed as a factor, which affects endometrium receptivity. 1,25-dihydroxy vitamin D3 (1,25$[OH]_2D_3$) is produced in endometriotic cells in response to interleukin B1, which is secreted by blastocyst. This enzyme binds to VDRs on the endometrium and regulates the expression of genes involved in implantation and placental development (32). Additionally, vitamin D plays a critical role in up-regulation of transcription of *HOXA10* gene, an important gene participating in both placentation and implantation (33). It is important to point out that *HOXA10* gene can be activated by interaction with vitamin D (34).

Secondly, the influence of vitamin D on development of follicles and embryo has been previously reported (23). This vitamin also stimulates the production of estradiol, estrone, and progesterone and the enzymes that are responsible for the production of these hormones have vitamin D response element in their promotors (24). Additionally, anti mullerian hormone (AMH), a marker of ovarian reserve, has an inhibitory effect on the primordial follicle recruitment during folliculogenesis. It has been shown that there is a functional VDR element (VDRE) in the promoter of *AMH* gene (24) and that AMH is positively affected by vitamin D. Therefore, defects in VDR or its deficiency in the body can retard follicle development and oocyte maturation (14). Thus, it is not surprising to see some reports regarding the relationship between level of vitamine D and low ovarian response, as well as the fact that viatmine D supplementation increases AMH level (24).

Finally, vitamin D plays a vital role in gestation and maintaining a healthy pregnancy. The association between a decreased level of vitamin D and a higher risk of gestational diabetes and preeclampsia has been investigated by several studies (35).

Considering all the above mentioned findings, it is not unexpected to observe that vitamin D plays a crucial role in fertility outcomes. Therefore, the discrepancy within published works can be explained by other confounding factors, such as a source of vitamin D (diet, exposure to the sun, former supplementation), lifestyle, ethnicity, age, BMI, seasonal effect, and involvement of other ovarian factors responsible for this procedure.

In this research certain limitations should be considered; although we investigated maternal vitamin D status, the paternal vitamin D concentration needs to be assessed simultaneously. There are numerous studies showing that deficiency in vitamin D not only effects sperm parameters but also affects sperm DNA integrity, which subsequently will affect embryo developmental competency (35). Additionally, there is a lack of monitoring and measurement of vitamin D levels during pregnancy until delivery. We analyzed our data in terms of biochemical and clinical pregnancy to help better understand these issues. And finally, although we provided sufficient results through this study, it is nearly impossible to measure all the various confounding factors.

Indeed, a possible association has been reported among vitamin D and small for gestational age (SGA) infants (37-39), preeclampsia (35), and gestational diabetes mellitus (GDM) (40). Therefore, the side effect of vitamin D supplementation during pregnancy should be considered before suggesting its widespread consumption.

Conclusion

The findings of this study revealed that there is a positive association between serum and follicular fluid vitamin D levels and the success rate of biochemical and clinical pregnancy. However, there was no significant relationship between vitamin D level of follicular fluid and embryo quality or fertilization rate. Vitamin D supplementation is suggested to increase the level of this vitamin to a normal range in women with an insufficient level of vitamin D for achieving successful biochemical and clinical pregnancy.

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Authors' Contributions

Gh.N; Participated in study design, data collection and drafting the manuscript. M.T; Participated in the conception and design of the presented idea, performed data analysis, revision the manuscript and drafting. M.H.N.-H.; Conducted experimental work, technical and material support and contributed to editing the manuscript. A.R., M.J.; Performed data analysis and interpretation, co-wrote the manuscript, and revised the final version of the manuscript. All the authors approved the final manuscript.

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Original Article

Treatment with Calcium Ionophore Improves The Results in Patients with Previous Unsuccessful Attempts at The Fertilization: A Cohort Study

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Abstract

Background: The objective of this study is to evaluate artificial oocyte activation (AOA) with calcium ionophore (CaI) in a subsequent attempt at fertilisation in patients after extremely low or failed fertilisation. We assessed improvements in fertilisation, implantation and pregnancy rates as well as cancellation rates in these patients. Finally, was evaluated the result testing in addition to delivery rate and obstetric outcomes in children born after AOA.

Materials and Methods: This was a retrospective observational study conducted in an IVF laboratory of an IVI clinic (IVIRMA Valencia, Spain). One group (509 mature oocytes from 66 patients) received a first intracytoplasmic sperm injection (ICSI) without AOA, which resulted in either a failed fertilisation or very low values (<30%). This group was compared with a second group (616 mature oocytes from the same 66 patients) that used AOA. Outcome was compared by McNemar's test and the dependent t tests.

Results: AOA plus CaI resulted in enhanced fertilisation (51 vs. 13.1%), ongoing pregnancy (47 vs. 21.7%), and implantation (31.1 vs. 13.1%) rates, and less chances for cancelling the cycle (22.7 vs. 69.3%). There were no observed adverse effects in obstetric and perinatal outcomes after the use of AOA.

Conclusion: Our findings support the use of AOA for a given population of patients where fertilisation was affected during previous attempts. After AOA, we observed a significant increase in reproductive success due to the increased number of embryos available for embryo selection and, therefore, enhanced chances for success. The use of this artificial technique is comforting after checking non-existence of detrimental effects on the offspring.

Keywords: Calcium Ionophore, Intracytoplasmic Sperm Injection, Male Factor

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Introduction

Implementation of intracytoplasmic sperm injection (ICSI) in our laboratories enabled us to overcome most problems related to male factor infertility that were attributed to low counts and diminished sperm motility. However, there are a low percentage of cycles where conventional ICSI does not work as expected; consequently, around 1-3% of cycles have complete fertilisation failure or a low fertilisation rate compared to the average of 70-80 % (1).

Naturally, oocyte activation occurs after sperm-oocyte fusion, which is induced by a series of intracellular calcium oscillations that are generated and released from endoplasmic reticulum stores after the sperm enters the cytoplasm. These oscillations pursue until pronuclear (PN) formation (2-4), and then they stop. Most of these calcium oscillations have been studied in mammals and identified as phospholipase PLC zeta (PLCζ), a protein located in the perinuclear theca of spermatozoa. Under usual conditions, this factor enters the oocyte's ooplasm and causes release of calcium (5) followed by activation of the egg and resumption of the cell cycle. It is proposed that many fertilisation failures in infertile men are due to a lack of oocyte activation because of deficient intracellular calcium release, especially the oscillation cascade (6, 7), with the exception of those cases that have incomplete cytoplasm oocyte maturation where the response to this sperm factor is inhibited (8).

One well-known option for rescuing these unsuccessful cycles consists of increasing the amount of calcium into the ooplasm through the use of ICSI followed by artificial oocyte activation (AOA), which is called ICSI-AOA - a modified technique of conventional ICSI (9). ICSI-AOA is induced by the use of chemical substances, calcimycin (A23187) and ionomycin, which are the most widely used because of excellent results (10-12).

The importance of calcium oscillation during the activation time becomes evident on successive pre- and post-implantation events - embryonic development and

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pregnancy outcomes (2). In contrast, AOA, by the use of electrical or other chemical methods in humans, causes an only one abnormal calcium gain without the posterior needed frequency of calcium oscillations.

Montag et al. (13) published a retrospective study with fertilisation rates close to 50% after the use of calcimycin (A23187) in previous cases with fertilisation problems. Similarly, patients with either minimal or nonexistent fertilisation values (<30 %) would benefit from the use of AOA with CaI (11, 13). Recently, another author (14) published a randomized clinical trial that found benefits in subsequent treatments with the use of AOA in couples with male factor and unsuccessful conventional ICSI in previous cycles. The published benefits and evidence for the use of AOA make this strategy a potential option for certain patients.

We evaluated the effect of CaI on patients who had previous failed attempts with conventional ICSI and, as a consequence, in the following cycle they were treated with ICSI-AOA. Analyses of the perinatal and obstetric effects during and immediately following birth in children derived from oocytes treated with AOA were also performed.

Materials and Methods

The Instituto Valenciano de Infertilidad (IVI Valencia) performed this retrospective study over the last four years. This study was approved by the Institutional Review Board, Ethical Committee of Clinical Research IVI Valencia (ref. 1506-VLC-045-MM), which regulates and approves database analysis and clinical IVF procedures for research at IVI Valencia. It also complies with the Spanish law governing assisted reproductive technologies (14/2006).

We included all patients registered in our electronic medical records system. There were 66 patients included in this study with a total number of 163 cycles. The first group consisted of 75 cycles, 509 oocytes and 41 embryos. We included patients with previous conventional ICSI treatments (without AOA) that resulted in compromised fertilisation (fertilisation failure or fertilisation below 30%). These patients presented with good response and oocyte quality (no significant morphological abnormalities) and non-severe male factor. Subsequently, they underwent conventional ICSI. The second group (with AOA) consisted of the same patients (n=66) who did not succeed in the first attempt with conventional ICSI treatment. After an unexpected low (<30%) or absent fertilisation rate, AOA treatment was indicated for the second attempt to improve the fertilisation outcome. This second group generated 88 cycles, 616 oocytes and 104 embryos. Both groups 1 and 2 had fresh and frozen embryo transfers.

The primary outcome of this study was ongoing pregnancy rate and the secondary outcomes included fertilisation, implantation, miscarriage, and live birth rates, usable embryos and percentage of cycles with surplus embryos in each group. Patients older than 39 years of age and those subjected to egg donation were excluded from the study.

Ovarian stimulation, oocyte pick up and denudation

The same stimulation protocol was used for both groups because they were the same patients. Gonadotrophin-releasing hormone (GnRH-a) antagonist protocols were employed from day 21 of the cycle by subcutaneous administration for controlled ovarian hyperstimulation (15, 16). Briefly, once the ovarian quiescence was confirmed, a combination of recombinant follicle stimulating hormone (rFSH, Gonal-F, Merck Serono) and human menopausal gonadotrophin (hMG, Menogon, Ferring) were administered at a 2:1 ratio, and the dose was adjusted according to the response. After three or more follicles reached 17 mm in diameter, final maturation was induced by administration of 10 000 IU human chorionic gonadotropin (hCG) and, after 36 hours, follicles were aspirated and washed and placed into Global HEPES medium (LifeGlobal) at 37°C by using a tube warmer. The encountered eggs were continuously kept in an incubator for 3-4 hours before they were denudated.

Between three and four hours after oocyte harvesting, denudation was conducted by mechanical pipetting in 1:1 Global hyaluronidase (80 IU/mL; LifeGlobal) and Global w/HEPES by passing the oocytes through increasingly smaller denuding pipettes from 275 to 150 µm). Once granulose cells were removed oocyte maturation was confirmed under a microscope, and we selected the metaphase II (MII) oocytes for microinjection.

Intracytoplasmic sperm injection-artificial oocyte activation and conventional ICSI procedures

Semen samples were collected by masturbation into non-toxic sterile plastic jars after 3-5 days of sexual abstinence. The samples were allowed to liquefy for 30 minutes at room temperature (22°C) before they were evaluated according to WHO criteria.

For the density gradient centrifugation (DGC) procedure, ALLGrad® (LifeGlobal®, Guelph, Canada) was diluted in medium for Global Fertilisation® (LifeGlobal®) to obtain dilutions of 45% and 90%. Two gradient columns were prepared in Falcon® tubes by gently layering 1 ml of each solution, starting with the 90% fraction at the bottom. One ml of the semen sample was centrifuged for 10 minutes at 350 g with Global Fertilisation® before it was stratified on top of the discontinuous gradient columns and centrifuged for 18 minutes at 300 g. After centrifugation, the pellet was collected and centrifuged twice at 350 g for 5 minutes. After DGC, a second evaluation of sperm parameters was carried out and the prepared samples were used for microinjection of the oocytes.

Approximately four hours after retrieval and two hours after denudation, ICSI was performed under a microscope at x40 x10 (magnifications) in both groups.

As mentioned above, the ICSI procedure for the study patients was somewhat different from the standard procedure. This combined method (ICSI-AOA) was performed as described. Briefly, a spermatozoon was injected into the oocyte in a conventional way. Consecutively, the oocytes were exposed to a pre-equilibrated calcium ionophore (CaI) (GM508 CultActive, GYNEMED) for 15 minutes.

The solution consisted of NaCl, KCl, KH_2PO4, $MgSO_4$, $7H_2O$, $NaHCO_3$, $CaCl_2.2H_2O$, D(+)-glucose anhydrous, Na-lactate, Na-pyruvate, EDTA, alanyl-glutamine, water, non-essential and essential amino acids, DMSO, and Ca+2 ionophore A23187. This medium (GM508 CultActive) is a bicarbonate-buffered reagent designed for oocytes of patients with failed fertilisation after previous ICSI cycles. The spermatozoa were selected according to morphological criteria without selecting borderline or abnormal sperm whenever possible, and then immobilized in the same way as conventional ICSI (fracturing the flagellum with the ICSI needle).

Immediately after the injection, the oocytes were incubated for 15 minutes in pre-equilibrated (4 hours) Ca^{+2}-ionophore GM508 at 37ºC, 6% CO_2 and 20% O_2 atmosphere. Next, the oocytes were washed in clean Global (LifeGlobal) media drops and placed in new culture dishes (Vitrolife) that contained Global media pre-incubated overnight at 37ºC, 6% CO_2 and 20% O_2 atmosphere until embryo transfer or vitrification were conducted.

In the control group, ICSI was performed as usual, without any contact between sperm or oocytes with the CaI solutions, and according to the standard process. The microinjected oocytes were cultivated in the same culture conditions as the study group (Global media dishes at 37ºC, 6% CO_2 and 20% O_2 atmosphere).

Fertilisation and embryo assessment

Fertilisation assessment and embryo classification were conducted on days 1, 3 and 5 of culture according to established methods (17, 18). Embryo selection for transfer or cryopreservation was performed on day 3 or day 5 depending on the embryo's features and quality.

Depending on the patient's history, single embryo transfer (SET) or double embryo transfer (DET) was offered with a maximum of two embryos transferred and, rarely, three embryos when the previous history promoted to transfer them. Wherever possible, elective SET was performed.

Supernumerary embryos were vitrified for further frozen embryo transfers (17, 19).

Assessment of clinical outcomes

A serum pregnancy test was conducted for β-hCG levels at 13 days post-transfer. Clinical pregnancy was defined as the presence of at least one intrauterine gestational sac with a foetal heartbeat detected by ultrasound examination, up to 12 weeks after the pregnancy test. Both live births and neonatal outcomes were reported.

Fresh and deferred cycles

In the standard group, 41 embryos were transferred as follows: 18 fresh cycles with 28 cleavages and 3 blastocyst embryos; 4 frozen cycles with 7 cleavages and 1 blastocyst embryo; one mixed cycle with two cleavage embryos (1 frozen embryo joined to 1 fresh embryo); and 52 total cancelled cycles. Of these, 48 fresh cycles were cancelled because neither the embryo nor the zygote were obtained after injection. In addition, four cases (the embryos were vitrified for deferred transfer) were cancelled after warming due to a lack of embryos for transfer or no surviving embryos.

In the AOA group, 104 total embryos were transferred as follows. There were 52 embryos transferred in 31 fresh cycles: 21 blastocyst embryos in 14 cycles and 31 cleavage embryos in 17 cycles. There were 52 frozen embryos transferred in 37 deferred cycles: 44 blastocyst embryos in 31 cycles and 8 cleavage embryos in 6 cycles. There were 20 cycles terminated: 15 fresh cycles were cancelled due to either poor embryo quality or no fertilisation was achieved and five thawed cycles were cancelled after verification that there were no surviving embryos.

Both 37 cycles from AOA and 4 cycles from the standard group were treated as deferred embryo transfers because the endometrium was not receptive for embryo transfer as well as hydrometra or ovarian hyperstimulation syndrome (OHSS) were observed. In these cases, the embryos were frozen for the next endometrium preparation.

In both the standard and AOA group, the embryos were replaced either at the cleavage or blastocyst stages according to medical indication, previous history and after counselling by the specialist team.

Statistical analysis

The total number of retrieved oocytes was 1348. Of these, we obtained 1125 mature oocytes (83.5%) that were divided into two groups; 616 treated with AOA (study group), and 509 treated by conventional ICSI (control group). Patients were compared (repeated) with or without CaI, and then related to the comparisons. We used McNemar's test for categorical variables while the t test was used for dependent samples, differing between variables. $P<0.05$ was considered statistically significant. The analysis included fertilization, pregnancy, ongoing pregnancy, and implantation rates as well as cancellation and delivery rates to determine the effectiveness of the AOA procedure. All analyses were performed using the Statistical Package for the Social Sciences 24 (SPSS, Chicago, IL, USA). Finally, multivariable logistic regression that included the generalised estimating equations (GEE) procedure to overcome the comparison within the same patients was conducted to assess the effect of AOA on ongoing pregnancy per cycle as well as per transfer.

Results

A total of 1125 microinjected oocytes were analysed in terms of fertilisation rate (normal or abnormal), degeneration rate, ongoing and implantation rates, and cancellation rate as well as embryo development. A comparison and analysis of the data in general terms showed that the AOA group (51%) had a higher fertilisation rate than the control group (13.1%, P<0.001). The AOA group also had a better ongoing pregnancy rate (47 vs. 21.7%, P<0.05) and implantation rate (31.1 vs. 13.1%) compared to the control group; however, there were no significant differences. Lower chances to cancel the cycle (22.7 vs. 69.3%, P<0.001) were found by applying AOA with CaI. The average embryo transfer was similar: 1.72 for the standard group and 1.66 for the AOA group.

As reflected in Table 1, the average sperm count, maternal age and number of matured oocytes, as well as the percentage of blastocyst transferred in each group and viable embryos (frozen or transferred) between the two groups were analysed by observing differences in the percentages provided by each group. In the AOA group, 66% of the cases were transferred during the blastocyst stage whereas in the conventional group, only 17.4% of the cases (P<0.01). The AOA group reached 33.1% of viable embryos and the control group reached the double 61.2% (P<0.01).

Table 1: Sperm, oocyte and embryo data

Type of technique	Conventional ICSI	AOA	P value
Paternal age (Y)	35.8 (35.1-36.5)	36.1 (35.4-36.7)	NS
Sperm count (10^6/ml)	18.2 (0.01-89.0)	24.5 (0.01-170.0)	NS
Sperm count (mill/ml after DGC)	3.93 (0.01-21.0)	4.01 (0.01-25.0)	NS
Maternal age (Y)	35.1	35.6	NS
Number of MII per cycle	6.73 (2-16)	7.68 (1-20)	NS
Number of embryos transferred	1.72 (1-3)	1.66 (1-3)	NS
Average embryos frozen	0.21 (0-2)	1.35 (0-8)	<0.001
Blastocyst transfer	17.4% (4/23)	66% (45/68)	<0.01
Good quality blastocysts	17.6%	19.9%	NS
% of usable embryos (transferred or frozen)	61.2% (41/67)	33.1% (31/104)	<0.01

Data are presented as average with a confidence interval (CI) of 95% between brackets. Average of sperm count in fresh and after DGC, mean age, average number of oocytes, and number of embryos transferred in each group as well as percentage achieved in terms of frozen or viable embryos according to the applied different technique. ICSI; Intracytoplasmic sperm injection, AOA; Artificial oocyte activation, DGC; Density gradient centrifugation, NS; Non-significant, and MII; Metaphase II.

Table 2 shows the variables related to both normal (two polar body and two pronuclei) and abnormal fertilization (one pronuclei or more than two pronuclei), and the degeneration rate after microinjection according to the method used.

Table 2: Maturation and fertilisation rates

Type of technique	Conventional ICSI	AOA	P value
Oocyte numbers	605	743	-----
Matured oocytes (1125)	509	616	-----
2PN/MII rate	67/509 (13.1%)	314/616 (51.0%)	<0.001
Abnormal fertilization rate	40/509 (7.8%)	27/616 (4.4%)	NS
Degeneration rate	31/509 (6.1%)	52/616 (8.4%)	NS
% non-fertilized oocytes	371/509 (73%)	223/616 (36.2%)	<0.001

Data are presented as average with a confidence interval (CI) of 95% between brackets. Normal and abnormal fertilisation rate and non-fertilisation rate as well as degeneration rate after microinjection according to both techniques used in this study. ICSI; Intracytoplasmic sperm injection, AOA; Artificial oocyte activation, 2PN; 2 pronuclear, MII; Metaphase II, NS; Non-significant, Normal fertilization or 2PN; Zygotes with two polar bodies and two pronuclei, and Abnormal fertilization; Zygotes that are unipronuclear or with more than two pronuclei, in addition to those without extrusion of the second polar body and without pronuclei due to lack of oocyte activation.

Table 3 shows the other analysed variables regarding outcome of the cycle, cancellation rate and development cycle rates. Group 1 consisted of 18 fresh cycles, 5 frozen cycles and 52 cancelled cycles due to poor embryo quality, no zygotes obtained or no embryos that survived post-thawing.

Table 3: Logistic regression analysis by cycle and transfer

Type of technique	Conventional ICSI	AOA	P value
Cycles (163)	75	88	---------
Cancellations	52/75 (69.3%)	20/88 (22.7%)	<0.001
Transferred-fresh cycles	18/75 (24%)	31/88 (35.2%)	NS
Transferred-frozen cycles	5/75 (6.6%)	37/88 (42%)	<0.05
Total cycles with embryo transfer	23	68	------
Ongoing pregnancy	5/23 (21.7%)	32/68 (47%)	<0.05
Ongoing pregnancy rate (fresh cycle)	2/18 (11.1%)	13/31 (42%)	<0.05
Ongoing pregnancy rate (frozen cycle)	3/5 (60%)	19/37 (51.35%)	NS
Transferred embryos	41	104	------
Implantation rate (100%)	5/41 (12.2%)	32/104 (30.8%)	NS
Miscarriage rate	3/5 (60%)	3/32 (9.3%)	NS
Babies born	3	22	-----
% of cycles with surplus embryos	1/75 (1.3%)	15/88 (17%)	<0.05

Data are presented as average with a confidence interval (CI) of 95% between brackets. Cancellation and development cycle rates were compared between the two groups, as well as ongoing pregnancy and implantation rates that resulted from embryos obtained by either technique. ICSI; Intracytoplasmic sperm injection, AOA; Artificial oocyte activation, and NS; Non-significant.

Group 2 consisted of 31 fresh cycles and 37 frozen cycles. Unfortunately, 15 fresh cycles were cancelled due to the lack of an embryo for transfer and 5 frozen

cycles were cancelled because embryos did not survive or inadequate embryo development post-warming. The overall ongoing pregnancy rate was higher in the AOA group (47%) compared to the conventional group (21.7%, P<0.001). When separated according to type of cycle (fresh or frozen), we noted that the ongoing pregnancy rate was higher in the AOA group (42%) compared to the conventional group (11.1%) in the fresh cycles. However, in the frozen cycle, the results were quite similar (60% in the conventional group and 51.3% in the study group, P<0.05). The implantation rate was higher for the AOA group (31.1%) compared to the conventional group (13.1%). In terms of cycles with frozen surplus embryos, the standard group achieved one cycle with one frozen embryo (1/75, 1.3%), whereas there were 15 cases with surplus frozen embryos (15/88, 17%) in the AOA group (P<0.05).

The number of babies born according to technique and the neonatal and obstetric data are shown in Table 4. In the control group, two pregnancies resulted from fresh embryo transfer and three pregnancies occurred after frozen embryos were transferred. Although there were 5 successful cases out 15, 3 ended in clinical pregnancy lost. As a result, three babies were born (1 from a singleton delivery and 2 from twins) via vaginal delivery. Out of 21 ongoing pregnancies achieved by AOA, 13 newborns originated from 13 frozen embryo transfers (10 blastocyst stage transfers and 3 cleavage stage transfers). Eight newborns resulted from 7 fresh embryo transfers (4 blastocyst stage transfers and 3 cleavage stage transfers). As a result, 20 of them gave rise to 21 babies born (one twin pregnancy) and one was lost in early pregnancy. Of these, 9 were born by caesarean section and 12 delivered vaginally.

Perinatal and obstetric outcomes were studied after the births of the babies from both techniques (Table 4). There were no minor or major adverse effects noted in the offspring. It was remarkable that there were low birth weight babies in the conventional group; however, the sample size was limited and might not be considered (three babies).

Although no differences were observed between ICSI and ICSI-AOA patients in terms of the day of transfer and maternal age, a further analysis was performed by multivariable logistic regression in which those variables were included as potential bias factors. The study was performed to weigh the effect of AOA on ongoing pregnancy per cycle as well as per transfer. As demonstrated, the application of AOA in our patients increased by more than four times odds ratio (OR=4.57) per cycle, but not per transfer. When embryos were available for transfer, AOA did not increase the chances of a viable pregnancy (Table 5).

Table 4: Neonatal data

Newborns data	Conventional ICSI	ICSI-AOA
Live birth rate	7.3% (3/41)	20.2% (21/104)
Weeks at delivery	37.4 (37.1-37.6)	39.2 (36.6-41.5)
Preterm births (<37 weeks)	0 (0%)	1 (5%)
Very preterm births (<34 weeks)	0 (0%)	0 (0%)
Caesarean section	0 (0%)	8 (38.1%)
Vaginal delivery	3 (100%)	12 (60.0%)
Neonatal outcome	N=3	N=21
Gender Female Male	 1 (33%) 2 (66%)	 8 (38%) 13 (62%)
Birth weight (g)	2253 (2115-2365)	3361 (2500-4300)
LBW (<2500 g)	3 (100%)	0 (0%)
Neonatal height (cm)	45.3 (44-46)	49.8 (45-53)
Apgar <7 at 5 minutes	0 (0%)	0 (0%)
Apgar score at 1 minute	9 (9-9)	9 (9-9)
Apgar score at 5 minutes	9 (9-9)	9.9 (9-10)
Malformations	0	0
Major malformations	0 (0%)	0
Minor malformations	0 (0%)	0 (0%)
Neonatal intensive care	0	2
Perinatal mortality	0 (0%)	0 (0%)

Data are presented as average with a confidence interval (CI) of 95% between brackets. Neonatal data and birth defects were retrieved for all children born through both techniques as well as preterm birth data, weeks of delivery and type of birth. The number of cases and/or range (between brackets) is included. ICSI; Intracytoplasmic sperm injection, AOA; Artificial oocyte activation, and LBW; Low birth weight.

Table 5: Sperm, oocyte and embryo data

Model effect	Value	OR	P value
Ongoing pregnancy rate per cycle			
ICSI	AOA versus conventional	4.57 (1.47-13.97)	0.008
Day of transfer	Day 3 versus day 5	0.93 (0.84-1.04)	NS
Maternal age	Years	0.80 (0.50-1.26)	NS
Ongoing pregnancy rate per transfer			
ICSI	AOA versus conventional	0.964 (0.23-4.06)	NS
Day of transfer	Day 5 versus day 3	0.85 (0.51-1.41)	NS
Maternal age	Years	0.73 (0.10-4.91)	NS

Logistic regression analysis of ongoing pregnancy after week 12 as affected by ICSI-AOA. The effect was considered by cycle and by transfer. As co-variables we included the day of transfer (day 5 vs. day 3) and age of the patient (years) because co-variables were included. OR is shown with 95% confidence intervals in brackets. OR; Odds ratio, ICSI; Intracytoplasmic sperm injection, and AOA; Artificial oocyte activation.

Discussion

The ICSI procedure must be performed by experienced personnel because an improperly performed ICSI

technique in any *in vitro* fertilization (IVF) laboratory would imply a decrease in fertilisation rate. Occasionally, there are very low number of collected oocytes that might not yield embryos for transfer. In any case, both of the above mentioned options are not responsible of this issue at hand. That is particularly the case where the ICSI is not successful, as we expected, even when the technique is carried out by senior embryologists and uses a good number of oocytes. Although the majority of technologies employed in assisted reproductive techniques have largely resolved infertility problems, there are still unsuccessful ICSI cycles due to improper oocyte activation. Oocyte activation did not occur in approximately 70% of oocytes unfertilised after sperm injection. In most of these cases, activation deficiency was due to PLCζ impairment (aberrant function), as shown by the reduced levels or absence of its expression (20). Nevertheless, some studies have reported that, punctually PLCζ protein seems to be unstable and could not be as effective as expected; therefore, the application of AOA is a requirement for cases with previous unsuccessful fertilisation attempts in order to achieve pregnancy (21). Our current method, which we reported as modified ICSI, used an AOA methodology with proven improvement in terms of fertilisation, embryo development and success rates (22).

The AOA technique has not resulted in any minor or major problems in offspring that resulted from this burgeoning technique (21). In the last decade, AOA treatment has been successfully tested in IVF laboratories in cases of highly altered sperm quality like globozoospermia or teratozoospermia (21, 23, 24).

However, the benefit has also found for patients with normozoospermia (as in our case) where were not obtained embryos for transfer in the first attempt, but after AOA implementation were achieved better results and it was reported in consequence (25).

Tavalaee et al. (25) found a benefit with the use of AOA for cases with extreme changes in semen (globozoospermic) where inappropriate levels of PLCζ was the reason for oocyte inactivation and subsequent fertilisation failure. Their results showed that fertilisation rates after AOA in globozoospermic patients matched our results (53.14 vs. 51); however, they used globozoospermic patients as a study group whereas our study population consisted of patients with normal semen (apparently no impaired), which probably masked compromised levels of PLCζ.

Other researchers performed a more detailed assessment that checked the levels of PLCζ in infertile men who had histories of failed oocyte activation (26). They found a decreased percentage of relative expression of this protein in infertile men as well as globozoospermic men compared to fertile men or patients with high fertilisation rates.

In our study, we observed an improvement in cases where the oocyte activation was harmed (most probably due to sperm, despite normal morphology and count parameters). Although we did not study the phospholipase levels in the oocytes, the data caused us to ponder PLCζ dysfunction in the study group and, therefore, this dysfunction might be liable for this fertilisation failure. Although the proportion of this alteration is extremely rate (1% of men), it was published by some authors (27). By taking into consideration the number of treatments performed in our centre over four years (approximately 10000 retrievals, excluding severe male factor cases), this very low rate of approximately 80 cycles matches the failed cases without AOA due to hidden male factor.

Therefore, we can recover egg activation, resume the first cell cycle and consequently improve oocyte fertilisation, and obtain more embryos after treatment, which would increase the chances for success. The most common CaI suppliers are ionomycin and calcimycin (1, 3, 28, 29), both for injection and incubation for 10 minutes or for injecting oocytes according to conventional ICSI and incubating them for 15 minutes in a CaI solution. Both options provoke the flow inlet of extracellular calcium. In our study, we preferred to use the second option (conventional injection and culture) to guarantee the success of the procedure without potential injury after injection.

This increase of intracellular calcium would re-establish normality and overcome the first calcium wave that should be caused by PLCζ. This calcium release caused by the effect of A23187 can mimic the repeated natural oscillations by calcium that are necessary to complete the cell cycle (30).

Another aspect to consider is the asynchrony between cytoplasmic and nuclear maturation. It has been proposed that oocytes with incomplete cytoplasmic maturity lead to poor clinical outcome, because of the association with a lower than expected proportion of MII oocytes (31). In our study, the percentage of matured oocytes (MII) obtained in the AOA group was similar to the control group (82.9 vs. 84.1%), and did not affect the fertilisation rate by the poor cytoplasmic maturity.

The implementation of AOA in the laboratory daily routine has allowed us to obtain a double benefit: on the one side, the pregnancy chances of the couple were increased due to more zygotes and, consequently, more embryos and, on the other side, we allowed the use of homologous gametes (bypassing unneeded sperm donation), especially when the couple wished to repeat the treatment with guarantees after a fertilisation failure.

Different authors reported high fertilisation levels (74%) and acceptable pregnancy rate limits (33%) after using AOA in couples with previous failed fertilisation. Regarding the fertilisation rate, our values were lower (74 vs. 51.3%); however, our pregnancy rate was superior to those found by Heindryckx et al. (11) (33 vs. 45.7%). Our results are in line with those found by Ebner et al. (30), who performed a study similar to ours and reported the following benefits after AOA in previous untreated cycles: fertilisation rates (56.9 vs. 51.3%), implantation rates (33.3 vs. 32.6%), and

pregnancy rates (29.7 vs. 45.7%). Even when taking into account the existing limitation of frozen embryos, the live birth rate between both groups significantly increased (10.7 vs. 29.4%) and 23 babies were born after both techniques: 16 singleton and 2 twin pregnancies, which resulted in 20 healthy children after AOA; one twin pregnancy and one singleton gave rise to three babies in the conventional ICSI group. There were no observed malformations.

An interesting paper from Belgium (32) described the neonatal and developmental outcome in 21 babies born following AOA. Their conclusions were reassuring, since the children born following AOA had normal neonatal, developmental and behaviour outcomes and there were no serious unfavourable effects observed. Another paper reported 22 babies born after different types of AOA where they studied 10 babies born after the use of CaI A23187 and 12 following $SrCl_2$; the infants had similar health and growth. Other authors assessed the weights and heights of 21 babies (up to six years) born after AOA, and found physical growth within the 10-90% percentile (32).

We obtained frozen surplus embryos that resulted from AOA (17 patients obtained extra embryos and 10 patients stored frozen embryos for future use), while in the control group, there was only one frozen embryo; thus, these results would be more promising. Thus far, the published studies found the same results as the current study results (no deleterious effects in the children born) with a similar number of babies born. Secondly, AOA is a safe option for couples, especially after failed conventional ICSI, as long as there is no oocyte factor hidden like immature oocyte factor (33, 34).

The difference in blastocyst transfer achieved with the two groups (17.4 vs. 66%) was remarkable. This difference was probably due to the lower number of embryos obtained by the conventional group, which promoted us to advance the embryo transfer on day 3. In this context, it should be noted that the higher proportion of usable embryos in favour of the conventional group demonstrated that once the embryos were obtained, the chances of pregnancy were the same.

Conclusion

A limitation of this study was the absence of PLCζ analysis in the study group. A more detailed study should analyse PLCζ levels in patients with compromised fertilisation to confirm the current study findings.

Both ongoing pregnancy and implantation rates, the number of embryos frozen per cycle and the percentage of cycles with surplus embryos were improved using AOA. Therefore, based on these results, we encourage the scientific community to develop more studies to confirm the effectiveness of AOA. The existing evidence recommends us to offer this type of treatment to patients with previous poor fertilisation procedures where we know or we are almost certain that the previous fertilisation failure was attributed to the sperm.

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Authors' Contributions

A.T.; Participated in the study design and drafted the manuscript. L.A.F.; Performed the statistical analysis, interpreted the data and helped to draft the manuscript. P.G.I., D.B.T.; Assisted with patient recruitment and data collection. M.M.E., J.A.R.; Participated in its coordination and helped to draft the manuscript. All authors participated performing oocyte collection, conventional ICSI and AOA-ICSI. Also, they read and approved the final version of manuscript for submission.

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Original Article

Dual Trigger Compared with Human Chorionic Gonadotropin Alone and Effects on Clinical Outcome of Intracytoplasmic Sperm Injection

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Abstract

Background: This study compared outcomes of the standard 6000 IU human chorionic gonadotropin (hCG) trigger with a dual trigger comprised of 6000 IU hCG and 1 mg leuprolide acetate for final oocyte maturation in an intracytoplasmic sperm injection (ICSI) cycle. By convention, ICSI was performed in most cases at the clinic.

Materials and Methods: In this retrospective study, a total of 50 women were included in each arm. Participants were matched for age, indication and number of prior assisted reproduction technology (ART) cycles. Women at risk for ovarian hyperstimulation syndrome (OHSS) were excluded. A flexible gonadotropin releasing hormone (GnRH) antagonist protocol was used and final oocyte maturation was triggered when two leading follicles were >17 mm. Distribution of variables was evaluated visually with histograms. Continuous variables were defined by mean (standard deviation) or median (25^{th}-75^{th} percentile) depending on distribution characteristics. Categorical variables were defined by numbers and percentages. Continuous variables were compared between the groups with the t test or Mann-Whitney U test as appropriate. Categorical variables were compared by the chi-square test and its derivatives as appropriate. A two-sided $P<0.05$ indicated statistical significance.

Results: Both groups had similar antral follicle counts, median parity (0) and number of previous failed cycles (0). The median number of oocytes (8 vs. 7), metaphase-two oocytes (6 vs. 5.5), blastocysts (1 vs. 1), clinical pregnancy rates (CPR) (28% vs. 22%), ongoing pregnancy rates (OPR) (22% vs. 20%) and pregnancy rate per transfer (53.3% vs 53.8%) were similar between the dual trigger and hCG only groups, respectively.

Conclusion: Dual trigger for oocyte maturation stimulation failed to improve the ICSI outcome.

Keywords: Dual Trigger, GnRH Agonist, Human Chorionic Gonadotropin, Infertility, *In vitro* Fertilisation Outcome

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Introduction

Ovarian stimulation for assisted reproduction technology (ART) has three components: induction of multifollicular growth with gonadotropins, suppression of luteinizing hormone (LH) surge to prevent ovulation before egg retrieval and replacing the suppressed LH activity to induce oocyte maturation, which is also known as triggering. It is believed that the mode of triggering has a significant impact on the efficacy and safety of the ART treatment (1).

Human chorionic gonadotropin (hCG) is the traditional agent used to trigger oocyte maturation. Similarity between the beta subunits of LH and hCG molecules enable the latter to stimulate LH receptors on granulosa cells. However, the half-life of hCG is longer than LH and it induces longer stimulation of multiple corpora lutea following oocyte retrieval. This is associated with an increased risk of ovarian hyperstimulation syndrome (OHSS), a major risk of ovarian stimulation (2-6).

A single bolus of a gonadotropin releasing hormone agonist (GnRH-a) also induces an endogenous LH surge. The short duration of the GnRH-a induced LH surge leads to luteolysis and significantly decreases the risk of OHSS. However, luteolysis is associated with

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decreased pregnancy and increased miscarriage rates following fresh embryo transfer in GnRH-a triggered cycles (7-10).

The addition of a small dose of hCG for luteal phase support restores clinical outcome to some extent, but may increase the risk of OHSS, which justifies triggering without hCG in women at risk for OHSS (11).

Another suggested advantage of GnRH-a is the induction of an endogenous follicle stimulating hormone (FSH) surge simultaneous with the LH surge. The use of a GnRH-a to induce both endogenous LH and FSH surges, and hCG trigger simultaneously known as the "dual trigger", has been suggested to improve ART outcomes (12-15).

It is suggested that addition of a GnRH agonist to the hCG trigger in women with low ovarian reserve could improve the *in vitro* fertilisation (IVF) outcome (16, 17). However, whether the dual trigger is beneficial over the traditional hCG trigger for the common ART patient is uncertain.

The present study aims to compare the laboratory and clinical outcomes of standard dose hCG trigger with a dual trigger of hCG and 1 mg leuprolide acetate.

Materials and Methods

The Koc University Clinical Research Ethics Committee, Istanbul, Turkey approved the protocol of this retrospective cohort study (2019.269.IRB1.049). All the patients had signed an informed consent for study participation.

Between January 2018 and September 2018, all women who planned to undergo egg retrieval for IVF/intracytoplasmic sperm injection (IVF/ICSI) at the Koc University Assisted Reproduction Centre, except for those at high risk for OHSS, were given the dual trigger in the context of another study on granulosa cell function. These patients constituted the dual trigger group. Women who received only the recombinant hCG (rhCG) trigger within three months immediately before and three months immediately after the dual trigger period constituted the hCG group. The authors were blinded to the pregnancy outcomes at the time of matching.

The dual trigger consisted of 1 mg leuprolide acetate (Lucrin Daily, Abbott, USA, equivalent to 0.1 mg Decapeptyle) and 250 mcg (6000 IU) rhCG (Ovitrelle, Merck, Germany), while the conventional trigger was 250 mcg (6000 IU) rhCG.

Women >45 years of age and with a history of recurrent pregnancy loss were excluded.

Gonadotropins were started on the 2nd or 3rd day of the patient's menstrual cycle after ruling out ovarian or endometrial pathology by a transvaginal ultrasound (TVUS) scan. The starting (rFSH) (Gonal F, Merck, Germany) dosage ranged between 225 and 300 IU/day, according to ovarian reserve and body weight. Ovarian response to gonadotropins was evaluated by TVUS and serum oestradiol levels on the 5th or 6th day of stimulation and every 1-3 days afterwards, based on clinical judgment. Daily administration of 25 mg GnRH antagonist (Cetrotide, Merck KGaA, Germany) was started when the leading follicle diameter reached 14 mm or serum oestradiol level exceeded 200 ng/ml. Final oocyte maturation was triggered when two leading follicles were >17 mm. Transvaginal egg retrieval was performed 36 hours after the trigger. Conventional ICSI was carried out and all embryos were cultured until the blastocyst stage. Luteal phase support with 90 mg vaginal micronized progesterone gel twice a day (Crinone 8%, Merck, Germany) was started on the evening of egg retrieval and continued until a negative pregnancy test or the 6th week of gestation.

Clinical pregnancy was defined as visualization of a gestational sac with a foetal heart beat by ultrasound at 6-7 weeks after embryo transfer. Ongoing pregnancy was defined as a pregnancy that proceeded beyond the 20th gestational week. Oocyte maturation rate referred to the proportion of metaphase II (MII) oocytes to all collected oocytes per cycle. Implantation rate (IR) was calculated per cycle as the number of embryos with heart beat divided by the number of blastocysts transferred.

Statistical analysis

Continuous variables were defined with mean (standard deviation) or median (25th-75th percentile), and were compared between the groups with the t test or Mann-Whitney U test depending on distribution characteristics. Categorical variables were defined with numbers and percentages, and were compared between the groups with the chi-square test and its derivatives as appropriate. $P<0.05$ were considered statistically significant.

Results

The study included 50 women in each group. Baseline characteristics of both groups were similar (Table 1).

As shown in Table 2, the median number of oocytes collected (8 vs. 7, P=0.33), MII oocytes (6 vs. 5.5, P=0.41), blastocysts (1 in both groups), fertilisation (70% vs. 77%) and blastulation (30% vs. 28%) rates were similar in the dual trigger and hCG groups, respectively.

Fresh embryo transfer was performed in 30 out of 50 (60%) women in the dual trigger group and in 26 out of 50 (52%) women in the hCG group (P=0.43). Clinical pregnancy rate (CPR, 28% vs. 22%, P=0.49) and ongoing pregnancy rate (OPR, 22% vs. 20% P=0.63) per woman were similar in the dual and hCG trigger groups, respectively. Pregnancy rate per transfer was 53.3% in the dual group and 53.8% in the hCG trigger group (P=0.96). CPR per transfer was 46.7% in the dual group and 42.3% in the hCG group (P=0.74). Both groups had a miscarriage rate of 8% and there were no cases of OHSS during the course of the study.

Table 1: Baseline characteristics of women in the dual trigger and hCG only groups

Baseline characteristics	Dual trigger (n=50)	hCG only (n=50)	P value
Age (Y)	33 (29-38)	33.5 (30-38)	0.90
Parity	0 (0-0)	0 (0-0)	0.18
Number of previous miscarriages	0 (0-0)	0 (0-0)	0.71
Number of previous failed IVF cycles	0 (0-1)	0 (0-1)	0.46
Duration of infertility (Y)	2 (1.5-4)	2.5 (2-4)	0.22
Cause of infertility			0.36
Ovulatory disorder	2	2	
Low ovarian reserve	14	10	
Tubal factor	2	2	
Endometriosis	3	3	
Male factor	13	8	
Unexplained	10	20	
Secondary infertility	6	5	
Body mass index (kg/m^2)	23.7 (22.1-26.5)	23.1 (20.4-26-6)	0.34
Antral follicle count	11.5 (5.7-17.2)	8 (5-12)	0.13
Oestradiol level on trigger day (pg/ml)	1188 (678.8-1842.8)	1103 (780.8-1690)	0.61
Progesterone level on trigger day (ng/ml)	0.50 (0.25-0.63)	0.42 (0.29-0.70)	0.71
LH level at trigger day	2.6 (1.55-4.1)	3.5 (1.47-6.97)	0.34
Endometrial thickness (mm)	10 (7.9-11.8)	9.9 (8.5-11.9)	0.75
Gonadotropin starting dose (IU)	300 (281.2-300)	300 (300-300)	0.34
Total dose of gonadotropin	2329 (1950-2700)	2400 (2100-2944)	0.22
Duration of gonadotropin (days)	8 (7-10)	8 (8-10)	0.15

All values are median (25th-75th percentile). hCG; Human chorionic gonadotropin, IVF; *In vitro* fertilisation, and LH; Luteinizing hormone

Table 2: Comparison of outcomes between the dual trigger and hCG only groups

Outcome	Dual trigger (n=50)	hCG only (n=50)	P value
Number of oocytes*	8 (4.75-8)	7 (4.75-10)	0.33
Number of MII oocytes*	6 (3-6)	5.5 (3-9)	0.41
Oocyte maturation rate*	0.80	0.75	0.90
Number of two pronuclear fertilised oocytes*	4 (2-4)	4 (2-7)	0.72
Fertilisation rate	0.70	0.77	0.47
Number of blastocysts*	1 (0-1)	1 (0-3)	0.77
Blastulation rate	0.30	0.28	0.33
Number of embryos transferred*	1 (0-1)	1 (0-1)	0.39
Number of frozen embryos*	1 (0-1)	0 (0-2)	0.92
Positive pregnancy test (pregnancy rate)	16/50 (32%)	14/50 (28%)	0.66
Pregnancy pate per transfer	16/30 (53.3%)	14/26 (53.8%)	0.96
CPR	14/50 (28%)	11/50 (22%)	0.49
CPR per transfer	14/30 (46.7%)	11/26 (42.3%)	0.74
IR	14/33 (42.4%)	11/28 (39.2%)	0.56
Number of miscarriages	4/50 (8%)	4/50 (8%)	0.64
LBR	11/50 (22%)	10/50 (20%)	0.80
LBR per transfer	11/30 (36.6%)	10/26 (38.4%)	0.42

***; Values are median (25th-75th percentile), hCG; Human chorionic gonadotropin, CPR; Clinical pregnancy rate, MII; Metaphase II, IR; Implantation rate, and LBR; Live birth rate.**

Fresh embryo transfer was performed in 30 out of 50 (60%) women in the dual trigger group and in 26 out of 50 (52%) women in the hCG group (P=0.43). Clinical pregnancy rate (CPR, 28% vs. 22%, P=0.49) and ongoing pregnancy rate (OPR, 22% vs. 20% P=0.63) per woman were similar in the dual and hCG trigger groups, respectively. Pregnancy rate per transfer was 53.3% in the dual group and 53.8% in the hCG trigger group (P=0.96). CPR per transfer was 46.7% in the dual group and 42.3% in the hCG group (P=0.74). Both groups had a miscarriage rate of 8% and there were no cases of OHSS during the course of the study.

Discussion

In our study, universal use of dual trigger did not seem to provide any benefit regarding oocyte yield oocyte maturation, fertilisation, blastulation, implantation or CPR/OPR compared to the hCG only trigger. However, the small number of samples is the shortcoming of this study.

Effectiveness of dual triggering compared to hCG only or GnRH a only triggering has been investigated in a number of studies that vary greatly in design, methods and outcomes. Two randomised clinical trials (RCT) studied the effect of dual trigger in normo-responders. In the first one, Decleer et al. (18) studied 120 women <38 years of age who did not have polycystic ovarian syndrome or endometriosis. The mean number of retrieved oocytes were similar between the dual trigger (5000 IU hCG and 0.2 mg triptorelin acetate) and 5000 IU hCG trigger alone groups, respectively. The shortcoming of their study was the focus on day-3 embryos that had excellent quality. This subjective perception of excellence did not translate into better clinical outcomes as IR and OPR did not meet statistical significance between the dual trigger and hCG only groups. Moreover, day-3 embryo quality could be a poor predictor of blastulation, and the number of good quality day-3 embryos is a questionable outcome measure (19-21).

Eftekhar et al. (22) randomized 192 normal responders to receive dual trigger or hCG only trigger. Although the mean number of oocytes (10.85 vs. 9.35) and embryos (6.86 vs. 5.34) were statistically higher in the dual trigger compared to the hCG only group, there were no significant differences between implantation or CPR between the dual trigger and hCG only groups, respectively. In another RCT, Kim et al. (23) compared dual trigger and hCG trigger alone for 60 women in each group. They observed that although the number of oocytes retrieved, fertilised oocytes and good quality embryos were similar in both groups, embryo IR (24.7% vs. 14.9%), CPR per cycle (53.3% vs. 33.3%) and live birth rate (LBR) (50.0% vs. 30.0%) were significantly higher in the dual trigger group compared to the hCG only group, respectively. They concluded that combined administration of GnRH a with rhCG might be beneficial in improving endometrial receptivity and pregnancy rates in GnRH antagonist cycles for IVF.

Ding et al. (24) conducted a systemic review and meta-analysis to investigate the efficacy of dual trigger compared to hCG alone. In their four eligible RCTs that included 527 women, they concluded that dual trigger was equivalent to hCG in triggering oocyte maturation and may be beneficial in improving reproductive outcomes; however, they emphasized that further intensive RCTs are needed to investigate the efficacy of dual trigger.

Lin et al. (25) retrospectively compared the hCG only trigger and dual trigger in 376 normo-responder women, and reported that dual trigger significantly improved LBR. In another study, they evaluated the outcome of dual trigger in 427 cycles with fresh embryo transfer in patients with diminished ovarian reserve (antral follicle count of <5 or serum AMH level of <1.1 ng/ml) (17). They reported significantly higher fertilisation rate, clinical pregnancy and LBR with dual trigger compared to hCG only triggering.

Schachter et al. (26) examined the effect of dual trigger in a RCT of 200 cycles in women with history of at least one failed IVF/ICSI cycle on the GnRH-a long protocol. Although the mean number of oocytes (7.9 vs. 9.9) and embryos (4.7 vs. 5.7) were similar between the dual trigger (5000 IU hCG plus 0.2 mg Triptorelin) and control (5000 IU hCG) groups, there was a higher rate of OPR per transfer reported in the dual trigger group with marginal significance.

Fabris et al. (27) studied 81 patients who had more than 50% immature oocytes in a previous rhCG only triggered ART cycle. The same women were given dual trigger in subsequent 81 cycles. Although they reported a significantly higher number of total and MII oocytes retrieved in the dual trigger group, it should be noted that any intervention almost always provides significant improvement in the second round of before-after studies where the first cycles are selected from those with particularly bad results. These findings are most likely explained by regression to the mean phenomenon, rather than a true biological effect (28, 29). Similarly, Griffin et al. (30) recruited 27 women with history of more than 25% immature oocytes (germinal vesicle or metaphase I) in their previous IVF cycles when triggered with hCG alone and compared the outcome of dual triggering with their previous cycle in a retrospective study. The proportion of mature oocytes retrieved was almost double with the dual trigger protocol compared to their previous hCG only trigger cycle (75% vs. 38.5%, OR: 2.51). However, similar to the Fabris et al. (27) study, the increase in oocyte maturation rate could likely be attributed to regression to the mean phenomenon.

Zhang et al. (31) compared dual trigger with hCG trigger only in a retrospective cohort study of 1350 poor responder patients diagnosed according to the Bologna criteria for poor responders. They reported increased numbers of mature oocytes with the dual trigger; however, fertilisation rate, number of viable embryos, implantation, and clinical pregnancy and miscarriage rates did not significantly differ between the groups.

In summary, most studies reported improved intermediate outcomes rather than clinically relevant endpoints such as

IR or OPR, whereas RCTs and our study reported similar clinical outcomes with dual and hCG only triggering. In addition, another RCT that assessed the isolated effect of FSH exposure on the day of ovulation trigger also failed to demonstrate a beneficial effect on OPR/LBR over hCG triggering alone (32).

In the present study, we used dual triggering for all women except those who were at high risk for OHSS on the trigger day, regardless of ovarian reserve or their previous IVF history. Moreover, the authors were blind to the cycle and clinical outcomes during matching of the controls. Thus, selection bias was reduced by avoiding patient selection or physician preference. Still, the retrospective nature and the size of the study are the weaknesses of this study. On the other hand, use of any hCG, alone or in combination with another agent, in patients at high risk for OHSS is not currently advised (33). Thus, this may not be a weakness but a choice that helps the study more aptly reflect clinical practice.

Conclusion

Based on our study and previous RCTs, universal use of dual triggering does not seem to result in improved oocyte yield, oocyte maturation, fertilisation, IR, and CPR or OPR. Studies on dual triggering show conflicting results on different patient groups; thus, its benefit for all women who undergo IVF/ICSI lacks robust evidence and large, well-designed trials should be conducted.

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Authors' Contributions

B.Sh.; Project development, data collection, and manuscript writing. E.T.; Data analysis and critical revision of the manuscript. S.G.C., Data collection, analysis and critical revision of the manuscript. S.Y.; Data analysis, interpretation of the results, critical revision of the manuscript. I.K.; Performing IVF laboratory procedures, data managment, interpretation of results. B.A.; Protocol development, clinical management of patients data analysis, interpretation of the results, critical revition of the manuscript. All authors read and approved the final manuscript.

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Case Report

A Feasible Option before Cycle Cancellation for Poor Responders; STOP-START Protocol

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Abstract

Despite the advances in controlled ovarian stimulation (COS), management of a subgroup of poor ovarian responder patients may still be challenging. We describe a feasible and simplified protocol, namely the STOP-START protocol, for poor responders defined as Patient-Oriented Strategies Encompassing Individualize D Oocyte Number (POSEIDON) groups 3 and 4, who are unresponsive to COS with maximum dose gonadotrophins. Data of 11 women unresponsive to COS were reviewed. Mean age of the patients was 36.5 ± 6.0 years. Unresponsiveness was defined as no follicular growth >9 mm and/or estradiol level less than 40 pg/ml after a week of recombinant follicle stimulating hormone (rFSH, 225-300 IU) administration. In that case, COS was stopped and each woman underwent weekly ultrasound assessment to catch a secondary follicular growth. All women showed at least one follicular growth within five to 20 days. Six women (54.5%) had spontaneous follicular growth and the other five required ovarian stimulation. At least one oocyte was retrieved from each one of seven patients (63.6%). The mean number of oocytes retrieved was 1.6 ± 1.4 and five women (45.5%) had at least one grade A embryo. Among all, two women became pregnant successfully and both gave live births (18.2%). In conclusion, STOP-START protocol may potentially be an effective, feasible, and time-saving management option for POSEIDON group 3/4 poor responders who are unresponsive to standard COS treatment with maximum dose gonadotrophins.

Keywords: Assisted Reproductive Techniques, Folliculogenesis, Ovarian Stimulation, Unresponsive

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Introduction

Despite advances in controlled ovarian stimulation (COS) protocols and laboratory technologies, the management of poor responder patients is still challenging. Recently, the Patient-Oriented Strategies Encompassing Individualize D Oocyte Number (POSEIDON) group suggested a detailed classification system to better identify the poor ovarian response patients who would be included in future studies investigating diagnosis and management (1). Several researches have been conducted and different strategies have been developed to improve the rate of success in assisted reproductive technology (ART) cycles of poor responder patients. However, there is still a subgroup of poor responders who require important decision making: POSEIDON groups 3 and 4 patients who are unresponsive to standard COS treatment with maximum dose gonadotrophins. Therefore, we present the cycle characteristics and outcomes of 11 women managed with the STOP-START protocol.

Case series

Data of poor responder patients who underwent COS and were unresponsive to stimulation at a university-based infertility clinic between July 2017 and July 2018 were reviewed. The study was approved by the Institutional Review Board of Ankara University (no.: E34690; date: 19.06.2019). POSEIDON group 3 and group 4 poor responders who were unresponsive to COS and were managed by STOP-START protocol were selected from the hospital database. POSEIDON group 3 was defined as patients <35 years, antral follicle count <5 or anti-müllerian hormone (AMH) <1.2 ng/mL and group 4 was defined as patients ≥35 years, antral follicle count <5 or AMH <1.2 ng/mL (1). The inclusion criteria were women aged 18-45 years, a starting dose of gonadotrophin stimulation with 225-300 IU/day, and unresponsiveness to the first COS. The exclusion criteria were body mass index (BMI) over 30 kg/m^2, and the presence of any untreated thyroid dysfunction or hyper-prolactinemia. Eleven patients were eligible for analyses and all data regarding COS, STOP-START protocol, and clinical outcomes were extracted from the hospital database. All women gave written consent for data sharing at the beginning of the COS cycle.

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COS was performed with administration of rFSH (Gonal-F, Merck-Serono, Turkey) beginning from the cycle day 2 with a starting dose of 225-300 IU/day. Dose adjustment was performed according to individual ovarian response. As there was no follicular growth, gonadotropin-releasing hormone (GnRH) antagonists were not introduced during the initial stimulation period. The cases were defined as unresponsive to COS when there was no follicular growth >9 mm and/or when the estradiol level was less than 40 pg/ml after a week of maximum dose stimulation by rFSH.

The rFSH treatment was then stopped and each patient underwent weekly ultrasound assessment and blood test beginning after one week to catch a secondary follicular growth (STOP period). When a new wave of follicles grew (at least one >10 mm follicle and/or oestradiol >150 pg/ml), a secondary ART cycle began with spontaneous follow-up (START period). The patients underwent ultrasound and estradiol test in three days and were followed up spontaneously in case of natural follicular development. COS was started with 150 IU gonadotrophin if the existing follicle/s did not show development within three days. GnRH antagonist (Cetrotide, Merck-Serono, Turkey) was commenced (0.25 mg/day) when the leading follicle reached 13-14 mm and continued to grow throughout ovarian stimulation. Transvaginal ultrasound guided oocyte retrieval was performed 35-36 hours after final oocyte maturation with recombinant human chorionic gonadotropin (rhCG, Ovitrelle, Merck, Turkey). A frozen-thawed embryo transfer (FET) was planned due to possible asynchronization of endometrium.

The mean age of the patients was 36.5 ± 6.0 years and all patients fulfilled the POSEIDON criteria for group 3 and 4 (Table 1). The mean AMH level was 0.23 ± 0.25 ng/mL and the mean antral follicle count was 2.4 ± 1.6. All of the patients were unresponsive to a standard initial COS. Hence, the gonadotrophins were stopped and all patients showed at least one follicular growth within at 5 to 20 days after stopping gonadotrophins.

Table 1: The demographics of the study population

Patients (n=11)	Mean ± SD	Min-Max
Age (Y)	36.5 ± 6.0	25-45
Body mass index (kg/m^2)	25.6 ± 4.3	22-36.8
Baseline E2 (pg/ml)	46.9 ± 30.1	20-113
Baseline FSH (IU/ml)	18.5 ± 8.2	9-36
Baseline AMH (ng/ml)	0.23 ± 0.25	0.01-0.68
Antral follicle count	2.4 ± 1.6	1-6
Duration of infertility (Y)	5.0 ± 3.5	1.5-12
Number of previous IVF attempts	1.3 ± 1.0	0-3

E2; Estradiol, FSH; Follicle stimulating hormone, AMH; Anti-müllerian hormone, IVF; *In vitro* fertilization, Min; Minimum, Max; Maximum, and SD; Standard deviation.

Following the STOP period six patients (54.5%) had spontaneous follicle development within three days and the other five required ovarian stimulation. We were able to retrieve at least one oocyte from each one of seven patients (63.6%) (Table 2). The mean number of oocytes retrieved was 1.6 ± 1.4 and five patients (45.5%) had at least one grade A embryo. As a result, two women got pregnant and both gave live birth (18.2%).

Table 2: The cycle characteristics of the study population

	Patients (n=11)	Mean ± SD	Min-Max
1-COS	Duration of stimulation (days)	7.9 ± 2.3	5-12
	Total dose of gonadotrophins (IU)	1955 ± 1033	900-3600
	E2 levels on the day of cancellation (pg/ml)	31.6 ± 10.4	17-42
2-STOP	Duration of cessation period (days)	9.3 ± 4.5	5-20
	Number of follicles >9 mm at return	2.4 ± 0.9	1-4
	E2 levels at return (pg/ml)	178.9 ± 100.5	41-390
	Number of patients with spontaneous follow up (%)	6 (54.5)	
3-START	Maximal E2 levels at follow-up (pg/ml)	332.0 ± 91.9	193-445
	Retrieved oocytes (n)	1.6 ± 1.4	0-3
	Number of MII oocytes	1.1 ± 1.1	0-3
	Fertilization rate (%)	64.2 ± 39.0	0-100
	Number of grade A embryos	0.9 ± 0.9	0-2
	Number of patients with at least one grade A embryo (%)	5 (45.5)	
	Ongoing pregnancy, n (%)	2 (18.2)	

1-COS; Controlled ovarian stimulation, at this step a conventional GnRH antagonist protocol is carried out, 2-STOP; All drugs are stopped, and the spontaneous follicular growth is followed up, 3-START; A new follicular legion had started to grow and followed up spontaneously, or a mild ovarian stimulation protocol was performed, E2; Estradiol, MII; Metaphase II, GnRH; Gonadotropin-releasing hormone, Min; Minimum, Max; Maximum, and SD; Standard deviation.

The first pregnancy was a spontaneous one following oocyte retrieval. The patient was 35 years old and her AMH level was 0.68 ng/ml. Following STOP-START protocol we retrieved two MII oocytes and both were fertilized. She had one frozen grade A embryo and she was called for endometrial preparation on the second day of her next cycle. However, she returned 20 days later with menstrual delay and her βhCG test was positive.

The second pregnancy was achieved following a fresh embryo transfer in a 29-year-old woman, whose AMH level was 0.09 ng/ml (POSEIDON group 3). Following STOP-START protocol we retrieved three oocytes and only one was MII. Although we opted FET, fresh embryo was transferred by patient demand and in the light of abovementioned patient who got spontaneous pregnancy. This woman received 90 mg/day vaginal micronized progesterone (Crinone 8% gel, Merck-Serono, Turkey) for luteal phase support from the day of oocyte collection until 10 weeks of gestation.

Discussion

Recently, the wave theory was proposed suggesting that the follicles may be recruited two to three times within

a single menstrual cycle, even in the luteal phase (2). Ovarian stimulation in luteal phase results in a longer duration of stimulation and higher total dose of rFSH when compared to conventional start COS (3). The other novel protocols identified by wave theory are random-start COS and double stimulation (DuoStim) protocols that all support the luteal phase ovarian stimulation (4-7). Hence, in the present series, one of the goals was to reach a new wave of follicular growth. The main difference between STOP-START protocol and DuoStim is the long drug-free interval for approximately one week. However, different from the other protocols evolved from the wave theory, in STOP-START protocol the patient had no ovulation before the spontaneous follicular growth and we actually performed the ovarian stimulation in a prolonged follicular phase. This situation makes fresh embryo transfer possible.

Theoretically, a hormone-receptor complex may be deactivated by external shedding or by internalization of the receptors into the cell. Excess concentrations of trophic hormones, such as FSH, stimulate the process of internalization, leading to a loss of receptors in the cell membrane and thus, a decrease in biological response. During a standard COS, a high dose of rFSH is utilized daily to stimulate the available follicular cohort. Poor responder patients most likely have a reduced number of FSH receptors. In addition, the high-dose rFSH administration in a non-pulsatile manner might reduce the active hormone-receptors by internalization, and at this point the patient becomes unresponsive. The internalized coated pits containing such hormone-receptor complexes are degraded by lysosomes. While the receptors may be reinserted and become functional again, the internalized FSH may mediate biological responses by influencing cellular organelles (8). The suggested mechanism by which the STOP-START protocol possibly works is the prevention of internalization by stopping the rFSH stimulation. Cessation of pushing by FSH (STOP period) probably prevents down-regulation of FSH receptors and allows for the development of some freshened follicles, in which the free receptors are reinserted to the cell membrane and slightly stimulated by the internalized hormone (START period). Protection from a similar down-regulation mechanism of FSH receptors can also be speculated in mild ovarian stimulation cycles and natural cycle *in vitro* fertilization (IVF) procedures for poor responders (9, 10).

The major strength of the present case series is reporting a feasible stimulation protocol for poor ovarian responder patients. We presented the clinical efficacy of START-STOP protocol with two live births among 11 poor responders. Another strength was presenting the possibility of fresh embryo transfer with this novel protocol. The main limitations of the present case series were the retrospective design and small sample size. The lack of a control group was also noted as a limitation.

Conclusion

STOP-START protocol might be an effective, feasible, and time-saving management option for POSEIDON group 3/4 poor responders who are unresponsive to COS. However, it's necessary to confirm the feasibility and effectiveness of this protocol through accomplishing future prospective trials.

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Authors' Contributions

C.S.A., Y.E.Ş., M.S.; Participated in study design, data collection and evaluation. C.S.A., Y.E.Ş.; Participated in drafting and statistical analysis. B.Ö., M.S., B.B., R.A.; Contributed extensively in interpretation of the data and the conclusion. C.S.A.; Was responsible for overall supervision. All authors read and approved the final manuscript.

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Letter to The Editor

Combined Fertility Preservation Technique before Gonadotoxic Treatments in Cancer Patients

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Although ovarian tissue cryopreservation is still considered as an experimental technique, several authors from around the world have reported successful and promising results. Currently, oocyte cryopreservation seems to be the most feasible technique for fertility preservation when there's some kind of a time constraint in adolescents and adults. However, it has been estimated that a young woman would be expected to have a 94% likelihood of having a live birth with 20 mature frozen oocytes (1). At age 34 years, however, this expectation is decreased to 90% with 20 mature frozen oocytes. In addition to age-related limitations, an immediate obstacle for obtaining oocytes in cancer patients is the fact that only one controlled ovarian hyperstimulation (COH) cycle can usually be performed in these women because of time constraints, yielding a relatively low number of oocytes and/or embryos. For this reason, results from egg donation programs cannot be extrapolated to cancer patients, nor can the quality of oocytes be guaranteed. Hence, a combined fertility preservation technique can be of valuable in increasing the chances of successful future pregnancies following gonadotoxic cancer therapies. Previously, Dolmans et al. (2) suggested that cryopreservation of bilateral ovarian cortex followed by COH is a feasible and safe approach to preserve fertility before gonadotoxic treatment, and that the number of cryopreserved embryos was similar to the controls.

We have been offering the option of the combined technique to fertility preservation patients for a couple of years and have performed it in a series of eight candidate patients. All patients had enough time for COH before oncology treatments. We first performed laparoscopic ovarian resection for ovarian tissue cryopreservation and then started COH on postoperative day 0 or 1 in each patient (Table 1). The main point in our findings is that ovarian resection is performed from the side with less antral follicle count of the patients. We suggest that this approach can increase the oocyte yield in a single available COH cycle.

The data is limited on the effectiveness of combined technique and more long-term follow-up studies are needed in larger groups with appropriate controls. According to our clinical experience, we believe that combined technique is a valid approach, which is expanding beyond the experimental stage and has become a clinical technique for fertility preservation. We particularly suggest selecting the ovary with a low antral follicle count for wedge resection to increase oocyte yield. The information gathered from large international multicenter reports would encourage physicians to agree that the method should complete the experimental phase and be ready for wider clinical use in female fertility preservation.

Table 1: Data of the patients who chose combined technique for fertility preservation

No	Age (Y)	Diagnosis	AMH (ng/mL)	Right AFC	Left AFC	OTC	No. of oocytes collected	MII oocytes cryopreserved	Embryos cryopreserved
1	26	Breast cancer	4.5	6	8	Right	21	19	-
2	34	NHL	0.94	3	1	Left	5	5	-
3	37	Breast cancer	1.7	2	5	Right	13	8	-
4	25	Breast cancer	3.9	4	7	Right	16	15	-
5	15	Ewing sarcoma	0.4	3	1	Left	6	5	-
6	30	AML	2.4	7	5	Left	12	7	7
7	28	Rectum cancer	5.2	8	5	Left	18	13	7
8	37	Breast cancer	2.0	1	4	Right	12	7	3

NHL; Non-Hodgkin lymphoma, AML; Acute myeloid leukaemia, AMH; Anti-Müllerian hormone, AFC; Antral follicle count, OTC; Ovarian tissue cryopreservation, and MII; Metaphase II oocytes.

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Authors' Contributions

K.G.S., Y.E.Ş., C.S.A.; Contributed to conception and design. K.G.S., Y.E.Ş.; Contributed to all work, data and statistical analysis. M.S., C.S.A.; Supervised the study design and revised the manuscript. All authors read and approved the final manuscript.

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Correction

Effect of Adding Human Chorionic Gonadotropin to The Endometrial Preparation Protocol in Frozen Embryo Transfer Cycles

Maryam Eftekhar, M.D.[1], Elham Rahmani, M.D.[2*], Tahereh Eftekhar, M.D.[3]

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In this article which was published in Int J Fertil Steril , Vol 6, No 3, Oct-Dec 2012, on Pages: 175-178, the authors found that Four samples from the control group were incorrectly included in the study. 4 cases were removed from the data and the data were re-analyzed. The results in Tables 1-3 are corrected.

The authors would like to apologies for any inconvenience caused.

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Results

A total of 126 couples participated: 65 in group 1 (HCG group) and 61 in group 2 (control group). The demographic and basic characteristics of patients are shown in Table 1.

There were no statistically significant differences between groups regarding age (P=0.696), duration of infertility (P=0.222), basal follicle stimulating hormone (P=0.061), BMI (P=0.526), and etiology of infertility (P=0.294). The cycle characteristics and outcome of vitrification are shown in Table 2.

There were no statistically significant differences between groups regarding the numbers of thawed embryos, numbers of transferred embryos, survival rates of thawing embryos and duration of freezing. Table 3 shows the outcome of ART cycles. Implantation, chemical pregnancy, clinical pregnancy, ongoing pregnancy, and abortion rates were similar in both groups.

Table 1: Basic patient characteristics in the two groups

Variables	HCG group	Control group	P value
Age (Y)	28.47 ± 4.14	28.74 ± 3.55	0.696
Duration of infertility (Y)	6.58 ± 2.9	5.96 ± 2.68	0.222
Basal FSH (IU/L)	5.15 ± 1.66	5.72 ± 1.70	0.061
BMI (kg/m^2)	23.67 ± 2.43	23.95 ± 2.39	0.526
Etiology of infertility			
Ovulatory	13 (20)	11 (18)	0.294
Tubal	9 (13.8)	10 (16.4)	
Unexplained	0 (0.0)	3 (4.9)	
Mixed	42 (64.6)	34 (55.7)	
Male	1 (1.5)	3 (4.9)	
Total	65 (100)	61 (100)	

Data are presented as mean ± SD or n (%). HCG; Human chorionic gonadotropin, FSH; Follicle-stimulating hormone, and BMI; Body mass index.

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Table 2: Cycle characteristics and outcome of vitrification

Variables	HCG group	Control group	P value
Duration of freezing (months)	6.58 ± 3.1	6.18 ± 2.74	0.445
Number of thawed embryos	2.96 ± 0.17	2.88 ± 0.32	0.068
Survival rate after thawing (%)	91.79 ± 0.20	94.54 ± 0.12	0.369
Numbers of transferred embryos	2.73 ± 0.44	2.57 ± 0.49	0.052

Data are presented as mean ± SD. HCG; Human chorionic gonadotropin.

Table 3: ART outcome in both groups

Variables	HCG group	Control group	P value
Implantation rate (%)	21.02	17.44	0.488
Chemical pregnancy rate, n (%)	27 (41.5)	25 (41.0)	0.950
Clinical pregnancy rate, n (%)	22 (33.8)	20 (32.8)	0.900
Ongoing pregnancy rate, n (%)	20 (30.8)	18 (27.7)	0.878
Miscarriage rate, n (%)	7 (25.9)	7 (28.0)	0.866
Endometrial thickness (mm)	8.83 ± 1.6	9.08 ± 1.10	0.427

Data are presented as mean ± SD or n (%) or %. HCG; Human chorionic gonadotropin, ART; Assisted reproductive technology.

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

Aims and scope

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

1. Types of articles

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

A. Original articles

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

B. Review articles

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

C. Systematic Reviews

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

D. Short communications

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

E. Case reports

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

F. Editorial

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

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

H. Letter to editors

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

I. Debate

2. Submission process

It is recommended to see the guidelines for reporting different kinds of manuscripts here. This guide explains how to prepare the manuscript for submission. Before submitting, we suggest authors to familiarize

themselves with Int J Fertil Steril format and content by reading the journal via the website (www.ijfs.ir). The corresponding author ensures that all authors are included in the author list and agree with its order, and they must be aware of the manuscript submission

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1. Hardy-Weinberg Equilibrium (HWE) calculations must be carried out and reported along with the P-values if applicable [see Namipashaki et al. 2015 (Cell J, Vol 17, N 2, Pages: 187-192) for a discussion].
2. Linkage disequilibrium (LD) structure between SNPs (if multiple SNPs are reported) must be presented.
3. Appropriate multiple testing correction (if multiple independent SNPs are reported) must be included.
Submissions that fail to meet the above criteria will be rejected before being sent out for review.
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is needed.
Title is providing the full title of the research (do not use abbreviations in title).
Running title is providing a maximum of 7 words (no more than 50 characters).

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.

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

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

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The authors must ensure that before submitting the manuscript for publication, they have to consider the following parts:

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3. Tables should be in a separate page. Figures must be sent in color and also in GIF or JPEG format with 300 dpi resolutions.
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