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Contents

Male Infertility during Antihypertensive Therapy: Are We Addressing Correctly The Problem?

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Abstract

Male fertility significantly decreased in the last 50 years, as showed in several studies reporting a reduction of sperm counts per ml in the seminal fluid. Several "acute" pharmacological treatments, as antibiotics, could cause subclinical and temporary reduction of male fertility; conversely, long-term medical treatment may severely affect male fertility, although this effect could be considered transient in most of the cases. Thus, nowadays, several long-term pharmacological treatments may represent a clinical challenge. The association between several kind of antihypertensive drugs and reduction of male fertility has been showed in the mouse model, although the modification(s) which may alter this fine-regulated machinery are still far to be elucidated. Furthermore, well-designed observational studies and randomized controlled trials are needed to accurately define this association in human model, meaning a narrative overview synthesizing the findings of literature retrieved from searches of computerized databases. We strongly solicit future human studies (both observational and randomized clinical trials) on large cohorts with adequate statistical power which may clarify this possible association and the effects (reversible or permanent) of each drug. Furthermore, we suggest a close collaboration between general practitioners, cardiologists, and andrologists in order to choose the most appropriate antihypertensive therapy considering also patient's reproductive desire and possible risk for his fertility.

Keywords: Andrology, Infertility, Antihypertensive

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Introduction

Male fertility significantly decreased in the last 50 years, as showed in several studies reporting a reduction of sperm counts per ml in the seminal fluid (1). To date, male factors account for almost 35% of couple infertility. As widely accepted, sperm counts may vary among different ejaculates according to several pathological conditions, lifestyle and exposure to pollutants (2). In this regard, although well-known diseases such as cryptorchidism, varicocele, hypospadias, testicular tumors, Y chromosome microdeletion and endocrine alterations can cause azoospermia and/or oligozoospermia, iatrogenic risk factors may also play a detrimental role in male fertility. Several "acute" pharmacological treatments, as antibiotics, could cause subclinical and temporary reduction of male fertility; conversely, long-term medical treatment may severely affect male fertility, although this effect could be considered transient in most of the cases. Thus, nowadays, several long-term pharmacological treatments may represent a clinical challenge. To the best of our knowledge, association between several kind of antihypertensive drugs and

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reduction of male fertility has been showed in the mouse model (3-5), although the modification(s) which may alter this fine-regulated machinery are still far to be elucidated and human data are still lacking. Indeed, data from the following animal studies are not robust: several studies (6-8) showed that verapamil, nimodipine, and lisinopril worsen semen quality and testicular morphology, while others (9, 10) have found that nifedipine and lisinopril improve these parameters.

In this regards, Bechara et al. (11) have studied the effects of an angiotensin-converting enzyme (ACE) inhibitor (Enapril) on hypertension-induced morphological changes in the testis and spermatozoid production in spontaneously hypertensive rats. According to their data analysis, sperm concentration was greater in the treated group than in the nontreated group, testicular vascular volumetric density decreased in the nontreated group and, last but not least, volumetric density of the seminiferous epithelium in the treated group was higher than in the nontreated group. Although these results could not be de facto translated in humans, they have suggested a possible pivotal role of ACE inhibitors as first-line treatment when fertility is a relevant concern. Even if well-designed observational studies and randomized controlled trials are needed to accurately define this association in human model, daily clinical experience seems to confirm that in case of antihypertensive therapy with concomitant male infertility, the substitution of the drug with another one which does not affect semen parameters may improve male fertility.

In particular, beta blockers and calcium-channel blockers (CCBs) seem to play a detrimental role on male fertility, causing in several cases azoospermia and/or oligozoospermia. On the other hand, inhibitors of the funny channel, such as oral ivabradine, seem not to be associated with reduction of male fertility. In this regard, it was already demonstrated in the mouse model that CCBs, like amlodipine, can cause a reduction of testosterone, luteinizing hormone (LH) and follicular stimulating hormone (FSH), leading to affect spermatogenesis and sperm parameters (12, 13). However, these data do not seem to be surprising, since accumulating evidence have already suggested that Ca^{2+} plays a prominent role during fertilization in all animal species. On one hand, in mice, rats, pigs, hamsters and bovines, extracellular Ca^{2+} is necessary for epididymal acquisition of sperm motility (14-18).

Furthermore, it is known to regulate both activated and hyperactivated motility (19-21). In addition, flagellar motility is controlled by calcium through the regulation of dynein-driven microtubule sliding and modulation of the sperm flagellar waveform (22, 23). Finally, calcium has a pivotal role during the acrosome reaction in invertebrates, such as echinoderms, and superior vertebrates (24, 25). Also, in this case, although these data demonstrated that it does have a direct impact on humans, they may underlie the possible detrimental effect of calcium antagonists administered for hypertension on male fertility.

Conclusion

The average life expectancy is increasing and even more older male patients refer to fertility experts, the association between antihypertensive therapy and male infertility is a growing area of interest. Based on this scenario, we strongly solicit future human studies (both observational and randomized clinical trials) on large cohorts and with adequate statistical power which may show this possible association and the effects (reversible or permanent) of each drug. Furthermore, we suggest a close collaboration between general practitioners, cardiologists, and andrologists in order to choose the most appropriate antihypertensive therapy considering also patient's reproductive desire and possible risk for his fertility.

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Review Article

Aromatase Inhibitors for Endometriosis-Associated Infertility; Do We Have Sufficient Evidence?

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Abstract

Orally active aromatase inhibitors (AIs) have gained attention for treatment of infertile women with endometriosis in whom aromatase p450 is aberrantly expressed. This review aimed to critically appraise and summarize the available evidence concerning the use of AIs for management of endometriosis-associated infertility. PubMed was searched to May 2015 with the following key words: endometriosis, infertility and aromatase. Priority was given for randomized controlled trials (RCTs) followed by other study designs. Main outcome measures were as follows: rates of clinical pregnancy, miscarriage and live birth as well as endocrine outcomes. Eighty-two abstracts were screened and six original articles were included. A RCT demonstrated that post-operative letrozole treatment did not improve spontaneous pregnancy rate. Another RCT reported no superiority of letrozole superovulation over clomiphene citrate (each combined with intrauterine insemination) in minimal–mild endometriosis and previous laparoscopic treatment. Anastrozole significantly inhibited the growth of endometriotic cells and their estrogen production in culture. In assisted reproductive technology (ART) cycles, dual suppression (Agonist/anastrozole) was tested in a pilot study with a pregnancy rate of 45% however, high pregnancy loss (30%) occurred. A retrospective study showed that letrozole may improve endometrial receptivity in endometriotic patients undergoing *in vitro* fertilization (IVF). An opposite view from an in vitro study showed lower estradiol production and aromatase expression in cultured granulosa cells from endometriotic women undergoing IVF and marked reduction under letrozole. In conclusion, current evidence is limited. More trials are warranted to enhance our knowledge and provide a clear and unequivocal evidence to guide our clinical management of infertile women with endometriosis using AIs.

Keywords: Endometriosis, Infertility, Aromatase, Assisted Reproductive Technology

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Introduction

Endometriosis is a complex clinical issue, in which the quote of Albert Einstein "The most incomprehensible thing of the world is that it is comprehensible" is merit worthy. In this context, endometriosis is not only classically defined as the presence of endometrial glands and stroma outside the uterine cavity (1), but also well known as three separate entities (peritoneal, ovarian, or deeply infiltrating) (2) as well as 4 distinct stages (from minimal or stage 1 to severe disease or stage 4) (3). Nevertheless, endometriosis remains enigmatic in its pathogenesis and controversial in therapy till now (4). It is estimated that 5 to 10% of reproductive age women have endometriosis with a higher prevalence rate up to 50% among infertile women (5). Importantly, endometriosis-associated infertility represents a therapeutic dilemma because of its illusive background including ovulatory dysfunction related to altered folliculogenesis as well as impaired steroidogenesis of granulosa cells, impaired fertilization, low quality embryos, defective implantation, sperm phagocytosis, the embryo toxic environment and pelvic adhesions in advanced stages (6). Aromatase p-450 enzyme is the key enzyme in the biosynthesis of estradiol (E_2) in the ovarian granulose cells of premenopausal women. Notably, unlike women without endometriosis, high levels of aromatase P450 enzyme expression has been shown in eutopic endometrial tissue as well as in ectopic endometrial

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implants in endometriotic patients (7). This abnormal aromatase expression results in local estrogen production by endometriotic implants. Together with other mechanisms such as altered immune responses, angiogenesis and apoptosis, the autonomously produced estrogen leads to inflammation, proliferation and survival of endometriotic implants (8-10). In view of their ability to inhibit estrogen biosynthesis, third generation aromatase inhibitors (AIs) mainly letrozole and anastrozole have challenged clomiphene citrate (CC) in management of infertile patients with polycystic ovary syndrome as well as those with unexplained infertility (11). Additionally, the use of AIs to suppress the locally produced E_2 by endometriotic deposits has gained ground as a potential therapy for correcting abnormal endocrine and reproductive function of patients with endometriosis (9-11). Noteworthy, letrozole and anastrozole have favorable pharmacokinetics and pharmacodynamics facilitating their use in clinical practice due to the following reasons: being selective, completely absorbed orally with absolute bioavailability of 99.9%, reversible with mean half-life of 48 hours, greater potency with reduction in serum estrogen by 97 to 99% and clearance mainly by the liver (Fig.1) (12). In that regard and given that this is a clinically important area to be addressed, this review was conducted to critically appraise and summarize the current evidence concerning the use of AIs for management of endometriosis-associated infertility in both non-assisted as well as assisted reproductive technology (ART) cycles.

Eighty-two abstracts were retrieved from an electronic search using the PubMed from inception to May 15, 2015 with the following search terms "endometriosis", "infertility" and "aromatase" without search filters. Studies in which reproductive outcome (rates of clinical pregnancy, miscarriage and live birth) as well as endocrine outcomes were included in the following order systematic reviews and meta-analyses of randomized controlled trials (RCTs), RCTs, prospective cohort studies and other observational studies. Manual screening of references of the retrieved articles was done to identify other pertinent studies. Finally, results of 6 original articles were included concerning the use of AIs for management of endometriosis-associated infertility in both non-assisted as well as ART cycles (Tables 1, 2) (13-18).

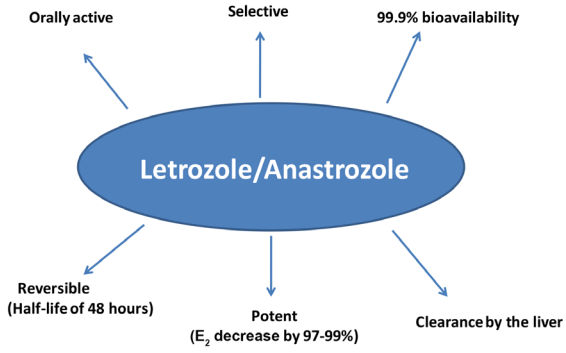

Fig.1: Favorable pharmacokinetics and pharmacodynamics of letrozole and anastrozole.

Table 1: Aromatase inhibitors in endometriosis-associated infertility in non-ART cycles

References	Study design/ sample size	Concept	Intervention	Outcome
Alborzi et al. (13)	RCT (n=144)	Post-operative suppression with letrozole and pregnancy outcome as well as the disease recurrence rate	Letrozole 2.5 mg/day vs. triptorelin 3.75 mg IM every month vs. no medication for 2 months after laparoscopic surgery, with a 12 months follow up	No significant differences among the three groups with regard to the pregnancy rate (23.4 vs. 27.5 and 28.1% respectively) as well as the disease recurrence rate
Abu Hashim et al. (14)	RCT (n=136)	Superovulation with letrozole+IUI in stage I-II endometriosis with no pregnancy 6-12 months after laparoscopy	Letrozole/IUI vs. CC/IUI	No significant differences between both groups for clinical pregnancy rate per cycle, cumulative pregnancy rate, miscarriage, or live birth rates.

ART; Assisted reproductive technology, CC; Clomiphene citrate, IM; Intramuscular injection, IUI; Intrauterine insemination, and RCT; Randomized controlled trial.

Table 2: Aromatase inhibitors in women with endometriosis-associated infertility undergoing ART

References	Study design/ sample size	Concept	Intervention	Outcome
Badawy et al. (15)	An *in vitro* study on cultured endometriotic cells	To demonstrate the effect of anastrozole, on the growth and E_2 production of endometriotic cells in culture	First addition of testosterone (10 µg/mL) to the culture medium then addition of anastrozole, in a dose of 200 µg/mL and 300 µg/mL,	Anastrozole produced significant decrease in endometriotic cell count as well as decrease in E_2 secretion and this effect was dose dependent.
Lossl et al. (16)	A prospective pilot study [n=20 with endometriomas undergoing IVF (n=16)/ICSI (n=4)]	Dual suppression	Prolonged down-regulation by combined 3-month GnRHa+1 mg anastrozole/day prior to IVF	Significant reduction of endometriomal volume (29%) and serum CA125 (61%). 45% clinical pregnancy rate and 15% live birth rate.
Miller et al. (17)	A retrospective cohort study (n=97 with endometriosis undergoing IVF)	Letrozole co-treatment might improve the IVF success rates by improving endometrial receptivity	29/79 women undergoing standard IVF lacked normal integrin expression. Other 18 integrin-negative women received letrozole early in IVF stimulation (5 mg, days 2-6).	Significantly higher clinical pregnancy and delivery rates observed in integrin-negative patients who received letrozole as compared to those who did not receive letrozole (61 vs. 14%, P<0.001 and 50 vs. 7%, P<0.001, respectively)
Lu et al. (18)	An *in vitro* study on cultured LGC	Letrozole may compromise aromatase activity of LGC resulting in a poor reproductive outcome in patients with stage III/IV endometriosis undergoing ART	Effect of different concentrations of letrozole on E_2 production and P450 aromatase mRNA expression in cultured LGC from women with (n=23) and without endometriosis (n=19)	Significantly lower E_2 production and P450 aromatase mRNA expression occurred in women with endometriosis and further reduction of these parameters were demonstrated following letrozole in a concentration of 1 µmol/L.

ART; Assisted reproductive technology, CA; Cancer antigen, E_2; Estradiol, GnRHa; Gonadotropin-releasing hormone agonist, ICSI; Intracytoplasmic sperm injection, IVF; *In vitro* fertilization, and LGC; Luteinized granulosa cells.

Discussion

The postoperative use of aromatase inhibitors in women who underwent laparoscopic surgery for endometriosis-associated infertility

The recent European Society of Human Reproduction and Embryology (ESHRE) Endometriosis Guideline demonstrated no evidence to support the use of postoperative hormonal therapy to improve spontaneous pregnancy rates in infertile women with endometriosis (19). This recommendation was based on the findings of a Cochrane meta-analysis by Furness et al. (20) including eight studies with 420 patients with endometriosis-associated infertility who were treated postoperatively by different modalities such as gonadotropin-releasing hormone agonist (GnRHa), medroxyprogesterone acetate, danazole and gestrinone [risk ratio (RR)=0.84, 95% confidence intervals (CI): 0.59-1.18]. Does the postoperative use of AIs increase the spontaneous pregnancy rate in women with endometriosis-associated infertility? This is a very relevant clinical question. Noteworthy, only one prospective RCT by Alborzi et al. (13) addressed this point among 144 patients who were diagnosed to have different stages of endometriosis ranging from minimal to severe disease by laparoscopy and histological confirmation. Patients were randomly allocated into the three following groups: group 1 who received letrozole 2.5 mg/day (n=47 cases), group 2 who had triptorelin (GnRHa) 3.75 mg intramuscular (IM) injection every 4 weeks (n=40 patients) and group 3 who received no medication for 2 months after laparoscopic surgery (n=57 patients) with a 12 months follow up period. The authors reported no significant differences among the three groups with regard to the pregnancy rate (23.4% in group 1 vs. 27.5% and 28.1% in groups 2 and 3 respectively) as well as the disease recurrence rate defined by recurrent symptoms in the form of dysmenorrhea, dyspareunia and pelvic pain (6.4% in group 1 vs. 5% and 5.3% in groups 2 and 3 respectively). Therefore, the authors did not recommend the post-operative use of letrozole or GnRHa in women undergoing surgery for endometriosis-associated infertility (13). The finding

from this RCT is in agreement with the aforementioned lack of beneficial effect of postoperative hormonal therapy on endometriosis-associated infertility (19).

Superovulation with aromatase inhibitors combined with intrauterine insemination for women with minimal or mild endometriosis-associated infertility

In view of the recent ESHRE Endometriosis Guideline, superovulation with intrauterine insemination (IUI) may be effective in increasing live birth rate, compared with expectant management in women with minimal to mild endometriosis (19, 21). In addition, superovulation with IUI may be more effective in increasing pregnancy rate than IUI alone and may be as effective in women with minimal or mild endometriosis within 6 months of surgical treatment as in women with unexplained infertility. In these women, there are potential benefits of superovulation including, improvement of the endocrine environment, correction of subtle ovulatory dysfunction as well as an increase in the number of mature oocytes. Thereby, it optimizes the chance per cycle of fertilization, embryo development and implantation (19, 22, 23). Unlike CC, letrozle has no downstream effects on the endometrium and cervical mucus. This could be attributed not only to its relatively shorter half-life (~2 days vs. ~2 weeks in CC), but also for its peculiar mechanism of action via down regulation of the E_2 synthesis rather than competitive inhibition of its action (11, 12). Furthermore, in ovulatory infertile patients, letrozole was reported to induce moderate ovarian hyperstimulation with E_2 levels similar to spontaneous cycles and higher midluteal progesterone, resulting in a normal endometrial histology and development of pinopodes, which could increase endometrial receptivity (24). Thereby, the biologic plausibility of the concept that superovulation with letrozole has the potential to increase pregnancy in women with minimal or mild endometriosis undergoing IUI is attractive, but is it clinically relevant?

In line with this concept, Abu Hashim et al. (14) in a RCT compared pregnancy rates following superovulation between letrozole and CC (each combined with IUI). The study included 136 women with primary infertility and no pregnancy following laparoscopic treatment for minimal to mild endometriosis over a 6-12-month follow-up period. Women were allocated to receive either 5 mg letrozole/day (69 women, 220 cycles) or 100 mg CC/day (67 women, 213 cycles) for 5 days, combined with IUI for up to 4 cycles. The authors demonstrated that the total number of follicles and serum E_2 on the day of hCG administration were significantly higher in the CC group; however, no significant differences were found with regard to the clinical pregnancy rate per cycle or the cumulative pregnancy rate after 4 cycles in both groups (15.9 vs. 14.5% and 64.7 vs. 57.2% in letrozole and CC groups, respectively). No significant differences were also found in miscarriage and live birth rates between both groups (11.4 vs. 12.9% and 44.9 vs. 40.3% in letrozole and CC groups, respectively). The authors concluded that superovulation with letrozole is not more effective than CC combined with IUI for women with minimal to mild endometriosis who did not achieve pregnancy after 6-12 months following laparoscopic treatment (14).

The effect of endometriosis on *in vitro* fertilization outcome

In a recent meta-analysis, Harb et al. (25) examined the effect of endometriosis on *in vitro* fertilization (IVF) outcome in twenty-seven observational studies that included 8984 women. The authors demonstrated that the presence of severe endometriosis (stage III/IV), but not minimal-mild endometriosis (stage I/II), was associated with reduced the implantation rate (RR=0.79, 95% CI: 0.67-0.93, P=0.006) and clinical pregnancy rate (RR=0.79, 95% CI: 0.69-0.91, P=0.0008) as compared to those of women with other causes of infertility. This finding may be attributed to defective embryo quality as well as defective endometrial receptivity (26, 27). High endometrial aromatase P450 mRNA expression in endometriotic women was also reported to be associated with poor IVF outcome (28). On the other hand, a reduction in fertilization rates was demonstrated in stage I/II endometriosis but not in stage III/IV disease as compared to controls (RR=0.93, 95% CI: 0.87-0.99, P=0.03). This interesting finding may be partially explained by impaired steroidogenesis of granulosa cells as well as poor oocyte quality in stage I/II disease (6). It is noteworthy that both of the third-generation AIs, letrozole and

anastrazole, have been used as adjuvant treatments in ART that is mainly for poor responders based on their ability to increase follicular sensitivity to follicle stimulating hormone (FSH) as a result of increased intrafollicular androgens (29). This point is outside the scope of this review.

The single and dual suppression concepts in women with endometriosis undergoing assisted reproductive technology

The merits of pituitary down-regulation for 3-6 months with a GnRHa in women with endometriosis before ART were previously demonstrated (30). Furthermore, this practice was recommended in the updated ESHRE Endometriosis Guideline (19). This recommendation was based on the findings of a Cochrane meta-analysis of three RCTs including 165 patients who had endometriosis-associated infertility (31). The authors concluded that this suppressive policy prior to ART increases the odds of clinical pregnancy by more than 4-fold (OR=4.28, 95% CI: 2.00-9.15, P=0.0001). This beneficial effect may be explained by increased activity of the immune system [increased natural killer (NK) cells, decreased auto-antibodies levels and reduction of elevated levels of peritoneal fluid interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α), stimulation of endometrial cell apoptosis as well as improved endometrial receptivity and implantation rates (30, 32). The latter action may be due to the suppression of endometrial aromatase expression by GnRHa (33). The concept of dual suppression (GnRHa+AI) prior to IVF has an appealing biological plausibility through the additional blockade of extraovarian aromatase enzyme aberrantly expressed in endometriotic implants as well as the eutopic endometrium of women with endometriosis (9-11). A more recent *in vitro* study demonstrated that anastrozole significantly suppressed endometriotic cells proliferation in culture as a result of marked reduction in the E_2 levels in these cells and this effect was dose dependent i.e. the suppression of growth and proliferation of the cultured endometriotic cells increased following increased anastrozole dosage (15). Thereby, dual suppression prior to IVF may result in a higher pregnancy rate rather than suppression with GnRHa alone. In a prospective pilot study, Lossl et al. (16) tested this concept among 20 infertile patients with endometriomas undergoing IVF/ Intracytoplasmic sperm injection (ICSI). A 3-month GnRHa course (3.6 mg goserelin administered through a subcutaneous injection on the treatment days 1, 28 and 56) combined with anastrozole (1 mg daily from the days 1 to 69) was used followed by controlled ovarian stimulation from the day 70. Interestingly, the authors demonstrated that prolonged dual suppression significantly reduces endometriomal volume by 29% (3-39%, P=0.007) and serum CA125 by 61% (21-74%, P=0.001). Nine of the 20 patients (45%) achieved pregnancy; however, only 3 patients (15%) delivered (two singletons and one twin). The authors speculated that the high pregnancy loss (6 patients) could be accidental or due to impaired quality of the oocytes and/or the endometrium (16). So far, there are no RCTs available in the literature comparing the use of goserelin+anastrozole suppression with single suppression by the GnRHa alone.

Aromatase inhibitors and endometrial receptivity in endometriotic women undergoing assisted reproductive technology

As endometrial aromatase expression was found to be associated with poor IVF outcome (28), it is theoretically plausible that the use of AIs in endometriotic women may be advantageous in improving their endometrial receptivity and the IVF outcome. Of note, reduced integrin expression, a marker of endometrial receptivity, has been shown in women with IVF failure (34). In a retrospective cohort study, Miller et al. (17) evaluated the effect of letrozole on endometriotic infertile women undergoing IVF and lacking normal integrin expression assessed by immunohistochemistry in mid-luteal endometrial biopsies. Out of 79 women undergoing standard IVF, 29 (36.7%) lacked normal integrin expression. Meanwhile other 18 integrin-negative women received letrozole early in IVF stimulation (5 mg, days 2-6). Notably, in women undergoing standard IVF, clinical pregnancy and delivery rates were significantly higher in women with normal integrin expression compared with integrin-negative women [20/50 (40%) vs. 4/29 (13.7%), P=0.02 and 19/50 (38%) vs. 2/29 (7%), P<0.01, respectively]. Additionally, among integrin-negative patients, significantly higher clinical pregnancy and delivery rates occurred in those who received letrozole as compared to women who did not receive letrozole [11/18 (61%)

vs. 4/29 (14%), P<0.001 and 9/18 (50%) vs. 2/29 (7%), P<0.001, respectively]. The authors concluded that letrozole co-treatment might improve the IVF success rates in a subset of women with endometriosis and implantation failure. However, they admitted the need for further prospective studies to confirm these findings (17).

The use of aromatase inhibitors in endometriotic women undergoing assisted reproductive technology; a counterintuitive view

Compared to women with tubal or unexplained infertility undergoing ARTs, reduced aromatase activity in granulosa cells as well as reduced expression of the cytochrome p450 family 19 subfamily A member 1 (*CYP19A1*) gene (that encodes aromatase) in cumulus cells has been shown in infertile women with endometriosis by *in vitro* studies (35, 36). Thereby, defective granulosa cell steroidogenesis could explain the impaired oocyte quality and the reduced fertilization rate associated with endometriosis. In that regard, an opposite view is that the use of AIs in patients with endometriosis undergoing ART may further compromise aromatase activity of granulose cells ending with a poor reproductive outcome. This possibility has been tested recently by Lu et al. (18) who compared E_2 production and P450 aromatase mRNA expression of cultured luteinized granulosa cells and the effect of letrozole on these parameters between women with (n=23) and without endometriosis (n=19). Of note, significantly lower E_2 production and P450 aromatase mRNA expression occurred in women with endometriosis and further reduction of these parameters were demonstrated following letrozole in a concentration of 1 µmol/L. Importantly, the results of this study should be interpreted with caution because it included women with advanced stage endometriosis (III-IV). So, whether early stage endometriosis (I-II) has a similar effect on *in vitro* E_2 production and P450 aromatase mRNA expression remains uncertain. The authors admitted the need for larger *in vivo* studies for further validation of these findings (18).

Safety of aromatase inhibitors

Data from the only abstract that did report increased rates of cardiac and bone malformations with letrozole treatment for infertility were subsequently unsubstantiated in lager trials (37, 38). More recently, in a large double-blind, multicenter RCT, Legro et al. (39) found no significant difference in the anomaly rates in infertile women with polycystic ovary syndrome who conceived with CC or letrozole (1.5 vs. 3.9%, respectively) and even these rates appear similar to that reported in healthy fertile women who conceived without undergoing ART(5.8%) (40).

Conclusion

In women with endometriosis, aromatase p450, the key enzyme in the biosynthesis of E_2 is not only present in the ovarian granulosa cells but also aberrantly expressed in the eutopic endometrium as well as in endometriotic deposits. Thereby, the orally active third-generation AIs letrozole and anastrozole have gained attention as a co-treatment for endometriosis associated infertility. In non-ART cycles, a RCT has demonstrated that post-operative treatment with letrozole or GnRHa for 2 months did not improve spontaneous pregnancy rates as compared to no treatment. Another RCT reported no superiority of superovulation with letrozole over CC followed by IUI in women with minimal-mild endometriosis who have not achieved pregnancy 6-12 months following laparoscopic treatment. Anastrozole significantly inhibited the growth of endometriotic cells and estrogen production in culture was also dose-dependent. In ART cycles, the concept of dual suppression (3-month GnRHa+1 mg anastrozole/day) was tested in a pilot study in 20 infertile women with endometriomas undergoing IVF. A significant reduction in endometrioma volume and serum CA-125 concentration was found. Of note, although the pregnancy rate was 45%, a high pregnancy loss was observed and the live-birth rate was 15%. So far, this concept has not been compared with single suppression by the agonist alone. Evidence from a retrospective cohort study has shown that letrozle could potentially improve endometrial receptivity in endometriosis patients undergoing IVF in whom aromatase is aberrantly expressed. On the other hand, an in vitro study reported lower E_2 production and aromatase expression of cultured granulosa cells in women with stage III-IV endometriosis undergoing IVF. Letrozole further reduced these parameters. Importantly, these findings should be interpreted with caution. No further validation has been made available so far in *in*

vivo studies. Overall, in view of the current limited body of evidence of 6 original studies, the research agenda for the future should address more questions concerning the use of AIs in patients with endometriosis such as the ideal timing for AIs co-treatment, optimum dosage, and definite effect on endometrial receptivity. In that respect, the quote of Albert Einstein "Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning" is merit worthy as evidence-based medicine is an evolving process. So, more trials are still needed to have a clear and unequivocal evidence to guide our clinical practice and to help achieve best outcomes.

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Original Article

Prevalence of Infertility Problems among Iranian Infertile Patients Referred to Royan Institute

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Abstract

Background: Few studies have been conducted on the infertility problems in Iran. This study aimed to investigate the prevalence of infertility problems and related factors in Iranian infertile patients.

Materials and Methods: In this cross sectional study, 405 infertile patients referred to Royan Institute, Tehran, Iran, between 2014 and 2015, were selected by simple random sampling. Participants completed the Fertility Problem Inventory (FPI) including 46 questions in five domains (social concern, sexual concern, relationship concern, rejection of parenthood, and need for parenthood). Mean difference between male and female was verified using independent-samples Student's t test. A generalized linear model (GLM) was also used for testing the effect of variables on the fertility problems. Data was analyzed using Stata software version 13.

Results: The mean age (SD) of participants was 31.28 (5.42). Our results showed that 160 infertile men (95.23%) were classified as very high prevalence of infertility problems. Among infertile women, 83 patients (35.02%) were as very high prevalence of infertility problems, and 154 patients (64.98%) were as high prevalence. Age (P<0.001), sex (P<0.001), a history of abortion (P=0.009), failure of previous treatment (P<0.001), and education (P=0.014) had a significant relationship with FPI scores.

Conclusion: Bases on the results of current study, an younger male with lower education level, history of abortion and history of previous treatments failure experienced more infertility problems.

Keywords: Infertility, Assisted Reproductive Technology, Iran

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Introduction

Infertility as a critical concept threatens individual stability and social relations (1). Although infertility is not considered as a medical issue, it critically affects the life of infertile couples in all aspects (2). Couples experiencing this crisis situation are more at risk for depression, anxiety, low self-esteem, and dissatisfaction (3). Infertile couples face both the physical and psychological problems during the diagnosis and treatment which can disrupt their lifestyle. Infertility can influence the relationship between the infertile patients with their spouse, friends, and colleagues, while it has a negative impact on their adherence performance (4, 5). Impulsive angry behaviors, feelings of helplessness and worthlessness, anxiety (particularly in the long-term treatments), concerns of sexual attraction, feeling of isolation, physical complaints, difficulty in sexual relationships, and sexual dissatisfaction are the problems reported by two recent studies (6, 7).

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The prevalence of problems associated with infertility has also been reported in other studies. However, according to studies by Ashkani et al. (8) and Fassino et al. (9), only 20 to 60 percent of infertile couples experienced these types of problems. There is a remarkable infertility rate in Iran (10). In Iranian culture, fertility is considered as an important concept. Unfortunately, few studies have been carried out on the different infertility problems, and most of them have focused on the psychological aspects of the subject (11, 12). Assessment of the problems related to infertility with a comprehensive tool is necessary (13). Therefore, this study aimed to investigate the prevalence of infertility problems and related factors in Iranian infertile patients.

Materials and Methods

This cross-sectional study was performed on 405 infertile patients referred to Royan Institute, Tehran, Iran, between 2014 and 2015. Simple random sampling was applied using a random numbers table based on the medical records of patients in this center. Inclusion criteria were as follows: definitive confirmation of infertility (primary or secondary) by a reproductive specialist, male factor infertility/female factor infertility (or both), unexplained infertility, and willingness to participate in the study. The diagnosis of infertility, cause of infertility, and infertility were defined based on the recommendations of World Health Organization (WHO) as follows: clinical infertility is defined as the lack of clinical pregnancy after 12 months of regular intercourse to have children. No history of previous pregnancy as primary infertility and secondary infertility was defined as a couple who experienced successful fertility in the past, and face infertility after previous pregnancies. A phone call was made to all infertile patients using simple random sampling, as mentioned. In a meeting with the patients, purpose of the study and the confidentiality of the data were clearly explained. Eligible individuals were also assured that acceptance or refusal to participate in the research had no influence on their treatment procedures. Filling the questionnaire was considered as signing a written informed consent. The study was approved by the Ethics Committee of Royan Institute. Participants completed two questionnaires. Firstly, a questionnaire about the socio-demographic and clinical characteristics was filled out that includes age (years), sex (male or females), educational levels (primary, secondary, and university), duration of infertility (years), type of infertility (primary, secondary), duration of marriage (years), cause of infertility (male factor, female factor, both, or unexplained infertility), the number of previous abortion (gestational age <20 weeks), failure of previous treatment (0,1, 2, 3, ≥4), and a history of abortion (yes, no). Secondly, the Fertility Problem Inventory (FPI) was completed. This questionnaire, which was made by Newton in 1999 to detect problems associated with infertility, includes 46 questions and five domains (social concern, sexual concern, relationship concern, rejection of parenthood, and need for parenthood). It is a 6-point Likert scale, from strongly disagree=1 to strongly agree=6. In this tool, 19 questions are scored inversely. The scores are between 46 and 276, indicating that the highest numerical values represent very high prevalence of problems related to infertility. In men, the scores range from 0 to 87 as less prevalence, 88 to 113 as moderate prevalence, 114 to 146 as high prevalence, and more than 147 as very high prevalence. In women, scores 0 to 97 are classified as low prevalence, 98 to 132 as moderate prevalence, 133 to 167 as high prevalence, and over 168 as very high prevalence (13). In a study by Ramezanzadeh et al. (6), the validity and reliability of the Iranian version of FPI were evaluated in Iranian infertile patients and Cronbach's alpha coefficient was greater than 70% for all domains.

Statistical analysis

Categorical data were presented as numbers (percent) and continuous data as mean (SD). Mean difference between male and female was verified by independent-samples Student's t test. A generalized linear model (GLM, family: Gaussian, link: identity) was used to test the effect of age, sex, educational level, duration of infertility (years), cause of infertility, type of infertility, failure of previous treatment, and history of abortion on the fertility problem score. Results were presented as standardized coefficients with 95 percent confidence intervals (CIs). Values of P less than 0.05 were considered statistically significant. Data was analyzed using Stata software (StataCorp, USA).

Results

A group of 410 participants completed both questionnaires. Five questionnaires (1.21%) were excluded due to the completion rate of less than

70%. The mean (SD) age was 31.28 (5.42). Socio-demographic and clinical characteristics of the participants are shown in the Table 1.

Table 1: Socio-demographic and clinical characteristics of the participants

Age (Y)	31.28 ± 5.42
Duration of infertility (Y)	4.93 ± 4.01
Duration of marriage (Y)	7.07 ± 4.23
Number of previous abortion	1.04 ± 0.87
Sex	
Male	168 (41.5)
Female	237 (58.5)
Cause of infertility	
Male factor	146 (36)
Female factor	88 (21.7)
Both	71 (17.5)
Unexplained	100 (24.8)
Type of infertility	
Primary	287 (70.9)
Secondary	118 (29.1)
Educational level	
Primary	90 (22.2)
Secondary	150 (37)
University	165 (40.8)
Failure of previous treatment	
0	208 (51.35)
1	81 (20)
2	61 (15.06)
3	36 (8.88)
≥4	19 (4.69)
History of abortion	
No	316 (78)
Yes	89 (22)

Values are given as mean ± SD or number (percentage) unless otherwise indicated.

Table 2 shows the mean scores of FPI domains compared between males and females. Our findings showed that a very high prevalence of fertility problems in infertile patients, meaning among infertile men, 160 individuals (95.23%) were classified as very high prevalence and the remaining 8 individuals (4.77%) as high prevalence. None of them were as less or moderate prevalence. Among infertile women, 83 patients (35.02%) were as very high prevalence and 154 (64.98%) as high prevalence. Like infertile men, no women were as less or moderate prevalence.

The GLM results showed that five out of eight predictors were statistically significant. The model was statistically significant [F (8, 397) =11.13, P<0.001] and accounted for approximately 16% of the variance of scores (R^2=0.185, Adjusted R^2=0.168). Basic descriptive statistics, crude and standardized regression coefficients are shown in Table 3. The strongest effect belongs to age followed by sex, a history of abortion, failure of previous treatment, and education. The results suggested that patients who had a history of abortion showed very high prevalence of fertility problem as compared to patients without a history of abortion (P<0.001). Similarly, patients with a history of failure in previous treatments had very high prevalence of fertility problems (P=0.001). As seen in Table 3, age was a significant predictor of prevalence of fertility problems. When the other predictors were ignored, age was negatively correlated with prevalence of fertility problem, meaning that an increase of one SD of age resulted in a decrease of 0.231 in mean scores of FPI. Like crude analysis, female had lower mean scores of FPI as compared to the related value of male. Also, mean scores of FPI decreased with an increase in the education level.

Table 2: Comparison of the mean scores of fertility problem inventory domains between males and females

Scale	Total (n=405)	Male (n=168)	Female (n=237)	P value
Social concern	34.36 ± 5.46	34.05 ± 5.64	34.58 ± 5.32	0.345
Sexual concern	29.82 ± 4.35	31.11 ± 4.02	28.9 ± 4.35	<0.001
Relationship concern	36.9 ± 3.58	36.81 ± 3.54	36.96 ± 3.61	0.678
Rejection of parenthood	25.66 ± 5.3	27.46 ± 5.56	24.38 ± 4.71	<0.001
Need for parenthood	37.37 ± 5.09	36.37 ± 6	38.08 ± 4.2	0.002
All scales	164.12 ± 10.42	165.82 ± 11.27	162.91 ± 9.61	0.007

Values are given as mean ± SD.

Table 3: GLM results for FPI scores

Variables	Standardized coefficient β	Unstandardized coefficient β	95% CIs for β		P value
			Lower bound	Upper bound	
Age (Y)	-0.231	-0.426	-0.631	-0.222	<0.001
Sex	-0.225	-4.72	-6.82	-2.63	<0.001
Education	-0.12	-0.951	-1.71	-0.191	0.014
Failure of previous treatment	0.155	3.27	1.31	5.23	0.001
History of abortion	0.181	4.6	1.13	8.06	0.009

All P values are reported as two-tailed. Significance is defined as P<0.05. CIs; Confidence intervals, GLM; Generalized linear model, and FPI; Fertility problem inventory.

Discussion

Our findings revealed that the prevalence of infertility problems is very high in infertile patients. In our study, 95.23% of male patients and 35.02% of female patients showed very high prevalence of infertility problems, so none of them was classified as less or moderate prevalence. After adjusting confounder variables (including age, sex, educational level, failure of previous treatment, and a history of abortion), a significant increase in prevalence of infertility problems was seen in younger male patients with lower education level, a history of previous abortion, and failure of previous treatment.

Considering the cause of infertility, our findings showed that the male factor (36%) was more prevalent than the female factor (21.7%). In a study by Kamali et al. (14), they have found that 50.5% of participants suffered from the male factor, while 28.6% had the female factor. In another study by Noorbala et al. (15) they have reported prevalence values of 44 and 28% for psychiatric disorders in infertile and fertile women, respectively. Furthermore, the most important stress factors in infertile women were feedback from others (81.3%), loneliness (74%), treatment of infertility (60.7%), incomplete families (53%) and the identity disorder (50.7%).

In a study by Hussain et al. (16) on psychological disorders among women with polycystic ovary syndrome (PCOS), as one of the major causes of infertility, they have reported a prevalence of 23% for major depression in women with PCOS. They have also reported 15.4% suffering from panic disorder and 15.4% diagnosed with generalized anxiety disorder. The prevalence of suicide is also more among women with PCOS than others (16, 17). In a study by Ramazanzadeh et al. (6) on 370 infertile women, they have found that the prevalence values of depression and anxiety in infertile women were 40.8 and 86.8%, respectively. These findings were consistent with our results. Furthermore, the prevalence of psychiatric disorders in Iranian infertile women was higher as compared with the Western countries (15, 18). Odden et al. (19) have reported that the prevalence of 35.2% for psychiatric disorders in infertile women.

In our relationship between the age and total scores of FPI, suggesting, as age increased, the total scores decreased. It means that younger infertile patients experienced very high prevalence of problems related to infertility as competed to older infertile patients. Ramazanzadeh et al. (6) have also found that the prevalence of anxiety and depression is more common in 6-4 years after infertility. Another study has indicated that anxiety and depression improve with increasing age.

Various researches have showed different findings about the relationship between prevalence of infertility problems with age and education level. In a study by Beutel et al. (20), there was a significant correlation between prevalence of infertility problems with age and education level, while such a relationship was reported by two other studies (19, 21). In our study, the prevalence of infertility problems was significantly higher in the participants with a history of previous treatment failure. This may be due to the failure of fertility treatment, medical expenses, and the reaction of others. A history of abortion was known as one of the factors in prevalence of infertility problems. One of possible limitations of this study was the patients with more complex infertility problems who are mostly referred to Royan Institute from

other centers. These patients are more likely to experience longer exposure to the problems related to infertility. Therefore, our findings can only be generalized to patients with more complex infertility problems referred to a fertility center. Finally, in all cross-sectional study, such as our study, there is no temporality. Therefore, determination of the exposure and the outcome is not easily performed and causality will be not detected.

Conclusion

The results showed that a very high prevalence of fertility problems in infertile couples. Bases on the results of current study, a younger male with lower education level, history of abortion and history of previous treatments failure experienced high prevalence of infertility problems.

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Original Article

Assisted Reproductive Technology in Iran: The First National Report on Centers, 2011

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Abstract

Background: Due to the worldwide increase in infertility, it is both necessary and important to have assisted reproductive technology (ART) registries. In Iran, donation and surrogacy programs are approved by decrees from religious scholars. ART has been used since 1984 in Iran and the first Iranian infant conceived by gamete intra-fallopian transfer (GIFT) was born in 1989. This report, however, is the first national report on Iranian ART centers.

Materials and Methods: This cross-sectional study, conducted under the supervision of the Iranian Ministry of Health, presented a summary of the numbers and percentages of centers that provided infertility services in Iran, as well as the status of ART in Iran during 2011.

Results: A total of 52 centers reported treatment cycles and performed approximately 29000 intrauterine insemination (IUI), in addition to 35000 *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI) cycles.

Conclusion: Iran has considerable potential to provide IVF services for both Iranians as well as other nationalities throughout the region. This proves the need for a national center that will implement a registry system.

Keywords: Iran, Assisted Reproductive Technology, Monitoring

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Introduction

With the expansion of infertility worldwide, the importance of assisted reproductive technology (ART) registries is critical. This registry information can assist health authorities, patients seeking medical assistance, the medical profession, and laboratory professionals in providing optimal patient care. A registry can provide the public a better understanding of ART procedures (1).

Reports of ART from European countries are presented annually with a four-year delay (2). The latest report of the European *in vitro* fertilization (IVF)-monitoring (EIM) has presented results of treatments initiated during 2010. Data is collected from existing national registries in the participating countries and directly entered by each national coordinator into the EIM database (3). ART data from the United States is also presented annually in surveillance papers (4). Data collected by the Latin American Registry of Assisted Reproduction (RLA) is also obtained from ART treatments performed in 155 institutions in 14 countries (5). Nevertheless, there is little information regarding the status of ART in Middle Eastern countries (6).

Iran is the only Islamic country in which donation and surrogacy programs are practiced. These

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programs have been accepted and approved by decrees from clergy scholars (7, 8). ART was first used in 1984 in Iran and the first Iranian infant conceived by gamete intra-fallopian transfer (GIFT) was born in 1989. Since then, more than 50 centers have been established in Iran. However, scant attention has been paid to a national registry and this is the first report on Iranian IVF monitoring.

This paper aims to present data gathered from the Iranian infertility centers in 2011. It proceeds by tracing the accessibility, procedure, cost, and some challenges of IVF in Iran. While definitions used in medically assisted reproduction are different in various settings, this paper has used the definitions suggested by the International Committee for Monitoring Assisted Reproductive Technology (ICMART).

Materials and Methods

We gathered current registry information under the supervision of the Iranian Ministry of Health. This cross-sectional study dealt with the treatment outcomes during 2011 while the population of the country approximated 75.149 million. To collect data for this registry, we designed a form that used published literature and surveillance reports from American Society for Reproductive Medicine (ASRM), National Institutes of Health (NIH), and European Society of Human Reproduction and Embryology ESHRE (9, 10). The content of the questions was evaluated and approved by health and medical experts using the nominal group method and also by the Ethics Committee of the Iranian Ministry of Health.

In Iran, although infertility centers provide ART, clinics and hospitals also offer a portion of reproductive services. In Iran, all medical services provided in a hospital are under the supervision of a medical university. Thus, in order to obtain information from each center for completing the designed form, we sent there an expert familiar with basic concepts and definitions of ART from the medical university affiliated with the respective center. In this way, we contacted all centers and gathered data via the questionnaire.

Iran is divided into 31 provinces. This report provides data on the numbers of infertility centers, the numbers and types of provided services for ART, and the distribution of admitted clients according to the provinces of Iran in 2011. Specifically, this report provides summarized information of the numbers and percentages of centers that have provided infertility services based on the source of the egg (patient or donor) and the status of the embryos (fresh or thawed). Data also includes the number of treatments from standard IVF, intra-cytoplasmic sperm injection (ICSI), and intrauterine insemination (IUI) performed during 2011 in Iran.

Infertility clinics are divided according to their level of expertise: primary (level I), secondary (level II), and tertiary (level III). This report only focuses on level III infertility centers located throughout Iran. These centers have an ART laboratory which includes day care centers or centers in the hospitals that perform diagnostic and therapeutic functions at the highest level of specialization. In order to assess the level III criteria adjustment of these centers, data on the number of specialist staff or lack of specialists is provided in this report.

Hospitals and level III infertility clinics are integrated with medical universities in Iran. Iranian medical universities undergo an annual evaluation on the basis of research activities using criteria such as the numbers of faculty members and researchers, knowledge production and budget, and leadership and governance. Medical universities are then ranked according to three categories: type 1 with large numbers of academic staff and high levels of research funds, type 2 university with less staff and research funds compared to type 1, and type 3 with still fewer numbers of academic staff and lower research funds than type 2 universities (11). This report also presents the distribution of fertility centers based on the type of medical universities.

Results

In 2011 there were 52 infertility centers in Iran, with most located in major cities. The number of infertility centers that performed ART procedures varied by province and the type of university hospitals (Fig.1).

A total of 34 centers were affiliated with type 1 and 18 centers with type 2 university hospitals. Table 1 shows the distribution of infertility centers by the type of university hospital. Among all type 2 medical universities, Zahedan, Qazvin, Ardabil, and Gorgan did not have any level III infertility centers. From 46 medical universities in Iran, 18 had at least one level III infertility center (Table 1).

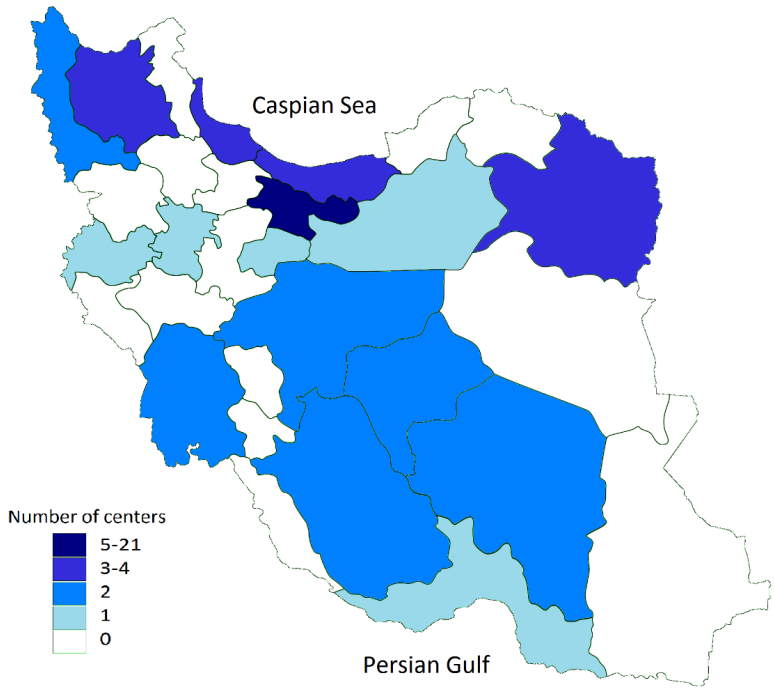

Fig.1: Distribution of infertility centers that performed assisted reproductive technology (ART) procedures in Iran during 2011.

Table 1: List of medical universities and numbers of their affiliated centers

Type 1 (n=34)	Type 2 (n=18)
Shahid Beheshti (16)	Gilan (3)
Tehran (5)	Mazandaran (2)
East Azerbaijan (4)	West Azerbaijan (2)
Mashhad (3)	Kerman (2)
Isfahan (2)	Yazd (2)
Shiraz (2)	Babol (2)
Khuzestan (2)	Hamadan (1)
	Kermanshah (1)
	Semnan (1)
	Qom (1)
	Bandar Abbas (1)

Medical university (No. of centers).

The infertility centers were divided into: private, government, and Academic Center of Education, Culture and Research [ACECR or the former Jihad Daneshgahi (non-government organizations)]. Among the 52 centers, there were 27 (52%) private, 21 (40%) government, and 4 (8%) ACECR.

Medical universities of Iran provide personal, educational, and research services. Thus infertility centers can be categorized as having one or all of the following: educational, clinical, and research departments. The percentage of centers with respect to their services is presented in Figure 2.

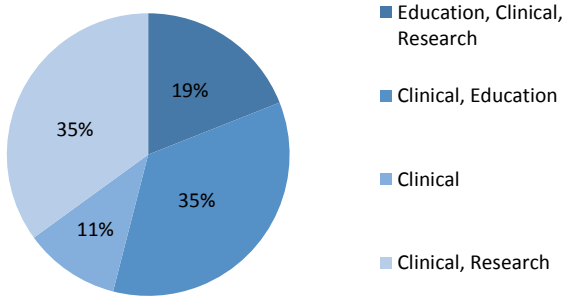

Fig.2: Percentage of clinics according to provision of services.

We have classified the centers according to the duration of their work. The oldest center is the government center of Yazd with 22 years of experience, followed by Shariati Hospital with 21 years, both of which are educational centers; after which are the private centers of Royan Institute with 20 years, Isfahan with 18 years, and Sarem and Navid centers, each with 17 years of experience (Fig.3).

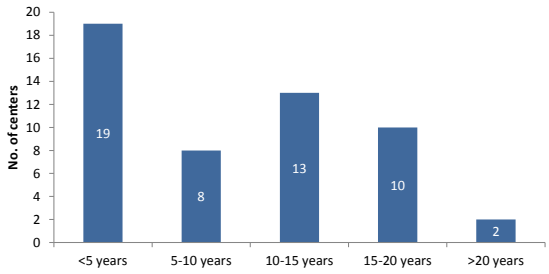

Fig.3: Frequency of clinics according to years of experience.

There were approximately 29000 IUI, as well as 35000 IVF and ICSI cycles in Iran during 2011. The sum of average number of visits in tertiary infertility centers for any type of diagnostic and therapeutic process per month was 39063; the mean number of new admissions among the centers was 641 per month. The minimum number of admissions in centers was three per month. The maximum numbers of admissions occurred in three private centers in Tehran and Isfahan. A total of 21 centers were located in Tehran, the capital of Iran, with a total number of 20786 clients per month, which was the highest proportion (53%) in the country (Fig.4).

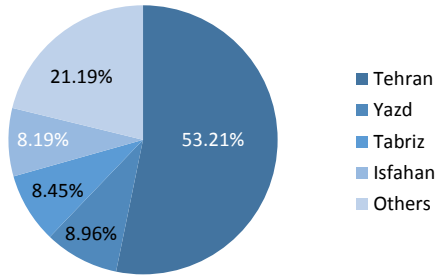

Fig.4: Percentage of visited clients in infertility centers of Iran during 2011.

Table 2 shows the number of centers according to provided services. All centers had documented records of patients' general information; however, 17 (33%) had electronic records. The monthly rate of ART services provided by centers is shown in Table 3.

Table 2: Numbers of centers that provide different ART services

Type of ART service	Number of centers (%)
IUI	52 (100)
IVF	49 (94)
ICSI	50 (96)
Surrogacy	35 (67)
Egg donation	43 (83)
Embryo donation	35 (67)
Sperm donation	1 (2)
Sperm bank	6 (12)
Embryo freezing	48 (92)
Egg freezing	35 (67%)
Sperm freezing	40 (77%)
Diagnostic laparoscopy and hysteroscopy	44 (85%)
Therapeutic laparoscopy and hysteroscopy	42 (81%)
Male infertility surgeries	43 (83%)

ART; Assisted reproductive technology, IUI; Intrauterine insemination, IVF; *In vitro* fertilization, and ICSI; Intra-cytoplasmic sperm injection.

In 2011, the mean cost of IVF ranged from $2250 to $3600 in government and private centers.

In Iran, technical managers of all centers should be fellowship of infertility or qualified by having three years of experience in an infertility center. Therefore, having infertility fellowship was assessed in all centers as criteria. In 5 centers, an infertility fellowship did not exist, 3 had no urologists, and 2 did not have any embryologists. In numerous cases, one embryologist simultaneously cooperated with more than one center. In 16 centers, midwives were used as alternatives to nurses and 2 centers had no midwives.

Table 3: Monthly rate of assisted reproductive technology procedures in 2011

Type of service	Mean	Minimum	Maximum
Intrauterine insemination (IUI)	47	2	300
In vitro fertilization (IVF)	57	0	400
	Total in all centers*	Minimum*	Maximum*
Surrogacy	165	1	61
Egg donation	997	1	150
Embryo donation	545	2	300

*; Mar. 21st to Sept. 22nd, 2011.

A total of 36% of centers had neither an internal specialist nor an endocrinologist. Patients who needed specialized medical counseling (i.e., urology, endocrinology, and genetics) were referred to another clinic or hospital when there was no specialist located at that center. Some infertility centers presented their needs for specialists (Table 4).

Table 4: Number of centers which required different medical specialists

Type of required specialist	Number of centers (%)
Gynecologists with infertility focus/infertility fellowship	8 (15)
Urologist	3 (6)
Embryologist	11 (21)
Internal specialist or an endocrinologist	19 (36)
Psychologist	32 (62)
Genetics specialist	30 (58)
Nutrition specialist	25 (48)

In order to assess level III criteria adjustment for diagnostic tests, we checked the sonohysterography and hysterosalpingography performances. In 31 centers, sonohysterography and hysterosalpingography were performed. All centers with the exception of one center, were equipped with a microinjection device.

Among 28 infertility research centers, 7 had scientific research journals: Vali Asr, Royan Institute, Avicenna Infertility Clinic, Family Hospital, Mehr Infertility Center, Sarem Hospital, and Mother Infertility Center.

Discussion

The increased use of reproductive science developments, as well as the least legal or religious barriers to ART in Iran as an Islamic country, have raised the hopes of infertile couples.

The availability of infertility services, as a product of public and private health policies, could determine the allocation of personnel, equipment, and facilities (12). According to our data, the number of infertility centers that performed ART varied considerably by province. Residents of Tehran with 21 infertility centers had the easiest access to infertility centers followed by East Azerbaijan, Khorasan Razavi, Gilan, and Mazandaran with 4 or 3 centers. The remainder of provinces had none to 2 centers. Lack of geographic access to level III infertility services has obviated the necessity for increasing the availability and utilization of these services in different areas of the country. Establishing at least one level III center in all provinces rather than multiple centers in specific areas could provide extensive facilities to cover the entire country.

Most medical universities that had an infertility center were type I. All centers provided clinical services, however 35% also had educational and research departments. Numerous centers solely focused on clinical activities (35%), which have suggested that stronger links among research, education, and practice are needed.

Although 12 centers had more than 15 years of experience, most had less than 5 years of experience in providing ART services. Compared with the most experienced infertility clinics in the US, which were established in the early 1980s, our first infertility center had almost a decade delay in establishment.

The estimated number of IVF per million population in 2011 showed that the national utilization of IVF was less than the equivalent reported from European countries in 2010 and the US in 2011 (10, 13). In 2011, most centers offered IUI, IVF, and ICSI. The number of centers that offered egg and embryo donation was almost half compared with clinics in the US. Only one center reported that they offered sperm donation to clients, which was entirely different from similar percentage of this service in the US (14).

It seems that due to lack of a supporting law in the country, some centers did not claim their donation practice. Due to legal and legislative approval of embryo donation in Iran, as well as the absence of laws regarding sperm donation, our data about embryo donation might be more realistic (8, 15). This could cause centers to perform embryo donation instead of sperm donation and as a result the portion of embryo donation cycles might increase compared with other donation programs. The low percentage of centers that provided sperm banks could be another consequence of a vague law and legislations on this program.

The mean number of new monthly admissions among centers was 641. As far as we know this has not been reported in other countries' studies. Usually the number of cycles is reported for each infertility clinic. Lack of a referral system in Iran may lead to overestimation of the number of new admissions be-

cause a person as a new admission of a specific infertility center may refer to another center after a period of time and be counted in more than one center.

Worldwide, ethical issues in reproductive medicine and assisted reproduction are influenced by religions. In Iran, Islamic primary sharia. In Latin America, the Catholic Church applies considerable pressure to prevent access to IVF and third party assisted reproduction is banned (8). Studies have mostly focused on the view of Sunni Islam on ART, as they comprise 90% of the Muslim population. Shia Islam which is concentrated in Iran, Azerbaijan, Iraq, and Afghanistan relies on clergy scholars' decrees on different new issues including ART. Although there are differences in the decrees, all are correct for the scholars' followers. Some Shia scholars have approved ART using third party and the embryo donation law was passed by the Iranian parliament in 2003. In contrast, in 1986, the Islamic Fiqh Academy based in Jeddah (Majmaal-Fiqh al-Islami) considered all types of third party assisted reproduction forbidden (haram) (8).

Ethical conditions may lead to legislative rules that are adopted by legal experts, ethicists, religious scholars, and politicians. Therefore, some people may obtain their desired infertility treatment outside of their own countries. In this way, they travel from a restrictive country to a permissive one (16, 17). Infertility is highly stigmatized in developing countries and leads to profound social consequences for these couples. Hence, infertility is sometimes kept secret. Due to losing both social support and social capital, patients seek reproductive services in neighboring countries (18, 19). Since Iran is a Muslim country in which gamete and embryo donation are practiced as well as surrogacy, it can be the first choice for Muslims from other countries who seek infertility treatment. We have not gathered international patients' data, so no conclusion could be made in this regard.

In most countries, cost is a serious concern for couples who receive infertility services. The cost of ART treatment is different worldwide due to the costliness of underlying health care systems and the level of patients' subsidization. As all infertility centers in Iran operate outside of government-financed health facilities, they actually provide services only for patients that can pay out of pocket for ART treatments. Although ART is cost prohibitive in Iran, the cost is relatively lower than neighboring countries with better economic situations and stronger currencies. The relative cost differences encourage infertile couples to travel to Iran to undergo ART (20, 21).

Considering the rate of lifetime experience of infertility (6.4%), as well as the rate of primary (21.1%), and secondary infertility (7.8%) in Iran (22), it is essential to draw up a national guideline. This guideline can offer the most practical advice on assisting couples with infertility problems, and take into account individual needs and preferences (14). Lack of national auditing, supervision, and a registry are the major drawbacks of this system. A national center is required to implement a registry system that reports important national outcomes of infertility centers such as success rates, numbers of embryos transferred, numbers of frozen-thawed eggs, and the woman's age at the time of retrieval, in addition to an introduction to the infertility centers and costs for ART cycles. A national registry and monitoring can lead to improvement in quality of aspects of the structures, processes, and outcomes of infertility centers. Establishing this registry system can be initially implemented by developing audit activity and outcome committees in the centers.

Conclusion

This paper has presented the status of ART in Iran during 2011. The most obvious finding to emerge from this study is that Iran has great potential to provide IVF services for both Iranians and other nationalities throughout the region. Therefore the implementation of a registry system seems to be vital.

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Original Article

Comparison of Quality of Life, Sexual Satisfaction and Marital Satisfaction between Fertile and Infertile Couples

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Abstract

Background: Fertility plays an important role in sexual and psychological function in families. Infertility can result in major emotional, social, and mental disorders, including a reduction in satisfaction with marital life and quality of life. The present study aimed to compare the quality of life and marital satisfaction and sexual satisfaction between fertile and infertile couples.

Materials and Methods: This analytical cross-sectional study was conducted on 250 couples at the Fatemiyeh Educational Research Center affiliated to Hamadan University of Medical Sciences, Hamadan, Iran, from May to August in 2014. The subjects were randomly selected from the patients referred to this center using a table of random numbers. They were then allocated into two groups of infertile group (n=125) and fertile group (n=125). The study participants completed World Health Organization Quality of Life-BREF (WHOQOL-BREF) questionnaire, Linda Berg's Sexual Satisfaction Scale, and Enrich Marital Satisfaction Scale. Then, the data were entered into the SPSS version16 for statistical analysis. The Chi-square and Mann-Whitney tests were also applied to compare the data between the groups.

Results: The results revealed no significant difference between the two groups regarding demographic and general health variables. The mean scores of sexual satisfaction were 63.67 ± 13.13 and 46.37 ± 7.72 in the fertile and infertile couples, respectively. Furthermore, the mean scores of marital satisfaction were also 44.03 ± 9.36 and 36.20 ± 4.03 in the fertile and infertile groups, respectively. Our finding demonstrated that the fertile couples obtained significantly higher mean scores of quality of life as well as lower mean scores of sexual satisfaction and marital satisfaction as compared to the infertile ones ($P<0.001$).

Conclusion: According to the results, the fertile couples obtained significantly higher quality of life and lower sexual satisfaction and marital satisfaction as compared to the infertile ones. Therefore, holding consultation programs and conducting more studies are necessary for improving the quality of life and promoting sexual and marital satisfaction in infertile couples.

Keywords: Infertility, Quality of Life, Sexual Satisfaction, Marital Relationship

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Introduction

Quality of life is a complex concept that is related to physical health, psychological status, level of independence, social relations, personal beliefs, and environmental factors (1). It is also affected by age, culture, sex, education level, social status, disease, and social environment (2). Infertility is among the difficult conditions affecting quality of life. It is also among the major medical problems whose rate has increased by 50% since 1955. To date, 10-15% of couples suffer from infertility (3). Infertility status and its related factors affect the quality of life through creating psychosocial stress, reduction of life satisfaction, increase of marital conflicts, and decrease of sexual satisfaction and marital satisfaction (4, 5).

In fact, infertility is considered as a personal and social problem affecting couple's life and family's performance, so exposes couples to mental pressure and various psychological disorders (6, 7). Infertility, as an emotional shock, can even have an impact on couples' communication, occupational, and sexual skills. Overall, infertility, as a serious medical problem, can have destructive effects on the quality of life (8). Evidence has indicated that infertility is a destructive or painful experience that leads to more disappointment, prostration, and anger in infertile couples as compared to their fertile peers. Besides, infertile couples have disturbed relationships with their spouses, families, and friends that make them more vulnerable to psycho-emotional disorders, depression, anxiety, low self-confidence, and dissatisfaction that subsequently lead to low quality of life (9). Nevertheless, some researches have demonstrated that in case of cooperation and sharing responsibilities between couples, treatment procedures can increase their intimacy and improve the quality of their marital life (10).

In a study by Nourani et al. (11) that was conducted at the Majidi Treatment Center, Tabriz, Iran, 12% of women reported low life quality, while more than half of them had desirable quality of life. In addition, familial and social pressure had a negative influence on infertile women's quality of life. Marital and sexual satisfaction considerably affect couples' physical and mental health. On the other hand, incompatibility in a marital relationship disturbs social relations, leads to tendency towards social deviations, and declines cultural values between couples. Thus, sexual satisfaction is necessary for solidity of marital life. Some researchers believe that sexual dissatisfaction accounts for 80% of marital conflicts (12). On the other hand, fertility status is one of the effective factors in sexual satisfaction. Based on various studies, infertility could result in several psychological disorders, including sexual dissatisfaction (13). However, some studies have shown no difference between the couples under infertility treatment and fertile couples in the context of marital satisfaction. In other words, marital satisfaction was not affected by infertility (14, 15). Tao in a systematic review has investigated marital relationship in infertility and reported that sexual satisfaction had impact on marital satisfaction (15). The problem of infertility has become deeper, especially in the Iranian culture in which there is extended family type. Because in this type of families having children is important. Infertility can be regarded as life crisis, identity crisis, chronic disease, or a combination of them (16).

Quality of life in infertile couple differs from one society/culture to another. Fertility is of utmost sociocultural importance, while controversial results have been obtained regarding the effect of infertility on quality of life in western countries. Besides, only limited studies have been performed in this regard in the Eastern Counties, including Iran. Due to inconsistencies in the available studies, this study aimed to compare the quality of life, sexual satisfaction and marital satisfaction between fertile and infertile couples.

Materials and Methods

The present analytical cross-sectional study was conducted on fertile couples and infertile couples referred to the Fatemieh Educational Research Center affiliated to Hamadan University of Medical Sciences, Hamadan, Iran, from May to August in 2014. The study protocol was approved by the Institutional Review Board and the Human Research Ethics Committee of the Hamadan University of Medical Sciences. Sample size was estimated using the following formula:

$$n = \frac{(Z_{1-\frac{\alpha}{2}} + Z_{1-\beta})^2(\sigma_1^2 + \sigma_2^2)}{(\mu_2 - \mu_1)^2}$$

To achieve power of 90 and level of significance of 0.05, 125 couples were determined for each group.

At first, a list of eligible couples, referred to the Fertility Center of Hamadan University of Medical Sciences, was prepared, among whom 125 infertile couples were selected using a table of random numbers. Then, 125 fertile couples were selected from those referring to other clinics (like women's health care, oncology, and children's health care centers) using the same method, meaning 25 fertile couples were chosen from each clinic. The two groups in terms of age, socio-economic status and lack of acute or chronic diseases were matched.

The inclusion criteria of the infertile group were as follows: not conceiving after 5 years of trying, primary infertility, male factor infertility/female factor infertility (or both), unexplained infertility, and literacy skills in Farsi. The inclusion criteria for the fertile group were as follows: not suffering from infertility, having at least one child, willingness to cooperate in the study, and literacy skills in Farsi. On the other hand, the exclusion criteria of the study were as follows: use of medications other than those used for infertility treatment, physical or mental disorders, death of close relatives during the past two months, child adoption, and unwillingness to cooperate in the study . After the study objectives and procedures were explained to them, all participants signed a written informed consent.

Measurement tools

All participants completed demographic questionnaire, World Health Organization Quality of Life-BREF (WHOQOL-BREF) questionnaire, Linda Berg's Sexual Satisfaction Scale, and Enrich Marital Satisfaction Scale.

WHOQOL-BREF questionnaire prepared by the WHO contains 24 items regarding physical health (7 items), mental health (6 items), social relationships (3 items), and environmental health (8 items) dimensions. This questionnaire also includes 2 other items to evaluate health status and quality of life generally. Thus, the questionnaire has 26 items. The items are responded through a 5-option Likert scale and a score between 0 and 100 is assigned to each dimension (17). Assessment of psychometric properties was done by the WHO (18). In Iran, the reliability and validity of this scale were approved by Nejat et al. (19). Besides, Keramat et al. (20) have evaluated the reliability of WHOQOL-BREF and reported the Cronbach's alpha (reliability) of 0.78, 0.77, and 0.79 belonging to physical health, mental health, and environmental health dimensions, respectively.

Enrich Marital Satisfaction Scale designed by Olsun, Furnier, and Druckman contains 14 subscales. The reliability of this questionnaire was already confirmed (α=0.92), while Pazandeh and Sharghi (16) have reported a reliability of α=0.95. This scale is responded using a 5-option Likert scale and a score between 1 and 5 is allocated to each item. Accordingly, the scores are below 30 indicating severe dissatisfaction, between 30 and 40 indicating dissatisfaction, between 40 and 60 indicating relative and moderate satisfaction, between 60 and 70 indicating great satisfaction, and above 70 indicating very great satisfaction. Keramat et al. (20) have confirmed the reliability of this scale with Cronbach's alpha of 0.91.

Linda Berg's Sexual Satisfaction Scale designed by Linda Berg and Cresy in 1997 consists of 17 items with the following options: always, often, sometimes, rarely, and never receiving 5, 4, 3, 2, and 1 scores, respectively. Thus, the minimum and maximum scores of the scale are 17 and 85, respectively. Accordingly, the scores are 17-51 indicating weak, 52-67 indicating moderate, and 68-85 indicating good sexual satisfaction. The validity and the reliability (Cronbach's alpha=0.94) of this scale were also approved by Keramat et al. (20). Assessment of psychometric properties of all questionnaire were done in many studies (18).

Statistical analysis

All data analyses were performed using the Statistical Package for the Social Sciences (SPSS, SPSS Inc., USA) version 16. Chi-square test and correlation analysis were used to assess the relationship between the study variables. Besides, student's t test was employed for comparison of the study groups. A value of $P<0.05$ was considered as statistically significant.

Results

According to the results, the mean score of physical dimension of WHOQOL-BREF was significantly higher in the fertile group (15.46 ± 2.66) compared to the infertile group (14.86 ± 2.66, $P<0.05$). Also, in the environmental dimension, the fertile couples obtained a significantly higher mean score (13.90 ± 2.41) in comparison to the infertile ones (13.13 ± 2.49, $P<0.05$). In the mental

dimension, the fertile group gained a higher mean score (13.71 ± 2.73) compared to the infertile group (13.42 ± 2.64), indicating the difference was not statistically significant. Considering the social dimension, although no significant difference was observed between the two groups, the mean score of the infertile group (14.27 ± 2.85) was higher than that of the fertile group (13.97 ± 2.85).

The mean score of sexual satisfaction was significantly higher in the infertile group compared to the fertile group (63.67 ± 13.13 vs. 46.37 ± 7.72). The mean score of marital satisfaction was also significantly higher in the infertile couples compared to the fertile ones (44.03 ± 9.36 vs. 36.20 ± 4.03) (Table 1). The results showed weak sexual satisfaction in 21.6% of infertile women, 79.2% of fertile women, 15.2% of infertile men, and 62.4% of fertile men. In other words, weak sexual satisfaction was less common in infertile couples (Table 2). Moreover, relative and moderate marital satisfaction was observed among 48% of infertile women, 12% of fertile women, 52% of infertile men, and 9.6% of fertile men. Moreover, very great marital satisfaction was found neither in the infertile nor in the fertile group, and great satisfaction was also not observed in the fertile couples (0 vs. 32% in infertile women and 8.8% in infertile men). In other words, marital satisfaction was higher in the infertile couples compared to the fertile ones (Table 3).

Table 1: Comparison the means scores of different dimensions belonging to WHOQOL-BREF questionnaire between two groups using t test

Variables	Group	Frequency	Mean	SD	t	P value
Physical dimension	Fertile	250	15.46	2.66	2.56	0.01
	Infertile	250	14.86	2.66		
Mental dimension	Fertile	250	13.71	2.73	1.20	0.23
	Infertile	250	13.42	2.64		
Social dimension	Fertile	250	13.97	2.74	-1.20	0.231
	Infertile	250	14.27	2.85		
Environmental dimension	Fertile	250	13.90	2.41	3.50	<0.001
	Infertile	250	13.13	2.49		
Sexual satisfaction	Fertile	250	46.37	7.72	-17.96	<0.001
	Infertile	250	63.67	13.13		
Marital satisfaction	Fertile	250	36.20	4.03	-12.14	<0.001
	Infertile	250	44.03	9.36		

WHOQOL-BREF; World Health Organization Quality of Life-BREF questionnaire.

Table 2: Comparison of frequency distribution of different sexual satisfaction categories between fertile and infertile couples

Sexual satisfaction categories	Infertile couples n=125						Fertile couples n=125					
	Infertile women		Infertile men		Total		Fertile women		Fertile men		Total	
	n	%	n	%	n	%	n	%	n	%	n	%
Weak	27	21.6	19	15.2	46	36.8	99	79.2	78	62.4	177	141.6
Moderate	52	41.6	45	36.0	97	77.6	26	20.8	47	37.6	73	58.4
Good	46	36.8	61	48.8	107	85.6	0	0	0	0	0	0
Total	125	100	125	100	250	100	125	100	125	100	250	100

WHOQOL-BREF; World Health Organization Quality of Life-BREF questionnaire.

Table 3: Comparison of frequency distribution of marital satisfaction categories between fertile and infertile couples

Marital satisfaction categories	Infertile couples n=125						Fertile couples n=125					
	Infertile women		Infertile men		Total		Fertile women		Fertile men		Total	
	n	%	n	%	n	%	n	%	n	%	n	%
Severe dissatisfaction	7	5.6	5	4.0	12	9.6	14	11.2	4	3.2	18	14.4
Dissatisfaction	54	43.2	44	35.2	98	78.4	96	76.8	109	87.2	205	164
Relative and moderate satisfaction	60	48.0	65	52.0	125	100	15	12	12	9.6	27	21.6
Great satisfaction	4	32	11	8.8	15	40.8	0	0	0	0	0	0
Very great satisfaction	0	0	0	0	0	0	0	0	0	0	0	0
Total	125	100	125	100	250	100	125	100	125	100	250	100

Discussion

Quality of life is the general well-being of individuals and societies, outlining negative and positive features of life. Sexual satisfaction is defined as an individual's judgment about pleasure of one's sexual behavior (21). The most important goal of sexual desire is reproduction and childbearing. Thus, sexual satisfaction is highly influenced by infertility (22). Compatibility and marital satisfaction are referred to a status in which a couple feels happy and satisfied that is created through mutual interest, caring, acceptance, understanding, and meeting each other's needs, including sexual needs (23).

Throughout the recent two decades, quality of life has been considered as a major concern. In 1978, WHO indicated quality of life improves when an individual receives mental and physical care. Fertility has social, psychological and physiological aspects. In most cultures, reproduction is of great importance concept (24). The results of the present study showed that the fertile women obtained higher mean scores in all dimensions of quality of life compared to the infertile ones. This difference was statistically significant in physical and environmental dimensions, but not in mental and social dimensions. Infertility and other related issues, like treatment process, have a negative impact on physical and mental health of infertile couples.

Physical dimension (general health, physical role, and bodily pain) is a highly important aspect of life quality. Stress-related infertility leads to physiological stress that results in serious health problems (25). Infertile couples seeking treatment also experience a lot of physical problems (26). In current study, physical dimension of infertile couples is lower than fertile couples. In a study by Kamkary and Shokrzadeh (27), they have showed that control of the environmental factors, which is among psychological components, is higher in couples with higher mental functions. Mental pressures resulting from infertility affects couples' attitude towards the environmental factors and reduces their determination to achieve their personal goals (28). According to the study by Direkvand Moghadam et al. (3), infertile women showed a lower mean score of physical role limitation due to physical problems as compared to the fertile ones. In addition, in a study by Hatamloye Saedabadi and Hashemi Nosratabad (29), he has indicated that control over the environment was lower among infertile women compared to fertile women. These results were consistent with those of the current study, showing that environment dimension of the quality of life in infertile couples was lower than fertile couples.

Evidence has demonstrated that psychological stress due to infertility treatment affect patients' quality of life through disturbing their psychological, social, and welfare conditions (30). Infertility is a source of social pressure that is exerted by a traditional culture surrounding the infertile couples (31). A study has shown that infertile couples have more feelings of helplessness and disappointment (32). One study has revealed that almost one thirds of all women and their partners experienced a lack of social support (30). Nevertheless, some researchers have reported higher

social support among infertile women. A study performed in Turkey has also showed that in spite of lower scores in mental dimension, infertile women had better social support (33). In our study, no significant difference was also found between fertile and infertile couples regarding mental and social dimensions. Unlike, another study in Iran has revealed statistically significant relationship between duration of infertility and mental disorders and marital conflicts (34). The findings of the current study demonstrated significantly higher sexual satisfaction among the infertile couples compared to the fertile ones, indicating the couples may be closer emotionally and psychologically to each other because of the conditions and continuation of treatment.

A previous study has indicated that infertile couples had lower sexual function in orgasm, arousal, and desire dimensions (23). On the other hand, a study by Jamali et al. (22) conducted in Iran has showed that infertility had no impact on couples' sexual function. Also, another study has indicated that sexual dysfunction was only detected in 11% of infertile couples (35). Similarly, Monga et al. (36) has reported no significant difference between infertile and fertile couples with respect to sexual function, which was attributed to the need for large number of sexual relationships for treatment of infertility and getting pregnant. Yet, this can also be associated with an increase in intimacy of infertile couples (22). In the present study, the infertile couples showed higher marital satisfaction compared to the fertile ones. In a study by Lotfi Kashani and Vaziri (37), they have concluded that marital satisfaction was accompanied by sexual satisfaction, which in return, higher sexual satisfaction resulted in higher marital satisfaction. Up to now, a large number of researches have shown that infertility declined marital satisfaction (38). However, many studies have indicated that children play a major role in decreasing marital satisfaction (39). Although having children strengthens the marital relationship, it may decrease marital satisfaction with passage of time and growing number of children (40, 41). Overall, the findings of our study showed that despite a decrease in life quality, infertile couples had high sexual and marital satisfaction. There were a number of limitations in this study. Due to randomly selected samples, there was no choice to select all samples properly. Therefore, it is recommended that in future, a study should be conducted with a larger sample size to show the improvement of quality of life and marital satisfaction of infertile couples as the basis of family and society.

Conclusion

According to the results, the fertile couples obtained significantly higher quality of life and lower sexual satisfaction and marital satisfaction as compared to the infertile ones. This might have resulted from disturbance in couples' marital relationships due to children, financial problems, etc. Therefore, holding consultation programs and conducting more studies are necessary for improving the quality of life and promoting sexual satisfaction and marital satisfaction in infertile couples.

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Original Article

Cord Blood Karyotyping: A Safe and Non-Invasive Method for Postnatal Testing of Assisted Reproductive Technology Children

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Abstract

Background: To verify the hypothesis that the incidence of chromosomal abnormalities increases in babies conceived by different assisted reproduction procedures. The availability of the umbilical cord blood encouraged us to study this hypothesis via this method.

Materials and Methods: This is a descriptive study, umbilical cord blood samples of assisted reproductive technology (ART) children were analyzed with standard cytogenetic techniques (G banding). Karyotyping was possible in 109 cases.

Results: The number of abnormal cases was four (3.7%), among which, three cases (2.8%) were inherited and only 1 case (0.9%) was a de novo translocation. In total, the incidence of de novo chromosomal abnormalities was in the range observed in all live births in the general population (0.7-1%).

Conclusion: No significant difference in the incidence of chromosomal abnormality was found between ART and naturally conceived babies. To date, several studies have examined the medical and developmental outcome of ART children and still have not reached a definite conclusion. Genetic counseling is recommended as an integral part of planning of treatment strategies for couples wishing to undergo ART.

Keywords: Assisted Reproductive Technics, Chromosomal Abnormality, Umbilical Cord Blood, karyotype

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Introduction

Assisted reproduction technology (ART) has provided great benefit for millions of couples who struggle with infertility. The definition of ART varies widely, but the US Center for Disease Control and Prevention (CDC) defines it as all fertility treatment in which both eggs and sperms are handled (1). Accordingly, in this study, we defined intrauterine insemination (IUI) babies along with *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI) babies as one group. The growing use of ART has dramatically increased the possibility of conceiving babies from infertile couples. Since then, the safety of these methods alongside the associated long-term impacts on the health of children has been a major concern. There are evidences of greater risks of low birth weight, preterm delivery (2, 3), cerebral palsy (4), and major birth defects (5) after ART, although the causes remains unknown. Obviously the genetic problem plays a considerable role in these debates (6, 7). Some researchers have questioned the genetic implications for offspring of couples having ART and

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suggested higher incidences of fetal sex-chromosomal aberrations (8) and de novo chromosomal anomalies (9) after ICSI procedures.

The advent of IVF technique provided a unique opportunity to analyze human pre- implantation embryo (10), moreover, cytogenetic analysis of product of conception can be helpful to determine the cause of the pregnancy loss and brings valuable information in the setting of infertility and assisted reproduction (7). However, advanced maternal age, altered karyotype, multiple assisted reproductive technologies failure, repeated miscarriages and spermatozoa obtained by Simon et al. (11) and Magli et al. (12) are characteristics that expose the couples to an increased risk of generating chromosomally abnormal embryos (6).

The main purpose of this study was to evaluate the risks of chromosomal aberrations in ART offsprings of normal karyotype parents by karyotyping these children using their cord blood. Cord blood is widely usable and easy to access; collection is relatively non-invasive and painless. This means for all newborns conceived through ART procedures, cord blood karyotyping may be performed to ensure their normal chromosomal status. On the other hand, there could be some disadvantages such as maternal blood contamination and missing some genetic conditions. Although there are reports based on peripheral blood samples, no study has been reported on such children using their cord blood.

Materials and Methods

This is a descriptive study that was conducted in the Department of Genetics of Royan Institute, Iran, during January 2009 to January 2012. We considered preparation of umbilical cord blood to be a safe method and therefore, karyotyping was conducted on these samples.

From 88 participating infertile couple candidates for ART procedures, 109 umbilical cord blood samples (68 cases had a singleton, 19 cases had twins and 1 case had a triplet birth) were obtained and analyzed. Prior to commencing the study, ethical approval was received from the local institutional Ethics Committee. When pregnancy happens, a written informed consent was obtained from each couple who participated in the study. A genetic counselor visited all pregnant patients and a pedigree was recorded for each of them. Briefly, data on pregnancies and deliveries (information about ectopic pregnancies, miscarriages, preterm births, stillbirths, live births, multiple pregnancies and terminations) were obtained. Additional clinical findings including history of infertility and reported use of ARTs, maternal ages, date of delivery and presence of congenital abnormalities in the members of the family were also recorded.

At the time of delivery, the umbilical cord blood samples were collected during cesarean section in sterile heparinized containers and delivered to genetic laboratory within 2 hours. Because of high risks and emergencies in the field of infertility, all samples were obtained by cesarean section based on the gynecologist preference. General pediatric examinations were performed at birth to identify any apparent anatomical abnormalities of the children. In the cytogenetic lab, a trained technician prepared karyotype slides using the Giemsa (G-banding) technique (500-550 bands per karyotype). It should also be mentioned, a drawback of blood-based G-banding karyotyping is that it can miss extremely subtle chromosome abnormalities that are at the limit of resolution of light microscopy. In brief, approximately 0.5 ml of heparinized whole blood was placed/poured into a glass or plastic tube and inoculated with 10 ml of PB-MAX medium (Gibco, USA). The culture was then incubated at 37°C for 48 hours and thymidine was added at a final concentration of 0.22 μg/ml (0.92 mM) and further incubated for 16 hours in incubator. The culture was subsequently transferred to a centrifuge tube and at 500 xg for 10 minutes. Afterwards, 5 ml of fresh medium without Phytohemagglutinin (PHA) was added and the culture was incubated for an additional 5 hours. Next, the cells were centrifuged as before and washed in fixative for a second time and incubated for 10 minutes. 0.2 μg/ml of KaryoMAX™ Colcemid Solution (Gibco, USA) was added to each culture tube then the culture was incubated for an additional 15 minutes. Afterwards, the culture was transferred to a centrifuge tube and spun at 500 xg for 10 minutes, then the supernatant was removed and the cells were re-suspended in 10 ml of hypotonic 0.075 M KCl (Gibco, USA) and incubated at

37°C for 15 minutes and then spun at 500 xg for 10 minutes. Subsequently the supernatant was removed, the cellular sediment was agitated and 5-10 ml of fresh, ice-cold fixative made up of 1 part acetic acid to 3 parts methanol was added drop-by-drop, and left in -20°C for 1 hour then spun at 500 xg for 10 minutes. The cell pellet was re-suspended in a small volume 0.5-1 ml of fresh fixative, dropped onto a clean slide and allowed to air dry. At this stage, the slide could be stained with Orecin or Giemsa. Giemsa banding has become the most widely used technique in cytogenetic analysis, and the most common method to obtain this staining is to treat slides with Trypsin-EDTA 10X (Gibco, USA). At least 15 metaphases were analyzed per baby and in the case of mosaicism or abnormal karyotype, 50 metaphases were analyzed. The chromosomal anomalies were reported in accordance with the current international standard nomenclature (13). From 109 newborns, in nine cases prenatal tests and amniocentesis eliminated the need for cord blood karyotyping.

Statistical analysis

This is a descriptive study and the reported rate is within the range reported in the literature. Abnormal karyotype rates were compared by Fisher's exact test between ART children and $P<0.05$ was considered statistically significant.

Results

The age of females ranged from 26 to 42 years (mean age of 34 years). Table 1 summarizes family histories of all participating couples in this study (consanguinity, history of spontaneous abortions, ART failure, Intrauterine fetal death (IUFD) in each couple, cleft lip/club foot and mental retardation (MR) in 1st or 2nd cousins). As shown in Table 1, failed ART and IUFD were the most and least frequent features (40.4 and 1.83% respectively) observed in the patients and their extended family.

Table 1: Medical history of couples participating in this study

Medical factors	n*	Percentage**
Spontaneous abortions	8	9.1
Consanguinity	28	31.8
ART failure	44	50
IUFD	2	2.3
Cleft lip/club foot in 1st or 2nd cousins	5	5.7
MR in 1st or 2nd cousins	12	13.6

ART; Assisted reproductive technology, MR; Mental retardation, IUFD; Intra Uterus Fetal Death, *; Some of the patients showed more than one medical factor, and **; Percentage of medical factors among 88 studied couples.

Table 2: Descriptive characteristics of couples

Characteristics		n	Percentage
Infertility factor	Female factor	24	27.2
	Male factor	43	48.9
	Male and female factor	14	16
	Idiopathic	7	7.9
Type of infertility	Primary	84	95.45
	Secondary	4	4.55
ART method	IVF	2	2.27
	ICSI	69	78.41
	IUI	17	19.32

ART; Assisted reproductive technology, IVF; *In vitro* fertilization, ICSI; Intra-cytoplasmic sperm injection, and IUI; Intrauterine insemination.

Descriptive characteristics of couples are summarized in Table 2.

About half of infertility factors were male-based (48.9%) and almost 8% were idiopathic infertiles. The most common infertility treatment was ICSI (78.41%), while IVF is the least used method (2%). And finally, the ratio of primary and secondary infertility was 84% and 4%, respectively. The most and least common sperm retrieval methods were masturbation (53.4%) and retrograde method (2.3%) respectively (Table 3).

Table 3: Type of sperm retrieval and their percentages

Type of sperm retrieval	Masturbation	Coitus	PESA	TESE	R.G
Number	47	27	7	5	2
Percentage	53.4	30.7	7.9	5.7	2.3

PESA; Percutaneous epididymal sperm aspiration, TESE; Testicular sperm extraction, and R.G: Retro grade.

Table 4: The percentage of chromosome abnormalities (de novo or inherited) in ART children

ART Children	Normal karyotype (%)	Abnormal karyotype (%)		Total P value
All	105 (96.3)	4 (3.7)		109
		De novo abnormality (%)	Hereditary abnormality (%)	
		1 (0.9)	3 (2.8)	
46,XX	54 (51.43)	2 (1.85)		56
		De novo abnormality (%)	Hereditary abnormality (%)	0.486
		0	2 (100)	
46,XY	51 (48.57)	2 (1.85)		53
		De novo abnormality (%)	Hereditary abnormality (%)	0.486
		1 (50)	1 (50)	

Chromosome analysis was successfully carried out for 109 ART children. As shown in Table 4, for 109 cord blood samples analyzed, the overall rate of abnormality was 3.7% (four cases), and among which, three cases (2.8%) were inherited (one marker chromosome and two inversions) and one case (0.9%) was a de novo chromosome abnormality (structural aberrations). In particular, the inherited cases were a non-identical twin who both showed inversion of chromosome 3 [(46, XX, inv (3) and 46, XY, inv (3)] and a baby who showed a marker chromosome with unknown source, inherited from the mother. In the case of the de novo abnormality, there was a non-identical twin of which the first one was normal (46, XX) and the second one showed translocation between chromosomes 18 and Y [(46, X, t(Y, 18) (q11.2; p11.3)]. The father had a normal karyotype (46, XY) and the baby's external genitalia was normal. The observed translocation involved Yq11.2 which includes AZF genes, thought to be essential for normal spermatogenesis, thus investigation after puberty was recommended to the parents. Overall, our finding confirmed that there is no significant difference regarding de novo chromosomal abnormality rate in ART children in comparison with naturally conceived babies. Also, ICSI is shown to be applied more in ART babies with chromosomal abnormality.

Discussion

There is a growing belief that ART children are phenotypically and somehow genetically different from naturally conceived children. However, the mechanism(s) leading to these possible changes have not been elucidated and may include parental factors, maternal medications, culture media, as well as egg and embryo manipulation (14). The present study included 109 cord blood samples of pregnancies achieved by IVF, ICSI and IUI and was undertaken to examine whether the rate of chromosomal abnormalities is increased among ART conceived children. In our study, we found 0.9% de novo chromosomal abnormality in ART children. This rate compared to the prevalence of this kind of abnormality among naturally conceived newborns in the general population is within the range of 0.7-1% (15). This demonstrates that ART children do not show a higher cytogenetic risk compared to the natural conceived one or in comparison with data from literature in the normal population (7, 16, 17). There are some studies showing the conflicting conclusions in this area (7, 9, 10, 17). Although the incidence of genetic anomalies are high in countries with the higher rate of consanguineous marriage(18-20), our results do not show any increase in the rate of chromosomal abnormalities in babies conceived by consanguineous couples studied here. Also our results are limited to live-born infants and do not involve stillbirths and aborted fetuses.

Several studies suggest abnormal karyotypes in infertile patients and also meiotic aberrations in their germ cells may be considered as the origin of abnormal karyotypes in ART children. These studies reported that the rate of chromosomal abnormalities in infertile male population has risen above the population baseline and others found a higher incidence of sex chromosome aneuploidy in sperm of men that underwent ICSI (21-23). Ca-

sio supposed that an increased incidence of XY spermatozoa was noted in chromosomally normal infertile males, perhaps, due to testicular mosaicism not detected in peripheral blood (6). In 1989, a European survey showed that IVF, compared with natural conception, does not increase the incidence of abortions due to chromosomal abnormality (24). Some authors postulated that the presence of an unbalanced translocation in some gametes may predispose to pre or post implantation failure of embryo development and as a result, the risk of chromosomal abnormalities of ART treatment may be increased (25-28). Genetic chromosomal abnormalities may arise de novo or derive from a familial anomaly present in one of the parents (29).

Chromosomal aberrations of ART children have been most extensively studied by the Belgian group (9). The limited available data on ICSI fetal karyotypes in comparison with general neonatal population revealed that there is: i. A slight but significant increase in de novo sex chromosome aberrations and structural autosomal abnormalities and ii. An increased number of inherited (mostly from the infertile father) structural aberrations (30-33). A survey has provided data on the frequency of chromosomal anomalies in newborns after ICSI (23) and found two de novo chromosomal abnormalities (3.6%). They presumed that the other live born children were normal because they noticed no typical malformations consistent with chromosomal defects and that is a percentage of 1.2% (2 out of 167). This was compatible with the prevalence of de novo chromosome abnormalities after ICSI reported in Bonduelle's study (9).

According to some other studies reporting increased risk of imprinting disorders (34, 35) and malignancies (36) in ART children, these kind of studies at least sound an alarm about the genetic alterations of ART offspring and these procedures should thus be used cautiously.

Conclusion

Comparing with some reports, our data showed that children born via ART were not subjected to a higher cytogenetic risk than naturally conceived babies in the general population. However, there is conflicting opinion on this area. Since the number of newborns conceived through ART procedures is growing, reports like this must be considered as a pilot study and prenatal tests must be performed for all pregnancies through ART. In lack of amniocentesis, cord blood karyotyping could be perform immediately after birth to find those aberrations which do not have phenotypic alterations such as sex chromosome aneuploidies. Further investigations by array based techniques and epigenetic tests are undergoing to evaluate possible subtle genetic alterations and different epigenetic modifications in ART conceived children.

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Original Article

Mitochondrial Genetic Variation in Iranian Infertile Men with Varicocele

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Abstract

Background: Several recent studies have shown that mitochondrial DNA mutations lead to major disabilities and premature death in carriers. More than 150 mutations in human mitochondrial DNA (mtDNA) genes have been associated with a wide spectrum of disorders. Varicocele, one of the causes of infertility in men wherein abnormal inflexion and distension of veins of the pampiniform plexus is observed within spermatic cord, can increase reactive oxygen species (ROS) production in semen and cause oxidative stress and sperm dysfunction in patients. Given that mitochondria are the source of ROS production in cells, the aim of this study was to scan nine mitochondrial genes (*MT-COX2*, *MT-tRNA^{Lys}*, *MT-ATP8*, *MT-ATP6*, *MT-COX3*, *MT-tRNA^{Gly}*, *MT-ND3*, *MT-tRNA^{Arg}* and *MT-ND4L*) for mutations in infertile patients with varicocele.

Materials and Methods: In this cross-sectional study, polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing were used to detect and identify point mutations respectively in 9 mitochondrial genes in 72 infertile men with varicocele and 159 fertile men. In brief, the samples showing altered electrophoretic patterns of DNA in the SSCP gel were sent for DNA sequencing to identify the exact nucleotide variation.

Results: Ten type nucleotide variants were detected exclusively in mitochondrial DNA of infertile men. These include six novel nucleotide changes and four variants previously reported for other disorders.

Conclusion: Mutations in mitochondrial genes may affect respiratory complexes in combination with environmental risk factors. Therefore these nucleotide variants probably lead to impaired ATP synthesis and mitochondrial function ultimately interfering with sperm motility and infertility.

Keywords: Infertility, Varicocele, Mutation, Mitochondrial Genes

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Introduction

The major concern among married couples when they are unsuccessful to conceive after one year of regular unprotected intercourse is that they may be infertile. Male factors can be attributed to half of these cases (1, 2). The most common surgically reversible cause of infertility is varicocele. Its prevalence is about 4.4-22.6% in the general population, 21-41% in men with primary infertility and 75-80% in men with secondary infertility (3, 4). Despite the advances in molecular medicine, the pathophysiology of varicocele induced infertility remains unknown. Several proposed mechanisms include venous pressure changes and increased testicular temperature due to dilation and tortuosity of the pampiniform plexus of veins, oxidative stress, retrograde flow of

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renal or adrenal products, Leydig cell dysfunction and hyperthermia (5, 6). In addition, a number of patients with varicocele have genetic abnormalities like Yq-microdeletions (7). Among them, oxidative stress-induced DNA damage appears to be a more likely cause which may severely affect sperm quality leading to infertility (8). This damage is one of the potential etiological factors in varicocele. A major source of partially reduced derivatives of molecular oxygen (O_2) is mitochondria (9). The variety of reactive oxygen species (ROS) that mitochondria produce principally include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and the hydroxyl radical (OH^-) (10, 11). In normal physiology, ROS perform several roles in regulating cellular functions by interacting with cellular components (12). In fertile men, physiological levels of ROS play important roles in sperm function, acrosome reaction, capacitation, hyper-activation and the penetration of oocyte by spermatozoa. However, in varicocele patients ROS generation is abnormally enhanced (13, 14).

Specific point mutations and deletions of mitochondrial DNA (mtDNA) have been associated with poor sperm motility and semen quality in several studies. Sperm mtDNA is highly sensitive to mutations due to increased ROS by-products generated during oxidative respiration (15). When large amounts of mutant mtDNA accumulate in the testes, reduction in ATP production, mitochondrial respiratory dysfunction and meiotic arrest are induced in spermatogonia cells (16). Each mitochondrion has 2-10 mitochondrial genomes responsible for coding the subunits of the OXPHOS complex. The OXPHOS machinery is made up of over 80 different polypeptides, of which the mtDNA encodes 13 polypeptides including complex I, III, IV and V subunits (17).

In this study, for first time, we further analyzed nine genes (*MT-COX2, MT-tRNA^{Lys}, MT-ATP8, MT-ATP6, MT-COX3, MT-tRNA^{Gly}, MT-ND3, MT-tRNA^{Arg}* and *MT-ND4L*) in the mitochondrial genome by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) assay and direct sequencing techniques to identify the possible association between mtDNA variation with varicocele in the Iranian population.

Materials and Methods

Patients

This study was a cross sectional study. Seventy two Iranian infertile men with clinical varicocele were recruited in the study. The varicocele diagnosis was made by the urologists for the patients by physical examination in standing position and via scrotal palpation in a temperature controlled room (23°C). Semen analysis was performed according to the WHO laboratory manual (18). Patients with varicocele were in 3 grades: i. Grade I (n=12), ii. Grade II (n=27) and iii. Grade III (n=33). The control group (healthy volunteers) consisted of 159 fertile and normospermic men from the Yazd Infertility Center who fathered at least one child. The ethnic and geographical origin of all patients and controls was the same. All participants were fully informed of the objectives of the study and those that signed the consent form were assigned to the study. All infertile men in the age group ranging from 22 to 36 years (mean, 29 years) were referred for evaluation of their infertility (1 year of unprotected intercourse and not leading to conception). The Yazd University Ethics Committee approved recruitment of patients and laboratory protocols in this study.

DNA extraction and mutation analysis

Peripheral blood samples were obtained from varicocele patients and the DNA was extracted using a standard salting-out procedure. Purified DNA samples from leukocytes were used for the PCR reactions. To amplify *MT-COX2, MT-tRNA^{Lys}, MT-ATP8, MT-ATP6, MT-COX3, MT-tRNA^{Gly}, MT-ND3, MT-tRNA^{Arg}* and *MT-ND4L* mitochondrial genes, four pairs of PCR primers were designed, which were located in the flanking regions of each gene (Table 1). Primer Design was based on the human mitochondrial sequence by primer design software (Primer Premier 5.0; Premier Biosoft Inc., Canada), and their secondary structure was examined with Gene Runner version 3.05 (Hastings Software Inc. Hastings, NY, USA, http://www.generunner.com). Each reaction was prepared to a final volume of 25 µl containing 1XMasterMix PCR (Yekta Tajhiz Azma Co., Iran), 0.2 mM of each primer and 0.5 µg DNA template. The PCR conditions were an initial denaturation of 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, the annealing temperature (Table 1) for 30 seconds and extension at 72°C for 30 seconds, which was extended for 5 minutes in the final cycle. The PCR products were electrophoresed on an ethidium bromide-stained 2% agarose gel.

Table 1: Primers used for mitochondrial genes

Segment	Primer sequence (5'-3')	Primer position	Tm (°C)	Size (bp)	Gene
Seg. 1	F: CTACGGTCAATGCTCTGAAA R: TAGGTGGTAGTTTGTGTTTA	8161-8180 84708451	56.5	309	*MT-COX2, MT-tRNA^{Lys}, MT-ATP8, MT-ATP6*
Seg. 2	F: AGCCCACTTCTTACCACAAG R: TACTATATGATAGGCATGTGA	8901-8920 9239-9219	56	338	*MT-ATP6*
Seg.3	F: CACTATCTGCTTCATCCGCC R: ATGTAGCCGTTGAGTTGTGG	9851-9870 10150-10131	57		*MT-COX3, MT-ND3*
Seg. 4	F: TCTGGCCTATGAGTGACTAC R: AGTATTATTCCTTCTAGGCA	10361-10380 10582-10380	57	221	*MT-ND4L, MT-tRNA^{Arg}*

Tm; Temperature melting.

For the SSCP assay, PCR products were heat-denatured at 95 ºC for 5 minutes and chilled on ice for 5 minutes, and then loaded onto an 8% non-denaturing polyacrylamide/TBE 0.5x gel. Gels were stained with silver nitrate to reveal the bands of single strand DNA. Various band patterns of the amplified PCR products were marked and scored. The typical gene variants got sequenced using a commercial company (Macrogen, South Korea). All the data obtained from automated sequencing was checked with Sequencher. The online multiple sequence alignment software ClustalW2 (http://www.ebi.ac.uk/tools/msa/clustalw2/) and BLAST analysis were used to determine the nature of mutations and percent homology of the sequences that have been obtained in the study with all other sequences of five other species (chimpanzee, monkey, cattle, zebrafish and drosophila).

Software and databases

We used the tool PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) for prediction of the functional consequences of mutations and damaging effect of missense mutations on protein structure. The sequence alignment was performed using the blastp program available at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/Blastp) and the ClustalW program (http://bioinfo.hku.hk/services/analyseq/cgi-bin/clustalw_in.pl). For detection of structural features of mammalian mitochondrial tRNAs and human diseases linked to point mutations in mitochondrial tRNA genes, we used Mamit-tRNA (http://mamit-trna.u-strasbg.fr).

Statistical analysis

The GraphPad Prism software (GraphPad Software, Inc. USA) was used for statistical analysis. Distributions of continuous variables in groups were expressed as mean ± SD, and compared with unpaired Student's t test. $P<0.05$ were regarded as statistically significant.

Results

The age difference between the 72 Iranian infertile men with varicocele (mean age of 30.76 ± 6.47) and 159 normal controls (mean age: 28.8 ± 6.01) was not significant (P=0.785). Mutation analyses for the mitochondrial *MT-COX2, MT-tRNA^{Lys}, MT-ATP8, MT-ATP6, MT-COX3, MT-tRNA^{Gly}, MT-ND3, MT-tRNA^{Arg}* and *MT-ND4L* genes were carried out in all of patients and healthy controls by PCR-SSCP. Mobility shift of single strand DNA on polyacrylamide gel electrophoresis was the criterion for sequencing and the identification of DNA variation (Fig.1). We found ten different nucleotide substitutions of which 4 caused an amino acid change, of which one occurred in tRNAArg. None of the ten mutations were found in healthy controls. All the mutations identified are summarized in Table 2. In addition, 6 were novel mutations of which four were silent mutations. Four reported polymorphisms, including m.8258T>C, m.9911C>A, m.9932G>A and m.10463T>C were found in six patients. The m.9911C>A variant in *MT-COX3* was heteroplasmic. The novel 9 bp heteroplasmic insertion was found in the non-coding MT-NC7 locus in one patient.

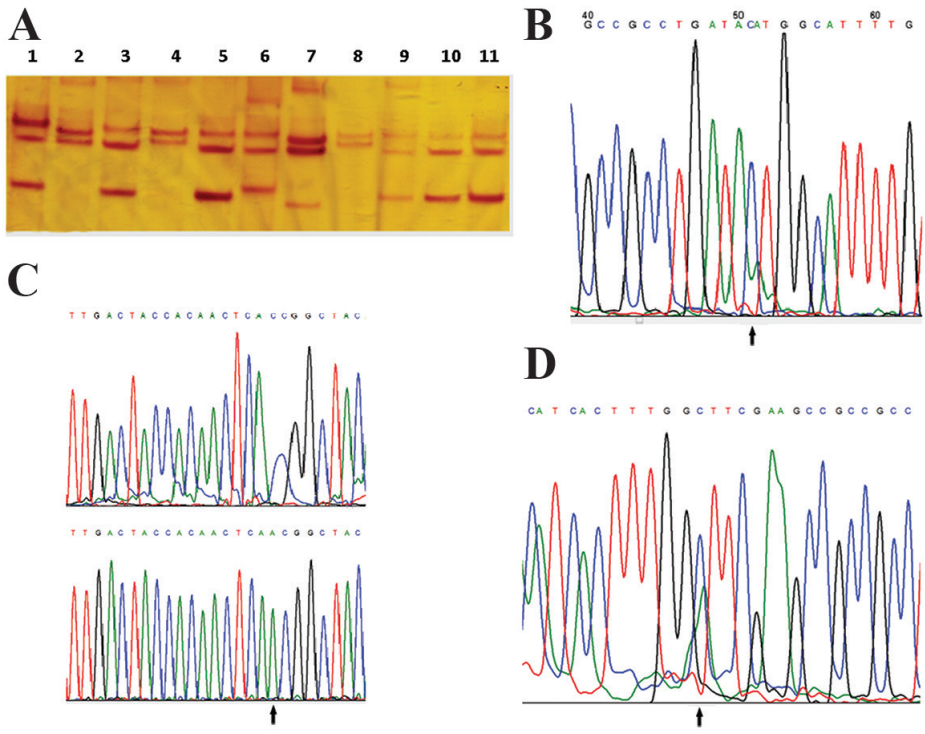

Fig.1: Silver staining SSCP analysis of fragment 3. **A.** Polyacrylamide gel electrophoresis. Lanes 1, 3 and 5 show 3 patients who did not have mutations, **B.** Lane 6 shows a patient with the m.9929 C>A mutation, **C.** Lanes 2, 4 and 8 show 2 patients with the m.10141A>C mutation, and, **D.** Lane 7 shows a patient with m.9911C>A and lanes 9, 10 and 11 are men without varicocele.

The m.9911C>A mutation, an aromatic amino acid phenylalanine codon (TTC), changes to leucine codon (TTA), a hydrophobic amino acid at position 235 (designated F235L) in 1 patient (Fig.2) and the novel 9929C>A mutation changes a polar tyrosine to threonine.

Two novel mutations were detected in 5 patients with one (9929C>A) being a nonsense mutation and changes tyrosine to stop codon (Y241X) and the other (10141C>G) being a missense mutation that changes Asparagine to Lysine (N27K). Also, three synonymous polymorphisms were found that were not reported previously (Table 2). The other variation was the m.10463T>C substitution (homoplasmic state) in the $tRNA^{Arg}$ gene that was found in 3 patients.

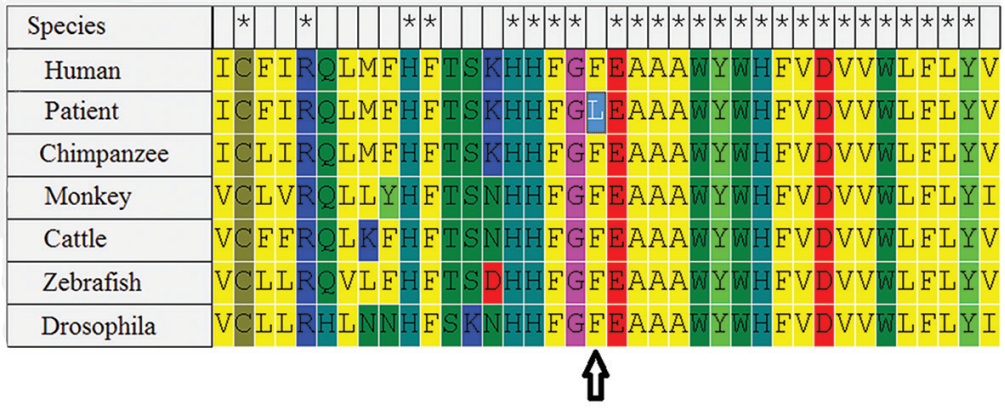

Fig.2: Protein alignment of m.9911C>A missense mutation *MT-COX3* and the arrow indicate the site of the F235L mutation.

Table 2: Mitochondrial variation found in infertile men with varicocele

Locus	Position	Nucleotide change	Amino acid position	No. of individuals	Hetero/Homo	Previously reported
MT-COX2	8258	T→C	F225L	1	Homo	Yes (19)
MT-NC7	Ins8288	9 bp	Non-coding	1	Hetero	No
MT-COX3	9911	C→A	F235L	1	Hetero	Yes (20)
MT-COX3	9929	C→A	Y241X	2	Hetero	No
MT-COX3	9932	G→A	W242W	1	Homo	Yes (21, 22)
MT-ATP6	9063	A→G	L179L	1	Homo	No
MT-ND3	10103	A→G	L15L	1	Homo	No
MT-ND3	10141	C→A	N27K	3	Homo	No
MT-TR	10463	T→C	$tRNA^{Arg}$	6	Homo	Yes (23, 24)
MT-ND4L	10550	A→G	M27M	12	Homo	No

Discussion

One of the most frequent causes of male infertility is varicocele, however, the pathogenic mechanisms by which it leads to changes in spermatogenesis are not clear (25). Some of these mechanisms may be related to mutations in mitochondrial complexes that affect flagellar movement and cause sperm dysmotility.

DNA alterations including point mutations and deletions of mtDNA have been reported in infertile patients with low sperm motility (26). The effect of mtDNA mutations on male infertility has also been studied. Shamsi et al. (27) reported that generation of ROS and mtDNA mutations are associated with pathogenic molecular mechanisms. Agarwal et al. (28) showed an increased oxidative stress in varicocele patients. Thangaraj et al. (29) demonstrated that sperm mitochondrial mutations is one of the causes of low sperm motility which is strongly dependent on ATP biosynthesis which is carried out by the mitochondrial OXPHOS system. Furthermore, it has been demonstrated that cells with some base substitutions in mtDNA can greatly influence semen quality (9, 30, 31).

It has been established that mitochondrial dysfunction caused by mtDNA mutations and oxidative damages is one of the important reasons for most types of infertility such as Varicocele (32). The mtDNAs alterations may accumulate in the spermatids or during gametogenesis and thereby impair the respiratory function and motility of spermatozoa (33).

We observed three heteroplasmic variations in 4 patients. A nine base pair heteroplasmic insertion in the non-coding MT-NC7 locus were found in 1 patient. Although this insertion (5′-CCCCCTCTA-3′) has been found in a non-coding region, it may cause mitochondrial rearrangements and DNA strand break affected by topoisomerases or DNA recombinase (34).

The heteroplasmic m.9911C>A and m.9929C>A transversions in *MT-COX3* alter two conserved codons. Given that these variants change highly conserved amino acids and were not identified in normal controls, they may be considered as pathogenic mutations for the following reasons. First, these missense mutations are found in several patients. Second, these mutations are not reported as polymorphisms in the general population and are not detected in the control individuals from the same ethnic background. Third, the mutations are heteroplasmic in the lymphocyte cells. Fourth, we propose that these mutations may affect the polarity of the protein due to the replacement of a natural amino acid with a polar amino acid. Using PolyPhen2, we found that these mutations are expected to change protein function.

Here, we describe seven homoplasmic variants in 25 patients: two missense mutations in *MT-COX2* (8258T>C) and *MT-ND3* (10141C>A), 4 synonymous polymorphisms in *MT-COX3*, *MT-ATPase6*, *MT-ND3* and *MT-ND4L* and one mutation (10463T>C) in *MT-$tRNA^{Arg}$*. This mutation is located at a moderate conserved region of the acceptor stem of tRNA arginine. This mutation was

not observed in healthy control subjects but was previously reported as a polymorphism in mitochondrial encephalomyopathy (35) and may be one of the several predisposing factors for varicocele.

Conclusion

Because sperms require an optimal energy to reach the oviduct during fertilization, the appropriate bioenergetic function of mitochondria is critical for male infertility. Therefore, any changes in mitochondrial genome can cause improper functioning of respiratory chain that in combination with environmental risk factors lead to infertility in men. This first Iranian study revealed that some Iranian infertile men carry variants in the nine mitochondrial genes and suggests that variants in these genes may be associated with varicocele.

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Original Article

Expression Profile of Developmentally Important Genes in pre- and peri-Implantation Goat Embryos Produced *In Vitro*

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Abstract

Background: Little is understood about the regulation of gene expression during early goat embryo development. This study investigated the expression profile of 19 genes, known to be critical for early embryo development in mouse and human, at five different stages of goat *in vitro* embryo development (oocyte, 8-16 cell, morula, day-7 blastocyst, and day 14 blastocyst).

Materials and Methods: In this experimental study, stage-specific profiling using real time-quantitative polymerase chain reaction (RT-qPCR) revealed robust and dynamic patterns of stage-specific gene activity that fall into four major clusters depending on their respective mRNA profiles.

Results: The gradual pattern of reduction in the maternally stored transcripts without renewal thereafter (cluster-1: *Lifr1, Bmpr1, Alk4, Id3, Ctnnb, Akt, Oct4, Rex1, Erk1, Smad1* and *5*) implies that their protein products are essential during early cleavages when the goat embryo is silent and reliant to the maternal legacy of mRNA. The potential importance of transcription augment at day-3 (cluster-2: *Fzd, c-Myc, Cdc25a, Sox2*) or day-14 (cluster-3: *Fgfr4, Nanog*) suggests that they are nascent embryonic mRNAs which intimately involved in the overriding of MET or regulation of blastocyst formation, respectively. The observation of two expression peaks at both day-3 and day-14 (cluster-4: *Gata4, Cdx2*) would imply their potential importance during these two critical stages of pre- and peri- implantation development.

Conclusion: Evolutionary comparison revealed that the selected subset of genes has been rewired in goat and human/goat similarity is greater than the mouse/goat or bovine/goat similarities. The developed profiles provide a resource for comprehensive understanding of goat preimplantation development and pluripotent stem cell engineering as well.

Keywords: Goat, Developmental Stage, Gene Expression, Preimplantation

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Introduction

Mammalian preimplantation embryonic development encompasses the period from fertilization to implantation. During this period, the embryonic stages and critical developmental events assessed are transition from germinal vesicle stage (GV) to metaphase-II (MII) oocyte, maternal-to-embryonic transition (MET), and the first lineage dif-

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ferentiation to the inner cell mass (ICM) and trophectoderm (TE) during blastocyst formation (1). Notably, implantation in ungulates, unlike human and mice, occurs with a delay of around 7 days. During this "peri-implantation" period, the rapid development of TE dramatically alters the blastocyst morphology from a sphere to a day 14 hatched blastocyst (2).

An improved understanding of gene activity that regulates preimplantation development is crucially important for assisted reproduction techniques and for derivation of embryonic stem cells (3). This goal has been largely achieved in mouse and human (4, 5). For example, it has been shown that embryonic developmental program is regulated by intricate cooperation of several important genes in the context of cell-signaling pathways. It was initially presumed that the developmental genes regulating early embryonic events are conserved across all mammalian species. Researchers attempted to extrapolate mice and human knowledge databases to the embryonic development of other species as well. However, further comparative studies revealed that species-specific differences exist between gene regulatory networks regulating embryo development in mammals (3, 5-7), which will provide a roadmap for differentiating definitive species-specific differences.

The goat is a valuable livestock animal with promising importance in agriculture, biomedicine and transgenesis (8, 9). However, the molecular basis of goat early embryonic development is poorly understood. Yan et al. (10) for the first time demonstrated that the expression of Oct4 and Nanog proteins were not restricted to the ICM of goat blastocysts. To date, four studies have reported derivation of goat "putative" embryonic stem cells (ESCs) from embryos produced either *in vivo* or *in vitro* (11-14). However, chimera production and germ line transmission of ESC have yet remained to be established in the goat (15).

An improved understanding of expression profiles of developmentally important genes in pre- and peri-implantation goat embryos would improve current attempts to establish ESC in this valuable farm species. Therefore, this study for the time investigated the expression profile of 19 genes, known to be critical for early embryo development in mouse and human, at five different stages of goat in vitro embryo development (oocyte, 8-16 cell, morula, day-7 and 14 blastocysts).

Materials and Methods

In this experimental study , unless otherwise stated, all chemicals and media were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA), respectively.

Selection of genes set

Nineteen candidate genes for the investigation were selected from the human and mouse data bases if i. They were only present in ESCs and either in the oocyte or blastocyst and ii. Their gene ontology applications indicated a critical role in transcription regulation, pluripotency and differentiation. This gene set included *Lifr1, Bmpr1, Alk4, Id3, Ctnnb, Akt, Oct4, Rex1, Smad1, 5, Fzd, c-Myc, Cdc25a, Sox2, Fgfr4, Nanog, Erk1, Gata4,* and *Cdx2*. Since sequence data of some genes was not available in the goat, we designed specific primers based on ortholog conserved regions in other studies. The registered cDNA for *Erk1, Alk4, Bmpr1, Fgfr4* and *Lifr1* were deposited into NCBI database under accession numbers KC687077, KF039752, KF039753, KF039754, and KF356183, respectively). Sequences and characteristics of successful polymerase chain reaction (PCR) primers can be found in Table 1.

In vitro production of goat embryos

The procedure used for *in vitro* production of goat embryos was as described previously (16). In brief, cumulus-oocyte complexes (COCs) were obtained from abattoir-derived goat ovaries. COCs were cultured in maturation medium comprised of tissue culture medium-199 (TCM199) supplemented with 10% fetal calf serum (FCS), 2.5 mM sodium pyruvate, 100 IU/mL penicillin, 100 µg/mL streptomycin, 10 µg/mL follicle-stimulating hormone (FSH), 10 µg/mL luteinizing hormone (LH), 1 µg/mL estradiol-17β, and 0.1 mM cysteamine under mineral oil for 20-22 hours at 39ºC, 6% CO_2, and maximum humidity. Matured oocytes were used for *in vitro* fertilization (IVF) and presumptive zygotes were cultured in groups

of six in 20 µl droplets of modified formulation of synthetic oviductal fluid (mSOF) at 39ºC, 6% CO_2, 5% O_2, and maximum humidity (16, 17).

For real time-quantitative PCR (RT-qPCR) experiments, oocytes and embryos at five different stages of goat *in vitro* embryo development (MII-oocyte, 8-16 cell, morula, expanded blastocyst, and day 14 blastocyst) were used. MII oocytes were collected at 20-22 hours post maturation. The 8-16-cell embryos, expanded blastocysts and day 14 blastocysts were collected during different days post embryo culture (days 3, 7 and 14.post embryo culture). Therefore, variation effect was removed from samples. After through washing in phosphate buffer saline (PBS), oocytes and embryos in pools of 60 (oocyte), 35-40 (D3), and 20 (D7) were collected in 500 µL microtubes containing 75 µl RLT buffer, frozen and stored at -70ºC until RNA extraction.

Derivation of *in vitro* D14 embryos

For extended *in vitro* culture of goat day-7 blastocysts until day-14, we prepared a co-culture system using a feeder layer of caprine fetal fibroblasts (CFF) as described by Behboodi et al. (12). Accordingly, CFF cell-line was prepared using fetal tissues of three 40-day goat fetuses. Single-cell suspension was prepared by mincing fetal tissues and culturing them in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FCS, 0.25% amphotericin-B, 1% penicillin-streptomycin, 1% gentamycin in 25 cm^2 culture flasks and cultured at 37ºC, 6% CO_2 Confluent monolayer was appeared from day 4 of culture onwards. The monolayer was trypsinized and passaged for proliferation of CFF cell-line. Each round of cell proliferation took around 3-4 days. The CFF cell-line at passages 2-to-4 was treated with mitomycin (10 mg/mL) for 2 hours. Treated cells were seeded at 1×10^5 cells/mL in drops of 100 µl DMEM which was placed in close proximity to a feeder-free 100µl droplet of DMEM supplemented with 10% FCS, 1% L-glutamine, 1% non-essential amino acid, and 0.1% β-mercaptoethanol under mineral oil. Five to six D7-blastocysts were transferred to each 100 µl droplet of feeder-free DMEM. Then, the DMEM drops containing blastocysts were gently connected to their adjacent DMEM containing CFF monolayer using a mouse pipette. This culture system provided beneficial effects of feeder layer for extended *in vitro* embryo culture while preventing attachment the day 14 blastocysts to the feeder layer (Fig.1). The culture medium was refreshed every other day until D14 of embryo development. Then, groups of 7-10 well-developed D14 embryos were pooled for RNA extraction as described above. A range of 50-65% of the developed blastocysts could progress to day 14.

RNA extraction and real time-quantitative polymerase chaine reaction

The procedure for RT-qPCR was as described previously (18). In brief, total RNA of oocytes and embryos was extracted suing RNeasy Micro kit (Qiagen, Mississauga, ON, Canada) followed by treatment with DNase I (Ambion, Streetsville, ON, Canada) according to the manufacturer's protocol. The RNA quality and quantity was determined using WPA Biowave spectrophotometer (Cambridge, United Kingdom). For reverse transcription, 10 µl of total RNA was used in a final volume of 20 µl reaction containing 1 µl of Random Hexamer, 4 µl RT buffer (10 x), 2 µl of dNTP, 1 µl of RNase inhibitor (20 IU), and 1µl of reverse transcriptase (Fermentas, Glen Burnie, Ontario, Canada). Reverse transcription was carried out at 25°C for 10 minutes, 42°C for 1 hour and 70°C for 10 minutes.

The selection of appropriate reference gene is of crucial importance in the accuracy and fidelity of the data of RT-qPCR results. Accordingly, we searched the literature to find the best suitable candidate reference gene for the goat. We observed that in almost infield studies, ACTB has been used as the choice reference gene in several similar studies in the goat (19), bovine (3, 9, 20). Moreover, ACTB was selected as a suitable internal control for study of gene expression in cryopreserved egg and embryo because it efficiently withstands cryoshocks and oocyte manipulation (9, 17, 21). After ascertaining that the expression of ACTB was stable among different development stages of embryos (data not shown), relative quantification of the target genes was undertaken with ACTB as the reference gene. For RT-qPCR, total RNAs of oocytes and embryos were extracted and used for cDNA

synthesis. RT-qPCR was carried out using 1 µl of cDNA (50 ng), 5 µl of the SYBR Green/0.2 µl ROX qPCR Master Mix (2X) (Fermentas, Germany) and 1 µl of forward and reverse primers (5 pM) adjusted to a total volume of 10 µl using water nuclease-free. Three technical replicates of RT-qPCR were conducted for each primer. CT samples of each target gene were normalized to the CT of ACTB and represented as $2^{-\Delta\Delta CT}$ (22). The primer sequences, annealing temperatures and the size of amplified products are shown in Table 1.

Table 1: Specific real-time primers were designed for gene sequences

Gene	Primer sequences (5´-3´)	Length of PCR product	TM
Lifr1	F: ATTTTTCGGTGTATGGGTGC R: CAGATGTATCCTCAACGGTA	117	56
Bmpr1	F: CCTGTTCGTCGTGTCTCAT R: CTGGTGCTAAGGTTACTCC	116	58
Alk4	F: TCTCCAAGGACAAGACGCTC R: ACGCCACACTTCTCCAAACC	152	62
Smad1	F: TCACCATTCCTCGCTCCCT R: AAACTCGCAGCATTCCAACG	140	60
Smad5	F: ACAGCACAGCCTTCTGGTTC R: GGGGTAGGGACTATTTGGAG	136	60
Id3	F: CGGCTGAGGGAACTGGTA R: CCTTTGGTCGTTGGAGATG	198	58
Ctnnb	F: AGTGGGTGGCATAGAGG R: CACAGGTAGCCCGTAG	160	54
Akt	F: TTCAGCAGCATCGTGTGGCA R: TCATCAAAATACCTGGTGTCCG	98	60
Oct4	F: GCCAGAAGGGCAAACGAT R: GAGGAAAGGATACGGGTC	96	56
Rex1	F: GCAGCGAGCCCTACACAC R: ACAACAGCGTCATCGTCCG	94	61
Fzd	F: CATCGGCACTTCCTTTATCC R: GCTTGTCCGTGTTCTCCC	89	59
c-Myc	F: CAACACCCGAGCGACACC R: GCCCGTATTTCCACTATCCG	160	61
Sox2	F: ATGGGCTCGGTGGTGA R: CTCTGGTAGTGCTGGGA	182	54
Fgfr4	F: GCTGACTGGTAGGAAAGG R: AGTGGCTGAAGCACATCG	193	56
Nanog	F: GATTCTTCCACAAGCCCT R: TCATTGAGCACACACAGC	137	54
Erk1	F: TCAAGCCGTCCAACATCCT R: CGACCGCCATCTCAACC	204	58
Gata4	F: TCCCCTTCGGGCTCAGTGC R: GTTGCCAGGTAGCGAGTTTGC	128	64
Cdx2	F: CCCCAAGTGAAAACCAG R: TGAGAGCCCCAGTGTG	144	53
Cdc25a	F: TGGCAAGCGTGTTATCGTG R: GGTAGTGGAGTTTGGGGTA	119	58
Actb	F: CCATCGGCAATGAGCGGT R: CGTGTTGGCGTAGAGGTC	146	60

PCR; Polymerase chain reaction and TM; Melting temperature.

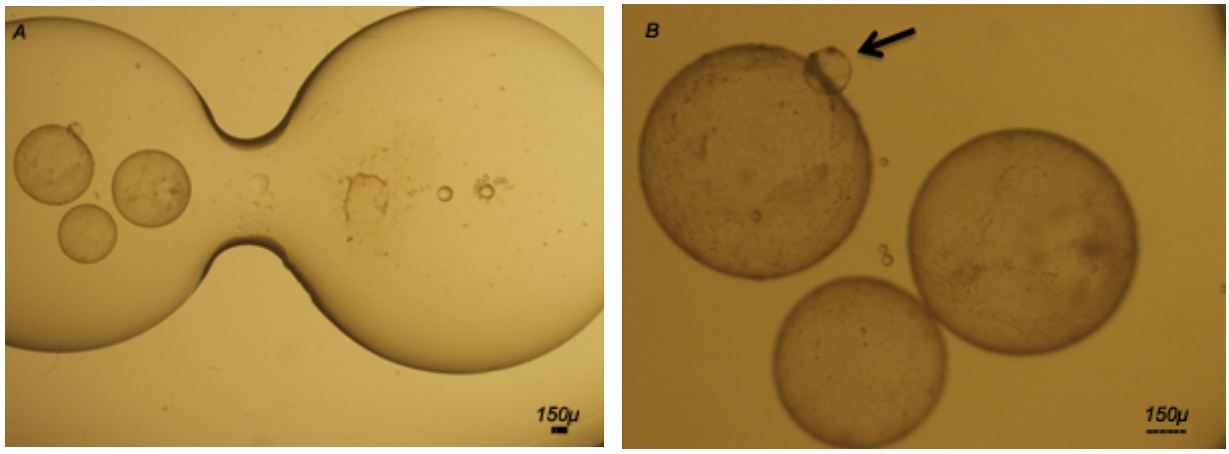

Fig.1. Extended in vitro culture system for goat embryos. **A.** The modified system for culture of expanded goat embryos in medium conditioned by feeder cells and **B.** Comparison between D7 expanded blastocyst (arrow) with D14 blastocysts developed in the modified culture system.

Statistical analysis

Statistical analysis were carried out using SPSS software. For the analysis of developmental data and real-time PCR data a two-tailed Students t test with equal variance was used to determine significance data. Statistical significance was accepted at $P<0.05$.

Results

All the 19 genes examined were expressed throughout embryo development, from MII-oocyte to D14 developing blastocysts (23, 24). Even though, the levels of expression of all genes varied during different developmental stages as no gene was found to be stably expressed throughout embryo development. Moreover, different genes had different levels of expression with respect to a certain stage of development. Stage profiling revealed robust and dynamic patterns of stage-specific gene activity that fall into four major clusters depending on the respective mRNA profile (Figs.2-6).

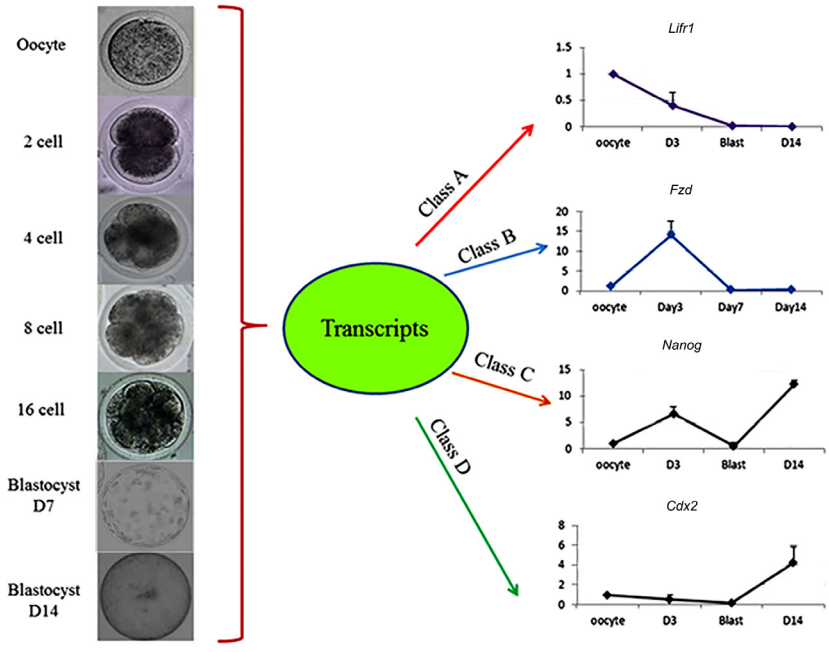

Fig.2. Schematic design for classification of profiles of gene expressions.

The first gene cluster (Figs.2, 3) exhibited highest levels of mRNA in MII-oocyte which gradually and consistently decreased during subsequent stages of embryo development. This cluster encompassed 11 genes including *Lifr1, Bmpr1, Alk4, Smad1, Smad5, Id3, Ctnnb, Akt, Oct4, Erk1* and *Rex1*. The speed and extent of the stepwise decreases in the transcripts varied between the genes.

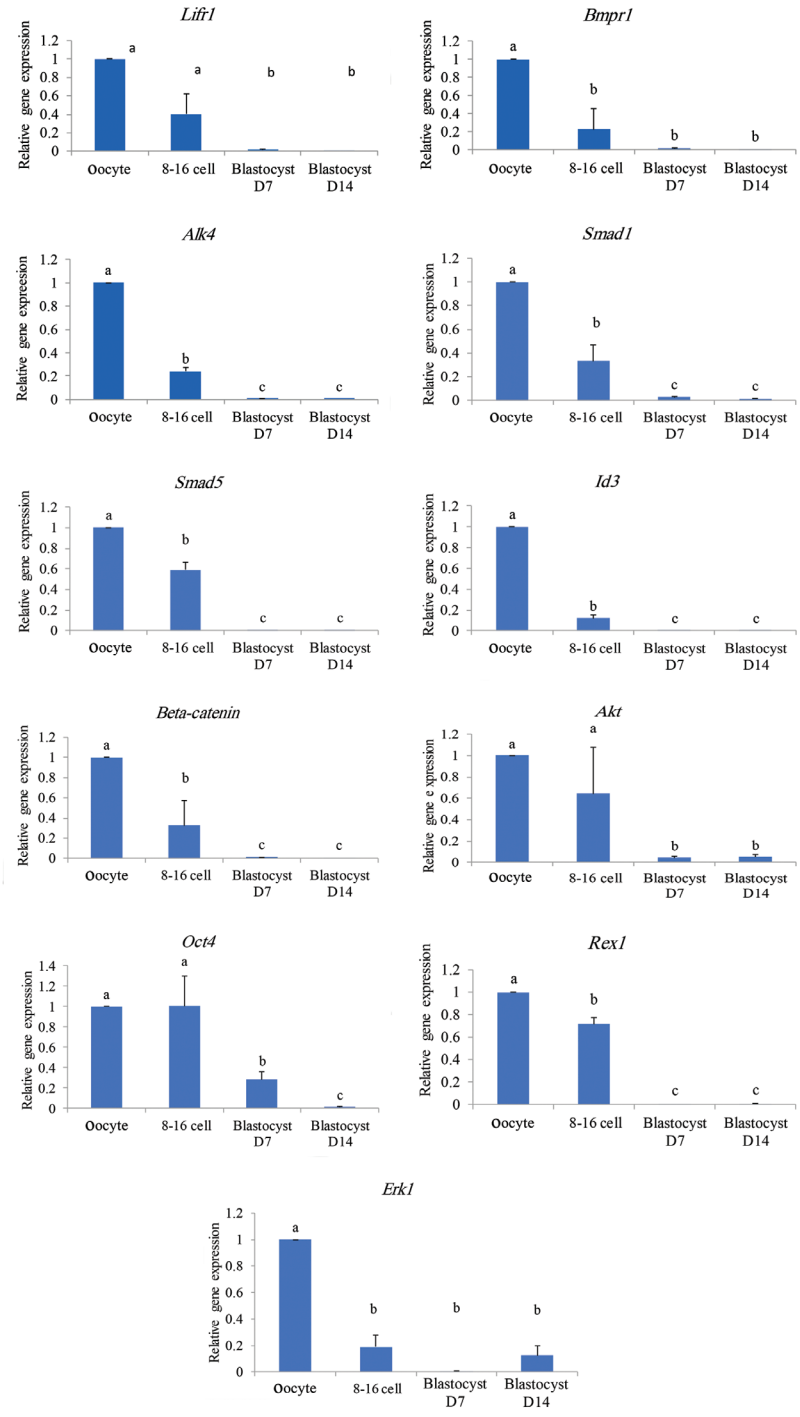

Fig.3. Profiles of expression of genes categorized in the first class based on Figure 2. a, b, and c; Significant difference at P<0.05%.

The second gene cluster (Figs. 2, 4) showed low levels of the transcripts in MII-oocytes, reached their highest relative mRNA levels in D3 embryos and significantly decreased thereafter with no sign of regain in transcription in D7 and D14 blastocysts. This gene set encompassed 4 genes including *Fzd, Sox2, c-Myc,* and *Cdc25a* which showed 14-, 22-, 60-, and 3- fold increase in their D3 transcripts compared to MII-oocytes.

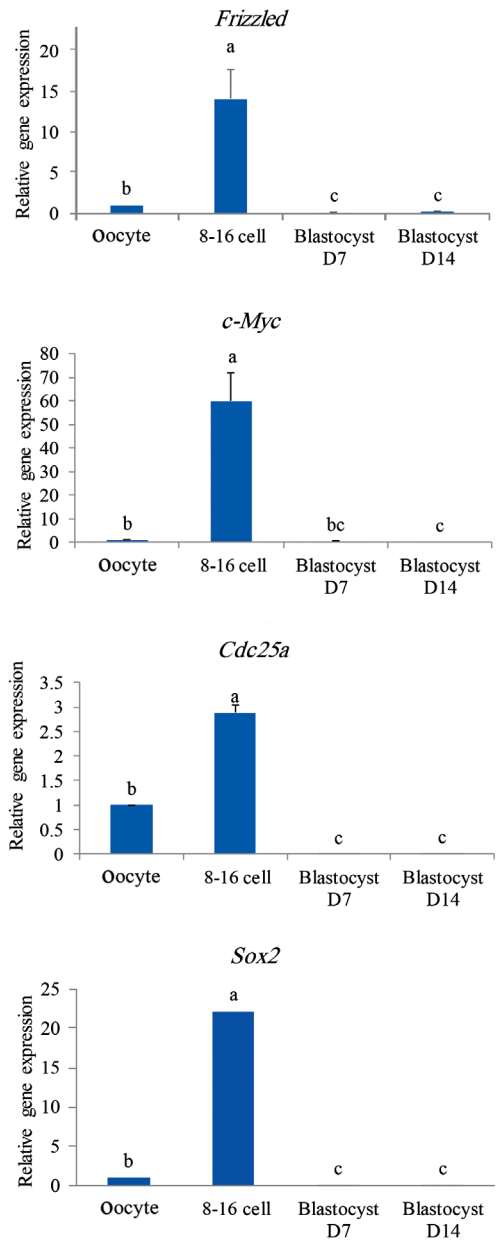

Fig.4. Profiles of expression of genes categorized in the second class based on Figure 2.
a, b, and c; Significant difference at P<0.05%.

In the third gene cluster (Figs.2, 5), the original levels of transcripts of MII-oocyte gradually decreased in developing embryos with a significant reduction in D7 blastocysts. However, these genes initiated transcription from D7 onwards which resulted in a peak of expression in D14 blastocysts. This gene set composed of 2 genes, *Gata4* and *Cdx2*. Despite similar pattern of expression, the magnitude of D14 gain in transcription was different between *Gata4* and *Cdx2* (4- and 2-fold, respectively) compared to the relative mRNA levels initially found in MII-oocytes.

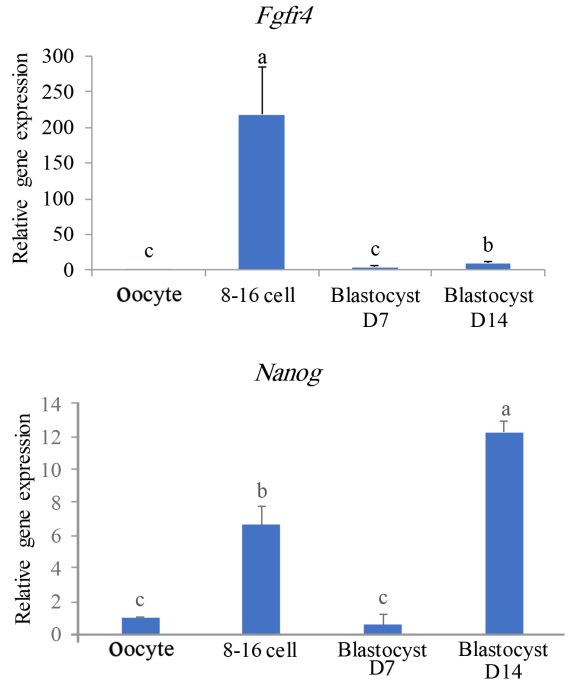

Fig.5. Profiles of expression of genes categorized in the third class based on Figure 2.
a, b, and c; Significant difference at P<0.05%.

The fourth gene cluster (Figs.2, 6), showed low levels of the transcripts in MII-oocytes, reached their first the peak of expression in D3 embryos and followed by a significant decrease in transcription in D7 blastocysts. However, this group of genes reinitiated transcription from D7 onwards which resulted in the second peak of expression in D14 blastocysts. This gene set encompassed 2 genes including *Fgfr4* and *Nanog*.

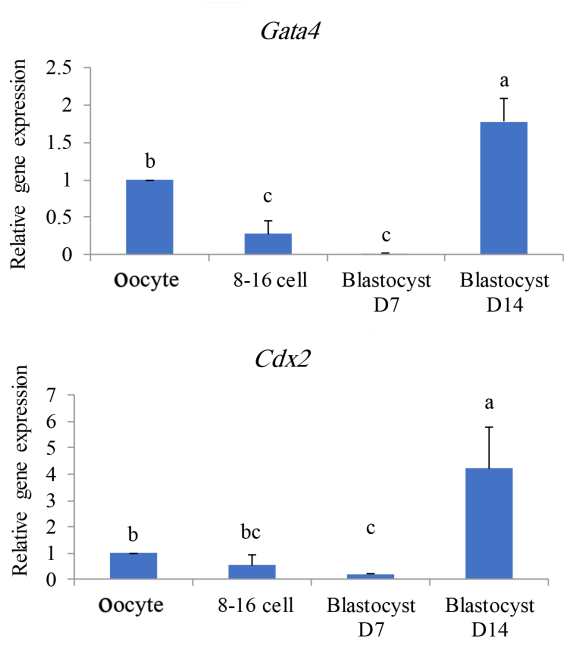

Fig.6. Profiles of expression of genes categorized in the fourth class based on Figure 2.
a, b, and c; Significant difference at P<0.05%.

Discussion

This study demonstrated that all developmental genes assessed are present throughout the pre and peri implantation stages of goat *in vitro* embryo development. Even though, none of these genes could exhibit significantly stable and ubiquitous expression patterns throughout these five developmental stages. Instead, all genes showed fluctuations in expression levels, and if we exclude *Oct4* and *Rex1*, the source of the greatest variations in relative mRNA expression was between MII-oocyte and D3 stages of embryo development. To better explain the quantitative results, schematic patterns of gene expression were categorized in the context of four groups based on the actual patterns of gene expression observed.

The first set of genes, which importantly comprised two-third of the examined genes, revealed a consistent trend of gradual mRNA reduction as the highest and lowest levels of transcripts were observed in MII-oocyte and D14 blastocysts, respectively. This may suggest that the transcripts of these set of genes have been transcribed and accumulated during oocyte growth phase. Because MII-oocyte and early embryo are considered transcriptionally silent (25) and since there is no evidence of active transcription during meiosis resumption, these maternal transcripts should be produced during earlier stages of oocyte growth preceding germinal vesicle breakdown stage.

Theoretically, the steady state of mRNA reduction without renewal (gene cluster 1) may suggest the potential importance of these mRNA for production of proteins that are required during early stages of embryo development, especially to support maternal embryonic transition (MET), when the goat embryos are self-reliant in their transcription. Mechanistically, the distinctive processes have been associated with the declines of maternal mRNA in the eukaryotes including deadenylation, degradation, and protein translation or synthesis (26). Moreover, it has been suggested that the oocyte unlikely would keep useless products (27). Therefore, the quick reduction in relative mRNA abundance could be associated with the protein production.

The second group of genes revealed a significant elevation in their transcripts at D3 compared to MII-oocyte. But, this elevation in the transcripts did not continue and gradually declined and reached the lowest levels in D14 blastocysts . The potential importance of D3 burst-in-the-transcription is indicative of their crucial importance at the MET stage. Accordingly, the lower abundances of these transcripts during post-MET period may not play an important role in the regulation of blastocyst formation and further stages.

The third group of genes revealed a gradual decrease in the maternal mRNA abundances similar to the first group, but their transcripts increased from D7 and reached their highest levels at D14. This would imply that this set of genes is of critical importance during pre- and post- MET phases and underscores the facts that: i. The exact time windows that the second and third sets of genes are in demand for the embryo development are different, and ii. The maternal stockpiles of the third, but not second, set of genes are quite enough to support MET without any need for the additional source of embryo-specific transcripts.

The fourth group of genes was those showing two peaks in embryo-specific mRNA transcription at two distinctive time points of MET and day 14 blastocyts. This may suggest that these transcripts

are crucially needed during both stages. This group can be considered as the combined model of the second and third groups, and correspondingly, the genes in this final group may be of theoretical alternative capacity to cover the duties of genes in both second and third groups.

One of the shortcomings of this study was usage of *in vitro* derived embryos while *in vivo* -derived embryos provide the best source of samples for gene expression studies. However, this was not possible due to technical limitations. A wide range of infield studies have used *in vitro* -derived embryos for similar analyses in the goat (9), equine (28), and bovine (3, 20). In ungulates, embryos are migrating within the uterus for about 7 days before implantation (12). This delay in implantation has provided a unique opportunity to extended *in vitro* culture of ungulates (28-30). At this stage, we expected to see day 14 blastocysts but a search in filed studies revealed that at this time window, embryos look similar to the blastocysts observed on day 14 in this study. For better clarifying this issue and also confirm the quality of these embryos to continue to development, we had previously transferred a number of *in vitro* derived day-7 blastocysts into uterine horn of synchronized goat recipients as routine. The transferred embryos were then flushed from the uteruses at day 20-21. Surprisingly enough, we observed that the embryos at day 21 start to form ovoid or tubular like structures with sizes ranged 0.5 to 5 mm in diameter. One may suggest that the elongation process of goat embryos possibley begins on day 14 onwards.

Conclusion

The results obtained through this work highlight the fact that transcription factors involved in the regulation of early events of pluripotency and differentiation are present through pre- and peri-implantation in the goat embryos. Since the blastocysts of ungulates, unlike human and mice, implant with a delay of around 7 days, the obtained results in D7 and 14 blastocysts may provide useful information to figure out the expression profiles of developmentally important genes between these two stages. The profiles obtained may be useful for derivation of ESC in this valuable farm species.

Acknowledgements

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A. You can send your article via online submission system which is available at our website: **http://www.ijfs.ir.** If the article is not prepared according to the format of ***Int J Fertil Steril***, it will be returned to authors.

B. The order of article appearance in the Journal is not demonstrating the scientific characters of the authors.

C. Int J Fertil Steril has authority to accept or reject the articles.

D. The received articles will be evaluated by one epidemiologist. Then associate editor will determine its reviewers. If three reviewers pass their judgments on the article, it will be presented to the editorial board of ***Int J Fertil Steril.*** If editorial board has a positive judgment about the article, reviewers' comments will be presented to corresponding author (the identification of the reviewers will not be revealed). The executive member of journal will contact the corresponding author directly within 7-8 weeks by email. If authors do not receive any reply from journal office after the specified time, they can contact journal office. Executive manager will respond promptly to authors' message.

The Final Checklist

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

1. Title page should contain title, name of the author/coauthors, their academic qualifications, designation & institutions they are affiliated with, mailing address for future correspondence, email address, phone, and fax number.

2. Text of article and References prepared as stated in the "guide for authors" section.

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

4. Covering Letter

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