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Contents

Review Article

The Role of Arginine-Phenylalanine-Amide-Related Peptides in Mammalian Reproduction

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Abstract

Until 2000 it was believed that gonadotropin-releasing hormone (GnRH) was the sole regulator of hypophyseal gonadotropes. In 2000, the discovery of a gonadotropin inhibitory hormone (GnIH) initiated a revolution in the field of reproductive physiology. Identification of GnIH homologues in mammals, the arginine-phenylalanine-amide (RFamide)-related peptides (RFRPs), indicated a similar function. Subsequently, further works conducted in various laboratories worldwide have shown that these neuropeptides inhibit the hypothalamic-hypophyseal axis. This review discusses the role of RFRPs in mammalian reproductive processes.

Keywords: RFamide-Related Peptide, Gonadotropin Inhibitory Hormone, Reproduction, Mammals

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Introduction

Gonadotropin-releasing hormone (GnRH), the main stimulator of gonadotropes and secretion of gonadotropins, was first purified from the pig and sheep hypothalami in the 1970s (1, 2). For years GnRH has been considered the only regulator of the hypothalamic-hypophyseal-gonadal axis. Gonadal steroids and inhibin regulate gonadotropin secretion via negative/positive feedback mechanisms. Although existence of a hypothalamic inhibitor of gonadotropin secretion was suspected earlier (3), in 2000 researchers discovered a 12 amino acid peptide (SIKPSAYLPLRFamide) in the quail brain which could directly inhibit GnRH release. It was subsequently named the gonadotropin inhibitory hormone (GnIH) (4). During the last 13 years, avian homologues of GnIH have been identified in several mammalian species and named arginine-phenylalanine-amide (RFamide)-related peptides (RFRP). In this review we describe the chemical structure, biosynthesis and functions of RFRPs related to mammalian reproduction and their possible roles in other physiologic events.

History, biosynthesis and chemical structure of RFamide-related peptides

The RFRPs are a family of peptides with an arginine-phenylalanine (RF-NH_2) sequence at their carboxyl terminals. Researchers have discovered the first peptide of this family in shell ganglions (FMRFamide) (5). The first RFRP in vertebrates was discovered in the avian brain (LPLRFamide) (6). In 2000 researchers reported that one of the RFRPs inhibited the secretion of gonadotropins. Since then, GnIH homologues have been identified (Table 1) in several species of mammals, including humans (7), monkeys (8), cattle (9), sheep (10, 11), rats, mice (12) and hamsters (13).

Following transcription and translation of the *RFRP* gene, a prepeptide is synthesized which routinely separates into two mature peptides, RFRP-1 and RFRP-3 (Table 1). The carboxy terminals of RFRPs contain a sequence of leucine-proline-

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XXX-arginine-phenylalanine (LPXRF, X=leucine or glutamine) followed by glycine (G) as an amidation signal, and arginine (R) or lysine (K) that act as endoproteolytic basic amino acids (14). However, in humans, monkeys, and cattle, RFRP-2 is also built from a prepeptide which differs from LPXRF. This prepeptide contains RS-amide sequences or an RS-amide in the carboxyl terminal. RFRP-1 and RFRP-3 bind the same receptor, named GPR147 (also known as OT7T022 and NPFF-1) with similar affinity (15).

Table 1: Amino acid sequence of the RFamide-related peptide (RFRP) prepeptide in different mammalian species

Species	Amino acid sequence	No. of amino acids
Human	MEIISSKLFILLTLATSSLLTSNIFCADELVMSNLHSKENYD-KYSEPRG	49
Monkey	MEIISSKLFILLTLATSSLLTSNISCADELMMSSLHNKENYD-KYSEPRG	49
Cow	MEIISLKRFILLMLATSSLLTSNIFCTDESRMPNLYSKKNYD-KYSEPRG	49
Sheep	MEIISLKRFILLMLATSSLLTSNIFCTDESR I PSLYSKKNYD-KYSEPRG	49
Rat	MEIISSKRFILLTLATSSFLTSNTLCSDELMMPHFHSKEGYG-KYYQLRG	49
Mouse	MEIISLKRFILLTVATSSFLTSNTFCTDEFMMPHFHSKEGDG-KYSQLRG	49
Hamster	MEIISSKRFILLTLATSSLLTSN IFCTEELMMPHFHS KE KED-KYSQPTG	49
Human	YP--KGERSLNFEELKDWGPKNVIKMSTPAVNKMPHSFANLPLRFGRNVQ	97
Monkey	YP--KRERSLNFEELKDWGPKNVIKMSTPAVNKMPHSVTNLPLRFGRTTE	97
Cow	DLGWEKERSLTFEEVKDWAPK--IKMNKPVVNKMPPSAANLPLRFGRNME	97
Sheep	DLGWEKERSLTFEEVKDWGPK--IKMNTPAVNKMPPSAANLPLRFGRNME	97
Rat	IPKGVKERSVTFQELKDWGAKKDIKMSPAPANKVPHSAANLPLRFGRNIE	99
Mouse	IPKGEKERSVTFQELKDWGAKNVIKMSPAPANKVPHSAANLPLRFGRTID	99
Hamster	ISKGEKERSVSFQEVKDWGAKNVIKMSPAPANKVPHSAANLPLRFGRTLE	99
	RFRP-1	
Human	EERSAGATANLPLRSGRNMEVSLVRRVPNLPQRFGRTTTAKSVCRMLSDL	147
Monkey	EERSTGAIANLPLRSGRNMEVSLVRQVLNLPQRFGRTTTAKSVCRTLSDL	147
Cow	EERSTRAMAHLPLRLGKNREDSLSRWVPNLPQRFGRTTTAKSITKTLSNL	147
Sheep	EERSTRVMAHLPLRLGKNREDSLSRRVPNLPQRFGRTIAAKSITKTLSNL	147
Rat	DRRSPRARA----------NMEAGTMSHFPSLPQRFGRTT-ARRITKTLAGL	140
Mouse	EKRSPAARV----------NMEAGTRSHFPSLPQRFGRTT-ARS-PKTPADL	139
Hamster	EDRSTRART----------NMEARTLSRVPSLPQRFGRTT-ARSIPKTLSHL	140
	RFRP-3	
Human	CQGSMHSPCANDLFYSMTCQH-QEIQNPDQKQSRRLLFKKIDDAELKQEK	196
Monkey	CQGSLHSPCANDLFYSMTCQH-QEIQNPDQKRSRRLVFQKMDDAELKQEK	196
Cow	LQQSMHSPSTNGLLYSMACQP-QEIQNPGQKNLRRRGFQKIDDAELKQEK	196
Sheep	LQQSMHSPSTNGLLYSMTCRP-QEIQNPGQKNLRRLGFQKIDDAELKQEK	196
Rat	PQKSLHSLASSELLYAMTRQH-QEIQSPGQEQPRKRVFTET DDAERKQEK	189
Mouse	PQKPLHSLGSSELLYVMICQH-QEIQSPGGKRTRRGAFVET DDAERKPEK	188
Hamster	LQRFLHSMATSEVLNAMTCQH-GEIQSPGGKQPRRQAFMETDDEEGKHEK	189
Rat	IGNLQPVLQGAMKL	203

Extension of RFamide-related peptide neuronal bodies and fibers in the mammalian brain

RFRP neuronal bodies have been detected in the rat dorsomedial hypothalamus and proven by a number of research studies using different antibodies. These antibodies included an antibody produced against the sparrow GnIH produced in rabbits (16) and an antibody produced against the sequence 119-132 of prepeptide RFRP (17). We also reported similar findings in rats (18) by using an antibody against the quail GnIH produced in rabbits (supplied kindly by Professor K. Tsutusi). The same neuronal extensions were also found in the brains of hamsters (13, 16) and mice (16). In sheep, neurons that expressed RFRP were identified in the dorsomedial hypothalamic area (DMH), paraventricular nucleus (PVN), the area between these nuclei (11) and the preoptic area (POA) (19). We showed that agouti-related peptide (AgRP) and RFRP coexpressed in 19 to 32% of the arcuate (Arc) neurons during various phases of the estrous cycle in the ewe (20). In addition, we observed similar neuronal extensions in the brains of native Fars goats (21). Positive cells were found in the monkey periventricular nuclei (8) and human DMH (7).

In rodents, fibers and terminals of RFRP neurons were observed in the middle areas of the brain, limbic areas (POA, septal and amygdala), rostral hypothalamus and Arc (13, 16). In the monkey brain, RFRP fibers were observed in most parts of the brain, including the hemispheres or telencephalon, septal nuclei and accumbens, hypothalamus and particularly POA, Pe, PVN and ARC, habenular nuclei, thalamus, upper calculi of the midbrain, Raphe nuclei and the pons (8).

The inhibitory role of RFamide-related peptides

In all vertebrate species studied from fish (22) to humans, the RFRP/GnIH peptides decreased the secretion of gonadotropins, particularly luteinizing hormone (LH), via actions on GnRH neurons and/or gonadotropes. This showed the possibility of a protective role in various species (16, 23, 24). Recently published reports indicated that these peptides in certain situations did not affect LH secretion or even have a stimulatory effect, which in the following they also be explained.

The effect of RFamide-related peptides on the gonadotropin-releasing hormone neuronal system

Any direct effect of RFRP on GnRH neurons necessitates a direct connection between RFRP neuronal terminals and GnRH neurons. In the POA of male rats, research has shown that RFRP fibers formed a close association with approximately 75% of GnRH neuronal bodies (24). Similar finding was reported in female hamsters (more than 40%), mice and rats (16). In another study, there was communication of RFRP fibers with GnRH neurons observed in the anterior hypothalamic area, MBH (approximately 30%) and POA of sheep (25). In sheep, co-expression of RFRP and GnRH during proestrus and estrus (follicular phase) and the luteal phase has been reported. During the luteal phase of sheep, more POA neurons expressed RFRP compared to the follicular stage, while there were no differences in the number of GnRH neurons in the hypothalamus, which indicated a direct effect of RFRP neurons in POA on GnRH neurons and an indirect effect on LH secretion (23).

In the POA of monkeys, 67.9% of GnRH neurons established connections with RFRP fibers (8), with similar connections observed in the human brain (7). More than 80% of GnRH neurons of POA in the Siberian hamster expressed GPR147 receptor (13). In adult male and female diestrus mice, there was a close relation between RFRP-3 neuron terminals with 25% of the body of GnRH neurons in the medial septum and 27% in the rostral part of the POA, and 33% of GnRH neurons which expressed *GPR147* mRNA (26). Addition of RFRP into GnRH neurons *in vitro* decreased the firing rate of 41% of the neurons. However, electrophysiologic evaluations in that study showed that RFRP treatment had a stimulatory effect on 12% of the neurons and no effect on 47% of neurons (27). In the same study, RFRP treatment caused hyperpolarization of more than 50% of GnRH neurons (28).

Intraventricular administration of RFRP rapidly decreased plasma LH concentration in male rats (24), ovariectomized hamsters (16) and Siberian hamsters maintained on a long-day photoperiod; however, injection of RFRP in hamsters on a short-day photoperiod stimulated LH release 30 minutes after the injection (13). In another research, intraventricular injection of RFRP-3 stimulated expres-

sion of c-Fos in GnRH neurons and increased both LH and testosterone secretion (29). In contrast, intraventricular injection of RFRP in ovariectomized rats had no effect on the mean plasma LH concentration or frequency of LH pulses (30). Intraventricular injection of RFRP in ovariectomized rats following induction of the GnRH/LH surge by estradiol (E_2) and progesterone decreased the activity of GnRH neurons (evaluated based on *c-Fos* gene expression) by 50 to 60% (31). However, in that study, central injection of RFRP in ovariectomized rats treated with E_2 implant had no effect on LH pulse and amplitude or mean concentration of LH. Recently, it was observed that intraventricular RFRP-3 injection in ovariectomized ewes had no effect on plasma LH concentration (32). Intraventricular administration of RF9, a potent and specific antagonist of the RFRPs receptor (33), resulted in a rapid, dose-dependent increase in gonadotropin secretion in male and female rats (34). Collectively, these finding suggested that RFRP could change GnRH secretion via a direct action on the GnRH neuronal system [for more information see the review by Anderson (35)].

Effect of RFamide-related peptides on hypophysis

In order to generate a physiologic effect on gonadotropin secretion, the hypothalamic RFRP neuronal terminals must either form a close association with GnRH neurons in the median eminence (ME) and/or RFRP receptors must be located on gonadotropes. The RFRP neuronal terminals are found in the external layer of the ME in hamsters (13, 16, 36), sheep (11), monkeys (8) and humans (7). GPR147 expression is reported in the hypophysis of hamsters (36), rats (37, 38) and humans (7). The presence of RFRP in the hypothalamic-hypophyseal portal vein of sheep has been reported by Smith et al. (39). In rats, while some researchers did not observe RFRP fibers in ME (17, 24), others reported the presence of RFRP fibers in male (40) and female (18) Sprague-Dawley rats.

Fluorogold is a retrograde tracer that does not cross the blood-brain barrier but can be absorbed from portal arterioles of hypophysis by neurons terminals in the external area of ME. Intraperitoneal injection of this tracer has been used to detect central hypophysiotropic cells. The results indicated that more than 90% of GnRH neurons and only 3 out of 234 RFRP neurons in the POA of rats stained with Fluorogold (17).

Intravenous injection of RFRP decreased LH secretion in several mammals; however, the mode of action might differ in various species. Intravenous injection of RFRP in ovariectomized ewes decreased the amplitude of LH pulses; but had no effect on pulse frequency (11). Intravenous injection of RFRP in castrated bulls decreased the frequency of LH pulses, however a single injection had no effect (41). Intravenous injection of RFRP in ovariectomized rats (30) and ovariectomized hamsters decreased mean concentrations of LH (16).

The addition of RFRP to cultures of hypophyseal cells of rats (30), cows (41) and sheep decreased GnRH-induced LH secretion. Interestingly, the addition of GnRH to hypophyseal cells increased expression of *LHβ* mRNA in rams (4 times) and ewes (2.5 times), but RFRP inhibited LHβ subunit expression (42). On the other hand, it was also reported that treatment with RFRP (31) or RF9 (an RFRP receptor antagonist) (34) had no effect on GnRH-induced LH secretion in a hypophyseal cell culture in rats.

Effect of RFamide-related peptides on gonads

In addition to expression in the brain, expression of RFRPs and their receptors in mammalian gonads have been reported. In male hamsters, cells that expressed RFRP were observed in the seminiferous tubules. Its receptor, GPR147, was observed in spermatocytes and spermatids (43). In the monkey, RFRP and its receptor were expressed in Leydig cells, spermatogonia and spermatocytes (44). RFRP was also found in the granulosa and luteal cells of mice ovaries (45).

RFRP peptides in granulosa cells of preovulatory follicles and corpus luteum along with GPR147 receptors in granulosa cells, theca cells and the corpus luteum have been observed in women. RFRP-3 could inhibit the effect of gonadotropins on progesterone production and expression of StAR protein (46). Thus, it was postulated that RFRP might have autocrine/paracrine roles in gametogenesis and steroidogenesis (44).

Effect of sex steroids on the RFamide-related peptide system

A low concentration of E_2 secreted during the majority of the ovarian cycle in most mammals ex-

erts a negative feedback effect on GnRH neurons by keeping GnRH/LH secretion at a basal level. During the preovulatory period, high levels of estrogen secreted from mature follicles results in a GnRH/LH surge via a positive feedback effect. The GnRH neurons do not express alpha E_2 receptors (ER_α) which are essential for the positive and negative feedback effects of E_2 (47). Therefore, it seems that other steroid sensitive neurons are intermediaries of the estrogen effect on regulation of GnRH (and LH) secretion.

Approximately 40% of RFRP neurons in the brain of female hamsters (16) and 18% in ovariectomized mice expressed ER_α (48). Therefore, RFRP neurons might intermediate the E_2 feedback effect. We studied the expression of *RFRP* mRNA and peptides during the estrous cycle of rats. Expression of *RFRP* mRNA in proestrus was less than in diestrus and the numbers of neurons that expressed the RFRP peptides during proestrus and early estrus was less than during estrus and diestrus. Increased secretion of E_2 in the evening of proestrus from dominant follicles in addition to the positive feedback effect on GnRH/LH surge might facilitate GnRH/LH secretion by exerting an inhibitory effect on RFRP expression in DMH (18). In another study we evaluated the numbers of neurons that expressed RFRP in DMH/PVN during the follicular and luteal phases of goats. The numbers of positive cells in the follicular phase (periovulatory period) was less than in the luteal phase (21). Consistent with this finding, the numbers of RFRP neurons decreased during the preovulatory period in hamsters (36). It was also reported that the number of POA neurons that expressed RFRP was greater during the luteal phase compared with the follicular phase in sheep (19).

E_2 implants (100 µg/ml) for 4 days in ovariectomized mice decreased the number of RFRP cells and the expression of *RFRP* mRNA per cell (48) as determined by in situ hybridization. Possibly, only high or long term levels of E_2 could decrease the expression of RFRP in rodents because only once subcutaneous injection of E_2 in ovariectomized hamsters sufficiently increased the activity of RFRP neurons (evaluated by c-Fos expression) at 3 and 6 hours after injection (16). In contrast to these findings, during the breeding and non-breeding seasons for sheep, there was no difference in the numbers of neurons that expressed *RFRP* mRNA and *RFRP* mRNA levels per cell between ovariectomized ewes and ovariectomized ewes that received E_2 implants (25).

RFRP neurons in the brain of male hamsters expressed an androgen receptor (16), however castration of male hamsters or treatment with testosterone implants for 4 weeks had no significant effect on the number of cells that expressed RFRP (49). Therefore, more studies should be conducted to clarify the mechanism of sex steroid action on RFRP neurons.

The effect of a photoperiod on the RFamide-related peptide system

Reproductive activity in several mammalian species shows salient seasonal alterations due to basal alterations in secretion of reproductive hormones. In compliance with the action of RFRP mammalian reproduction, it is logical that RFRP expression in seasonal breeders will be harmonized with changes in the photoperiod. Contrary to the expectation in Syrian and/or Siberian hamsters (long-day breeders), there were fewer neurons that expressed *RFRP* mRNA and RFRP peptide during the short-term photoperiods (8 hours light) compared with the long-term (16 hours light) photoperiods (13, 49, 50). These findings were not related to the specific time of day since gene expression was the same during 24 hours (49).

Aggregation of RFRP fibers in POA and rostral hypothalamus (aggregation area of GnRH neurons) and the percent of GnRH neurons that established connections with RFRP fibers were less during the short-term compared to the long-term photoperiod (13, 50). On the other hand, pinealectomy prevented a decrease in RFRP expression during short days (13, 49). A 60-day melatonin injection administered to hamsters maintained under long-term photoperiods remarkably decreased *RFRP* mRNA expression and produced the same response as in the short-term photoperiod (49). Administration of melatonin for 13 weeks to pinealectomized hamsters kept under a short-term photoperiod decreased *RFRP* gene expression (13). Therefore, melatonin appeared to decrease the activity of RFRP neurons during short-term photoperiods.

Coordination of these findings with the inhibitory role of RFRP was difficult because when the

lowest level of expression was seen, the reproductive system was inactive. In Siberian hamsters, the relative expression of *RFRP* mRNA during average days (13.5 hours light) was more than 40 times the long days (16 hours light) (51). Therefore, it was possible that a considerable increase in RFRP expression during the early period of reproductive system regression (average days) would be necessary to inhibit the reproductive axis. However, this level of expression in hamsters whose reproductive axis did not completely regress was not necessary. Because intraventricular injections of RFRP in hamsters maintained under short-term photoperiod conditions had a stimulatory effect (13) it was possible that the decrease in RFRP expression during short-term photoperiods was important for inhibition of reproduction.

Unlike hamsters, sheep and goats are short-day breeders. The number of RFRP that expressed neurons during the non-breeding season in sheep (long-term photoperiod) was approximately 40% more than during the breeding season, but there was no difference in the number of *RFRP* mRNA per cell (25). However, in another study, *RFRP* mRNA expression was highest during the long days (10). Communication of RFRP fibers with GnRH neurons in POA and rostral hypothalamus was highest during the non-breeding season (25). In addition to the seasonal change of RFRP expression in DMH/PVN nuclei, *RFRP* mRNA expression in epithelial or ependymal cells around the ventricle was seen only in long days (10).

Recently we evaluated RFRP expression in DMH/PVN nuclei in goats during the breeding season (follicular and luteal phases) and anestrus. In both nuclei, the number of cells that expressed RFRP was higher in during anestrus compared to the follicular phase. However, there was no difference between the anestrus and luteal phases. We also determined the number of positive neurons in the rostral, middle and caudal parts of the DMH/PVN. In the rostral areas, more RFRP neurons were observed during anestrus than during the follicular phase; however, there was no effect of the reproductive stage recorded in middle and caudal parts of these nuclei (21). These results in sheep and goats were in accordance with the inhibitory role of RFRP on the reproductive axis.

The probable action of RFamide-related peptides on other physiologic events

The diffuse distribution of RFRP neuronal processes in the brain is suggestive of additional roles for this neuropeptide in physiology. The RFRP neuronal terminals in sheep brain are extended to neurons of orexin, melanin, proopiomelanocortin and neuropeptide Y; therefore, RFRP neurons may have a role in the regulation of appetite and energy balance, and possibly function as a link between nutrition and reproduction (52). Long term malnutrition (2 weeks) has been shown to increase *RFRP-3* mRNA expression in DMH of the hypothalamus in ovariectomized female rats (53).

Furthermore, in the monkey brain, RFRP fibers had a close relation with neurons of dopamine, beta-endorphin and GnRH-II. Since dopamine neurons express GPR147, it was suggested that RFRP might stimulate prolactin secretion by inhibition of dopamine neurons (8). Consistent with this idea, we showed that the numbers neurons that expressed RFRP in suckling rats (in which plasma prolactin is at its highest level) was higher than in non-suckling rats (54). Increased *RFRP-3* mRNA expressions in DMH of the hypothalamus while increasing milk production in rats might be the inhibitory factor for GnRH secretion (55).

Based on the findings that prolactin (56, 57) and oxytocin (58) secretion increased during the refractory period after ejaculation in men, we proposed a hypothesis that increased RFRP expression after ejaculation might be the cause of the post-ejaculation refractory period in men (59). Intracerebroventricular injection of RFRP in rats also increased the activity of oxytocin neurons in the hypothalamus and oxytocin concentrations in plasma. It was shown that the supraoptic and PVN nuclei of the hypothalamus expressed *GPR147* mRNA (60). Therefore, RFRP peptides might also participate in the regulation of oxytocin secretion.

Coexpression of RFRP and AgRP in the Arc neurons of the ewe has been reported which indicated a probable role of these two peptides in control of the ewe reproductive cycle. This study also showed that ovarian steroids affected expression of these peptides in the Arc of the hypothalamus

and might be a link between energy homeostasis and reproduction (23).

RFamide-related peptides and treatment of reproductive disorders

As mentioned before, RFRP peptides have an opposite effect against GnRH in numerous situations and inhibit secretion of gonadotropins. However in some cases they may have an effect on LH release and a stimulatory effect (please see the previous sections). GnRH analogs (agonists and antagonists) have been applied in the treatment of a wide spectrum of reproductive disorders, including precocious puberty, endometriosis, uterine fibroids, prostatic hyperplasia, prostatic and breast cancers. By 2000, more than 2 billion dollars in sales of these compounds was recorded (61). Therefore, considering the potential effect of RFRP in inhibition of gonadotropins, the use of these peptides in the future for the treatment of reproductive disorders would be expected (62).

The inhibitory effect of stress on reproductive performance has been demonstrated. Stress leads to activation of the hypothalamus-pituitary-adrenal axis which inhibits GnRH secretion. It seems that the effects of stress on the hypothalamus-pituitary-gonad axis is mediated by adrenal steroid hormones (glucocorticoids). Since neurons of GnRH do not express glucocorticoid receptors, it is possible that these steroid hormones affect neurons upstream of GnRH neurons and change the release of GnRH. Reports have shown that RFRP neurons mediate the effects of stress on reduction of GnRH/LH secretion and stop of the reproductive axis (38, 63). Therefore, it is possible that using RFRP antagonists or antibodies against RFRP safeguard reproductive performance in stressful situations. Also, as noted above, increase in RFRP expression may be involved in the post-ejaculatory refractory period (59) Hence disabling the RFRP system may shorten this period.

Conclusion

Based on the findings in mammals, RFRPs are homologues of GnIH in birds and can inhibit LH secretion and the reproductive axis; however, their mode of action is not yet clearly established. For example it is not known whether they inhibit the GnRH system and/or have a direct effect on hypophyseal gonadotropes in preventing gonadotropin secretion. Amongst studied species, the most contradictory data have been reported in the rat. Based on extensive connection between the RFRP neurons and other neurons, more studies will be required to identify the exact role of these peptides in reproduction and other physiologic functions.

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Original Article

In Vitro Fertilization, Levels of Pro-Inflammatory Factors and Lipid Peroxidation

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Abstract

Background: Infertility is a problem concerning 10-15% of the individuals in the fertile period. This study investigated effects of proinflammatory factors as well as lipid hydroperoxides (LPO) levels upon *in vitro* fertilization (IVF) success.

Materials and Methods: In this prospective, non-randomized, controlled clinical study, sera obtained from 26 fertile (group-1), 26 infertile women before (group-2) and after (group-3) IVF treatment were analyzed. Leptin, leptin receptor, resistin, tumor necrosis factor-alpha (TNF-α), and C-reactive protein (CRP) were analyzed using enzyme-linked immunosorbent assay (ELISA). LPO was determined spectrophotometrically. Mann-Whitney U test, paired samples t test, Wilcoxon signed-rank test as well as Pearson correlation analysis by SPSS were performed for statistical analysis.

Results: TNF-α, resistin and LPO levels increased (P=0.020, P=0.003, P=0.001, respectively) in group-3 compared to group-2. A significant increase in LPO was noted both in group-2 and -3 compared to controls (P=0.000). LPO were higher in non-pregnants than pregnants in group-2. For pregnants, significant correlations were observed between leptin and resistin in group-2 and TNF-α and leptin in group-3. None of these correlations were found for the women, who could not conceive.

Conclusion: LPO, leptin-resistin correlation, associations with TNF-α may be helpful during the interpretation of IVF success rates.

Keywords: Infertility, Leptin, Resistin, TNF-α, Lipid Peroxidation

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Introduction

Infertility can be defined as inability to conceive despite sexual relationship without contraception for 1 year. This problem concerns about 10-15% of the individuals in the fertile age (1).

To have a family and a child is well-accepted and a desired status all over the world. They are important for the development of the community and continuity of the generation. Couples with an enthusiasm of having a child but facing the problem of infertility, experience a decrease in communication, belief of health, social esteem and self-confidence, and become disappointed about expectations for the future. After the diagnosis of

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infertility is made, people ignore all areas of their lives and concentrate on the matter and the methods used in the processes of diagnosis and treatment, and particularly for the women, it becomes a painful chain of physical and emotional events (2).

In vitro fertilization (IVF) can be defined as one of the assisted reproductive techniques medically applied on oocyte, sperm or embryo cells *in vitro* in order to develop pregnancy (3, 4). Cytokines as key modulators of the immune system appear to modulate other regulatory systems. They also contribute to regulation of the ovarian cycle (5). A proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) and C-reactive protein (CRP), one of the acute phase reactants, can increase resistin expression (6-9). CRP is a negative regulator of functions of human leptin (10). Resistin is synthesized mostly by inflammatory cells such as macrophages and correlated with TNF-α (9). Resistin levels are capable of increasing expression of TNF-α via nuclear factor (NF)-κβ-dependent pathway (6, 8, 11-13). Leptin, a proinflammatory factor, regulates food intake and energy expenditure (14). It is also linked to reproductive functions (15). Leptin levels may be used as predictive markers of assisted reproductive technology (ART) (16, 17). It has been demonstrated that combined exposure of human monunuclear cells to high concentrations of insulin and leptin for 24 hours *in vitro* stimulates resistin and TNF-α protein expression (12). Leptin level is elevated in cases associated with high levels of TNF-α that increases serum leptin concentrations (14, 18).

Studies on reactive oxygen molecules (ROM) during the course of this process and their relationship with proinflammatory factors have gained importance in recent years (19, 20). Shift of the equilibrium between pro-oxidants and anti-oxidants towards pro-oxidants results in oxidative stress. Effects of oxidative stress on the stages of reproduction like oocyte maturation and follicular development are important from the IVF success point of view (21). Its importance is emphasized by the conditions providing low oxygen during IVF application. Since the role of oxidative stress on infertility has not been fully cleared yet, effects of lipid peroxidation upon various stages of IVF process and the contribution of some cytokines and hormones upon the process are noteworthy.

The aim of this study was to investigate the profiles of some pro-inflammatory factors, cytokines and hormones, such as CRP, TNF-α, leptin as well as resistin, known to be involved in the process of inflammation, to evaluate their relationship with lipid hydroperoxides, the markers of early lipid peroxidation and to assess their associations with female infertility.

Materials and Methods

In this prospective, non-randomized, controlled clinical study, the blood samples from 70 women, who consulted to the IVF Center, Obstetrics and Gynaecology Department, Cerrahpasa Medical Faculty, University of Istanbul, Istanbul, Turkey, with the complaint of infertility were used. They also had the features of being between the ages of 23 and 40, being married for 3 years, having social security for 5 years and having two times of intrauterine insemination before, while they were taken for analysis prior to the beginning of the treatment in order to determine the suitability for the participation into the study.

Patient history and gynaecological exam, routine biochemical tests, ultrasonography, serology, basic infertility tests [spermiogram, hormonal tests and hysterosalpingography (HSG)] were performed to evaluate the causes of infertility before the treatment in order to enlighten the source of the problem.

Causes of reduced female fertility included decreased ovarian reserve, anovulation, uterine disorders other than endometriosis, fertility-sparing surgery with unilateral salpingo-oophorectomy, methylene tetrahydrofolate reductase gene mutation, unexplained reasons and presence of more than one factor. Patients with polycystic ovary syndrome (PCOS) were also included in the study population, with the result that both pregnant and non-pregnant groups had a proportionate distribution to eliminate the effects of their possible contribution in terms of inflammation.

A signed written informed consent was obtained from all participants prior to the study. Procedures were carried out in accordance with Declaration of Helsinki. This project was approved by the Ethics Committee and Institutional Board of Cerrahpasa Medical Faculty, Istanbul University, Istanbul, Turkey.

Twenty-six women, who had given a birth without any medication constituted the control group

(group 1). The blood samples from 70 infertile women were collected during the early follicular phase (the 3rd day of the cycle) before the onset of the intervention. A total of 26 individuals managed to complete the process with the appropriate respond to treatment. Their "pre-IVF samples" (group 2) out of 70 were included into the study to constitute the paired data with the "post-IVF samples" (group 3) taken on the 15th day of the application of embryo transfer from these 26 women. There was no statistically significant difference between the age and body mass index (BMI) values of the control and patient groups (P=0.909, P=0.431, respectively).

Anthropometric measurements and demographic characteristics of the women participated in the study were recorded. Blood samples were taken into sterile vacuum operated tubes at 08:00 – 10:00 am while fasting before the IVF treatment on the 3rd day of the menstruation (follicular phase) and on the 15th day after the embryo transfer, and were centrifuged in 2000 rpm for 10 minutes. Serum samples were stored at -80˚C until assayed.

Serum anti-mullerian hormone (AMH), inhibin B, follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E_2), prolactin, and thyroid stimulating hormone (TSH) levels were determined in all women. The levels of TNF- α (Human TNF-α Elisa Kit, Assaypro, USA), resistin (Human Resistin Elisa Kit, Assaypro USA), leptin (Human Leptin Elisa Kit, Assaypro, USA), leptin receptor (Human Leptin Receptor Elisa Kit, BioVendor, EU), and high sensitive - C reactive protein (hs-CRP) (CRP HS ELISA Kit, DRG Int, Inc. USA) were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits. Lipid hydroperoxide levels, one of the important markers of oxidative stress, were determined by a spectrophotometric method [Lipid Hydroperoxide (LPO) Assay Kit, Cayman Chem Comp., USA].

All samples were assayed using the AssayMax Human TNF-alpha ELISA kit (AssayPro, USA). The intra-assay and inter-assay coefficients of variation (CV) were 5.5 and 7.0%, respectively, using the AssayMax Human Resistin ELISA kit (Assaypro, USA). The intra-assay and inter-assay CV were 4.0 and 7.2%, respectively, using the AssayMax Human Leptin ELISA kit (Assaypro, USA). The intra-assay and inter-assay CV were 4.0 and 7.7%, respectively, using the Human Leptin Receptor ELISA kit (BioVendor Research and Diagnostic Products, EU). The intra-assay and inter-assay CV were 7.2 and 9.8%, respectively, using the hs-CRP ELISA kit (DRG International, Inc., USA). The intra-assay and inter-assay CV were 4.1 and 7.5%, respectively, using the Lipid Hydroperoxide (LPO) Assay kit (Cayman Chemical Company, USA).

Statistical analyses

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, SPSS Inc., Chicago, IL, USA) software package. Data were analyzed using descriptive-analytic tests. Parametric variables were represented as mean and standard error (SE), and categorical data were represented by number (n) and percentage (%). The values for arithmetical mean, standard deviation (SD) and SE were calculated for the pregnant and non-pregnant women in pre-IVF and post-IVF groups as well as for the participants in control group. Mann-Whitney U test, paired sample t test, Wilcoxon signed-rank test as well as Pearson correlation analysis were performed. P values less than 0.05 were considered significantly.

Results

IVF-applied group had a mean age (mean ± SE) of 31.2 ± 1.4 years and BMI of 25.4 ± 2.8 kg/m^2. Control group was consisted of healthy women who took no medication, had no illnesses, were spontaneously-conceived and volunteered. Mean age of this group was 31.4 ± 1.5 years and BMI value was 24.1 ± 1.1 kg/m^2. There was no statistically significant difference between the ages and BMI values of the groups.

Eight of 26 women conceived after application of IVF treatment (30.8%). Two of them were concluded with medical abortion due to unembryonic/empty sac pregnancy. Six of 26 women finished the period with live birth (23.1%).

In table 1, some demographical and clinical parameters in non-pregnant and pregnant women are summarized.

Values for mean ± SE for the parameters of control, pre- and post-IVF groups as well as P values that define the statistical differences between the groups are shown in table 2.

The values of LPO (P=0.000) and leptin receptor

(P=0.01) between control and pre-IVF groups showed statistically significant differences.

There were statistically significant differences between LPO (P=0.000), CRP (P=0.023) and resistin (P=0.002) levels obtained in control and post-IVF groups.

The differences between pre-IVF and post-IVF levels of resistin, LPO (P=0.003, P=0.001, respectively) and TNF-α (P=0.020) were statistically significant.

As far as the values for the parameters in the pregnancy (+) and pregnancy (-) groups of pre- and post-IVF women were considered, TNF-α showed a statistically significant increase after IVF in pregnancy (-) women (P=0.026). Similar post-IVF profiles were observed for the resistin (P=0.028, P=0.016, respectively) and LPO (P=0.046, P=0.003, respectively) levels in pregnancy (+) and pregnancy (-) groups. Table 3 shows the mean ± SE and p values of the non-pregnant and pregnant women in pre-IVF and post-IVF groups.

BMI had a positive correlation with leptin (r=0.615, P=0.001), a negative correlation with leptin receptor (r=-0.505, P=0.008) and a positive correlation with CRP (r=0.464, P=0.039). Also, leptin had a negative correlation with leptin receptor (r=-0.534, P=0.005) and a positive correlation with CRP (r=0.639, P=0.002) in the control group. In this group, resistin had also positive correlation with age (P≤0.05).

Before IVF, statistically significant correlations were observed between BMI and leptin (r=0.486, P=0.012) with resistin (r=0.517, P=0.007); between leptin and leptin receptor (r=-0.574, P=0.002) with resistin (P=0.047) and between TNF-α and LPO (P=0.016). After IVF, a significant correlation between BMI and leptin (r=0.641, P=0.000) was found. Strong correlations were detected for leptin (r=0.599, P=0.001) and LPO (r=0.715, P=0.000) between pre- and post- IVF values.

Important correlations were determined between BMI and leptin before (r=-0.547, P≤0.05) and after IVF (r=0.771, P≤0.01) for the women who were in the pregnancy (-) group. Before IVF, also, a statistically important relationship was observed between BMI and resistin (r=0.686, P≤0.01). An inverse association was noted between leptin and leptin receptor (r=-0.617, P≤0.01).

Women, who could conceive following IVF treatment showed significant correlations between leptin and resistin (r=0.874, P≤0.01) before IVF as well as leptin and TNF-α (r=0.841, P≤0.01) after IVF. None of these correlations were detected in the pregnancy (-) group.

Table 1: Comparison of some demographical and clinical parameters (mean ± SE) in non-pregnant and pregnant women

	Non-pregnant	Pregnant	P value
Age (Y)	31.3 ± 1.3	33.2 ± 1.5	≥ 0.05
Infertility period (Y)	7.4 ± 1.1	5.0 ± 1.2	≥ 0.05
BMI (kg/m^2)	25.8 ± 1.7	24.9 ± 2.9	≥ 0.05
AMH (ng/ml)	4.2 ± 0.8	4.2 ± 1.0	≥ 0.05
LH (mIU/ml)	4.6 ± 1.1	3.3 ± 0.8	≥ 0.05
FSH (mIU/ml)	5.8 ± 0.3	4.9 ± 0.7	≥ 0.05
Prolactin (ng/ml)	16.7 ± 1.9	16 ± 3.2	≥ 0.05
Estradiol (pg/ml)	43.0 ± 4.0	37.5 ± 5.3	≥ 0.05
TSH (μIU/ml)	1.6 ± 0.2	1.8 ± 0.4	≥ 0.05
Inhibin-B (pg/ml)	100.2 ± 19.0	99.7 ± 3.5	≥ 0.05
Gonadotropin dose (IU)	2025 ± 190	1958 ± 346	≥ 0.05
Total oocytes (n)	8.6 ± 1.1	9.3 ± 1.9	≥ 0.05
Fertilized oocytes (n)	4.2 ± 0.7	6.0 ± 0.9	≥ 0.05
Transferred embryos (n)	2.0 ± 0.2	2.2 ± 0.3	≥ 0.05

SE; Standard error, BMI; Body mass index, AMH; Anti-mullerian hormone, LH; Luteinizing hormone, FSH; Follicule stimulating hormone and TSH; Thyroid stimulating hormone.

Table 2: The mean ± SE and P values of the control and IVF groups

Parameter		Control (Group 1)	Pre-IVF (Group 2)	Post-IVF (Group 3)	P value
TNF-α (pg/ml)	Mean ± SE	9.7 ± 0.8	7.2 ± 0.5	9.8 ± 0.9	≤0.05 G2 vs. G3 ≤0.05 G1 vs. G2
Leptin (ng/ml)	Mean ± SE	42.0 ± 6.4	62.6 ± 10.5	52.7 ± 6.8	≥0.05
Leptin receptor (ng/ml)	Mean ± SE	42.3 ± 3.0	32.0 ± 2.4	42.7 ± 5.7	≤0.05 G1 vs. G2
CRP (mg/L)	Mean ± SE	3.8 ± 0.5	3.4 ± 0.4	7.0 ± 0.8	≤0.05 G1 vs. G3
Resistin (ng/ml)	Mean ± SE	12.2 ± 1.1	12.5 ± 1.5	22.2 ± 2.9	≤0.01 G1vs. G3 ≤0.01 G2 vs. G3
LPO (nmol)	Mean ± SE	1.5 ± 0.3	4.4 ± 0.3	5.3 ± 0.2	≤0.001 G1vs. G2 ≤0.001 G1vs. G3 ≤0.01 G2 vs. G3

SE; Standard error, IVF; *In vitro* fertilization, TNF-α; Tumor necrosis factor alpha, CRP; C-reactive protein and LPO; Lipid hydroperoxides.

Table 3: The mean ± SE and P values of the non-pregnant and pregnant women in pre-IVF and post-IVF groups

		Non-pregnant			Pregnant		
Parameter		Pre-IVF	Post-IVF	P value	Pre-IVF	Post-IVF	P value
TNF-α (pg/ml)	Mean ± SE	7.1 ± 0.5	10.1 ± 0.9	≤ 0.05	7.8 ± 0.5	8.4 ± 0.7	≥ 0.05
Leptin (ng/ml)	Mean ± SE	64.9 ± 10.5	51.2 ± 6.7	≥ 0.05	54.6 ± 6.9	57.3 ± 7.5	≥ 0.05
Leptin receptor (ng/ml)	Mean ± SE	31.6 ± 2.5	43.2 ± 5.5	≥ 0.05	33.1 ± 2.7	40.8 ± 5.1	≤ 0.05
CRP (mg/L)	Mean ± SE	3.8 ± 0.5	8.4 ± 1.0	≥ 0.05	2.8 ± 0.4	7.2 ± 0.8	≥ 0.05
Resistin (ng/ml)	Mean ± SE	12.9 ± 1.7	20.6 ± 2.6	≤ 0.05	11.2 ± 1.1	27.8 ± 3.4	≤ 0.05
LPO (nmol)	Mean ± SE	4.5 ± 0.3	5.3 ± 0.3	≤ 0.01	4.1 ± 0.2	5.3 ± 0.3	≤ 0.05

SE; Standard error, IVF; *In vitro* fertilization, TNF-α; Tumor necrosis factor alpha, CRP; C-reactive protein and LPO; Lipid hydroperoxides.

Discussion

Infertile couples have to face emotional and economical sides of the problem, because success rates of IVF trials have not reached to the desired level, yet. Immunologic factors may contribute to unexplained losses and thus, studies on the matter are being accelerated.

Cytokines are polypeptides that occur at the crossroads of immunological pathways. Maternal inflammatory response plays an important role in the early stages of pregnancy; however, there is no consensus on the roles of inflammatory parameters within this period (22).

In this study, mean TNF-α levels were found higher (P≤0.05) in the pregnancy (-) group than pregnancy (+) group after IVF. This situation reminds us a question whether the success rates of IVF applications in infertile women can be increased by use of TNF-α blockers.

It was reported that use of TNF-α inhibitors and intravenous immunoglobulins (IVIG) in young infertile women improved the result of IVF application and increased the success rates of IVF. TNF-α/interleukin-10 (IL-10) elevation before pregnancy

might relate with the risk of failure in IVF (23-25).

Serum resistin levels might be a good predictor of ovarian response in infertile women during IVF (7). In the present study, resistin levels were almost the same in the control and pre-IVF groups (12.2 ± 1.1 ng/ml vs. 12.5 ± 1.5 ng/ml). This level increased to 22.2 ± 2.9 ng/ml after IVF. This increase suggested that the profile of this parameter could be important.

Resistin shares several features with proinflammatory cytokines in humans and can partially contribute to regulation of inflammation and immunity. Macrophages incubated with recombinant resistin caused elevated production of TNF-α via the transcription factor NF-κβ dependent pathway (6, 13). Elevated TNF-α and resistin levels may contribute to increased inflammation, which may lead to poor quality oocytes and embryos (7, 26).

In the previous study, resistin was reported to increase the expression of TNF-α (27). In this study, post-IVF levels of TNF-α also increased in a parallel manner with resistin. CRP levels were similarly increased but much higher than the levels of TNF-α.

In a similar manner, CRP levels of post-IVF group were statistically higher than those of control and pre-IVF groups. In Pre-IVF group, a statistically significant difference was found between pregnancy (+) and pregnancy (-) groups (2.8 mg/L vs. 3.8 mg/L). It was noted that pre-IVF pregnancy (+) group had lower levels of CRP than the other group.

Probable effects between leptin and systemic inflammation are on-going discussion subject. Studies on culture cells and mouse models reported that human CRP prevented binding of leptin to its specific receptor and blocked the signal transduction. Thus, this parameter may weaken the physiologic function of leptin that contributes to "leptin resistance" (10, 28, 29).

Leptin, an adipocyte-derived hormone, does not only take role in the regulation of food intake, but is also involved in many reproductive functions including steroideogenic potential of ovary (15). Ovary is a target organ for leptin because leptin, its mRNA as well as its receptors are found in reproductive tissues (15, 30). Since leptin may influence follicular growth as well as oocyte development, leptin and leptin receptors were also investigated in this study.

In general terms, investigation of the effects of hormones that were applied in the extent of IVF treatment protocol showed decreases in leptin levels of the post-IVF group compared to pre-IVF values (52.7 ± 6.8 ng/ml vs. 62.6 ± 10.5 ng/ml). Pre-IVF levels of leptin decreased significantly in the pregnancy (+) group compared to the pregnancy (-) group (54.6 ng/ml vs. 64.9 ng/ml). Our results were consistent with the report stating that elevated leptin may exert adverse impacts on pregnancy success (15). Several investigations reported that high leptin is associated with low pregnancy rates in IVF cycles (16, 31). The effect of leptin on embryo quality is currently a controversial topic (30, 32, 33). However, it remains elucidated how elevated leptin concentrations negatively impact IVF outcome (31).

Certain studies (34-36) showed that soluble leptin receptor levels were inversely correlated with BMI. A similar relationship was found for the control group in this study. Levels of leptin receptor were higher in the post-IVF period (42.7 ± 5.7 ng/ml) compared with the pre-IVF period (32.0 ± 2.4 ng/ml), but there was no statistically significant difference between the levels in pre-IVF and post-IVF pregnancy (+) and pregnancy (-) groups.

In this study a positive correlation was found between levels of leptin and TNF-α in post-IVF pregnancy (+) women. This suggested that leptin may have a relationship with some other inflammatory parameters.

The best investigated adipocytokin up-till-now is resistin. It is claimed that resistin takes role as an acute phase reactant due to its up-regulation in patients with severe sepsis and septic shock (10, 28, 37). However, there is little knowledge about its potential association with leptin. A finding that may contribute to this subject was obtained in this study. A strong pre-IVF relationship was determined between leptin and resistin in pregnancy (+) women following IVF application.

On the other hand, in a recently published article, leptin and resistin are reported as negative and positive outcome predictors, respectively, in women undergoing IVF (38). Our results were consistent with these findings. In pregnancy (+) group, as pre-IVF samples showed significant decreases

in leptin concentrations, increased resistin levels were observed for post-IVF samples.

Oxidative stress may be used as a predictive marker in controlled ovarian stimulation success (39). Although it is not sufficient to measure LPO levels alone to interpret oxidative stress, they give some notion about the matter as the markers of early lipid peroxidation. Significant differences were found between LPO levels of the control group and pre-IVF as well as post-IVF groups. Also, a strong correlation was found between pre-IVF and post-IVF values of this parameter. Levels for this parameter were determined lower in pre-IVF pregnancy (+) group compared to pregnancy (-) group.

Conclusion

In women, who ended up the IVF attempt with a successful pregnancy, a relationship between leptin and resistin is noted beside the association of these parameters with TNF-α. Relationships between leptin and resistin as well as TNF-α are expected, because double-sided effects are being observed among them. Resistin increases TNF-α, which in turn induces resistin. Leptin stimulates both resistin and TNF-α, that increases serum leptin concentrations. Association of TNF-α as well as resistin levels with quality of oocytes and embryos, and also influence of leptin upon follicular growth as well as oocyte development support the leptin-resistin and leptin-TNF-α correlations, which appear to be effective upon IVF outcome. Monitoring the levels of these parameters within the period, which follows IVF attempt may reveal more significant relationships.

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Original Article

Comparison of The Effectiveness of Clomiphene Citrate versus Letrozole in Mild IVF in Poor Prognosis Subfertile Women with Failed IVF Cycles

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Abstract

Background: Our objective was to evaluate the effectiveness of clomiphene citrate (CC) vs. letrozole (L) plus human menopausal gonadotropin (hMG) in gonadotropin releasing hormone (GnRH) antagonist protocol in poor prognosis women with previous failed ovarian stimulation undergoing intracytoplasmic sperm injection (ICSI).

Materials and Methods: This retrospective cohort study included cycles with CC and L plus hMG/GnRH antagonist protocols of 32 poor responders who had failed to have ideal follicles to be retrieved during oocyte pick-up (OPU) or embryo transfer (ET) at least for 2 previous *in vitro* fertilization (IVF) cycles with microdose flare protocol or GnRH antagonist protocol from January 2006 to December 2009. Main outcome measures were implantation, clinical pregnancy and live birth rates per cycle. Duration of stimulation, mean gonadotropin dose used, endometrial thickness, number of mature follicles, serum estradiol (E_2) and progesterone (P) levels on the day of human chorionic gonadotropin (hCG) administration, number of retrieved oocytes and fertilization rates were also evaluated.

Results: A total number of 42 cycles of 32 severe poor responders were evaluated. Total gonadotropin consumption was significantly lower (1491 ± 873 vs. 2808 ± 1581 IU, P=0.005) and mean E_2 level on the day of hCG injection were significantly higher in CC group than L group (443.3 ± 255.2 vs. 255.4 ± 285.2 pg/mL, P=0.03). ET, overall pregnancy and live birth rates per cycle were significantly higher in CC than L protocol (27.2 vs. 15%, 13.6 vs. 0% and 4.5 vs. 0%, respectively, P<0.05).

Conclusion: Severe poor responders who had previously failed to respond to microdose or GnRH antagonist protocols may benefit from CC plus hMG/GnRH antagonist protocol despite high cancellation rate.

Keywords: ICSI, Ovarian Response, Clomiphene Citrate, Letrozole, Ovarian Stimulation

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Introduction

A poor responder has been defined as an infertile woman that develops ≤3 follicles after controlled ovarian hyperstimulation with conventional stimulation protocols in *in vitro* fertilization (IVF) (ESHRE consensus). The management of poor responders with a history of recurrent failure in conventional microdose protocol or antagonist IVF cycles is difficult and controversial. Recurrent poor response is associated with high financial costs and emotional distress in these couples. There is still no sufficient data and standard accepted treatment protocol in recurrent poor responders. The current treatment strategies in poor responders include

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higher doses of gonadotropins (over 450-600 IU/day) (1), use of antagonists (2-4), microdose flare (4-6) and growth hormone (7, 8). Adjuvant therapies such as dehydroepiandrosterone (DHEA) (9), oral contraceptive pills, progestins (10), steroids (11), L-arginine (12) and low dose aspirin (13) have also been used in order to improve ovarian response and pregnancy rates in poor responders. Modifying controlled ovarian hyperstimulation (COH) with clomiphene citrate (CC) or letrozole (L) in addition to gonadotropins is promising and has gained acceptance for use in these cases (14-17). CC binds hypothalamic estrogen receptors and induces gonadotropin releasing hormone (GnRH) secretion by altering the negative feedback effect of estrogen on the hypothalamus. Triggered GnRH secretion increases pituitary gonadotropin release and finally results in stimulated ovarian follicular activity. The main benefits of adjunctive use of aromatase inhibitors (AI) in cycles of poor responders were reduced costs and cycle cancellation rates with comparable pregnancy outcomes (18,19). However, in the literature, there is one report that compares the effectiveness of CC and AI in poor responders in intracytoplasmic sperm injection (ICSI) cycles (16) and yet there is no study comparing these agents in recurrent poor responders.

In this study, we attempted to clarify the effectiveness of CC or L adjunctive to antagonist cycles stimulated with human menopausal gonadotropin (hMG) in poor prognosis IVF women who failed previous cycles with microdose or antagonist protocols.

Materials and Methods

Cases

One thousand and one hundred IVF cycles at Gazi University School of Medicine-based infertility clinic, Ankara, Turkey, from January 2006 to December 2009 were reviewed and 42 cycles of 32 infertile women who underwent IVF with at least 2 cycles of microdose flare or GnRH antagonist protocol and who failed to have ideal follicles to be retrieved during ovum pick-up (OPU) as a result of poor response to gonadotropin stimulation were retrospectively evaluated in this study. The Institutional Review Board and Ethics Committee of Gazi University School of Medicine approved this retrospective cohort study.

Ovarian stimulation protocols

Women (n=32) were equally divided into two groups, as CC and L groups, based on receiving CC (Serophene®, Serono, Turkey) 100 mg/day and L (Femara®, Novartis, Turkey) 2.5 mg/day, beginning on day 2 of the cycle and continued for 5 days. On day 4 of the cycles, hMG (Merional®, IBSA, Turkey) 300-450 IU/d administration was initiated. Daily GnRH antagonist (0.25 mg of cetrorelix acetate, Cetrotide®, Serono, Turkey) was started when the leading follicle exceeded ≥13 mm in diameter and continued until the day of human chorionic gonadotropin (hCG) administration. Recombinant hCG (250 mcg prefilled syringe, Ovitrelle®, Merck Serono, Turkey) was administered subcutaneously (SC) for final oocyte maturation when two or more leading follicles were ≥ 17 mm in diameter. The endometrial thickness was also documented via transvaginal ultrasonography (TVU) on the day of hCG administration. Schematic representation of the CC/L+hMG+antagonist protocols was shown in figure 1.

Oocyte retrieval, embryo transfer and luteal support

Oocyte retrieval was performed under TVU guidance 35-36 hours after hCG administration and all women had intravenous sedation with midazolam (Dormicum®, Roche, Turkey). Metaphase II (M2) oocytes were fertilized with ICSI instead of conventional IVF to minimize the risk of fertilization failure. Depending on the women's age, quality and number of available embryos, 1-4 embryo transfer (ET) was performed under TVU guidance 48-72 hours after OPU. Luteal phase was supported with 90 mg intravaginal progesterone gel (Crinone 8% gel®, Merck Serono, Turkey).

Detection of pregnancy

Pregnancy testing was performed by determining the quantitative serum hCG level at 12 days after ET, while intrauterine pregnancy was confirmed using TVU 2 weeks after a positive pregnancy test. A clinical pregnancy was defined as a positive serum beta hCG (βhCG) test result with the presence of a gestational sac on TVU or by histologic examination of products of conception in women who were aborted.

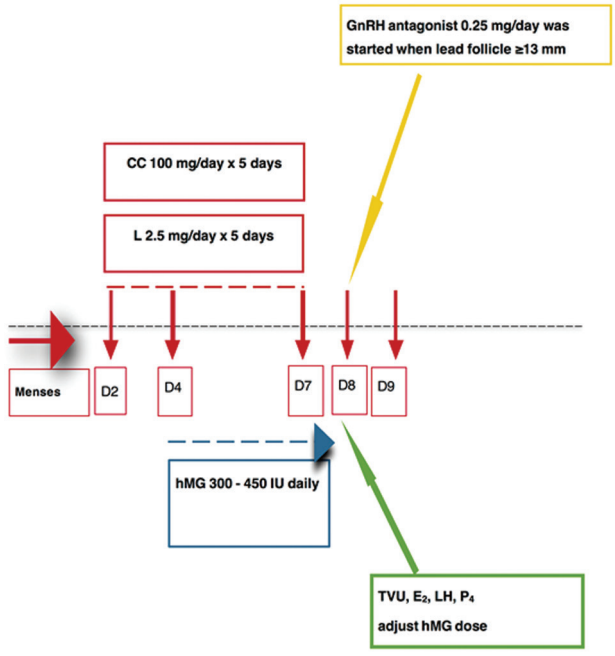

Fig.1: Schematic representation of CC vs. L+hMG+antagonist protocols.
CC; Clomiphene citrate, L; Letrozole, hMG; Human menopausal gonadotropin, GnRH; Gonadotropin releasing hormone, TVU; Transvaginal ultrasound, LH; Luteinizing hormone, D; Day, E_2; Estradiol, and P_4; Progesterone.

Outcome measures and statistical analysis

Main outcome measures were overall pregnancy, clinical pregnancy and live birth rates per cycle. The duration of stimulation, mean gonadotropin dose used, endometrial thickness, number of mature follicles, serum estradiol (E_2) and progesterone (P) levels on the day of hCG administration, the number of retrieved oocytes and fertilization rates were also evaluated. The statistical analysis was performed using the Statistics Package for Social Sciences version 12.0 (SPSS, SPSS Inc., Chicago). The Chi square (χ^2) test and Fisher's exact test were used to analyze nominal variables in the form of frequency tables. Normally distributed (Kolmogorov-Smirnov test) parametric variables were tested by independent Student's t test. Non-normally distributed metric variables were analyzed by Mann-Whitney U test. A value of $P<0.05$ was considered statistically significant. Values were expressed as mean ± standard deviation (SD) unless otherwise stated.

Results

A total number of 42 cycles of 32 severe poor responders were evaluated in this study. There were 22 cycles of 16 cases in the CC group and 20 cycles of 16 cases in the L group. The baseline characteristics of both groups were given in table 1. The overall cancellation rate was 78.5% and the pregnancy rate per attempted cycle was 7.1%.

The women in both CC and L protocol groups were comparable regarding age (37.7 ± 6 vs. 36.3 ± 4.2, respectively), basal FSH level (13.3 ± 4.9 vs. 14.6 ± 4, respectively) and antral follicle count (2.1 ± 1.1 vs. 2.1 ± 1.1, respectively). Mean total dose of FSH used was significantly lower (1491 ± 873 vs. 2808 ± 1581.1 IU, P=0.005) and mean E_2 level on the day of hCG injection was significantly higher (443.3 ± 255.2 vs. 255.4 ± 285.2 pg/mL, P=0.03) in the CC when compared to the L group. Other cycle characteristics and cancellation rates were similar in both groups. However, the ET rate was significantly higher in CC protocol (27.2%) when compared to that of the L protocol (15%, P<0.05, Table 1).

The overall pregnancy and live birth rates per attempted cycles were significantly higher in CC protocol than L protocol (13.6 vs. 0% and 4.5 vs. 0%, respectively, P<0.05, Table 1).

Table 1: Comparison of baseline characteristics, COH response and pregnancy outcomes between CC and L+ GnRH antagonist protocols

Variable	CC n=16	L n=16	P value
No. of cycles	22	20	0.16
Female age (Y)	37.7 ± 6	36.3 ± 4.2	0.07
Day 3 serum FSH (mIU/mL)	13.3 ± 4.9	14.6 ± 4.2	0.56
Antral follicle count	2.1 ± 1.1	2 ± 1.2	0.32
Duration of stimulation (days)	12 ± 3.4	11.6 ± 2.8	0.43
Total dose of FSH used (IU)	1491 ± 873	2808 ± 1581.1	0.005
E_2 level on the day of hCG injection (pg/mL)	443.3 ± 255.2	255.4 ± 285.2	0.03
P level on the day of hCG injection (ng/mL)	0.6 ± 0.7	0.9 ± 1.1	0.29
Endometrial thickness on the day of hCG administration (mm)	9.1 ± 2.4	8.6 ± 3.7	0.07
Follicles ≥17 mm on hCG (day)	1.1 ± 0.7	1.1 ± 0.7	0.96
Follicles 12-16 mm on hCG (day)	1.8 ± 1.5	1.6 ± 1.5	0.91
No. of canceled cycles %	72.7	85	0.1
No. of canceled cycles due to poor ovarian response %	63.6	70	0.2
No. of oocyte-cumulus complexes	2.5 ± 1.4	3.3 ± 1.3	0.52
No. of M2 oocytes	2.0 ± 1.4	2.6 ± 1.7	0.83
M2/no. of oocyte-cumulus complexes %	80	80	0.59
Fertilization rate %	70.7	80	0.65
ET rate %	27.2	15	0.04
No. of ET	1.6 ± 0.8	2.3 ± 1.1	0.42
No. of ET with less than 10% fragmentation and blastomere number ≥7	0.8 ± 1.1	1.0 ± 0.1	0.09
Pregnancy rate per cycle attempt %	13.6	0	<0.05
Pregnancy/ET %	50	0	<0.05
Biochemical pregnancy rate per cycle attempt %	4.5	0	<0.05
Biochemical pregnancy/ ET %	16.6	0	<0.05
Clinical pregnancy rate per cycle attempt %	9	0	<0.05
Clinical pregnancy/ET %	33.3	0	<0.05
Miscarriage rate %	33.3	0	<0.05
Live birth rate per cycle attempt %	4.5	0	<0.05
Live birth/ET %	16.6	0	<0.05

Data presented as mean ± standard error (SE).
CC; Clomiphene citrate, L; Letrozole, ET; Embryo transfer, COH; Controlled ovarian hyperstimulation, GnRH; Gonadotropin releasing hormone, FSH; Follicle stimulating hormone, hCG; Human chorionic gonadotropin and M2; Metaphase II.

Discussion

We used CC and L in cases of IVF with previous attempts resulting with cancelation due to poor response to gonadotropin stimulation and an ovum pick up was not completed under either flare or antagonist protocol. Although the definition of "severe poor responder" did not exist in the literature, we used this term to indicate very poor prognostic cases before an adoption or oocyte donation were advised to the couples. Our study revealed that the adjunctive use of CC is more effective in reducing hMG dose, increasing the number of embryos transferred and achieving better pregnancy rates than AI in severe poor responders. Both groups were comparable in the number of retrieved oocytes and cancellation rates. Unlike previous reports regarding adjunctive use of CC or L in poor responders, our higher cancellation rates (78.5%) might be attributed to allocation of more severe, recurrent poor prognostic cases into our study.

Microdose flare and GnRH antagoists are mostly accepted as first line protocols in poor responders (20). The adjunctive use of AI or CC may be helpful in their subsequent ICSI cycles. There is little but encouraging evidence for using these agents in poor responders (16, 21). In a subgroup analysis of a study performed by Jovanovic et al. (16) there were comparable improvements in COH response and cycle cancellation rates (39.8 ± 8.5% vs. 24.8 ± 7.6%, respectively) with the adjunctive use of CC vs. L plus high dose gonadotropins in 29 poor responders, only 2 clinical pregnancies and one live birth were reported in group L but none in group CC.

Regarding our data, it should be stated that the adjunctive use of L has little advantage in improving pregnancy outcomes in severe poor responder women. In the current study, we observed that adjunctive use of L failed to increase pregnancy rates despite its useful effects on ovarian response. L increases local androgen levels in the follicle and this hyperandrogenic environment in the follicle might impair oocyte quality and be responsible for poor pregnancy outcome (18, 22, 23). However, different outcomes in terms of quantity of the oocytes retrieved, quality of the embryos and pregnancy success concerning the use of L were previously reported (16, 18, 24-27). CC stimulates ovarian follicle development and maturation by inducing endogen gonadotropin secretion and aromatase activity, indirectly (28). The opposite effects of CC and L on aromatase enzyme activity may be the main cause of different pregnancy outcomes. AI treatment as an adjunctive therapy has been administered at a standard dose for a standard duration. It is possible that different infertile women with different aromatase activities require an individualized dosage in order to attain the desired effect and maximize the benefit of AI.

It must be noted that the retrospective design and low number of cycles weakened the power of our results. The burden of financial costs and the psychological aspect of recurrent failure lead to a high drop-out rate in these couples (29). For this reason, it is difficult to find high number of severe poor responder cases and perform a more powerful prospective randomized study. Therefore, most previous similar analyses in the literature were also in retrospective design with low number of cycles (16, 17). In another retrospective study, Yarali et al. (15) compared the effectiveness of L/antagonist protocol with microdose flare in 885 poor responder women and concluded that L plus antagonist has similar efficiency in terms of cycle characteristics and pregnancy outcome. However, the women had more than 4 M2 oocytes in each group, which indicates a population with more favorable prognosis as compared to our population. In fact, bias cannot be eliminated without randomization as a nature of retrospective studies (30). However, in a recent randomized study L/antagonist protocol was found better than microdose flare up in decreasing the days of stimulation and doses of used gonadotropin in poor responders' ICSI cycles (31).

CC significantly improves COH response by decreasing the doses of used gonadotropin and duration of stimulation without altering endometrial development in gonadotropin plus antagonist protocols in poor responders (32). Although pregnancy rates of adjunctive use of CC to gonadotropin were comparable with microdose flare up or antagonist protocols in poor responders, addition of CC seems to be beneficial for reducing costs (32, 33). In a recent report from a group of women with severe poor response to gonadotropin stimulation, high doses of gonadotropins were used on the subsequent cycle and clinical pregnancy rate was 5.6% with a mean costs per cycle and per live birth of €5597 and €124,540, respectively (29).

In that analysis, some women preferred a milder stimulation with CC and authors concluded that all results were similar with CC as compared to gonadotropins.

Conclusion

Severe poor responders who had previously failed to respond to microdose flare protocol or GnRH antagonist protocol may benefit from CC+GnRH antagonist protocols despite a high cancellation rate. CC+GnRH antagonist protocols may provide an alternative option for severe poor responders with low costs. Further prospective randomized studies are needed to confirm these results or to determine better one in severe poor responder women.

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Original Article

Increasing The Number of Embryos Transferred from Two to Three, Does not Increase Pregnancy Rates in Good Prognosis Patients

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Abstract

Background: To compare the pregnancy outcomes after two embryos versus three embryos transfers (ETs) in women undergoing *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycles.

Materials and Methods: This retrospective study was performed on three hundred eighty seven women with primary infertility and with at least one fresh embryo in good quality in order to transfer at each IVF/ICSI cycle, from September 2006 to June 2010. Patients were categorized into two groups according to the number of ET as follows: ET2 and ET3 groups, indicating two and three embryos were respectively transferred. Pregnancy outcomes were compared between ET2 and ET3 groups. Chi square and student t tests were used for data analysis.

Results: Clinical pregnancy and live birth rates were similar between two groups. The rates of multiple pregnancies were 27 and 45.2% in ET2 and ET3 groups, respectively. The rate of multiple pregnancies in young women was significantly increased when triple instead of double embryos were transferred. Logistic regression analysis indicated two significant prognostic variables for live birth that included number and quality of transferred embryos; it means that the chance of live birth following ICSI treatment increased 3.2-fold when the embryo with top quality (grade A) was transferred, but the number of ET had an inverse relationship with live birth rate; it means that probability of live birth in women with transfer of two embryos was three times greater than those who had three ET.

Conclusion: Due to the difficulty of implementation of the elective single-ET technique in some infertility centers in the world, we suggest transfer of double instead of triple embryos when at least one good quality embryo is available for transfer in women aged 39 years or younger. However, to reduce the rate of multiple pregnancies, it is recommended to consider the elective single ET strategy.

Keywords: Embryo Transfer, Sperm Injections, Intracytoplasmic, Live Birth Rate

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Introduction

An important issue in assisted reproductive techniques (ART) is how many embryos could be transferred for each couple. A number of variables considered for a high success rate in *in vitro* fertilization (IVF) treatment may be followed by a high rate of multiple pregnancies. Over 30% of IVF pregnancies are multiples which are associated with increasing maternal and infant morbidity and mortality such as (1, 2) hypertension, polyhydramnios, premature labor, (1) low birth weight, higher perinatal mortality and congenital anomalies (1). Therefore, it is crucial to find proper methods to reduce multiple pregnancies without reducing the overall pregnancy rate. Despite recent recommendations for achieving acceptable pregnancy rate with few multiple pregnancies, one or two good quality embryos needs to be considered for transfer (3-6), but still in some countries including Iran, patients have an impression that increasing the number of embryos transferred is associated with increased pregnancy rate.

Some studies have reported elective single-embryo transfer (eSET) in IVF-intracytoplasmic sperm injection (ICSI) cycles prevents multiple pregnancies without reduction of overall pregnancy rate (3, 4, 6), while some other studies believe that eSET could be associated with a lower pregnancy rate per cycle, especially in an unselected population (5, 7, 8). Despite the efforts have been made in order to limit the incidence of multiple pregnancies after ART (e.g. by SET), the average IVF treatment includes transfer of two, three or sometimes even more embryos into the uterus, while use of eSET in clinical practice has not yet been performed. This may be due to the factors influencing the number of embryos transferred such as cost-effectiveness of eSET technique, professional attitudes and the financial situation of couples. In a recent systematic review, Pandian et al. (8) reported that insufficient data are available on the outcome of two versus three and four ETs policies. In Iran and some other parts of the world where no legal restrictions exist, this is the responsibility of infertility specialists and patients to make decision about the number of embryos transferred with respect to the risks associated with multiple gestations and acceptable pregnancy rates. A recent guideline stating the suitable number of embryos to transfer following an IVF cycle suggests that a maximum of three or four embryos can be transferred in women over the age of 39 (9).

The primary purpose of the present study was to investigate whether increasing the number of embryos transferred from two to three leads to an increase in the overall pregnancy and live birth rates in women undergoing ICSI cycles, then the secondary objective was to evaluate the impact of maternal age on the outcome of IVF/ICSI according to the number of embryos transferred.

Materials and Methods

This retrospective study was performed at Reproductive Biomedicine Research Center, Royan Institute, Tehran, Iran, from September 2006 to June 2010. The Institutional Review Board of Royan Institute was approved the study. All patients signed a consent form in their initial visit giving permission to use their results without using their names in the future studies. The study population consisted of 387 women with primary infertility and with at least one fresh ET in good quality. Exclusion criteria were as following: use of clomiphene citrate; use of human menopausal gonadotropin (hMG) only in antagonist protocols; women with advanced age (≥40), women with body mass index (BMI) ≥30, as well as history of ovarian hyperstimulation syndrome, uterine factor infertility, severe endometriosis, hydrosalpinges and repeated implantation failure. All patients according to number of ETs were categorized into two groups: ET2 and ET3 groups, indicating two and three embryos were transferred.

In this study, all of the patients underwent a standard long protocol using 500 μg gonadotropin-releasing hormone (GnRH)-a (Buserelin, Superfact, Aventis Pharma Deutshlan, Frankfurt, Germany). Once down-regulation was confirmed by linear endometrium thickness in ultrasonography and serum estradiol concentration <50 pg/ml, the Superfact dose was reduced by one-half (250 μg), and gonadotrophin stimulation with recombinant follicular stimulating hormone (rFSH, Gonal-F, Serono Laboratories Ltd., Geneva, Switzerland) was applied and continued until the day of human chorionic gonadotropin (hCG, IBSA, Switzerland) administration. The first ultrasound scan was performed on day 6 and the dose of rFSH was adjusted according to the ovarian response. When at least two follicles >18 mm were seen, 10000 IU urinary hCG (uhCG, Choriomon, IBSA, Switzer-

land) was injected intramuscularly and oocyte retrieval was performed 34-36 hours later. The presence of two pronuclei and two polar bodies was assessed 16-18 hours after ICSI. Approximately, 48 hours after injection, embryos were classified based on morphological criteria (10). Embryos with the best morphology and with the most advanced stage of development were selected for transfer. In our institute, the grading of embryos was performed by two embryologists with same background. Two expert clinicians performed the ETs and the difficult ETs were excluded from study.

In our institute, a number of factors, including the patient's age, cause and duration of infertility, the number and grade of the available embryos and requests of the couples, were taken into consideration in order to decide how many embryos to transfer. ET performed on day 2 or 3 after oocyte retrieval and two or three embryos per patient were transferred. Luteal-phase support was provided with 400 mg vaginal progesterone (Aburaihan co., Tehran, Iran) twice a day until the day of beta-hCG (β-hCG) assay. If the result of β-hCG assay was positive, the same dosage of progesterone was continued up to 10 weeks of gestation. Clinical pregnancy was defined as a positive pregnancy test followed by the presence of fetal sac on transvaginal ultrasound 4 weeks later.

Data were analyzed using the Statistical Package for the Social Sciences 16.0.0 (SPSS, SPSS Inc. Chicago, IL, USA). Demographic factors, reproductive history, ART cycle-specific parameters, and pregnancy outcomes were compared between two groups using the chi-square test for categorical variables and Student t test for continuous variables when data was normally distributed, whereas Mann-Whitney test was used for abnormal cases. All tests were done two tailed. Descriptive statistics are presented as mean ± standard deviation (SD) and percentage. Multiple logistic regression analysis was used to evaluate the association between the number of ET and live birth rate, adjusting for potential confounding variable (age). We used Receiver Operating Curve (ROC) analysis to find the best cut point of age for prediction of live birth by regression equation. A value of $P<0.05$ was considered to be statistically significant.

Results

A total of 387 patients were included in this study, among whom 193 patients with two ETs and 194 patients with three ETs. Pregnancy outcomes were compared between ET2 and ET3 groups.

The demographic characteristics are demonstrated in table 1. Two groups had no difference in terms of infertility diagnosis, women's BMI, infertility duration and the number of previous ART cycles. The mean of maternal age in ET2 group was significantly higher than ET3 group ($P<0.001$).

Table 1: Demographic characteristics of women according to the number of embryos transferred (ET)

Variables	Two ET (n=193)	Three ET (n=194)	P value
Age mean (SD)	29.6 (5.3)	27.5 (3.5)	<0.001
BMI mean (SD)	24.6 (3.4)	24.7 (3.1)	0.48
Infertility duration n (%)	7.0 (5.0)	6.6 (3.8)	0.4
Infertility reason n (%)	-	-	0.59
Male factor	105 (45.4)	113 (58.2)	-
Tubal factor	9 (4.7)	21 (10.8)	-
Ovulatory factor	25 (13)	19 (9.8)	-
Unexplained	20 (10.4)	15 (7.8)	-
Multiple factors	34 (17.6)	26 (13.4)	-
No. of previous cycles n (%)	0.8 (0.5)	0.7 (0.7)	0.2

SD; Standard deviation and BMI; Body mass index.

The mean total dose of rFSH, duration of gonadotropin administration, and the number of MII oocyes were similar between two groups (P=0.7, P=0.6 and P=0.3, respectively). Furthermore, the mean number of oocytes retrieved and total embryos were significantly higher in ET3 group compared to the ET2 group (P=0.001, Table 2). Chi-square test showed that the percent of patients with Grade C ET in ET3 group was higher significantly (P=0.02).

Our results showed that pregnancy rates in patients with two and three ETs were similar (P=0.7). There was also no significant differences in terms of live birth (P=0.4), miscarriage and intrauterine death rate between two groups. Multiple pregnancy rate was significantly higher in the ET3 group

compared to ET2 group (Table 3).

The cut point for maternal age obtained by ROC analysis for clinical pregnancy rate was 33 years. Logistic regression analysis revealed that only age was predictable for clinical pregnancy rate in stimulating ICSI cycles [Odds ratio (OR): 1.6, Confidence interval (CI):1.05-2.8] (Table 4). Furthermore, logistic regression analysis for predictive factors of live birth rate showed that the quality of transferred embryos and number of ET were significantly predictable, so that the quality of ET was directly related to the live birth, but the number of ET had an inverse relationship with this variable (Table 5).

As shown in table 6, live birth rates were similar between ET2 and ET3 in women younger and older than 33 years old. Therefore, a reduction in number of embryos transferred did not decrease the clinical pregnancy and live birth rates in both age levels.

Table 2: Characteristics of ICSI cycles of study population according to the number of embryos transferred (ET)

Variables	Two ET (n=193)	Three ET (n=194)	Overall P value
No. of total gonadotropins (75 IU/Amp)	26.0 (12.7)	25.7 (9.2)	0.7
Duration of stimulation (days)	10.2 (2.0)	10.3 (2.1)	0.6
No. of oocytes retrieved	9.5 (6.1)	11.8 (5.8)	<0.001
No. of M2 oocytes	7.5 (5.4)	8.0 (4.5)	0.3
No. of embryos	4.8 (3.9)	6.2 (3.2)	<0.001
Quality of transferred embryos	-	-	0.02
Two or three ET (grade A) n (%)	81 (42)	79 (40.7)	-
Two or three ET (grade B) n (%)	110 (57)	100 (51.5)	-
One ET (grade A or B) and one or two ET grade C	2 (1)	15 (7.8)	-
Day of embryos transferred	2.2 (0.4)	2.3 (0.5)	0.01

Data are presented as mean ± SD. ICSI; Intracytoplsamic sperm injection, SD; Standard deviation and M2; Metaphase II.

Table 3: ICSI outcomes according to the number of embryos transferred (ET)

Variables	Two ET (n=193)	Three ET (n=194)	Overall P value
Fertilization rate mean (SD)	61.5 (30.5)	61.2 (35.0)	0.9
Implantation rate mean (SD)	26.1 (35.7)	20.0 (29.5)	0.07
Clinical pregnancy rate n (%)	78 (40.4)	73 (37.6)	0.7
Multiple pregnancy rate n (%)	21 (27)	33 (45.2)	0.01
Miscarriage rate n (%)	6 (3.1)	10 (5.1)	0.3
Live birth rate n (%)	69 (35.7)	60 (30.9)	0.4
Intrauterine fetal death n (%)	3 (1.5)	3 (1.5)	NS*

*NS; Not significant, ICSI; Intracytoplsamic sperm injection and SD; Standard deviation.

Table 4: Logistic regression analysis for predicting the clinical pregnancy rate in ICSI cycles

Variable	OR	95% CI	P value
Age			
<33	1.6	(1.05-2.8)	0.05
≥ 33 years old	Reference group	-	-

ICSI; Intracytoplsamic sperm injection, OR; Odds ratio and CI; Confidence interval.

Table 5: Logistic regression analysis for predicting the live birth rate in ICSI cycles

Variable	OR	95% CI	P value
Quality of transferred embryos			
Grade A	3.1	(1.1-9.0)	0.02
Grade B	2.2	(1.07-7.0)	0.05
Grade C	Reference group	-	-
Number of embryos transferred			
Two embryos	3.1	(1.09-9.2)	0.03
Three embryos	Reference group	-	-

ICSI; Intracytoplsamic sperm injection, OR; Odds ratio and CI; Confidence interval.

Table 6: Age related results according to number of embryos transferred (ET)

	Two ET	Three ET	P value
<33 years: No. of cases	**n=136**	**n=132**	
Implantation rate mean (SD)	29.0 (36.2)	20.8 (30.2)	NS*
Pregnancy rate n (%)	61 (44.8)	57 (43.1)	NS
Live birth rate n (%)	53 (38.9)	46 (34.8)	NS
Multiple pregnancy rate (%)	17 (27.8)	30 (52.6)	0.001
≥33 years: No. of cases	**n=57**	**n=62**	
Implantation rate mean (SD)	19.2 (33.7)	11.7 (20.2)	NS
Pregnancy rate n (%)	17 (29.8)	17 (28)	NS
Live birth rate n (%)	16 (28.0)	14 (22.5)	NS
Multiple pregnancy rate n (%)	4 (23.5)	3 (17.6)	NS

*NS; Not significant and SD; Standard deviation.

Discussion

Our study indicated that in good prognosis patients aged 39 years or younger, two and three ETs have same pregnancy and live birth rates, while the multiple pregnancy rate was significantly higher in ET3 group; therefore, it is recommended to transfer two instead of three embryos.

Based on a recent guideline, individual IVF-ET centers should evaluate their own data to identify patient-specific, embryo-specific, and cycle-specific to determine factors of implantation and live birth in order to develop ET protocols minimizing the occurrence of multi-fetal gestation, while preserving acceptable overall pregnancy and live birth rates (11).

In a recent study, Min et al. (9) presented a guideline for the number of embryos transferred considering the maternal age; however, this numbers can be different in various infertility centers according to the laws of those countries. In a number of countries, including Norway, Sweden, Denmark, Belgium, England, Italy, Germany, and Australia, the complications associated with multiple pregnancies are reduced through use of SET by legal restrictions, while many other European countries have bordered to a maximum of two ETs.

In other parts of the world like Iran, there is no legal restriction in this regard, and it is the responsibility of infertility specialists and patients to make decision about the number of embryos transferred. Various strategies for eSET depend on different funding methods of infertility treatments. There are countries where the public sector covers the majority of costs, whereas in some other courtiers, patients have to undertake the costs directly or indirectly through private insurance systems. Despite the recent emphasis and supports on eSET (3-6, 12, 13), this technique in not generally used in Iran because of heavy treatment costs. SET in our institute is necessarily for some patients with special conditions such as poor ovarian response and male factor cases.

In agreement with the previous studies (14-17), we found similar clinical pregnancy and live birth rates in patients with two versus three ETs, but the multiple pregnancy rate in our study was significantly greater in group with three embryos than two embryos transferred. Despite the fact that women in ET3 group were younger than ET2 group, but due to higher number of couples with male infertility in the ET3 group, the number of patients with grade C embryos transferred were significantly higher than ET2 group. Therefore, no significant difference was observed between the two groups in terms of implantation, clinical pregnancy and live birth rates.

We evaluated the influence of maternal age on IVF/ICSI outcome for the number of ET and the obtained data indicated lower rate of multiple pregnancies in group with two embryos transferred as compared to ET3 group with individuals of different ages (younger or older than 33 years), while the pregnancy and live birth rates were similar. In contrast to our results, Giannini et al. (18) showed that in older women (≥35 years), a reduction in the number of embryos transferred significantly decreased the chances of pregnancy.

Logistic regression analysis showed that one significant prognostic variable for clinical pregnancy: maternal age (as categorical variable); it means that in women younger than 33 years old, the chance of clinical pregnancy increased 1.6-fold. Our results were in line with Chuang et al. (19) that age is a good predictor for pregnancy potential. Implantation, pregnancy and live birth rates in women younger than 33 years old in both groups (ET2 and ET3) were higher than older women.

On the other hand, logistic regression analysis indicated two significant prognostic variables for live birth that were quality and number of transferred embryos; it means that the chance of live birth following ICSI treatment increased 3.2-fold when the embryo with top quality (grade A) was transferred. Our result is in agreement to Dennis et al. (20) study, in which they suggested that embryo grade is a significant predictor for live birth rate. But the number of ET had an inverse relationship; it means that probability of live birth in women with transfer of two embryos was three times greater than those who had three embryos transferred.

One of the limitations of this study comes from its retrospective nature; however, we tried with regression test to control confounding factors.

Present study was performed in fresh ET cycles, and because the probability of no synchronization

between the time of fresh ET and the window of implantation in the endometrium is existed (21), we suggest future study to evaluate the association between number of embryos transferred and live birth rate in cycles with frozen and/or blastocyst stage ETs.

According to a recent guideline, women aged 35 years or younger, in first or second IVF attempt, with at least 2 good quality embryos transferred should be considered as good-prognosis patients, and the eSET strategy should be used in order to avoid multiple pregnancies (9). In our study, the rate of twin pregnancy in women with double ET was 27%, which is not acceptable; therefore, it is recommended that the eSET strategy be considered, although there are no executive arrangements to enable our institution to enforce the eSET strategy. Moreover it is important to remind the patients about the high risk nature of multiple pregnancies that requires equipped labor which may not be available in some rural or smaller areas. Concerns in relation to multiple pregnancies and even twin pregnancy resulting from ART specify the importance of counseling before ET for IVF patients (22).

Conclusion

Due to the difficulty of implementing the eSET strategy in some infertility centers in the world including Iran, we suggest transferring of a maximum of two embryos with at least one good quality embryo for good prognosis women aged 39 years or younger. However, further clinical trials are required to evaluate the effect of the number of embryos transferred (single versus double ET) for women in different age groups using fresh or frozen ET technique.

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Original Article

Evaluating The Effective Factors in Pregnancy after Intrauterine Insemination: A Retrospective Study

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Abstract

Background: Controlled ovarian hyperstimulation (COH) in conjunction with intrauterine inseminations (IUI) are commonly used to treat infertile couples. In this study we evaluated the relationship between IUI outcome and special causes of infertility. We also aimed to examine parameters that might predict success following IUI.

Materials and Methods: In this cross-sectional study, we included 994 IUI cycles in 803 couples who referred to the infertility Institute. All statistical analyses were performed by using SPSS program, t tests and chi-square. Stepwise multiple linear regression analysis was performed to compare the association between dependent and independent variables. Logistic regression was conducted to build a prediction model of the IUI outcome.

Results: Overall pregnancy rate per completed cycle (16.5%) and live birth rate per cycle (14.5%). The mean age in the pregnant group was significantly lower than that of the non-pregnant group (P=0.01).There was an association between cause of infertility and clinical pregnancies (P<0.001). Logistic regression identified four significant factors in determining the success of the IUI [menstrual irregularites (OR:2.3, CI:1.6-3.4, P<0.001), duration of infertility (OR:0.8, CI:0.8-0.9, P<0.001), total dose of gonadotropin (OR:1.02, CI:1.003-1.04, P=0.02) and semen volume (OR:1.1, CI:1.008-1.2, P=0.03)] which were the most predictive of IUI success.

Conclusion: Our study defined prognostic factors for pregnancy in COH+IUI. These variables can be integrated into a mathematical model to predict the chance of pregnancy rate in subsequent COH+IUI cycles.

Keywords: Pregnancy Rate, Infertility, Prognostic Factors

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Introduction

Controlled ovarian hyperstimulation (COH) in conjunction with intrauterine inseminations (IUI) are commonly used to treat infertile couples (1). The most important indications for IUI are male subfertility, unexplained infertility, ovulatory dysfunction and cervical factor infertility (2). Several prognostic factors that determine IUI treatment outcome have been identified and include the woman's age, duration of infertility, follicle number, endometrial thickness, numbers of sperm inseminated, sperm morphology, progressive motile sperm count, and cause of infertility (3-5). Tomlinson et al. (6) found no differences in age, duration of infertility, number of follicles, body mass index (BMI) and sperm quality in the pregnancy rates

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of patients undergoing IUI. Although IUI with or without ovarian stimulation is widely used, its success rate is lower than that of the assisted reproductive technologies (ART) (7). Nevertheless, in comparison to ART controlled ovarian hyperstimulation combined with intrauterine (COH+IUI) requires less frequent clinic visits, and is simple, relatively less invasive and comparatively inexpensive (8). Regardless of the method of assisted conception utilized, the couples always desire to know their chances of success. Thus, identifying the factors which are influential in the success rate is highly crucial. The purpose of this study was to identify the parameters which were most influential in the success rate using COH+IUI treatment modality. Using logistic regression, we were able to devise a mathematical model to predict the success rate in COH+IUI. The data presented, will enable the healthcare providers to counsel their patients about their chances of getting pregnant by using COH+IUI.

Materials and Methods

In this cross-sectional study, we included 994 IUI cycles in 803 couples who referred to the infertility Institute between 2010-2012. This study was approved by the Institutional Review Board of the Royan Institute Research Center and the Royan Ethics Committee according to the Helsinki Declaration, signed informed written consent was obtained from all participants. All couples had attempted to conceive for at least one year prior to undergoing COH+IUIs. The women completed the self-administered questionnaire which was used to collect data about demographic, menstrual and obstetrical characteristics. A menstrual interval shorter than 21 days and longer than 35 days is defined as menstrual irregularities. Amount of bleeding is varied (9).

The study population comprised of all couples who were candidates for COH+IUI and had diagnoses of subfertile male infertility, polycystic ovary syndrome (PCOS), mild or minimal endometriosis or unexplained infertility and various ovulatory disorders. Ovulatory disorders included diminished ovarian reserve, PCOS and hypothalamic amenorrhea. Subfertile male infertility was defined as per criteria outlined by Molinaro et al. (10).

The following evaluations were performed prior to the initiation of COH+IUI. The women underwent cycle day 3 hormone evaluation [follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E_2), thyroid-stimulating hormone (TSH) and prolactin] and assessment of tubal patency by using hysterosalpingogram (HSG) and/or laparoscopy. Tubal patency of at least one tube was mandatory. In case of either a tubal abnormality in HSG or dysmenorrhea and dyspareunia, a laparoscopy was performed.

Inclusion criteria were: male factor, combined causes, Ovulatory disorder (Pco, diminished ovarian reserve, and hypothalamic amenorrhea), unexplained disorder, and all patients with normal TSH and prolactin levels. The couples with testicular atrophy, hydrosalpinx, anatomical abnormalities, infection, uterine fibroids, and systemic disease were excluded from participation.

All IUI cycles were performed with ovarian stimulation and included either clomiphene citrate (Iran Hormone Pharmaceutical Company, Iran), gonadotropin only, or letrozol (Femara, Novartis Pharma AG, Switzerland) or the combination of either clomiphene citrate or letrozol with gonadotropin. On days 11-12 of the menstrual cycle, we assessed follicular development and endometrial thickness by transvaginal ultrasound. If the endometrial thickness was <7 mm, 4 mg/per day E_2 was administered (2 mg, Aburaihan Co., Iran) and continued during the luteal phase. Once a leading follicle of ≥18 mm was identified, human chorionic gonadotropin (5,000 IU IM, Pregnyl®, Darou Pakhsh Pharmaceutical, Iran) was administored to induce the final stage of oocyte maturation and a single IUI was planned 36-38 hours later. If more than five follicles ≥18 mm in size developed, the cycle was cancelled.

Sperm preparation

Semen samples were obtained from patients who attended the unit for infertility treatments. Semen samples (n=994) were collected following 3-7 days of sexual abstinence. They were allowed to liquefy at (add room temperature or 37˚C) for 15-30 minutes and each was subject to an analysis according to the 2010 World health Organization (WHO) guidelines (11). After analysis, samples were prepared for IUI using to discontinuous density gradient centrifugation (DGC). For this purpose, we prepared a two layer gradient consisted

of solutions of 100 and 50% Allgrade® (LifeGobal, Belgium). The 50% concentration was made by diluting 100% Allgrade® with Ham's F10 (Sigma, USA) medium. The density gradients were performed by layering 2 mL of each concentration into a conical tube (15 mL, Falcon, Becton Dickinson, NJ, USA). These tubes were pre-incubated for at least 2 hours in a 37˚C incubator. After semen liquefaction, 2 mL of ejaculation was layered on the top of the Allgrade® gradient and centrifuged for 30 minutes at 300 x g. After centrifugation, the sperm was collected at the bottom of the tube by a clean Pasteur pipette and transferred to a 5 mL clean tube (Falcon), and washed twice with Ham's F10 medium by using centrifugation at 300 x g for 5 minutes. The pellet was resuspended in 1 mL of Ham's F10 medium, then the sperm concentration and motility were evaluated. In our study the sperm analysis data haven't been recorded after processing and sperm analysis data before processing was available.

Intrauterine inseminations was performed by a soft catheter (INDOVASIVE, Biorad, India) with an insemination volume of 0.6 mL. The IUI catheter was passed gently through the cervical canal until the tip passed the internal os. Then, the sperm suspension was deposited slowly through the uterine cavity. All patients were provided with luteal support by using cyclogest according to the treatment physician's preference. Clinical pregnancy was defined as a positive pregnancy test followed by the presence of a gestational sac visualized by transvaginal sonography 4weeks after IUI.

In order to build a prediction model, we used stepwise logistic regression analysis, in which a P value of 0.15 was used as an entry criterion, whereas a P value of 0.10 was the threshold for a variable to stay in the model. We check the performance of the model by the area under the receiver operating characteristic (ROC) curve (AUC). An AUC of 0.5 indicates no discriminative performance, whereas an AUC of 1.0 indicates perfect discrimination.

Calibration of the model was assessed by comparing the predicted probability of pregnancy in a category of patients and the observed percentage of pregnant woman in that category. We first categorized the predicted probabilities of pregnancy in 10 groups, then we compared the mean predicted probability of pregnancy in that particular category with the observed probability, i.e. pregnancy rate in that category.

Statistical analysis

All statistical analyses were done by using SPSS software (version 20, USA). Chi-square and t tests were used for analyses. We performed univariate logistic regression for each factor and reported the odds ratio (OR) and 95% confidence interval (CI). In order to predict the IUI result, we used multiple logistic regression analyses. Data were expressed as mean ± standard deviation (SD). A Pvalue of <0.05 was considered to be statistically significant.

Results

We studied a total of 994 IUI cycles in 803 couples. Each couple underwent 1.23 ± 0.4 (mean ± SD) COH+IUI cycles (range: 1-3). Causes for infertility were: unexplained disorder (290, 29.2%), male factor (395, 39.7%), combined causes (108, 10.9%), and ovulatory disorder (201, 20.2%). In our study population, ovulatory disorders included diminished ovarian reserve, 0.5% (n=1), PCOS, 93.5% (n=188) and hypothalamic amenorrhea, 6% (n=12).

In our study combined cause including; ovulatory disorder and male factor 83.3% (n=90), tub peritoneal and male factor 6.5% (n=7), uterine factor and ovulatory disorder 3.7% (n=4), uterine factor and male factor 2.8% (n=3), male factor and recurrent abortion 1.9% (n=2), ovulatory disorder and recurrent abortion 0.9% (n=1), uterine factor & recurrent abortion 0.9% (n=1).

Table 1 compares the demographic characteristics between pregnant and nonpregnant women. The pregnancy rate in younger women was significantly higher than those of older women. In addition, an infertility duration of ≤4years was associated with a significantly higher pregnancy rate (OR:1.5, CI:1.1-2.2, P=0.01). Infertility type (primary or secondary) did not significantly affect the outcome. With regards to the diagnosis of infertility, the highest pregnancy rate (27.8%) was achieved in couples with combined infertility, whereas the lowest (13.4%) rate was observed in couples who suffered from male factor infertility (P<0.001, Table 1).

Table 1: Characteristics of study patients who underwent IUI

	Pregnant	Nonpregnant	OR (95% CI)	P value
Female age (Y) †*	27.80 ± 3.69	28.62 ± 3.94	0.97 (0.90-0.98)	0.01
Male age (Y) †	32.95 ± 4.57	32.41 ± 4.41	0.97 (0.93-1.01)	0.16
Menstrual irregularities n (%)*				
No	93 (13.5)	596 (86.5)	0.48 (0.34-0.67)	<0.001
Yes	72 (24.5)	222 (75.5)	1§	
BMI (kg/m^2) †	25.23 ± 4.32	25.41 ± 14.36	0.99 (0.98-1.01)	0.88
Type of infertility-n (%)				
Primary	142 (16.6)	708 (83.4)	1§	
Secondary	24 (16.7)	120 (83.3)	1.00 (0.62-1.61)	1.00
Duration of infertility (Y) †*	3.65 ± 2.41	4.38 ± 2.82	0.89 (0.83-0.96)	0.002
Etiology of infertility-n (%)*				
Male factor	53 (13.4)	342 (86.6)	1§	<0.001
Unexplained disorder	40 (13.8)	250 (86.2)	1.03 (0.66-1.60)	
Ovulatory disorder	42 (20.9)	159 (79.1)	1.70 (1.09-2.66)	
Combined	30 (27.8)	78 (72.2)	2.48 (1.48-4.13)	

IUI; Intrauterine insemination, OR; Odds ratio, CI; Confidence interval, BMI; Body mass index,†; Values are mean ± SD, §; Reference category and *; P<0.05 was considered as statistically significant.

The pregnancy rates according to female characteristics and sperm parameters (according to Strict Criteria) are summarized in table 2. Pregnancy rate was not related to sperm count. There were no significant differences in total sperm concentration among the pregnant and nonpregnant study population. Sperm parameters did not significantly affect the outcome of COH+IUI treatment. Seminal volume did not significantly affect the success of COH+IUI. The total dose of gonadotropin in nonpregnant women was significantly lower than that of the pregnant women (P=0.03, Table 2).

No significant difference was found between the two groups in different types of gonadotropins (data not shown).

Pregnancy outcome, in our study included, there were 3 (1.8%) ectopic pregnancies, 9 spontaneous miscarriages of which 5 (3%) occurred during the first trimester and 4 (2.4%) during the second trimester; 8 (4.8%) cases of blighted ovum, and 145 (87.9%) live births. This corresponded to an ongoing pregnancy rate of 14.9% (149/994) per IUI cycle. Of the 165 clinical pregnancies (ongoing pregnancies and early pregnancy loss), 22 were twin pregnancies (13.3%). There were 7 (4.2%) triplet pregnancies, of which one ended with a late abortion and another terminated at 24 weeks from which no fetus survived. Of the remaining triplet pregnancies, two mothers gave birth at 32 and 34 weeks (two healthy sets of one girl and two boys) and 3 triplet pregnancies were reduced to twins. The mean birth weight was 1488 ± 395 g and mean gestational age at delivery was estimated to be 32 weeks for the triplet pregnancies that reduced to twins. Of these, all neonates were well and healthy. The mean birth weight of singletons was 3000 ± 525.7 g, twins weighed 2081 ± 557.4 g and triplets weighed 1588.3 ± 549.1 g. All singletons ended at 28-40 weeks and twins at 30-38 weeks.

We used linear-by-linear test for calculation, the correlation between age of women and clinical, ongoing and multiple pregnancies rate. When one of the variables is ordinal and the other variable is ordinal or nominal with 2 level, this trend test can be used (12).

The proportion of clinical, ongoing and multiple pregnancies, decreased with age (P=0.041, P=0.044 and P=0.046, respectively, Table 3).

Table 2: Cycle parameters of the patients who underwent IUI

	Pregnant	Nonpregnant	OR (95% CI)	P value
Total dose of gonadotropin †*	589.21 ± 554.61	508.43 ± 420.62	1.00 (1.00 ± 1.0001)	0.03
Serum FSH level on day 3 (IU/ml) †	6.36 ± 3.91	6.38 ± 2.50	0.99 (0.94-1.05)	0.93
Serum LH level on day 3 (IU/ml) †	5.84 ± 3.52	5.94 ± 3.73	0.99 (0.94-1.03)	0.75
Serum estradial level on day 3 (Pg/ml) †	51.78 ± 40.66	63.98 ± 106.69	0.99 (0.99-1.007)	0.72
Sperm count ($\times 10^6$/ml) †	53.10 ± 25.32	51.71 ± 28.92	1.002 (0.99-1.008)	0.55
Total sperm count †	175.93 ± 100.57	161.07 ± 107.43	1.001 (0.99-1.003)	0.10
Total motile sperm ($\times 10^6$/ml) -n (%)				
<10	1 (14.3)	6 (85.7)	0.81 (0.09-6.78)	
10-20	6 (9.5)	57 (90.5)	0.51 (0.21-1.20)	
>20	158 (17)	766 (83)	1§	0.29
Normal morphology-n (%)				
≤10	116 (16.5)	589 (83.5)	1§	0.89
>10	48 (16.8)	238 (83.2)	1.02 (0.70-1.48)	
Semen volume †	3.59 ± 1.82	3.31 ± 1.64	1.09 (0.99-1.20)	0.054
Endometrial thickness (mm)-n (%)				
<6	3 (25)	9 (75)	1§	0.40
≥6	162 (16.5)	820 (83.5)	0.57 (0.15-2.13)	

IUI; Intrauterine insemination, OR; Odds ratio, CI; Confidence interval, FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, †; Values are mean ± SD, §; Reference category and *; Significant statistical differences between the two groups.

Table 3: Clinical and ongoing pregnancy rates per couple and the frequency of multiple pregnancies for women according to age group

Age (Y)	Clinical pregnancy/couple % (n)*	Nonpregnant % (n)*	Multiple pregnancy/clinical pregnancy % (n)*
≤30	22.2 (124/558)	20.1 (112/558)	20.1 (25/124)
31-35	18 (37/206)	16.5 (34/206)	10.8 (4/37)
36-40	10.3 (4/39)	7.7 (3/39)	0
Total	20.5 (165/803)	18.6 (149/803)	17.5 (29/165)
P value	0.041	0.044	0.046

*; Significant statistical differences between the groups.

Of the 145 (87.8%) live births, 139 resulted in live deliveries at term, 123 (88.5%) patients underwent caesarean sections and 16 (11.5%) had normal vaginal deliveries. There were no major congenital anomalies reported. The live birth rate/cycle was 14.5% (145/994).

The clinical pregnancy rate per couple was 20.5% (165/803) with an ongoing pregnancy rate per couple of 18.5% (149/803). Pregnancy rates per cycle were as follows: first (21%), second (19.4%) and third (15.3%).

Stepwise multiple linear regression analysis was performed to compare the association between dependent (total dose of gonadotropin) and independent (age, BMI, menstrual irregularities, duration of infertility, type of infertility, endometrial thickness, number of dominant follicle, etiology of infertility) variables. Age (P<0.001), menstrual irregularities (P<0.001), and duration of infertility (P=0.01) were the main variables that significantly influenced the total dose of gonadotropin in couples undergoing IUI (Table 4).

Table 4: Variables influencing total dose of gonadotropin in couples undergoing IUI

Variable	Coefficient	SE	P value *
Age	26.16	3.44	<0.001
Menstrual irregularities	-118.36	29.56	<0.001
Duration of infertility	10.97	4.94	<0.01

*; P value multiple regression, IUI; Intrauterine insemination and SE; Standard error.

According to logistic regression, female age, duration of infertility, menstrual irregularities, seminal volume and total dose of gonadotropin were significantly associated with pregnancy outcome. Higher female age, prolonged duration of infertility and regular menstruation showed a negative association with pregnancy outcome, while seminal volume and total dose of gonadotropin were positively associated with pregnancy outcome (Table 5).

Table 5: Result of logistic regression analysis

Variable	OR	95% CI	P value *
Duration of infertility (Y)	0.86	0.80-0.93	<0.001
Menstrual irregularities			
No	1§	1§	
Yes	2.37	1.65-3.40	<0.001
Semen volume	1.11	1.008-1.22	0.03
Total dose of gonadotropin	1.02	1.003-1.04	0.02
Constant	0.11		<0.001
AUC:0.65 (95% CI: 0.60-0.70)			

OR; Odds ratio, CI; Confidence interval, §; Reference category, *; Significant statistical differences and AUC; Area under curve.

The ROC curve was used to assess the discriminative performance of the fitted logistic model (Fig.1). An AUC equal to 0.5 indicates no discriminative power whereas an AUC of 1.0 shows a perfect discrimination. In our study, the AUC for the fitted logistic model was found to be 0.65 with the 95% CI of 0.60 - 0.70, indicative of a reasonable prognostic potency for predicting pregnancy following COH+IUI.

With the data obtained, we were able to construct a formula for calculation of the probability of pregnancy following COH+IUI as carried out in our study (see below).

In this formula, the duration of infertility is the number year each couple has been attempting to conceive without success. The menstrual history is=1 if history of menstrual is irregular and 0 if history of menstrual is regular. The performance of the prediction model for pregnancy following COH+IUI was calibrated as shown in figure 2. The predictive performance appears to be acceptable because the 95% confidence intervals of the observed pregnancy rates overlap with the predicted pregnancy rate.

$$\text{Probability of pregnancy after IUI} = \frac{e^{(-2.154-0.147\times\text{Durationofinfertility}+0.865\times\text{Menstrualhistory}+0.105\times\text{Volume}+0.022\times\sqrt{(\text{Total dose of gonadotropin})})}}{1+e^{(-2.154-0.147\times\text{Durationofinfertility}+0.865\times\text{Menstrualhistory}+0.105\times\text{Volume}+0.022\times\sqrt{(\text{Total dose of gonadotropin})})}}$$

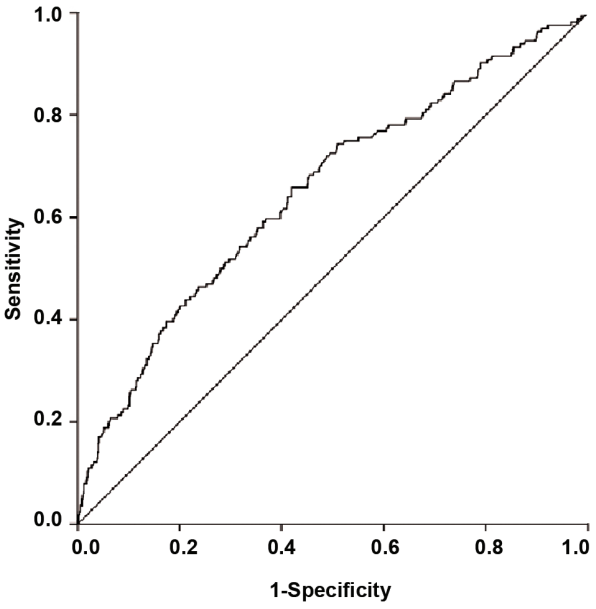

Fig.1: ROC curve for assessment of logistic regression discriminative preformance.
ROC; Receiver operating characteristic.

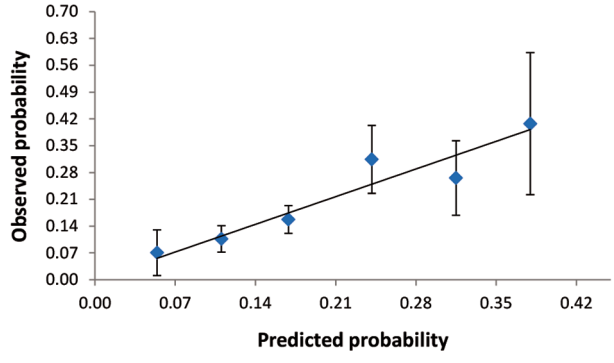

Fig.2: Calibration plot, showing the relationship between predicted and observed rate of pregnancy after intrauterine insemination (IUI).

Discussion

Among the various parameters that were studied, female age, duration of infertility, menstrual irregularities, semen volume, cause of infertility and the dose of gonadotropin significantly affected treatment success.

In the current study, we have shown a statistically significant association between reduced COH+IUI success rate and increased age. Several studies have illustrated the decline in pregnancy with advancing age, (13-15); however, Erdem et al. (15) did not find female age to be a prognostic factor in the prediction of a live birth in ovarian stimulation and IUI cycles. The current study, IUI was offered for women over the age of 40 years. According to studies, woman over the age of 40 are not good candidates for IUI (16, 17).

We observed a significant decrease in pregnancy rate with increased duration of infertility (OR: 0.8, CI: 0.8-0.9, P<0.001). This result was also supported by the results observed in a study by Kamath et al. (18). In another study (17), a significantly higher pregnancy rate (14.2%) was observed in couples with the duration of infertility of less than 6 years compared to 6.1% rate for those with the duration of more than 6 years. Merviel et al. (14) did not observe this difference. However, our findings indicate that the duration of infertility must be considered when counselted patients on their chances of a successful pregnancy.

Infertility type (primary or secondary) did not significantly affect the outcome of COH+IUI the result of which has been shown in some studies (14, 15). Our study found a significant effect of the total gonadotropin dose on outcome in pregnancies conceived by COH+IUI (P=0.03). The data were also evaluated to determine the variables which may influence the total dose of gonadotropins. It appears that women with higher age, those with menstrual irregularities and low number of dominant follicles as well as those with shorter duration of infertility should not be given high doses of gonadotropins. I argue with such a strong statement based on the findings presented in table 4. The data presented are simple correlation data. The only time you can make such a statement is when for example you give the same doses of gonadotropins to women of younger and older age and assess the outcome.

A total of 17.5% of the recorded clinical pregnancies after COH+IUI at our center were multiple pregnancies; no case of hyperstimulation was documented during the study period. Other studies have reported an incidence of twins (20%) and higher-order (39%) multiple pregnancies that were the result of ovulation induction (19, 20). Thus, centers should choose appropriate stimulation pro-

tocols and attempt to achieve a balance between the search for advanced success rate and suitable multiple pregnancy rates. The present study, no significant difference was found in endometrial thickness between pregnant women and those who did not become pregnant. This finding is similar to the result of Kamath et al. (18).

The information available at present study indicates that COH+IUI can be considered prior to more expensive IVF in patients that have combined (27.8% per cycle) and ovulatory disorder (20.9% per cycle) infertilities. The success rate was higher for ovulatory cases and for those who suffered from more than one etiological factor. The patients of this group have been diagnosed as the combination of mild male infertility and PCOS. Compared result has been reported regarding the highest success rate in an ovulatory patient (13, 15, 17). The clinical pregnancy rate was significantly higher in patients who had irregular menstruation. All of these patients were diagnosed with PCOS, according to the cause of infertility as discussed, the success rate was higher in an ovulatory patient. When the effect of the infertility etiology was assessed, there was a significantly lower pregnancy rate observed in endometriosis patients compared with women who had unexplained infertility (16). Peterson et al. (21) have found the average pregnancy rate for unexplained infertility to be 18%. Our result showed a 13.8% average pregnancy rate for unexplained infertility; however, Basirat and Esmaeilzadeh (22) determined that the etiology of infertility was not significantly different between pregnant and nonpregnant women (P=0.63).

Predictive sperm parameters for successful IUI have been controversial (23, 24). Total motile count (TMC) is a potential predictive factor for a successful COH+IUI (15). The pregnancy rates according to Kamath et al. (18) were as follows: a significantly higher pregnancy rate (18.2%) was observed when TMC was in the range of 10-20 million. TMC at a range of 5-10 million resulted in a 5.6% pregnancy rate, whereas in cases where TMC was <5 million, the rate was 2.7%. A TMC of <1 million was associated with poor pregnancy rates. When the TMC was <5 million, sperm morphology appeared to play an important role. A pregnancy rate of 18.4% was observed with a normal morphology compared to a rate of <5.4% with <30% morphology (18). In our study, although the sperm analysis data was not recorded after processing, the data prior to sperm processing was available. Sperm parameters did not significantly affect the success of COH+IUI. These results also confirm the findings achieved by other researchers (16, 25). In contrast, other studies have described several semen parameters that correlated with IUI outcome, such as the number of motile sperm (24, 26) and normal morphology (24). In the current study there was a lower pregnancy rate when the TMC was in the range of 10-20 million (9.5%). The pregnancy rate was 17% when the TMC was >20 million. The pregnancy rate increased when there was higher normal morphology (OR=1.02, 95% CI:0.7-1.4, Table 2). Generally, published studies have been inconsistent related to the association between the morphology readings and the success in IUI (21, 22).

We observed four parameters that significantly affected success: duration of infertility, menstrual irregularities, seminal volume and total dose of gonadotropin. In a previous study there were four prognostic factors: etiology and duration of infertility, number of treatment cycles, and number of pre ovulatory follicles (16). Kamath et al. (18). found a significant effect of the duration of infertility and TMC on outcome of pregnancies conceived by IUI.

Our study was a retrospective, no documents were available about the number of follicles and there was a limitation to our study. On the other hand, we did not have information about semen quality after processing and it was another ristriction.

Conclusion

With the data obtained, we were able to construct a formula for to calculate the probability of pregnancy following COH+IUI as carried out in the present study. These results suggested that female age, duration of infertility, cause of infertility, menstrual irregularities, ejaculatory volume and total dose of gonadotropin be the most important prognostic factors in predicting successful outcome of IUI. A larger study population might assist with the formulation of a better predictive model for IUI success. Such information could be used by couples and clinicians during counseling participants to arrive at a decision with regards to their treatment options.

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Original Article

Evaluation of Some Plasma Coagulation Factors in Women with Spontaneous Miscarriage

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Abstract

Background: It has been reported that 15-20% of parous female have experienced at least one miscarriage, while 3% of them have experienced two miscarriages. The goal of this study was to evaluate the plasma level of coagulation factors in women with a history of spontaneous abortions.

Materials and Methods: In this case-control study, 82 women with a history of two or more abortions referred to the six private gynecologic clinics in Gorgan city without any structural abnormality were recruited during 2011-2012. Plasma levels of antithrombin III (ATIII) using colorimetric assay, protein C, protein S, factor V Leiden and lupus anticoagulant (LAC) using coagulation method were measured. The control group was women with a history of normal delivery and no abortions. Those under anti-coagulant therapy were excluded from the study. Data were entered into the computer using the Statistical Package for the Social Sciences (SPSS, SPSS Inc., Chicago, IL, USA) version 16 and analyzed by Chi-square, t test and non-parametric tests.

Results: At least one abnormality was reported in 35 cases (42.7%). Among them, protein C deficiency was the most prevalent (30.5%). ATIII was abnormal in 17.1% and lupus anti-coagulant was abnormal in 8.5%. Factor V Leiden was normal in all cases and protein S deficiency was only seen in one case.

Conclusion: We suggest to perform these tests in regards to the thrombophilia in cases with spontaneous abortions in order to find an early cure for this treatable disorder.

Keywords: Thrombophilia, Abortion, Protein C, Protein S, Factor V Leiden

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Introduction

It has been reported that 15-20% of all parous women have experienced at least one miscarriage, 3% have experienced two miscarriages and finally 1% have experienced three or more miscarriages (1). Recurrent pregnancy loss (RPL) is defined as two or more consecutive pregnancy losses before the 28th week of pregnancy with fetus weighing less than 1000 g. RPL affects 2-4% of reproductive age couples worldwide (2, 3). Several different etiologies have been defined for RPL such as: genetic factors, structural abnormalities, endocrine disorders like thyroid hormone alteration, immunologic factors, infective causes and unexplained

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causes (3).

There are still debates on the role of immunologic factors as the pathogenesis of recurrent miscarriages (1, 4). Several risk factors as definitive or probable causes of RPL have been reported for women with recurrent pregnancy loss, like acquired maternal thrombophilia and inherited thrombophilia that include factor V Leiden deficiency, activated protein C resistance, prothrombin G20210A and protein S deficiency (4, 5).

There are few data about the spontaneous pregnancy loss in Iranian women in regards to thrombophilia markers. Therefore, we conducted this study to evaluate the plasma level of thrombophilia markers in women with a history of two or more abortions.

Materials and Methods

In this case-control study, 100 women with pregnancy loss history were referred to the academic and six private gynecologic clinics in Gorgan city, during 2011-2012. The inclusion criteria were a history of at least two pregnancy losses and no structural abnormality in the genital tract. Those undergoing anti-coagulant therapy were excluded from the study. Considering the inclusion criteria, 82 women were enrolled in our study. This study was confirmed by the Ethics Committee of Golestan University of Medical Sciences, Gorgan, Golestan Province, Iran. After obtaining informed consent from participants, the checklist was filled and they were referred to the Immunohematology Laboratory of Paramedicine Faculty of Golestan University of Medical Sciences. Control group included 86 age-matched women who experienced a normal pregnancy history for less than a year.

Plasma levels of anti-thrombin III (ATIII) was evaluated using colorimetric assay (Stago Kit, Diagnostica Stago, Inc., France). Protein C, protein S, factor V Leiden and lupus anticoagulant (LAC) were also evaluated using coagulation methods (Hyphen Biomed Kit, Amelung coagulometer Instrument, France). All laboratory tests were done in a single center.

The lower limit of the reference interval values for protein C, protein S, ATIII and factor V Leiden are 70, 55, 80 and 1.8%, respectively. Reference interval value for LAC was normal, less than 40 seconds.

Data were entered into the computer using the Statistical Package for the Social Sciences (SPSS, SPSS Inc., Chicago, IL, USA) version 16. Mean values of factors were reported. P value<0.05 was considered significant.

Results

Mean (±SD) age was 28.97 (±5.19) years in case group and 29.18 (±5.93) years in control group. The median pregnancy loss in cases was 2 (±0.85), minimum 2 and maximum 7 abortions. In the control group, the minimum values belonging to protein C, protein S, ATIII and factor V Leiden were 70.1, 60, 80, and 1.9%, respectively, while maximum value for LAC was normal less than 39.8 seconds.

Factor V Leiden was normal (>1.8) in all cases. LAC was abnormal (≥40) in 7 (8.5%), ATIII (<80) in 14 (17.1%), protein S (<55%) in 1 and protein C (<70%) in 25 (30.5%) (Table 1).

In about 35 cases (42.7%), at least one abnormality was reported in the lab tests. No one had all abnormalities at once, but there was a case with triple abnormalities in protein C, ATIII and LAC. One case had both abnormalities in protein C and protein S. In 3 cases, there was an abnormality in both protein C and LAC, and in 8 cases, protein C and ATIII were abnormal at once.

Table 1: Comparison of mean values of five thrombophilia factors between case and control groups

Factors	Case	Control	P value
LAC (s)	34.1 ± 14.7	32.0 ± 6.5	0.323
ATIII (%)	111.4 ± 52.1	123.1 ± 52.3	0.152
Factor V Leiden (Ratio)	2.8 ± 0.7	3.0 ± 0.8	0.231
Protein C (%)	90.4 ± 38.6	119.6 ± 35.7	<0.001
Protein S (%)	105.2 ± 23.7	102.0 ± 20.2	0.353

All data were presented as mean ± standard deviation (SD). LAC; Lupus anticoagulant and ATIII; Anti-thrombin III.

Discussion

Abnormality in protein C has been reported in a relatively high percent of the cases with a history

of abortion in the present study. Abnormal Factor V Leiden and protein S were not seen almost at all, but in other studies, they were reported to have a high prevalence.

D'Uva et al. (6) from Italy reported thrombophilia in 78% of cases with history of recurrent abortion and 16% of controls. They reported that abnormal ATIII was not seen in their population. Furthermore their findings showed that Factor V Leiden heterozygosity was present in 5.2%, protein C deficiency in 1.7% and protein S deficiency in 13%, indicating that protein C and protein S levels are lower and higher, respectively, than what we found in our study group.

Bellver et al. (5) from Spain designed a study on 4 groups including control group, without a history of abortion; unexplained infertility (UI) group, a history of fertility more than one year without any autoimmune endocrine diseases; *in vitro* fertilization (IVF) group; normal Caucasian women group aged under 38 years and with unsuccessful IVF; and recurrent abortion group. Combined thrombophilia (abnormality in all five factors) was seen in 3 (9.4%) of controls, 3 of UI groups (9.7%), 5 in IVF group (19.2%) and 2 of recurrent abortions (6.7%) . However, in the present, there was no case of combined abnormality.

Mitic et al. (7) reported at least one congenital thrombophilia alteration in 54 (36.7%) women with a history of abortion, while protein S deficiency was the most prevalent one among them. Our results showed that protein S deficiency is present in just one case and protein C deficiency is the most prevalent one. Our finding indicated that alteration in at least one factor was reported in 35 cases (42.7%).

Jyotsna et al. (8) from India reported a significantly lower protein C, protein S and ATIII in cases with a history of abortion. The level of protein C was lower than normal in 33.3% of their cases. Their findings showed that levels of protein C, protein S and ATIII were lower as compared to related values of our findings, while the percentage of abnormal tests are more than the present study. In a study by Saadati et al. (9) in Iran, 3 cases (8.4%) and no control had LAC positive results.

In a study performed in Pakistan, opposite results have been reported. Their study included 52 women with a history of recurrent miscarriage and 268 healthy controls. The values of protein factors C (5.7 in patients vs. 6.7% in the control group), protein S (3.8 in patients vs. 4.5% in the control group) and Leiden factor (19.2 in patients vs. 10.0% in the control group) were significantly different between patient and control groups. However, antithrombin deficiency in the control group was significantly greater than the patient group (1.9 in patients vs. 15.2 % in the control group) (10), suggesting that there may be a problem in their selection of subjects recruited into the control group.

Conclusion

We suggested to perform these tests, specially protein C, in regards to the thrombophilia in cases with spontaneous abortions in order to find an early cure for this treatable disorder.

Acknowledgements

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Original Article

Relationship between Serum Leptin, Ghrelin and Dietary Macronutrients in Women with Polycystic Ovary Syndrome

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Abstract

Background: Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women. It may involve an impairment in physiologic regulation of leptin and ghrelin. There is limited, controversial data on the relation of dietary components with leptin and ghrelin in PCOS, so the current study has been conducted to explore the effects of different macronutrients on serum levels of leptin and ghrelin in PCOS and healthy subjects.

Materials and Methods: In this case-control study, we randomly choose 30 PCOS patients and 30 healthy age and body mass index (BMI) matched controls. Intake of macronutrients [protein, total fat, saturated, monounsaturated and polyunsaturated fatty acids (PUFA), carbohydrate, dietary fiber] and energy were assessed using 3-day, 24-hour food recall and food frequency questionnaires (FFQ). Fasting hormonal status was measured for each participant.

Results: PCOS women had higher levels of serum leptin, insulin, testosterone, and luteinizing hormone (LH), whereas sex hormone-binding globulin (SHBG) was lower compared to healthy women. There was no significant difference in mean ghrelin concentrations between the groups. Among PCOS women, independent of BMI and total energy intake, we observed an inverse association between leptin concentration and total dietary fat (β=-0.16, $P<0.05$) and saturated fatty acid (SFA) intake (β=-0.58, $P<0.05$). This relationship was not seen in the healthy subjects. There was no significant association between ghrelin and macronutrients in PCOS and healthy participants.

Conclusion: Certain habitual dietary components such as fat and SFA may decrease serum leptin, whereas ghrelin is not influenced by these in PCOS women. More studies are needed to better clarify the effects of dietary macronutrients on serum leptin and ghrelin.

Keywords: Leptin, Gherkin, Habitual Diet, Polycystic Ovary Syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous collection of signs and symptoms that form a spectrum of a mild disorder in some but a severe disturbance of reproductive endocrine and metabolic functions in others. PCOS is diagnosed by amenorrhea/oligomenorrhea, clinical or biochemical signs of hyperandrogenism and/or polycystic ovaries. It is one of the most common endocrinopathies. The prevalence of PCOS has risen substantially, from 6-8% to 12-20%, as a result of the adoption of the Rotterdam criteria for diagnosis. This criteria has introduced the following different phenotypes: classic (characterized by hyperandrogenism and oligoanovulation, with or without PCO morphology, and corresponding to the previous National Institutes of Health definition), ovulatory (hyperandrogenism and PCO), and normoandrogenic (oligoanovulation and PCO) (1-4).

Leptin, a 167-aminoacid peptide hormone, is a product of the human obese (*OB*) gene. It is related to the circulatory system by adipose tissue as a function of energy stores (5). Leptin has a number of important effects on eating behavior, energy expenditure and body weight (6, 7). Studies have indicated leptin's role in reproduction and its involvement in the regulation of gonadotropin-releasing hormone (GnRH), follicle stimulating hormone (FSH), luteinizing hormone (LH), adrenocorticotropic hormone (ACTH), cortisol, and growth hormone (GH) concentrations. Leptin plays a major role in interactions that take place between nutritional status of the body and the hypothalamic-pituitary-ovarian axis (8-10).While data in other groups is high, there is relatively little information about the influence of specific dietary factors on circulating leptin concentrations among PCOS women. A study has shown that reduced carbohydrate intake rather than reduced fat or protein intake lowered serum leptin in obese subjects (11). Total fat and polyunsaturated fatty acid (PUFA) intakes had a positive association with plasma leptin level in American men with normal weight (12). Reduction of 24-hour circulating leptin concentrations in women by the consumption of high-fat meals was reported (13). Intake of dietary fiber showed an inverse association with serum leptin in a group of young Japanese women (14). Leptin had a positive relation with saturated fatty acids (SFA) in healthy women (15). Another study reported a negative association of leptin with energy intake from carbohydrates and a positive association with energy from dietary fat in healthy subjects (16).

Ghrelin is an acylated 28 amino acid peptide originally isolated from the rat stomach and later found in the intestines, pancreas, testes, and ovaries. Ghrelin enhances the appetite, stimulates food intake, and reduces fat utilization. It is involved in ovarian function. Its low levels are probably associated with insulin resistance and positive energy balance or obesity (17-22). Studies have shown impaired homeostasis of ghrelin in PCOS patients (22, 23). This situation may disturb the normal relation of different dietary factors and serum ghrelin levels, resulting in an altered food intake pattern and energy homeostasis. Studies on the effect of various dietary factors on fasting ghrelin levels in PCOS patients are limited and have conflicting results. A recent study on PCOS women has shown that protein intake suppressed postprandial ghrelin significantly longer compared to glucose (24). Another study on obese and overweight postmenopausal women with elevated serum insulin levels showed a negative association between dietary fat and carbohydrates with leptin. This association was positive with ghrelin (25).

With attention to the scarcity of data and conflicting results of studies on PCOS women, the objectives of this study were to examine the relationship between habitual dietary components (protein, total fat, saturated, monounsaturated and PUFA, carbohydrates and dietary fiber) with these peptides in PCOS women compared to healthy women.

Materials and Methods

Study participants

This case-control study enrolled 30 PCOS patients and 30 healthy age and body mass index (BMI) matched controls at Alzahra Hospital, Tabriz, Iran, from 2009 to 2010. The study participants were selected from an outpatient setting in the Gynecology and Obstetrics Ward. Participants were selected by the accidental (convenience) sampling method.

After being informed on the purpose and procedures of the study, all subjects signed an informed

consent. Diagnosis of PCOS was made by a gynecologist using Rotterdam criteria (4). The study protocol was approved by the Ethics Committee at Tabriz University of Medical Sciences. Health status of the control group women were determined by medical history, physical and pelvic examinations and complete blood test. Control group participants had no signs of PCOS such as hyperandrogenism, menstrual dysfunction or polycystic ovaries.

Exclusion criteria for all subjects included pregnancy, hypothyroidism, hyperprolactinemia, Cushing's syndrome, congenital adrenal hyperplasia, current or previous (within the last 3 months) use of oral contraceptives, glucocorticoids, anti-androgens, ovulation induction agents, anti-diabetic and anti-obesity drugs or other hormonal drugs.

Dietary intake and anthropometry

Dietary intake and habits were assessed using 3-day 24-hour food recall and food frequency (FFQ) questionnaires. Participants were asked to recall the type and amount of food and beverage consumed using standard household measures (cups, tablespoons, etc.). The interviewers reviewed the questionnaire entries with the subject in order to clarify servings, recipes and forgotten foods. Food intake data obtained from the PCOS and healthy control groups were analyzed for protein, total fat, SFA, monounsaturated fatty acids (MUFA), PUFA, carbohydrates, dietary fiber and energy using nutritionist III diet analysis software.

In each woman we measured weight and height to calculate BMI. Body height was measured to the nearest 0.1 cm with the subject standing without shoes. Body weight in light indoor clothing was measured to the nearest 0.1 kg. The BMI was calculated using the standard formula of weight (kg)/height (m^2).

Biochemical assays

After undergoing a history and physical examination, venous blood sampling was performed for the hormonal assays. Blood samples were taken in the morning at 09:00 hours after a 12-hour overnight fast. The serum was separated and frozen at -70˚C until assayed.

The analyses were carried out during the early follicular phase in women who had menstrual cycles and in any phase of the cycle in PCOS patients. Serum leptin levels were measured using the Human Leptin ELISA kit (BioVendor GmbH, Im Neuenheimer Feld 583, D-69120 Heidelberg, Germany). In all subjects we measured plasma immunoreactive ghrelin levels with a commercially available radioimmunoassay that uses ^{125}I-labeled bioactive ghrelin as a tracer and a rabbit polyclonal antibody raised against full-length octanoylated human ghrelin (Phoenix Parmaceuticals Inc., Belmont, CA, USA) that recognizes both acylated and des-acylated ghrelin.

Levels of serum LH and FSH were determined by the direct immunoenzymatic method [DiaMetra Srl, Folingo (PG) Italy]. The measurement of serum sex hormone-binding globulin (SHBG) was performed using an enzyme-linked immunosorbent assay (ELISA) kit (IBL Immuno-Biological Laboratories, Flughafenstrasse52A, D-22335, Hamburg, Germany). Total testosterone levels were determined using a commercially available ELISA kit (Monobind Inc., Lake Forest, CA, USA).

Statistical analyses

Results are expressed as mean ± SD. Comparisons between two groups were made using the independent samples t test. Pearson correlation analyses were performed to define correlations between parameters. Simple regression modeling was applied to analyze the impact of independent variables on dependent variables. Statistical evaluations were performed by running the SPSS/PC software package (SPSS, Inc., Chicago, IL, USA). P values of less than 0.05 were regarded as statistically significant.

Results

Anthropometric and biochemical data

Baseline characteristic of the study groups are presented in table 1. We observed no significant difference in BMI between the two study groups. Analysis of biochemical data showed significantly higher mean leptin concentrations in PCOS women compared to healthy subjects. There was no significant difference in mean ghrelin concentrations between the two groups.

Analysis of dietary variables revealed dietary energy intake was lower in the PCOS group (1334.9

± 143.4 kcal/d) compared to controls (1716.1 ± 142.07 kcal/d, P=0.007), but the relative macronutrient contribution to the daily energy intake did not differ between groups (Table 1).

Table 1: Anthropometric and biochemical data of study participants

	PCOS (n=30)	Controls (n=30)
Age (Y)	25.83 ± 4.00	26.06 ± 4.44
Height (cm)	160.1 ± 6.01	162.4 ± 6.52
Weight (kg)	64.4 ± 10.46	62.4 ± 8.82
BMI (kg/m^2)	25.00 ± 3.61	23.68 ± 3.07
Leptin (ng/ml)	21.68 ± 4.49**	17.96 ± 0.54
Ghrelin (pmol/l)	210.33 ± 58.50	216.00 ± 80.84
Insulin (mU/l)	14.91 ± 1.78**	7.90 ± 1.16
Total testosterone (ng/ml)	0.75 ± 0.60*	0.45 ± 0.26
SHBG (ng/ml)	31.81 ± 14.29*	52.34 ± 23.41
LH (mIU/ml)	12.50 ± 2.33**	4.86 ± 2.12
FSH (mIU/ml)	6.03 ± 1.64	5.74 ± 1.10
Nutrient intake		
Energy intake (kcal/d)	1334.9 ± 143.4*	1716.1 ± 142.07
Carbohydrate (g/d)	171.6 ± 9.3	222.57 ± 20.4
Fat (g/d)	50.72 ± 2.72*	65.73 ± 6.24
Protein (g/d)	49.9 ± 2.43*	67.28 ± 5.89
Total dietary fiber (g/d)	6.0 ± 1.0	6.7 ± 0.6
Energy from carbohydrates (%)	51.13 ± 8.86	51.76 ± 8.67
Energy from fat (%)	34.06 ± 8.41	32.86 ± 6.55
Energy from protein (%)	14.8 ± 3.01	15.36 ± 3.45

Data are presented as means ± SD. BMI; Body mass index, FSH; Follicle stimulating hormone, LH; Luteinizing hormone, PCOS; Polycystic ovary syndrome, SHBG; Sex hormone-binding globulin, *P<0.05 with unpaired t test and **P<0.001 with unpaired t test.

Correlation of habitual dietary intake with serum leptin and ghrelin levels

We observed a positive, significant association between leptin and BMI in the PCOS and control groups (Table 2). The PCOS group had a significant negative correlation between total dietary fat, MUFA and SFA and leptin concentration. However no significant correlation was found between dietary intakes and leptin levels in the control group. There was no significant association observed for ghrelin with dietary variables in either group.

The findings from bivariate correlation analyses were further examined using linear regression analysis in order to control for potential confounding factors. The unadjusted model (model 1), energy adjusted model (model 2) and fully adjusted model (model 3) for the association between main macronutrients (carbohydrate, protein, fat) and leptin, and fully adjusted model for the association between macronutrient subtypes (total fiber, water soluble and insoluble fiber, PUFA, MUFA, SFA) and leptin are presented. In the PCOS group, there was a statistically inverse association between fat intake and leptin (β=-0.43, P=0.02) which remained significant after adjustments for BMI and total energy intake (β=-0.16, P=0.04). This suggested that for every one gram increase in fat intake, there was a 0.226 ng/ml decrease in leptin among PCOS women who were similar in energy intake and BMI. For other macronutrients and macronutrients subtypes, there was a significant inverse association between SFA intake and serum leptin levels after adjusting for BMI and energy intake (β=-0.579, P=0.036). In healthy subjects, we did not observe a significant association between nutrients and leptin concentration (Table 3).

Ghrelin relationships with dietary factors are shown in table 4. We used three models to assess the ghrelin relationship with dietary factors. Model 1 presents unadjusted, model 2 shows the adjusted model (controlling for energy intake), and model 3 shows the adjusted model (controlling for both energy intake and BMI). Overall, there were no statistically significant associations between ghrelin and macronutrients or macronutrients subtypes in the two study groups. The association between carbohydrate intake and serum ghrelin was near to significant (β=-0.7, P<0.1).

Table 2: Pearson correlation tests of dietary variables with leptin and ghrelin concentrations

	PCOS (n=30)		Controls (n=30)	
	Leptin	**Ghrelin**	**Leptin**	**Ghrelin**
Age (Y)	0.05	0.49	0.11	0.05
Weight (kg)	0.74**	-0.24	0.80**	-0.24
BMI (kg/m^2)	0.84**	-0.04	0.93**	-0.22
Total energy intake (kcal/d)	0.09	-0.17	0.10	-0.01
Carbohydrate (g/d)	0.18	-0.10	-0.03	0.08
Protein (g/d)	-0.002	-0.27	0.21	-0.04
Fat (g/d)	-0.43*	-0.05	0.18	-0.11
Total fiber (g/d)	0.17	-0.10	0.23	-0.16
Water soluble fiber (g/d)	0.17	-0.22	0.20	-0.24
Insoluble fiber (g/d)	0.13	-0.05	0.21	-0.08
PUFA (g/d)	-0.08	-0.13	0.12	-0.10
MUFA (g/d)	-0.37*	0.01	0.23	-0.16
SFA (g/d)	-0.54*	-0.40	0.15	-0.11

BMI; Body mass index, MUFA; Monounsaturated fatty acids, PCOS; Polycystic ovary syndrome, PUFA; Polyunsaturated fatty acids, SFA; Saturated fatty acids, *; P<0.05 with correlation test and **; P<0.001 with correlation test.

Table 3: Relationship between leptin concentration to macronutrient and macronutrient subtypes in study groups according to regression analysis

		PCOS (n=30)			Control (n=30)	
	Model	**β**	**P value**	**Model**	**β**	**P value**
CHO (g/d)	1[a]	0.014	0.947	1[a]	-0.135	0.522
	2[b]	-0.219	0.522	2[b]	0.312	0.573
	3[c]	0.158	0.195	3[c]	0.073	0.382
Fat (g/d)	1[a]	-0.431	0.02	1[a]	-0.18	0.362
	2[b]	-0.556	0.02	2[b]	0.360	0.270
	3[c]	-0.160	0.04	3[c]	-0.130	0.504
Protein (g/d)	1[a]	0.096	0.657	1[a]	0.268	0.501
	2[b]	0.084	0.699	2[b]	0.410	0.344
	3[c]	-0.014	0.907	3[c]	0.037	0.807
Macronutrient subtypes						
Total fiber (g/d)	3[c]	0.169	0.373	3[c]	0.235	0.211
Water-soluble fiber (g/d)	3[c]	0.177	0.355	3[c]	0.126	0.588
Insoluble fiber (g/d)	3[c]	0.133	0.484	3[c]	0.140	0.548
PUFA (g/d)	3[c]	0.143	0.555	3[c]	-0.149	0.647
MUFA (g/d)	3[c]	-0.042	0.902	3[c]	0.500	0.367
SFA (g/d)	3[c]	-0.579	0.036	3[c]	-0.165	0.807

[a]; Unadjusted model, [b]; Energy-adjusted model, [c]; Energy- and body mass index (BMI) adjusted model, MUFA; Monounsaturated fatty acids, PCOS; Polycystic ovary syndrome, PUFA; Polyunsaturated fatty acids and SFA; Saturated fatty acids.

Table 4: Relationship between ghrelin concentration to macronutrient and macronutrient subtypes in the study groups according to regression analysis

	PCOS (n=30)			Control (n=30)		
	Model	**β**	**P value**	**Model**	**β**	**P value**
Carbohydrate (g/d)	1[a]	0.040	0.881	1[a]	0.119	0.574
	2[b]	-0.589	0.085	2[b]	0.057	0.920
	3[c]	-0.701	0.095	3[c]	0.190	0.737
Fat (g/d)	1[a]	0.031	0.885	1[a]	-0.342	0.396
	2[b]	-0.259	0.266	2[b]	-0.401	0.534
	3[c]	-0.024	0.917	3[c]	-0.185	0.776
Protein (g/d)	1[a]	-0.297	0.205	1[a]	0.211	0.599
	2[b]	-0.329	0.127	2[b]	0.191	0.665
	3[c]	-0.276	0.246	3[c]	0.324	0.469
Macronutrient subtypes						
Total fiber (g/d)	3[c]	-0.101	0.596	3[c]	-0.159	0.400
Water-soluble fiber (g/d)	3[c]	-0.218	0.246	3[c]	-0.240	0.201
Insoluble fiber (g/d)	3[c]	-0.049	0.791	3[c]	-0.420	0.672
PUFA (g/d)	3[c]	-0.318	0.265	3[c]	0.074	0.813
MUFA (g/d)	3[c]	0.374	0.345	3[c]	-0.315	0.504
SFA (g/d)	3[c]	-0.217	0.479	3[c]	0.117	0.756

[a]; Unadjusted model, [b]; Energy-adjusted model, [c]; Energy- and body mass index (BMI) adjusted model, MUFA; Monounsaturated fatty acids, PCOS; Polycystic ovary syndrome, PUFA; Polyunsaturated fatty acids and SFA; Saturated fatty acids.

Discussion

We took into consideration the metabolic and endocrine importance of leptin and ghrelin in PCOS patients and investigated the dietary predictors of these hormones in this study. The results indicated a significant, inverse association of habitual dietary fat and SFA with leptin concentrations in PCOS patients after adjustment for total energy intake and BMI. In healthy individuals we observed no significant association between dietary intakes and serum leptin concentration. Ghrelin was not influenced by habitual dietary components.

Studies on the association of dietary factors with leptin concentration were conducted on healthy, obese or other than PCOS subjects, with contradictory results.

Havel et al. (13) in a study on healthy and normal weight women showed that high fat meals reduced circulating leptin concentrations which supported the results of the current study. In another study by Larsson et al. (26) on 64 healthy postmenopausal women, the authors reported that leptin levels negatively correlated with total fat (r=-0.36, P=0.004) as well as SFA (r=-0.31, P=0.014).

Kong et al. (25) found that higher habitual intake of dietary fat was associated with lower leptin

in overweight and obese postmenopausal women. They observed an inverse association between leptin concentration and percentage of energy from carbohydrates.

In contrast to these studies, other studies failed to show any association between dietary fat and its subtypes with serum leptin levels (11, 14, 27). Numerous studies showed a positive correlation between serum leptin and dietary fat intake (12, 15, 16). Others showed a different association between serum leptin and dietary fat in men and women (28, 29). However, these studies were all conducted healthy subjects or other than PCOS patients.

The difference among PCOS women and healthy controls in term of leptin and its association with habitual dietary fat and SFA might be explained by varying hormone levels such as testosterone and particularly insulin. In our study, there were higher testosterone and insulin levels observed in PCOS women compared to the control group.

Androgen excess plays an important role in the development of PCOS (30). A number of studies have shown the role of fat and fatty acids in androgen synthesis (31, 32). Insulin modifies the hormone - nutrient associations and has a potential impact in the regulation of both leptin and ghrelin (33, 34). Although inconsistent, a stimulating effect of insulin on *OB* gene expression has been reported in several studies (35, 36). Another factor is plasma free fatty acids. Plasma leptin is sensitive to free fatty acids. An *in vitro* study has shown that free fatty acids inhibit leptin transcription in adipocytes (37). Donahoo et al. (38) found that increasing free fatty acids in humans led to a decreased leptin concentration. The reduction of leptin secretion after high fat meals might be attributed to decreased insulin release, which resulted in lowered glucose metabolism in adipose tissue. The consumption of high fat meals which consequently decrease total leptin, could demonstrate the adipogenic effect of diets high in fats (13, 39).

The present study found no significant association between ghrelin concentration and habitual dietary macronutrients in PCOS and healthy controls. Similar results were obtained in a study on fasting and postprandial ghrelin levels in PCOS women (40). In contrast to our study, Kong et al. (25) studied overweight and obese postmenopausal women. They reported that habitual dietary intake of carbohydrate and fat were associated with higher ghrelin concentrations. Barber et al. (41) showed that ghrelin levels were suppressed by oral glucose in women with PCOS. Kasim-Karakas et al. (24) reported that in PCOS patients protein intake suppressed ghrelin longer than glucose.

In our study the negative association between carbohydrate intake and serum ghrelin was near to significant. Ghrelin fluctuations have been shown to inversely correlate with those of insulin, when insulin concentrations rise, ghrelin concentrations fall (42-44). It is generally accepted that carbohydrate is the most effective macronutrient for ghrelin suppression, because of its rapid absorption and insulin secreting effect. Protein induces a prolonged effect and fat exhibits weak ghrelin suppressing capacity (45).

Our study had some limitations. First, was the use of self-reported nutrient intake which has the potential for tremendous recall bias and under-reporting. This was likely the case for the PCOS group that had a significantly lower total energy intake. In addition, single fasting hormone measures were more limited than a 24 hour profile or hormone response to given macronutrient test meals. The sample size of our study might be considered too low. Blood samples from PCOS women were collected at any time, hence it was not possible to determine which cycle stage they were at.

Conclusion

The results of the current study showed that in PCOS patients some components of habitual dietary intake such as fat and SFA were inversely associated with serum leptin concentrations, independent of BMI and total energy intake. There was no significant association between habitual dietary intake of macronutrients or their subtypes and serum ghrelin concentrations.

Based on these findings, limiting food stuffs high in fat (especially SFA) would be beneficial in appropriate control and management of either the metabolic or endocrine status of PCOS patients.

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Original Article

Flow Cytometric DNA Analysis and Histopathologic Re-Evaluation of Paraffin Embedded Samples from Hydatidiform Moles and Hydropic Abortions

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Abstract

Background: Distinction of hydatidiform moles (HMs) from non-molar abortions and sub-classification of HMs are important for clinical practice; yet, diagnosis based solely on morphology is affected by interobserver variability. The objective of this study was to determine the role of DNA flow cytometry in distinguishing molar from non-molar pregnancies.

Materials and Methods: This retrospective study was conducted at the Department of Pathology, Women's Hospital, Tehran University of Medical Sciences, Tehran, Iran, between 2006 and 2010. DNA ploidy analysis and histopathologic re-evaluation were performed on paraffin-embedded tissue from 36 (17 complete and 19 partial) molar and 24 hydropic abortus (HA) cases which were previously diagnosed based on histomorphologic study.

Results: Of the 17 cases initially diagnosed as complete HM (CHM), 9 were diploid, 2 were triploid, 5 were tetraploid and 1 was aneuploid. Of the 19 initial partial HMs (PHMs), 2, 8, 1 and 8 cases were diploid, triploid, tetraploid and aneuploid, respectively. In the initial HA category (n=24), 14 diploid, 1 triploid, 5 tetraploid, and 4 aneuploid cases existed. Following flow cytometry and histopathologic re-evaluation, 1 case with previous diagnosis of HA was reclassified as PHM, 2 initial PHMs were reclassified as CHM and 2 initial CHMs were categorized as PHM.

Conclusion: The results show that correct diagnosis of PMH is the main challenge in histological diagnosis of gestational trophoblastic disease (GTD). DNA flow cytometric analysis could be an informative supplement to the histological interpretation of molar and hydropic placentas.

Keywords: Partial Hydatidiform Mole, Complete Hydatidiform Mole, Hydropic Abortion, Flow Cytometry

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Introduction

Hydatidiform mole (HM) is a complication of gestation observed in approximately 0.5-1/1000 pregnancies in the western world and most prevalent in South-East Asia with rates ranging from 1-2/1000 pregnancies in Japan and China to 12/1000 pregnancies in Indonesia, India and Turkey (1-3). HM is classified as either complete or partial based on morphological, histopathological, and cytogenetic studies (4). Genetically, complete HM (CHM) has diploid 46XX karyotype. Although chromosomes are entirely of paternal origin, mitochondrial DNA is of maternal origin. By contrast, partial HM (PHM) has diandric monogynic triploid karyotype (69 chromosomes), which is the "gold standard" for the ultimate diagnosis (4-6). Most reports on the pathological evaluation of early abortions have focused on differentiating between PHM and CHM and between PHM and non-molar hydropic abortus (HA). It is of importance to distinguish these entities because PHMs and CHMs known as risk factors for developing an aggressive clinical and biological behavior, whereas HAs don't. Persistent gestational trophoblastic disease (GTD) is predicted to occur in 10-30% of CHMs, but only in 1-7% of PHMs (7, 8) have been identified.

In many cases, distinction between HM and HA can be made based on only morphological examination. However, absence of sufficient published standard morphological criteria, presence of atypical cases and early evacuation of molar pregnancy cause major difficulties in the histopathological diagnosis of HM with significant interobserver and intraobserver variability (9, 10). To help differentiate between partial and CHMs, some ancillary techniques such as genotyping, DNA ploidy analysis and p57 immunohistochemistry have been developed and used. However, their application and interpretation are not without drawbacks. For example, p57 is the gene product of the paternally imprinted, maternally expressed gene, while immunostaining of p57 is helpful in confirming the diagnosis of CHM, but this useful marker cannot differentiate between PHM and other non-molar gestations (4, 11). Several studies have recently applied this commercially available and cost-effective molecular genotyping method in cases of molar gestations to identify and compare the parental genetic contribution in the chorionic villi and in the maternal decidua. Flow cytometry is widely accepted as a rapid and easy test for ploidy evaluation with ability to analyze a large number (10,000-20,000) of random nuclei and with sufficient sensitivity to distinguish diploidy from triploidy, tetraploidy and non-tri/tetraploid aneuploidy (4, 5, 7, 8, 12, 13). However, ploidy analysis cannot differentiate between non-molar digynic triploid gestations and PHM. Correlation between histology and ploidy improves diagnostic accuracy and concordance among pathologists.

In the present study, previously diagnosed samples from molar and non-molar aborted pregnancies were histopathologically reviewed and analyzed by flow cytometry. The correlation between histological diagnosis and ploidy status was then evaluated.

Materials and Methods

We carried out a retrospective study of 60 selected specimens from all aborted conceptions at the Department of Pathology, Women's Hospital, Tehran University of Medical Sciences, Tehran, Iran, between 2006 and 2010. Present study was a retrospective research, so we were not able to obtain a written informed consent from the participant. The research protocol was approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran. Specimens were obtained from spontaneous abortions or from curettages carried out after detection of intrauterine death or HM by ultrasound examination. A 4-µm section of all specimens was stained using hematoxylin and eosin (H&E) stain. Number of slides ranged from 1 to 10 (average of 3 slides per case). All of the slides were reviewed regarding the histological criteria described by Paradinas et al. (14). Diagnosis of CHM was made by microscopic finding of enlarged edematous villi, prominent central cistern formation, and moderate to marked circumferential trophoblastic hyperplasia, often

with cytologic atypia. Diagnosis of PHM was made based on presence of dual population of villi (large, irregular, and hydropic villi as well as small and fibrotic villi), cistern formation in some enlarged villi, markedly irregular villi with scalloped borders, trophoblastic hyperplasia and presence of inner cell mass (15).

Flow cytometric DNA analysis was performed on formalin-fixed, paraffin-embedded tissue blocks. The selection criterion was the presence of both placental and maternal (decidual) tissue in such amount that DNA histograms could be obtained. For each case, a tissue block containing at least 90% chorionic villi and no more than 10% decidua and blood was selected. Maternal decidual tissue had to be present as the internal diploid control. Sixty blocks were analyzed. The technique of Hedley was used for DNA analysis. Briefly, 30 µm-sections were cut from each case and then deparaffinized with xylene and rehydrated. Sections were subsequently incubated at 37˚C for 45 minutes with 0.05% pepsin in normal saline to disaggregate the tissue and yield the nuclei. The cell suspension was filtered through a 50-µm steel mesh and centrifuged at 1500 rpm for 5 minutes. The pellet was washed and resuspended in propidium iodide solution (Sigma, USA). After 30 minutes of incubation at room temperature, the processed tissue was analyzed by flow cytometry (Coulter Electronics, Hialeah, FL, UK). Fluorescence intensity measured on a linear axis was regarded as proportional to the DNA content of individual cells, 10,000 of which were analyzed to produce each histogram.

Cellular DNA content was determined with an Epics C flow cytometer (Coulter Electronics, Hialeah, FL, UK). Histograms of 10,000 cells were recorded and analyzed. Placenta was classified as tetraploid if the peak in the G2/M region represented greater than 25% of the cells and the DNA index (DI) was between 1.90 and 2.10. Furthermore with DI between 1.40 and 1.60, placenta was considered to be triploid. In this study, we use the simple qualifier "aneuploid" to define cases with non-tri/tetraploid aneuploidy (7, 16). The gathered data was analyzed by Statistical Package for Social Sciences version 18.0 (SPSS, SPSS Inc., Chicago, IL, USA) software.

Results

Patients' age ranged from 15 to 50 years (mean: 29.6 ± 2.3), while gestational age ranged from 6 to 24 weeks (mean: 9.5 ± 2.1). Based on histopathological review of the sections, diagnoses were made as CHM in 17, PHM in 19 and HA in 24 cases. DNA ploidy analysis in the 60 cases showed 25 diploid, 11 triploid, 11 tetraploid and 13 aneuploid (non tri/tetraploid) cases. Of the 17 cases histologically diagnosed as CHM, 9 were diploid, 2 triploid, 5 tetraploid and 1 aneuploid according to the yielded histograms. Two diploid, 8 triploid, 1 tetraploid and 8 aneuploid histograms were produced by the 19 histologically-diagnosed PHM cases. At last, of the 24 cases with histological diagnosis as HA, 14, 1, 5 and 4 cases yielded diploid, triploid, tetraploid and aneuploid histograms, respectively. After the release of flow cytometric results, all of the slides from cases in which the primary histological diagnosis did not match the flow cytometry analysis result in terms of expected ploidy were reviewed. Finally, according to both flow cytometric analysis results and histological criteria, out of total 60 cases, 17 cases were diagnosed as CHM (15 cases from the initial histopathology-based diagnostic category of CHM and 2 cases from the initial diagnostic category of PHM). Twenty cases were finally diagnosed as PHM (17, 2, and 1 cases from the initial diagnostic categories of PHM, CHM and HA, respectively). Twenty-three out of the 24 cases with preliminary diagnosis of HA were confirmed after incorporation of flow cytometric data into the diagnostic criteria. Comparing the initial histomorphology-based diagnoses with the results obtained from flow cytometric analysis, five cases were found to have discordant results. In these cases, the original H&E stained sections and clinical data were reviewed along with the ploidy status. Finally, the histological diagnosis was revised in all of the 5 cases. A summary of the results is presented in table 1.

Table 1: Summary of the results of study

Initial histo-morphological Diagnostic categories	Flow cytomery result		Definite diagnosis considering flow cytometry result and histopathological re-evaluation			
	Ploidy	**No. of cases**	**CHM n=17**	**PMH n=20**	**HA n=23**	**No definite diagnosis**
CHM, n=17	Diploid	9	9	-	-	-
	Triploid	2	-	2*	-	-
	Tetraploid	5	5	-	-	-
	Aneuploid	1	1	-	-	-
PHM, n=19	Diploid	2	2*	-	-	-
	Triploid	8	-	8	-	-
	Tetraploid	1	-	-	-	1
	Aneuploid	8	-	-	-	8
HA, n=24	Diploid	14	-	-	14	-
	Triploid	1	-	1**	-	-
	Tetraploid	5	-	-	5	-
	Aneuploid	4	-	-	4	-

CHM; Complete hydatidiform mole, PHM; Partial hydatidiform mole, HA; Hydropic abortion, *; The number of cases with discordant initial and definite results are underlined and **; Case of tubal pregnancy.

Discussion

It is well known that CHM, PHM and HA represent three independent conditions in terms of etiology, pathology, genetic characteristics, morphology and clinical aspects. In most cases, the diagnosis is straightforward (17). Biological variability and scarcity of available tissue, however, will sometimes cause difficulties in clinical and morphological differentiation between different pathologies, mainly between CHM versus PHM or PHM versus HA (18). No single criterion is enough to make this distinction. Therefore, methods that evaluate the ploidy (such as karyotyping as well as flow and image cytometry) have been used to distinguish HM from HA (19). Flow cytometry permits evaluation of cellular DNA content and is employed to determine the ploidy status of different lesions. In this regard, a significant further advantage has been developed as a technique for extracting DNA from formalin fixed-tissue (20). When it comes to molar pregnancies, flow cytometry has confirmed that the majority of CHMs are diploid and that most PHMs are triploid. Thus, flow cytometric analysis of hydropic abortions can be used as an adjunct to histopathologic criteria to differentiate among PHM, CHM and HA. The current study, using flow cytometry as an ancillary techniques to improve the accuracy of histopathological diagnosis, further reveals the difficulty in making the correct diagnosis in hydropic abortions (especially in early gestation) based on histopathology. This difficulty in distinction among PHM, CHM and HA was demonstrated by 5 (8.3%) of the cases in our study, in which the primary diagnoses changed following the acquisition of flow cytometric results. In a study done by Fukunaga et al. (21), nuclear DNA content of 219 hydropic and 68 nonhydropic placentas (as a control) were analyzed by flow cytometry in paraffin-embedded tissue. Based on flow cytometry and review of the histology, 10 PHM diagnoses were reclassified as HA, 1 PHM diagnosis was revised as CHM, 4 HA diagnoses were changed to PHM and 1 CHM diagnosis was corrected as PHM. They had 16 dis-

crepant diagnoses and their results showed that the main difficulty in histological diagnosis of hydropic abortions is due to the diagnosis of PHM. There is a considerable overlap in the histological features of PHM and HA as well as of PHM and CHM which result in discordant diagnoses. In another study of Fukunaga et al. (22), 76 cases of hydropic placentas were retrieved and analyzed by flow cytometry. Out of 23 specimens originally diagnosed as CHM, 21 diagnoses were confirmed and 2 were revised as PHM; out of 22 initially diagnosed PHMs, the primary diagnosis was confirmed in 20 cases and was changed to HA in two; and out of 31 firstly diagnosed HAs, 20 diagnoses were confirmed, while 9 and 1 diagnoses were revised as PHM and CHM, respectively. Also most of the cases with discordant diagnoses were definitively or initially diagnosed as PHM. They also concluded that PHM is a common condition that goes underdiagnosed because of the usual subtle histologic changes.

In our study, nine from the 17 cases initially diagnosed as CHM were diploid; therefore, the primary diagnosis was confirmed. Two cases out of 17 were triploid which ended in having the revised diagnosis as PHM based on re-evaluation of histological features. Five and one cases out of 17 were tetraploid and aneuploid, respectively. In the latter cases, we did not change the initial diagnosis of CHM. It is very important to interpret the ploidy result in conjunction with histomorphologic study (20). Although the great majority of CHMs and PHMs have been reported to be diploid and triploid, respectively, exceptional cases including non-molar digynic triploid abortion, triploid androgenetic CHMs, and tetraploid CHMs and PHMs have been seen as well. Ploidy analysis is not helpful in evaluation of these exceptional cases of molar and non-molar gestation, so a correct diagnosis can be made through karyotype analysis by cytogenetic study and/or p57 immunostaining (23).

In the present study, eight from the 19 cases with primary histological diagnosis as PHM were triploid; therefore, the primary diagnosis was confirmed. Two out of the 19 cases were diploid in which review of H&E slides and clinical data resulted in change of diagnosis to CHM. One and eight out of the 19 cases with primary diagnosis of PHM were tetraploid and aneuploid, respectively. In histomorphological review, these cases failed to show all the characteristic features of PHM; however, all showed the combination of dual villous population, round or oval trophoblastic pseudoinclusions, and cistern formation. As known from previous studies, several conditions including early CHM, trisomies, non-molar HAs, digynic triploid abortions, and placental mesenchymal dysplasia can mimic PHM at the morphologic level (6). In these cases, accurate diagnosis is not possible without genotyping. Therefore, we could not make any definitive diagnosis in the nine tetraploid and aneuploid cases which were primarily diagnosed as PHM. Fourteen out of the 24 cases with histological diagnosis as HA were diploid with the same diagnosis of HA on histological re-evaluation. One case out of the 24 was triploid which, after the histological review, was reclassified as PHM. In our study, similar to those mentioned above, the most discordant results were seen in the diagnosis of PHM. Moreover, our flow cytometry analysis in 9 cases with initial diagnosis of HA showed abnormal ploidy (5 tetraploid and 4 aneuploid) which could not be related to any form of molar gestation with the regard to the histopathologic criteria for HMs. This is supported by the fact that a great proportion of early abortions with hydropic changes are due to chromosome abnormalities. Since karyotyping was not performed in these cases, we were unable to verify or refute this possibility.

In present study, one case with original diagnosis of HA with triploidy in flow cytometry and revised diagnosis as PHM was a tubal pregnancy. In addition, 4 other cases of tubal pregnancy existed in HA group, diagnosis of which was confirmed by flow cytometry. Regarding these findings, there might be some relationship between abnormality in DNA ploidy with occurrence of ectopic pregnancy and presence of hydropic changes in these abnormal pregnancies.

In equivocal cases, ploidy analysis in association with histomorphologic study may be useful. Techniques including immunohistochemical analysis of p57 expression and molecular genotyping are also helpful in improving the diagnosis of hydatidiform moles, but have the limitation of not being able to establish maternal/paternal contributions of chromosome complements. For example, a dilpoid result by karyotyping or DNA flow cytometry analysis cannot distinguish a CHM (androgenetic diploidy) from a diploid NM (biparental diploidy),

and a triploid result cannot distinguish a PHM (diandric triploidy) from a triploid NM (digynic triploidy). Similarly, p57 immunostain cannot distinguish a PHM from a diploid (biparental) or triploid (digynic) non-molar (due to the presence of a maternal chromosome complement, all share the same pattern of p57 expression) (24).

In planning our study, we considered the high incidence of gestational trophoblastic disease in our country as well as the challenge encountered in their correct diagnosis, particularly in differentiating between PHM and non-molar hydropic changes, even based on immunohistochemistry as a routine procedure. Furthermore, molecular techniques could not be routinely implemented due to high cost. Thus, although flow cytometry is not a novel technique, we think that this study demonstrates its potential role as a cost effective as well as an efficient adjunct diagnostic tool to differentiate between the etiologies of molar/hydropic abortion, especially between PHM and non-molar hydropic abortion.

Conclusion

As a rapid and accurate means for determination of nuclear ploidy, DNA flow cytometric analysis can surely contribute to confirmation of histopathological diagnosis in most cases of molar pregnancy. This would provide valuable information, regarding the characteristics related to the persistent disease, which cannot always be obtained by macroscopic or microscopic inspection alone.

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Original Article

Evaluation of Tumor Necrosis Factor Alpha Polymorphism Frequencies in Endometriosis

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Abstract

Background: The pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α), is a pathogenic element for a number of disorders. Previous studies have reported that the -1031 T/C and -238 G/A polymorphisms in the promoter region of the *TNF-α* gene are important factors in reproductive-related disorders. One of the most common gynecological diseases of women during the reproductive years is endometriosis. This study aims to assess an association between the -1031 T/C, -238 G/A and -308 G/A polymorphisms of the *TNF-α* gene promoter region to endometriosis.

Materials and Methods: In this case-control study, we enrolled 65 endometriosis patients and 65 matched healthy control women by simple sampling. Polymerase chain reaction (PCR) analysis was used to analyze -1031 T/C, -238 G/A and -308 G/A polymorphisms in the *TNF-α* gene promoter region. Statistical analysis was performed using the chi-square test. P values less than 0.05 were considered statistically significant.

Results: We found a strong association between the -1031 T/C polymorphism in the promoter region of the *TNF-α* gene with endometriosis (P=0.001). There were no significant associations between the -238 G/A (P=0.243) and -308 G/A (P=1) polymorphisms with endometriosis and again endometriosis stages have no association with these polymorphisms.

Conclusion: The -1031 T/C polymorphism and CC genotype can be used as a relevant marker to identify women at risk of developing endometriosis.

Keywords: Endometriosis, Tumor Necrosis Factor, Polymorphism

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Introduction

Endometriosis, which is the presence of endometrial cells at ectopic sites outside the uterine cavity, is a common, complicated problem in women (1, 2). Although extensive research has been performed to improve our understanding of endometriosis, its natural history remains uncertain, with an indefinite etiology, unpredictable clinical presentation, problematic diagnosis, and poorly standardized treatment. Endometriosis is a cause of morbidity attributed to pelvic pain and infertility among 15-25% of women during their reproductive years (3). The prevalence of endometriosis varies from 109 to 247 per 100,000 (4).

Endometriosis is usually limited to the pelvis.

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This benign disease is characterized by peritoneal inflammation, fibrosis, adhesions, and ovarian cysts, but displays features of malignancy, such as neovascularization, local invasion, and distant metastasis (5). Hormonal, immunological, environmental, genetic factors and reflux as a mechanical factor have been implicated in its etiology but provide inconclusive explanations. More recently, an association between infectious factors and initiation of endometriosis has been proposed (3). The theory that endometrial tissue and cells reach the peritoneal cavity through retrograde menstruation along the fallopian tubes is widely conventional (6). However, although retrograde menstruation is found in 90% of women, only 10-20% of women are inflicted with endometriosis, which suggests the possibility that genetic and immunologic factors are involved (5).

Recently, an increasing body of evidence has reported a genetic basis for endometriosis. This evidence has stimulated and motivated researches toward the genes involved in its pathogenesis. However, even with significant efforts, the exact genetic mechanisms remain unknown. Until now, numerous genes such as detoxification enzyme genes, as well as estrogen and progesterone receptor genes have been considered in relation to endometriosis (7).

The inflammatory response in endometriosis may be mediated by proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α). For the first time, TNF-α has been recognized in peritoneal fluid of women with endometriosis, which introduced an association between endometriosis and disorders of the immune system (8). Study has shown that several products of the immune system such as interleukin-1 (IL-1), IL-6 and TNF-α play an important role in the establishment and maintenance of endometriosis (9). Several lines of evidence support the involvement of TNF-α as an important factor in the development of inflammatory pathologies such as increased levels of TNF-α in the peritoneal fluid of women with endometriosis which is correlated with disease severity (10) and the introduction of TNF-α as a motivator of ectopic endometrial tissue implantation (11). In addition, anti-TNF therapy is an essential part of endometriosis treatment (12) and an association between *TNF-α* gene polymorphisms with endometriosis has been reported in in several ethnic populations (13).

TNF-α is a potent immunomodulator and proinflammatory cytokine that plays an important role in the initiation and regulation of immune responses. It has been implicated in the pathogenesis of autoimmune and infectious diseases (14). TNF-α receptors such as TNFR1 and TNFR2 perform and manage its function (15). TNF-α plays a critical role in cellular proliferation, differentiation, inflammation, apoptosis, tumorigenesis and viral reproduction. Although numerous cell types produce TNF-α, it is typically produced by monocytes and macrophages (7, 16). IL-1, IL-6 and TNF-α are produced by stimulated macrophages and activated leukocytes (17). TNF-α is one of the 20 genes of the HLA system which maps to chromosome 6p21.3; it spans approximately 3 kb and has 4 exons. The last exon codes for more than 80% of the secreted protein (18).

TNF-α is also a significant source of genetic variability. Many single nucleotide polymorphisms (SNPs) in the promoter region of the *TNF-α* gene can play a part in the transcriptional regulation of this gene. SNPs are defined as genomic variations or differences among individuals (16). The substitution of G to A at position -238, G to A substitution at position -308, C to T substitution at position -857, C to A substitution at position -863, and the T to C substitution at position -1031 have been described in the proximal promoter of the *TNF-α* gene (19-24). For example, chronic inflammatory diseases such as rheumatoid arthritis, ulcerative colitis, and Crohn's disease show significant correlations with -G308A and -C850T polymorphisms (25, 26). In pregnant women with preeclampsia and eclampsia, a significant association with -C850T polymorphism has been observed (27).

Although studies evaluated the association between *TNF-α* gene polymorphisms and endometriosis, their results were incompatible (28, 29). Incidental positive findings, an insufficient statistical power that yielded negative results due to small study populations or genetic variation that led to heterogeneity of the study populations might explain the differences between studies (16). Therefore, additional studies from different geographical areas might shed light on the role of TNFα- polymorphisms in endometriosis. The aim of this study was to assess the associations of -1031 T/C, -238 G/A and -308 G/A polymorphisms in the *TNF-α* gene promoter region with endometriosis.

Materials and Methods

Subjects and sampling

This case-control study was conducted at

Beheshti Hospital, affiliated with Isfahan University of Medical Sciences, Isfahan, Iran. The study was approved by the Ethical Committee of Isfahan University of Medical Sciences. The study enrolled 130 individuals, 65 surgically confirmed cases of endometriosis and 65 healthy women who presented for normal deliveries. Control women had normal vaginal deliveries, as well as no family history, clinical symptoms, or diagnostic evidence suggestive of endometriosis. All individuals were informed about the study and signed an informed consent. The case and control groups were individually matched for age. The sample size was calculated using the proportion comparison formula by taking into consideration a confidence coefficient of 95% and statistical power of 80%. In order to participate in this study, cases had to be diagnosed with stages II, III, or IV endometriosis via a laparoscopy. Women with myoma and any benign or malignant mass were excluded from the study. A total of 2 ml of peripheral blood was collected from all participants along with clinical data, personal and family histories.

DNA isolation and genotype analysis

DNA was isolated from peripheral white blood cells using the PrimePrep Genomic DNA Isolation Kit (GeNet Bio, Korea) according to the manufacturer's instructions. The DNA was stored at -20˚C until processed. Genotyping for the TNF-α polymorphism was performed by polymerase chain reaction (PCR) with specific primers, followed by restriction fragment length polymorphism (RFLP) analysis. A three-step PCR was performed using a TC-512 thermal cycler (Techne Company, UK). PCR amplification was performed in a volume of 20 μL for each sample. The reaction was done using 10 mM tris-HCl (pH=8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each primer (forward and reverse), one unit Taq polymerase, and 50 ng of genomic DNA. Briefly the PCR conditions included an initial denaturation at 95˚C for 5-10 minutes, followed by 35 cycles at 94˚C for 30 seconds, annealing steps that were dependent on each primer, extension steps at 72˚C for 60 seconds, and a final extension at 72˚C for 10 minutes. PCR products were digested using 1 μL of BbsI, BamHI or NcoI restriction enzymes for each sample in a volume of 10 μL. For the TNF-α -1031 polymorphism T allele, the enzyme cut the PCR product into 251 and 13 bp fragments. For the C allele 251, the PCR product was cut into 71 and 13 bp fragments. Samples that had the G allele at position -238, BamHI digestion produced 123 and 42 bp fragments. Undigested 165 bp fragment illustrated A allele. For the TNF-α -308 polymorphism G allele, the NcoI enzyme cut the PCR product into 107 and 24 bp fragments, For the A allele, the 131 bp fragment remained undigested (Table 1).

TNF-α polymorphisms were detected after separation of enzyme-treated PCR products on a 2% agarose gel, followed by GelRed™ staining.

Table 1: Primer sequences and PCR-RFLP analysis data from TNF-α polymorphisms

Polymorphism	Primers	PCR product	Restriction enzyme	Annealing temp.	Alleles
-1031 T/C	F: 5´- TATGTGATGGACTCACCAGGT-3´	264 bp	BbsI	55˚C for 1 minute	251 bp+13 bp (T) 180 bp+71 bp+13 bp (C)
	R: 5´- CCTCTACATGGCCCTGTCTT-3´				
-238 G/A	F: 5´- AAA CAG ACC ACA GAC CTG GTC -3´	165 bp	BamHI	64˚C for 1 minute	123 bp+42 bp (G) 165 (A)
	R: 5´- CTC ACA CTC CCC ATC CTC CCG GAT -3´				
-308 G/A	F: 5´- GAGGCAATAGGTTTTGAGGGCCAT -3´	131 bp	NcoI	60˚C for 30 seconds	107 bp+24 bp (G) 131 (A)
	R: 5´- GGGACACACAAGCATCAAG -3´				

PCR-RFLP; Polymerase chain reaction-restriction fragment length polymorphism and TNF-α; Tumor necrosis factor-alpha.

Statistical analysis

Data are reported as mean ± SD. The chi-square test was used for statistical comparisons between group means. The odds ratio (OR) (2) and 95% confidence interval (CI) were estimated. Comparisons of the allele and genotype distributions were performed by SPSS version 19 software. P values less than 0.05 were considered significant.

Results

In this study, we compared 130 subjects in terms of the -1031T/C, -238G/A and -308G/A polymorphisms. There were 29 (22.3%) patients with stage II endometriosis, 31 (23.8%) with stage III, and 5 (3.8%) patients diagnosed with stage IV endometriosis. The mean age for cases was 30.53 ± 7.18 years and the mean age for controls was 29.04 ± 7.25 years, which was not statistically significant (P=0.145).

Totally, in both case and control groups, the homozygous TT genotype was seen in 84 (64.62%), TC in 33 (25.38%), and CC in 13 (10%) blood samples for the -1031 T/C polymorphism. We observed the homozygous GG genotype in 63 (48.46%), GA in 53 (40.77%), and AA in 14 (10.77%) blood samples for the -238G/A polymorphism. There was no evidence of any genotype related to the -308G/A polymorphism in either the patient or control group.

We observed significantly more of the -1031 CC genotype in the endometriosis group (20%) compared with the control group (0%, P=0.001). The TT and TC genotypes were more prevalent in the control group. This difference for the TT genotype was significant (P=0.001). There were fewer cases of the homozygous TT genotype in blood samples of patients with endometriosis (55.38%) compared to healthy women (73.85%). There were 24.65% of samples from the endometriosis group with the TC genotype compared to 26.15% of samples from the control group (P>0.05, Fig.1).

Although the -238 GG genotype was less frequent in the endometriosis group (41.54%) compared to the control group (55.38%) and the -238 TC genotype was more frequent in the endometriosis group (47.7%) compared to the control group (33.85%), these differences were not significant (P>0.05, Fig.2). Table 2 shows the genotype distributions of SNPs in the case and control groups.

In terms of the -308 polymorphism, none of the control and patient group samples contained the GA or AA genotypes. All samples from both groups were of the GG genotype (Fig.3).

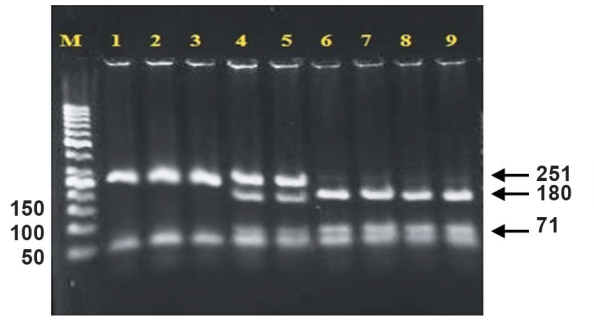

Fig.1: Agarose gel electrophoresis of polymerase chain reaction (PCR) assays for identification of the -1031 (T/C) single nucleotide polymorphism (SNP).

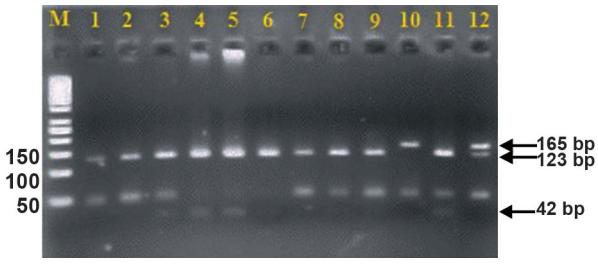

Fig.2: Agarose gel electrophoresis of polymerase chain reaction (PCR) assays for identification of the -238 (G/A) single nucleotide polymorphism (SNP).

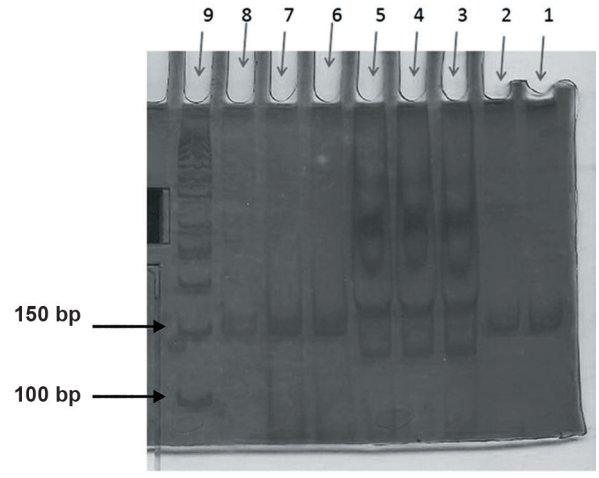

Fig.3: Agarose gel electrophoresis of polymerase chain reaction (PCR) assays for identification of the -308 (G/A) single nucleotide polymorphism (SNP).

Logistic regression analysis with the backward conditional method showed that only different genotypes of the -1031 polymorphism showed significant relations with risk of endometriosis (P<0.001). The risk of endometriosis in the CC genotype was significantly more than the other genotypes (OR=0.39, 95% CI=0.22-0.7, P=0.002).

The frequency of different genotypes according to the disease stage is shown in table 3. There was no significant difference in the frequency of these three polymorphisms in the *TNF-α* gene promoter region noted in relation to disease stage.

Table 2: Genotype distribution of single nucleotide polymorphisms (SNPs) in the case and control groups

Genotype	Group (%)		Total	P value
	Control	Case		
-1031 T/C				
TT	48 (73.85)	36 (55.38)	84 (64.62)	
TC	17 (26.15)	16 (24.65)	33 (25.38)	0.001
CC	0	13 (20)	13 (10)	
-238 G/A				
GG	36 (55.38)	27 (41.54)	63 (48.46)	
GA	22 (33.85)	31 (47.7)	53 (40.77)	0.245
AA	7 (10.77)	7 (10.77)	14 (10.77)	
-308 G/A				
GG	65 (100)	65 (100)	130 (100)	
GA	0	0	0	1.00
AA	0	0	0	

-1031 T/C (P=0.001, χ^2=19.7, df=2, OR=0.39, 95% CI=0.22-0.7), -238 G/A (P=0.245, χ^2=2.8, df=2), -308 G/A (P=1.00).
χ^2; Chi-square, OR; Odds ratio, df; Degree of freedom and CI; Confidence interval.

Table 3: Genotype distribution of TNF-α single nucleotide polymorphisms (SNPs) based on endometriosis stages

Genotype	Control (65)	Stage II (29)	Stage III (31)	Stage IV (5)	Total (130)	P value
-1031 T/C						
TT	48	17	15	4	84	
TC	17	8	7	1	33	0.42
CC	0	4	9	0	13	
-238 G/A						
GG	36	11	15	1	63	
GA	22	17	10	4	53	0.076
AA	7	1	6	0	14	
-308 G/A						
GG	65	29	31	5	130	
GA	0	0	0	0	0	1.00
AA	0	0	0	0	0	

-1031 T/C (P=0.42, χ^2=3.9, df= 4), -238 G/A (P=0.076, χ^2 = 8.5, df=4), -308 G/A (P=1.00).
χ^2; Chi-square, df; Degree of freedom and TNF-α; Tumor necrosis factor-alpha.

Discussion

In this study, we investigated the association of endometriosis with three common polymorphisms in the promoter region of the *TNF-α* gene in an Iranian population according to PCR-RFLP analysis. We observed no significant differences in the frequencies of the -238 and -308 promoter polymorphisms of the *TNF-α* gene between endometriosis patients and controls. However there was a significantly lower frequency of the -1031T allele observed in patients compared to controls.

We can explain the association in different ways. For example the -1031T polymorphism may protect patients from the most severe forms of endometriosis. Also linkage disequilibrium between another gene and this polymorphism may cause this association.

Minimal/mild endometriosis represents a normal physiological process usually found in asymptomatic women. In this study, we have performed a separate analysis on patients related to the stage of their disease. When the endometriosis cases were divided into subgroups with stages II, III, and IV disease, we observed no significant difference in the frequency of these three polymorphisms in the *TNF-α* gene promoter.

It is well known that several molecular entities play a role in establishing and maintaining endometriosis. The relationship between TNF-α and endometriosis has been indicated in several studies making it a good candidate gene. TNF-α is produced during inflammatory processes. It is a pro-inflammatory cytokine involved in numerous infectious and inflammatory processes (30). Different researches have explained the nature of endometriosis as an inflammatory disorder (1). A number of studies assessed different TNF-α polymorphisms in endometriosis patients (13, 30).

Wilson et al. (21) emphasized the importance of several polymorphisms in the promoter region of the *TNF-α* gene. Many studies have confirmed the association between the polymorphisms at positions -238, -308, -857, -863 and -1031, increased transcriptional activity and production of TNF-α (23, 31). There is also evidence for association of the -238 G/A polymorphism with insulin resistance syndrome and obesity (32), as well as an association of -308 G/A polymorphism with various inflammatory and autoimmune diseases (33). Previous studies have illustrated relations between certain immune-mediated diseases and -857, -863, -1031 polymorphisms (20, 34). Two studies performed in Australia and Korea reported -238G/A and -308G/A polymorphisms in endometriosis patients but did not describe any association. Regionally and geographically, the rates of -238A and -308A alleles in TNF-α have been shown to significantly differ (35), hence there is a need for large sample sizes in order to study relationships between diseases and these polymorphisms.

The -1031T/C polymorphism is reported to be associated with Behcet's disease (31), Crohn's disease (34) and Crohn's extra-intestinal manifestations that include uveitis, erythema nodosum and large joint arthropathy (36). Studies have shown increased frequency of the -1031T allele in patients with hyperandrogenism and in those with ulcerative colitis (34, 37). In contrast, a lower frequency of the -1031T allele is reported in patients with Crohn's and Behcet's diseases compared to controls (31, 34).

The findings of this study were in concordance with the outcomes of studies by Ahmad et al. (31) and Negoro et al. (34) who reported a low frequency of the -1031T allele in the mentioned diseases compared to normal population, but this polymorphism had no significant correlation with other disorders. In order to describe the importance of the TNF-α -1031T/C polymorphism, additional research would be necessary to clarify the relationship between this multi-functional proinflammatory cytokine with endometriosis.

In the current study, differences in the frequency of the -1031T/C alleles was not associated with the severity of endometriosis. Asghar et al. (16) reported that a decreased frequency of the -1031C allele was related to severe endometriosis but they did not find an association with less severe disease. The low number of patients in the current study, in particular the severe forms of endometriosis could explain this difference. The results implied that the T allele could provide protection from endometriosis.

Our data showed an increase in the frequency of the genotypes -1031 C/C and -238 G/A as well as a decrease in the frequency of the -1031 T/T,T/C and -238 G/G genotypes in patients with endome-

triosis. However only the -1031 T/T,C/C polymorphism changes were significant. There was no association between the stage of endometriosis and different genotypes.

Although there was no association between the TNF-α polymorphism and endometriosis in the majority of similar studies (38, 39), we did find associations reported in some studies. In a study performed on Indian women, there was a significant increase of endometriosis in women carriers of the -850 T/T genotype and TT genotype increased the risk of endometriosis. In the Japanese population, TNF-α -1031T/C polymorphism was associated with reduction of endometriosis risk (39). In the Korean and Japanese populations, advanced stage endometriosis was seen mostly in patients who had the TNF-α -1031T/C polymorphism (13, 30) but there were no significant differences in frequencies between endometriosis cases and controls for TNF-α -238G/A, -308G/A, -857C/T, and -863C/A polymorphisms (13). TNF-α polymorphisms showed no significant association with endometriosis in Australian, Chinese, Taiwanese, and Austrian populations (1). Asghar et al. (16) assessed the -238C/T polymorphism of TNF-α with inflammatory diseases and endometriosis. However, most did not report any significant associations with endometriosis.

Elevated TNF-α levels in peritoneal fluid have been associated with up-regulated TNF-α production in peritoneal macrophages and peripheral monocytes of women with endometriosis (11). The functional role of TNF-α in endometrial tissue is unknown. It has been assumed that a TNF-α polymorphism that alters its transcription/expression subsequently enhances its level, leading to increased proliferation and decreased apoptosis as seen in the inflammatory cascade.

The results of a recent meta-analysis study implies that TNF-α -1031C is associated with a higher risk of endometriosis in Asian individuals in homozygote comparisons and the recessive genetic model (16). However in another study the TNF-α -1031T/C polymorphism has shown a relation with endometriosis in the Iranian population. They reported that the -1301C allele might have a protective role in the development of endometriosis (40).

In this study, an association was demonstrated between the -1031C/T TNF-α polymorphism and endometriosis, which indicated that it could be used as a relevant molecular marker to assess the risk of endometriosis.

Due to the small number of studies (39, 40) that have focused on *TNF-α* gene polymorphisms and the limited number of cases and controls in these studies, it is necessary to perform additional studies with larger sample sizes and well-matched controls stratified by stage, ethnicity, or other risk factors. These studies may assist in explaining the possible roles of the *TNF-α* gene polymorphisms in endometriosis, particularly in other ethnic populations.

Conclusion

The TNF-α promoter -1031 T/C polymorphism is associated with decreased risk of endometriosis in an Iranian population. Our data have demonstrated decreased frequency of the -1031T polymorphism in the promoter region of the *TNF-α* gene in the most severe cases of endometriosis in our studied population. This finding has suggested that the -1031T polymorphism may play a protective role in endometriosis progression. Although this model is biologically acceptable, we recognize that our conclusions are based on relatively small numbers and require confirmation from additional independent studies.

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Original Article

Comparison of Protamine 1 to Protamine 2 mRNA Ratio and *YBX2* gene mRNA Content in Testicular Tissue of Fertile and Azoospermic Men

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Abstract

Background: Although aberrant protamine (*PRM*) ratios have been observed in infertile men, the mechanisms that implicit the uncoupling of *PRM1* and *PRM2* expression remain unclear. To uncover these mechanisms, in this observational study we have compared the *PRM1/PRM2* mRNA ratio and mRNA contents of two regulatory factors of these genes.

Materials and Methods: In this experimental study, sampling was performed by a multi-step method from 50 non-obstructive azoospermic and 12 normal men. After RNA extraction and cDNA synthesis, real-time quantitative polymerase chain reaction (RT-QPCR) was used to analyze the *PRM1, PRM2*, Y box binding protein 2 (*YBX2*) and JmjC-containing histone demethylase 2a (*JHDM2A*) genes in testicular biopsies of the studied samples.

Results: The *PRM1/PRM2* mRNA ratio differed significantly among studied groups, namely 0.21 ± 0.13 in azoospermic samples and -0.8 ± 0.22 in fertile samples. The amount of *PRM2* mRNA, significantly reduced in azoospermic patients. Azoospermic men exhibited significant under expression of *YBX2* gene compared to controls (P<0.001). mRNA content of this gene showed a positive correlation with *PRM* mRNA ratio (R=0.6, P=0.007). *JHDM2A* gene expression ratio did not show any significant difference between the studied groups (P=0.3). We also observed no correlation between *JHDM2A* mRNA content and the *PRM* mRNA ratio (R=0.2, P=0.3).

Conclusion: We found significant correlation between the aberrant *PRM* ratio (*PRM2* under expression) and lower *YBX2* mRNA content in testicular biopsies of azoospermic men compared to controls, which suggested that downregulation of the *YBX2* gene might be involved in *PRM2* under expression. These molecules could be useful biomarkers for predicting male infertility.

Keywords: Protamines, *YBX2, JHDM2A*, Azoospermia

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Introduction

Protamines (*PRMs*) comprise the largest amount of nucleoproteins in mature human sperm. These proteins are transcribed in steps 1-4 of spermatids (1) while synthesis of the corresponding proteins starts, with temporal delay, in step 4 spermatids (2). During spermiogenesis, *PRMs* replace somatic histones in a step-by-step manner, and cause higher DNA packaging in sperm compared to somatic cells. On the other hand, the condensed and insoluble nature of the sperm chromatin protects the genetic integrity of the parental genome during its transport through the male and female reproductive tracts (3).

Various studies reported abnormal expressions of *PRM* genes in sperm of infertile men. In addition,

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correlation of the altered *PRM1/PRM2* ratio has been shown with low sperm counts, decreased sperm motility and morphology, decreased fertilization ability and increased sperm chromatin damage (4-7). Several factors have been postulated and studied as possible causes of *PRM1/PRM2* deregulation (8-11). One of these candidate mechanisms is *PRM* gene polymorphisms that have also been reported for *PRM1, 2* genes. However most of these studies suggest that none of the *PRMs'* single nucleotide polymorphisms (SNPs) and transition protein genes is likely to be a common cause of *PRM* abnormalities. Other factors that have attracted attention in this regard are transcription and translation regulatory genes of *PRM*. Several genes and proteins involved in *PRM1/PRM2* expression regulation have been identified and presented (12-16); until now, modification of these factors in infertile men with *PRM* deficiency attracted less attention. In this study we have proposed two *PRM* regulatory factors -Y box binding protein 2 (*YBX2*) gene and JmjC-containing histone demethylase 2a (*JHDM2A*) gene. These genes encode two important proteins involved in regulation of *PRM1/PRM2* expressions (17, 18). *YBX2* is the human homologue of Xenopus DNA/RNA-binding and mouse *MSY2* proteins (16), animal model studies show that this protein exists abundantly in testis tissue and is expressed in meiotic and post-meiotic germ cells (17). *YBX2* acts as an mRNA stabilizer and a transcription factor of *PRM* genes (18, 19). Consequently, *YBX2* loss of expression is likely to contribute to the nuclear condensation defects that occur in Msy2-null late-stage spermatids (20). *JHDM2A* specifically regulates the expression of genes that encode transition protein 1 (*Tnp1*) and *PRM1*; this is necessary for proper chromatin reorganization during spermatid maturation by directly promoting transcription of *TNP1* and *PRM1* genes (21). The current study analyzes the *PRMs* ratio in testicular tissue of azoospermic men. As *YBX2* and *JHDM2A* are involved in expression regulation of these genes, we have additionally evaluated whether PRM deficiency is related to downregulation of these genes.

Materials and Methods

Testicular tissue

This experimental study was approved by the Ethical Committee of the Faculty of Medical Sciences of Qazvin Medical Science University (Qazvin, Iran). After patients gave their informed written consent, testicular biopsies were obtained from 50 infertile men with a mean age of 31.3 ± 3.7 years; these patients were candidates for assisted reproductive technique (ART) and exhibited impaired spermatogenesis. In 12 patients with obstructive azoospermia after vasectomy, biopsies were performed out for diagnostic reasons during vasectomy reversal. These biopsies revealed normal spermatogenesis which served as controls; the mean age of these individuals was 35 ± 2.9 years. In this study patients were excluded if they had the following criteria: Y chromosome microdeletion, cystic fibrosis, varicocele, Klinefelter syndrome, or exposure to chemotherapy and radiation. In non-obstructive azoospermia patients, one part of the testicular tissue specimen was used for testicular sperm extraction, while the other part was cut into two pieces. One piece was immediately prepared and frozen for the RNA extraction procedure and the other piece was fixed in Bouin's fixative, then embedded in paraffin.

Histological evaluation

We stained 5 μm paraffin sections in hematoxylin and eosin, and then scored the sections according to the modified Johnsen scoring system for histological evaluation (22). In this system of classification, all tubular sections in each piece of the testicular biopsy are evaluated systematically, and each is given a score from 1 to 10. Complete spermatogenesis with numerous spermatozoa is evaluated as score 10; slightly impaired spermatogenesis, numerous late spermatids, disorganized epithelium as score 9, less than five spermatozoa per tubule and few late spermatids as score 8, no spermatozoa, no late spermatids, and numerous early spermatids as score 7, no spermatozoa, no late spermatids, and few early spermatids as score 6; no spermatozoa or spermatids, and many spermatocytes as score 5, no spermatozoa or spermatids, and few spermatocytes as score 4; spermatogonia only as score 3, no germinal cells and Sertoli cells only as score 2; no seminiferous epithelium as score 1 (11). To follow this classification method, we divided the samples into two groups based on the above scoring: normal spermatogenesis (scores 9-10) and impaired spermatogenesis (scores 1-8) (Table 1, Fig.1A-E). It should be mentioned that our samples with score 8 had severe hypospermatogenesis.

Table 1: Characterization of patient candidates for ICSI

	Fertile men	Azoospermic men	P value
Age (Y) (Mean ± SD)	35 ± 2.9	31.3 ± 3.7	
Histology (Score)	10	1-8	
Serum FSH (mIU/ml) (Mean ± SD)	5.2 ± 2.3	14.26 ± 7.3	
$\Delta Ct_{(PRM2\text{-}PRM1)}$, (Mean ± SD)	-0.8 ± 0.22	0.21 ± 0.13	<0.0001
$\Delta Ct_{(YBX2\text{-}GAPDH)}$, (Mean ± SD)	3.3 ± 0.99	5.1 ± 1.2	<0.0001
$\Delta Ct_{(JHDM2A\text{-}GAPDH)}$, (Mean ± SD)	6.1 ± 1.1	6.3 ± 0.89	0.5

FSH; Follicle stimulating hormone and ICSI; Intracytoplasmic sperm injection.

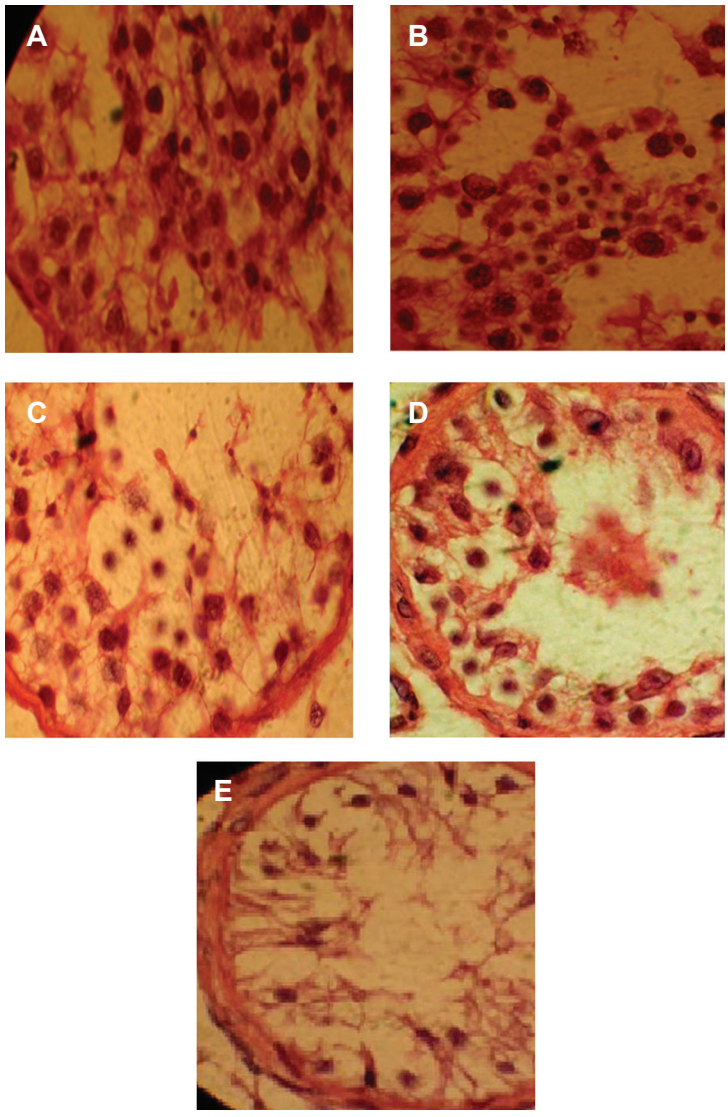

Fig.1: Results of hematoxylin and eosin staining of testis tissues. **A.** Hypospermatogenesis, **B.** Maturation arrest in round spermatid stage, **C.** Maturation arrest in spermatocyte stage, **D.** Maturation arrest in spermatogonial stage and **E.** Sertoli cell only. (magnification: ×1000).

RNA extraction and first strand cDNA synthesis

After homogenizing frozen testis tissues using an Ultrasonic Processor UP100H (Hielsher, Germany), RNA was extracted with an RNeasy Mini Kit (Qiagene, Germany). The extracted RNA was frozen at -80˚C. We used a Nano Drop 2000c (Thermo, USA) to evaluate the quantity of isolated total RNAs. In this regard, RNA samples with A260/A280 ratios of >2 were selected for quantitative analysis. First strand complementary DNA (cDNA) synthesis was also performed using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas, Waltham, MA, USA).

Real time quantitative polymerase chain reaction (RT-QPCR)

We designed four target genes primers and probes (*PRM1, 2*, *YBX2* and *JHDM2A*) using Gene Runner Softw are (Version 3.05, Table 2). A Taq Man RT- QPCR assay was carried out in final reaction volumes of 20 µl with 10 µl of Taq Man Master Mix (Takara, Shiga, Japan), 0.2 µM of forward and reverse primers, and 2 µl of cDNA. Thermal cycling was performed on the ABI-7500 (Applied Biosystems, Foster, CA, USA) sequence detection system by using the following cycling condition: 30 seconds at 95˚C as the first denaturation step, followed by 40 cycles at 95˚C for 5 seconds and 60˚C for 34 seconds. Each assay was repeated at least twice. The log-ratio of the transcript content in the samples was determined by the ΔCt method of relative quantification. The log-ratio of *PRM1* and *PRM2* was also calculated by $\Delta Ct = Ct_{PRM2} - Ct_{PRM1}$, for *YBX2* and *JHDM2A*, these were $\Delta Ct = Ct_{YBX2} - Ct_{GAPDH}$ and $\Delta Ct = Ct_{JHDM2A} - Ct_{GAPDH}$. To study the correlation between *YBX2* and *JHDM2A* mRNA content and *PRM* log concentration, we used the calculating pattern of Steger et al. (6). Since Ct of *PRM1* did not change in the different groups, log-concentration of *YBX2* and *JHDM2A* were normalized to *PRM1* ($\Delta Ct = Ct_{YBX2} - Ct_{PRM1}$ and $\Delta Ct = Ct_{JHDM2A} - Ct_{PRM1}$) (23).

Table 2: Primer and probe sequences of target and internal control genes

Target and internal control genes	Sequence	Amplicon size (bp)
PRM1	F: TGACTCACAGCCCACAGAGT	124
	R: CTGCGACAGCATCTGTACCT	
	P:AGGCCAAGCCCATCCTGCAC	
PRM2	F:GCAAGAGCAAGGACACCAC	98
	R:GACACTGCTCTCGAAGGAGG	
	P: CGGAGCACGTCGAGGTCT	
YBX2	F:CCCTACCCAGTACCCTGCT	150
	R:CCTTCCTTCAACCCTTGATAA	
	P:CAGGAGGACCAAAGCAGCAGC	
JHDM2A	F: GTTCCACAAGCATTGACTGG	145
	R :CTGGTGCATTTGAAACATCC	
	P:TGCCAATCCTCCTGAACTGCAG	
GAPDH	F: TCAAGAAGGTGGTGAAGCAG	93
	R:CGCTGTTGAAGTCAGAGGAG	
	P: CCTCAAGGGCATCCTGGGCT	

YBX2; Y box binding protein 2, *JHDM2A*; JmjC-containing histone demethylase 2a, *PRM*; Protamin and *GAPDH*; Glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

In order to determine the significant differences between the studied groups, statistical analysis that included mean, standard deviation (SD), correlation coefficients (R^2) and unpaired t test were performed with Prism (version 3) software. Additionally, linear correlations were tested using the Pearson coefficient of correlation. All tests were performed at a confidence level of 95%.

Results

The mean Ct of *PRM1* in testis tissues were almost identical, 23.4 ± 3.6 (azoospermic) and 23.3 ± 1.5 (fertile). The mean Ct of *PRM2* in azoospermic men was 23.6 ± 1.8 and in the fertile group, it was 22.5 ± 0.41. Hence the expression ratio of *PRM2* was lower than the *PRM1* expression ratio in azoospermic men. The logarithm of the *PRM1/PRM2* mRNA ratio in azoospermic men was 0.21 ± 0.13 and for fertile men, it was -0.8 ± 0.22. This difference of ratios between fertile and azoospermic men was statistically significant (P<0.0001, Table 2).

In testicular tissues of azoospermic men with impaired spermatogenesis, the fold change of *YBX2* transcripts was 0.02 ± 0.019, and the log ratio of *YBX2* expression between azoospermic (5.1 ± 1.2) and fertile men (3.3 ± 0.99) significantly differed (P<0.0001, Table 2). In terms of *JHDM2A* gene expression, the differences of expression ratio were 6.1 ± 1.1 (fertile) and 6.3 ± 0.89 (azoospermic), which was not significant (P=0.5, Table 2).

The *YBX2* mRNA content revealed a positive linear correlation (R=0.6, P=0.007) with the *PRM1/PRM2* mRNA ratio or with *PRM2* deficiency (Fig.2A). However, we observed no linear correlation between *JHDM2A* mRNA content and the *PRM1/PRM2* mRNA ratio (R=0.2, P=0.3, Fig.2B).

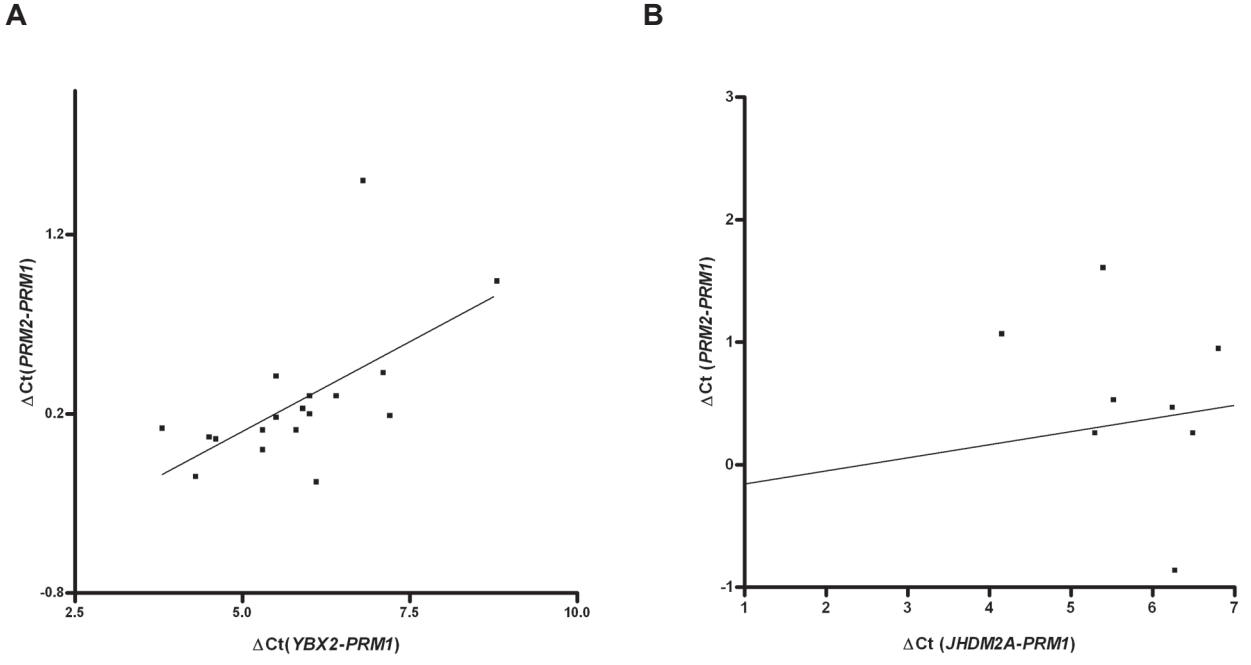

Fig.2: A. Correlation between the protamine-1 *(PRM1)* to *PRM2* log-ratio ΔCt *(PRM2-PRM1)* and normalized *YBX2* log-concentration ΔCt(*YBX2-PRM1,* R=0.6, P=0.007) and **B.** Correlation between the *PRM1* to *PRM2* log-ratio ΔCt (*PRM2-PRM1*) and normalized *Jhdm2a* log-concentration ΔCt (*JH-PRM1,* R=0.2, P=0.3).

Discussion

In this research we observed a significant difference in *PRM1/PRM2* ratio between azoospermic and fertile men testicular biopsies (0.21 ± 0.13, -0.8 ± 0.22). *PRM2* changes were more than *PRM1*, which suggested different mRNA stabilities for the two molecules. A variety of studies reported a relationship between abnormal *PRM1/PRM2* ratios and male infertility (4-6, 21, 23). On the basis of the studies which showed high *PRM1/PRM2* ratios, it has been supposed that a reduction in *PRM2* expressions was responsible for aberrant *PRM1/PRM2* ratios in infertile males. Along this line, two studies reported complete selective absence of *PRM2* in infertile men (4, 24, 25). Lewis et al. (26) observed that in sperm of infertile men, *PRM2* downregulation occurred much more frequently than *PRM1* deregulation, because *PRM2* expression was more sensitive to the variation of regulatory controlling mechanisms than those for *PRM1*.

Our results confirmed the results from the above studies; we have observed significant downregulation of *PRM2* in the studied azoospermic men. *PRMs* genes are only transcribed in round spermatids and stored as silent mRNAs for later translation in elongating spermatids, in which transcription is no longer active. Since it is well justified to consider altered *PRMs* mRNA levels as a potential origin of altered protein levels (1, 11, 27), we have evaluated *PRM* expression at the mRNA level. Generally speaking, the mechanisms that underlie the uncoupling of *PRM1* and *PRM2* expression remain unclear, but generally four pathways in regulation of *PRM* gene expression have received more attention: the *PRM* genes themselves, transcription regulation, translation regulation, and downstream protein processing.

The *PRM1* and *PRM2* genes exist in a single chromatin domain in human sperm, and their transcription is regulated by the same upstream regulatory elements, thus making transcriptional and translational regulation a possible cause for aberrant *PRM1/PRM2* expression (28, 29). There are a number of regulatory proteins identified which are involved in repression or activation of *PRM* expression (29). In this regard, various animal models and *in vitro* studies have been performed, but scientists emphasize that future investigations should focus on aberrant expression, activation, and function of these regulatory factors in patients with deregulated *PRM1/PRM2* ratios (30, 31). To this end, we have focused on the expression ratio of two factors of *PRM* gene expressions.

YBX2 is a transcription and translation regulatory factor, and a germ-cell-specific molecule essential for the production of functional spermatozoa. This gene is expressed in meiotic and post-meiotic germ cells, but its functional form is in round spermatids. Inactivation of *YBX2* can lead to male infertility. *YBX2*, also known as Contrin, is the human homologue of Xenopus DNA/RNA-binding and mouse MSY2 proteins (32, 33). To clarify the functional role of MSY2 in germ cells, Yang et al. (19) have generated Msy2-null mice. They found that mutant males had an abnormally high numbers of apoptotic meiotic spermatocytes, lacked spermatozoa in the epididymis, and were sterile. Their results emphasized the major role of this protein in male fertility (33). Hammoud et al. (34) investigated *YBX2* gene alterations in men with severe defects in spermatogenesis that included azoospermia, severe oligozoospermia, and *PRM* deregulation samples. Their results showed 15 polymorphic sites, of which 7 polymorphisms were present at a statistically higher frequency in patients with infertility, particularly in men with abnormal *PRM* expression. On the same path they showed that some SNPs in the *YBX2* gene occurred at a significantly higher incidence in men with *PRM* abnormalities than the control group. Our results, accordingly, showed significant downregulation of this gene in testicular tissues of azoospermic men compared to fertile men.

In terms of the molecular function of this protein, animal models and *in vitro* assay studies have shown that MSY2 acts as a transcription factor and an mRNA stabilizer which regulates expression of some testis specific genes at the transcription and translation levels, such as *PRM1, 2*. MSY2 marks specific mRNAs (those transcribed from Y-box promoters) in the nucleus for cytoplasmic storage, and thereby links mRNA transcription and storage/translational delay. In this process, MSY2 recognizes the CTG ATTGGC/TC/TAA sequence, a DNA motif in the promoter of numerous genes specifically expressed in male germ cells. After binding MSY2 to its consensus promoter

sequence, it binds to transcripts of this gene, and stabilizes and represses their translation in cytoplasmic RNA-protein complexes (19, 35-38). Since *PRM1, 2* are regulated by the same upstream regulatory elements, we have expected significant under expression of *YBX2* to cause simultaneous downregulation of *PRM1, 2* in our azoospermic samples compared to fertile samples.

Unlike our expectation *PRM2* downregulated in the studied samples. We observed a positive linear correlation between downregulation of *PRM2* and *YBX2* genes. Statistically, R=0.6 exhibited an intermediate or good correlation, but this correlation was not perfect or 100%. This probably indicated that factors other than *YBX2* were involved in *PRM2* downregulation. Consistent with our results, one study has shown that decreased *PRM1* protein level is usually linked with post-translational deregulation, but decreased *PRM2* is associated with low *PRM2* mRNA (30). *PRM2* transcripts are more susceptible to variations other than *PRM1* transcripts, downregulation of *YBX2* affects *PRM2* transcripts more than *PRM1* (26).

Another regulatory factor studied in this research was *JHDM2A*, also known as Jmjd1a or Kdm3a, was identified as an H3K9 demethylase (for monomethylation and dimethylation). *JHDM2A* was originally cloned as a testis-specific gene transcript. Results of immune histochemical analysis using anti- *JHDM2A* antibody showed an intense nuclear expression of this gene in round spermatids and a sub-nuclear distribution. Co-expression of *JHDM2A* gene with RNA polymerase II indicated that *JHDM2A* might contribute to transcriptional activation of some testis specific genes. *JHDM2A* has been shown to stimulate the transcriptional activation of transition nuclear protein1 and *PRM1* genes by bonding to the core promoter and removing H3K9 methylation (21). Histone demethylase *JHDM2A* is critical for Tnp1 and *PRM2* transcription and spermatogenesis. *JHDM2A* -deficient mice have infertility and smaller testes.

Despite the fact that animal model studies show the role of this protein in male infertility, we have not observed any significant difference in expression ratio of this gene between azoospermic and fertile men samples. Probably the role of this gene is not very influential in human spermatogenesis, and more samples must be studied. Regarding the correlation between this gene expression and *PRM2* downregulation our results have shown no positive linear correlation. Statistically, shows a weak or no correlation. Other studies have shown that this gene acts as a transcriptional activator of the *PRM1* gene. In addition, our samples displayed downregulation of the *PRM2* gene; therefore, expression of *JHDM2A* in our samples did not have an important role.

Conclusion

We found significantly aberrant *PRM* mRNA ratios and a lower *YBX2* mRNA content in testicular spermatids of infertile men. In future studies the exact role of these molecules (*YBX2* and *JHDM2A*) and other *PRM* expression regulatory factors must be determined in human spermatogenesis.

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Original Article

Chromosomal Aneuploidies and Early Embryonic Developmental Arrest

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Abstract

Background: Selecting the best embryo for transfer, with the highest chance of achieving a vital pregnancy, is a major goal in current *in vitro* fertilization (IVF) technology. The high rate of embryonic developmental arrest during IVF treatment is one of the limitations in achieving this goal. Chromosomal abnormalities are possibly linked with chromosomal arrest and selection against abnormal fertilization products. The objective of this study was to evaluate the frequency and type of chromosomal abnormalities in preimplantation embryos with developmental arrest.

Materials and Methods: This cohort study included blastomeres of embryos with early developmental arrest that were biopsied and analyzed by fluorescence in-situ hybridization (FISH) with probes for chromosomes 13, 16, 18, 21 and 22. Forty-five couples undergoing IVF treatment were included, and 119 arrested embryos were biopsied. All probes were obtained from the Kinderwunsch Zentrum, Linz, Austria, between August 2009 and August 2011.

Results: Of these embryos, 31.6% were normal for all chromosomes tested, and 68.4% were abnormal. Eleven embryos were uniformly aneuploid, 20 were polyploid, 3 were haploid, 11 displayed mosaicism and 22 embryos exhibited chaotic chromosomal complement.

Conclusion: Nearly 70% of arrested embryos exhibit chromosomal errors, making chromosomal abnormalities a major cause of embryonic arrest and may be a further explanation for the high developmental failure rates during culture of the embryos in the IVF setting.

Keywords: Fluorescence In Situ Hybridization, Blastomeres, Embryonic Development, Aneuploidies, Chromosomes

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Introduction

The earliest stages of human development are highly prone to error because of chromosomal abnormalities which may occur during the critical steps of meiosis, fertilization and the early cleavage stage (1, 2). *In vitro* fertilization (IVF) allows the study of early embryonic development. Even with significantly improved culture conditions, approximately 10 to 15% of IVF embryos exhibit a permanent cell cycle arrest state, and 40% of IVF patients show at least one arrested embryo per cycle (3). Cleavage stage embryos generally show high levels of chromosomal aneuploidies, and statistically, only 1 of 5 has the capacity to

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implant (4). Humans are not very fertile compared to other species (5, 6). Mantzouratou and Delhanty (4) postulate that on the third day of development, 60% of all IVF embryos show at least one aneuploid cell. Even embryos from patients younger than 35 years displaying the best morphology and development show an aneuploidy rate of 56% (7). Human embryos have higher aneuploidy rates than those observed during prenatal analysis and postnatal life; therefore, there appears to be strong selection pressure against chromosomally abnormal embryos. This negative selection seems to occur primarily during the pre-implantation period, most likely through developmental arrest or the degeneration of chromosomally abnormal embryos (8).

This study specifically analyzed embryos that were not suitable for transfer. In these embryos, the aneuploidy rates are expected to be higher than in normal IVF embryos. Many studies have analyzed the correlation between morphological and developmental abnormalities in IVF embryos. A number of abnormalities are notably well correlated, such as giant oocytes (>220 µm), which are nearly always diploid, whereas other abnormalities show no direct correlation (9).

Arrested and slow cleaving embryos are hypothesized to have higher than average rates of chromosomal abnormalities. Embryos with accelerated cleavage are also associated with a higher rate of chromosomal abnormalities, and the time frame is an important factor (2).

Fluorescence in situ hybridization (FISH) was the method of choice used in this study. Using FISH, it was possible to distinguish between polyploidy and aneuploidy. When a representative number of blastomeres from an embryo could be analyzed, it was possible to diagnose mosaicism (9, 10).

The objective of this study was to determine whether chromosomal abnormalities are a common cause of embryonic arrest, which may be an additional explanation for the notably high developmental failure rates of IVF embryos during culture, even after continuous improvements of the culture conditions.

Materials and Methods

This cohort study included hundred nineteen embryos with early developmental arrest that were obtained during 45 cycles of IVF treatment. The number of embryos analyzed per cycle ranged from 1 to 9, with an average of 3 arrested embryos per cycle. All probes were obtained from the Kinderwunsch Zentrum Linz, Austria, between August 2009 and August 2011, and the genetic analyses were performed at the Department of Human Genetics, Landes- Frauen- und Kinderklinik Linz, Austria. All the arrested embryos used in this study were donated by patients undergoing conventional IVF treatment for infertility, and written consent was obtained from each patient. The study was approved by the local Ethical Review Board and was performed in accordance with the Austrian legal regulations, because only fertilization products without development potential, which would usually be discarded during normal IVF cycles, were used for analysis. The age of the patients ranged from 26 to 47 [mean age: 35 years, standard deviation (SD): 4.9 years]. Embryos were considered arrested when no cleavage had occurred during a 24-hour period (<5 cells on day 3 post fertilization, <8 cells on day 4 and <12 cells on day 5) (6, 11). A percentage of all arrested embryos (47%) showed additional morphological abnormalities, such as multinucleation and uneven blastomere size.

For the biopsy procedure, a infrared diode laser "Fertilaser" 1.48 µm (MTG, Bruckberg, Germany) was used. A hole was drilled into the zona pellucida (12, 13) and the blastomeres were then separately aspirated and transferred onto glass slides. Embryos were biopsied on days 3, 4, 5 or 6, and the time of arrest ranged from day 1 to 4. Fixation was performed with ice-cold Carnoy's fixative (3:1 methanol: acetic acid).

Depending on the number of blastomeres per glass slide, 10-15 µl fixative was applied twice or until the cytoplasm dissolved, and the glass slide was air-dried for at least 15 minutes before the FISH procedure.

The FISH process was performed in one hy-

bridization round with the MultiVysion DNA Probe Panel (Vysis, Abbott Molecular Inc., Des Plaines, USA) for chromosomes 13 (LSI 13, SpectrumRed, 13q14), 16 (CEP 16 satellite II, SpectrumAqua, 16q11.2), 18 (CEP 18 alpha satellite, SpectrumBlue, 18p11.1-q11.1), 21 (LSI 21, SpectrumGreen, 21q11.2 – q22.2) and 22 (LSI 22, SpectrumGold, 22q11.2). The probes were denatured at a melting temperature of 69˚C for 8 minutes and hybridization was performed at 37˚C overnight. Next the hybridization coverslips were removed and the slides were washed for 7 minutes at 72˚C in 0.7×saline sodium citrate (SSC)/0.3% Nonidet® P-40 (NP-40: Abbott Molecular Inc., Des Plaines, USA, SSC, Invitrogen, life technologies, LifeTech Vienna, Austria) followed by a 1 minute incubation at room temperature in 2×SSC/0.1% NP-40. The slides were mounted with Antifade Solution without DAPI (Vector Laboratories, CA, USA) and fluorescence microscopy was performed using an Axioplan 2 microscope equipped with specific filters for each fluorochrome. In some cases, the split signals were problematic and usually correlated with bad blastomere morphology. The probe panel was tested on lymphocyte slides prepared by standard cytogenetic procedure, scoring for 25 metaphases and 100 interphases. The probe efficiency was 97% (Fig.1D).

The embryos were categorized into the following 6 subgroups:

I. Normal diploid (euploid) embryos with all cells showing two signals for the analyzed chromosomes.

II. Homogeneously abnormal (aneuploid) embryos with either monosomy (same chromosome missing in all cells) or trisomy (three chromosomes of the same type in all cells).

III. Mosaicism with embryos containing two cell lines, each representing >20% of the cells.

IV. Embryos with more than two abnormalities affecting multiple chromosomes and varying from cell to cell (uncontrolled division) were categorized as chaotic (complex) (14).

V. Polyploid embryos with three or more signals for each analyzed chromosome in all cells. VI. Haploid embryos with only one signal for each analyzed chromosome in all cells (15).

Results

Forty-five couples donated their arrested embryos for this study. A total of 649 blastomeres were biopsied from 119 arrested embryos. The percentage of arrested embryos per cycle ranged from 7 to 51.4%. During the biopsy procedure 422 nucleated (65%) and 227 anucleated blastomeres were counted.

An average of 5.5 blastomeres per embryo was biopsied. Only 384 cells (59.2%) showed to be clearly analyzable because the remainder were either damaged or lost during the biopsy and fixation procedure, covered with too much cytoplasm and not analyzable, showed no detectable signal or unclear signals (spots to close to the vicinity, lysed signals, overlapping signals/cells, or probe inefficiency), and could not be located on the glass slides.

Table 1 presents the observed chromosomal abnormalities according to the maternal age. Thirty-two percent of all analyzable embryos showed a normal (euploid) result and 68% were abnormal. Twenty-one embryos displayed inconclusive results.

A total of 67 embryos showed abnormal results, 11 (16.4%) were aneuploid for all analyzed blastomeres (5 monosomies, 4 trisomies and 2 double trisomies), 11 showed mosaicism (3 with trisomy/euploid mosaicism, 1 with polyploid/euploid mosaicism, 2 with chaotic/euploid mosaicism, 3 with monosomy/euploid mosaicism, and 2 with trisomy/monosomy mosaicism), 3 were haploid, 20 (29.9%) were polyploid (ranging from 3 N to 25 N) and 22 (32.8%) embryos showed a chaotic chromosomal complement.

There was no preferential malsegregation of one of the analyzed chromosomes, and no statistically significant difference in the frequency or type of abnormality was observed.

Figure 1 shows the FISH images of different blastomeres displaying various types of chromosomal abnormalities.

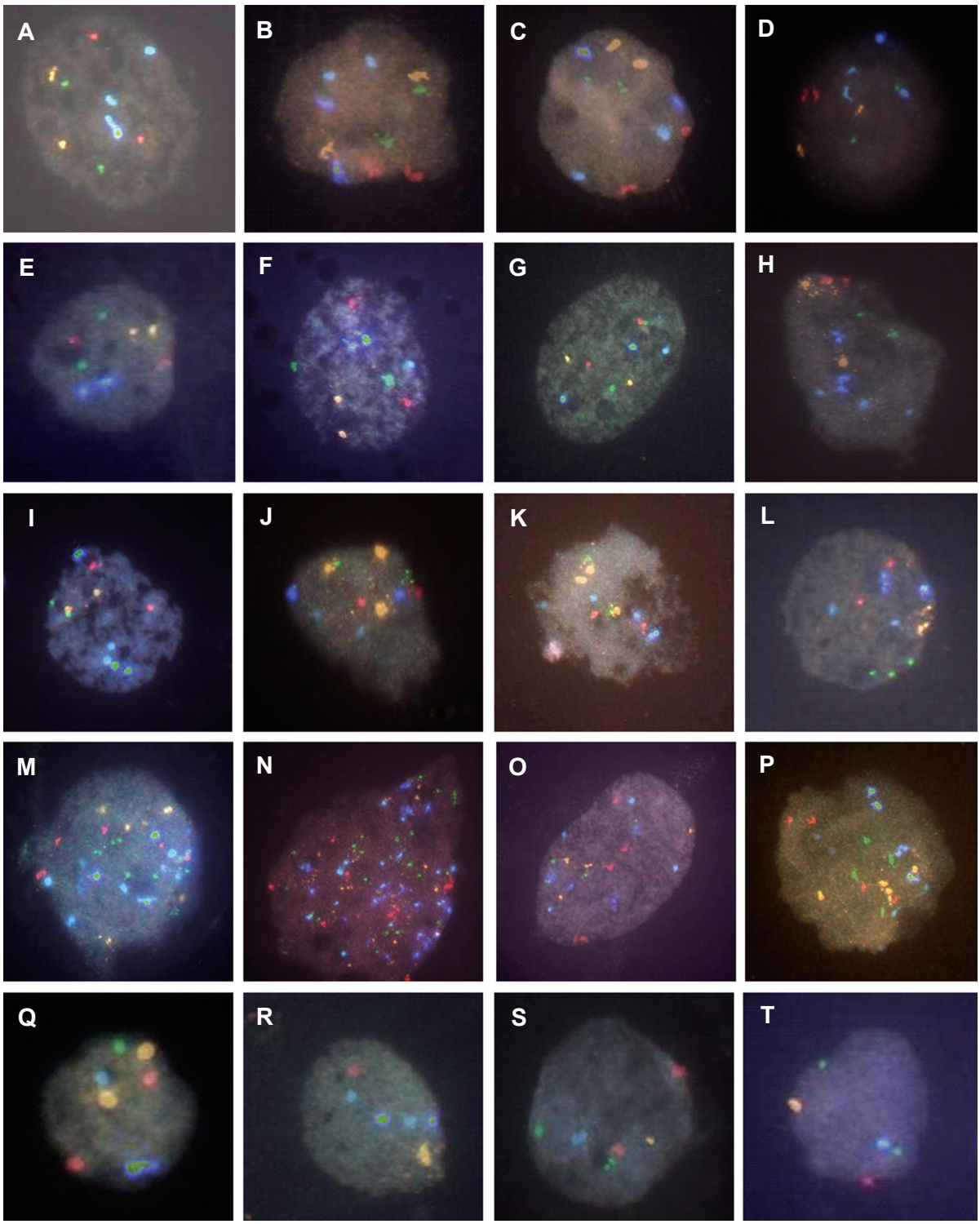

Fig.1: FISH images of different blastomeres displaying various abnormalities. **A-C.** Euploid blastomeres with normal FISH signal patterns for chromosomes 13 (red), 16 (aqua), 18 (blue), 21 (green) and 22 (gold), **D.** Interphase cell of a control lymphocyte, **E.** Nullisomy 16 - no signal for chromosome 16 (aqua), **F-G.** Monosomy 16-only one signal for chromosome 16 (aqua), **H.** Trisomy 16-three signals for chromosome 16 (aqua), **I.** Trisomy 18-three signals for chromosome 18 (blue), **J-K.** Trisomy 22-three signals for chromosome 22 (gold), **L.** Trisomy 21- three signals for chromosome 21 (green), **M-P.** Different types of polyploidy and **Q-T.** (chaotic) mosaic (haploid/diploid).

Table 1: Chromosomal abnormalities according to maternal age

Age (Y)	Cycles	Number of embryos	Mean value number of arrested embryos per cycle	Standard deviation	Number of embryos diagnosed	Inconclusive embryos	%	Normal embryos	% (of all embryos with result)	Abnormal embryos	% (of all embryos with result)	Aneuploid	% (of all abnormal embryos*)	Chaotic	% (of all abnormal embryos*)	Mosaic	% (of all abnormal embryos*)	Polyploid	% (of all abnormal embryos*)	Haploid	% (of all abnormal embryos*)
26 - 30	11	39	3.5	*1.9*	33	6	15.4	13	39.4	20	60.6	4	20	4	20	5	25	6	30	1	5
31 - 35	11	35	3.2	*2.7*	28	7	20	11	39.3	17	60.7	1	5.9	9	52.9	3	17.6	2	11.8	2	11.8
36 - 40	15	29	1.9	*1*	27	2	6.9	5	18.5	22	81.5	5	22.7	7	31.8	3	13.6	7	31.8	0	0
41 - 47	8	16	2	*1.1*	10	6	37.5	2	20	8	80	1	12.5	2	25	0	0	5	62.5	0	0
Total count∑	45	119			98	21	17.65	31	31.6	67	68.4	11	16.4	22	32.8	11	16.4	20	29.9	3	4.5

*; In the corresponding age group.

Discussion

The high rate of chromosomal abnormalities may either be an artefact of *in vitro* manipulation induced by artificial conditions in the IVF laboratory or a physiological state of embryonic development (16).

Munné et al. (17) stated that there may be a relationship between early chromosomal disorders and specific reproductive technologies, and found different rates of mosaicism at different IVF centres ranging from 11 to 52%.

IVF methods like ovarian stimulation may influence the aneuploidy rate. Verpoest et al. (18) conducted a small study analyzing unstimulated IVF cycles with patients with a low mean age (31.4 years), but even in this cohort, the aneuploidy rate was rather high (36.4%).

Labarta et al. (19) conducted a very elegant study with oocyte donors comparing chromosomal abnormalities in unstimulated and stimulated cycles in the same patient. Intrasubject comparison revealed abnormality rates of 34.8% in unstimulated and 38.2% in stimulated cycles, leading to the conclusion that moderate ovarian stimulation in young normo-ovulatory women does not significantly increase aneuploidy rates in embryos.

The overall fertility rate of the human population is low, the natural abortion rate is very high, and even during natural conception cycles, a number of chromosomal abnormalities occur. Aneuploidy occurs in approximately 0.3% of all newborns and 4% of stillbirths, while 35% of spontaneous abortions exhibit chromosomal errors, leading to the estimation that 5% of all human conceptions are aneuploid (20). The range of chromosomal abnormalities in human preimplantation embryos varies from 15% to over 85% (21, 22).

Aneuploidy may occur for a number of reasons, such as the inappropriate attachment of chromosomes to the mitotic spindle, partial inactivation of spindle checkpoint proteins or the amplification of centrosomes (23).

Vanneste et al. (24) showed in a study of normal fertile couples with a risk for inherited genetic diseases that only 9% of all generated IVF embryos had a normal chromosomal complement in all blastomeres and that nearly half of the embryos had no normal blastomeres. The study of normally conceived *in vivo* embryos is not possible; therefore, artificially produced chromosomal abnormalities cannot be excluded and the *in vivo* and *in vitro*

data cannot be compared. Moreover, mouse models are not very representative of humans because the aneuploidy rates of mice are low compared to humans (16).

In our cohort study, an abnormality rate of 68% was observed, and the percentage of abnormal embryos may actually be higher because only 5 chromosomes were analyzed and the apparently euploid embryos may be aneuploid for the other chromosomes not analyzed (25).

Of all the abnormal embryos, 29.9% were polyploid [more than two haploid (n) sets of chromosomes], ranging from 3N (triploid) to 25N, and there were many chaotic mosaic polyploidies. Reasons for this observation, such as polyspermic fertilization, are unlikely because only 2 PN were observed. Tri- and tetraploidy were the most commonly observed ploidies. Polyploidies may be a physiological phenomenon during preimplantation development (26). Tetraploidy can arise via different mechanisms, including cell fusion, endoreduplication and cytokinesis failure (23). FISH artefacts are unlikely because the polyploid chromosome patterns involve multiple chromosomes (25). A possible explanation is that these embryos stopped cell division, but continued DNA synthesis (9, 27).

Of the analyzed arrested embryos with abnormal results, 16.4% displayed two-cell line mosaicism and 32.8% displayed chaotic mosaicism. Human embryos display a high rate of mosaicism during all developmental stages (28) and this is of great importance for preimplantation genetic diagnosis, because the chromosomal status of an embryo is determined by only a single cell from the specific embryo (29). Meiotic errors lead to complete aneuploidies, whereas mitotic malsegregation results in mosaic aneuploidies, and postzygotic mitotic errors lead to aneuploid mosaics (25). The results of this study confirm previously published results demonstrating that post-meiotic abnormalities, such as polyploidies and mosaicism increase with decreasing embryonic development and that post-meiotic abnormalities and not aneuploidies are the most frequent outcome (7). A high percentage of mosaic embryos have diploid cells as the primary cell line (29). In their study, Daphnis et al. (28) describe diploid/aneuploid mosaicism as the predominant type of mosaicism. Chaotic mosaicism is more frequent in developmentally arrested embryos, and arrested embryos show higher proportions of abnormal cells in mosaics than non-arrested embryos. The developmental potential of mosaic embryos depends on the type and proportion of non-diploid cells with the higher the number of blastomeres containing abnormalities, the smaller the developmental capacity. Diploid-haploid mosaicism in fetal tissues has never been described, leading to the conclusion that these types of embryos are eliminated during the earliest stages of embryonic development (21).

Aneuploid mosaicism did not increase with maternal age, and the rate of mosaic embryos decreased by age instead. However, mitotic non-disjunction has been associated with maternal age (30), leading to mosaics with trisomic and monosomic cell lines, caused by a reciprocal gain or loss in daughter cells (28). Two of this type of mosaics were observed in our cohort study, both in the 35-40 year age group.

The high rate of chaotic embryos is noteworthy, because these embryos show various chromosomal imbalances that vary from cell to cell with no clear mechanisms for the malsegregation. Furthermore, the extensive imbalances are incompatible with normal preimplantation development (21). Chaotic embryos have very low developmental competence, and development beyond implantation and to the blastocyst stage is unlikely (14); therefore, the high rate of arrested embryos is not unexpected. The high rate of aberrations and the chaotic pattern somewhat resembles the chaotic situation of cancer cell lines and should normally be avoided by cell cycle checkpoints, which would ensure normal chromosomal numbers in daughter cells.

According to these results, evaluating only on a single cell of an eight-cell embryo is a poor representation of the entire embryo. Based on this one cell, embryos can only be graded as "normal" or "abnormal". It is not possible to distinguish between aneuploidy or mosaic embryos (7) because one cell cannot represent mosaicism (31).

Furthermore, the day of transfer plays an important role during the selection of the most suitable embryo. When transferring on day 2, it is not possible to distinguish between slowly developing and arrested embryos (32).

Euploid embryos have higher blastocysts rates

than chromosomally abnormal embryos (1); however, extended culture to the blastocyst stage is not reliable for selecting against chromosomally abnormal embryos, because even if there is strong selection against abnormal embryos, aneuploid embryos may also survive and develop into normal blastocysts (33, 34).

Conclusion

Arrested embryos are a good representation of the natural negative selection against aneuploid embryos prior to preimplantation; however, nearly 32% of euploid embryos in the arrested cohort demonstrate that embryonic development depends on many factors and that embryonic arrest is caused by a wide variety of factors (3), with genetics being only one of many.

In the context of preimplantation genetic diagnosis, our study vividly demonstrates how genetically heterogeneous human embryos can be, arrested or not, and that FISH analyses of single blastomeres have significant limitations. Array-based genotyping methods (35) on other embryonic tissues may be able to overcome these problems and identify the genetic composition of preimplantation embryos and its impact on embryonic development.

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Original Article

Follicle Development of Xenotransplanted Sheep Ovarian Tissue into Male and Female Immunodeficient Rats

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Abstract

Background: This study aimed to assess follicle survival after xenotransplantation of sheep ovarian tissue into male and female immunodeficient rats. We evaluated the effects of gonadotropin treatment on follicular development in the transplanted tissue.

Materials and Methods: In this experimental study, sheep ovarian cortical strips were transplanted into the neck back muscles of 8 male and 8 female immunodeficient, castrated rats. Fourteen days after surgery, each rat was treated with human menopausal gonadotropin (hMG) for 9 weeks. One day after the last injection, ovarian tissues were removed and fixed for histology assessment. Histology analyses were performed before and after grafting. Estradiol (E_2) levels were measured before and after gonadectomy, and at the end of the experiment. The control group consisted of 7 male and 7 female non-castrated/non-grafted rats and the sham group comprised 7 male and 7 female castrated/non-grafted rats for comparison of serum E_2 concentrations.

Results: The percentage of primordial follicles decreased after transplantation in male (25.97%) and female (24.14%) rats compared to the control group (ovarian tissue non-grafted; 37.51%). Preantral follicles increased in the male (19.5%) and female (19.49%) transplanted rats compared to the control group (11.4%). Differences in antral follicles between male ($0.06 \pm 0.0\%$) and female ($0.06 \pm 0.0\%$) rats were not noticeable compared to control ($1.25 \pm 0.0\%$) rats. We observed a significantly higher percent of mean E_2 secretion in grafted males compared to grafted females ($P<0.05$).

Conclusion: Despite significant differences in E_2 secretion between xenografted male and female rats, we observed no statistical differences in terms of follicular development.

Keywords: Follicle, Rat, Sheep, Xenotransplantation

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Introduction

Transplantation of a reproductive organ is one treatment for sterility. Transplantation of vitrified-warmed ovarian tissue from the donor female to herself can revive fertility. Inability to transplant ovarian tissue from the donor female or risks of disease relapse often prohibit autotransplantation (1-3). Xenotransplantation can remove transfer risk or relapse of cancer cells that may occur with autotransplantation. Xenotransplantation of ovarian tissue not only provides the possibility of access to gametes for reproduction

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from vitrified ovarian tissue, but also acts as a tool to understand the mechanism of follicular development (4-8). Van den Broecke et al. (9) have shown that follicular growth reduced 48 hours after xenotransplantation through hypoxia before angiogenesis. In order to resolve this problem, some researchers advocate the use of hormone therapy (2, 9). Different gonadotropins at several doses are used for resumption of follicle growth in transplants. In mammals, development of antral follicles during the ovulatory phase is dependent on gonadotropin secretions of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the anterior pituitary gland (9). However, other studies have reported the combined effect of gonadotropins exogenous injection in mice castrated have the same effect as those produced by the pituitary gland. Injections of gonadotropins have the same effect as those produced by the pituitary gland (2, 10-13). Maltaris et al. (14) reported that increasing the dose of human menopausal gonadotropin (hMG) hormone injection two weeks after transplantation in gonadectomy mice resulted in better growth of primary follicles up to the antral follicle stage.

The present study aimed to determine the optimal transplant conditions for maintenance, growth and maturation of follicles after xenotransplantation of fresh ovarian tissue from sheep into immunodeficient rats. We evaluated the ovarian tissue response to exogenous gonadotropin stimulation.

Materials and Methods

To follow-up the current experimental study, all chemicals were purchased from Sigma (USA) and Gibco (Life Technologies Ltd., UK), with the exception of hMG and human serum albumin (HSA) which were procured from Organon, Oss, Holland and from Biotest, Germany, respectively. All animal procedures were approved by the Ethical Committee at Royan Institute.

Sheep ovarian tissue preparation

Sheep ovaries were procured from a slaughterhouse in Shahriar (Iran). Ovaries were transported in phosphate buffered saline medium (DPBS, 14190, Gibco) supplemented with 50 mg/ml streptomycin and 60 IU/ml penicillin (Gibco) at 39˚C. In the laboratory, the ovaries were washed three times with fresh DPBS and placed on a sterile Petri dish that contained HEPES tissue culture medium (HTCM) supplemented with 10% HSA. Next, we separated the medulla from the cortex. The cortex was cut into slices with approximate dimensions of 1 mm (length)×2 mm (width)×2 mm (thickness). Sections were placed in fresh HTCM medium supplemented with 10% HSA. The ovarian pieces were washed three times with TCM. One or two pieces of ovarian cortex were fixed in Bouin's solution as a fresh control for morphological and functional analyses (15).

Experimental design

We evaluated the host gender (male vs. female rats) on follicular development in xenografted sheep ovarian tissue. In this study, 8 sheep ovaries were used. Each ovary was segmented into 3 parts and divided into 3 groups, a control (intact non-grafted) and two experimental (XM=xenografted in male and XF=xenografted in female). The control group consisted of 7 male and 7 female non-castrated/non-grafted rats and the sham group comprised 7 male and 7 female castrated/non-grafted rats for comparison of serum estradiol (E_2) concentrations.

Immunosuppressed rats

Sixteen female and male Wistar rats were housed in standard cages under a 12-hour light/dark regime at 24-27˚C. The rats were immunesuppressed by the addition of 210 mg/l of cyclosporine-A (Sandimmune, Novartis Pharmaceuticals, USA) to their drinking water for five days before xenotransplantation of the ovarian tissues. The rats remained on the same immunosuppressant throughout the experiment. Rats had free access to sterilized food and water. Serum levels of cyclosporine with the average of concentration 1750 ± 34 ng/ml were measured in a random sample of rats (16).

Castrated rats

The immunosuppressed rats were anesthetized with intraperitoneal (i.p.) injections of 50 mg/kg bodyweight of 10% ketamine (Alfasan,

Woerden, Holland) and 5 mg/kg xylazine (2%, Alfasan, Woerden, Holland). Gonadectomy was performed through a dorsomedian incision in female rats and through a ventromedial incision in male rats.

Transplantation

For transplantation, we used anesthetized, castrated rats. Surgery procedures were carried out under a laminar flow hood under aseptic conditions. One piece of ovarian tissue was placed in each rat's neck muscle. The muscle was subsequently sutured with absorbable thread.

Hormone stimulation

Each female and male rat received i.p. injections of hMG (5 IU FSH / 5 IU LH) every second day starting from day 14 after transplantation for 9 consecutive weeks. This dose was adjusted from an earlier study by Kagabu and Umezu (17).

Estradiol determination

We measured E_2 levels before and after the gonadectomy, and at the end of the experiment. Rats were anesthetized, after which 800-1000 µl of blood was obtained using an orbital sinus puncture technique for E_2 measurement. Blood was allowed to clot at room temperature (24˚C) for 30 minutes, and then centrifuged at 3000×g for 5 minutes for serum collection. E_2 was determined by an ELISA Kit (ABIN416279) according to the manufacturer's instructions.

Histological assessment

Ovarian tissue from the experimental (XM, XF) and control (intact non-grafted) groups were fixed in Bouin's solution, embedded in paraffin wax, serially sectioned at 6 µm, and stained with hematoxylin and eosin. The numbers of morphologically normal and degenerated follicles were counted in all prepared slides from each group, which included 24 total sections. All sections were studied using a light microscope (Olympus CX31, Philippines) at a magnification of ×200. According to Liu et al. (18) "follicles were classified as follows: i. Primordial follicles with one layer of flattened pregranulosa cells that surrounded the oocytes; ii. Primary follicles with one layer of cuboid granulosa cells, iii. Preantral follicles with two or more layers of granulosa cells and no antrum and iv. Antral follicles with an antral cavity (17)". Eosinophilia of the ooplasm, and wrinkling of nuclear membrane of the oocytes were considered as signs of atresia (15).

Statistical analyses

Percentages of growing follicles and E_2 concentrations in each group were analyzed using one-way ANOVA. Numbers of follicles between experimental groups were analyzed according to the Kruskal-Wallis test. A value of $P<0.05$ was considered statistically significant.

Results

Surgery recovery

Tissue from 16 sheep ovaries were transplanted into 16 gonadectomy rats (8 male and 8 female). However, after surgery, 7 male and 6 female rats survived. Therefore we evaluated tissue from 7 control group ovaries (ovarian tissue non-transplanted), 6 ovaries transplanted into female rats, and 7 ovaries transplanted into male rats.

Histological evaluation

Histological assessment of the ovarian tissues showed a non-significant decrease in the percentage of primordial follicles in the XM (25.97 ± 0.03%) and XF (24.14 ± 0.03%) groups compared to the control group (37.51 ± 0.05%). The percentage of primary follicles non-significantly increased in both the XM (47.7 ± 0.03%) and XF (48.85 ± 0.04%) groups compared to the control group (43.22 ± 0.04%). The lowest percentage of preantral follicles was in the control group (11.4 ± 0.03%) compared to the XM (19.5 ± 0.02%) and XF (19.49 ± 0.02%) groups. There were no significant differences in preantral follicles between the groups. There was no significant difference in terms of follicle degeneration among the groups (Table 1, Fig.1). Differences of antral follicles in XM (0.06 ± 0.0%) and XF groups (0.06 ± 0.0%) were not noticeable compared to the control group (1.25 ± 0.0%).

Table 1: Mean percentage of viable follicles from sheep ovarian tissue grafts at nine weeks after xenotransplantation among the control, male xenograft (XM), and female xenograft (XF) groups

Follicles	Primordial (%)		Primary (%)		Preantral (%)		Antral (%)	
Experimental groups	Normal	Degenerated	Normal	Degenerated	Normal	Degenerated	Normal	Degenerate
Control (non-grafted)	37.51 ± 0.05	2.07 ± 0.0	43.22 ± 0.04	3.28 ± 0.0	11.4 ± 0.03	0.85 ± 0.0	1.25 ± 0.0	0.0 ± 0.0
XM	25.97 ± 0.03	2.01 ± 0.01	47.7 ± 0.03	2.92 ± 0.01	19.5 ± 0.02	1.41 ± 0.0	0.06 ± 0.0	0.0 ± 0.0
XF	24.14 ± 0.03	1.66 ± 0.0	48.85 ± 0.04	4.0 ± 0.0	19.49 ± 0.02	1.31 ± 0.0	0.06 ± 0.0	0.0 ± 0.0

Analysis was performed using the Kruskal-Wallis test.

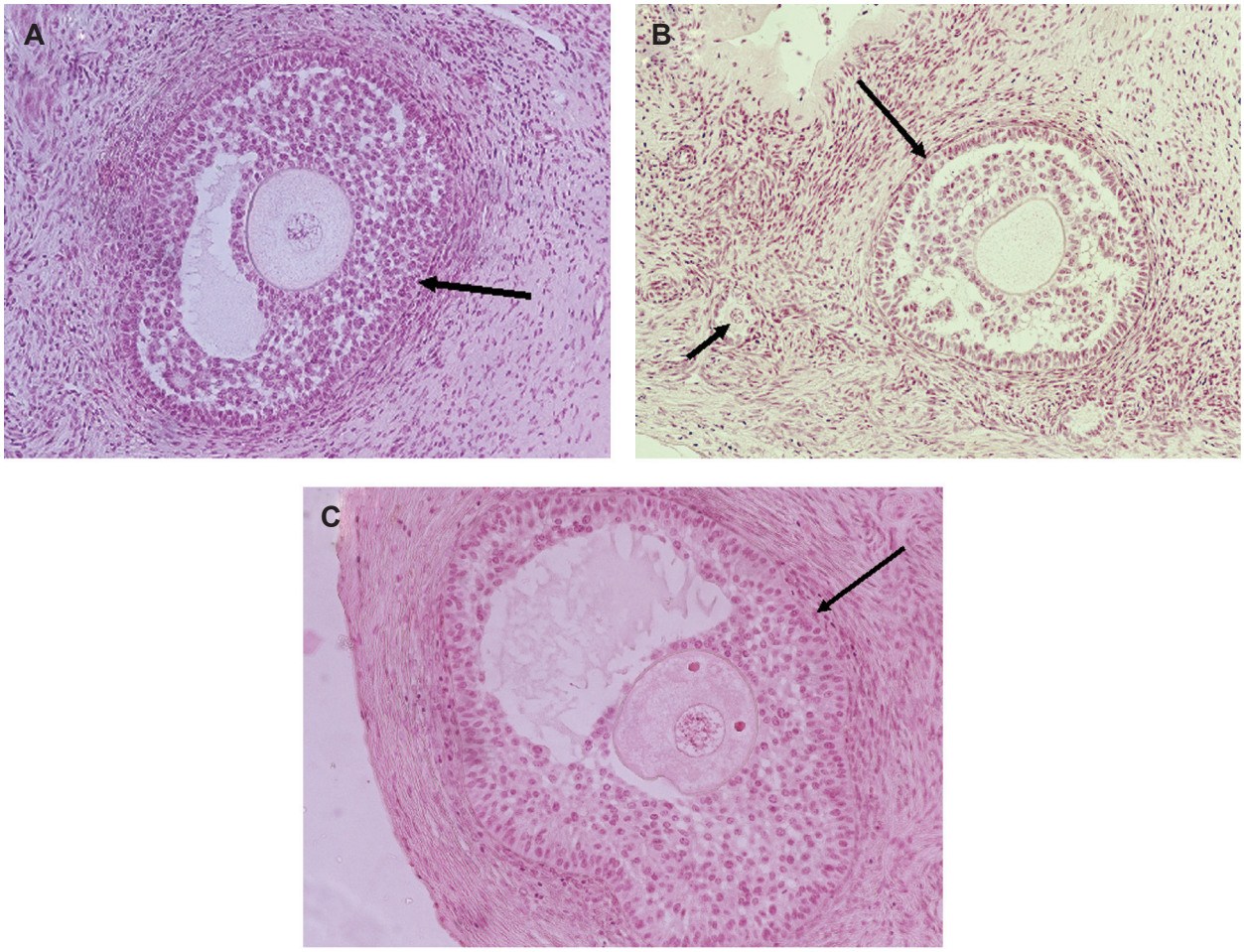

Fig.1: Hematoxylin–eosin staining. Histology staining of antral follicles in **A.** Control group (ovarian tissue non-grafted), **B.** Xenotransplanted sheep ovarian tissue into females (large arrows; Antral follicle and small arrow; Primary follicle) and **C.** Xenotransplanted sheep ovarian tissue into males (magnification: ×200).

Serum estradiol measurement

We observed a significant difference in E_2 levels (pg/ml) between the XM (45.44%) and XF (33.36%) groups. The XM group (45.44%) compared to the sham male (22.71%) and female (25.96%) rats significantly differed (P<0.05, Fig.2).

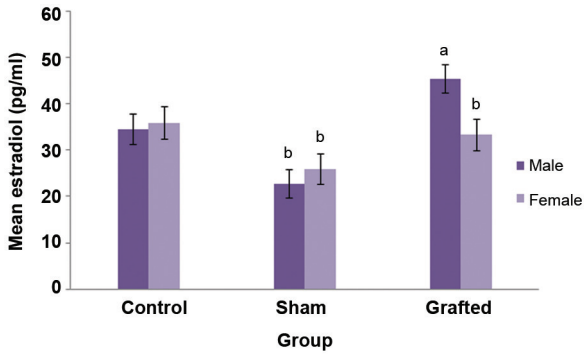

Fig.2: Estradiol (E_2) secretion rate in the control and sham groups. Control group included 7 male and 7 female non-castrated/non-grafted rats. Sham group included 7 male and 7 female castrated/non-grafted rats. In our xenograft model, there were 7 males and 7 females. Analysis was carried out using one-way ANOVA with a random effect. E_2 level with different superscripts is significantly different (a, b) (P<0.05).

Discussion

In this study, we have evaluated the survival and growth of sheep primordial follicles after xenotransplantation of sheep ovarian tissue into immunodeficient rats. Xenotransplantation of ovarian tissue is a promising procedure to preserve endangered animal species and a useful investigative tool to understand follicular development and ovarian physiology (12, 18). Several breeds of animals with suppressed immune systems can act as recipients for xenotransplantation (19-21).

In this study, we used rats as the host and incubator for follicular development. Rats were immunosuppressed following administration of cyclosporine A. To ensure the absence of rejection before xenotransplantation, each rat underwent blood testing. A study by Aubard (22) reported that the immune system of the recipient could be weakened during xenotransplantation by turning off the recipient's blood immune response. With the immune system of the rats weakened with cyclosporine A, the rats were susceptible to infection and various diseases which resulted in the deaths of a number of rats. The percentage of follicles that survived after transplantation decreased in both male and female rats compared to the control group. The percentage of deaths and illnesses were higher in female rats compared to males. Ischemia-perfusion injury caused during the revascularization process was assumed to be the principal factor responsible for a severe reduction in the primordial follicle population in transplanted ovarian tissue (2, 9, 12, 14, 23).

In an earlier study, primary follicles were expected to be found after xenotransplantation of human ovarian tissue to immunodeficient mice; however, antral follicles were observed just after injection of exogenous FSH (23). In fresh primate ovarian tissue xenotransplantation, FSH could improve the follicular number and their morphology by preserving the resting follicle pool. In another study administration of long-term FSH decreased the number of primordial follicles in xenografts of cryopreserved human ovarian tissue (24, 25). Therefore, in the present study, we used hMG, not only as an exogenous gonadotropin, but also as a factor in angiogenesis to promote follicular development in grafted ovarian tissues according to Maltaris et al. (14). We have shown that exogenous gonadotropin increases the number of primary and preantral follicles rather than the resting follicles. A number of studies reported positive effects of mouse gonad removal and exogenous gonadotropin injections on the development of follicles in the ovary (2, 12-14). Induction of revascularization and decrease in ischemia-perfusion are the best routes to maximize follicular survival. Recent studies (26, 27) have shown a direct effect of gonadotropins on induction of endothelial cells for revascularization. Gonadotropins, including human chorionic gonadotropin (hCG), LH and FSH are factors for tissue-specific angiogenesis and hormones that regulate the vascular system (26).

Other studies observed that male mice, with high concentrations of androgen, were better hosts for the development of growing follicles than female mice (28, 29). They assumed that production of endogenous androgens could act as a substrate for estrogen, which could protect the antral follicles (2, 12, 13, 28, 29).

However, this supposition did not apply in our study because the gonads were removed along with transplantation in both male and in female immunodeficient rats. In addition, hMG hormone injection in castrated male rats affected the hypothalamus and caused the secretion of gonadotropin-releasing hormone (GnRH). This hormone subsequently stimulates the pituitary gland and causes a normal rate LH and FSH secretion, whereas increasing hormone dosage in castrated female rats may stop or slow down the hormonal cycle. The percentage of follicles between transplanted groups and the control group showed no significant difference. The percent mean E_2 secretion in grafted males was significantly higher than grafted females ($P<0.05$).

Conclusion

Despite significant differences in E_2 value (pg/ml) between xenografted male and female rats, we observed no statistical difference in terms of follicular development. We found no justification for follicular development and increasing E_2 levels.

In general, castrated male rats, due to increased levels of E_2 and a better survival rate, are likely to be better candidates for supporting ovarian xenotransplantations compared to castrated female rats.

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Original Article

Activation and Inhibition of The *Wnt3A* Signaling Pathway in Buffalo (*Bubalus bubalis*) Embryonic Stem Cells: Effects of WNT3A, Bio and Dkk1

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Abstract

Background: This research studies the effects of activation and inhibition of *Wnt3A* signaling pathway in buffalo (*Bubalus bubalis*) embryonic stem (ES) cell-like cells.

Materials and Methods: To carry on this experimental study, the effects of activation and inhibition of *Wnt3A* signaling in buffalo ES cell-like cells were examined using Bio (0.5 mM) combined with WNT3A (200 ng/ml), as an activator, and Dickkopf-1 (Dkk1, 250 ng/ml), as an inhibitor, of the pathway. ES cells were cultured up to three weeks in ES cell medium without fibroblast growth factor-2 (FGF-2) and leukemia inhibitory factor (LIF), but in the presence of Bio, WNT3A, Bio+WNT3A and Dkk1. The effects of these supplements were measured on the mean area of ES cell colonies and on the expression levels of a number of important genes related to pluripotency (*Oct4, Nanog, Sox2* and *c-Myc*) and the *Wnt* pathway (*β-catenin*). ES cell colonies cultured in ES cell medium that contained optimized quantities of LIF and FGF-2 were used as the control. Data were collected for week-1 and week-3 treated cultures. In addition, WNT3A-transfected ES cells were compared with the respective mock-transfected colonies, either alone or in combination with Dkk1 for expression of *β-catenin* and the pluripotency-related genes. Data were analyzed by ANOVA, and statistical significance was accepted at $P<0.05$.

Results: Among various examined concentrations of Bio (0.5-5 mM), the optimum effect was observed at the 0.5 mM dose as indicated by colony area and expressions of pluripotency-related genes at both weeks-1 and -3 culture periods. At this concentration,the expressions of *Nanog*, *Oct3/4, Sox2, c-Myc* and *β-catenin* genes were nonsignificantly higher compared to the controls. Expressions of these genes were highest in the Bio+WNT3A treated group, followed by the WNT3A and Bio-supplemented groups, and lowest in the Dkk1-treated group. The WNT-transfected colonies showed higher expressions compared to both mock and Dkk1-treated mock transfected colonies.

Conclusion: WNT3A functions to maintain the pluripotency of ES cell-like cells both as an exogenous growth factor as well as an endogenously expressed gene. It complements the absence of FGF-2 and LIF, otherwise propounded essential for buffalo ES cell culture. WNT3A antagonizes the inhibitory effects of Dkk1 and acts in combination with its activator, Bio, to activate the *Wnt* signaling pathway.

Keywords: WNT3A, Buffalo, Embryonic Stem Cells, Bio, Dkk1

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Introduction

Buffalo embryonic stem (ES) cell-like cells are derived from the inner cell mass of the blastocysts and can be maintained in culture conditions that retain their pluripotency. Among the various intrinsic and extrinsic factors that maintain ES cell pluripotency, leukemia inhibitory factor (LIF) and fibroblast growth factor-2 (FGF-2) are well characterized (1). These are required either alone, for example LIF in case of mouse ES cells and FGF-2 in human ES cells, or in combination as in the case of buffalo ES cells.

Though feeder-dependent cultures are presumed to be better for long-term cultures, conditioned media (cm) from mouse embryonic fibroblast (MEF) can support the self-renewal of ES cells and eliminate the need for a feeder layer. It has been demonstrated that MEF inhibits ES cell differentiation via production of the IL-6 family cytokine and LIF (2). LIF binds the heterodimeric LIF receptor-glycoprotein 130 (gp130) complex and activates Jak kinases with recruitment of *Shp-2* and signal transducer and activator of transcription 3 (STAT3) (3) while FGF-2 signals are transduced through receptors with intrinsic protein tyrosine kinase activity (4-6).

FGF-2 supplementation is associated with pleiotropic-positive effects: impeding spontaneous differentiation, increasing human ES cell proliferation, enhancing attachment/survival, inhibiting earliest neural induction, and, more precisely, moderately stimulating *Nanog* gene expression. In contrast, the FGF/ERK cascade plays a role in the differentiation of mouse ES cells (7). Since increased telomerase activity is presumed to be pivotal for ES cell self-renewal, the study of the pathways that control telomerase activity has gained a considerable interest in stem cell studies. Among the various studies reported so far in this context, a molecular link between *Wnt/β-catenin* signaling and the expression of the telomerase subunit *Tert* has gained a considerable interest owing to contrasting associations of *Wnt* signaling with both proliferation and differentiation of ES cells.

WNT genes, of which the human genome harbors almost 20, occur throughout the animal kingdom (8). The proteins constitute a family of cysteine-rich secreted ligands essential for a wide array of developmental and physiological processes. The intracellular signaling pathway activated by WNT has been originally identified as a β-catenin-dependent pathway that is highly conserved among various species. WNTs act through the cytoplasmic protein Dishevelled (Dsh) to inhibit the activity of the serine-threonine kinase, GSK3-β, which otherwise bind to the β-catenin-APC complex through Axin, leading to β-catenin phosphorylation and rapid degradation. WNT-induced inhibition of GSK3-β causes β-catenin stabilization which results in its increased level in the uncomplexed soluble form. This latter form can interact with TCF/LEF transcription factors and, after translocation to the nucleus, activate target genes such as *Myc, CyclinD1, Axin2* and *Siamois*. Most of these genes have one or more TCF-binding elements near the transcription start site in their promoter region and play roles in the regulation of gene expression, cell proliferation, differentiation, and maintenance of cell polarity (9, 10).

Wnt signaling has been shown to play a role in the regulation of self-renewal of both mouse and human ES cells independently of LIF/STAT3 signaling. It is associated with both proliferation and differentiation of ES cells and therefore, the role of *Wnt* signaling in ES cells remains controversial (11).

Sato et al. (12) have found that *Wnt* pathway activation by Bio, a specific pharmacological inhibitor of GSK3-β, maintains the undifferentiated phenotype in both types of ES cells and sustains expression of the pluripotent state-specific transcription factors such as *Oct3/4, Rex-1* and *Nanog* (13). Hence, low GSK3 activity could be an absolute requirement for pluripotency and ES cell self-renewal (14). Using a high-throughput cell-based assay, Miyabayashi et al. (11) have identified the small molecule Iq-1 that allows for *Wnt/β-catenin*-driven long-term expansion of mouse ES cells and prevention of spontaneous differentiation.

In addition to the GSK3-β/Axin/APC destruction complex, the *Wnt* pathway is also controlled by extracellular antagonists such as Wnt inhibitory signaling factor-1 (WIF1), Cerebrus, Sclerostin, Dickkopf-1 (Dkk1) and SFRP2 (15). Cerebrus, WIF1 and SFRP2 interact directly with WNT proteins, however Sclerostin and Dkk1 bind to LRP5/6 and indirectly exert their antagonizing effects (16). Different Frizzled-related protein and Dkk family members have shown opposite effects in a variety of *in vivo* and *in vitro* assays (17).

In order to investigate the effects of *Wnt* signaling on ES cells, the present study was designed to examine the effects of *Wnt3A* signaling activation on buffalo (*Bubalus bubalis*) ES cell-like cells, which would provide a higher mammalian model, by addition of Bio as the activator. To ensure that the effects are due to activation of *Wnt* signaling pathway, we used Dkk1 as the pathway inhibitor to examine the contrary effects and *Wnt 3A*, in both exogenous and endogenous forms, to corroborate the primary results.

Materials and Methods

Chemicals

To carry on this experimental study, unless mentioned otherwise, all culture media, growth factors, fetal bovine serum (FBS), Bio (B1686) and other chemicals were purchased from Sigma (USA) and plastic ware was purchased from Falcon (UK). Recombinant human WNT3A (5036-wn) and recombinant human Dkk1 (5439-DK) were purchased from R and D systems.

In vitro embryo production

Buffalo ovaries were obtained from a local abattoir and transported to the laboratory in phosphate-buffered saline that contained penicillin (100 IU/mL) and streptomycin (50 mg/mL) at 30-34˚C within 5 hours of slaughter. Cumulus-oocyte complexes (COCs), from follicles 2-8 mm in diameter, were aspirated using an 18 G needle attached to a 10 mL disposable syringe. A group of 15 to 20 excellent quality COCs were transferred to a 100 mL droplet of the *in vitro* maturation (IVM) medium [TCM 199+10% FBS+5 µg/mL porcine follicle stimulating hormone (pFSH)+1 µg/mL estradiol-17 β+0.81 mM sodium pyruvate+5-10% buffalo follicular fluid+50 µg/mL gentamicin sulfate] under mineral oil in a petri dish and cultured at 38.5˚C in a humidified atmosphere of 5% CO_2 for 24 hours. The *in vitro* matured (IVM) oocytes were washed twice with Bracket and Oliphant's (BO) medium and transferred to 50 µL droplets (15-20 oocytes/droplet) of the medium. The spermatozoa were prepared for fertilization as per the protocol established by Chauhan et al. (18). Oocytes were then inseminated by addition of spermatozoa at a final concentration of $1.0\text{-}2.0\times10^6$ motile sperm/mL. Sperm and oocytes were incubated under paraffin oil at 38.5˚C under a humidified atmosphere of 5% CO_2 for 18 hours. At the end of the interval, groups of 10 oocytes stripped free from cumulus cells were transferred into modified Charles Rosenkrans medium with amino acids (mCR2aa) that contained 0.6% bovine serum albumin (BSA) and cultured in this medium for the first 2 days, which was then replaced by *in vitro* culture (IVC) medium (mCR2aa+0.6% BSA+10% FBS). The culture medium was changed every 2 days up to 8 days until the blastocysts were obtained.

Establishment of buffalo embryonic stem cells

Buffalo ES cells were derived from the *in vitro* fertilized embryos as described by Muzaffar et al. (19). Briefly, we mechanically dissected the inner cell mass from the embryos under a zoom stereomicroscope and seeded them onto a mitomycin-treated buffalo fetal fibroblast feeder layer in ES medium that consisted of knockout Dulbecco's modified eagle medium (KO-DMEM, Gibco/BRL) supplemented with 15% knockout serum replacement medium (Gibco/BRL), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids (all from Gibco/BRL), 1000 U/mL LIF, and 5 ng/mL FGF-2 (R & D Systems). The media was changed every alternate day and the resultant colonies were sub-cultured onto fresh feeders after every 7 days. LIF and FGF-2 were added only to the control group media while the treatment group media lacked these factors. Bio, WNT3A, and Dkk1 were added to the latter media at every media change at their optimized concentrations.

Characterization of the embryonic stem cells

Alkaline phosphatase (AP) staining and immunofluorescence were used for characterization of buffalo ES cells, as per a previously described protocol (20). The cell surface antigens used for characterization comprised glycolipids: SSEA-1 and SSEA-4; keratan sulfate antigens: TRA-1-60 and TRA-1-81 (Chemicon, Millipore, Cat. No. SCR002) and the pluripotency markers Nanog (Santa Cruz, Cat. No. SC 134218), Oct3/4 (Chemicon, Millipore, Cat. No. SCR002) and Sox2 (Chemicon, Millipore, Cat. No. SC1002). We used a 1:50 dilution of primary antibodies while the appropriate flourescein isothiocyante (FITC)-conjugated secondary antibodies were diluted 1:500 in Dulbecco's phosphate-buffered saline (Fig.1).

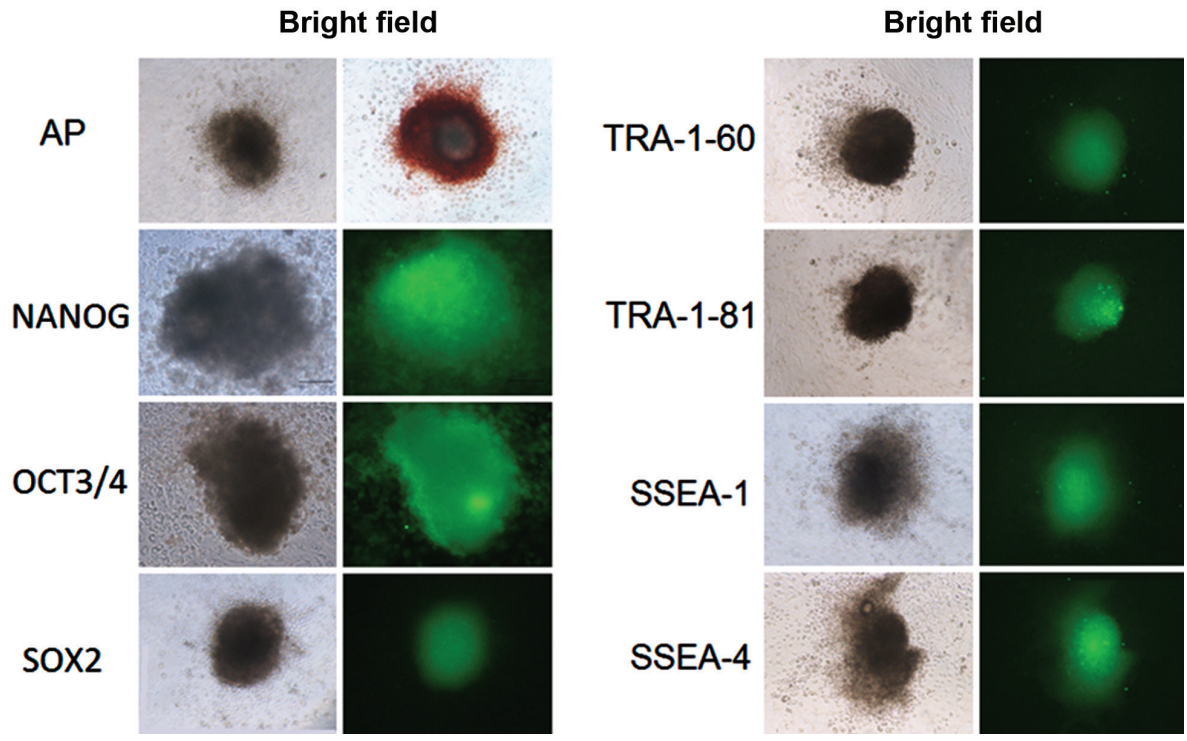

Fig.1: Alkaline phosphatase (AP) and immunofluorescence staining for characterization of buffalo embryonic stem (ES) cells at passage 20. Cell surface antigens SSEA-1 and SSEA-4, keratan sulfate antigens TRA-1-60 and TRA-1-81, and pluripotency markers Nanog, Oct3/4 and Sox2 were used to characterize ES cells.

Estimation of colony area

An inverted microscope (Nikon, Japan, Model Eclipse Ti 5), equipped with the software for calculation of the colony area was used to estimate the colony area.

RNA isolation, reverse transcription and quantitative real-time PCR (qPCR)

Total RNA was isolated with the Trizol reagent (Invitrogen) and subsequently treated with DNAse (Ambion, USA) to eliminate DNA contamination. Reverse transcription was performed with MMLV enzyme (USB) using oligo dT primers. qPCR was carried out with SYBR Green mix (ABI). Calculations were based on the ΔΔCt method taking GAPDH as the endogenous control. Primer sequences are listed in table 1.

Cloning of the *Wnt3A* gene

Primers were designed from *Bos taurus* full length *Wnt3A* gene sequence (Accession No. XM_002688509.1) using Primer 3 Software. The polymerase chain reaction (PCR) cycling conditions were 94˚C for 3 minutes, followed by a cycling program of 94˚C for 30 seconds, 60˚C for 45 seconds and 72˚C for 30 seconds for 36 cycles, and a final extension at 72˚C for 10 minutes. The amplified *Wnt3A* gene was analyzed on a 2% agarose gel. The PCR product was purified from the gel as per the manufacturer's protocol using the QIAquick Gel Extraction Kit (Cat. No. 28704) and ligated into a pDrive cloning vector according to the manufacturer's guidelines (Qiagen PCR Cloning Kit). The ligated product that equaled 5% of the cell volume was added to competent cells (*E.coli XL1*), the contents mixed and kept on ice for 30 minutes. Bacterial transfection was achieved by heat shock at 42˚C for 90 seconds, then chilled on ice for 2 minutes. Next, 800 µl of super optimal broth (SOC) was added to each tube followed by incubation in a shaking incubator at 37˚C for 42 minutes at 225 cycles/minute. The cells were then centrifuged at 3000 rpm for 3 minutes and the pellet was plated on an Luria Bertani (LB) agar plate for overnight incubation at 37˚C. The colonies which had the insert were verified by colony PCR and restriction enzyme

test digestion. The positive clones were propagated and subjected to plasmid isolation for sequencing of the insert. The insert in the correct reading frame was subsequently ligated into a pAcGFP1-N1 vector, as a GFP-fusion protein, employing *EcoR1* and *Bam H1* restriction sites for generation of cohesive ends. The ligation reaction was prepared as: vector (20-100 ng), insert DNA (3:1 to 5:1 molar ratio with vector), 10X T4 DNA ligase buffer (2 µL) and T4 DNA ligase (1 µL), and incubated for 10 minutes at 22˚C.

Overexpression of *Wnt3A* in buffalo embryonic stem cell-like cell colonies

ES cell colonies were mechanically divided into small parts, then cultured in 100 µL droplets of the ES cell culture medium for three days prior to transfection. The vector that contained the insert (pAcGFP1-N1+*WNT3A*, 2-5 µg) was diluted in 50 µL DMEM without serum and mixed with Lipofectamine™ 2000 (Invitrogen) also diluted in 50 µL of the medium. The mixture was incubated for 20 minutes at room temperature and used to replace the ES cell culture medium in which the ES cell colonies were growing. The cells were incubated at 37˚C in a CO_2 incubator for 18-48 hours, with daily media changes until GFP expression was evident. The colonies that expressed GFP were separated individually and used for further studies. The expression of Wnt3A in transfected colonies was further confirmed by RT-PCR (Fig.2).

Table 1: Real-time polymerase chain reaction (PCR) primers

Gene	Sequence	Annealing temp. (˚C)	Base pairs	Accession no.
Sox2	F: 5′CGTGGTTACCTCTTCTTCC3′	60	139	GQ85388
	R: 5′CTGGTAGTGCTGGGACAT3′			
Oct3/4	F: 5′TTGCAGCTCAGTTTCAAG3′	54	75	EU926737
	R: 5′GTTGTTGTCAGCTTCCTC3′			
Nanog	F: 5′CCGAAGCATCCAACTCTAGG3′	60	100	NM001025344.1
	R: 5′GAGACAGTGTCCGTGTCGAG3′			
c-Myc	F: 5′CTCCTCACAGCCCGTTAGTC3′	53	156	GU296437.1
	R: 5′ATTTGCGGTTGTTGCCTATC3′			
β-catenin	F: 5′ACAGAAAAGCAGCCGTCAGT3′	56	191	NM001076141.1
	R: 5′AGAAAACCCCTGTTCCCACT3′			
GAPDH	F: 5′TCAAGAAGGTGGTGAAGCAG3′	57	121	GU324291.1
	R: 5′CCCAGCATCGAAGGTAGAAG3′			

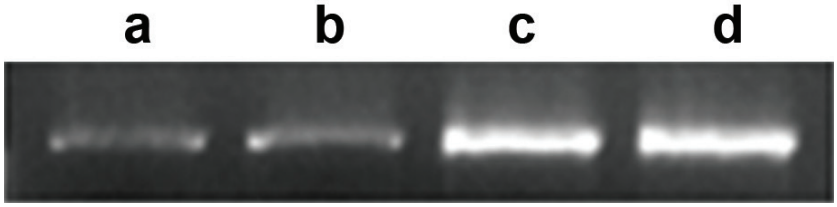

Fig.2: RT-PCR of transfected and non-transfected colonies. Agarose gel electrophoresis for *Wnt3A* expression (cDNA). Lane a; Control, Lane b; MOCK vector transfected colonies, Lanes c, d; *Wnt3A* transfected colonies and RT-PCR; Real-time polymerase chain reaction.

Experimental design

Experiment 1: Optimization of bio concentrations for buffalo embryonic stem cell culture

This experiment was performed with five doses to optimize the Bio concentration: 1. ES cell medium as a control, 2. ES cell medium+0.5 μM Bio, 3. ES cell medium+1 μM Bio, 4. ES cell medium+2 μM Bio and 5. ES cell medium+5 μM Bio.

Experiment 2: Effects of activation and inhibition of the *Wnt3A* signaling pathway on buffalo embryonic stem cells

Experiment 2.1

This experiment was performed to examine the effects of exogenous WNT3A and Wnt pathway activator and inhibitor on ES cell growth and stemness in1 and 3 week cultures. The treatments were as follows: 1. ES cell medium as a control, 2. ES cell medium+0.5 μM Bio, 3. ES cell medium+0.5 μM Bio+200 ng/ml WNT3A, 4. ES cell medium+250 ng/ml Dkk1 and 5. ES cell medium+200 ng/ml WNT3A.

Experiment 2.2

This experiment was performed to examine the effects of overexpression of WNT and its ability to counteract the inhibitory effects of Dkk1 on ES cell pluripotency and the *Wnt* pathway by comparing expression of pluripotency maintaining genes and β-catenin, respectively. The treatments were as follows 1. MOCK vector-transfected ES cells (control), 2. WNT3A-transfected ES cells and 3. Mock vector-transfected ES cells+Dkk1 250 ng/ml.

Statistical analysis

Data were analyzed with a statistical software program (SPSS 11.5, 2004). Comparisons between multiple numeric data sets were performed using one-way ANOVA followed by Duncan's multiple range test. The results were expressed as mean ± SEM and statistical significance was accepted at $P<0.05$.

Results

Effect of Bio on pluripotency and *Wnt* signaling

The ES cells exposed to Bio, in a dose-and time-dependent manner, showed optimum activity at a concentration of 0.5 μM for day 1 versus day 6 of week 1 as well as for day 1 versus day 6 of week 3 on cell proliferation (mean colony area), self-renewal (expression of pluripotency genes) and *Wnt* pathway activation (*β-catenin* expression). We observed no statistically significant difference in colony area at different concentrations of Bio (0.5, 1, 2 and 5 μM) on days 1 and 6 of the first week of culture, although a trend towards increase in the colony area was observed which indicated a stimulatory effect of Bio on cellular proliferation (Fig.3A). A similar trend which indicated an increase in the colony area was observed between days 1 and 6 of the third week of culture, with the exception of the 5 μM Bio concentration when mean colony area was smaller, with a statistically significant difference in the area (Fig.3A´). Real-time PCR analysis showed that the expressions of *β-catenin* and pluripotency genes were relatively higher at the 0.5 μM Bio concentration compared to the other doses. At this concentration of Bio, the treatment groups exhibited almost similar growth and stemness properties as the control group. *β-catenin* expression was highest at the 0.5 μM Bio concentration, equivalent to the control, while it showed a thorough decrease at the other concentrations (Fig.3B).

Effect of WNT3A (exogenous and endogenous), Bio and Dkk1 on embryonic stem cell growth and *Wnt* signaling pathway

We used 200 ng/ml exogenous WNT3A optimized in our earlier study (20). In this experiment, the effects of Bio (0.5 μM) combined with WNT3A (200 ng/ml) for activation of the Wnt3A signaling pathway as well as Dkk1 (250 ng/ml) for inhibition of the *Wnt3A* signaling pathway were studied on ES cell proliferation, *β-catenin* and pluripotency gene expression. Dkk1 treatment had no significant effect on mean colony area in the 1-week culture period (day 1 vs. day 6) relative to the control where as a significant decrease was observed in the 3-week culture period (day 1 vs. day 6, Fig.4A, A´). Real-time PCR analysis showed decreased expressions of *Nanog, β-catenin, Oct3/4* and *c-Myc* genes in Dkk1 treated cultures in comparison to all other treatments viz. Bio alone, Bio+WNT and WNT alone (Fig.4B). WNT3A transfected colonies showed increased expression of all the genes under study compared to both the mock transfected colonies as well as Dkk1 treated mock transfected colonies (Fig.4C). The band that corresponded to the β-catenin protein was virtually absent in Dkk1 treated ES cell colonies when visualized parallel to the control and WNT3A treated colonies for detection of the protein (Fig.4D).

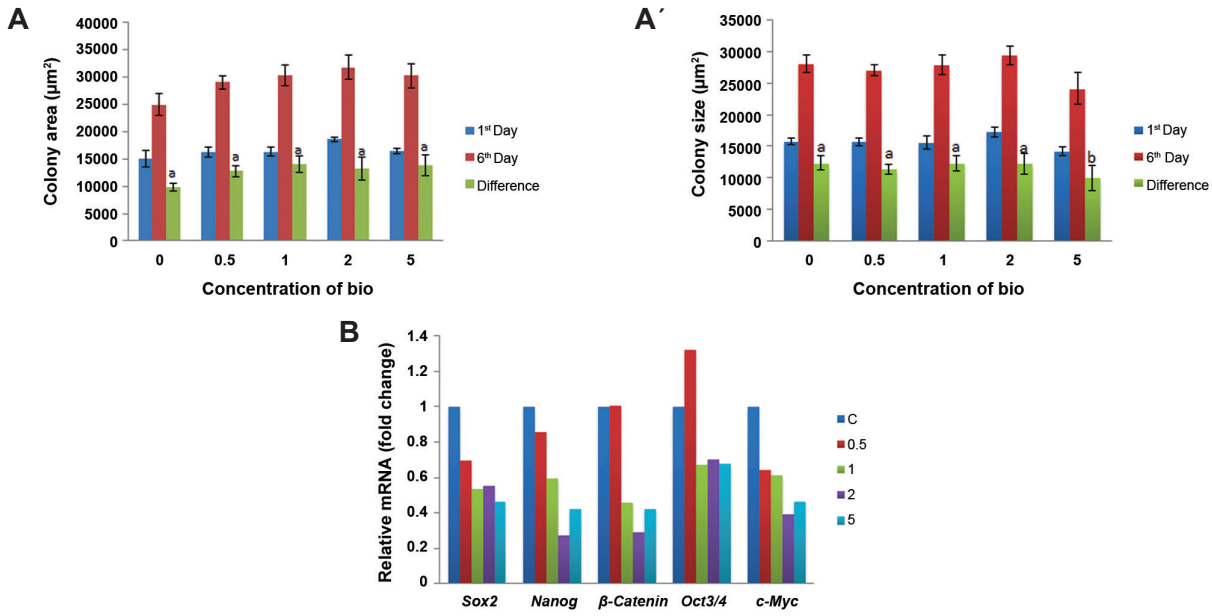

Fig.3: The effects of different concentrations of Bio (µM). Mean area of buffalo embryonic stem (ES) cell colonies at: **A.** First week of exposure. **A´.** Third week of exposure and **B.** Effect on expression of *β-catenin* and pluripotency genes after three weeks of exposure. Bars with different superscripts differ significantly while those with the same superscripts do not significantly differ at P<005.

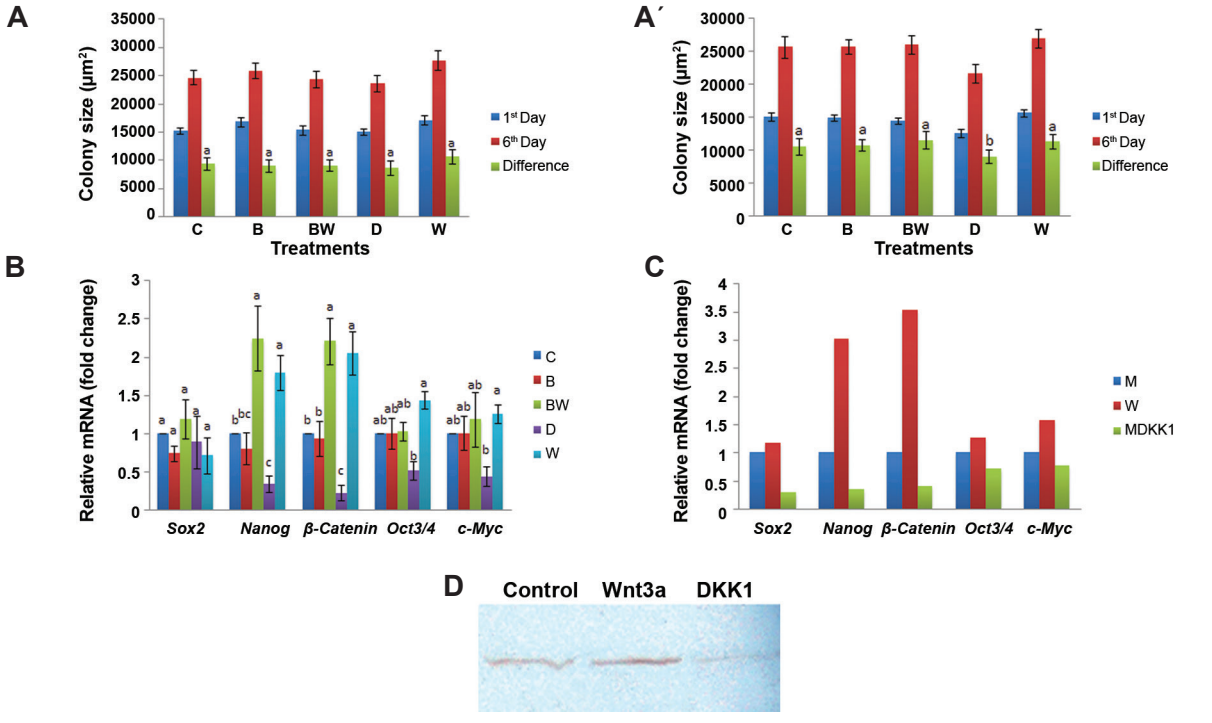

Fig.4: The effect of activation and inhibition of the Wnt3A signaling pathway. Mean area of buffalo embryonic stem (ES) cell colonies at: **A.** First week of exposure. **A´.** Third week of exposure. **B.** Expression of *β-catenin* and pluripotency genes. C; Control, B; Bio, BW; Bio+WNT3A, D; Dkk-1 and W; WNT3A. **C.** Expression of *β-catenin* and pluripotency genes from transfected colonies (W; *WNT3A* transfected colonies, M; MOCK vector transfected colonies and MDkk1; MOCK vector transfected colonies+Dkk1) and **D.** Western blot analysis of buffalo ES cells for β-catenin (Control; ES medium, Wnt3a; ES medium+Wnt3a and Dkk1; ES medium+Dkk1). Bars with different superscripts differ significantly while those with the same superscripts do not significantly differ at P<005.

Discussion

Our results have shown that supplementation with Bio in the absence of LIF and FGF-2 can maintain ES cell growth and pluripotency in both weeks 1 and 3 culture periods. However, at higher concentrations (5 µM), Bio adversely affected cellular proliferation in the 3-week culture period. The absence of a significant difference in the colony area between Bio-treated and feeder supported (controls) ES cell colonies may be due to the combined effects of Bio and feeder layer cells, since the feeder layer is thought to provide both LIF and FGF-2 (2).The equivalency in expression of *β-catenin* and pluripotency genes between the control and Bio-treatment group (0.5 µM) indicates up-regulation of these genes by Bio treatment. The decrease in gene expression at higher concentrations of Bio can be due to probable toxicity or feedback inhibition of the *Wnt* signaling pathway or activation of other mechanisms of differentiation induction in ES cell colonies. This is in accordance with the report of Sato et al. (12) who have shown that *Wnt* pathway activation by Bio, a specific pharmacological inhibitor of GSK-3, maintains the undifferentiated phenotype in both types of ES cells and sustains expression of the pluripotent state-specific transcription factors *Oct3/4, Rex-1* and *Nanog*. It has also been suggested that Bio may have a combinatorial effect on mouse ES cells, activating both the canonical *Wnt* signal and the LIF signal pathways simultaneously in order to maintain the cells in an undifferentiated state as in the case of the combination of recombinant WNT3A and LIF (21).

Doble et al. (22) have shown that the PI3K pathway, which is known to negatively regulate GSK3-β through serine phosphorylation of its amino-terminal domain, plays a role in the maintenance of mouse and primate ES cell pluripotency. Also, GSK3-β phosphorylation of *c-Myc* on *T58* has been implicated in the LIF/STAT3-mediated regulation of mouse ES cell pluripotency, since over expression of a *T58A* mutant of *c-Myc* in mouse ES cells promotes their self-renewal and pluripotency in the absence of exogenous LIF. Therefore, it seems that activation of the PI3K pathway by feeder layer secreting factors (FGF-2 or LIF) can lead to inactivation of GSK3-β, in conjunction with exogenous Bio in our treatment groups. This enables the cells to retain their growth and pluripotency almost equivalent to FGF-2 and the LIF supplemented control group (Fig.5).

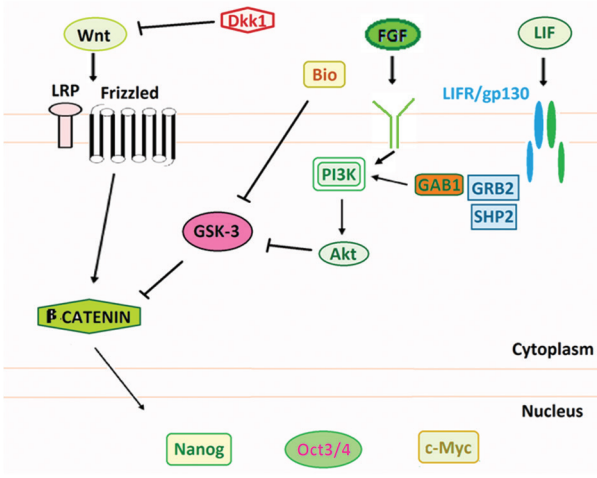

Fig.5: Predicted model for the action of GSK3-β in buffalo embryonic stem (ES) cells. Leukemia inhibitory factor (LIF) and fibroblast growth factor-2 (FGF-2) stimulation appear to cause inactivation of GSK3-β through the PI3K/Akt pathway in buffalo ES cells. Hence, Bio, as a specific pharmacological inhibitor of GSK-3β, was unable to further inactive GSK3-β.

It has also been predicted that suppression of GSK3-β activity by Bio, in the absence of *LIF/Wnt* signaling, can establish conditions where *c-Myc* is unphosphorylated on T58, leading to elevated c-*Myc* levels and hence, enhanced stem cell stability and self renewal (23).

Individual members of the Dkk family of secreted proteins could either antagonize or stimulate *Wnt* signaling through interaction with LRP6 (17). Our results showed that Dkk1 significantly ($P<0.05$) decreased the growth of buffalo ES cells based on the mean area of colonies and expression of *β-catenin* and other pluropotency-related genes. In agreement with our result, Beildeck et al. (15) showed that Dkk family members and Wise, a context-dependent secreted protein, both antagonize the *Wnt* pathway by binding to LRP5/6 and preventing WNTs from binding, thereby executing their function to maintain stem cell growth and pluripotency in absence of exogenous FGF-2 and LIF. It has also been reported that activation of the canonical *Wnt* signaling pathway could

be measured by accumulation of *β-catenin* in the presence of the WNT protein (24). Sclerostin and other BMP antagonists had no effect on accumulation, whereas Dkk-1, a WNT inhibitor, completely blocked WNT-induced *β-catenin* accumulation and activation of the *Tcf/Lef* reporter gene (25). These findings together have supported the virtual absence of β-catenin in Dkk1 treated ES cell colonies. Dkk1 is a prototypic *Wnt* signaling inhibitor that binds to and antagonizes the function of LRP6 (26). In addition, Dkk1 has also been shown to antagonize LRP6 function via LRP6 degradation with or without clathrin- dependent internalization (26, 27), thereby suppressing the *β-catenin* pathway. Thus both phosphorylation and internalization of LRP6 seem necessary to induce β-catenin accumulation. WNT3A exerts its antagonistic action against Dkk1 by binding to LRP6 and linking it to molecules that reside in lipid rafts and prevents its clathrin-mediated internalization, a prerequisite for its activity (27).

Our real-time PCR analysis showed that *Nanog, β-catenin, Oct3/4* and *c-Myc* were downregulated in buffalo ES cell colonies when supplemented with Dkk1. It has been demonstrated that overexpression of Dkk-1 leads to down regulation of *c-Myc* and *cyclin D1* expression, while reduction of Dkk-1 expression by RNA interference enabled upregulation of *β-catenin, c-Myc*, and *cyclin D1* in H7402 cells. It also promoted β-catenin translocation from the cytoplasm into the nuclei and increased cell migration (28). This would explain the reason behind decreased expression of pluripotency maintaining genes and smaller colony area in Dkk1 treated cells compared to other treatment groups.

Conclusion

In the absence of exogenous FGF-2 and LIF, activation of the *Wnt3A* signaling pathway with exogenous or endogenous WNT3A, either alone or in combination with an activator (Bio), could maintain pluripotency and growth of buffalo ES cells throughout the 3 week culture period. The decrease in ES cell colony area and expression level of pluripotency-related genes and *β-catenin*, in the presence of Dkk1 as a selective inhibitor of the *Wnt* signaling pathway, has indicated that the Wnt pathway complements FGF-2 and LIF, which are indispensable for ES cell self-renewal and pluripotency.

Acknowledgements

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Original Article

The Effects of Chlorpromazine on Reproductive System and Function in Female Rats

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Abstract

Background: Chlorpromazine (CPZ), an antipsychotic drug, is associated with increased risk of sexual dysfunction through increasing prolactin levels. The current study evaluates the effect of CPZ-induced hyperprolactinemia on ovarian follicular growth, gonadotropins, and alteration of ovarian source hormones.

Materials and Methods: In this experimental study, animals were divided into four groups, control and CPZ (n=8 per group). In the treated groups, CPZ was administered by gavage at doses of 3, 10 and 30 mg/kg per day for 28 days. On day 29 the animals were killed after which histopathological and histomorphometric analyses of the ovaries were performed. We evaluated the levels of prolactin serum, luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E_2) and progesterone.

Results: The ovaries of the test groups showed numerous atretic follicles of various sizes. CPZ caused a significant difference between the test groups and the control group ($P<0.05$) on the amount of atresia and the size of the normal corpora lutea (CL). The increased dysfunction of the ovaries from the different groups depended on the amount of CPZ administered. The serum concentrations of prolactin and progesterone significantly increased ($P<0.05$), while the serum concentrations of estradiol, LH and FSH notably decreased ($P<0.05$), depending on the CPZ dose. CPZ-induced animals had unsuccessful mating and decreased pregnancy rate.

Conclusion: The present findings suggest that CPZ-induced disturbances not only depend on prolactin level but the increased prolactin level is largely dose-dependent.

Keywords: Chlorpromazine, Hyperprolactinemia, Ovary, Atresia, Rat

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Introduction

Chlorpromazine (CPZ), an antipsychotic drug, has been widely used to treat schizophrenia and other psychotic disorders. CPZ is also used to control nausea, vomiting, long-term hiccups and as treatment for acute intermittent porphyria (1, 2). The antipsychotic effect of CPZ and other types of antipsychotic drugs is on the dopaminergic neurons of the mesolimbic system which is linked with psychotic symptoms (3).

Antipsychotic medications effectively diminish the intensity of psychotic hallucinations and allow most institutionalized patients with schizophrenia to be discharged into community treatment. The use of antipsychotic medications implicates a difficult trade-off between the benefit of alleviating psychotic symptoms and the risk of troubling, sometimes life-shortening adverse effects (4). All antipsychotic medications are associated with increased risk of sexual dysfunction, postural hypo-

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tension, cardiac arrhythmia, and sudden cardiac death (5-9). In order to successfully treat patients with schizophrenia, the adverse effect profiles of these medications should be taken into consideration. Physicians should be careful about the occurrence of adverse effects and be willing to adjust or change medications as needed or work with other psychiatrists to familiarize themselves with others' experiences to enable better and less dangerous treatments (4).

Until recently, increased prolactin rate (hyperprolactinemia) as a common side effect of antipsychotic treatments, has received little attention (10). Antipsychotic drugs block dopamine D2 receptors on lactotroph cells in the anterior pituitary gland and thus remove the inhibitory influence on prolactin secretion (11). Researchers have shown the adverse effect on fertility, sexual function, and bone mineral density of hyperprolactinemia (8, 12, 13).

Prolactin can suppress gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus and directly affect the physiological actions of the pituitary. Prolactin causes gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] to adversely affect the gonads (12). On the other hand, physiologic function of follicular growth and granulosa cells mainly depend on serum levels of FSH and LH. Therefore the dysregulation of hormones in which their source is ovarian, will lead to important problems in fertilizing potential (13, 14). This disorder in the function of gonadotropins is related to the pituitary gland and its feedback mechanisms.

Antipsychotic treatment is often initiated when patients are in their late teens or twenties. This treatment continues for years or decades (11). Although conventional antipsychotic drugs elevate prolactin rates (above the normal limit for both men and women), no reliable study that shows the relationship between these medications' doses and the effects of antipsychotic drug-induced follicular atresia is available. Thus, the present study evaluates the dose-dependent effects of CPZ on serum prolactin, sex hormone concentrations and ovarian tissue of adult female rats.

Materials and Methods

Animals

We conducted an experimental study on 32 female Wistar rats that were 70 days old and weighed 160 ± 5 g. Rats were obtained from the Animal House at the Faculty of Science, Urmia University, Iran and were allowed to acclimatize in an environmentally controlled room with a temperature of 22 ± 2˚C and a 12 hour light/12 hour dark schedule. Standard pellet food and tap water were available ad libitum. In this study all experiments conducted on the animals were in agreement with the Urmia University guidelines of the Ethical Committee for research on laboratory animals. Animals were allowed to acclimatize for one week before the experiments.

Drugs

CPZ (Sigma-Aldrich Co., Germany) was used at three dose levels - 3, 10 and 30 mg/kg based on previous study (14). The drug was dissolved in 0.5% methylcellulose solution (15) and administered to female rats by oral gavage.

Drug treatment

After a one-week acclimation, we assigned the animals to four groups (n=8 per group), as control and test groups. The control group rats received 5 ml/kg of 0.5% methylcellulose solution once daily for 28 consecutive days. The test subgroups received 3, 10 or 30 mg/kg/day CPZ for 28 consecutive days. In each group, we randomly chose 4 animals for potential fertility assessment. The remaining 4 animals were used for histological examinations.

One day after the last drug treatment, 4 animals from each group were killed by CO_2 inhalation and blood samples were collected from the jugular veins. Subsequently, the serum was harvested and frozen. The ovaries were removed surgically.

Prolactin is a stress hormone. Hence, in order to obtain unstressed levels of prolactin, we choose rapid decapitation as the method of sacrifice due to its decreased stress for the rodent. In addition, the animals were not in the presence of one another at the time of sacrifice (to smell the blood).

Potential fertility assessment

One week before the end of the treatment period, we randomly selected 4 females from each group to be placed in individual cages with one same-strain sexually active male. We considered the day

which sperm was detected in smears to be day 0 of pregnancy; after 21-23 days (pregnancy period in rats) the neonates were counted.

Histomorphologic analyses

On day 29, the ovaries were removed and fixed in formaldehyde acetic solution (IFAA, Merck, Germany) for 4 weeks. Ultimately, they were dissected free from ovarian tissues. Samples were processed through paraffin embedding and serially cut with a rotary microtome (Microm GmbH, Germany), then stained with hematoxylin and eosin (Merck, Germany).

We characterized the follicles in the ovarian sections according to size: under 100, 101-200, 201-300, 301-400, 401-500 and larger than 500 µm. Follicular morphology was examined by microscope under a ×40 objective lens (Olympus, Germany) magnification. Follicles with a complete layer of flattened granulosa cells, a normal nucleus, and oocytes with cytoplasm were considered normal follicles. Abnormal follicles were classified as follows: pyknotic nucleus, cytoplasmic damage, and combination of damaged nucleus and cytoplasm. Follicular number was estimated by counting follicles in all slides (16). The corpora lutea (CL) number per ovary was counted.

Hormonal assay

Blood sera were separated by centrifugation at 3000 g for 5 minutes, then subjected to assessments of serum levels of LH, FSH, progesterone, estradiol (E_2) and prolactin. Animals were killed and blood samples obtained in the morning hours.

Radioimmunoassays of prolactin, LH and FSH in sera

We added 100 µl of sera to tubes which contained 100 µl of hormones labeled with rabbit antisera in 0.01 M phosphate buffer (pH=7.6). Anti-rat prolactin (Cisbio Bioassays, France), LH and FSH were diluted to 1:5000, 1:10000 and 1:2500, respectively. Goat anti-rabbit IgG at a dilution of 1:10 (200 µl) was added to the mixture after which the mixture was allowed to remain for 18 hours at 40˚C, then centrifuged at 2000×g for 30 minutes. Radioactivity levels in the resultant pellets were measured by a gamma counter.

Radioimmunoassays of serum estradiol and progesterone

Concentrations of serum estradiol were measured by CIS kits (Cisbio Bioassays, France) according to the manufacturer's instructions. Serum (300 µl) was extracted with 3 ml ethyl ether. The layer of ether was evaporated under N_2 gas and the extract resuspended in 300 µl of 0.04 M phosphate buffer. After the addition of 100 µ1 17/3-estradiol (14000 cpm). Goat anti-rabbit r-globulin (1 ml) was added and the mixture was allowed to incubate for 15 minutes at room temperature. After centrifugation, the radioactivity in the pellet was counted. In order to evaluate serum levels of progesterone, we mixed serum (0.1 ml), l ml ethyl ether and 50 µ1 propylene glycol. After evaporating the ether under N_2 gas, 0.5 ml phosphate buffer and 0.1 ml (20000 cpm) of iodoprogesterone were added to the tube and the mixture was incubated with 0.1 ml anti-serum raised in rabbits for 18 hours at room temperature. Then, 0.1 ml bovine serum gamma globulin and polyethylene glycol were added to the mixture. The mixture was centrifuged for 10 minutes at 2000×g. The radioactivity was measured in the pellet (17).

Statistical analysis

Data are presented as mean ± SD. Experimental data were analyzed by analysis of variance and Duncan's multiple range test (SPSS version 16, Chicago, IL, USA).

Results

Fertilizing index and neonates

We analyzed the fertilizing index in the control and test groups. In CPZ-administered groups, the two high doses had a negative fertilizing index; these groups produced no neonates. In contrast, the control animals and the 3 mg/kg/day showed positive fertilizing indexes with 35 (control) and 21 (low dose) neonates (Table 1).

Hormone concentrations

Biochemical analyses showed that the serum levels of prolactin significantly ($P<0.05$) increased in CPZ-administered animals. This increase was dose-dependent. In contrast, control animals had constant prolactin levels. The serum levels of LH and FSH between the CPZ and control groups

showed that the serum levels of LH and FSH remarkably (P<0.05) decreased in animals that received CPZ. This reduction in LH and FSH levels was CPZ dose-dependent. The serum levels of estradiol significantly (P<0.05) decreased, while the progesterone level remarkably (P<0.05) increased in animals that received CPZ, which was dose-dependent. The data for hormonal analyses are presented in table 2.

Ovarian follicular growth, atresia and corpora lutea

Histological analyses in this study showed that in CPZ-administered groups, the total number of normal follicles significantly (P<0.05) decreased compared to control animals. Ovaries from the control group contained follicles in various developmental stages including primordial, primary, secondary, tertiary and graafian follicles with different sizes that ranged from <100 μm to >500 μm. There were no large antral follicles (>500 μm) in the two groups that received high doses of CPZ. Treatment with CPZ resulted in a significant (P<0.05) decline in follicular size in the CPZ groups compared to the control group. In the CPZ groups, there were more total numbers of atretic follicles compared to the control group. This finding was dependent on the dose of CPZ (Fig.1). Comparing the rate of normal follicles between the control and CPZ groups showed a significant (P<0.05) decrease in the CPZ groups. The highest rate of number of normal follicles between the test groups was observed in the low dose group. We observed that animals which received the two higher doses of CPZ exhibited significantly higher CL sizes compared to the control group (Tables 2-5).

Table 1: Fertilizing index (pregnant rats) and numbers of neonates in control and CPZ-treated groups

Parameters	Control	3 mg/kg	10 mg/kg	30 mg/kg
Number of animals examined	4	4	4	4
Mated animals (n)	4	4	0	0
Fertility index (%) [1]	100	75	0	0
Neonates (n)	35	21	0	0

[1]; Fertility index (%)=(number of pregnant animals/number of animals that copulated)×100 and CPZ; Chlorpromazine.

Table 2: Mean serum levels of prolactin, LH, FSH, progesterone and estradiol (E_2) in study groups

Hormones	Control	3 mg/kg	10 mg/kg	30 mg/kg
Prolactin (ng/ml)	55.75 ± 3.06	109.25 ± 13.37	223.75 ± 26.35 [a, b]	249.50 ± 25.82 [a, b, c]
LH (ng/ml)	0.56 ± 0.05	0.58 ± 0.06	0.30 ± 0.02 [a,b]	0.26 ± 0.02 [a,b]
FSH (ng/ml)	3.17 ± 0.48	1.97 ± 0.44	1.35 ± 0.27 [a,b]	1.13 ± 0.06 [a,b]
E_2 (pg/ml)	41.50 ± 2.62	29.00 ± 1.47	29.50 ± 2.59 [a,b]	24.00 ± 0.40 [a,b]
Progesterone (ng/ml)	18.12 ± 2.55	22.75 ± 3.11	32.07 ± 3.75 [a,b]	33.82 ± 3.71 [a,b]

[a, b, c]; Indicate significant differences (P<0.05) between data of chlorpromazine (CPZ) groups with control, 3 mg/kg and 10 mg/kg groups, respectively. All data are presented as mean ± SD.
LH; Luteinizing hormone, FSH; Follicle-stimulating hormone and E_2; Estradiol.

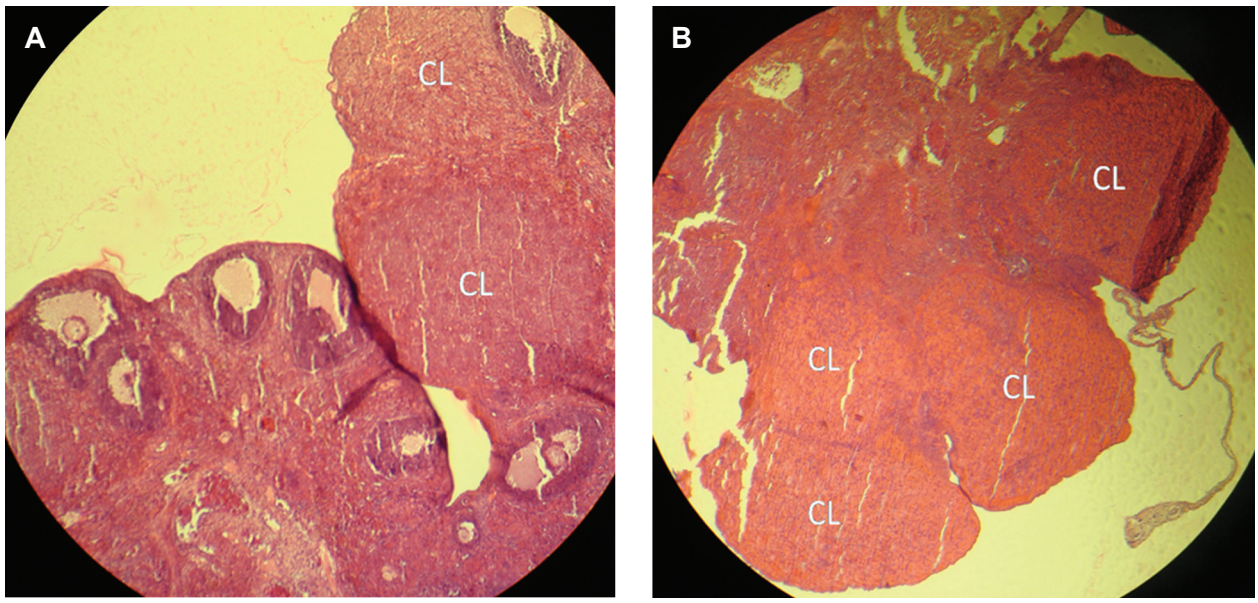

Fig.1: Cross-section from an ovary. **A.** Control group ovary presents with different size follicles and corpora lutea (CL) and **B.** 30 mg/kg dose chlorpromazine (CPZ) group show large, active CL without follicular growth. Hematoxylin-eosin staining, (×400 magnification).

Table 3: Mean numbers of normal and atretic follicles on ovaries of study groups

Parameters (n)	Control	3 mg/kg	10 mg/kg	30 mg/kg
Primordial follicles	287.50 ± 11.90	250.75 ± 13.47	137.50 ± 4.19 [a, b]	154.75 ± 9.46 [a, b, c]
Primary follicles	4.75 ± 0.48	3.75 ± 0.48	5.00 ± 0.57	6.50 ± 0.64
Secondary follicles	7.00 ± 0.70	4.50 ± 0.28	4.50 ± 0.28 [a, b]	3.75 ± 0.62 [a, b]
Tertiary follicles	6.50 ± 0.25	7.50 ± 0.28	5.75 ± 0.47	5.75 ± 0.75
Graafian follicles	8.25 ± 0.75	7.00 ± 0.40	5.50 ± 0.28 [a, b]	3.75 ± 0.85 [a, b, c]
Atretic follicles	1.25 ± 0.25	3.00 ± 0.41	11.50 ± 0.29 [a, b]	12.50 ± 0.64 [a, b, c]
Preantral atretic follicles	0.25 ± 0.25	1.25 ± 0.25	5.75 ± 0.48 [a, b]	7.00 ± 0.41 [a, b, c]
Antral atretic follicles	1.00 ± 0.00	1.75 ± 0.29	5.50 ± 0.57 [a, b]	5.50 ± 0.70 [a, b, c]
Corpora lutea	10.50 ± 0.28	10.75 ± 0.62	11.50 ± 0.57 [a, b]	10.50 ± 0.64 [a, b]

[a, b, c]; Indicate significant differences (P<0.05) between data of chlorpromazine (CPZ) groups with control, 3 mg/kg and 10 mg/kg groups, respectively. All data are mean ± SD.

Table 4: Sizes of follicles on ovaries of different groups

Follicles (µm)	Control	3 mg/kg	10 mg/kg	30 mg/kg
<100	294.00 ± 10.97	255.25 ± 13.82	148.00 ± 4.70 [a, b]	165.75 ± 9.93 [a, b, c]
100-200	5.50 ± 0.86	6.00 ± 0.70	12.50 ± 1.29	9.00 ± 2.27
201-300	6.50 ± 0.22	7.50 ± 0.64	9.75 ± 0.75	6.25 ± 0.63
301-400	6.75 ± 0.47	7.00 ± 0.70	8.50 ± 0.86	6.00 ± 0.12
401-500	2.50 ± 0.44	3.00 ± 0.58	2.25 ± 0.25	2.50 ± 0.86
500<	1.25 ± 0.62	0.75 ± 0.25	0.00 ± 0.00	0.00 ± 0.00

[a, b, c]; Indicate significant differences (P<0.05) between data of chlorpromazine (CPZ) groups with control, 3 mg/kg and 10 mg/kg groups, respectively. All data are mean ± SD.

Table 5: Sizes of corpora lutea (CL) in chlorpromazine (CPZ) and control groups. Control animals exhibited smaller CLs that remained from previous cycles, whereas treatment animals had larger CL per ovary

Corpora lutea (µm)	Control	3 mg/kg	10 mg/kg	30 mg/kg
301-400	0.75 ± 0.25	0.20 ± 0.00	0.25 ± 0.25 [a]	0.00 ± 0.00 [a]
401-500	2.00 ± 0.00	0.75 ± 0.25	0.25 ± 0.25	0.25 ± 0.25 [a]
501-600	2.00 ± 0.40	2.50 ± 0.50	2.00 ± 0.50	2.50 ± 0.50
601-700	2.00 ± 0.00	2.50 ± 0.50	1.75 ± 0.25	2.25 ± 0.75
701-800	2.25 ± 0.25	2.25 ± 0.50	2.25 ± 0.25	2.00 ± 0.40
801-900	0.50 ± 0.25	0.75 ± 0.25	2.00 ± 0.25 [a, b]	2.00 ± 0.40 [a, b, c]
900<	0.00 ± 0.00	0.00 ± 0.00	2.50 ± 0.50	1.50 ± 0.75 [a, b]

[a, b, c]; Indicate significant differences (P<0.05) between CPZ groups with the control, 3 mg/kg and 10 mg/kg groups, respectively. All data are mean ± SD.

Discussion

The present study attempted to reiterate and integrate the understanding of the well-known dose dependent adverse effects of a conventional antipsychotic agent (CPZ) on the reproductive system and functions in female rats mediated via the hypothalamic-pituitary-gonadal system.

Hormonal analyses showed increased serum prolactin and progesterone levels and decreased serum LH, FSH and E_2 levels in rats that received CPZ. This observation was dose-dependent. On the other hand, histological and histomorphometric examinations showed that CPZ significantly enhanced atretic follicle formation which was accompanied by a remarkable decrease in the rate of normal follicles and significantly larger sizes of normal CL at the two high doses. The results of this study have demonstrated decreased potential fertility at the high doses of CPZ.

It is well established that dopamine plays a crucial role in tonic inhibition of prolactin secretion (18, 19). Dopamine acts on lactotroph cells in the anterior pituitary gland and inhibits prolactin secretion (20). In one study, the use of a dopamine antagonist, haloperidol, as an antipsychotic drug to inhibit dopamine secretion, has resulted in increased prolactin levels in rats (19). The results from biochemical analyses in our study corrobo-

rated with the mentioned hypothesis. Serum prolactin levels significantly increased in groups that received CPZ. This observation was dose-dependent.

It has been reported that high prolactin levels inhibit the secretion of GnRH from the hypothalamus axis (21, 22). Prolactin can prevent luteolysis and cause increased numbers of persisting CL (23). The pulsatile secretion pattern of GnRH induces the cyclic release of LH and FSH. In female mammals, FSH induces follicle growth and subsequently E_2 secretion by granulosa cells (24, 25). It has been reported that inhibition of GnRH results in reduced LH and FSH levels (26). Histological observations demonstrated that CPZ-administered animals had significantly increased atresia of different sizes; these ovaries exhibited higher CL sizes. On the other hand, depending on dose, the serum level of E_2 decreased and the progesterone concentration increased in CPZ-administered groups. Thus, it could be proven that increased levels of prolactin with a simultaneous effect of progesterone resulted in a remarkable follicular atresia. These impairments might not only be caused by higher prolactin levels, they might be caused with resistance CL from previous cycles (which, in turn leads to severe follicular atresia). This resistant CLs did not let the estradiol secretion restart, and reduced serum level of E_2 in CPZ-administered animals proofed mentioned theory very well. It is known that E_2 directly stimulates prolactin synthesis in lactotrophs and prolonged E_2 administration is known to produce elevation of serum prolactin levels and induce hyperplasia of prolactin-secreting cells. Even with the low levels of E_2 that we have observed in the study rats that was attributed to hypogonadism, there was marked increase in prolactin secretion with CPZ treatment which showed the drug's effect on prolactin secretion (27).

As previously mentioned, the increased level of prolactin can largely affect gonadotropins. Our analyses have shown that serum levels of LH and FSH significantly decreased in the two high CPZ dose groups. In patients treated with antipsychotic-drugs reduced secretion of GnRH in the hypothalamus decreased stimulation for LH and FSH secretion in the pituitary gland (11). Thus, we could conclude that CPZ directly and indirectly with hyperprolactinemia blocked the hypothalamus-pituitary axis, which in turn inhibited gonadotropin secretion. Additionally, the E_2 positive feedback in the pituitary gland for LH hormone secretion was eliminated. Therefore the serum levels of LH and FSH decreased significantly in animals that received CPZ. Additionally, CL resistance delivered from the previous cycle caused decreased E_2 level that was related with reduced gonadotropins and ultimately occurred situation increased atresia in CPZ-administered animals. Inhibited follicular growth marked with reduced normal follicles in CPZ-induced groups proved this theory.

In order to evaluate the biological activity of CLs, we investigated the serum level of progesterone. Observations demonstrated that the serum level of progesterone remarkably increased in animals treated with CPZ. This finding showed that the observed CLs were considerably active. Due to increased progesterone levels and absence of appropriate feedback for androgens and E_2 secretion, in order to restart a new cycle (28, 29), follicular growth depression occurred in the ovaries of CPZ-administered animals. A study suggested that estradiol actions on the oocyte or pregranulosa cells associated with the primordial follicle inhibited the initial wave of primordial to primary follicle transition. This decrease in primordial follicles in treated animals might be related in decreased E_2 levels in these animals (30).

During the estrous cycle, E_2 levels increase at proestrus and are low during estrus, metestrus and diestrus. Therefore in this study, we have observed that lower serum E_2 levels in the treatment animals were consistent with the persistence of the diestrus phase (31). Hyperprolactinemia is known to be one of the causes of pseudopregnancy, namely continuous diestrus, by stimulating and maintaining CL in rodents since prolactin has a luteotropic activity (24). Thus, evidences can explain the reproductive disorders that have been observed in this investigation.

The luteotropic effect of prolactin, increase in progesterone and ovarian hormones, directly influence changes in the uterine wall. Remarkable ($P<0.05$) elevations have been observed in uterine horn endometrium, myometrium and perimetrium thicknesses along with remarkably higher gland number per mm^2 of the endometrium in animals that received CPZ, which will be reported in another paper.

Conclusion

Our results showed that rats treated with CPZ had mean serum prolactin levels several-fold greater than the upper limit of normal. Additionally, CPZ-induced hyperprolactinemia was associated with a disturbance in the levels of essential reproductive hormones, E_2 and progesterone. The prolactin-associated disturbances in gonadotropins and reproductive hormones exerted significant adverse effects on follicular growth in CPZ-administered rats. Accordingly, due to increased atresia at different follicle sizes (preantral and/or antral) in CPZ-treated rats and the absence of >500 μm follicles and increased CL size in the ovaries, it seemed that CPZ caused significant hypo-ovulation by increasing atresia. CPZ, as a prolactin-elevating antipsychotic drug, decreased the fertilizing index. This finding was particularly observed at higher doses. The mentioned impairments remarkably depended on CPZ doses.

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Original Article

Increased Litter Size and Suckling Intensity Stimulate mRNA of *RFamide-related Peptide* in Rats

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Abstract

Background: RFamide-related peptide-3 (RFRP-3) inhibits gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion in rats. This study evaluates the effects of litter size and suckling intensity on *RFRP* mRNA expression in the dorsomedial hypothalamic nucleus (DMH) of rats.

Materials and Methods: A total of 32 pregnant and 4 non-lactating ovariectomized (control group) Sprague-Dawley rats were used in this experimental study. Lactating rats were allotted to 8 equal groups. In 3 groups, the litter size was adjusted to 5, 10, or 15 pups upon parturition. Dams were allowed to suckle their pups continuously until 8 days postpartum. In the other 3 groups, the litter size was adjusted to 5 pups following birth. These pups were separated from the dams for 6 hours on day 8 postpartum, after which the pups were allowed to suckle for 2.5, 5, or 7.5 minutes prior to killing the dams. In 2 groups, lactating rats with 10 and 15 pups were separated from their pups for 6 hours on day 8 postpartum. In these groups, the pups were allowed to suckle their dams for 5 minutes before the dams were killed. All rats were killed on day 8 postpartum and the DMH was removed from each rat. We evaluated *RFRP* mRNA expression using real-time polymerase chain reaction (PCR).

Results: The expression of *RFRP* mRNA in the DMH increased with increased litter size and suckling intensity compared to the controls. The effect of suckling intensity on the expression of *RFRP* mRNA was more pronounced compared to the litter size.

Conclusion: Increased litter size and suckling intensity stimulated *RFRP* mRNA expression in the DMH which might contribute to lactation anestrus in rats.

Keywords: *RFRP* mRNA, Suckling Intensity, Dorsomedial Hypothalamic Nucleus, Lactation, Rat

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Introduction

Gonadotropin releasing hormone (GnRH) is a hypothalamic neuropeptide that acts as the primary signal for regulation of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion. It is well established that GnRH acts as a key neurohormone for vertebrate reproduction.

Gonadotropin-inhibitory hormone (GnIH) is a key inhibitory regulator of the hypothalamus-pituitary-gonads axis. This hormone has been shown to directly act on the pituitary gland and inhibit gonadotropin release (1). Initially identified in quails, this was the first demonstration that a hypothalamic neuropeptide could inhibit gonadotropin

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release in any vertebrate. GnIH has since been isolated as a mature peptide in starlings (2) and zebra finches (3).

RFamide-related peptides (RFRP) are GnIH orthologs that have been subsequently identified in a number of other vertebrates, including mammals. In mammals, cDNAs that encode LPXRFamide peptides (X=L or Q) similar to GnIH were investigated by a gene database search (4). The cDNAs identified from the mammalian brain encode RFRP-1, 2, and 3, in cattle and humans, as well as RFRP-1 and 3 in rodents (5-7). RFRP-3 has been shown to putatively modulate the negative feedback effect of estrogen on gonadotropin secretion (8). RFRP-ir cells cluster in the dorsomedial hypothalamic nucleus (DMH) and have been identified in hamsters, rats, and mice (8). The inhibitory effects of RFRP-3 on gonadotropin release were reported in rodents (9, 10) and sheep (11, 12).

Follicular maturation and ovulation are inhibited during lactation in various mammals (13). Inhibition of the estrous cycle in lactating rats mostly results from inhibition of LH and GnRH secretion (14). Although suckling is an important inhibitory cue for LH surge during the first 8 days of lactation in rats, separating pups from their dams restores LH secretion (15). Levels of LH pulsatile secretion are low in lactating rats (16) and humans (17). Administration of bromocriptine (a dopamine agonist used in the treatment of hyperprolactinemia) does not impact the inhibitory effect of suckling on LH pulse (18). Research has shown that endogenous opioid peptides do not mediate suppression of the LH release by the suckling stimulus. In the rat model, intravenous injection of naloxone does not increase LH secretion during early lactation (19). Neuroendocrine mechanisms that affect inhibition of LH secretion during lactation are unknown (20).

Suckling is an appropriate model for studying the reproductive endocrine hormones involved during lactation and to investigate the neuroendocrine pathways that regulate negative energy balance. Estrus and ovulation are delayed for approximately 20 days in lactating rats that suckle 6 to 10 pups. The delay period is dependent on the number of pups. If the number of pups is greater than 12 with a 2-day-long water and food withdrawal period, then the strength of suckling is increased (21). Therefore, the number of pups and strength of suckling may be critical stimulants in inhibiting LH surge. The aims of the present study are to evaluate the effects of litter size and suckling intensity on *RFRP* mRNA transcription in lactating rats. The findings will be beneficial to better clarify the underlying mechanism(s) involved in lower reproductive performance attributed to lactation anestrus.

Materials and Methods

Animals, experimental groups, and sampling

In the present experimental study, we randomly selected 32 pregnant and 4 ovariectomized (3-4 month-old) female Sprague-Dawley rats (*Rattus norvegicus*) that weighed 205.9 ± 10.7 g (mean ± SD). Rats were housed in individual cages under controlled temperature (22 ± 2˚C) and light (14 hours light/10 hours dark; lights on from 07:00 to 21:00) with free access to food and water in the Laboratory Animal Center of Shiraz University of Medical Sciences, Shiraz, Iran. The rats were treated humanely and in compliance with the recommendations of the Animal Care Committee at Shiraz University of Medical Sciences. The rats were randomly assigned to 9 groups (n=4 per group). The control group comprised 4 ovariectomized rats. Each rat assigned to the ovariectomized group received an intraperitoneal injection of ketamine (100 mg/kg, Netherlands) and xylazine (7 mg/kg, Alfazyne, Netherlands) as anesthesia. Control rats were ovariectomized through the ventral midline incision. Further procedures were carried out over a two-week recovery period.

Lactating rats were allotted to 8 groups (n=4 per group). These rats were allowed to suckle their pups until day 8 postpartum. In 3 groups, the litter sizes were adjusted to 5, 10, or 15 pups upon parturition. Rats from these groups were allowed to suckle their pups continuously. In an additional 3 groups of rats, the litter size was adjusted to 5 upon birth. The pups were separated from their dams on day 8 postpartum for 6 hours, after which they were allowed to suckle their dams for 2.5, 5, or 7.5 minutes before the dams were killed. This separation time was selected according to Marina et al. (22). This time period made the pups hungry which enabled them to intensively suckle their mothers' teats. The minimum of 2.5 minutes and 2.5-minute intervals were selected according to the minimum time in increase in RNA levels immediately detected after transcription stimulation of

a single cell (23). Two groups of lactating rats with either 10 or 15 pups were similarly separated from their pups for 6 hours on day 8 postpartum, after which the pups were allowed to suckle their dams for 5 minutes before the dams were killed. Rats were anesthetized with ether and killed via cervical dislocation at 15:00 to 16:00 on day 8 postpartum. Brains were immediately removed and the diencephalon was dissected out by an anterior coronal section, anterior to the optic chiasm, and a posterior coronal cut at the posterior border of the mammillary bodies. To separate DMH, a third coronal cut was made through the middle of the optic tract, just rostral to the infundibulum (24). The specimens were stored in liquid nitrogen until further analysis.

Real-time polymerase chain reaction (PCR)

Total RNA was extracted using RNX-Plus buffer (Cinnagen, Tehran, Iran). Briefly, the tissue (100 mg) was ground in liquid nitrogen, transferred to RNX-Plus buffer (1 mL) in an RNase-free microtube, mixed thoroughly, and kept at room temperature for 5 minutes. Chloroform (0.2 mL) was added to the slurry and mixed gently. The mixture was centrifuged at 12000×g (4˚C) for 20 minutes after which the supernatant was transferred to another tube, then precipitated with an equal volume of isopropanol for 15 minutes. The RNA pellet was washed with 75% ethanol, quickly dried, and re-suspended in 50 µL RNase-free water. The integrity and quantity of RNA was checked by visual observation of 28S and 18S rRNA bands on a 1.2% agarose gel. The purified total RNA was quantified by a Nano-Drop ND 1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). DNase treatment was carried out using a DNase kit (Fermentas, St. Leon-Roth, Germany) according to the manufacturer's instructions. The DNase-treated RNA (3 µg) was used for first strand cDNA synthesis with 100 pmol oligo-dT, 15 pmol dNTPs, 20 U RNase inhibitor, and 200 U M-Mulv reverse transcriptase (Fermentas, Germany) in a final volume of 20 µL. Primers were designed using Allele ID 7 software (Premier Biosoft International, Palo Alto, USA) for the reference gene and RFRP (NM_023952). The rat *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene (M32599) was used as a reference gene for data normalization (Table 1). Relative real-time PCR was performed in a 20 µL volume that contained 1 µL cDNA, 1X Sybr Green buffer and 4 pmol of primer. The amplification reactions were carried out in a Line-Gene K thermal cycler (Bioer Technology Co., Ltd., Hangzhou, China) under the following conditions: 2 minutes at 94˚C, 40 cycles of 94˚C (10 seconds), 57˚C (15 seconds), and 72˚C (30 seconds). After 40 cycles, the specificity of the amplifications was tested by heating from 50˚C to 95˚C, which resulted in melting curves. All amplification reactions were repeated three times under identical conditions, including a negative control and five standard samples. To ensure that the PCR products were generated from cDNA rather than genomic DNA, proper control reactions were implemented in the absence of reverse transcriptase. For quantitative real-time PCR data, the relative expression of *RFRP* mRNA was calculated based on the threshold cycle (C_T) method. The C_T for each sample was calculated, using Line-gene K software (25). Accordingly, the fold expression of the target mRNAs over the reference values was calculated by the equation $2^{-\Delta\Delta CT}$ (26), where ΔC_T was determined by subtracting the corresponding GAPDH C_T value (internal control) from the specific C_T of the target *(RFRP)*. The $\Delta\Delta C_T$ was obtained by subtracting the ΔC_T of each experimental sample from that of the calibrator one (non-lactating ovariectomized rats).

Table 1: Real-time polymerase chain reaction (PCR) primer sequences used to evaluate relative expression of *RFRP* gene in a rat model

Primer	Sequence	Amplicon length (bp)
RFRP-F	5′ CTCAGCAGCCAACCTTCC 3′	165
RFRP-R	5′ AAACCAGCCAGTGTCTTG 3′	
GAPDH-F	5′AAGAAGGTGGTGAAGCAGGCATC 3′	112
GAPDH-R	5′ CGAAGGTGGAAGAGTGGGAGTTG 3′	

GAPDH; Glyceraldehyde-3-phosphate dehydrogenase and *RFRP; RFamide-related peptide-3.*

Statistical analysis

Data from relative expression of the *RFRP* gene were subjected to the test of normality and analyzed by one-way ANOVA (SPSS for Windows, version 11.5, SPSS Inc., USA). Mean separation was performed by post hoc LSD test at P=0.05.

Results

The relative expression of *RFRP* mRNA in rats continuously housed with five pups was lower than those that suckled either 10 or 15 pups (P=0.02, Fig.1). The relative expressions of *RFRP* mRNA in the DMH of lactating rats continuously housed with 5 (P=0.009), 10 (P=0.01), or 15 (P=0.02) pups were respectively lower than in rats that were separated from their pups for 6 hours, then allowed to suckle for 5 minutes (Fig.1). The relative expression of *RFRP* mRNA in lactating rats with 5 pups that were separated from their pups, then allowed to suckle for 5 minutes was lower than those that suckled either 10 or 15 pups (P=0.02, Fig.1). The relative expression of *RFRP* mRNA in the DMH of lactating rats with 5 pups that were separated from their pups for 6 hours, then allowed to suckle for 2.5 (P=0.001) or 5 (P=0.03) minutes was lower than in rats that suckled for 7.5 minutes (Fig.2).

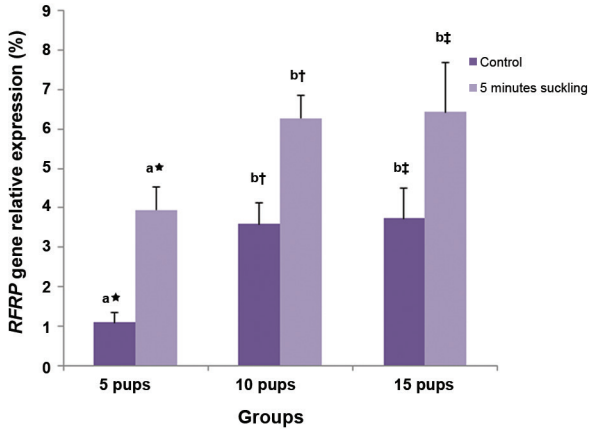

Fig.1: The effect of litter size and 5 minutes suckling duration (after a 6-hour separation period of the dam and pup) on relative expression of the *RFamide-related peptide-3 (RFRP)* gene (mean ± SE) in the dorsomedial hypothalamic nucleus (DMH) of lactating rats (n = 4) with 5, 10, or 15 pups. Control lactating rats were not separated from their pups. Different letters indicate significant differences between different litter sizes in each group and the same symbols indicate significant differences between the different suckling durations with the same litter size (P<0.05).

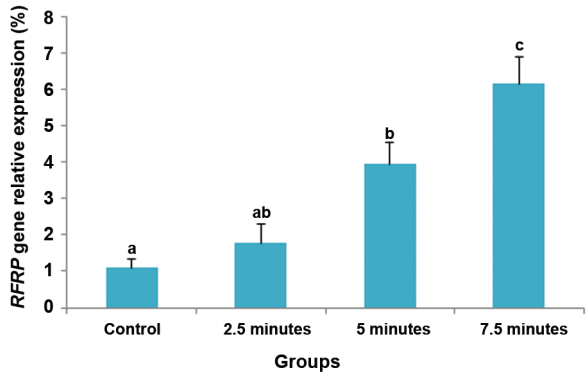

Fig.2: The effect of suckling intensity on relative expression of the *RFamide-related peptide-3 (RFRP)* gene (mean ± SE) in the dorsomedial hypothalamic nucleus of lactating rats (n=4) with 5 pups which were separated from their pups for 6 hours on day 8 postpartum, after which the pups were allowed to suckle their dams for 2.5, 5, or 7.5 minutes. Control lactating rats were not separated from their pups. Different letters indicate significant difference (P<0.05).

Discussion

In this study, *RFRP* mRNA levels greatly increased in the DMH during suckling. The *RFRP* mRNA in neurons of DMH from lactating rats increased with increased numbers of suckling pups and intensity of suckling. Consistent with our findings, *RFRP* mRNA expression (27) and RFRP-3-ir neurons According to immunohistochemistry analyses (28) in DMH of the hypothalamus of lactating rats was more than non-lactating rats. It has been shown that the effects of RFRP-3 were opposite to kisspeptin during the estrous cycle in the rat (29). In keeping with our findings, Yamada et al. (30) reported that suckling stimulus inhibited the expression of kisspeptin in neurons of the arcuate nucleus (ARC). These findings demonstrated the inhibitory effect of RFRP-3 on reproduction at transcription and translation levels during lactation in a rat model.

The present study showed that increased intensity of suckling resulted in higher expression of *RFRP* mRNA in the DMH. RFRP-1 is a secretion stimulator of prolactin in rats (4). A relationship was observed between the intensity of the suckling-induced prolactin increase and litter size in rats (31) and level of increase in prolactin secretion during lactation. Increased prolactin levels directly inhibited GnRH and LH (32) secretions. Prolactin did not mediate the suppressing effect of the suckling stimulus on LH secretion at the hypothalamic level in rats during early (33) and mid-lactation

(18). In support of our results, Hinuma et al. (4) reported that intracerebroventricular RFRP-1 administration caused increased prolactin release in humans. It was likely that the negative effects of prolactin on LH were exerted through RFRP-3.

Tuberoinfundibular dopaminergic (TIDA) neurons in ARC are known as the key regulators of prolactin release (34). Dopamine has been shown to inhibit prolactin. A close contact between RFRP neurons and dopamine was reported where RFRP receptors were expressed in dopamine neurons (35). Therefore, RFRP-3 might stimulate prolactin secretion by suppressing the activity of dopamine neurons.

Intensive suckling acutely increased *RFRP* mRNA expression, whereas this effect was not observed with continuous suckling. Lactating rats normally receive continuous suckling from pups rather than intensive suckling. The intensity of the suckling stimulus was reported to depend on the number of pups attached to the nipples, duration of attachment and the suckling intensity (36). In the present study, increased duration of attachment (5 and 7.5 minutes) of hungry pups acutely increased *RFRP* mRNA expression after 5 minutes of intensive suckling with the same litter size (5 pups). Increased numbers of hungry pups after 5 minutes of intensive suckling acutely increased *RFRP* mRNA expression. Consistent with our findings, suckling stimulus inhibited the expression of kisspeptin in ARC neurons (30). There was a negative correlation between expression of kisspeptin and RFRP in rat ARC neurons (29). Therefore, increased litter size and/or duration of suckling caused increased suckling intensity that led to increased *RFRP* mRNA expression in ARC neurons in lactating rats.

We showed that increased suckling stimulus caused more *RFRP* mRNA expression. Suckling has been shown to be an important inhibitor of LH secretion in lactating rats (37). Although, few data reported the relationship between lactation stress and expression of RFRP-3, it has been shown that stress increased RFRP expression in male rats (38). Cortisol levels were higher in lactating female rhesus macaques than in non-lactating females (37). Corticotrophin-releasing hormone was not critical in conveying the inhibitory inputs of the suckling stimulus in ovariectomized lactating rats (39), but cortisol treatment suppressed LH release in rats (40) and ewes (41). Consequently, increased glucocorticoid secretion due to increased litter size and/or suckling intensity might inhibit gonadotropin secretion through stimulation of RFRP-3.

Increased numbers of pups per lactating rat result in higher milk production, therefore the negative energy balance becomes more exacerbated (42). Adequate energy reserves are essential for reactivation of the reproductive axis. During periods of negative energy balance, GnRH release is suppressed (43). Regardless of the level of energy intake the efficiency of energy use substantially increases during lactation in rats. The mechanisms involved in negative energy balance play an important role in the change of energy expenditure (42). Melanin-concentrating hormone (MCH) and orexin, two appetite neuropeptides, have been reported to inhibit LH secretion during lactation (33). On the other hand, RFRP neurons project to MCH and orexin producing cells in the lateral hypothalamic area of sheep (44). Therefore, enhancement of *RFRP* mRNA expression with an increase in lactation is simultaneous with negative energy balance and inhibition of reproduction.

Conclusion

We demonstrated a relationship between *RFRP* mRNA expression, increased litter size, and suckling intensity in the DMH of rats.

Stimulation of RFRP-3 might be a factor in inhibition of LH secretion during lactation, although the mechanisms underlying this inhibition should be further addressed.

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Original Article

Development of A Questionnaire to Measure Attitude toward Oocyte Donation

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Abstract

Background: To our knowledge, there is no valid and comprehensive questionnaire that considers attitude toward oocyte donation (OD). Therefore this study has aimed to design and develop a tool entitled attitude toward donation-oocyte (ATOD-O) to measure attitude toward OD.

Materials and Methods: This methodological, qualitative research was undertaken on 15 infertile cases. In addition, we performed a literature review and search of various databases. Validity of this questionnaire was conducted by knowledgeable experts who determined indices such as relevancy, clarity, and comprehensiveness. Reliability of the questionnaire was assessed based on the opinions of experts and infertile couples referred to Royan Institute.

Results: ATOD-O was designed in 52 statements that covered various issues such as the OD process, donor and recipient characteristics, as well as family, emotional, psychological, legal, religious, and socio-economic dimensions. Results were scored as five points: 1 (strongly disagree), 2 (disagree), 3 (somewhat), 4 (agree), and 5 (strongly agree). The overall relevancy of the questionnaire was 97% and clarity was 96%. Overall comprehensiveness was 100%.

Conclusion: The findings from this preliminary validation study have indicated that ATOD-O is a valid measure for measuring and assessing attitude toward donated oocytes. This questionnaire can be used in studies regarding different groups of a society.

Keywords: Oocyte Donation, Attitude, Questionnaire, Infertility, ATOD-O

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Introduction

There has been significant progress worldwide in the development of assisted reproductive techniques (ARTs) to aid infertile couples in achieving their reproductive goals (1). One of these techniques is oocyte donation (OD), initially introduced by Buster. The first infant was born via OD in 1984 (2).

OD is the process of ovulation stimulation in which a woman other than the infertile female partner donates her oocyte for fertilization. The donated oocyte is fertilized by the sperm of the infertile woman's partner in the laboratory, after which the fertilized oocyte is subsequently transferred to the uterus of the infertile female partner (3). OD is a remarkably effective method of treatment, even in difficult cases (4). Challenges and problems associated with OD exist, such as disclosure of a child's genetic origin and other ethical issues (5-8). However this is the only way for infertile women who lack normal or high quality oocytes due to increased age, early menopause, birth defects, and genetic mutations, as well as chemotherapy and radiotherapy cancer treatments, despite the health of their other reproductive organs (9). The number of families that have been treated by this method is

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increasing. In the United States, the pregnancy rate through OD has reached 50% and the live birth rate has approximated this rate (10). A clear, exact and accurate rate for OD does not exist in Iran.

According to the Theory of Planned Behavior (TPB), attitude towards any behavior is one of the factors that help predict intentions to perform a given behavior (11). Therefore, it is necessary to measure and assess attitudes toward OD in the general population or other groups in a society. At present, OD is performed in Iran. To our knowledge, there is valid, comprehensive questionnaire regarding attitude toward OD. This study aims to design and develop a tool entitled attitude toward donation-oocyte (ATOD-O) to measure attitude toward OD.

Materials and Methods

This methodological research was performed to design and develop a questionnaire with a Likert type scale to assess attitudes toward OD among infertile couples, donors and recipients of oocytes, and general population. This was a part of a big research entitled " attitude toward donation and surrogacy".

Designing and developing attitude statements about oocyte donation

Qualitative research

We conducted a qualitative study in order to obtain attitude scale-items. Infertile couples referred to Royan Institute were included in this research using the quota method that took into consideration socioeconomics, age, and educational levels of the patients. Data saturation was accomplished after 12 couples. For assurance, we continued the interviews for a total of 15 couples. Content analysis was performed by two different researchers (M.Sc. and Ph.D. in Epidemiology) for better validity (member check).

Literature review

In order to identify the presence of an existing questionnaire, influencing factors, and other aspects on attitudes towards OD, we searched Iranian and international databases that included Magiran, Google Scholar, Science Direct, PubMed, and Iran Medex. Both internal and external related papers were studied. Therefore, other possible questions that related to any aspect of OD were designed. The questions were comprehensive to the best extent possible.

Face validity

This type of validity indicates whether a test is apparently valid for subjects, administrative factors, and untrained observers (12). The face validity of ATOD-O has been assessed by 10 experts familiar and unfamiliar with the donation process. Experts took into consideration the proper sequence of questions, simple and illustrative form of the questionnaire, grammar, syntax, organization, appropriateness, and logical sequence of the statements (13).

Content validity

Content validity determines the extent to which the questions of the tool are related to the objectives studied (14). In order to assess and evaluate content validity of this questionnaire, we have used 16 knowledgeable experts that included obstetricians and gynecologists (5 persons) and community medicine specialists (5 persons), as well as experienced managers, nurses, and experts familiar with the process of OD (6 persons). These experts determined indices such as relevancy (power and ability of statements that reflect content characteristics), clarity (clarity in correct spelling and statements' concepts), and comprehensiveness (the ability of this tool to cover all relevant areas studied). The indices were subsequently assessed and assigned scores from 1 to 4, where a score of 1 was inappropriate, scores 2 and 3 were considered partly inappropriate and appropriate, and score 4 was quite appropriate. These individuals were asked to modify the statements they considered inappropriate. It should be said that the inter-rater agreement (IRA) by experts was calculated for the indices as follows.

We determined IRA on clarity and relevancy by dividing the statements that all experts agreed were appropriate by the total number of statements. The acceptable ratio was considered 70%. To specify clarity and relevancy of each statement, the numbers of experts who determined the indices for each statement were divided by the total number of experts in the

study (15). As well, to delimit the overall clarity of the questionnaire, a dichotomous option (appropriate and inappropriate) was considered for each statement after merging inappropriate or partly inappropriate, and appropriate or quite appropriate options. The mean was used to calculate the overall relevancy of this tool, in which the total relevancy of each question was divided by the total number of questions. The overall clarity of the questionnaire was also obtained using the mean. In various studies, appropriate relevancy/clarity of a new tool was considered to be at least 80%. The overall comprehensiveness of the questionnaire was obtained by dividing the numbers of experts who recognized comprehensiveness of the questionnaire as appropriate by the total number of experts.

Reliability

In this study, since the statements were qualitatively produced, we assessed reliability of the questionnaire based on the opinion of experts and infertile couples. Therefore, the statements had no capability for measuring repeatability of the total score in pre- and post-tests by intraclass correlation (ICC) and internal consistency reliability, using Cronbach's alpha (16-18).

Data analysis

Statistical analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA) version 18. The significant level was considered 0.05.

Ethical issues

This study was approved by the Ethical Committee of Royan Institute. The main objective of study was explained to participants. Informed consent from participants was obtained. The questionnaire contained no identifying information.

Results

Questionnaire design

We used data collected from the qualitative study and aspects obtained from database searches to generate a structured questionnaire. From the qualitative study, 12 domains were extracted from interviews and 8 domains were added from the literature review. After merging, deleting, and editing the items, they were reduced to 58 statements distributed in 12 domains. The different stages of the study and the outcomes obtained at each stage are shown in figure 1.

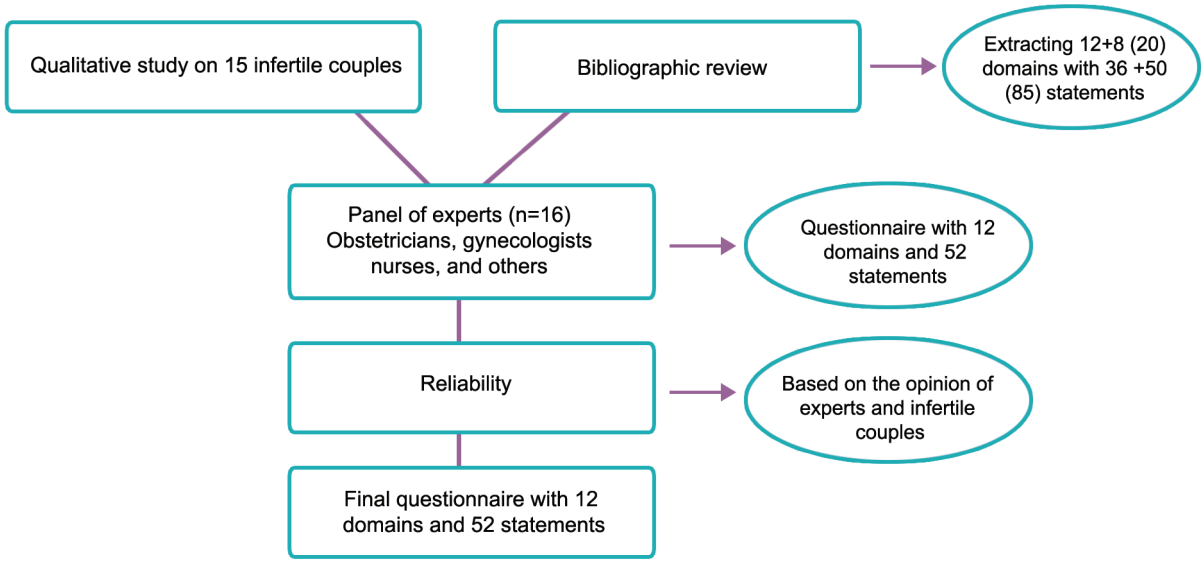

Fig.1: Flow chart related to the different stages of developing attitude toward donation-oocyte (ATOD-O).

ATOD-O questionnaire

In this study, we designed the questionnaire to include 58 statements according to various issues such as the OD process, donor and recipient characteristics, in addition to family, emotional, psychological, legal, religious, and socio-economic dimensions. According to the opinion of experts during content validity, we removed any unnecessary and less relevant questions. Some of the removed statements were as follows: "I agree to use oocytes from a living person"; the terms " IQ" and "morality" were deleted from "characteristics of the donor and recipient"; and "It is likely that the donor wants to see the child" was deleted. Finally, we reached a total number of 52 statements in 12 domains scored as follows: 1 (strongly disagree), 2 (disagree), 3 (somewhat), 4 (agree) and 5 (strongly agree). These domains included the importance of having children (2 statements), decision making and acceptance of OD (7 statements), playing the role of oocyte donor (5 statements), characteristics of the oocyte donor (8 statements), characteristics of the oocyte recipient (8 statements), being an anonymous child toward the donor (4 statements), disclosure of the use of this treatment method with others (3 statements), legal issues (4 statements), tendency to use different methods of OD (2 statements), the parent-child relationship (4 statements), and belonging of children (2 statements).

Validity

Considering the opinion of experts in assessing content validity, 11 statements in 3 domains were also modified for clarity, relevance, and comprehensiveness. Additional details about modified statements are shown in table 1.

Findings indicated that the IRA on clarity was approximately 70% (36/52). The IRA on relevancy was 71% (37/52). The questionnaire had high overall relevancy (97%) and clarity (96%). The overall comprehensiveness of the questionnaire was 100%.

Table 1: Modified statements by experts during assessing content validity of attitude toward donation-oocyte (ATOD-O)

Domains	Statements
Decision making about receiving donated oocytes	I am ready to use oocyte donation if there is no any other therapy for infertility problem.
	Mental conditions of my male partner are important for receiving oocyte donated.
	Relatives or friends' opinion is important for receiving oocyte donated for me.
	If my relatives or friends want to receive a donated oocyte, I would support their decision.
	Receiving oocyte donated is acceptable from my sister or relatives for me.
Decision making about donating oocytes	It is acceptable to give my oocyte my sister or relatives.
	I think that my male partner would agree on oocyte donation process for infertile couples.
	If my relatives or friends want to donate oocytes, I would support their decision.
Characteristics of an oocyte donor	The statement "beautiful appearance" was used instead of "a beautiful face".
	The statement "ethnicity and race" was used instead of "ethnicity".
	The statement "physical and mental health" was used instead of "physical health".

Discussion

A systematic review on OD conducted in 2009 showed 64 eligible studies; most lacked standardized and validated questionnaires that did not report reliability and validity (2). The lack of valid and reliable questionnaires could lead to greater heterogeneity of the results in the review. Thus, a comparison of the studies made it difficult to reach a conclusion. Hence, this study was undertaken in order to develop and evaluate a new instrument for measuring attitudes toward OD. The instrument was primarily developed according to a qualitative study on 15 infertile couples to ensure that this new instrument would cover all existing concepts that pertain to OD. In addition, according to experts' opinions, we removed any unnecessary and less relevant questions. The remaining questions were modified as statements. This tool included the following domains: OD process, donor and recipient characteristics, as well as family, emotional, psychological, legal, religious, and socioeconomic dimensions. We designed ATOD-O to be self-administered. However, in order to prevent selection bias due to illiterate participants and reduce missing data, this tool could also be used in an interview format.

Validity is requisite for a questionnaire because any defect or problem in the tool's structure leads to bias and confounding results (19). Content validity is the first and most crucial step in a questionnaire design process, and a prerequisite for other validities. The validity improves the quality, and increases questionnaire reliability. In other words, reliability of a questionnaire is useless without content validity (20). In this study, we have determined the overall relevancy and clarity of ATOD-O to be higher than 0.9, which indicated appropriate validity. Obtaining feedback and opinions, and developing a tool by experts has been shown to enhance content validity (21). Therefore, the relatively high number of specialists involved in developing ATOD-O (16 specialists), despite the greater variance, was an advantage of this study due to high generalizability and agreement. The overall comprehensiveness of the questions was 100%. This suggested that important aspects related to the topic of interest were asked.

To measure reliability in quantitatively developed questionnaires, indexes such as ICC and Cronbach's alpha are used. ICC assesses repeatability of the total questionnaire score by pre- and post-tests, whereas Cronbach's alpha coefficient is applied to measure internal consistency (22-24). These indexes are used when questions from each domain in a tool have a correlation with each other (25, 26). In the current study, the statements have been obtained from the qualitative assessment, therefore they had a qualitative nature, but no correlation. No correlation was seen among statements of each item. Therefore, reliability of ATOD-O was assessed based on the opinion of experts and infertile couples.

We designed the statements to include both important aspects (psychological, scientific, and legal issues) and more general details. To increase external validity and generalization of the instrument, we applied the terms "female or male partners" instead of the words "wife or husband", respectively. In conservative or religious societies such as Islamic countries, laws and rights are consistent with the religious orders or recommendations obtained from religious establishments. As a result, cohabitation for couples is illegal and not permissible for non-married couples. Therefore, only married couples can undergo infertility treatments in these countries. If this tool is applied in such societies, it can be modified by taking into consideration legal issues.

Finally, ATOD-O can assess attitude toward OD in the general population, donors and recipients of oocytes, infertile couples, and other groups in a society. It is necessary to update questions over time because this technique (OD) may be used more frequently in the future and information about OD will increase among individuals and the general population.

Conclusion

The findings from this preliminary validation study have indicated that ATOD-O is a valid tool for measuring and assessing attitude toward OD. It can be used in studies on different groups in a society. This newly developed scale can also be particularly useful and helpful to health professionals and authorities in order to assess the beliefs and attitudes of individuals regarding the OD process.

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Original Article

The Effectiveness of Emotionally Focused Couples Therapy on Sexual Satisfaction and Marital Adjustment of Infertile Couples with Marital Conflicts

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Abstract

Background: The purpose of this investigation is to determine the efficacy of emotionally focused couples therapy (EFT-C) on enhancement of marital adjustment in infertile couples.

Materials and Methods: This was a semi-experimental study with a pre- and post-test design. We selected 30 infertile couples (60 subjects) by purposive sampling. Couples were randomly assigned to two groups, sample and control. Each group consisted of 15 couples who had marital maladjustment and low sexual satisfaction. Couples answered the marital adjustment and sexual satisfaction questionnaires at baseline after which the sample group received 10 sessions of EFT-C.

Results: Results of pre-test and post-test showed that EFT-C significantly impacted marital adjustment and sexual satisfaction.

Conclusion: EFT-C had a significant effect on enhancement of satisfaction, cohesion and affectional expression. This approach impacted physical and emotional sexual satisfaction of infertile couples.

Keywords: Couples, Therapy, Adjustment, Sexual, Satisfaction

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Introduction

As marital life begins, couples expect to have children because with the birth of a baby, life will head toward another path. In contrast, in the case of infertility, serious psychological problems may be faced by partners (whether male or female) (1). Although in recent years infertile couples have greater opportunities to bear offspring due to advances in medical science and various fertility methods (2), this situation, as a tense crisis, influences different life aspects of infertile couples (3). For most people, children are the meaning of life and constitute an important part of their identity. Multiple researches on infertile couples that yearn for biological babies have shown that these couples experience tension in a deep, distressed manner (4). The medical definition of infertility is as follows: after one year of usual sexual activity without contraception if the woman is either not pregnant or fails to become pregnant, she is considered infertile (5).

Infertility brings about detrimental psychological effects (6) that include reduced self-confidence, disordered self-image, and impairment of masculine and feminine identities (7). Some researchers

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believe that infertility is a challenging experience which leads to problems in marital life (8).

Marital adjustment is a changing process that includes four aspects of couples' performance as a joint life: i. Marital life satisfaction, ii. Commitment to marriage, iii. Agreement and unanimity in marital life, and iv. Manifestation and expression of couples' emotions and feelings within the family (9). Couples who agree with each other are relatively satisfied with their marital life and express satisfaction with their partner's personality habits, enjoy companionship with their family and friends, solve problems together, and are highly satisfied with their marital and sexual life (10). One of the biggest problems that influence one's personal and social life is sexual satisfaction which plays a crucial role in personality development. Just like other fundamental motivations of human beings, sexual motivation and desire constitute an inevitable part of their biological, psychological, and social natures. The quality with which this a biological need is met plays a very important role in personal and social health, thereby achieving relaxation and comfort (11). Couples' degree of satisfaction with sexual relations and ability to take pleasure in and give pleasure to one another is called sexual satisfaction. Gratification from sexual relations is one of the important factors of marital life satisfaction, and those with greater sexual satisfaction considerably report a better life related to those without sexual satisfaction (12).

A desirable sexual relation can increase the possibility of fertility. Furthermore, infertility, in turn, can influence couples' sexual relations. It is thought that psychosexual disorders in infertile couples are more prevalent than other couples (13). Infertility leads to increased sexual disorders, maladjustment, reduced sexual satisfaction and sexual activity times (14). Infertility influences one's sense of self, sexual identity, self-confidence, and body-image, it inevitably impacts sexual relations, the importance of sexual relations, and sexual desire along with satisfaction (15). Researches show that men and women react differently toward infertility.

Gender is an important factor in assessing differences between men and women regarding the stress of infertility and sexual satisfaction. Duration of infertility and the reason and type of infertility are among important factors of sexual and marital satisfaction of infertile couples. Besides age, marriage length, education, income, and social class have been stated in different resources as factors that influence infertile couples' sexual and marital satisfaction (16). Infertility leads to couples' reduced sexual satisfactions (17). Anxiety and loss of self-confidence, shame, and depression that result from infertility harm infertile people's sexual performance. Moreover, diagnosis, examination and treatment of infertility affect their sexual satisfaction (18). Dyer et al. (19) have reported that dissatisfaction with sexual relations, stress and planning for sexual relations, and lack of sexual self-esteem in infertile women have the highest impact on their sexual satisfaction. Additionally, since women have reported that they only think about having a baby while having a sexual relation, thus this concern leads to increased stress (15).

Infertile couples experience tremendous stress at the first phase of treatment regarding their sexual activity, especially sexual desire and sexual arousal. Women suffer more than men. Therefore, doctors must pay attention to sexual problems of couples in order to prevent a vicious cycle which may decrease the possibility of pregnancy and cause permanent disorder to their sexual relations (20).

The relationship between infertility and sexuality can be seen from two angles: i. Infertility as a cause or ii. Infertility as a result of sexual dysfunction. However, sexual disorders are considered as minor reasons for infertility as approximately 5% of all infertile cases result from sexual disorders (21). In addition, men's sexual disorders include chronic erection dysfunctions and non-ejaculation while the only sexual disorder in women in terms of infertility is vaginismus (22). Since the label of infertility is worrisome, sexual intercourse can lose its spontaneity (23).

Examination and treatment of infertility in traditional social interactions leads to a high level of psychological distress that directly affects couples' marital and sexual relations (14, 24-26). The clinical effect and impact of this disease on couples' overall health, daily performance, social interaction and marital relations, and quality of life is partly underestimated (24).

In the examination of an infertile couple, it is highly important to note that emotional factors, as sexual disorders attributed to emotional factors, may lead to infertility. Emotions have a key role in the infertile couples' relations and must be given special attention. Therefore, one can use emotionally focused couples therapy (EFT-C) that consists of 9-20 short-term, structured sessions. This ther-

apy is both a branch of couple's therapy and regards emotions as a treatment axis. This treatment addresses relational disorders and maladjustment and encourages people to talk about and discuss their emotions. From the EFT-C point of view, the center of marital distress is created and continued by negative affection and attachment injuries (27).

In EFT-C, it is assumed that conflict in marital life occurs when spouses fail to meet each other's attachment needs for security, safety, and satisfaction. In other words, disturbed marital relations are suggestive of the couples' failure in establishing relations associated with a safe attachment pattern. By not meeting each other's attachment needs, these spouses experience secondary emotional responses such as anger, hostility, vengeance, or feelings of guilt.

Secondary emotional responses are also manifested in avoidant aggressive behaviors, which may ultimately lead to the creditor-creditor or avoidance-avoidance models. These inflexible interactional models which also fuel conflict occur repeatedly because spouses desperately want their genetic attachment needs to be met. Unfortunately, partners' efforts to get the spouses' attention are not made properly. Consequently, the partners are forced into relations that lead to continued failure of attachment needs (28). Accordingly, EFT-C seeks to solve couples' problems by focusing on their emotional relationships. The change process of EFT-C has been specified in three phases that include: i. Prevention from development of a vicious cycle; ii. Reconstruction of interactional situations and iii. Consolidation and integration (29). Therefore, the present study attempts to explain whether EFT-C can increase the adjustment of conflicting infertile couples that suffer from low sexual satisfaction.

Materials and Methods

This was a quasi-experimental study that used pre- and post-tests on two groups, control and sample.

To test the hypothesis of research, that is the rejection of null hypothesis (Ho) and the acceptance of (HA), values of 0.05 and 0.01 were used for alpha. In other words, for the level of significance, probability of error was determined to be less than $P<0.05$ or $P<0.01$ and if greater than 0.05 it was considered to be non-significant.

We assessed subjects' demographics after which they were measured before conducting the independent variable (EFT-C) as a pretest. Then, we administered EFT-C to each couple in the experimental group over a period of 10, 120 minutes sessions in accordance with Johnson's model (27). Subjects were subsequently re-evaluated (post-test).

The statistical population of the study included couples who visited infertility centers during 2013 and were identified by obstetricians and gynecologists as infertile. Couples were living with each other for at least 10 years. Individuals (men and women) completed the marital adjustment and sexual satisfaction questionnaires.

In this research, purposive sampling was used whereby 30 couples (60 subjects) were selected and randomly assigned to two groups, sample and control. Each group consisted of 15 couples who had marital maladjustment and low sexual satisfaction. Inclusion criteria for the study were marital conflict and low sexual satisfaction in infertile couples, interest in couples therapy sessions, present for all treatment sessions, having at least a high school level of education, no significant acute mental-physical disorders as self-reported in the demographic characteristics questionnaire, and loss of fertility after 10 years of marital life. Exclusion criteria included lack of marital conflicts and high sexual satisfaction in infertile couples, non-participation in all stages of measurement and intervention, having a significant acute mental-physical disorder through the self-reported demographic characteristics questionnaire, primary school level of education, and infertility less than 10 years. All participants expressed consent to participate.

In order to analyze data, we used descriptive statistical methods that measured the mean, maximum and minimum standard deviation. Analysis of covariance was adopted using SPSS version 18 in the inferential section.

Research tools

Questionnaire of demographic characteristics
This inventory included factors of age, sex, educational level, occupation, income level, and cause of infertility, period of infertility, quantity of surgeries, date of last surgery, history of attending psychological or counseling sessions, as well as histories of any chronic physical or psychological disorders (30).

Spanier's Dyadic Adjustment scale

This scale is constituted of 32 questions based on a Likert scale of responding which measures the total score of marital adjustment in a range of 0 to 15. Individuals who score 101 or less according to Spanier, are deemed as maladjusted and those with higher scores are supposed to be well-adjusted. In a study by Hasan shahi (28), well-adjusted couples had an average score of 114.7 ± 17.8 while the average score for maladjusted couples was 70.7 ± 23.8. Spanier categorized the data into four subscales of marital satisfaction, dyadic consensus, dyadic cohesion, and affectional expression with evaluated validities of 0.94, 0.90, 0.81 and 0.73 respectively. The entire scale had a validity of 0.96. Reliability was estimated to be 0.86 according to Pearson's correlation coefficients between Locke-Wallace Marital Adjustment Scale and Spanier's scale (30).

Index of Sexual Satisfaction

This scale was developed by Hudson et al. and revised by Javidi (15). In 1981 it contains 25 questions with a 5-point Likert response scale (1=never, 2=rarely, 3=sometimes, 4=most often, 5=always). This scale evaluates sexual satisfaction in two aspects of physical satisfaction and emotional satisfaction. The dimension of physical satisfaction includes sexual behaviors and sexuality whereas the emotional aspect includes intimacy and quality of sexual relations. Hudson believes that this scale assesses sexual satisfaction through the intensity and extent of sexual components. Internal consistency of this test by Cronbach's alpha coefficient was calculated to be 0.92. Studies have indicated that this questionnaire is significantly related to scales designed to measure similar constructs. The correlation coefficient of this scale with the Marital Satisfaction questionnaire was 66% (31). This was the first time this scale was used in Iran. Hence, we assessed the psychometric properties from two aspects, reliability and validity. The alpha value for the entire scale was calculated to be 0.88 and for emotional satisfaction this value was 0.90. The alpha value of the sexual behavior subscale was 0.84, for sexuality it was 0.78, sexual intimacy was 0.87, and quality of sexual relations was 0.74. The reliability coefficient for sexual satisfaction by the method of split-half was calculated to be 0.85 and the Spearman revised coefficient was 0.92 (32). Table 1 points out the treatment protocol used in this study, this protocol is emotionally-focused therapeutic approach, which have been provided to the couple during 10 sessions.

Ethical consideration

In order to observe ethical considerations, in this study, the researchers made a great importance to the confidentiality and preserving the couples' dignity. In addition, since the emotionally-focused treatment training has been effective in experiment group, the researchers also carried out such training for members of the control group in their training sessions after the completion of their work.

Results

The demographic description of the sample is provided in table 2. There were 30 participants of both genders. The maximum and minimum frequencies in terms of educational level were 19 (31.7%) individuals with diplomas and 10 (11.7%) people with secondary school certificates. The average age of participants was 33.8 ± 5.03 years.

First, we used the Kolmogorov-Smirnov test. The data was approved as normal for all variables ($P>0.05$). There was no significant difference between groups in the pre-test subscales of marital adjustment and sexual satisfaction.

Table 2 shows no significant difference between groups in marital adjustment and sexual satisfaction ($P>0.05$). Therefore both groups were the same at the pre-test stage. According to table 2, it could be inferred that no significant difference existed between groups in the pre-test sexual satisfaction subscales ($P>0.05$).

According to the covariance analysis test results in the dimensions of marital adjustment, there was a significant difference between the pre- and post-tests in terms of couples satisfaction, couples correlation, couples agreement, expression of love, and sexual satisfaction (physical and emotional, $P<0.001$).

In order to compare average sexual satisfaction of the sample group with the control group at the pretest stage, we statistically compared the two independent means. The Kolmogorov-Smirnov test was also employed to assess normal distribution of the variable of sexual satisfaction which, according to the results, was confirmed ($P=0.599$ and Z K-S=-0.526). Thus, comparison of two population means is practicable. Table 3 shows the results of ANCOVA of marital adjustment and sexual satisfaction subscales in couples.

Table 1: Johnson's protocol of emotionally focused therapy (EFT-C) for infertile couples (30)

Step	Session	To do
1 Identification	1	Collect general information about the couple; introduce the therapist to the partners; investigate grounds and expectations of participation; define the method of EFT-C in addition to concepts of infertility, conflict, marital adjustment, sexual satisfaction, and life quality; ask the couple for their opinion on the method and concepts; identify negative cycles; assess couple's way of dealing with issues; discover attachment blocks as well as personal and interpersonal tensions; evaluate status of marital relationship, sexual satisfaction and quality of life.
		Task: Pay attention to positive and negative emotions such as joy, happiness, anger, hate, sadness, jealousy, anxiety, etc.
	2	Appoint a separate session for each partner to discover significant events, and information that is not feasible to discuss in the presence of the other, such as commitment to marriage, extramarital relationship, exporter attachment trauma; assess the fear of revelation.
		Task: Pay attention to your partner's cycle of interaction.
2 Change	3	Ascertain interaction patterns and ease acceptance of the experienced emotion; discern every partner's fears of insecure attachment; help each partner with openness and self-disclosure, continue the therapy.
		Task: Discern pure emotions, thoughts, and sentiment.
	4	Restructure the bond through clarification of key emotional reactions; widen emotional experience of each spouse to create new ways of interaction, partners should accept new patterns of behavior.
		Task: Express pure emotions and sentiments.
	5	Task: Deepen the relationship by recognizing recently developed needs of attachment, improve personal health and relationship status, express pure emotions and sentiments
3 Stabilization	6	Establish a safe therapeutic alliance, develop new ways of interaction; promote acceptance of the other, discover deep-seated fears and express needs and wants.
	7	Restructure the emotional experiences of the couple, clear the needs and wants of each partner.
		Task: Underline strengths and weaknesses.
	8	Support couple in finding new solutions to past problems; change problematic manners of behavior, facilitate steps the couple can take to invest in their responsive and accessible positions, sync the inner feelings and concepts to the relationship, encourage positive reaction.
		Task: Find new solutions to past problems.
	9	Take advantage of therapeutic achievements within daily life to consolidate intimacy, keep going with the therapy and its direction, create secure attachment, discern and support constructive patterns of interaction; help the couple shape a story about their future together.
		Task: Practice the techniques in daily life.
	10	Ease the end of the treatment, keep the way of therapeutic changes, draw a comparison between the past and present cycles of interaction, keep on emotional involvement to the deepest status of the relationship.

Table 2: Pre-test comparison of the groups in the subscales of marital adjustment and quality of life

Subscales	Group	Mean	Standard deviation	t *	df **	P value ***
Satisfaction of dyadic	Control	22.18	4.34	0.813	58	0.425
	Sample	21.29	4.25			
Cohesion of dyadic	Control	7.94	2.05	1.087	58	0.283
	Sample	7.35	2.17			
Consensus of dyadic	Control	24.21	5.91	1.493	58	0.146
	Sample	21.80	6.52			
Affectional expression	Control	4.67	1.30	0.787	58	0.434
	Sample	4.40	1.33			
Physical sexual satisfaction	Control	30.06	7.38	0.99	58	0.58
	Sample	28.20	7.20			
Emotional sexual satisfaction	Control	21.20	8.30	2.269	58	0.027
	Sample	16.83	6.49			

*; Paired t test, **; Degrees of freedom and ***; Probability of rejecting the null hypothesis.

Table 3: ANCOVA of marital adjustment and sexual satisfaction subscales in couples

Aspects	Freedom	Mean square		F Value		P value		Effect size		Statistical power	
	Pretest	Pretest	Group member ship	Pretest	Group member ship	Pretest	Group member ship	Pretest	Group member ship	Pretest	Group member ship
Satisfaction of dyadic	1	141.82	5138.84	10.12	363.96	0.002	0.001	0.151	0.87	0.88	1
Cohesion of dyadic	1	54.07	62.478	15.38	15.86	0.001	0.001	0.212	0.93	0.96	1
Consensus of dyadic	1	427.14	16503.14	13.21	542.54	0.001	0.001	0.19	0.90	0.92	1
Affectional expression	1	10.126	703.516	8.14	565.79	0.006	0.001	0.125	0.91	0.80	1
Physical sexual satisfaction	1	82.338	18769.06	1.16	263.47	0.287	0.001	0.002	0.82	0.18	1
Emotional sexual satisfaction	1	141.48	22023.44	50.3	545.83	0.066	0.001	0.058	0.90	0.45	1

According to the results from Levene's test, equality of the variances in the sample and control groups was corroborated (P>0.05). Therefore, use of covariance analysis was permitted. The results of covariance analysis related to comparison of mean scores of studied dimensions in the sample and control groups are shown below.

As table 2 shows, there was a statistically significant difference between the groups (P<0.001). Thus, EFT-C positively affected sexual satisfaction and marital adjustment in infertile couples. The effective percentage of intervention was: dyadic satisfaction (86%), dyadic cohesion (92%), dyadic consensus (90%), affectional expression (90%), physical sexual satisfaction (82%) and emotional sexual satisfaction (90%0, which indicated the efficacy of EFT-C.

Discussion

Findings of the present study were consistent with related studies. In different studies (32-34) it was found that infertile couples did not have a clear sign and regardless of men's sexual role, obtained a higher score in the sexual satisfaction, sexuality, and orgasm items of the questionnaire. A comparison of the results of Experimental and control groups in terms of sexual arousal, satisfaction, lust, and orgasm, suggested that the experimental group was weaker than the fertile control group. Sexual life of infertile couples was poor due to infertility. In his study entitled "Sexual Disorders in Infertile Couples", Wischmann (35) concluded that sexual dysfunction, as the factor of reluctance to have children, was relatively abnormal. Instead, temporary sexual disorders in couples with infertility affected women more than men due to diagnostic complications and pharmacotherapy. Counseling for couples with unfulfilled desires to have children should include clear, appropriate clarification of sexual and gender disorders.

Findings of this research corresponded with the results of a study by Greil et al. (36) as well as another research by Martins et al. (37). Greil et al. (36) declared that EFT could have significant positive and constructive effects on the relationship of couples and their satisfaction with life which might have declined due to infertility. Greil et al., through a meta-analysis, stated that a variety of psychological interventions could improve both life quality and relationship of infertile couples. The single difference between these methods was the size of the effect. Couples therapy surpassed the other interventions in this respect. This achievement could be attributed to the fact that couples therapy during the intervention points out at the same time.

Tie and Poulsen believed that EFT was influential in the promotion of couples' marital adjustment. After intervention, the relationship of couples improved and they expressed increased satisfaction from their spouses when compared to the past. Marital disputes significantly decreased and an upturn was observed in marital adjustment. The results of Tie and Poulsen's research have been confirmed by the present study. Dyadic consensus is one the aspects of marital adjustment. Increase in this area denotes growth of dyadic consensus (38).

Bodur et al. (39) measured the effect of marital adjustment in infertile couples on stress related to infertility. This study included 104 couples with primary and/or secondary infertility and 44 fertile couples as the control group. Women in infertile groups reported more psychological symptoms and decreased marital adjustment than men in infertile groups, but there was no significant difference between partners in the control group regarding the aforementioned parameters. In general, infertile couples had decreased marital adjustment and increased depression and anxiety levels. Nevertheless, if infertile couples were mentally supported and received service from social systems, their marital adjustment would increase and psychological symptoms disappear.

Barani Ganth et al. (40) reported that fertile couples were more satisfied with their marital life than infertile. The effect of infertility on women's satisfaction appeared more concerning compared to men. Infertility in women has been shown to lead to a life with low marital satisfaction.

Jalil and Muazzam (41) found a significant relationship between emotional intelligence and marital satisfaction in both groups. A comparison of both groups showed that fertile women with higher emotional intelligent had greater levels of marital adjustment. On the contrary, infertile women with lower emotional intelligent suffered from poorer marital satisfaction.

Moura-Ramos et al. (42) showed that contextual factors such as socioeconomic status and urban or rural residence impacted affective anxiety in infertile couples. An Investigation into marital relationships in infertile couples showed that male infertility did not any negative effect on the marital relationship.

In Austria, Drosdzol and Skrzpulec (43) evaluated sexual and marital Interactive responses in infertile couples which showed that marital relations of infertile women were less than fertile women. As a result, the former were more prone to marital disorders than fertile women.

Mira (44) investigated the effect of EFT-C on knowledge, competence, emotional processing, and self-compassion of 76 participants. The results showed that knowledge and competence of participants in the experimental group increased as a result of therapy. He also stated that a significant relationship existed between competence and the emotional process of individuals as well as self-compassion. Javidi et al. (45) investigated the long-term effect of EFT-C on knowledge, competence, self-compassion, and secure attachment. Results showed the effectiveness of EFT-C in increasing knowledge, competence, self-compassion, secure attachment, and personal interactions.

Pinto-Gouveia et al. (46) investigated the efficacy of protective emotion-regulation training toward adjustment in infertile patients. They reported that infertile couples had problems with expression of emotions and mutual understanding of their partners' feelings which were notably resolved by training in emotion-regulation strategies. After intervention, couples had great success in affectional give and take with improvement in their marital adjustment (46). Javidi et al. (47) indicated that EFT-C could significantly increase sexual satisfaction of couples.

Finally, it can be said that EFT-C trains couples to correct their behavior through increasing security and support, availability, response to the spouse's need and creating safe behaviors, methods of increasing intimacy and relationships, learning proper communication skills, and establishment of desirable sexual relations.

This study has regarded sexual satisfaction as an important factor for controlling daily emotions. In explaining the reason for the increase in couples' sexual satisfaction due to EFT-C, it can be said that this therapy teaches couples to reveal the important problems of their life to their spouses, receive a positive response from spouses. In addition, they can also increase their verbal and non-verbal interactions, show sexual self-expression including touching, hugging, and kissing, express their thoughts, feelings, needs and tendencies, and have more physical closeness. Considering that sexual relation is among the most important matters in marital life and acts as the emotional barometer in relations, it can reflect couples' satisfaction with other aspects of the relationship. Thus, it is a good scale of the overall health of couples' relations.

Use of this treatment plan can help to increase couples' sexual satisfaction and intimacy, leading to improved overall relations for couples.

Conclusion

This treatment approach can increase adjustment and sexual satisfaction in couples. Results from this research can create a clear and practical outlook for counselors, psychotherapists, and family therapists. The results can provide couples with desirable applied and empirical guidance for creating self-esteem and detection, and revision of inconsistency in their way of giving messages to each other, their communication patterns and, as a whole, human growth which consequently reduces marital conflicts and increases adjustment. The results of the emotional sexual satisfaction dimension were consistent with those reported by Huppelschoten et al. (48).

EFT-C, through concentrating on the emotional relationship, manages to solve couples' problems. Accordingly, marital disputes that result from emotional problems and insecure attachment can be addressed by EFT-C. Sexual dissatisfaction generally appears in the form of complaining, blame, or rebuke and extremely endangers attachment. The sexual relationship is more than intercourse and is a way to achieve harmony and create positive emotions which thereby strengthens attachment (49). Application of this therapeutic plan can promote sexual satisfaction of couples and their intimacy.

By taking into consideration the effects of EFT-C on marital adjustment and sexual satisfaction, we propose that additional research should investigate

which technique from the EFT-C approach has the highest level of effectiveness.

As infertility has negative effects on marital satisfaction, it is recommended that necessary training be provided in fertility clinics to lessen social and psychological stress of clients and increase their marital adjustment. Since familial disputes are the most important cause for separation between infertile couples, training in marital relationships can prevent couples from divorcing and instead, reinforce their family's foundation.

Constraints of the study included the number of questionnaires and large numbers of questions which made the respondents tired. Additionally there was no follow-up due to participant refusal.

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Ethics, Legal, Social, Counseling Article

The Lineage of Children Born by Sperm Donation: A Shiite Perspective

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Abstract

Background: Despite the meager role of the masculine agent in infertility (the low number of infertile men than women infertile), there are men whose wives are unable to become pregnant due to the absence of sperm, decreased numbers of sperm or lack of sufficient motile sperm. Utilizing donated sperm is a method that enables these families to have children. The use of this method prompts us to ask different questions, among which is the quality of the child's relation to the sperm donor, the sterile man and his wife. In this research we intend to study the issue of lineage of those who use heterogeneous insemination.

Materials and Methods: This analytical-descriptive research gathered relevant data in a Literature search. After a description of the fundamentals and definitions, juridical texts were subsequently analyzed and one of the viewpoints regarding lineage related to infertility treatment by donated sperm was selected.

Results: There are three persons that have a possible legal relationship to the child born from this method - the sperm donor (biological father), the wife's husband (social father) and the wife (mother). In treating infertility with donated sperm, there is neither a third party to make the possibility of attribution of the child nor is there a doubt that the child is the result of insemination of the woman's egg with the donated sperm rather than the husband's sperm as he has a lack of sperm.

Conclusion: The child born by heterogeneous insemination only has a relation with the sperm donor and the woman contributing her egg. This child is eligible for all parental rights and obligations. These children are not related to the sterile man.

Keywords: Kinship, Lineage, Intimacyn

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Introduction

In order to comprehend fertility, it is important to understand how conception naturally occurs. First, the ovary must release (ovulate) an egg, which must be picked up by the fallopian tubes. Sperm must travel through the vagina into the uterus and into the fallopian tube in order to fertilize the egg. Fertilization usually takes place in the fallopian tube. Then, the fertilized egg or embryo travels down to the uterus, where it implants in the uterine lining and develops. Infertility occurs when there is a problem at any part of this process (1). Infertility is defined as one year of unprotected intercourse that does not result in pregnancy. This condition may be further classified as primary infertility, in which no previous pregnancies have occurred and secondary infertility where a prior pregnancy, although not necessarily a live birth, has occurred (2).

Infertility affects approximately 10 to 15% of

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couples and is a medical problem for 2.7 million women of reproductive age in the United States. Over the past few decades, successful treatments for all infertility types have been developed, providing hope for infertile couples (3).

The physician's initial visit with the infertile couple is the most important because it can serve as a guide. Factors from either or both partners may contribute to difficulties in conception; therefore, it is important to consider all possible diagnoses before pursuing invasive treatment. The main causes of infertility include male factor, decreased ovarian function and ovulatory disorders (ovulatory factor), tubal injury, blockage, or paratubal adhesions (endometriosis with evidence of tubal or peritoneal adhesions), uterine factors, systemic conditions (infections or chronic diseases such as autoimmune conditions or chronic renal failure), cervical and immunological factors, and unexplained factors (endometriosis with no evidence of tubal or peritoneal adhesions) (4).

Although 20% of infertility relates to men (male factor), it may be a contributing factor in as many as 30 to 40% of cases. Treatment of reversible endocrine or infectious causes of subfertility, such as sexually transmitted diseases and thyroid disorders, tends to be efficacious. Although the prevalence of infertility has not changed, the demand for infertility care has increased significantly over the past few decades (4).

Heterogeneous insemination is a type of assisted reproductive technique (ART). In this method, sperm from a man other than the husband is inseminated together with the wife's egg inside her womb. Although it is possible to use fresh sperm, in most cases frozen sperm stored in sperm banks are utilized.

Regardless of whether Islamic jurisprudence considers the use of donated sperm as permissible (javaz) for the fertilization of a man's wife or not (adam al-javaz), the most important question that confronts Muslim scholars is, as a result of this process, the child or children's legal status and to whom their lineage (nasab) should be linked.

Materials and Methods

The study of lineage of children born by sperm donation is an interdisciplinary research that includes both medical and Islamic jurisprudence sciences. Identification and specification that pertain to methods to conceive a child by heterogeneous insemination is an issue related to medicine. However the specification of religious statements on this issue which include familial relative or non-relatives of children born by sperm donation is related to Islamic jurisprudence. Islamic jurisprudence is the result of Muslim jurisprudents' attempts with specific religious beliefs.

Due to the extensiveness of Islamic perspectives, we conducted this research according to the ideas of Shi'ite jurisprudents among the five Islamic schools of thought, namely Shi'a, Hanafi, Maliki, Shafi'i, and Hanbali. The author studied law books and Shi'ite jurisprudence texts that pertained to children's lineage. After library research and analytical analyses, this study has proven the lack of existence of a familial relationship between the child born by sperm donation and his/her social father.

Results

If donated sperm is used for treating infertility, there will be three persons that have a possible legal relationship to the child born from this method-the sperm donor (biological father), the wife's husband (social father) and the wife (mother).

Sperm donor (biological father)

There are three different viewpoints on the fatherhood relationship between the sperm donor and the child produced from this donation.

First viewpoint

Discontinuity of lineage (nasab)

Adherents of this viewpoint have cited two points in proving discontinuity of lineage.

Absence of marriage bed (farash)

Some jurists believe that the child is not related to the sperm donor in terms of lineage (nasab) because this child has not been born in his farash (5).

The "farash rule" makes sense when there is doubt in attribution of the child to the legal husband of the wife or a third party (6). However, in treating infertility with donated sperm, there is neither a third party to make the possibility of attribution of the child nor is there a doubt that the child

is the result of insemination of the woman's egg with the donated sperm rather than the husband's sperm as he has a lack of sperm.

Lack of sexual intercourse (penetration)

In some juridical texts there are three conditions stated required for linking the lineage of the child to the husband of a woman: sexual intercourse (dokhul) of the man and woman, having past at least 6 months from the time of intercourse until the delivery date (minimum delivery period) and having past not more than 10 months from intercourse until the delivery date (maximum delivery period) (7-9). Therefore if the man did not have intercourse with his wife, the child born from that woman would not be linked to him. The husband would not be regarded as the father of this child (10, 11). Additionally, because the sperm donor did not have intercourse with the infertile woman, the resultant child could not be considered his child (5).

Answer: Although the issue of validity of sexual intercourse in different jurisprudent discussions such as the child's lineage in temporary (mowaqqat) and permanent (daim) marriage, the necessity of observing the waiting period, oath of imprecation (lian) and denial of the child (nafy al-walad) is described. However it seems that the validity of sexual intercourse in substantiation of lineage and establishment of kinship between the biological father and the child is only because in normal conditions and in most cases, sexual intercourse is the way to transfer sperm into the woman's womb (8, 12, 13). Therefore if a man transfers his sperm into his wife's womb without having sexual intercourse, no doubt the resultant child will be considered his child (14-17). For example, as in anal intercourse (watye fi al-dobor), if there is a possibility for sperm to reach the womb, the child will be attributed to the owner of the sperm (10, 18). This intercourse under normal conditions cannot be the cause for fertility (9, 19). Also if the husband has been away from his wife (ghaybat) for more than ten months or the couple are united in the absence of intercourse, the child would not be attributed to the husband since during this period intercourse was not possible (20-22) without a need for the husband to deny the attribution of the child to himself by liʿan va nafy al-walad (23).

Second viewpoint

Difference between cases (separation)

Followers of this doctrine contend that although there is a birth relationship between the child and sperm donor, this relationship legally does not always have equal consequences. A distinction should be made between the cases.

Known or unknown sperm donor

In heterogeneous insemination from a known donor, the child is attributed to the donor; the parental rights and duties are preserved between them. However if the sperm donor is unknown, since the basis of donating sperm is on not having a link with the resultant child, a legal judgment on realizing the legal effects of the paternal relationship between the donor and child will be an imposition of lineage on the sperm donor. Hence the social father is the real father of the newborn child (24).

Answer: There is no doubt that the sperm donor, whether known or unknown, has no motive for reproduction and consequently survival of generations. Thus the issue of imposition of lineage is not applied just to the unknown sperm donor. Furthermore, lineage (nasab) is a genetic link independent of the will of either the man or woman. For the same reason, children born without the will of either the wife, husband or both parents (unwanted child), have as much lineage link to their parents as the children who are born willingly (25, 26).

Unknown status does not negate lineage from the sperm donor but only prevents actualizing lineage to him. For this reason if the sperm donor is known, no doubt, the child will be attributed to him since a child without a father does not have any position in the legal system.

Awareness and unawareness of the sperm donor

Certain lawyers believe that awareness and unawareness of the sperm donor will affect the attribution and lack of attribution of the child to the donor. If the sperm donor, knowing that his donation will set the stage for producing a child unlawfully, proceeds to do such an action then the child born by sperm donation will be regarded as illegitimate (walad al-zina) and no legal relationship will exist between him and the donor. A child belongs to the father when it is born through legal and normal ways (marriage). Pregnancy through sperm donation is

not considered to be included in such methods.

However if the sperm donor is unaware that donating his sperm is the beginning for producing a human being or is not aware that donating sperm to others is not permissible, the child born in this way will be considered a child of suspicious intercourse (walad al-shobha) and therefore attributed to the sperm donor (27, 28).

Answer: The title illegitimate child (walad al-zina) applies to a child who is the product of illegal sexual intercourse (zina). On the other hand, procreation is a natural process which depends upon the man's sperm and woman's egg to join together normally through sexual intercourse. Naturally this genetic link cannot be legally taken away (29) although legislators can ignore certain consequences of such a genetic link.

Therefore there is no doubt that the sperm of an adulterer is not respectable (without legal value and consequences) (30) and the child who is born through an illegal relationship (adultery) is not a legitimate child (31). However this does not mean that the child born through an illegal relationship is without parents (32) because the performance of sexual intercourse itself is enough for this link (23). Although the legislator of Islam has excluded only the child's inheritance from parents (33-35), they have recognized other outcomes of this birth such as being intimate (mahram) and sanctity of marriage, as is the case for legitimate children (17, 29).

Hence, the child born by heterogeneous insemination is not illegal, and there is no reason for negation of their lineage (nasab) and the sperm donor (biological father) would be their real father.

Third viewpoint

Continuation of lineage

The child born through artificial insemination with donated sperm (AID) legally belongs to the sperm donor - attributed to him by name, biologically, genetically and customarily (24). Similarly, the sperm donor is considered to be the child's father and the child is deemed as his real and legal child. Consequently, overall parental rights and obligations apply to the sperm donor and the child.

To prove this claim, we cite a number of traditions (rawayat) as evidence such as the transfer of sperm by a method other than conventional sexual intercourse (tribadism/lesbian sex-musahiqa). These traditions emphasize that when a man has a type of sexual relationship with his wife that leads to ejaculation of sperm and the woman immediately leaves her husband and goes to another woman where contact between their genitals (musahiqa) causes the transfer of her husband's sperm to the second woman, which results in pregnancy of the second woman by this contact. The resultant child belongs to the husband of the first woman, as the owner of sperm, in terms of lineage (36-39).

Shiite jurists and lawyers based on these traditions have declared that once the sperm of a man enters the womb of a woman other than his wife with whom he has not had sexual relationship, thus making her pregnant, the resultant child belongs, in terms of lineage (nasab), to the owner of the sperm. The sperm of the man is considered the cause of producing this child, while no illegitimate sexual relationship has existed between the owner of the sperm and the woman who became pregnant by his sperm (6, 21, 40-53).

Social father

One of the most important issues discussed in AID is the relation of the baby born to an infertile man whose wife utilized donated sperm. Can the infertile man consider himself to be the father of the child and be entitled all paternal rights and obligations?

First viewpoint

Apparent link

A number of lawyers believe that the child does not have a real, genetic link to the infertile man (the mother's husband). However according to the farash rule, the child is apparently connected to the mother's husband and all paternal rights apply.

Answer: The principle of farash is considered a legal principle on which the Holy Prophet (pbuh) of Islam and his immaculate family have relied. The subject of these traditions is the married woman who, while married and has sexual intercourse with her husband, becomes pregnant through intentional or unintentional sexual deviation. Her husband may raise the question that whether his wife's pregnancy is out of his own sexual intercourse or is as a result of her illegitimate relation-

ship with others. The statement of the immaculate Imam (pbuh): "al-walad li -lfarash wa li -lahir al-hajar" (the baby is from the marriage bed and an adulterer should be stoned) deals with the issue that the child belongs to the husband and as a result its illegitimacy will be improbable.

Use of the word farash in this principle is because the Holy Quran and other religious sources sometimes directly refer to couples or with such implicit words as "garment (libas)" and "farash". "They are your garments and you are their garments" (54) "and on thrones (of dignity), raised high" (55). The couples are considered as "garments" that cover up each other's faults and as "beds" on which they sleep (alluding to intercourse).

In lexical texts there are two definitions for the word farash: the wife or anyone who plays this role as a slave-girl/kaniz (12) and couples (56). Jurists have also used this word with either of these two meanings: sexual intercourse (waty) and marriage with the possibility of intercourse (20, 21, 33, 47, 57, 58); because a question has often posed by Muslim jurists that whether the accomplishment of farash depends upon the husband's sexual intercourse with his own wife, or if the man and woman are married and the possibility of intercourse exists for them, the farash, although uncertain, has taken place.

In any case, the principle of farash is a jurisprudence rule when there is a suspicion as to whether the child was born as a result of the husband's intercourse or an illegitimate relationship (8, 32, 59). The implication is an external judgment about the legitimacy of the child and its association with the husband as well as apparent denial of the possibility of producing a child out of an illegitimate relationship (12).

Therefore, if the possibility of sexual intercourse between the husband and his wife exists even though one is not certain of its implementation, the child will belong to the husband unless he repudiates the child by liʿan (oath of imprecation) (18). If the husband is certain that he has not transferred his sperm to the womb of his wife - neither through sexual intercourse, nor further procedures, then according to farash principle the child cannot be attributed to him even the sexual relationship may have taken place (8). It is with regard to this aspect that in the legal Shiʿite literature, the possibility of the attribution of the child to the husband in normal situations is accepted as an assumption (60). However from the viewpoint of Sunni jurists the child is attributed to the husband even if he did not have sexual intercourse with his offender wife and did not transfer his sperm to her womb by any means (12).

Therefore citing the rule of farash in AID is completely inappropriate because the assumption is that the husband is responsible for infertility and because of this he needs donated sperm. We do not doubt that whether the child belongs to the husband or the sperm donor, but we are certain that the child belongs to the sperm donor.

If we are certain that the child has no biological relation to the infertile man, we cannot say that the child is apparently attributed to him. Clearly, acquiring an identification card under the name of his own family for a child that belongs to others is not to be taken as a justification for genetic attribution and establishing lineage (61). The husband's agreement with inseminating sperm donated by a stranger into his wife's egg would not indicate the child's apparent link to him.

Second viewpoint

Lineage discontinuity

According to jurisprudence rules and Islamic law there is no lineage relation between the child and the husband (infertile man) of the mother. None of the rights and responsibilities that exist between a father and his own child are applicable because alimony (nafaqa), heritage (werasat), custody (hizanat) and guardianship (welayat) do not exist between them (24). The exception is marriage, which is forbidden if the child is a girl according to the Quranic verse: "Forbidden to you are your mothers… and your step-daughters who are in your guardianship (born) of your wives to whom you have gone in, but if you have not gone in to them there is no blame on you (in marrying them)" (62). The husband cannot marry the daughter of his wife (rabibah) (24). For additional information on this subject, please refer to references (31, 63-66).

The reason for discontinuity of the lineage is that according to scriptural texts "… nor has He made those whom you assert to be your sons your real sons, these are the words of your mouths …" (67),

adoption (tabanni) is not permissible and has no social and legal efficiency (5, 68-71). Such children are only the children of their real, biological father. "Assert their relationship to their father, this is more equitable with Allah, but if you do not know their fathers, then they are your brethren in faith and your friends …" (72).

Mother

If a woman, due to infertility attributed to her husband uses donated sperm for having a child, will she be the mother of the child? To answer this question we have to reassess the discussion of illegal transfer of the husband's sperm to an unknown woman. Earlier we have explained that if a woman, by tribadism (lesbian sex) transfers her husband's sperm to another woman and the second woman becomes pregnant in this way, there is no doubt that the child will have lineage relation to the owner of sperm. The first woman who transferred her husband's sperm to the other women is, therefore, not the mother of the child (47). But with this assumption, is the second woman who has become pregnant from this sperm and delivered a child considered to be the child's mother? There are two possibilities offered in jurisprudence texts.

Discontinuity of lineage

A number of jurists maintain that for establishment of kinship and continuity of nasab there must be marriage (nikah) between the man and the woman or at least with the suspicion of the existence of a marriage relationship, they perform sexual intercourse and reproduction (watye bi shobha). However since none of these points are available in this case, the child is considered to be illegitimate (Haramzadeh) and does not have any lineage relation with the woman who has given birth (7, 73).

Lineage continuity

In contrast, some jurists maintain that the woman who receives the sperm that results in the birth of a child is considered the mother of the child. Therefore all legal effects of maternal relationship will apply (7, 51, 52).

The title of "offspring" (walad) applies to this baby, for it has developed in the womb of this woman (6, 17, 60). On the other hand, the only impediment to the accomplishment of a descendant relationship is sexual deviation, otherwise known as an illegitimate relationship (adultery). As the child has not been born from this relationship, thus there is no reason to reject the child's attribution to its mother and not to adhere to the legal consequences (7, 73).

By taking into consideration both possibilities, we comment about the relative correlation between the child and his mother in the issue of AID. As the studies show, there are two viewpoints among Muslim lawyers.

First viewpoint

Separation between awareness and unawareness of the woman

According to this viewpoint, if the woman based on her ignorance of this rule or subject uses donated sperm, hence the resultant child is considered to be a dubious child (walad al-shobha) and joins her in respect to lineage. However if she has knowingly used donated sperm for fertilization, the child will be regarded as an illegitimate child (Haramzadeh) and have no connection with her in terms of lineage (53).

Answer: Earlier we have explained that the relationship between the child and the woman or man who prepared the grounds for its existence, is a genetic relationship. The legislator has no role in its existence or nonexistence. However he may not recognize some of its outcomes.

Therefore, the child born through AID with by either willing or unwilling contribution of the sperm donor, is attributed to the woman. Since the relation between the woman and the sperm donor is not illegal (adultery), none of the legal outcomes of kinship correlation between them are accepted. Therefore awareness (ilm) or unawareness (jahl) of the woman about AID, if conceivable, has no effect on the continuity of lineage of the child with her.

Second viewpoint

Continuity of lineage (nasab)

According to this viewpoint which is the most accurate in terms of previous explanations, the child born through AID is the real and actual child of the woman whose sex cell contributed to the creation of this child who developed in her womb

and came to life in this world (5, 52).

Discussion

Citing the rule of farash for proving the relationship of a child is only permissible when there is the possibility of attribution of child to the husband. It is not possible to prove the lineage (nasab) of a child born through AID by farash because we have no doubt that the child has been born without contribution of the husband's sperm.

An infertile man whose wife has a baby by donated sperm, has no kinship with the child. The sperm donor is the real father of a child who is born by contributing his sperm and all paternal rights and responsibilities are applicable between them. Awareness or unawareness of the sperm donor about his germ cell being the origin of producing of a human being has no effect on the attribution of the child to the sperm donor. The use of donated sperm of known or unknown origins does not affect the attribution of the child to the sperm donor.

A woman whose egg has been fertilized by a stranger's sperm is the real mother of the child. Paternal rights and responsibilities are applicable between them. A child born by AID is a legitimate child and entitled to all rights for legitimate children.

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