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Contents

Original Articles

Original Article

The Effect of Coasting on Intracytoplasmic Sperm Injection Outcome in Antagonist and Agonist Cycle

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Abstract

Background: Coasting can reduce the ovarian hyperstimulation syndrome (OHSS) risk in ovulation induction cycles before intracytoplasmic sperm injection (ICSI). This study aimed to investigate the effect of gonadotropin-releasing hormone (GnRH) agonist and GnRH antagonist protocols to controlled ovarian hyperstimulation (COH) cycles with coasting on the parameters of ICSI cycles and the outcome.

Materials and Methods: In a retrospective cohort study, 117 ICSI cycles were performed and coasting was applied due to hyperresponse, between 2006 and 2011. The ICSI outcomes after coasting were then compared between the GnRH agonist group (n=91) and the GnRH antagonist group (n=26).

Results: The duration of induction and the total consumption of gonadotropins were found to be similar. Estradiol (E_2) levels on human chorionic gonadotropin (hCG) day were found higher in the agonist group. Coasting days were similar when the two groups were compared. The number of mature oocytes and the fertilization rates were similar in both groups; however, the number of grade 1 (G1) embryos and the number of transferred embryos were higher in the agonist group. Implantation rates were significantly higher in the antagonist group compared to the agonist group. Pregnancy rates/embryo transfer rates were higher in the antagonist group; however, this difference was not statistically significant (32.8% for agonist group vs. 39.1% for antagonist group, P>0.05).

Conclusion: The present study showed that applying GnRH-agonist and GnRH-antagonist protocols to coasted cycles did not result in any differences in cycle parameters and clinical pregnancy rates.

Keywords: Ovarian Hyperstimulation Syndrome, GnRH Agonist, GnRH Antagonist

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Introduction

Ovarian hyperstimulation syndrome (OHSS) is the most important and potentially life-threatening iatrogenic complication of ovulation induction (1). Coasting is the stopping of gonadotropin administration when OHSS risk develops during controlled ovarian hyperstimulation (COH) and the withholding of human chorionic gonadotropin (hCG) administration until Estradiol (E_2) levels reach a plateau or drop to a safe range with a significant reduction (2). Follicular growth is generally correlated with follicle-stimulating hormone (FSH) threshold. Large follicles are more resistant to apoptosis and atresia; thereby, larger follicles continue developing while

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immature follicles undergo a selective regression when the FSH level drops. Coasting works with this principle to reduce the functional granulosa cell mass available for luteinization and prevent an increase in vasoactive substances involved in OHSS pathogenesis (3). Coasting does not completely eliminate the OHSS risk in high-risk patients, but may reduce incidence and OHSS severity (4, 5).

OHSS risk is lower in gonadotropin-releasing hormone (GnRH) antagonist cycles, so this protocol should be preferred in high-risk patients. Shifting from agonist protocols to antagonist protocols is a good alternative for preventing OHSS since it ensures the proper maintenance of granulosa functions (6). Coasting in agonist cycles has been in use since the 1980s (7), while there is a broad range of publications about its outcomes (8). Coasting in antagonist cycles was started in 2001 as case reports (9), and coasting was shown to have no adverse effects on *in vitro* fertilization (IVF) outcome nor in antagonist cycles in subsequent studies (10).

In a study by Farhi et al. (11), they compared coasting practices in agonist and antagonist cycles and did not find any difference in cycle parameters, number of retrieved oocytes or pregnancy rates. They have also reported that the same coasting criteria could be applied to agonist and antagonist protocols. In a study by Tarlatzis et al. (12), they have reported a lower pregnancy rate in agonist cycles with coasting as compared to agonist cycles.

The present study aimed to investigate the effect of applying agonist or antagonist protocols to COH cycles with coasting on the parameters of intracytoplasmic sperm injection (ICSI) cycles and the outcome.

Materials and Methods

The present retrospective cohort study was conducted by the IVF Unit of Turgut Ozal University, Ankara, Turkey. During the study period, 1140 ICSI cycles were performed in our IVF center. Coasting was applied for 117 (11%) cycles, meaning in 92 (78.6%) cycles after GnRH-agonist protocol and in 26 (22.2%) cycles after GnRH-antagonist protocol. The study investigated retrospectively 117 cycles (11% of total) in this unit between 2006 and 2011, in which ICSI was performed and coasting was applied due to hyperresponse. Cycles were divided into two following groups according to the preferred stimulation protocol: i. GnRH agonist group (n=91) and ii. GnRH antagonist group (n=26). GnRH agonist protocol was initiated with leuprolide acetate 1 mg daily (Lucrin, Abbott, Turkey) in midluteal phase. Down-regulation was confirmed after 13-15 days (no ovarian cysts>18 mm, E_2<50 pg/mL) that was followed by gonadotropin stimulation. After down regulation, the dose of leuprolide acetate was reduced to 0.5 mg daily until hCG day. The antagonist protocol consisted of daily gonadotropin stimulation from day 3 or 4 of menstruation followed by daily injections of Cetrotide 0.25 mg (Serono, Switzerland) or Orgalutran 0.25 mg (N.V. Organon, The Netherlands) once the leading follicle reached 14 mm and until the day of hCG injection.

Gonadotropin stimulations was performed by recombinant FSH (rFSH), as follitropin-alfa (Gonal F, Merck- Serono, Switzerland) or follitropin-beta (Puregon, N.V. Organon, Oss, The Netherlands), or in combination with urinary gonadotropins (Menogon, Ferring, Germany). The choice of agonist or antagonist protocol for COH was made according to the patient characteristics [age, antral follicle count (AFC), and body mass index (BMI)] or previous IVF cycles responses if available. The following infertility factors were found in patients undergoing coasting: 45% male factors, 24% polycystic ovary syndrome (PCOS), 16% unexplained infertility, and 15% others.

The study included women aged <38 years who underwent ovarian stimulation for ICSI with the GnRH agonist down-regulation protocol or GnRH-antagonist protocol and were subsequently coasted for risk of severe OHSS. Azoospermia and premature ovarian insufficiency were the exclusion criteria. Being retrospective in nature with anonymous data collection, this study did not require Ethical Committee approval. A written consent form was signed by all participants.

Throughout the treatment, the ovarian response was evaluated by measuring follicles via transvaginal ultrasound and measuring serum levels of E_2 once every 1 to 3 days from day 4 of stimulation. The treatment was maintained by adjusting the gonadotropin dose according to these outcomes.

Coasting was applied to COH cycles when the serum E_2 concentration was 4,000 pg/mL or when at least 20 follicles, each measuring 10 mm in diameter and 20% measuring 15 mm in diameter, were present (7). The minimum coasting days was 1.7 ± 1.1, and the maximum coasting days was 4.1 ± 1.0.

Cycles were canceled if coasting period was more than 4 days. During the coasting period, GnRH antagonist in antagonist protocol and leuprolide acetate in agonist protocol were administered in the same dosage until the E_2 concentrations dropped. When the E_2 concentration dropped below 4,000 pg/mL or when at least two follicles reached 18 mm in diameter, ovulation was triggered by administration of 5,000/10,000 IU hCG (Pregnyl, Organon, The Netherlands). Since E_2 levels were not decreased during the coasted period, 7.6 % of cycles (9 cycles) were abandoned in the study.

Oocyte retrieval was performed 35-36 hours after hCG trigger. Oocytes were fertilized by ICSI. After oocyte retrieval, all patients were prophylactically administered 50 mL human albumin intravenous (Human Albumin, Octapharma, Germany). Three days after oocyte retrieval, embryos were transferred transcervically under ultrasound control. Luteal phases were supported by micronized progesterone 200 mg three times a day (Progestan, Koçak, Turkey). Clinical pregnancy was defined by a demonstrable gestational sac accompanied by fetal heart activity on ultrasound.

Statistical analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPSS, SPSS Inc., USA) for Windows 11.5 software package. The compatibility of discrete and continuous numeric variables with normal distribution was analyzed using the Shapiro-Wilk test. Descriptive statistics were expressed in mean ± SD or median (minimum-maximum) for discrete and continuous variables, while categorical variables were expressed in number of cases (N) and percentage (%). The significance of the difference between the groups was analyzed using Student's t test, and the significance of the difference for mean values was analyzed using the Mann-Whitney U-test. Categorical variables were evaluated using Pearson's Chi-squared or Likelihood ratio Test. The value of $P<0.05$ was considered statistically significant.

Results

Agonist protocol was applied to 91 patients and antagonist protocol was applied to 26 patients in the present study. There were no significant differences noted for BMI, duration of infertility, basal FSH and E_2 levels in groups. In the antagonist group, patients' ages were significantly higher compared to the agonist group. Ovarian volume was significantly higher in the agonist group compared to the antagonist group (Table 1). There is a significant difference in the age of women in both groups, as this could affect selection in treatment method, which is applied in our daily practice. The significant difference in ovarian volume is declared by the age difference and the high rate of PCOS patients in the agonist group.

Table 1: Demographic characteristics and basal hormonal parameters of both groups

Characteristic	Agonist group	Antagonist group	P value
Age (Y)	29.3 ± 4.6	31.6 ± 4.3	0.024*
BMI (kg/m^2)	24.2 ± 4.5	23.1 ± 3.7	0.277
Duration of infertility (Y)	5.3 ± 3.7	4.5 ± 3.9	0.311
FSH (mIU/mL)	6.8 ± 1.2	8.1 ± 1.1	0.540
Basal E_2 level (pg/mL)	38 ± 22.9	48.1 ± 19.7	0.057
Ovarian volume (cm^3)	16.6 ± 10.6	9.8 ± 7.9	0.001*

*; Significant at P<0.005, FSH; Follicle-stimulating hormone, E_2; Estradiol, and BMI; Body mass index.

Cycle characteristics and cycle outcomes of both groups are presented in Table 2. Initiation dose of gonadotropin (220.9 ± 64.1 vs. 193.7 ± 72.3), duration of induction (8.4 ± 3.8 vs. 8.9 ± 2.9), and total consumption of gonadotropin (1793 ± 830 vs. 2004.3 ± 1677.6) were similar between the groups. The mean serum E_2 levels on day of hCG were significantly higher in the agonist group compared to the antagonist group (3950.2 ± 300.3 vs. 3600 ± 250.2, $P<0.05$). Endometrial width on day of hCG was also similar between the groups (10 ± 2.1 and 9.7 ± 2.0).

Coasting days were found to be similar for both groups (3.1 ± 1.0 and 2.8 ± 1.1). There was no significant difference in the number of mature (M2) oocytes and the number of 2-pronucleus (PN) embryos (8.7 ± 3.8 vs. 9.8 ± 3.6 and 6.7 ± 3.2 vs. 7.8 ± 3.1, respectively). However, the number of grade 1 (G1) embryos and the number of transferred embryos were significantly lower in the antagonist group compared to the agonist group (4 ± 2.2 vs. 3.8 ± 2, $P<0.05$ and 2.4 ± 0.8 vs. 1.6 ± 0.7, $P<0.05$, respectively).

There was no significant difference in fertilization rates between two groups (72.5 ± 22.4 vs. 79.6 ± 19.7). However, implantation rates were significantly higher in the antagonist group compared to the agonist group (19.7 vs. 26.3, $P<0.05$). Pregnancy rates per embryo transfer were found to be similar in both groups (32.8 vs. 39.1).

Table 2: Cycle characteristics and outcome for coasted cycles

	Agonist group	Antagonist group	P value
Initial dose of rFSH (IU)	220.9 ± 64.1	193.7 ± 72.3	0.060
Duration of induction (day)	8.4 ± 3.8	8.9 ± 2.9	0.773
E_2 level on hCG day (pg/ml)	3950.2 ± 300.3	3600 ± 250.2	0.044*
Endometrial width (mm)	10 ± 2.1	9.7 ± 2.0	0.662
Total consumption of gonadotrophins	1793 ± 830	2004.3 ± 1677.6	0.571
Coasting days	3.1 ± 1.0	2.8 ± 1.1	0.736
M2 oocytes (n)	8.7 ± 3.8	9.8 ± 3.6	0.180
Transfer day	3 ± 1.1	3.0 ± 1.0	0.530
G1 embryo (n)	4 ± 2.2	3.8 ± 2	0.024*
Number of transferred embryos (n)	2.4 ± 0.8	1.6 ± 0.7	0.001*
Fertilization rate (%)	72.5 ± 22.4	79.6 ± 19.7	0.134
Implantation rate (%)	19.7	26.3	0.008*
Pregnancy rate/embryo transfer (%)	32.8	39.1	0.057

***; Significant at P<0.005, G1; Number of grade 1 embryo, hCG; Human chorionic gonadotropin, E_2; Estradiol, rFSH; Recombinant follicle-stimulating hormone, and M2; Number of mature oocytes.**

Discussion

The present study, which investigated whether applying coasting to agonist and antagonist cycles had any differences, found no significant differences in cycle characteristics and clinical pregnancy rates. The M2 oocytes and the fertilization rates were similar in both groups; however, the number of G1 embryos and the number of transferred embryos were higher in the agonist group. Implantation rates were significantly higher in the antagonist group compared to the agonist group. Pregnancy rates/embryo transfer rates were higher in the antagonist group; however, this difference was not statistically significant.

The higher age in the antagonist protocol group is an indicator of a tendency toward antagonist protocol with increasing age at the clinical decision stage. This opinion was shared by the articles of Lainas et al. (13) and Al-Inany and Aboulghar (14). The lower ovarian volume of the antagonist group compared to the agonist group is a result of the fact that ovarian volume decreases with increasing age (15).

The present study found similar initial rFSH doses and total consumptions of gonadotropin in both groups, which is consistent with the study by Huirne et al. (16). There was no significant difference in induction times for both protocols. However, a number of studies have reported that induction times are generally shorter in antagonist protocol (14, 17). Our hyperresponsive patients may experience shorter induction time in agonist protocol.

The present study found higher E_2 levels on hCG day in the agonist group. The studies by Farhi et al. (11) and Tarlatzis et al. (12) comparing agonist and antagonist coasted cycles reported lower E_2 levels on hCG day in the antagonist groups. Similarly, Elter et al. (18) investigated the pattern of E_2 change in agonist and antagonist coasting protocols and found lower E_2 levels on hCG day in antagonist cycles.

There are several studies about the coasting time in cycles coasted due to OHSS risk; however, the optimal coasting time that would not affect the pregnancy rate has not yet been defined (19, 20). It is recommended that the coasting time to be no more than four days (21, 22). In the present study, coasting times did not exceed 4 days and no difference was found in coasting time between two groups.

The number of post-coasting mature oocytes was found similar in the agonist and antagonist groups. The study by Elter et al. (18) obtained a higher number of mature oocytes in the antagonist coasting group compared to the agonist group. The authors attributed this finding to the shorter coasting time in the antagonist group. Farhi et al. (11)

compared the number of collected oocytes and the coasting days together and showed that a coasting time of more than 3 days reduced the number of mature oocytes in the antagonist group. They demonstrated that the number of oocytes was reduced when the coasting time was 1-2 days in the agonist group, whereas it did not change when the time was 4 days and more.

The present study found that there was no difference in fertilization rates between agonist and antagonist coasting groups. In the studies by Bahceci et al. (10) and Mansour et al. (22), fertilization rates did not differ in antagonist coasting cycles compared to control groups. Likewise, in the studies by Farhi et al. (11) and Tarlatzis et al. (12), they compared agonist and antagonist coasting cycles and found no significant difference in fertilization rates.

Although there were no differences in the number of mature oocytes and the fertilization rates between two groups in the present study, the number of G1 embryos and the number of transferred embryos were higher in the agonist group. A similar study in the literature reported that the quality of embryos was not affected by the protocol applied to the coasting cycles, but an extreme drop in the E_2 level and the prolonged duration of this condition were shown to affect the quality of embryos (10, 11, 23).

The studies on pregnancy rate investigated coasting practices in agonist and antagonist protocols individually, while most of them showed no effect on implantation and pregnancy rates after coasting. Their finding reported that the implantation and pregnancy rates are reduced only in the cycles with a coasting time exceeding 4 days, regardless of the protocol used (10, 22, 24). The study by Grace et al. (25) compared the cycles with and without coasting and reported that coasting reduced the fertilization rates, the quality of embryos and pregnancy rates. The study by Farhi et al. (11) comparing agonist and antagonist coasting did not find any difference in pregnancy rates, and Tarlatzis et al. (12) found a lower pregnancy rate in the antagonist coasting group than the agonist coasting group. In the present study, the implantation rates were significantly higher in the antagonist group, whereas the pregnancy rates/embryo transfer rates were also higher in the antagonist group, indicating there were no statistically significant in this regard. The increased implantation rates in the antagonist group may be attributed to the impaired endometrial receptivity due to the higher levels of E_2 in the agonist group.

The present study is one of the rare studies investigating the effect of a selected protocol on the cycle outcome in coasted cycles. The most important drawbacks of the study were the differences between the participants in the groups, leading to bias in the study. The reason of this difference mostly depended on our preferences, as we used GnRH agonist protocols in ICSI cycles routinely till 2009. After that time, we increased our experience with GnRH-antagonist protocol, and we then preferred one of these protocols, according to patients' characteristics. Another important limitations of the present study were its retrospective nature and the lack of a control group without coasting. The present study may indicate the OHSS rates in the selected protocols, and at which rates coasting was required in agonist and antagonist cycles. The present study was also limited by not evaluating the effect of E_2 drop rate on cycle outcome during the coasting (8) and the correlation between the coasting days and the number of oocytes obtained (26), which are considered as controversial topics in the literature. In addition, we wanted to declare that in all cases of infertility, whether of female, male or "unexplained" nature, regardless of sperm function, ICSI bypasses most dysfunctions, eliminating the majority of barriers to fertilization. As the compelling evidence of ICSI, we prefer ICSI procedure routinely in all cases rather than IVF.

Conclusion

The present study showed that applying GnRH-agonist or GnRH-antagonist protocols to coasted cycles did not result in any differences in cycle parameters and clinical pregnancy rates. It is suggested that future prospective randomize controlled studies about coasted cycles in IVF.

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Original Article

Low-Dose Urinary Human Chorionic Gonadotropin Is Effective for Oocyte Maturation in *In Vitro* Fertilization/ Intracytoplasmic Sperm Injection Cycles Independent of Body Mass Index

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Abstract

Background: Currently, there is no agreement on the optimal urinary derived human chorionic gonadotropin (u-hCG) dose requirement for initiating final oocyte maturation prior to oocyte collection in *in vitro* fertilization (IVF), but doses that range from 2500-15000 IU have been used. We intended to determine whether low dose u-hCG was effective for oocyte maturation in IVF/intracytoplasmic sperm injection (ICSI) cycles independent of body mass index (BMI).

Materials and Methods: We retrospectively evaluated a cohort of 295 women who underwent their first IVF/ICSI cycles between January 2003 and December 2010 at the Division of Reproductive Endocrinology and Infertility, Wayne State University, Detroit, MI, USA. Treatment cycles were divided into 3 groups based on BMI (kg/m^2): <25 (n=136), 25- <30 (n=84), and ≥30 (n=75) women. Patients received 5000, 10000 or 15000 IU u-hCG for final maturation prior to oocyte collection. The primary outcome was clinical pregnancy rates (CPRs) and secondary outcome was live birth rates (LBRs).

Results: Only maternal age negatively impacted (P<0.001) CPR [odds ratio (OR=0.85, confidence interval (CI: 0.79-0.91)] and LBR (OR=0.84, CI: 0.78-0.90).

Conclusion: Administration of lower dose u-hCG was effective for oocyte maturation in IVF and did not affect the CPRs and LBRs irrespective of BMI. Women's BMI need not be taken into consideration in choosing the appropriate dose of u-hCG for final oocyte maturation prior to oocyte collection in IVF. Only maternal age at the time of IVF negatively influenced CPRs and LBRs in this study.

Keywords: Body Mass Index, Urinary Human Chorionic Gonadotropin, *In Vitro* Fertilization, Pregnancy Rate, Live Birth Rate

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Introduction

Extremes in body weight have a significant impact on overall health and fertility (1). However, lower fertility rates in obese women may not be exclusively related to ovulatory dysfunction (2-5) as there is evidence to suggest that excess weight and its associated hyperglycemia increase intrafollicular glucose levels (6) that may adversely affect oocyte (7), embryo (8, 9), and endometrial (10, 11) quality. The follicular fluid composition of patients who undergo assisted reproductive technology (ART) with different body mass indexes (BMIs, kg/m^2) has been analyzed and compared to oocyte collection, embryo development, and pregnancy rates (PRs) in a study by Robker et al. (12). The authors reported a significant effect on oocyte quality by increased BMI with less oocytes collected and less embryos produced from obese patients (BMI≥30). This has led some (13), but not all (14), experts to suggest that BMI should influence ART protocols.

Urinary derived human chorionic gonadotropin (u-hCG) is used instead of luteinizing hormone (LH) to trigger final oocyte maturation prior to oocyte collection in ART because of its long half-life and stronger impact on the follicle. The hCG concentrations in serum that reach the follicles should be at a level capable of initiating meiosis and triggering the release of the cumulus-oocyte complex into the follicular fluid. While some authorities (15, 16), including our group (17), have reported a significant negative correlation between BMI and serum hCG levels post u-hCG administration, others report no difference in these levels in relation to BMI (18). Currently there is no agreement on the optimal u-hCG dose requirements for initiating final oocyte maturation prior to oocyte collection in *in vitro* fertilization (IVF); doses that range from 2500-15000 IU have been used (17, 19, 20) and often depended on the number of follicles that developed, the peak serum estradiol (E_2) level, and the perceived risk of ovarian hyperstimulation syndrome (OHSS).

These studies did not specifically include obese patients. The present manuscript has sought to determine the effectiveness of lower dose u-hCG (5000 IU) compared to higher doses (10000 or 15000 IU) for final oocyte maturation in IVF/ intracytoplasmic sperm injection (ICSI) cycles independent of BMI. Our null hypothesis was that the commonly administered lower dose of u-hCG (5000 IU) compared with 10000 and 15000 IU would not adversely affect clinical pregnancy rates (CPRs) and live birth rates (LBRs) across the different BMI strata [i.e., <25, 25 - <30 (overweight), and ≥30 (obese)]. The results of this study might provide a better understanding of the effect of BMI on lower u-hCG doses when used in lieu of the LH surge.

Materials and Methods

After obtaining approval for this retrospective cohort study from the Institutional Review Board of Wayne State University, we conducted a study of 295 women who had their first IVF/ICSI cycles (out of a total 467 cycles) in our institution between January 2003 and December 2010. Reasons for exclusion of cycles from analysis included: cycles other than the first IVF/ICSI cycles (n=114), missing data such that BMI could not be calculated (n=24), hCG levels that were not drawn 12-14 hours after administration of the u-hCG dose (n=17), and cycles in which booster u-hCG doses were given after the initial trigger u-hCG dose (n=17). Booster doses of u-hCG were administered when the patient's 12-14 hour levels were perceived to be low by the care provider. All 295 women had oral contraceptive pill administration following which they underwent ovarian stimulation with gonadotropins (Gn) - mainly recombinant follicle-stimulating hormone (FSH) and preparations that contained equal amounts of FSH and LH, and gonadotropin releasing hormone (GnRH) agonists [6 (2.0%)] or antagonists [289 (98.0%)].

We administered u-hCG when a minimum of two follicles with a mean diameter >18 mm were identified on transvaginal ultrasound and there were at least four follicles between 16-20 mm. Patients received varying doses of u-hCG (5000 IU, 10000 IU, or 15000 IU) administered intramuscularly depending on the number of follicles that developed and their peak E_2 levels. The lower dose (5000 IU) was often administered when there was a higher perceived risk of OHSS (E_2≥3000 pg/mL). Poor responders who barely attained our minimum criteria for proceeding to oocyte collection (4 follicles between 16-20 mm and appropriate E_2 levels for the size and number of follicles) received the 15000 IU u-hCG dose. The patient's BMI was not a factor in determining the doses of u-hCG given to trigger ovulation in any of these cases. Blood for serum hCG levels were drawn 12-14 hours after u-hCG ad-

ministration, mainly to ascertain that patients appropriately self-administered the drug. This time point was based on a study by Chan et al. (21) where they reported that serum hCG levels peaked at 12 hours after the injection and decreased thereafter. Oocyte collection was performed 36 hours after the hCG trigger. Embryo transfer was performed on days 2 to 5 after oocyte collection. We defined the implantation rate as the number of gestation sacs per embryo transferred, while CPR was defined as number of cycles with intrauterine gestational sac(s) with fetal heart pulsation at 6-8 weeks from the day of transfer. Luteal support was with intramuscular 100 mg progesterone in oil that began on the evening of oocyte collection. When pregnant, patients continued progesterone supplementation until 12 weeks of pregnancy (10 weeks after retrieval). Treatment cycles were divided into 3 groups based on BMI (kg/m^2): <25 (n=136), 25- <30 (overweight, n=84) and ≥30 (obese, n=75).

Data were analyzed using SPSS version 22.0 and presented as mean ± SE. Most of our independent variables did not have a normal distribution. Hence, in order to satisfy the normality assumptions of ANOVA, we used log algorithm to transform infertility duration, the total dose of Gn used for superovulation, and hCG levels at 12-14 hours. The baseline FSH (drawn on day 3 of the menstrual cycle), the total number of follicles that developed, follicles ≥14 mm and E_2 on the day of u-hCG trigger, number of mature oocytes and hence subjected to ICSI, number of oocytes that fertilized, and endometrial thickness on the day u-hCG trigger was administered were transformed using square root algorithm. Patients' ages, the number of days patients received Gn stimulation, and the number of embryos transferred had normal distributions and were not transformed.

First, we performed a one-way ANOVA to compare the mean dose of u-hCG administered in relation to BMI categories as well as the mean BMI in the different u-hCG doses administered. Next, we performed two-way ANOVA to determine the main effects and interaction between the independent variables (demographics, superovulation parameters, dose of u-hCG administered, and BMI) and the dependent variables (CPRs and LBRs). Tukey's post hoc tests were performed for mean separation following the detection of a significant main effect. Gravidity, parity, and antral follicle count (AFC) were analyzed using non-parametric Friedman's two-way ANOVA. Log linear analysis was conducted to examine the association among diagnosis, BMI, and u-hCG categories.

We used binary logistic regressions to model CPRs and LBRs with forward stepwise selection according to the likelihood ratio method based on inclusion/exclusion criteria of $P \leq 0.05/P > 0.10$. The BMI, u-hCG doses given for ovulation trigger along with all significant factors in Tables 1, 2 and 3 were included in the model. Mother's age was also included based on significant correlations with both CPRs ($r=-0.28$ and $P<0.001$) and LBRs ($r=-0.028$ and $P<0.0001$). Statistical significance was defined as $P<0.05$.

Results

Initially we conducted log linear analysis to examine the association among etiology of infertility, BMI, and u-hCG categories. No association was detected. Etiologies of infertility were thus presented as frequencies and percentages for the whole population. Infertility causes included: sperm dysfunction in 141 (47.8%), tubal disease in 71 (24.1%), ovulatory dysfunction in 48 (16.3%), and unexplained in 18 (6.1%) patients. The remaining 17 (5.8%) patients had other causes for their infertility.

Next, we conducted two-way ANOVAs that contained demographic variables because no interaction between BMI and HCG was detected using a full model. Patients' demographic variables in relation to BMI and u-hCG dose categories are shown in Table 1. Obese women had a significantly higher infertility duration compared to overweight individuals and those with BMI <25. However, a significantly lower baseline day 3 FSH existed in obese women compared to those with BMI <25 and overweight women. These values were not different between the latter two groups of women. No differences in maternal age, gravidity, parity, and AFC existed in relation to weight distribution. Similar to weight distribution, we observed significantly lower baseline FSH in those who received 5000 and 15000 IU u-hCG for ovulation trigger compared with those who received 10000 IU. No difference in baseline FSH existed between the former two groups. However, there was a significantly higher mean AFC in those who received 5000 IU u-hCG compared with patients who received 10000 and 15000 IU. We observed no difference in the latter two groups in terms of mean AFC (Table 1).

Table 1: Demographic variables in relation to BMI categories and u-hCG dosages (n=295)

Variable	BMI				u-hCG			
	<25	25 - <30	≥30	P value	5000	10000	15000	P value
Age (Y)	33.8 ± 0.4	34.1 ± 0.5	33.9 ± 0.6	NS	33.1 ± 0.8	34.2 ± 0.4	33.5 ± 0.5	NS
	n=136	n=84	n=75		n=39	n=193	n=63	
Infertility duration (months)	38.1 ± 2.5	38.3 ± 3.4	50.0 ± 5.5	0.09	34.6 ± 5.8	42.9 ± 2.5	39.8 ± 5.0	NS
	n=109	n=66	n=64		n=32	n=176	n=31	
Day 3 FSH (mIU/L)	7.2 ± 0.2[a]	7.6 ± 0.5[a]	6.4 ± 0.3[b]	0.031	6.4 ± 0.3[b]	7.4 ± 0.2[a]	6.8 ± 0.6[b]	0.003
	n=128	n=81	n=72		n=36	n=190	n=54	
Gravidity	0.7 ± 0.1	1.2 ± 0.3	1.2 ± 0.2	NS	0.8 ± 0.2	1.0 ± 0.1	1.1 ± 0.3	NS
	n=136	n=84	n=75		n=39	n=193	n=63	
Parity	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	NS	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	NS
	n=136	n=84	n=75		n=39	n=193	n=63	
AFC	13.5 ± 0.8	12.5 ± 0.9	15.4 ± 1.2	NS	18.8 ± 1.9[a]	13.1 ± 0.6[b]	12.0 ± 1.6[b]	0.006
	n=107	n=74	n=65		n=31	n=187	n=28	

Results are means ± SE from two-way ANOVAs presented with Tukey post hoc analysis as appropriate. No interactions were detected between BMI and u-hCG. Different letters denote difference among groups ([a] vs. [b]; P<0.05).
BMI; Body mass index, u-hCG; Urinary derived human chorionic gonadotropin, FSH; Follicle-stimulating hormone, AFC; Antral follicle count, and NS; Nonsignificant.

Next, we performed a two-way ANOVA to evaluate the effect of both BMI and u-hCG dose categories on superovulation variables, CPRs and LBRs (Tables 2, 3). As expected, a significantly decreased trend existed in serum hCG levels 12-14 hours after u-hCG administration as the strata of BMI increased even though the doses of u-hCG administered did not differ in relation to BMI (Tables 2). Analysis of the BMI groups showed that obese patients had significantly more total numbers of developed follicles and numbers of follicles ≥14 mm on the day the u-hCG trigger was administered compared with the other BMI groups (Table 2).

Table 2: Superovulation variables, serum hCG, oocytes and embryo parameters, CPRs and LBRs in relation to BMI categories and u-hCG dosages

Variable	BMI			
	<25 (n=136)	≥25 - <30 (n=84)	≥30 (n=75)	P value
Dose of HCG administered	10547.9 ± 3169.0	10333.3 ± 2784.4	10166.7 ± 2849.2	0.623
Serum hCG (IU) 12-14 hours after hCG trigger	312.7 ± 16.5[a]	230.4 ± 12.0[b]	162.8 ± 9.6[c]	0.001
Gn stimulation (days)	10.2 ± 0.2	10.6 ± 0.2	10.2 ± 0.2	NS
Total Gn dose (IU)	4454.0 ± 286.5	5342.9 ± 404.9.5	4592.9 ± 415.5	NS
Total follicles developed	19.7 ± 0.9[b]	19.4 ± 1.1[b]	22.5 ± 1.1[a]	0.029
Follicles ≥14 mm day of hCG	10.9 ± 0.4[b]	10.6 ± 0.6[b]	12.2 ± 0.6[a]	0.049
E_2 day of hCG (pg/mL)	2508.7 ± 84.1	2396.3 ± 194.2	2363.5 ± 107.8	NS
Endometrium thickness on day of hCG (mm)	10.2 ± 0.2	10.6 ± 0.2	10.7 ± 0.3	NS
Oocytes collected	13.7 ± 0.7	13.9 ± 1.1	15.4 ± 0.9	NS
Mature oocytes	10.9 ± 0.6	11.4 ± 0.8	12.0 ± 0.7	NS
Oocytes fertilized	8.4 ± 0.5	9.2 ± 0.7	9.3 ± 0.6	NS
Embryos transferred	2.6 ± 0.1	2.5 ± 0.1	2.8 ± 0.1	NS
CPR (%)	61/136 (44.9)	35/84 (41.7)	28/75 (37.3)	NS
LBR (%), n=292*	54/136 (39.7)	30/81 (37.0)	24/75 (32.0)	NS

Except otherwise stated results are means ± SE from two-way ANOVAs with Tukey post hoc analysis as appropriate. No interactions were detected between BMI and hCG. Different letters denote differences among groups ([a] vs. [b], [a] vs. [c], and [b] vs. [c]; P<0.05).
BMI; Body mass index, u-hCG; Urinary derived human chorionic gonadotropin, Gn; Gonadotropin, E_2; Estradiol, CPR; Clinical pregnancy rate, LBR; Live birth rate, NS; Nonsignificant, and *; 3 cycles were lost to follow up hence the outcome of pregnancy in these 3 cases was not known.

However, BMI categories did not impact other superovulation parameters: the number of days Gn were administered and the total dose of Gn used for superovulation; E_2 levels and endometrial thickness on the day the u-hCG was administered; and the number of oocytes collected, the number of mature and fertilized oocytes, and the number of embryos transferred.

As expected, there was a significant increased trend in the serum hCG levels at 12-14 hours in relation to the amount of u-hCG given to trigger ovulation even though the BMI did not differ among patients in the three administered doses of u-hCG (Table 3). In addition, on the day we administered the u-hCG trigger, the total number of follicles that developed, follicles ≥14 mm, and serum E_2 levels were significantly higher in those who received 5000 IU compared with the other two u-hCG groups. Those who received 10000 IU had significantly higher E_2 compared with those who received 15000 IU. We observed similar trends with the number of oocytes collected, number of mature oocytes, and number of fertilized oocytes (Table 3).

With two-way ANOVA, although the CPRs and LBRs were lowest in obese patients, the differences were not statistically significant. CPRs and LBRs were also not influenced by the different doses of u-hCG administered to trigger ovulation. We performed forward stepwise logistic regression in order to determine whether any of the significant differences (age, based on significant correlations with both CPRs and LBRs) seen in the BMI groups and the doses of u-hCG administered influenced the CPRs and LBRs. Only maternal age (P<0.001) influenced CPRs (OR=0.85, CI: 0.79-0.91) and LBRs (OR=0.84, CI: 0.78-0.90). These findings suggested that for a one-unit increase in maternal age, we would expect approximately 15% decrease in the odds of a clinical pregnancy and 16% decrease in the odds of a live birth.

There were 3 patients in the entire cohort who developed moderate to severe OHSS; all were in the subgroup of patients that received 10000 IU of u-hCG for trigger. These patients had a peak E2 of 2604, 2662, and 3881 pg/mL. They developed 23-26 follicles and had 13-20 oocytes collected. All were pregnant. One delivered a singleton and the other two delivered triplets.

Table 3: Superovulation variables, serum hCG, oocytes and embryo parameters, CPRs and LBRs in relation to u-hCG dosages

Variable	u-hCG			
	5000 (n=39)	10000 (n=193)	15000 (n=63)	P value
BMI	27.0 ± 5.7	27.8 ± 6.5	26.0 ± 6.1	0.11
Serum hCG (IU) 12-14 hours after hCG trigger	129.2 ± 10.1[c]	229.2 ± 8.5[b]	394.0 ± 27.0[a]	0.001
Gn. stimulation (days)	10.4 ± 0.3	10.2 ± 0.1	10.6 ± 0.2	NS
Total Gn dose (IU)	4019.6 ± 551.0	5150.1 ± 246.0	5218.5 ± 429.3	NS
Total follicles developed	30.7 ± 1.6[a]	18.6 ± 0.7[b]	19.5 ± 1.2[b]	0.001
Follicles ≥14 mm day of hCG	17.1 ± 0.9[a]	10.2 ± 0.3[b]	10.2 ± 0.6[b]	0.001
E_2 day of hCG (pg/mL)	3981 ± 88.0[a]	2293.5 ± 57.1[b]	1930 ± 80.9[c]	0.001
Endometrium thickness on day of hCG (mm)	10.7 ± 0.4	10.4 ± 0.2	10.5 ± 0.3	NS
Oocytes collected	22.6 ± 1.6[a]	13.4 ± 0.6[b]	11.4 ± 0.8[b]	0.001
Mature oocytes	18.8 ± 1.0[a]	10.5 ± 0.5[b]	9.0 ± 0.6[b]	0.001
Oocytes fertilized	14.8 ± 0.9[a]	8.2 ± 0.4[b]	7.2 ± 0.5[b]	0.001
Embryos transferred	2.5 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	NS
CPR (%)	17/39 (43.6)	79/193 (40.9)	28/63 (44.4)	NS
LBR (%), n=292*	16/39 (41.0)	67/190 (35.3)	25/63 (39.7)	NS

Except otherwise stated results are means ± SE from two-way ANOVAs with Tukey post hoc analysis as appropriate. No interactions were detected between BMI and hCG. Different letters denote differences among groups ([a] vs. [b], [a] vs. [c], and [b] vs. [c]; P<0.05).
BMI; Body mass index, u-hCG; Urinary derived human chorionic gonadotropin, Gn; Gonadotropin, E_2; Estradiol; CPR; Clinical pregnancy rate, LBR; Live birth rate; NS; Nonsignificant, and *; 3 cycles were lost to follow up hence the outcome of pregnancy in these 3 cases was not known.

Discussion

The results of this study have shown the effectiveness of low dose u-hCG compared with higher doses for final oocyte maturation in IVF/ICSI cycles. This finding was associated with similar CPRs and LBRs independent of BMI. We believed these results were valid given that we found no interaction between BMI and the u-hCG categories. Our results, however, concurred with other researchers who found an inverse relationship between serum hCG concentration and BMI (15-17, 21-23) which might be a volume distribution phenomenon. While a previous study by our group (17) reported on the impact of lower u-hCG doses in obese patients, we did not report on the CPRs. Others (15, 16, 18) reported on the CPRs in a small number of patients (≤50), but did not report on the LBRs. Carrell et al. (24) observed that patients with a BMI >30 had significantly lower follicular fluid hCG levels and CPRs after the administration of 10000 IU of u-hCG compared to patients with BMIs of 20-30 and <20, which suggested that BMI could influence CPRs at a dose of 10000 IU u-hCG. However, they used arbitrary BMI categories that did not conform to the World Health Organization definition. In the current study, we used robust statistical analyses and determined that neither BMI nor the different doses of u-hCG influenced oocyte maturation, CPRs, and LBRs, which indicated that these doses were adequate for successful treatment outcome.

Drug metabolism and distribution depend on hepatic clearance, rate of excretion, and the volume of adipose tissue (25). Therefore, it is understandable that concerns exist as to the suitability of lower doses of u-hCG in initiating and completing final oocyte maturation in obese patients. The threshold for the lowest levels of serum hCG 12-14 hours after u-hCG administration required for final oocyte maturation (i.e., a serum hCG level when no oocyte would be collected) remains a contentious issue. Initial trials of early hCG formulation (Profasi) have shown that a mean serum level of ≥129 mIU/mL one day after injection sufficiently induced follicular maturation and adequate luteinization (26). This was in keeping with findings in the current study which showed that despite lower levels of serum hCG in those who received 5000 IU u-hCG, forward logistic regression indicated that the number of mature oocytes collected and fertilized, and CPRs and LBRs were not affected. Levy et al. (27) measured serum hCG levels the morning after u-hCG administration and reported that similar numbers of mature oocytes were achieved when cycles were in the lower 5th percentile of serum hCG levels (range: 27-50 mIU/mL) compared to those in the top 5th percentile (range: 300-700 mIU/mL). Stefanis et al. (28) found no correlation between BMI, number of oocytes retrieved, or fertilized and serum concentrations of hCG at 12 and 36 hours following administration of 5000 IU u-hCG. They concluded that neither serum concentrations of hCG nor BMI influenced IVF outcome. They did not categorize BMIs; and used correlation coefficients to assess relationships between hCG levels and BMI, and used fertilization and biochemical pregnancy as their main outcome measures.

Conclusion

We have concluded that BMI need not be taken into consideration in choosing the appropriate dose of u-hCG to effect final oocyte maturation prior to oocyte collection in IVF as low dose u-hCG is equality effective. The common practice of administering lower doses of u-hCG to trigger final oocyte maturation in patients at high risk of OHSS does not compromise CPRs and LBRs irrespective of BMI. Our study could not confirm the need for the use of longer needles to reach the muscles rather than adipose tissue for administration of the lower dose of hCG (5000 IU) in obese women. Only maternal age negatively impacted CPRs and LBRs in the present study, a phenomenon that has been shown to be associated with an age dependent increase in aneuploid embryos (29). The common practice of administering lower doses of u-hCG to trigger final oocyte maturation in patients at high risk of OHSS should not and does not compromise CPRs and LBRs irrespective of BMI.

Finally, this study had several limitations. Our study was not randomized, hence has the inherent limitations of a retrospective study such as incomplete information for some of the patients as can be deduced from the tables. There were 10 patients with BMI 17.3-18.4 included in the <25 BMI group but inclusion of these patients did not influence the results of this study. The overall sample size was small (n=295) and could have influenced

the findings. An adequately powered multicenter randomized study would confirm the current study findings; however, such a study might not be feasible because of the sample size. In the absence of results from randomized studies, management of obese patients that undergo ART will likely need to be based on results of observational studies such as the current study.

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Original Article

Renal and Hepatic Functions after A Week of Controlled Ovarian Hyperstimulation during *In Vitro* Fertilization Cycles

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Abstract

Background: One the main aspects of *in vitro* fertilization (IVF) cycle is to avoid any possible systemic damage on women undergoing a controlled ovarian hyperstimulation (COH). The aim of this work is to evaluate renal and hepatic function blood tests in patients undergoing controlled ovarian hyperstimulation during IVF cycles.

Materials and Methods: We performed a prospective cohort analysis. All patients received a long stimulation protocol with gonadotropin-releasing hormone (GnRH) analogues by daily administration, since the twenty-first day of the previous ovarian cycle followed by COH with recombinant follicle-stimulating hormone (FSH). The daily dose of exogenous gonadotropins for every single patient was modified according to her follicular growth. The oocytes were retrieved during the oocyte pick up and fertilized by standard procedures of intracytoplasmic sperm injection (ICSI). The blood samples to evaluate renal and hepatic functions were taken at the 7th day of ovarian stimulation.

Results: We enrolled 426 women aged between 19 and 44 years, with a mean body mass index (BMI) of 24.68 Kg/m^2. The mean value of blood urea nitrogen was 14 ± 3.16 mg/dl, creatinine: 1 ± 0.45 mg/dl, uric acid: 4 ± 1.95 mg/dl, total proteins: 7 ± 3.93 mg/dl, aspartate aminotransferase: 18 ± 6.29 mU/ml, alanine aminotransferase: 19 ± 10.41 mU/ml, alkaline phosphatase: 81 ± 45.25 mU/ml, total bilirubin 1 ± 0.35 mg/dL. All of the results were considered as a normal range following the Medical Council of Canada.

Conclusion: Our data suggest that, unlike ovarian hyperstimulation syndrome (OHSS), COH patients did not show any alteration to renal and hepatic functions.

Keywords: Infertility, Ovarian Hyperstimulation, Intracytoplasmic Sperm Injection, *In Vitro* Fertilization

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Introduction

The correlation between renal and hepatic damages and ovarian stimulation is not well understood, and so far data about ovarian hyperstimulation syndrome (OHSS) are not enough robust. As widely evidenced, OHSS is a rare iatrogenic complication of ovarian stimulation, that usually happens during an *in vitro* fertilization (IVF) cycle, luteal phase or early pregnancy. OHSS has been known since 1943, when recombinant gonadotrophins recombinant follicle-stimulating hormone (rFSH), and recombinant luteinizing hormone, (rLH) were used for the first time to induce ovulation (1, 2). OHSS generally occurs

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only after exposure to human chorionic gonadotropin (hCG) and its mortality rate is between 1 in 45.000 and 1 in 500.000 (3), and it has a morbidity even higher though not accurately quantified. Based on the clinical presentation, laboratory and ultrasound findings, OHSS is classified into three categories (mild, moderate and severe) and five grades (1 ± 5) of severity (4). The initial symptoms are abdominal bloating and pain; the ovarian size usually is <8 cm. In severe clinical presentations, the patients suffer from ascites, oliguria and haemoconcentration; in these cases, ovarian size is usually >12 cm. The mild form has rather high incidence considering that it affects up to 33% of woman undergoing to IVF cycles, while the moderate or severe OHSS complicates 3-8% of IVF cycles (5). Altered liver function tests have been considered to be a rare expression of the severe form of OHSS, because it may induce microvascular thrombosis and liver tissue ischemia leading to hepatic dysfunction (6).

An accurate evaluation of the patient before an IVF technique includes an hysteroscopy for diagnostic as well as therapeutic purpose (7-9), but also the study of any thrombophilic genetic nucleotide polymorphisms (10); it is mandatory to study male partner, evaluating accurately semen parameters (11).

The main risk of a stimulation protocol for an IVF cycle could be considered OHSS, so to date it is recommended to accurately check renal and hepatic functions through blood tests, in order to suspect this condition as early as possible. Considering also that most of the women try repeated cycles of IVF, due to the common failure of the technique, it is important to know whether the normal stimulation protocol (which does not hesitate into OHSS) could determine renal and/or hepatic damages. To the best of our knowledge, no study has yet investigated this point, since most of the available data were focused on OHSS. In the light of this evidence, we think that it is extremely important to know if even normal stimulation protocols with controlled ovarian hyperstimulation (COH) may lead to renal and/or hepatic altered functions, also taking into account the "basal risk" before the start of another IVF cycle. Thus, the aim of this work is to evaluate renal and hepatic function blood tests in patients undergoing COH during IVF cycles.

Materials and Methods

We performed this single-center prospective cohort study at the Department of General Surgery and Medical Surgical Specialties of the University of Catania (Italy), between July 2012 and August 2015. We enrolled women IVF for primary or secondary infertility, considering the development of OHSS as exclusion criteria. Each patient was informed about the procedures and signed an informed consent allowing data collection for research purposes.

All patients received a long stimulation protocol, which involved the pituitary desensitization with gonadotropin-releasing hormone (GnRH) analogues by daily administration since the twenty-first day of the previous ovarian cycle. When we reached pituitary desensitization, indicated by serum estradiol (E_2) level <40 pg/ml and follicular diameter <10 mm, we started ovarian stimulation with rFSH (GONAL-f, Merck Serono Europe, UK). We modified the daily dose of exogenous gonadotropins for every single patient according to her follicular growth.

We administrated 10000 UI of hCG (Gonasi HP, IBSA Farmaceutici Italia, Italy) when the two largest follicles reached a minimum diameter of 16 mm with a peak of E_2 of about 1600 pg/ml. We performed oocyte pick-up about after 36 hours by transvaginal ultrasound-guided needle aspiration of the follicles.

The oocytes were fertilized by standard procedures of intracytoplasmic sperm injection (ICSI). After 72 hours embryos were transferred into the uterus. All patients underwent luteal phase support with progesterone and low-dose of acetylsalicylic acid. The pregnacy test was performed after 12 days.

We evaluated renal and hepatic function blood tests (creatinine, blood urea nitrogen, total protein, uric acid, transaminases, total bilirubin, alkaline phosphatase) at the day 7th, since ovarian stimulation with rFSH.

The study was designed in accordance with the Helsinki Declaration, confirming the Committee on Publication Ethics (COPE) guidelines and it was approved by the Institutional Review Board (IRB) of the university hospital, where this work was performed. All designs, analyses, interpreta-

tion of data, drafting and revisions followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies, available through the EQUATOR (Enhancing the QUAlity and Transparency Of health Research) network. All the results were considered as normal range following the standard values defined by Medical Council of Canada (12).

Results

We recruited 426 women aged between 19 and 44 years (mean age: 32.88 years) that met inclusion and exclusion criteria. We excluded from the study four patients that have developed OHSS during IVF procedures. Mean body mass index (BMI) of the cohort was 24.68 Kg/m^2. As reported in Table 1, we measured aspartate aminotrasferase (AST) in 393 patients (93.1%), alanine aminotrasferase (ALT) in 393 patients (93.1%), alkaline phosphatase in 402 patients (95.3%), total bilirubin in 400 patients (94.8%), blood urea nitrogen in 392 patients (92.9%), creatinine in 400 patients (94.8%), uric acid in 350 patients (82.9%), total proteins in 390 patients (92.4%).

Table 1: Renal and hepatic blood tests. Standard values refer to Medical Council of Canada, Appendix C - Clinical Laboratory Tests (2010)

Variables	Mean ± SD	Standard values
Blood urea nitrogen (mg/dL)	14.26 ± 3.16	7.0-22.0
Creatinine (mg/dL)	0.78 ± 0.45	0.57-1.02
Uric acid (mg/dL)	3.77 ± 1.95	3.0-7.0
Total protein (mg/dL)	7.41 ± 3.93	6.7-8.7
Aspartate aminotrasferase (mU/mL)	18.37 ± 6.29	18-40
Alanine aminotrasferase (mU/mL)	19.06 ± 10.41	17-63
Phosphatase alkaline (mU/mL)	81.49 ± 45.25	38-126
Total bilirubin (mg/dL)	0.64 ± 0.35	<1.5

Discussion

Pregnancy is a physiological condition that may predispose itself to abnormal renal and hepatic functions (13). During pregnancy, the increased blood flow in the liver should reduce transaminases (14), but there are several pathologic conditions such as chronic intrahepatic cholestasis and HELLP (Hemolysisis, Elevated Liver enzyme levels and Low Platelet) syndrome in which there is an increase in AST and ALT (15, 16). Although several studies already investigated renal and hepatic alterations in women who developed OHSS, to the best of our knowledge this is the first report of liver and kidney function during COH. As previously evidenced by Kopylov et al. (17), IVF pregnancies had more elevated AST values compared to spontaneous ones. Generally, liver blood tests are elevated in about 3-5% of all pregnancies (18). Giugliano et al. (19) reported a case of liver failure after four cycles of COH and subsequent intrauterine insemination (IUI): in this case, the patients developed severe HELLP syndrome during the third trimester, allowing authors to hypothesize a correlation between COH and liver failure. Considering HELLP syndrome, hepatic damage could be due (at least in part) by endothelial dysfunction, since it was already demonstrated that increased angiotensin and pro-inflammatory cytokines may lead to hepatic ischemia (20). Furthermore, as documented by Obrzut et al. (21), liver damage severity could be foretold by high value of estradiol during ovarian stimulation, in direct proportion with risk of developing OHSS. Based on these data, we could hypothesize a link between high estradiol values during ovarian hyperstimulation and histopathological liver changes in woman with severe OHSS, similar to those already determined during oral contraceptive therapy (22-24).

Recent data (25, 26) demonstrated that the trends in liver and renal function tests could be affected by single- or double-dose methotrexate in case of ectopic pregnancy after fresh IVF embryo transfer cycles; for this reason, liver and renal function tests have to be carefully evaluated in these patients.

Another specific condition is represented by cases of IVF treatment in renal transplanted patients (who have already altered renal function) and nephrotic syndrome: these patients have higher risk to develop OHSS (27), so they have to be monitored strictly to avoid it.

Conclusion

The ovarian hyperstimulation, even if controlled, determines physiological modifications and could lead to hemodynamic changes in kidney and liver. Based on that, in our study we expected changes on the parameters of liver and kidney function in women undergoing COH. Our data demonstrated that no patient developed hepatic and/or renal damage, suggesting a good safety profile for COH. Nevertheless, several limitations of this study should be taken into account: first of all, we did not measure all parameters in every patients, although rate of evaluation was above 90% in 7/8 of them; second, we modified the daily dose of exogenous gonadotropins for every single patient according to her follicular growth; finally, our cohort included patients with different ages (between 19 and 44 years) and different types of infertility (primary or secondary). For these reasons, it is required to further study on larger cohorts, with greater statistical power, to accurately clarify the risk of hepatic and/or renal damage during COH.

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Original Article

Identification of Reproductive Education Needs of Infertile Clients Undergoing Assisted Reproduction Treatment Using Assessments of Their Knowledge and Attitude

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Abstract

Background: In order to empower infertile individuals and provide high quality patient-centered infertility care, it is necessary to recognize and meet infertile individuals' educational needs. This study aims to examine infertility patients' knowledge and subsequently their education needs given their attitudinal approach to infertility education in terms of patients who undergo assisted reproduction treatment.

Materials and Methods: This descriptive study enrolled 150 subjects by convenience sampling of all patients who received their first assisted reproductive treatment between July and September 2015 at a referral fertility clinic, Royan Institute, Tehran, Iran. We used a questionnaire that measured fertility and infertility information (8 questions) as well as attitude toward education on the causes and treatment of infertility (5 questions). Chi-square, independent sample t test, and one way ANOVA analyses were conducted to examine differences by sex. $P<0.05$ was considered statistically significant.

Results: Total mean knowledge was 3.08 ± 0.99. Clients' responses indicated that the highest mean knowledge scores related to knowledge of factors that affected pregnancy (3.97 ± 1.11) and infertility treatment (3.97 ± 1.16). The lowest mean knowledge scores related to knowledge of the natural reproductive cycle (2.96 ± 1.12) and anatomy of the genital organs (2.94 ± 1.16). Most females (92.1%) and males (83.3%) were of the opinion that infertility education programs should include causes of infertility and types of treatment associated with diagnostic and laboratory procedures. No statistically significant difference existed between male and female participants ($P=0.245$).

Conclusion: Most participants in this study expressed awareness of factors that affect pregnancy and infertility treatment. It is imperative to educate and empower infertile individuals who seek reproduction treatment in terms of infertility causes and types of treatment, as well as diagnostic and laboratory procedures to enable them to make informed decisions about their assisted reproductive procedures.

Keywords: Education, Training, Knowledge, Attitude, Infertility

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Introduction

One of the key responsibilities of health care providers is recognizing and meeting patients' educational needs. It is not only considered as fundamental to patient or client empowerment, but can also promote standards of care and provide high quality patient-centered care (1, 2). Satisfaction with care can originate from adequate patient education, which enables patients to give greater understanding of and participation in medical decision making which often result in better health outcomes (2-4). Bennett et al. (2) have reported that a demand existed for further knowledge in 87% of 212 infertile Indonesian female patients about the causes and treatment of infertility. This finding underlines the need and importance of patient education within the infertility field, especially in developing countries (5, 6). Although there is an enormous gap in educational needs of infertile patients in infertility care centers within resource poor settings, many attempts have been made to investigate knowledge and awareness, in addition to attitude and experiences regarding infertility among various populations (7-9). Relatively little is known about infertility education (6, 8), particularly in patients who receive reproductive treatment (2). To the best of our knowledge no study has investigated these needs according to the perspective of the infertile patient. This was the first study that examined the knowledge and educational needs of infertility patients undergoing assisted reproduction treatment, given their attitudinal approach to infertility education.

Materials and Methods

This descriptive study recruited 150 subjects by convenience sampling of all infertile clients who received assisted reproductive treatment for the first time between July and September 2015 at a referral fertility clinic (Royan Institute, Iran). This referral clinic assesses people from all socio-economic and ethnic backgrounds.

We measured patients' knowledge of infertility and educational needs with a questionnaire, designed for Iranian context and validated by a group of 18 gynecologists, embryologists, and conducted face validity of the questionnaire. A graphics expert designed the questionnaire's font and graphics. The final version of the questionnaire developed by researchers comprised the following two constructs. Initially we requested participants to complete the following demographic information: age (years), sex (male or female), education levels (under diploma, diploma, and academic), occupational status (employed or unemployed), and duration of marriage (years). Then, the questionnaire included two domains that pertained to fertility and infertility information (8 questions), in addition to attitude toward education about the causes and treatment of infertility (5 questions). Question types included yes/no; a Likert scale (too little, little, moderate, much, too much) that ranged from 1 to 5 for knowledge assessment; and the choice of one option and a 5-point Likert scale (too little, little, moderate, much, too much) for attitude assessment.

The Ethics Committee of Royan Institute approved the study (code no: EC/1390/1136). All participants received a complete explanation of the research aims prior to the onset of the study. Voluntary completion of the questionnaire was considered as consent. Eligible individuals were assured that their confidentiality and anonymity, as well as their decision to participate in or withdraw from the study would not impact their current or future relationship with the clinic.

Statistical analysis

Statistical analyses were carried out using the Statistical Package for Social Science (SPSS, version 15.0 for Windows; SPSS, Inc., Chicago, IL). Continuous variables were expressed as mean ± SD and categorical variables as numbers (percentages). We did not compare the knowledge responses (5-point Likert scale; range: 1 to 5) by sex through the Chi-square test for categorical data. Instead, we used the independent samples t test because it is robust when one may encounter ordinal scaled data. The statistical issue was demonstrated by Heeren and D'Agostino, in 1985 as previously explained (10). The mean differences in infertility knowledge between female and male participants were measured with one-way ANOVA. Chi-square tests of independence were used to assess relationships between categorical variables asked from participants for attitudinal approach. $P<0.05$ was considered statistically significant.

Results

Participants had a mean age of 30.93 ± 5.56 years. Females comprised 54% of the study population compared to 46% for males. Only 34% had an academic education. Approximately two-thirds were employed. Demographic characteristics of study participants are presented in Table 1.

Table 1: Demographic characteristics of study participants (n=150)

Socio-demographic variables		Number	Percentage
Age (Y)	18-26	25	16.67
	27-32	71	47.33
	33-44	52	34.67
	45-75	2	1.33
Sex	Male	69	46
	Female	81	54
Education level	Under diploma	57	38
	Diploma	42	28
	Graduated	51	34
Occupation	Unemployed	57	38
	Employed	93	62

Table 2 lists participants' total mean knowledge score of fertility and infertility for each item. As shown, factors that affected pregnancy (3.97 ± 1.11) and infertility treatment (3.97 ± 1.16) had the highest mean knowledge scores. Knowledge of the natural reproductive cycle (2.96 ± 1.12) and anatomy of the genital organs (2.94 ± 1.16) had the lowest mean knowledge scores. We determined the total mean knowledge to be 3.08 ± 0.99.

Table 2: A description of knowledge items from study participants (n=150)

		Mean	SD
K1	Natural reproductive cycle	2.96	1.12
K2	Anatomy of the genital organs	2.94	1.17
K3	Diagnostic tests and procedures	3.07	1.18
K4	Diagnostic surgery	3.12	1.31
K5	Factors affecting pregnancy	3.97	1.11
K6	Infertility treatment	3.97	1.16
K7	Success in infertility treatment	3.08	1.07
K8	Effective factors in the success of infertility treatment	3.20	1.05

Range: 1 (minimum) to 5 (maximum).

As seen in Figure 1, the highest mean knowledge scores according to gender showed that males scored 3.28 ± 1.07, whereas females had a score of 3.14 ± 1.04 in the question that pertained to effective factors in the success of infertility treatment. This was a nonsignificant difference between males and females (P=0.444). A question on diagnostic surgery showed greater mean knowledge scores of 3.25 ± 1.29 (males) and 3.01 ± 1.32 (females), which was not statistically significant between male and female responders (P=0.266).

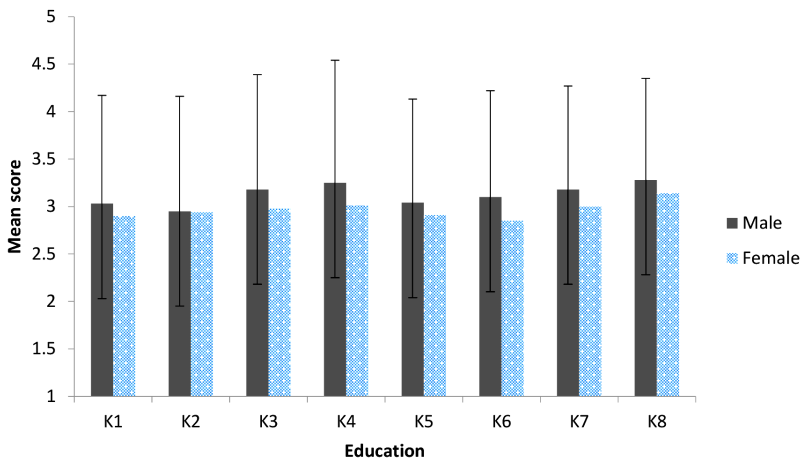

Fig.1: Fertility and infertility knowledge of the 150 respondents by sex.
K1; Natural reproductive cycle, K2; Anatomy of the genital organs, K3; Diagnostic tests and procedures, K4; Diagnostic surgery, K5; Factors affecting pregnancy, K6; Infertility treatment, K7; Success in infertility treatment, and K8; Effective factors in the success of infertility treatment. Range: 1 (minimum) to 5 (maximum).

One-way ANOVA showed that infertile participants who had an education level of under diploma had a significantly higher (P=0.042) mean knowledge score of 3.27 ± 1.19 compared to those with diploma (2.80 ± 1.10) and graduates (2.76 ± 1.01). Table 3 lists additional details about fertility and infertility knowledge of the respondents according to level of education. One-way ANOVA showed that infertile participants who had an education level of under diploma had a significantly higher (P=0.042) mean knowledge score of 3.27 ± 1.19 compared to those with diploma (2.80 ± 1.10) and graduates (2.76 ± 1.01). Table 3 lists additional details about fertility and infertility knowledge of the respondents according to level of education.

Attitude of respondents toward education regarding infertility treatment by sex is presented in Table 4. Of note, approximately 40% of females and 30% males believed in the effectiveness of group education. However 35.4% of males and 25.3% of females preferred individual infertility education. These differences were not statistically significant (P=0.226). Most females (92.1%) and males (83.3%) were of the opinion that infertility education programs should include causes of infertility and types of treatment associated with diagnostic and laboratory procedures. However, no statistically significant difference was found between male and female participants (P=0.245). Views did not differ in terms of the best time for education - whether the first clinic visit or not. The majority thought that education, on average, effectively decreased stress and encouraged cooperation during treatment. Table 5 presents the patients' attitudes about infertility treatment education in detail.

Table 3: Fertility and infertility knowledge of respondents (n=150) by education

		Education level	Mean	SD	P value
K1	Natural reproductive cycle	Under diploma	3.27	1.19	0.042*
		Diploma	2.80	1.10	
		Graduated	2.76	1.01	
K2	Anatomy of the genital organs	Under diploma	3.15	1.35	0.214
		Diploma	2.73	1.06	
		Graduated	2.90	1.01	
K3	Diagnostic tests and procedures	Under diploma	3.18	1.36	0.586
		Diploma	2.93	0.98	
		Graduated	3.06	1.11	
K4	Diagnostic surgery	Under diploma	3.20	1.47	0.537
		Diploma	2.93	1.27	
		Graduated	3.20	1.15	
K5	Factors affecting pregnancy	Under diploma	3.05	1.21	0.426
		Diploma	2.78	1.01	
		Graduated	3.04	1.06	
K6	Infertility treatment	Under diploma	3.13	1.16	0.346
		Diploma	2.78	1.23	
		Graduated	2.94	1.08	
K7	Success in infertility treatment	Under diploma	3.20	1.12	0.476
		Diploma	2.93	1.15	
		Graduated	3.08	0.96	
K8	Effective factors in the success of infertility treatment	Under diploma	3.32	1.19	0.592
		Diploma	3.13	1.00	
		Graduated	3.14	0.94	

*; P<0.05 was considered statistically significant. Range: 1 (minimum) to 5 (maximum).

Table 4: Attitude of respondents (n=150) toward education about infertility treatment by gender

		Group	Male n (%) n=69	Female n (%) n=81	P value
1	Which was the best way to improve awareness of infertility education?	Group	18 (27.7)	32 (40.5)	0.226
		Individual	23 (35.4)	20 (25.3)	
		Does not matter	24 (36.9)	27 (34.2)	
2	Education and counseling should be provided in what context?	Cause of treatment	2 (3.2)	4 (5.1)	0.245
		Type of treatment	1 (1.6)	6 (7.7)	
		Types of treatment cycles	0 (0)	2 (2.6)	
		Diagnostic and laboratory methods	2 (3.2)	1 (1.3)	
		All items	58 (92.1)	65 (83.3)	
3	When is the best time for the education?	First visit	36 (56.3)	42 (53.2)	0.105
		Before starting treatment	16 (25)	30 (38)	
		During treatment	12 (18.8)	7 (8.9)	
4	How much does education reduce your stress effectively?	Too much	17 (26.6)	21 (26.9)	0.919
		Much	12 (18.8)	18 (23.1)	
		Moderate	17 (26.6)	19 (24.4)	
		Little	7 (10.9)	10 (12.8)	
		Too little	11 (17.2)	10 (12.8)	
5	How much education is effective in your cooperation during the course of treatment?	Too much	19 (30.2)	18 (23.4)	0.491
		Much	11 (17.5)	21 (27.3)	
		Moderate	13 (20.6)	15 (19.5)	
		Little	7 (11.1)	12 (15.6)	
		Too little	13 (20.6)	11 (14.3)	

Table 5: Attitude of respondents (n=150) toward education regarding infertility treatment by educational status

		Group	Under Diploma n (%) n=57	Diploma n (%) n=42	Graduated n (%) n=51	P value
1	Which was the best way to improve awareness of infertility education?	Group	20 (37.7)	14 (35)	16 (31.4)	0.408
		Individual	16 (30.2)	8 (20)	19 (37.3)	
		Does not matter	17 (32.1)	18 (45)	31.4 (16)	
2	Education and counseling should be provided in what context?	Cause of treatment	4 (8)	2 (5)	0 (0)	0.181
		Type of treatment	2 (4)	0 (0)	5 (9.8)	
		Types of treatment cycles	0 (0)	1 (2.5)	1 (2)	
		Diagnostic and laboratory methods	2 (4)	0 (0)	1 (2)	
		All items	42 (84)	37 (92.5)	44 (86.3)	
3	When is the best time for the education?	First visit	30 (57.7)	18 (45)	30 (58.8)	0.704
		Before starting treatment	15 (28.8)	16 (40)	15 (29.4)	
		During treatment	7 (13.5)	6 (15)	6 (11.8)	

Table 5: Continued

		Group	Under Diploma n (%) n=57	Diploma n (%) n=42	Graduated n (%) n=51	P value
4	How much does education reduce your stress effectively?	Too much	10 (19.2)	9 (22.5)	17 (34)	0.079
		Much	7 (13.5)	6 (15)	10 (20)	
		Moderate	14 (27.9)	5 (12.5)	17 (34)	
		Little	9 (17.3)	11 (27.5)	4 (8)	
		Too little	12 (23.1)	9 (22.5)	2 (4)	
5	How much education is effective in your cooperation during the course of treatment?	Too much	11 (22)	6 (15)	18 (36)	0.141
		Much	6 (12)	11 (27.5)	13 (26)	
		Moderate	12 (24)	8 (20)	8 (16)	
		Little	8 (16)	5 (12.5)	8 (16)	
		Too little	13 (26)	10 (25)	3 (6)	

Discussion

Interestingly, all participants from both sexes gave limited information about their reproductive systems and anatomy of the genital organs. There is scant mention of infertility and sexual health in compulsory secondary school curricula in Iran. From this study, we have determined that males had better knowledge than females on the natural reproductive cycle, diagnostic tests and procedures in infertility, risk factors that affected fertility and pregnancy, and infertility treatment and its success. In contrast, in a survey on infertility knowledge and attitudes in urban high school students, about 20% of students (mostly males) did not recognize that infertility could result from both male and female factors (11). It is well established that awareness of infertility risk factors is essential for fertility preservation (12). Infertility knowledge of male and female risk factors is a critical first step for fertility preservation through lifestyle modification (13-17). Female factor does not always cause infertility, but male factor infertility is responsible of some other cases (15). However, in traditional societies, infertility is known as a female problem (18, 19).

Research has highlighted that infertility knowledge is associated with education; health promotion strategies are effective when they begin with educational interventions (20). There is an important gap in the literature regarding infertility education. Education about fertility and infertility issues is also needed to prevent fear and unnecessary delay in seeking help and treatment when faced with problems of conception (21, 22).

The present study was the first to investigate patients' attitudes toward the effect of education on infertility treatment. Numerous studies aimed to determine knowledge and awareness of infertility among their study population (high school students, medical students, adults, infertile couples, etc.) and to explore an attitudinal trend toward various aspects of infertility (11, 23, 24). On the basis of our results, the majority of women believed group education to be more effective, while most men preferred individual infertility education or neither of the two methods. Therefore, infertility care providers must take this into consideration when designing infertility education. According to the opinion of the vast majority of both sexes, an education program should include causes of infertility and types of treatment associated with diagnostic and laboratory procedures. This education should be conducted at the first visit in order to be more effective in decreasing stress and encouraging cooperation during treatment. Hence, less knowledge about all aspects of infertility, as well as patients' attitudes toward conditions of infertility education should be taken into account in developing infertility education programs in referral infertility clinics. Very few studies have deter-

mined whether public education about infertility is warranted and ultimately effective in prevention. It is recommended that the extent of people's knowledge of infertility and attitudes about education on infertility should be specified because this would be useful for planning public education programs related to the prevention of infertility, even for the entire society.

The strength of this study was the collection of data on infertile patients' attitudes toward education on infertility, which thus far has not been considered. Study limitations included the reliance on clients that presented to only one center - a referral clinic for infertility in Iran which limited the generalizability of these findings. This study was cross-sectional and therefore only suggested associations rather than causal relationships.

Conclusion

Most participants in this study have expressed awareness of factors that affected pregnancy and infertility treatment. It is imperative to educate and empower infertile individuals seeking reproduction treatment in terms of infertility causes and types of treatment, as well as diagnostic and laboratory procedures in order for them to make informed decisions about assisted reproductive procedures.

Information gathered from this study could be useful for public health educators, health care providers in the clinic, and for government policy makers in order to prepare educational services and programs that meet patients' needs It seems necessary to provide effective public education on infertility through multiple sources such as media, schools, family, community, health care workers, and the government.

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Original Article

The Frequency of *Staphylococcus aureus* Isolated from Endocervix of Infertile Women in Northwest Iran

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Abstract

Background: Infertility is one of the major social issues. Due to the asymptomatic cervical infection associated with *Staphylococcus aureus (S. aureus)*, the majority of patients remain undiagnosed. The present study intended to assess the frequency of *S. aureus* isolated from infertile women's endocervix in northwest Iran.

Materials and Methods: In a descriptive cross sectional study, specimens were randomly collected during vagina examination using a sterile speculum and swabbing. After performance of antibiotic susceptibility testing, polymerase chain reaction (PCR) was used to identify methicillin-resistance *S. aureus* (MRSA) and toxic shock syndrome toxin-1 (TSST-1).

Results: About 26 (26%) and 9 (9%) women's urogenital tracts were colonized by S. aureus and Candida spp., respectively, of which three (11.5%) patients were infected with fungi and S. aureus, simultaneously. Antibiotic susceptibility results showed high activity of vancomycin and co-trimoxazole on isolates. Regarding PCR results, mecA sequences were detected in 7 (26.9%) strains, whilst the *tst* gene encoding TSST-1 was not detected in any of clinical strains.

Conclusion: The prevalence of *S. aureus* was very high in infertile women. Therefore, it demands all patients undergoing infertility treatment to be investigated thoroughly for this type of infection.

Keywords: Infertility, *S.taphylococcus aureus*, Endocervix, *mecA*

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Introduction

One of the most common reproductive health issues in developing countries is the high rate of infertility (1). It becomes a globally important subject for clinical research and practice because infertility affects 60 to 168 million individuals, both women and men, in reproductive age. It has been reported that of 10 couples, one couple incurs the early or secondary stages infertility (2, 3). Infertility is characterized as inability to conceive during one year, despite normal cohabitation (4), as indicated by the European Society of Human Reproduction and Embryology (ESHRE), which is evidenced in 10-15% of all couples (5).

There are a number of factors which are responsible for infertility in females. According to a study by Vayena et al. (3), the rates of primary infertility are generally between 1 and 8% with rates of secondary infertility reaching as high as

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35%. Infection of reproductive organs is one of the most important factors affecting infertility, while the determination of the type of infection has a significant contribution to the treatment of this issue. However, the significance of these infections in the genital tracts is not well known. Many microorganisms, including bacteria, viruses, parasites and fungi, seem to be able to interfere with the reproductive function in both genders. Bacterial vaginosis is a prevalent issue among women with changing the balance of normal vaginal flora such as lactobacilli (6, 7). However, some related pathogenic impacts have been evidenced such as increased rates of premature rupture of the membranes (PROM), late miscarriage in first trimester, preterm labor, endometriosis and delivery (6). Bacterial vaginosis is the most common lower genital tract disorder among reproductive age of women (pregnant and non-pregnant) and the most common cause of vaginal odor and malodorous discharge (8).

The bacteria encountered in the female genital tract can be divided into aerobic and anaerobic organisms. Among the aerobic Gram-positive organisms, there are several species of *Streptococcus (S.)*, such as group B S. (GBS), and among Gram-positive facultative anaerobic organisms, there are several species of *Enterococcus (E.)* (9). *S. aureus* is an infrequent but one of the most successful human pathogens. It has the ability to cause a number of infections in various environmental corners within the host. *S. aureus* has additionally been reported to be commonly isolated microorganism from cervical specimens (10). It is found in the genital tract of approximately 9 to 10% of asymptomatic women, approximately 10% of patients with postoperative wound abscess after gynecologic or obstetric procedures, and 5 to 20% of genital tract cultures of women with pelvic infection. It is also detected in virtually 100% of women who have toxic shock syndrome toxin-1 (TSST-1) (9). This study was, therefore, designed to assess the frequency of S. aureus isolated from infertile women's endocervix in northwest Iran.

Materials and Methods

Sampling

Descriptive cross sectional study was carried out at the Tabriz University of Medical Science (TUMS), Tabriz, Iran, between April and July 2015. The cervical samples were randomly taken from 100 women who attended the Department of Obstetrics and Gynecology of Milad Infertility Center affiliated to TUMS for unexplained infertility during the mentioned-period. Patients with infertility due to unrelated reasons to *S. aureus* infections, such as ovarian cancer, lazy ovary, ovarian cysts and polycystic ovary syndrome (PCOS), were excluded from the study.

The Ethic Commission of TUMS approved this study (Number: 5/412912-2015). After obtaining a written consent form from all patients, the specimens were collected using a sterile vaginal speculum and swab.

Isolation and identification

Samples were streaked on blood agar and mannitol salt agar (Merck, Germany) plates and incubated aerobically at 37°C for 24-48 hours. After overnight incubation, the isolates were examined by Gram staining technique using catalase, DNase and coagulase tests (11).

Antibiotic susceptibility testing

The susceptibility of *S. aureus* isolates to antimicrobial agents was measured *in vitro* using the disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) protocols (12). The tested antibiotics included penicillin, gentamicin, ciprofloxacin, vancomycin, trimethoprim/sulfamethoxazole and cefoxitin (MAST Diagnostics, UK).

Detection of *mecA* and *tst* genes

Bacterial DNA was extracted from the isolates according to tissue buffer boiling method (13). Firstly, 20 µl tissue buffer [0.25% sodium dodecyl sulfate (SDS)+0.05M NaOH (CinnaClon, Iran)] were mixed with one colony of bacterial isolate, the combination was incubated for ten minutes in 95°C, the mixture was centrifuged for one minute in 13000 g, 180 µl Milli-Q water (CinnaClon, Iran) were slowly added, and finally extracted DNA was frozen in -20°C for durable storage.

DNA isolates with the concentration of 0.1 ng/µl

were used as the templates for polymerase chain reaction (PCR) analysis. Multiplex PCR was carried out by CinnaGen MsterMix (CinnaClon, Iran) using the *mecA* and *tst* primers, as described previously (14).

The sequences of the *mecA* primers used were: 5′-ACTGCTATCCACCCTCAAAC-3′ and 5′- CTGGTGAAGTTGTAATCTGG -3′, while the sequences of the *tst* primers used were: 5′-ACCCCTGTTCCCTTATCATC-3′ and 5′-TTTTCAGTATTTGTAACGCC-3′ (synthesized at the CinnaClon, Iran).

The strains 92-S-1344 (*tst*) and 95-S-739 (*mecA*) were used as positive control in this study. Amplification was carried out using a thermocycler (Eppendorf, Germany) as follows: i. Initial denaturation at 94°C for 5 minutes, ii. 35 cycles of denaturation at 94°C for 2 minutes, annealing at 57°C for 2 minutes, and primer extension at 72°C for 1 minutes, and iii. Terminal extension at 72°C for 7 minutes. Electrophoresis of PCR products was performed on 1% agarose gel (CinnaClon, Iran). The gel staining was performed in ethidium bromide for 20 minutes and visualized using a gel documentation system (UVP, USA).

Results

During the study period, a total of 100 infertile women were included in this study. All participants underwent intrauterine insemination (IUI). About 26 (26%) and 9 (9%) women's urogenital tracts were colonized by *S. aureus* and *Candida* spp., respectively, which were identified by mycology methods. Among them, three (8.5%) patients were infected with fungus and *S. aureus*, simultaneously. The average age of these patients was 30.96 years, ranging between 18 and 56 years. Of the patients who were colonized by *S. aureus*, 11 (42.1%) were under 30 years and 14 (53.7%) were over 30 years of age. After examining the wives of the patients who were colonized by *S. aureus*, the semen samples of four (15.38%) couples were positive for *S. aureus*. In addition, penicillin-resistant strains of *S. aureus* were colonized in the reproductive system of a couple. Of those *Candida* spp. carriers, three (3 of 9) wives were also infected with Candida spp. The results of antibiotic susceptibility testing are summarized in Table 1.

Table 1: The results of antibiotic susceptibility testing

Antibiotic	Sensitive (%)	Intermediate (%)	Resistant (%)
Penicillin	3 (11.53)	0 (0)	23 (88.46)
Gentamicin	19 (73.07)	0 (0)	7 (26.09)
Ciprofloxacin	19 (73.07)	0 (0)	7 (26.09)
Vancomycin	25 (96.15)	1 (3.84)	0 (0)
Co-trimoxazole	25 (96.15)	0 (0)	1 (3.84)
Cefoxitin	19 (73.07)	0 (0)	7 (26.09)

The data were presented as N (%).

In general, vancomycin and co-trimoxazole showed high activity against the isolates. Regarding PCR results, *mecA* sequences were detected in 7 (26.9%) isolates, whilst the *tst* gene encoding TSST-1 was not detected in any of clinical isolates.

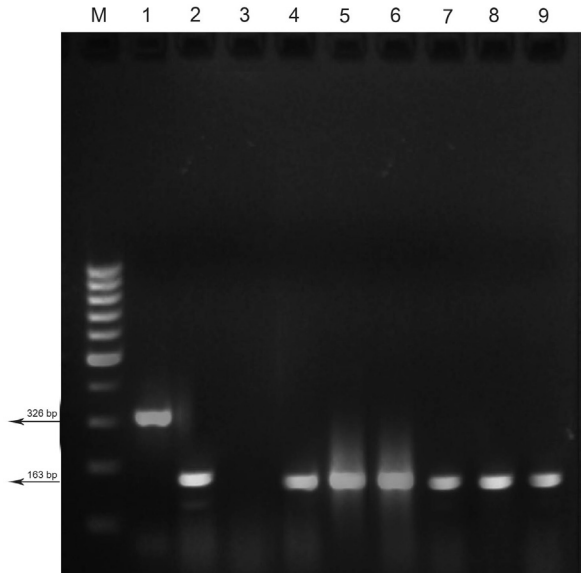

Fig.1: Multiplex PCR product for the *mecA* and *tst* genes. M; 100 bp molecular weight marker, Lane 1; *tst* gene positive control (ATCC 92-S-1344), Lane 2; *mecA* gene positive control (ATCC 95-S-739), Lane 3; Negative control, and Lane 4-9; mecA positive .

Discussion

The study investigated the prevalence of *S. aureus* in infertile women. An in-depth study on these genera has not yet been conducted in Iran. Generally, infectious vaginitis is a prevalent disorder with noteworthy clinical results if left untreated. Infertility is an important health issue with far-reaching consequences on couple, family planning program,

health system and spread of sexually transmitted diseases (STD) and acquired immunodeficiency syndrome (AIDS). It can be characterized as the lack of a conception after at least one year of constant, unprotected sexual intercourse (15).

In a study by Okonofua et al. (10), *Candida albicans* (25%), *S. aureus* (21.7%) and *Neisseria gonorrhoeae* (17.4%) were the most commonly isolated microorganisms; however, there was no difference between fertile and infertile women in the rates of isolation of these pathogens. In most cases, resistance to penicillin is attributable to β-lactamase production. We found that the most antibiotic resistance was against penicillin, which is not supported by a study conducted by Ghiasi et al. (16), in which they have indicated 100% of S. aureus were sensitive. Our findings also indicated that none of the *S. aureus* strains were resistant to vancomycin. In a study by El-Ghodban et al. (17), they have reported that less than 50% of S. aureus strains were β-lactamase producers and resistant to penicillin. However, almost 75% of the strains originating from food were positive for β-lactamase and resistant to penicillin. In our study, all isolates were positive for *mecA* genes, while they were resistant to the gentamicin, ciprofloxacin and cefoxitin. de A Trindade et al. (18) have also reported that among methicillin-resistant *S. aureus* (MRSA) isolated from blood samples, twenty (13%) individuals were susceptible to four or more antimicrobials.

The incidence of TSST-1-producing strains has been registered worldwide (19). Colonization with *S. aureus* is generally highest (20 to 30%) in the oropharynx or nose of non-healthcare workers. Vaginal colonization with *S. aureus* has been determined to be lower (10 to 20%) in the United States, Europe, and Asia (20). Similarly, TSST-1-producing strains of *S. aureus* have been isolated vaginally from 1 to 4% of healthy, menstruating women in the general population (19). Due to a higher immune response to TSST-1, *S.* infections are more common in developing countries than developed countries (21). We decided to use tst in the present study because of limited reports from many developing countries. In a study by Parsonnet et al. (19) carried out on Japanese women, of the 159 *S. aureus* isolates recovered, 14 (9%) were TSST-1 positive, suggesting that only 47% of women had positive titers of anti-TSST-1 antibody, which is significantly lower than the reported seropositivity rates in the Europe and United States (20). In the same study by El-Ghodban et al. (17), TSST-1 was detected in only three (7.5%) of 40 *S. aureus* clinical strains and in none of the food strains. In another study by Tsen et al. (22), they have firmly reported the comparative discoveries, in which only three (4.8%) of 62 strains of S. aureus from clinical sources as tst-carrying strains were identified using PCR, but none of their food strains carried this gene.

In the etiologies of infertility, the most contributed factors are related to female (40 to 55%) followed by male factors (30 to 40%), both partners (10%) and unexplained factors (10%) (1). There are several factors which increase risks for acquisition of bacterial vaginosis, while bacterial vaginosis is more prevalent in women who smoke (23), black women (24), women who are sexually active compared with virginal women (25), and women who utilize vaginal douches (26).

The infertility leads to decreased levels of personal well-being, while for many individuals, it causes more serious consequences (27). Subsequently, it appears that screening is a reasonable approach that is likely to be cost effective. However, all physicians must have a high index of suspicion and utilize promptly accessible screening methods to detect and treat the patients with infectious vaginitis adequately (28). A limitation of the present study was the lack of evaluation of *S. aureus* in fertile women. However, it is suggested that further studies will be conducted on a larger sample.

Conclusion

The exact knowledge of *S. aureus* colonization rate in infertile women has a great importance. Therefore, it demands all patients undergoing infertility treatment to be investigated thoroughly. Such screening and treatment during the course of infertility treatment increase the pregnancy rate extensively. However, randomized studies with larger number of participants are needed to reach more validated conclusions.

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Original Article

Follicle Stimulating Hormone and Anti-Müllerian Hormone among Fertile and Infertile Women in Ile-Ife, Nigeria: Is there A Difference?

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Abstract

Background: Reduced ovarian reserve predicts poor ovarian response and poor success rates in infertile women who undergo assisted reproductive technology (ART). Ovarian reserve also decreases with age but the rate of decline varies from one woman to another. This study aims to detect differences in ovarian reserve as measured by basal serum follicle stimulating hormone (FSH) and anti-Müllerian hormone (AMH) between a matched cohort of fertile and infertile regularly menstruating women, 18-45 years of age.

Materials and Methods: This case-control study involved 64 fertile and 64 subfertile women matched by age at recruitment. Peripheral blood samples were taken from the women recruited from the Gynecological and Outpatient Clinics of Obafemi Awolowo University Teaching Hospital, Ile-Ife, Nigeria. Serum FSH and AMH were quantified using ELISA at the Metabolic Research Laboratory of LAUTECH Teaching Hospital, Ogbomoso, Nigeria.

Results: A significant difference existed in the mean FSH of fertile (6.97 ± 3.34) and infertile (13.34 ± 5.24, P=0.013) women. We observed a significant difference in AMH between fertile (2.71 ± 1.91) and infertile (1.60 ± 2.51, P=0.029) women. There was a negative correlation between FSH and AMH in both fertile (r=-0.311, P=0.01) and infertile (r=-0.374, P=0.002) women.

Conclusion: The difference in ovarian reserve observed in this study suggests that reduced ovarian reserve in regularly menstruating women may be associated with early ovarian ageing or subfertility.

Keywords: Infertility, Ovarian Reserve, Follicle Stimulating Hormone, Anti-Müllerian Hormone

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Introduction

Infertility is the inability of a couple to conceive despite adequate unprotected sexual intercourse within one year (1). Infertility affects 10-30% of couples in sub-Saharan Africa (2, 3). Infertility and its management place substantial psychosocial demand on the couple, especially the woman (4). The physician therefore needs to be well equipped in order to manage couples with infertility.

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The number of oocytes in a female reaches its peak at 20 weeks during fetal life at 7 million primordial follicles. At birth, human ovaries contain approximately 1 million primordial follicles which arrest at the prophase of the first meiotic division (5, 6). This further reduces to 400,000 at puberty and only about 400 follicles will eventually acquire gonadotrophin receptors and the possibility of ovulation. Follicle depletion occurs before and after menarche, during use of oral contraceptives, pregnancy, and whether menstruation is regular or not. As the depletion of the follicular pool continues during the reproductive life, there is regular escape of the primordial follicles from the resting phase by entering into meiosis (6).

Longitudinal studies in fertile women have shown declines in anti-Müllerian hormone (AMH) levels with age; it is the earliest marker of decline in ovarian reserve in young women (7). The purposes of assessing ovarian reserve are to predict reproductive age; detect early ovarian ageing (currently affecting 10% of the general population); predict chances for conception in women desirous of pregnancy; and in counseling women desirous of delaying childbearing (8, 9). There is a large individual variability that exists in age at which ovarian aging commences. Factors that contribute to biological ovarian aging and reduction in ovarian reserve include ovarian toxicants, chromosomal abnormality, cigarette smoking, alcohol abuse, nutritional deficiencies, oxidative stress, and auto-immunity. Gynecological conditions and treatments such as pelvic surgery, chemotherapy, and radiotherapy also affect the rate of decline in ovarian reserve (10). The possibility thereby exists that exposure to the factors that accelerate ovarian aging associated with reduction in ovarian reserve is associated with subfertility.

The aim of this study was to detect differences in ovarian reserve as assessed by AMH and follicle stimulating hormone (FSH) between fertile and infertile women in Ile-Ife, Nigeria. We hypothesized that a difference exists in ovarian reserve as measured by basal serum FSH and random serum AMH levels in infertile women compared to fertile women.

Materials and Methods

Study population and participants

A study by Kalaiselvi et al. (11) compared mean AMH levels between fertile women (3.7 ± 1.6 ng/ml) and subfertile women (2.9 ± 0.98 ng/ml); this was used to calculate the sample size according to the formula for comparison of means (12). Assuming a minimum detectable difference of 0.8 ng/ml, 95% confidence interval (CI), study power of 90% with attrition rate of 10%, we required 65 participants in each group to ensure statistically significant results. This case-control study enrolled 65 subfertile women recruited from the Gynecology Clinic of Obafemi Awolowo University Teaching Hospitals Complex, Ile-Ife, Nigeria and 65 fertile women matched by age with the infertile group recruited from the general Outpatient Clinic of this hospital from November 2014 to January 2015. All women recruited were between the ages of 18 and 45 years and had regular menstrual cycles that ranged from 21 to 35 days. The fertile participants also had proven natural fertility with at least one pregnancy carried to term within the preceding 2 years; each pregnancy haven arisen spontaneously following unprotected sexual intercourse within 1 year. Subfertile participants had at least a 12-month history of inability to conceive despite adequate sexual intercourse. We excluded women with any history, radiological or biochemical parameters suggestive of polycystic ovary syndrome (PCOS) or evidence of endocrinological diseases, and those that used hormonal contraceptives.

The study proforma was then completed to document demographic and gynecological information. Study outcomes included basal serum FSH and random serum AMH levels. A venous blood sample was taken for serum AMH measurement. Each woman was instructed to alert the investigator at the onset of her next menstrual cycle in order to make arrangement for collection of the day 3 FSH sample. Peripheral blood samples were collected through a venipuncture by a doctor who collected 5 ml for each assay. Samples were collected into plain sterile sample bottles and left to stand for 1 hour for clot retraction and then centrifuged for 10 minutes at 5000 rpm. Serum was then separated into another unheparinized sterile sample using a pipette. The serum was then stored in a -20ºC freezer until analysis within 3

weeks. The samples were transported to the laboratory in ice packs.

Follicle stimulating hormone and anti-Müllerian hormone assays

Serum samples were thawed at room temperature and processed at the Metabolic Research Laboratory of Ladoke Akintola University of Technology (LAUTECH) Teaching Hospital, Ogbomoso. Serum FSH was quantified in duplicate with Follicle Stimulating Hormone Test System (Monobind, Inc., USA) using the direct enzyme linked immunosorbent (ELISA) assay according to the manufacturer's manual. After incubation, the absorbance was read at 450 nm within 30 minutes using a microplate ELISA reader (LT 4000). The precision of the assay was 0.134 mIU/ml.

AMH was quantified in duplicate with the Human Anti-Müllerian Hormone ELISA kit (Span Biotech Ltd., Hong Kong) using a double-antibody sandwich ELISA according to the manufacturer's manual. After incubation, the absorbance was read as above. The sensitivity of the assay was 0.01 ng/ml.

Statistical analysis

We analyzed data from 128 women with Stata version 13 (StataCorp). Pearson's correlation was used to determine the relationship between age, body mass index (BMI), AMH, and FSH while the paired t-test was used to compare means between the two groups.

Ethical consideration

The Ethics and Research Committee of Obafemi Awolowo University Teaching Hospitals Complex, Ile-Ife approved the study (Ethical clearance certificate number: ERC/2014/05/01). Informed consent was obtained from each participant before enrollment.

Results

We recruited 130 women into the study from November 2014 to January 2015; sixty five women in the subfertile group and sixty five women in the fertile group. However, two participants, one from each group, did not complete the study. Therefore, we analyzed the data from 128 women who completed the study.

Baseline characteristics

We compared the baseline characteristics of the recruited women between the two groups (Table 1). The fertile group had a mean age of 31.16 ± 5.78 years; for the subfertile group, the mean age was 31.52 ± 4.35 years. The mean age difference between the two groups was 0.36 (P=0.58, 95% CI: 4.65-0.88). The mean BMI of the fertile group was 26.31 ± 4.48 vs. 26.03 ± 5.74 for the subfertile group. The mean difference in BMI difference between the two groups was 0.27 (P=0.77, 95% CI: 1.45-1.99). The mean parity of the fertile group was 1.95 ± 1.08 compared to 0.48 ± 0.97 for the subfertile group. The mean difference in parity was 1.48 (P=0.00, 95% CI: 1.12-1.83, Table 1).

Among the subfertile women, 27 (42.2%) had primary infertility while 37 (57.8%) had secondary infertility. There were 44 (68.8%) women in the subfertile group diagnosed with tubal factor infertility. There were 5 (7.8%) anovulatory cases and 4 (6.3%) with male factor infertility. Both tubal and male factors were present in one (1.6%) participant while another participant (1.6%) also had both tubal factor and anovulation. A third participant (1.6%) had both male factor and anovulation. However, 15 (23.4%) remained unexplained at the conclusion of the study. Participants aged 18-24 years, 25-34 years and 35-45 years were 6, 39 and 20 respectively.

Table 1: Comparison of age, parity, and body mass index (BMI) between fertile and subfertile women

	Fertile (mean ± SD)	Infertile (mean ± SD)	Mean difference	95% CI	t statistics	P value
Age (Y)	31.16 ± 5.78	31.52 ± 4.35	-0.36	-4.65-0.88	0.83	0.58
BMI (kg/m^2)	26.31 ± 4.48	26.03 ± 5.74	0.27	-1.45-1.99	0.31	0.77
Parity	1.95 ± 1.08	0.48 ± 0.97	1.48	1.12-1.83	8.20	0.00*

CI; Confidence interval and *; Statistically significant.

Correlation of ovarian reserve markers in fertile women

Fertile women had moderately negative correlations between FSH and AMH, as well as AMH and age whereas we observed a positive correlation between age and FSH (Table 2). However, neither FSH nor AMH had any significant association with BMI (Table 2). Figure 1 depicts the association between FSH and AMH. The Pearson's rho coefficient for the correlation between FSH and AMH after controlling for age was -0.24 (P=0.04).

Table 2: Correlation between anti-Müllerian hormone (AMH), follicle stimulating hormone (FSH), body mass index (BMI), and age in fertile women

Parameters	FSH		AMH	
	Pearson correlation coefficient	P value	Pearson correlation coefficient	P value
Age (Y)	0.258	0.038*	-0.332	0.007*
BMI (kg/m^2)	0.14	0.28	-0.044	0.726
FSH (IU/L)	1	-	-0.311	0.01*

*; Statistically significant.

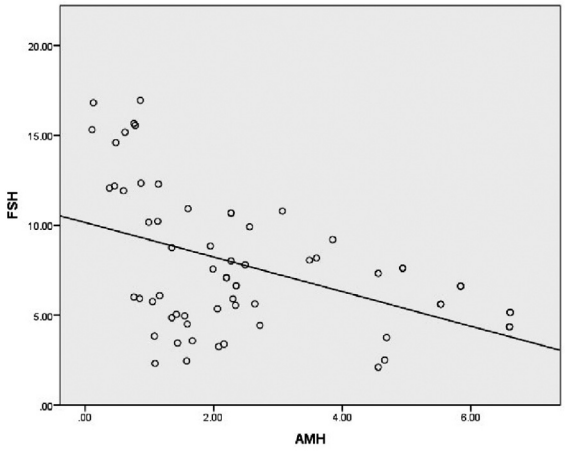

Fig.1: Relationship between FSH and AMH among fertile women. FSH; Follicle stimulating hormone and AMH; Anti-Müllerian hormone.

Correlation of ovarian reserve markers in subfertile women

Subfertile women had negative correlations between FSH and AMH, as well as between AMH and age. A positive correlation existed between age and FSH (Table 3). Also, neither FSH nor AMH had any significant association with BMI (Table 3). Figure 2 depicts the association between FSH and AMH. The Pearson's rho coefficient for the correlation between FSH and AMH after controlling for age was -0.311 (P=0.012).

Table 3: Correlation between anti-Müllerian hormone (AMH), follicle stimulating hormone (FSH), body mass index (BMI), and age in subfertile women

Parameters	FSH		AMH	
	Pearson correlation coefficient	P value	Pearson correlation coefficient	P value
Age (Y)	0.292	0.01*	-0.323	0.009*
BMI (kg/m^2)	0.01	0.93	0.005	0.972
FSH (IU/L)	1	-	-0.374	0.002*

*; Statistically significant.

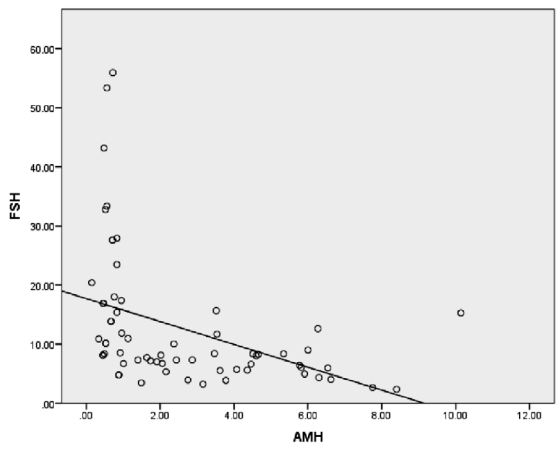

Fig.2: Relationship between FSH and AMH among infertile women. FSH; Follicle stimulating hormone and AMH; Anti-Müllerian hormone.

Table 4: Comparison of anti-Müllerian hormone (AMH) and follicle stimulating hormone (FSH) between fertile and infertile women

	Fertile (mean ± SD)	Infertile (mean ± SD)	Mean difference	95% CI	t statistics	P value
FSH (IU/L)	6.97 ± 3.34	13.34 ± 5.24	-6.37	-11.36- -1.38	-2.55	0.013*
AMH (ng/ml)	2.71 ± 1.91	1.60 ± 2.51	1.11	1.06-1.83	1.21	0.029*

CI; Confidence interval and *; Statistically significant.

Table 5: Sub-analysis by age groups

Age (Y)		Fertile (mean ± SD)	Infertile (mean ± SD)	Mean difference	95% CI	t statistics	P value
25-34	FSH (IU/L)	6.26 ± 2.25	7.41 ± 7.85	-0.97	-5.94-3.38	-0.45	0.65
	AMH (ng/ml)	3.20 ± 1.84	1.37 ± 2.63	1.82	0.36-2.72	1.52	0.043*
35-45	FSH (IU/L)	8.87 ± 4.46	28.48 ± 10.42	19.60	6.67-32.20	-3.20	0.004*
	AMH (ng/ml)	1.76 ± 1.92	0.83 ± 2.31	1.08	0.43-3.66	1.49	0.031*

CI; Confidence interval, FSH; Follicle stimulating hormone, AMH; Anti-Müllerian hormone, and *; Statistically significant.

Table 6: Sub-analysis using clinical groups

		Fertile (mean ± SD)	Infertile (mean ± SD)	Mean difference	95% CI	t statistics	P value
Tubal factor	FSH (IU/L)	7.16 ± 3.53	9.48 ± 12.80	-2.32	-4.97--2.32	-2.73	0.19*
	AMH (ng/ml)	2.64 ± 1.84	1.55 ± 2.77	1.10	0.54-1.99	1.28	0.024*
Unexplained	FSH (IU/L)	6.67 ± 2.59	19.53 ± 15.91	-12.74	-27.36-1.87	-2.87	0.043*
	AMH (ng/ml)	2.97 ± 2.29	2.14 ± 2.15	0.83	-0.88-2.53	1.03	0.32

CI; Confidence interval, FSH; Follicle stimulating hormone, AMH; Anti-Müllerian hormone, and *; Statistically significant.

Comparison of ovarian reserve markers between fertile and subfertile women

Fertile women had a mean FSH value of 6.97 ± 3.34, whereas this value was 13.34 ± 5.24 for subfertile women. The mean difference was -6.37 (P=0.013, 95% CI: -11.36 to -1.38). The fertile group had a mean AMH value of 2.71 ± 1.91. The subfertile group had a mean AMH value of 1.60 ± 2.51. Their mean difference was 1.11 (P=0.029, 95% CI: 1.06 to 1.83;,Table 4).

Sub analysis performed after categorizing the participants into age groups showed significant differences in both mean FSH and AMH levels in women aged 35-45 years, while only AMH showed a significant difference in women aged 25-35 years (Table 5).

Women segregated according to clinical conditions showed that tubal factor forms the majority of cases A statistically significant difference existed between the mean FSH and AMH levels in women with tubal factor infertility, whereas serum AMH did not differ in patients with unexplained infertility (Table 6).

Discussion

This research work showed significantly higher basal serum FSH and lower random serum AMH levels in subfertile women compared to fertile women in Ile Ife, Southwestern Nigeria. The strength of this study was the participation of both young and older women. However, the hormonal levels did not correlate with number of oocytes retrieved, pregnancy rate, or live births. In addition, we did not include other ovarian reserve markers such as antral follicle count in the study.

No statistically significant difference existed in the mean age and BMI between the fertile and subfertile groups. The subfertile group had significantly lower parity. We have expected this finding because it is the major difference between these two groups. Zaidi et al. (9) reported a significant difference in the BMI among the older fertile and subfertile women aged 30-39 years. The discrepancy between this study and other studies might be due to the difference in the age groups compared in both studies. The result obtained here, however, was comparable to the study by Kalaiselvi et al. (11).

There was a moderate negative correlation between FSH and AMH among the fertile women, which was similar to the reports (13). Random serum AMH level reduced as the basal serum FSH increased. This could be explained by the fact that increased basal serum FSH and reduced random serum AMH depicted a decline in ovarian reserve which tended to occur with increasing age. However, a stronger positive correlation between age and FSH was reported by another study; this might be attributed to a larger sample size (14). BMI did not correlate significantly with both basal FSH and random AMH which was comparable to findings

from other studies (15, 16).

The negative correlation between AMH and age among the subfertile group compared to other studies in infertile women (17, 18). In this study, the basal serum FSH increased with increased age. There was no correlation between AMH, FSH, and BMI among the subfertile women. There were conflicting reports about the correlation between BMI and ovarian reserve tests in subfertile women such as the study by Buyuk et al. (19) that reported lower serum AMH levels among overweight and obese women with reduced ovarian reserve.

Subfertile women had statistically significant higher basal serum FSH levels which compared to the results reported by Kalaiselvi et al. (11). This further corroborated the findings by other researchers that reported a decline in ovarian reserve among regularly menstruating infertile women (11, 20). Erdem et al. (21) however did not find any difference in basal serum FSH between fertile and subfertile women. This might be due to patient selection in their study, which consisted of older women.

In addition, random serum AMH also differed significantly between the two groups of women. We observed significantly lower random serum AMH in the subfertile women. This supported other studies about AMH (11, 22). Kalaiselvi et al. (11) reported significantly lower AMH in subfertile women. This difference in AMH between both groups also supported a decline in ovarian reserve in subfertile women. Therefore, ovarian reserve might be reduced in regularly menstruating subfertile women.

Younger infertile women had reduced AMH and normal serum FSH levels, whereas older infertile women had both reduced AMH and elevated FSH levels. This suggested that older women with reduced ovarian reserve were more likely to show both elevated FSH and reduced AMH levels while younger women with diminished ovarian reserve were likely to have normal FSH but reduced AMH levels. This finding supported previous studies where elevated FSH was a late indicator of diminished ovarian reserve (23).

Mean serum AMH did not differ among the unexplained infertility group, whereas we have observed a difference in mean serum FSH levels. This could be due to the fact that serum FSH is secreted from the anterior pituitary and depends on other factors such as serum estrogen while AMH is secreted directly from the preantral follicles (24). Women with unexplained infertility may therefore have other factors responsible for elevated FSH levels.

Conclusion

Ovarian reserve, as assessed by basal serum FSH and random serum AMH, significantly reduced in regularly menstruating subfertile women. A statistically significant difference existed in ovarian reserve of infertile women compared to fertile women in Ile-Ife, Nigeria. Therefore, reduction in ovarian reserve might be associated with early ovarian ageing or subfertility.

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Original Article

Age as A Predictor of Embryo Quality Regardless of The Quantitative Ovarian Response

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Abstract

Background: One determining factor of a successful *in vitro* fertilization (IVF) cycle is embryo quality. The aim of the present study was to evaluate associations of embryo quality and reserve markers like age, FSH and AMH.

Materials and Methods: In this prospective study, 120 infertile women, aged 21-44 years, undergoing routine exploration during an unstimulated cycle preceding assisted reproductive technology (ART) at our center were studied prospectively, from February 2011 to December 2014. Descriptive parameters and patient characteristics were reported as mean (SD) or median (range) depending on the distribution. Student's t test was performed for continuous variables, Wilcoxon and Pearson's Test were used for not distributed variables and Fisher's Test was performed for categorical variables. $P<0.05$ was considered statistically significant.

Results: Overall, at the time of investigation, patients had a mean age of 33.03 ± 4.15 years old. On cycle day three, serum anti-Mullerian hormone (AMH) level was 3.50 ± 1.54 ng/mL, serum follicle-stimulating hormone (FSH) level was 6.29 ± 1.53 mUI/mL, at baseline, women had 16.57 ± 7.0 antral follicles. The mean of collected oocytes was 11.80 ± 5.25, embryo I+II was 2.46 ± 2.11. A greater number of embryos I+II was observed in young patients. By evaluating 120 patients, a significant relationship was observed between age and FSH (r=0.24, P=0.01), age with AMH (r=-0.22, P=0.02), age with collected oocytes (r=-0.23, P=0.03) and age with embryo I+II (r=-0.22, P=0.03). A significant relationship was also observed between antral follicle count (AFC) and AMH (r=0.29, P=0.01), AFC and the number of transferred embryo (r=-0.18, P=0.03), AFC and total dose of the drugs (r=-0.23, P=0.03). Significant relationship of FSH with total dose of drugs (r=0.19, P=0.02) was also observed. In addition, we determined significant relationships between AMH and the number of collected oocytes (r=0.38, P=0.01), AMH and the number of metaphase II oocytes (r= 0.35, P=0.01), AMH and the number of embryo (r=0.19, P=0.04) as well as AMH and total dose of the drugs (r=-0.25, P=0.01).

Conclusion: Commonly used clinical markers of ovarian reserve are reflection of the ovarian reserve, while the outcome measurements of ART and age are the best predictors of embryo quality.

Keywords: Age, Anti-Mullerian Hormone, Follicle-Stimulating Hormone

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Introduction

A classic report on the effect of female age on fertility found that the percentage of women, who using no contraception remained childless, were increased steadily according to their age of marriage: 6% at the age of 20-24 years, 9% at the age of 25-29 years, 15% at the age of 30-34 years, 30% at the age of 35-39 years, and 64% at the age of 40-44 years (1).

According to the 1999 Assisted Reproductive Technology Success Rates (ARTSR), the percentage of clinical pregnancies (gestational sac as imaged with sonography) which is failed to result in a live birth was 14% for women with younger than 35 years of age, 19% for those with 35-37 years of age, 25% for those with 38-40 years of age, and 40% in those with older than age of 40 years (2).

The age-associated decline in female fecundity as well as increased risk for spontaneous abortion, are largely attributable to abnormalities in the oocyte. The meiotic spindle in the oocytes of older women frequently exhibits abnormalities in chromosome alignment and microtubular matrix composition (3). Higher rates of single chromatid abnormalities in oocytes (4), as well as aneuploidy in preimplantation embryos (5) and ongoing pregnancies, are observed in older women. The higher rate of aneuploidy is a major cause of increased spontaneous abortion and decreased live birth rates in women of advanced reproductive age.

Evaluation of ovarian reserve has been the focus of a substantial amount of clinical research during the past several years (6-10). A number of tests have been proposed and evaluated that may be used to prognosticate ovarian responsiveness to exogenous gonadotropin stimulation, quality of the oocytes, subsequent implantation and pregnancy rates (PR) (6-9). The prognostic value of these tests has been clearly demonstrated by a number of investigators in a wide variety of settings (9, 10). The main markers of ovarian reserve are age, basal follicle-stimulating hormone (FSH), anti-Mullerian hormone (AMH) and/or basal antral follicle count (AFC) that are valuable for determining stimulation protocols and predicting assisted reproductive technology (ART) outcome (11-22).

One decisive factor of a successful *in vitro* fertilization (IVF) cycle is embryo quality. Currently, embryo quality is determined by direct visualization of an embryo by an embryologist, who assesses the morphological appearance or markers, to evaluate embryo health and quality (23).

The aim of the present prospective study was to evaluate the associations of embryo quality and ovarian reserve markers like age, FSH, AMH after stimulation with gonadotropin-releasing hormone agonist- for the respective treatment.

Materials and Methods

Subjects

120 infertile women (aged 21-44 years) undergoing routine exploration during an unstimulated cycle and preceding ART were studied at IBRRA, Brazil prospectively from February 2011 to December 2014. All patients met the following inclusion criteria: i. Both ovaries present, ii. No current or past diseases affecting ovaries, gonadotropin or sex steroid secretion, clearance or excretion, iii. No current hormone therapy, iv. Adequate visualization of ovaries at transvaginal ultrasound scans, and v. Total number of small antral follicles (3-12 mm in diameter) between 1 and 32 follicles, including both ovaries. All patients signed an informed consent form for this analysis.

Protocol

The patients received leuprolide acetate (Lupron, Abbott, France), and the gonadotropin-releasing hormone (GnRH)-agonist was initiated at a dose of 2.0 mg/day during the midluteal phase with approximately a 5-days overlap with the OCP (Diane 35, Schering, Brasil). Pituitary down-regulation was monitored and patients with adequate pituitary desensitization started their recombinant FSH regime (Gonal-F, Merck-Serono Pharmaceuticals, Italy) and dose of the GnRH-agonist was reduced to 1.0 mg/day. FSH was started with dosages between 150 and 300 IU daily for 4 days, with or without human menopausal gonadotropin (hMG, Menopur, Ferring Pharmaceuticals, Germany). Thereafter, dose of the FSH was individually adjusted according to

the estradiol (E_2) response and vaginal ultrasound findings.

When two follicles reached to ≥16-18 mm, 250 mg, recombinant human chorionic gonadotropin (Ovidrel, Merck-Serono Pharmaceuticals, Italy) was administered and oocyte retrieval occurred 35-36 hours later.

Intracytoplasmic sperm injection (ICSI) was routinely performed in all of the fertilization procedures. Fertilization was evident when two pronuclei were observed. Embryos were cultured until the day of transfer (day 3) in IVF Global® media (Life Global, Canada) supplemented with 10 % synthetic serum substitute (SSS) and graded by Veeck's criteria (24) before transfer.

Veeck's morphological grading system was modified and adopted for day 3 embryo scoring, as follows: grade I=8 cells, blastomeres of equal size and no cytoplasmic fragments; grade II=8 cells, blastomeres of equal size and <20% cytoplasmic fragments; grade III=8 cells, uneven blastomere sizes and no cytoplasmic fragments; and grade IV=4 or 8 cells with >20% fragmentation. The same embryologist performed all embryology and embryo scoring, in this study.

Embryo transfer (ET) number was determined using the Federal Council of Medicine-Brazil (FCM) guidelines. Embryo grade I, II and III was transferred. Luteal phase was supported with micronized P4, 600 mg/day, administered continuously by vaginal route, starting on the evening of ET.

Hormonal measurements and ultrasound scans

On the third day of cycle preceding COH, each woman underwent blood sampling by venipuncture for serum AMH, and FSH measurement and a transvaginal ovarian ultrasound scan was performed for follicle measurement.

Serum levels of AMH and FSH were determined using an automated multianalysis system with chemiluminescence detection (ACS-180, Bayer Diagnostics, Puteaux, France). Serum AMH levels were determined using a second generation enzyme-linked immunosorbent assay. Intra- and inter-assay coefficients of variation were <6% and <10%, respectively, with the lower detection limit at 0.13 ng/mL and linearity up to 21 ng/mL for AMH. For FSH, functional sensitivity was 0.1 mIU/mL, and intra-assay and inter-assay CV were 3 and 5%, respectively. Ultrasound scans were performed using a 3.7-9.3 MHz multifrequency transvaginal probe (RIC5-9H, General Electric Medical Systems, France) by a single operator who was blinded to the results of hormone assays.

The objective of ultrasound examination was to evaluate the number and size of small antral follicles. Follicles measuring of 3-12 mm in mean diameter (mean of two orthogonal diameters) in both ovaries was considered.

To optimize the reliability of ovarian follicular assessment, the ultrasound scanner was equipped with a tissue harmonic imaging system, which allowed improved image resolution and adequate recognition of follicular borders. Intra-analysis CV for follicular and ovarian measurements was <5%, and their lower limit of detection was 0.1 mm. In an effort to evaluate the bulk of granulosa cells in both ovaries, we calculated the mean follicle diameter (cumulative follicle diameter divided by the number of follicles measured 3-12 mm in diameter in both ovaries) and the largest follicle diameter.

Ethical approval

Written informed consent was obtained from all participants before inclusion. The study was approved by Brazilian Institute of Assisted Reproduction Ethical Committee, Brazil.

Statistical analysis

Descriptive parameters and patient characteristics were reported as mean (SD) or median (range) depending on the distribution. Student's t test was performed for continuous variables, Wilcoxon and Pearson's Test were used for not distributed variables and Fisher's Test was performed for categorical variables. $P<0.05$ was considered statistically significant.

Results

Overall, at the time of the investigation, patients had a mean age of 33.03 ± 4.15 years old, body mass index (BMI) 22.78 ± 4.01 kg/m^2, and infertility length of 3.1 ± 2.36 years. 67% of in-

dividuals had regular cycles. On cycle day 3, serum AMH level was 3.50 ± 1.54 ng/mL, serum FSH level was 6.29 ± 1.53 mUI/ mL, at baseline, women had 16.57 ± 7.0 antral follicles. The mean day of stimulation was 12 ± 1:41 days and the mean total dose of drugs was 3382.29 ± 778.06 IU. The mean collected oocytes was 11.80 ± 5.25, metaphase II oocytes was 10.64 ± 5:07, embryo grade I was 0.32 ± 0.63, grade II was 2.14 ± 1.90 embryo, embryo I+II was 2.46 ± 2.11, embryo III was 3.16 ± 2:11, embryo IV was 1.74 ± 2.18, the number of transferred embryo was 2.22 ± 0.61. A greater number (SD) of embryos I + II was observed in young patients (Table 1, Fig.1, P=0,027).

Table 1: Number of embryos (SD)/patients by age

Age	Embryos I+II/ patients
21-24	4.20
25-29	2.83
30-34	2.75
35-39	1.86
40-44	1.38
Total	4.2

We studied Pearson's correlation coefficient for the markers of ovarian reserve. Evaluation of 120 patients showed a significant relationships between age and FSH (r=0.24, P=0.01), age and AMH (r=-0.22, P=0.02), age and collected oocytes (r=-0.23, P=0.03), age and metaphase II oocytes (r=-0.23, P=0.04), age and embryo I+II (r=-0.22, P=0.03), age and the number of transferred embryo (r=0.26, P=0.01, Tables 2, 3, Fig.1). We also determined significant relationships between AFC and AMH (r=0.29, P=0.01), AFC and the number of transferred embryo (r=-0.18, P=0.03), AFC and total dose of the drugs (r=-0.23, P=0.03). Significant relationship was also observed between FSH and total dose of drugs (r=0.19, P=0.02) (Table 3).

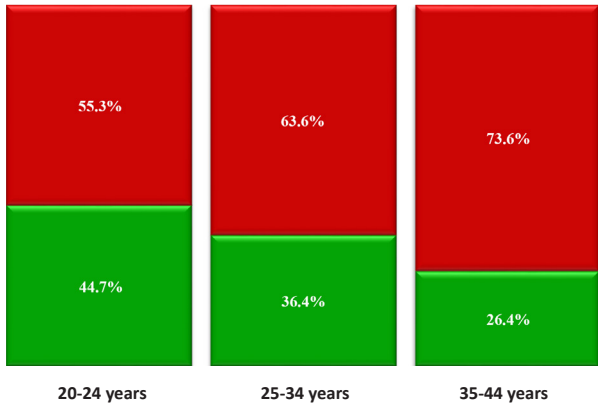

Fig.1: Comparison of different groups. Fisher's Test (P=0.027). Green: embryos I+II and Red: embryos III+IV

Table 2: Spearman's correlation of variables

Variables	Embryos* I	Embryos II	Embryos I+II	Embryos III	Embryos IV
Age	-0.11	-0.21**	-0.22**	-0.12	0.09
FSH	0.00	0.07	0.07	-0.03	0.03
AFC	0.01	0.05	0.05	0.01	0.14
AMH	0.12	0.08	0.11	0.04	0.10

*; Graded by Veeck's criteria, **; Significant<5%, P<0.05, FSH; Follicle-stimulating hormone, AFC; Antral follicle count, and AMH; Anti-Mullerian hormone.

Table 3: Spearman's correlation of variables

Variables	FSH	AMH	Number of collected oocytes	Number of metaphase II oocytes	Number of embryos	Number of embryo transferred	Total dose of drugs' s stimulation
Age	0.24***	-0.22**	-0.23**	-0.23**	-0.13	0.26***	0.04
FSH		0.04	-0.05	0.00	0.02	0.10	0.19**
AFC		0.29***	0.16	0.09	0.11	-0.18**	-0.23**
AMH			0.38***	0.35***	0.19**	-0.13	-0.25***

; Significant<5%, P<0.05, *; Significant<1%, P<0.05, FSH; Follicle-stimulating hormone, AFC; Antral follicle count, and AMH; Anti-Mullerian hormone.

Other significant relationships were between AMH and the number of collected oocytes (r=0.38, P=0.01), AMH and the number of metaphase II oocytes (r=0.35, P=0.01), AMH and the number of embryo (r=0.19, P=0.04), AMH and total dose of the drugs (r=-0.25, P=0.01, Table 3).

Discussion

In this investigation, we have validated the relationship between commonly used ovarian reserve clinical measures and outcome measures.

Our observation, indicating that basal AMH, FSH and AFC are not related to embryo quality, could contribute to an explanation for the low correlation with pregnancy probability, because embryo quality is crucial for clinical success (25).

Although ovarian reserve markers have been shown to have some predictive power in the ART, there is consensus that they provide only general approximations of stimulation quantity (e.g. the number of oocytes retrieved in ART treatment cycles). The major limitations of these tests include their poor sensitivity and, in most cases, dependency on cycle stage. Furthermore, once a woman test is abnormal, her poor prognosis in ART is already established.

Currently, there is no reliable test of ovarian reserve for an individual woman that could accurately predict her remaining reproductive life span. Integration of more than one marker improves the results, and repetition of some markers might be needed.

An interesting point of this study is that average age of the group is 33 years, with 62.5% under 35 years, implicating that perhaps the oocyte quality can be impaired even before the age of 35.

Age was found to be predictive for the number of collected oocytes, number of metaphase II oocytes and embryo quality. This correlation of age and embryo quality could possibly be happened since oocyte is the major determinant of embryo developmental competence in women. It delivers half of the chromosomal complement to the embryo, but the maternal and paternal genomes are neither symmetrical nor equal in their contributions to embryo fate. Unlike the paternal, the maternal genome carries a heavy footprint of parental aging. This marker of ovarian reserve is the single best predictor of reproductive outcome in women, and oocyte is the locus of reproductive aging in women. The incidence of both whole chromosomal nondisjunction and precocious chromatid separation were correlated to maternal aging. Disturbance in sister chromatid cohesion might be a causal mechanism predisposing to premature chromatid separation and subsequently to nondisjunction in female meiosis. In addition, the asymmetry of female meiosis division could favor a nonrandom meiotic segregation of chromosomes and chromatids.

An overall age-related change in the expression of certain genes and proteins, involved in mitochondrial function, was observed in many studies. Mitochondria play a primary role in cellular energetic metabolism, homeostasis, and cell death, while it is directly involved in oogenesis and folliculogenesis. Their functional status influences the quality of oocytes and sperm. It also contributes to the success of fertilization and embryonic development. Oocytes rely on ATP produced by the mitochondria via oxidative phosphorylation to generate energy. In the aging, there is increased mitochondrial DNA damage, a decrease in oxidative phosphorylation and ATP production for oocyte. Furthermore, mitochondrial mutations in follicular cells, surrounding the oocyte, have been correlated with maternal age, suggesting that oxidative phosphorylation in the follicle is compromised (26).

Embryo quality may be affected by oxidative stress (27), but even morphologically normal embryos could show an abnormal number of chromosomes and low pregnancy rates (28). But perhaps the major factor in the etiology of age-related female infertility is decline in the oocyte quality associated with factors including, but not limited to, chromosomal aneuploidy and mitochondrial dysfunction (29, 30). However, the underlying mechanisms still remain poorly understood.

Some limitations of the current investigation should be noted. First, relatively small sample size in this investigation may have limited our ability to demonstrate the additional value of these markers, related to embryo quality. Second, there are many published embryo scoring systems (31-36). Despite the systematic approach of such scoring methods to compare and contrast embryos, em-

bryo morphology and assigning of a grade is, by default and design, a subjective process subject to interobserver and intraobserver variability, although all embryos were evaluated by the same embryologist.

We agree that the force of these clinical results described in a transparent manner are in the non-intentionality to fit a trended question. Instead of this, we took the results in order to analyse the presented data and make some recommendations based on it. The need for more simplified clinical treatments, cost reduction studies and dose of ovarian stimulation in countries without social coverage becomes imminent. Recent studies have aimed their proposal to focus on only markers of embryo quality (including age, AMH) and reduce the use of quantitative response markers (like FSH).

More studies have to be done to improve the accuracy and interpretation of the current ovarian reserve markers to state clear cut-off levels for each marker and find another markers which could more correlate with the number of ova retrieved, embryo quality and clinical pregnancy rate. Determining the etiology of maternal aging on oocyte competence could lead to improve patient care and fertility outcome.

Conclusion

We have demonstrated that commonly used clinical markers of ovarian reserve reflect true ovarian reserve and outcomes measures of ART, while age is the best predictor of embryo quality.

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Original Article

Changes of The Uterine Tissue in Rats with Polycystic Ovary Syndrome Induced by Estradiol Valerate

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Abstract

Background: Polycystic ovary syndrome (PCOS) is one of the most common hormonal disorders that can lead to irregular menstrual cycles and hyperandrogenism. Reduced levels of progesterone and increased estrogen in these women can perpetually stimulate the endometrial tissue of the uterus. In this study, we assess the effect of PCOS induction by estradiol valerate (EV) in a rat model.

Materials and Methods: In this experimental study, adult female Wistar rats that weighed approximately 200 g were divided into control, sham, and experimental groups (n=6 per group). The experimental group received subcutaneous injections of 2 mg EV for induction of PCOS. We confirmed the presence of PCOS in the experimental group rats. Rats from all groups were subsequently killed, after which their uteri were removed and fixed for histological and cytological analyses. The uterine tissue sections were stained with hematoxylin and eosin (H&E) and iron hematoxylin (iron-H). We examined epithelium height, thickness of the uterus wall, and frequency of the mitotic cells. The data were assessed at α=0.05.

Results: Uterine tissue findings from the experimental group showed significant increases in the height of the uterus luminal epithelium, the thickness of the uterus wall, and the frequency of eosinophils in the endometrial stroma. We observed an increased frequency of mitotic cells in the experimental group in both luminal and glandular epithelia of the uterus. An increased rate of the glandular epithelium region was noticeable and significant.

Conclusion: Induction of PCOS by EV could change the proliferation rate in the endometrial tissue of the uterus.

Keywords: Uterus, Estradiol Valerate, Polycystic Ovary Syndrome, Mitosis, Rat

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Introduction

Polycystic ovary syndrome (PCOS) is a hormonal imbalance disorder (1, 2) that occurs in approximately 4-18% of reproductive-aged women (12 to 45 years) (3). PCOS is a metabolic and reproductive disorder with characteristic features that include hyperandrogenism, irregular menstrual cycles, insulin resistance, obesity, hirsutism, and acne (4). Anovulation that results from PCOS is the most common cause of infertility in women (5). Features of PCOS may manifest at any age and range from childhood (premature puberty), teenage (hirsutism, menstrual abnormalities), early adulthood and middle life (infertility, glucose intolerance), to later life (diabetes mellitus and cardiovascular diseases) (6).

Numerous evidences affirm the fact that endocrinologic and metabolic abnormalities in PCOS may have complex effects on endometrial tissue, thus contributing to infertility and endometrial disorders in women with this syndrome (7). Long-term PCOS increases the risk of hyperplasia, endometrial cancer (EC), and metabolic syndrome (8). Endometrial hyperplasia is a premalignant condition that usually heralds EC (9). It has been reported that women with PCOS and endometrial hyperplasia have a four times greater risk of developing EC than women without PCOS (10). Hyperplasia and uterine cancer have been observed in women with

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PCOS who received no treatment (11).

The two main types of EC are estrogen-dependent type I and estrogen-independent type II (12). It is widely believed that PCOS is one of the most impressive risk factors that promote type I EC (10, 13, 14). Prolonged exposure of the endometrium to estrogen, as a consequence of anovulation, is suggested to be the prime cause of this increased risk (15). Therefore, the hormonal imbalance associated with PCOS can alter endometrial tissue homeostasis and promote cell proliferation (16). In humans, continuous exposure of the endometrium to estrogen can lead to endometrial hyperplasia (17). Progesterone acts as a protective factor against estrogen-driven uterine growth and proliferation (18).

Steroid hormone levels regulate the cycle of cellular proliferation and apoptosis in the endometrial tissue. Therefore, a firm balance between these two processes would secure the normal function of the endometrium (19). Endocrine-metabolic situations associated with abnormalities in plasma hormone concentrations, as seen with PCOS, can affect the processes that occur in the endometrium, which includes cell proliferation, differentiation and response to biological stimuli (20). Estrogen is a hormone that affects the uterus. Strong activation of proliferative activity is the most important physiological effect of estrogen hormones in the uterus (21). Significant consequences of (particularly long-term) endometrial exposure to estrogen are morphogenetic alterations that include modified type of luminal and glandular epithelia, glandular shape, and the glandular to stromal ratio (22, 23).

Estradiol valerate (EV) is used to create PCOS by inducing hormone abnormalities (24). EV, which is introduced as a prodrug, is an ester derived from 17β-estradiol. EV is normally cleared in blood plasma and the liver into 17β-estradiol by esterase activity (25). The 17β-estradiol metabolizing procedure includes an array of reversible and non-reversible enzyme-mediated reactions (26). The metabolites 17β-estradiol and estron may predict the risk of breast (27) and other hormone-related cancers (28). Studies show that hormonal abnormalities attributed to EV can create a phenotype similar to PCOS (29). In this study we focus on tissue changes and proliferation activity of the uterus in a rat model of PCOS induced by EV.

Materials and Methods

Animals

The present experimental study used 18 adult female Wistar rats that weighed 200 ± 20 g. Animals were obtained from the Pharmacology Department of Tehran University and maintained in special cages under standard conditions of 22ºC, a 12-hour dark/light cycle, and free access standard chow and water. In order to conduct a comparative evaluation, we divided the rats into three groups of 6 animals per group: control (normal rats), experimental group or PCOS (rats that received EV), and sham (rats that received EV solvent). Before the induction, we confirmed the rats' normal estrous cycles through daily vaginal smears over two weeks. Animals that had at least two normal estrus cycles were selected for PCOS induction.

The Ethics Committee of the Biological Sciences Faculty at Kharazmi University, Tehran, Iran approved this study.

Induction of polycystic ovary syndrome

We used EV to induce the polycystic condition. Each experimental rat received 2 mg of EV, dissolved in 0.2 ml sesame oil, through a single subcutaneous injection at the inguinal region. Rats in the sham group received an equal volume of sesame oil. Subsequently, vaginal smears of these rats were monitored for 60 days, until the time when abnormal estrus cycles and persistent vaginal cornification (PVC) occurred as a sign of the presence of ovarian cysts and early confirmation of PCOS induction (30). Rats in the sham group that received sesame oil showed no evidence of abnormalities in estrus cycles or vaginal smears. Hence, further experiments were concentrated mainly on control and PCOS rats.

Histological and cytological studies

On the 60th day after the EV injection, rats from all groups were sacrificed and the uterine specimens were fixed in 10% formaldehyde. The tissue samples were dehydrated by graded series of ethanol, embedded in paraffin, then sectioned into 5-7 µm sections prior to microscopic analysis.

Histological evaluations of the uterus and determination of mitosis

As mentioned, the effect of estrogens on the uterus

tissue is chiefly related to its strong invigorating impact on cell proliferation. Long-term exposure to estrogen leads to uterus endometrium overgrowth and hyperplasia. We used hematoxylin and eosin (H&E) in addition to iron hematoxylin (iron-H) staining to conduct in-depth assessments of histological changes, the occurrence of mitosis, and proliferating cells.

For histological evaluations, tissue sections were stained with H&E. We measured the height of the epithelial cells, uterus wall thickness, accumulation of uterine glands, and the number of eosinophil cells in the uterine stroma as visualized by a light microscope at ×100, ×400, and ×1000 magnifications. The longitudinal measurements were obtained by Microstructure Measurement software ver.1.04 (Scalor, Crop Toky, Japan).

For iron-H staining, we stained the tissue sections with Heidenhain's iron hematoxylin color. In this hematoxylin solution, iron salts are used both as an oxidizer and a mordant. This staining method can be used to demonstrate numerous structures, such as nuclear chromatin, according to the degree of differentiation (31). This staining method shows the presence of cells during the mitotic cycle. In order to measure the percentage ratio of proliferating cells to the total number of epithelial cells, we separately counted both the total and mitotic cell numbers in the uterus luminal and glandular epithelia in 10 microscopic fields of view for each tissue specimen at ×1000 magnification with a light microscope. Overall, we assessed 4359 cells.

Statistical analysis

Comparative assessments of the aforementioned parameters are reported as mean ± SE. Assessment between PCOS and the control group was performed through one-way ANOVA (Tukey post hoctest) by SPSS Statistics software ver. 20.0 (IBM), at α=0.05. Charts were drawn with Excel software.

Results

Histology of the uterus

Microscopic study of the uterine tissue in the group treated by EV (PCOS) showed an increase in luminal epithelium height, accumulation of endometrial glands and their luminal diameter, and also the number of eosinophils in the endometrial stroma (Fig.1). Statistical comparison among the groups also revealed that luminal epithelium height and the thickness of the uterine wall in the PCOS group increased significantly compared to the control group (Fig.2A, B, Table 1). In addition, the percentage of eosinophils significantly increased in the experimental group (Fig.2C, Table 1).

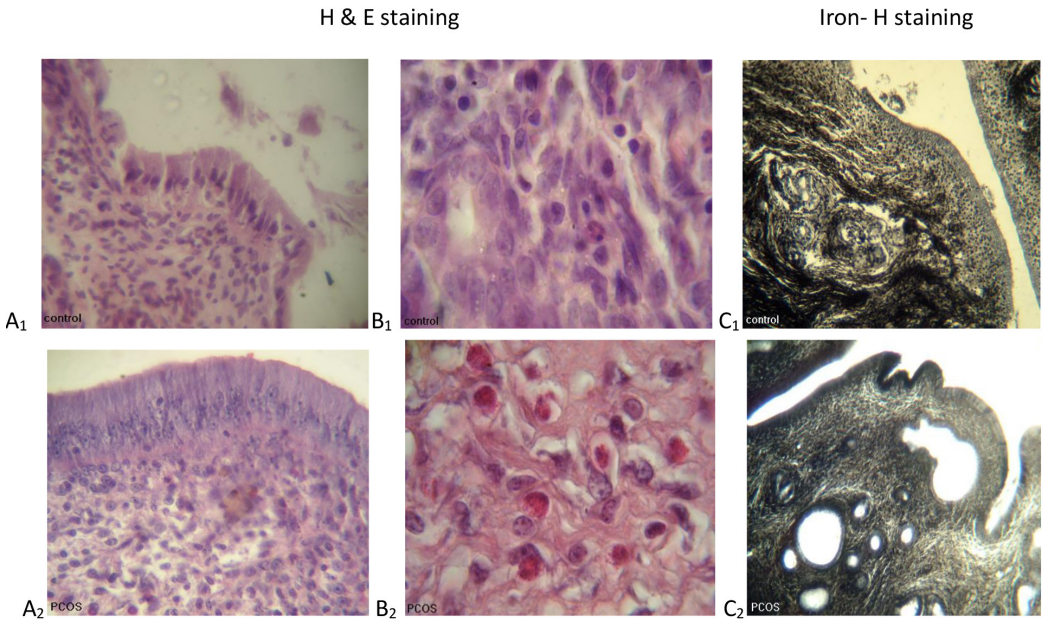

Fig.1: Histological sections of the uterus from control and estradiol valerate (EV)-treated polycystic ovary syndrome (PCOS) rats following hematoxylin and eosin (H&E) and iron hematoxylin (iron-H) staining. **A_1, A_2**. The uterine epithelium (×400), **B_1, B_2**. Eosinophil cells in the endometrial stroma (×1000), and **C_1, C_2**. The endometrial glands (×100).

Table 1: The height of the epithelial cells, uterine wall thicknesses, and the numbers of eosinophil cells in uterine stroma in control and estradiol valerate (EV)-treated polycystic ovary syndrome (PCOS) rats

Group	Cell height (µm)	Wall thickness (µm)	Eosinophils (%)
Control	31.81 ± 3.38	781.11 ± 53.59	5.49 ± 3.01
PCOS	48.57 ± 2.81*	989.96 ± 22.07†	21.06 ± 4.97*

Values are mean ± SE. *; P<0.05 and †; P<0.01.

Researchers have reported that eosinophilic infiltration may be under the control of different hormones in rats. Eosinophilic infiltration is dependent upon the continued presence of elevated levels of estrogen in the blood and 17β-estradiol stimulates eosinophilic invasion (32). Therefore, in the present study, we have documented changes in the numbers of eosinophils after the injection of EV as a hormonal mechanism.

Proliferation

We examined and counted the epithelial cells in order to assess the frequency of mitotic cells in uterine luminal and glandular epithelia among the samples stained with iron-H. The numbers of mitotic cells were compared to the total numbers of epithelial cells. We assessed a total number of 2164 cells in the uterine luminal and 2195 cells in the glandular epithelia. A comparison between the groups revealed that the PCOS group had a nonsignificant increase in percentage of mitotic epithelial cells in the luminal region (Fig.3A, Table 2). On the other hand, the percentage of mitotic cells increased significantly in its glandular counterpart (Fig.3B, Table 2). Animals that received EV had remarkably more mitotic cells compared to animals in the control group (Fig.3C, D).

Table 2: Percentage of mitotic cells in luminal and glandular epithelia in control and estradiol valerate (EV)-treated polycystic ovary syndrome (PCOS) rats

Group	Luminal epithelium (%)	Glandular epithelium (%)
Control	0.97 ± 0.49	0.00 ± 0.00
PCOS	1.82 ± 0.67	9.86 ± 3.53*

Values are mean ± SE. *; P<0.001.

It can be concluded that, as a sign of proliferation, the increase in numbers of mitotic cells leads to the development of a uterus with a thicker wall and dilated glands. This result can be considered as an overture for hyperplasia.

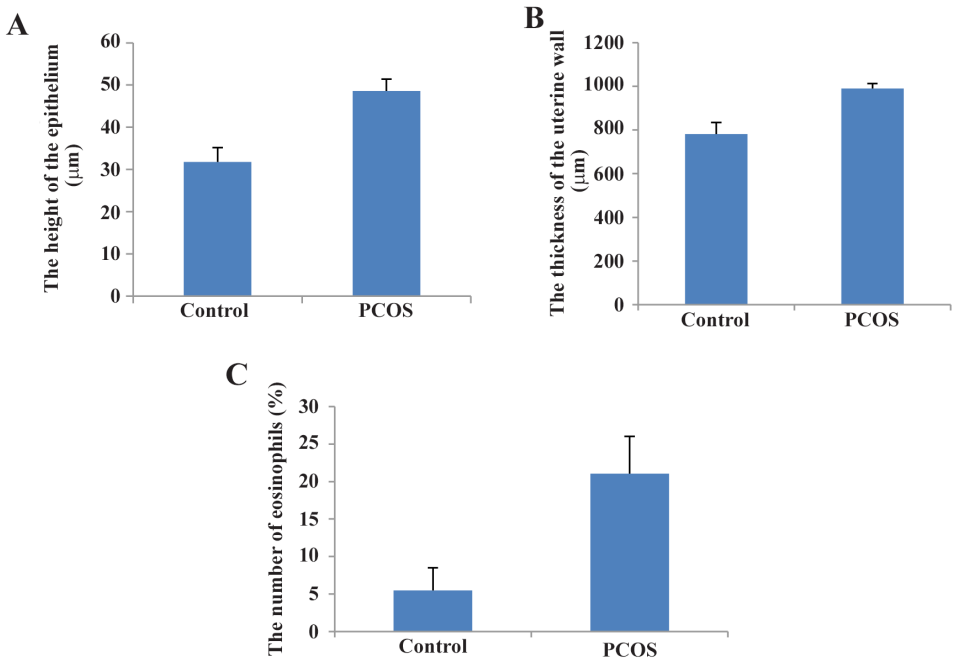

Fig.2: Statistical comparison between control and estradiol valerate (EV)-treated polycystic ovary syndrome (PCOS) rats. **A.** The height of the uterine epithelium (P<0.05), **B.** The thickness of the uterine wall (P<0.01), and **C.** The number of eosinophil cells in the endometrial stroma (P<0.05).

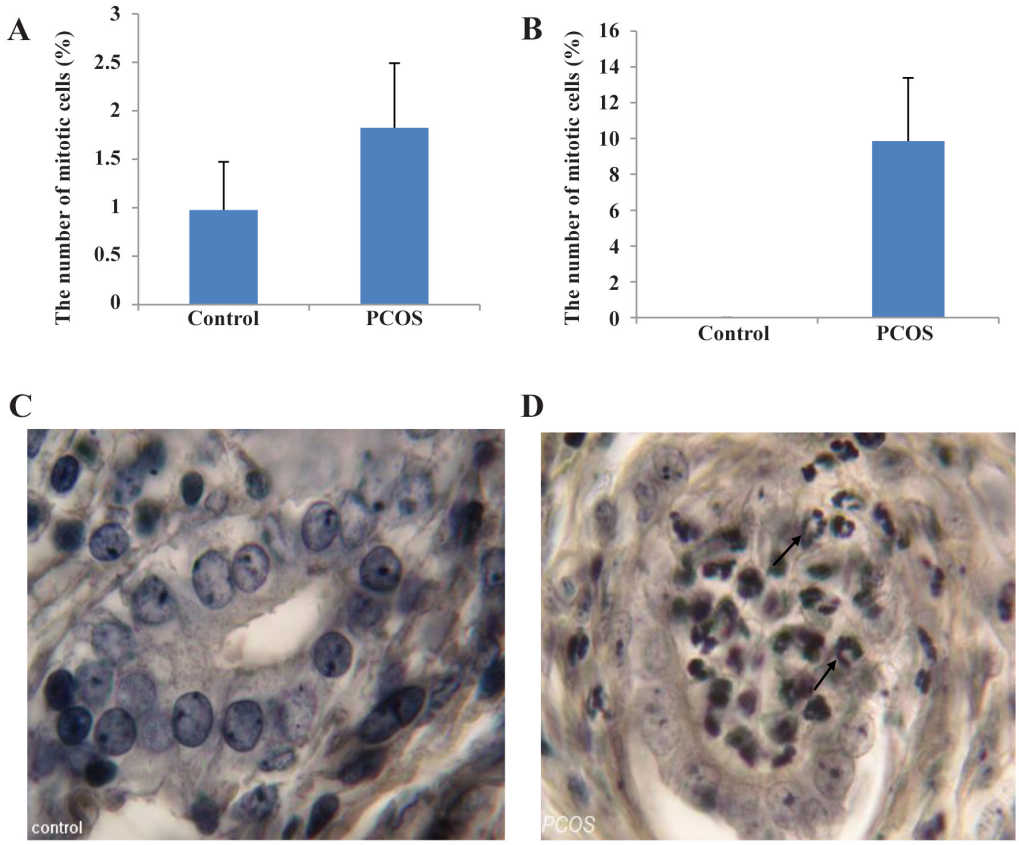

Fig.3: Up-Statistical comparison between control and estradiol valerate (EV)-treated polycystic ovary syndrome (PCOS) rats. **A.** The number of mitotic cells in the luminal epithelium (P<0.05), **B.** The number of mitotic cells in the glandular epithelium (P<0.001). Down-Histological sections of the uterus from control and EV-treated PCOS rats following iron hematoxylin (iron-H) staining, **C**, and **D.** The glandular epithelium (×1000). ↗; Mitotic cells

Discussion

The present study assessed the proliferative activity and histological changes in uterine tissue of an EV treated PCOS female rat model. Histological observations of uterine tissue sections showed a statistically reasonable increase in wall thickness of the uterus of EV treated (PCOS) rats in comparison with the control group. There was a significant rise in the average of the height of epithelial cells in PCOS rats compared to normal control rats. It has been shown that estrogen mediated stimulation of the uterus results in morphogenetic changes that include alterations in the type and morphology of luminal and glandular epithelia (24, 25). Similarly, the current study has proven that the stromal uterine glands of PCOS rats have larger luminal space and higher accumulation. *In vitro* studies of radiothymidine uptake by endometrium suggest that the maximal proliferation in uterine glands and stroma is chiefly associated with high concentrations of estradiol (33) and that ovarian steroids are among the most significant factors that affect both morphology and motility of the uterus (34). The results of this study have also supported the idea that noticeable changes in the epithelial surface, gland accumulation, and overall thickness of the uterus wall due to an abnormality at the level of ovarian steroids.

Based on the results, we observed a significantly higher eosinophil quantity in the endometrial stroma in the experimental group compared to the control rats. Experiments on the effect of hormonal perturbations on reproductive tissues suggested that the leukocyte invasion into these tissues have mainly occurred under hormone control. Eosinophil invasion is related to the continued presence of elevated blood estrogen levels as it is stimulated by estrogen (35). It has been reported that the immune system and inflammation are involved in the pathophysiological process of PCOS

(36). Additionally, polymorphonuclear leukocyte infiltration may be relevant to an immunological process (37).

Results of the changes in proliferative activity in various regions of the uterus tissue showed a higher percentage of mitotic cells in luminal and glandular epithelia among rats of the experimental (PCOS) group compared to control rats. Estrogen has been well recognized as a strong factor which intensifies the proliferative activity of the uterus, with its major impact on uterine tissue (38, 39). The maximal proliferation in uterine glands and stroma occurs in the presence of high levels of estradiol (36). Studies have shown that the mitotic activity of estrogen in the endometrium of rodents is restricted to the luminal and gland neck epithelia (40, 41). In response to estrogen injection into ovariectomized mice, mitotic activity is first observed in the luminal, followed by the glandular region, while progesterone application can inhibit the mitotic response (42). Luminal epithelia have been suggested to undergo proliferation in the presence of 17β-estradiol (43, 44). In this study, we have shown that while mitotic activity was, to some extent, elevated in luminal epithelia in PCOS rats that received EV, this was not a statistically significant finding compared to the control group. We found that EV administration in PCOS rats had a surge in mitotic proliferation in the uterine glandular epithelia, which provided a probable explanation for the enlarged glands and thickened uteri wall.

Increased estrogenic environment may favor mitogenic activity in the breast and/or other reproductive tissues (45, 46). Estrogens lead to a reduction in the duration of all the stages of the cell cycle and drive cells from the G0 to the G1-phase; this is followed by an increase in the number of cells in passing the G1-and S- phases, as well as the quantity of dividing cells (47-49). Hyperplasia is an early response to an abnormal stimulation in the cell proliferation process which leads to an increase in the numbers of cells. Hyperplasia can cause the organ size to increase. It has been suggested that the development of estrogen related morphogenetic changes in the uterus can be considered as an early step towards endometrial hyperplasia and cancer (50). The persistent stimulation of endometrial tissue by estrogen (mainly estrone) in PCOS patients without the progesterone-induced inhibition leads to uterine hyperplasia as a preliminary step to carcinoma (1). Cellular proliferation and apoptosis in the human endometrial tissue take place in a cyclic procedure as they are regulated by steroid hormone levels (20). In the normal endometrium, pro-apoptotic and anti-apoptotic factors are under fine regulation that leads to tissue homeostasis which can be disturbed by hormonal alterations (51, 52). The uterus response to estrogen requires changes in the expression of genes whose products regulate successive and functionally interlinked cellular processes. Researchers suggest that the earliest changes after 17β-ethinyl estradiol treatment occur in the expression genes whose products are involved in transcriptional regulation and signal transduction, followed by those involved in mRNA and protein synthesis, cell cycle regulation, DNA replication, cell proliferation and differentiation, apoptosis, tissue remodeling, and immunological responses (53).

Conclusion

Administration of EV to induce an animal model of PCOS caused changes in epithelial height, uterus wall thickness, and the quantity of eosinophil cells. Additionally, PCOS rats showed considerably higher rates of proliferation in the glandular epithelium region of their uteri. Hence, it could be concluded that excessive estrogen content attributed to EV administration, caused an increase in the mitogenic activity of the uterus, which could be a prologue to endometrial hyperplasia and carcinoma.

Acknowledgements

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Original Article

Bull Fertility and Its Relation with Density Gradient Selected Sperm

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Abstract

Background: Sperm selection method is usually used to collect these cells for *in vitro*-assisted reproduction. Few studies reported the relationship of *in vivo* fertility and semen parameters after sperm selection; hence, the present study attempted to assess different semen parameters after post-thaw or sperm selection, using density gradient separation BoviPure®, to predict *in vivo* fertility.

Materials and Methods: In this experimental study, frozen semen quality of four Montbeliarde bulls were assessed after post-thaw (PT) or after sperm selection (SSp), using density gradient separation BoviPure®, to predict the fertility rate *in vivo*. In addition to PT or SSp, semen was examined for concentration, motility, morphology abnormalities, viability, acrosome and plasma membrane integrities. Fertility was measured as non-return rates within 56 days after the first insemination (NRR) or as corrected NRR, expressed as CNRR, to the factors influencing fertility using linear mixed model. Non-parametric Kruskal-Wallis test was performed to compare semen parameter variables. Fertility rates were compared using Chi-square test. Pearson correlation analysis was used to test the relationship between CNRR and semen parameters. Data was analysed using SPSS package program, version 21.0.

Results: Most of the examined bulls exhibited a high fertility rate (3/4 bulls, 62.1-81.8% for NRR or 67.2-98.5% for CNRR). Fertility rate, expressed as CNRR, was significantly related to semen parameters after SSp, but not after PT. Thus, CNRR was increased with decrease of total motility, progressive spermatozoa and abaxial implantation frequencies after SSp (r=-0.999, P=0.001; r=-0.990, P=0.010; r=-0.988, P= 0.012, respectively); while, CNRR was decreased with decrease of SSp immotile spermatozoa (r=+0.995, P=0.005), underlying that maximal limit of determined immotile spermatozoa is 47%.

Conclusion: High frequencies of total and progressive motility spermatozoa, and abaxial implantation in gradient selected sperm appear to be not favorable for fertility *in vivo*.

Keywords: Frozen Semen, Fertility, Bull

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Introduction

Artificial insemination (AI) is the cheapest and most applicable method of reproductive biotechnology around the world to select superior genetic of sires and dams (1). In semen production centers, quality control should be considered before selling it to livestock producers (2). Considering that usage of AI allows semen from one bull to be used for insemination into thousands of females, bull effects are paramount on herd genetics, dynamics, and production. Use of sperm from a low fertility (or infertile) bull leads to lower pregnancy rates, which then results in greater economic costs of housing these bulls and non-pregnant cows (3). Even though semen assay *in vitro* would be of great benefits in AI programs for determining bull fertility, it is unlikely feasible to evaluate a single sperm characteristic reflecting the real sperm fertilization capacity of the semen sample.

Until now, no single laboratory test was able to accurately predict *in vivo* fertility; hence, potential bull fertility can be estimated from laboratory semen assessment with higher accuracy when a combination of several sperm analyses are performed *in vitro* (4). However, spermatozoa require capacitation before fertilization; mammalian spermatozoa must undergo epididymal maturation, capacitation and the acrosome reaction to fertilize an oocyte. Capacitation is possible even *in vitro* in the absence of reproductive-tract fluids and several compounds are known to induce capacitation *in vitro*. During capacitation, several biochemical modifications occur on the sperm surface; such changes are essential in permitting sperm-oocyte binding and the acrosome reaction (5).

In the mid-1980s, it was not always clear, how specific sperm procedures impacted sperm to enhance *in vitro* fertilization (IVF) in the bovine. Effect could have been on capacitation, acrosome reaction, or both. Compositions of different media are used in oocyte handling, sperm preparation and IVF (6). Sperm selection is a term with many interpretations; however, it is generally used to describe methods for separation of spermatozoa for *in vitro*-assisted reproduction (1). The techniques of "swim-up" and "swim-down" were often used for sperm selection after washing by extension and centrifugation, filtration/gradient separation, or self-motility (7).

Many scientists investigated the relationship between post-thaw sperm parameters and fertility (8-15); but, few studies reported results on the relationship between fertility *in vivo* and semen parameters after selection of fertile spermatozoa (15, 16). Thus, our research attempted to assess different semen parameters after post-thaw (PT) or after sperm selection (SSp), using density gradient separation BoviPure®, to predict fertility *in vivo*.

Materials and Methods

Chemicals

All of the used chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Semen source and examination

In this experimental study, frozen semen quality of four Montbeliarde bulls (1-4) was examined. The examined straws were provided from the same batch which was used for AI and purchased from National Center for AI and Genetic Improvement (Algiers, Algeria).

For each bull, four straws from one freezing batch, two straws after PT and two straws after SSp, were assessed for concentration, motility, morphology abnormalities, viability, acrosome and plasma membrane integrities.

Semen straws were thawed for AI analysis at 37°C for 30 seconds, to assess different sperm parameters. As few spermatozoa are available after SSp, some manipulations were adapted to have enough spermatozoa for this observation. All sperm parameters were performed by the same operator.

Density gradient selected sperm

Sperm selection was performed using a commercial product BoviPure® (Nidacon Laboratories AB, Göothenborg, Sweden) according to manufacturer's instruction. In sterile graduated centrifuge tube of 10 ml, 2 ml of BoviPure 80% Layer was placed. Next, 2 ml of BoviPure 40% was carefully added and incubated at 37°C. After thawing semen, straw was cut in one side and fixed in syringe, followed by cutting the

second side. Semen was placed gently on the prepared gradient of BoviPure®. After centrifugation for 15 minutes at 300x g, supernatant was carefully removed up to 0.3 ml, and remaining semen suspension was subsequently mixed and evaluated.

Motility evaluation

Thawed semen was diluted 1:4 in pre-warmed phosphate buffer saline (PBS, NaCl 0.138 M, KCl 0.0027 M, pH=7.4) containing freshly prepared 1% bovin serum albumin (BSA) in our laboratory. Spermatozoa were incubated at 37°C for 3 minutes, before motility assessment, in the laboratory of semen production of Wallonne Breeding Association (AWE, Belgium).

For assessment of individual motility, one drop was placed between slide and coverslip and observed on Sumsung monitor PC via a 295 camera Leica connected to a trinocular phase-contrast Leica DM 1000 microscope (Germany), equipped with a Leica warm stage (37°C). Spermatozoa were examined at magnification of ×400 after PT or ×200 after SSp. A total of 200 spermatozoa were observed in at least 10 different fields, each spermatozoa was categorised in one of the following three motility classes: progressive, non-progressive or immotile spermatozoa. The proportion of each motility class was then calculated regarding the total number of spermatozoa. Total motility frequency is the sum of progressive motility and non-progressive motility frequencies.

Sperm concentration

An aliquot of thawed semen was diluted 1:20 in 1% formaldehyde solution. Spermatozoa were counted in duplicate using a hemocytometer.

Examination of spermatozoa viability and morphology

Viability and morphology of spermatozoa were assessed by mean of eosin-nigrosin staining. The stain was prepared using 3.3 g of eosin Y, 20 g nigrosin, 1.5 g sodium citrate and they were dissolved in 300 ml of warmed distilled water adjusting to pH=6.8-7. The stain was then filtered and preserved at 4°C (17, 18).

Two drops (40 µl) of thawed semen were mixed with one drop (20 µl) eosin-nigrosin on the pre-warmed slide and incubated for 2 minutes at 37°C. A thin smear was made and air dried. At least 200 spermatozoa were observed under bright field and oil immersion (magnification: ×1000) using Leica DM 1000 phase contrast microscopy.

Abnormal spermatozoa were classified, according to the guideline of the previous report (19): primary abnormalities (proximal cytoplasmic droplets, pyriform heads, strongly folded or coiled tails, midpiece defects, maldeveloped, and craters), and secondary abnormalities (distal droplets, tailless heads, simple bent or terminally coiled tails, small or giant heads, abaxial implantations, and abnormal acrosomes) (17, 20). However, frequency of abnormal acrosome was assessed in other smear, according to description in the following procedure.

Hypo-osmotic swelling test and acrosomal evaluation

Frequencies of normal acrosome and positive hypo-osmotic swelling (HOS) test for spermatozoa were determined as previously reported (21). In addition, plasma membrane integrity of spermatozoa was assessed using HOS test (22). HOS solution was prepared by dissolving 0.735 g sodium citrate and 1.351 g fructose in 100 ml distilled H_2O (23).

For assessment of positive spermatozoa HOS test after PT or SSp, 30 µl of semen sample was mixed to 300 µl pre-warmed HOS solution and incubated at 37°C for 45 minutes. After incubation, a drop of semen sample was placed in clean slide, covered with cover slip and examined under phase-contrast microscope. Positive spermatozoa for HOS were considered as coiled tail, due to intact plasma membrane. A total 200 spermatozoa were counted in different fields and percentage of positive spermatozoa for HOS test was then determined.

However, assessment of normal acrosome was performed by fixing 50 µl of semen sample in 500 µl (PT) or 250 µl (SSp) of 1% formal citrate containing 2.9% (w/v) trisodium citrate dehydrate before capacitation *in vitro* (24). Thick smear was performed and at least 200 spermatozoa were observed at ×1000 magnification under oil immersion

using a Leica DM 1000 phase-contrast microscopy to determine the frequency of normal apical ridge spermatozoa.

Data collection and fertility measures

In this research, animal care protocol and all used procedures were approved by Algerian animal welfare laws and policies (law 88-08 of 1998, article 58).

In Algeria, dairy cattle are mostly present as small herds. Data were collected from dairy farms situated in Setif region, North-Eastern part of Algeria, involving 110 inseminations. Montbeliarde cows were inseminated after oestrus observation and estrus-insemination interval (EI) was then recorded for each cow. All cows included in this study were inseminated between 14.11 and 15.25 hours after estrus observation according to the routine insemination (12-24 hours) from estrus onset to avoid altering fertility. Inseminations were realized in both season (summer and fall), where maximum temperatures ranged between 44 and 45°C in summer and from 23 to 36°C in fall. Fertility was measured as non-return rates within 56 days after the first insemination (NRR) or as corrected NRR (CNRR) when NRR was statistically corrected for the factors influencing fertility.

Statistical analysis

Semen variables are presented as means ± standard error and fertility as frequencies. Homogeneity of variance was examined by Levene's test. As variances were unequal, non-parametric Kruskal-Wallis test was performed to compare semen parameter variables. Fertility was analysed as binary trait (yes or no) and compared using Chi-square test. NRR was collected and linear mixed model was conducted to correct NRR, expressed as CNRR, to the following factors: cow age (<3, 3-4 and >4 years), parity (1, 2 and ≥3), inseminator (1, 2), season (summer, fall) and proven AI service (4 bulls). Pearson correlation analysis was performed to test the relationship between CNRR and semen parameters; data normality was checked with Kolmogorov-Smirnov test.

Differences were considered significant when $P<0.05$ and trends were discussed when $P<0.10$. All statistical analyses were performed using SPSS package program, version 21.0.

Results

Sperm motility and concentration

Table 1 shows no significant difference between bulls for PT sperm concentration values and different motility frequencies. For SSp, semen concentration remains similar between bulls; the lowest total and progressive motility frequencies were observed in the bull 1 compared to the others, albeit these evident decreases are not statistically significant.

Table 1: Semen concentration and motility (means ± SE) after post-thaw and selected sperm

			Motility frequencies (%)			
	Bulls (n)	**Concentration ($\times10^6$)/Straw**	**Total motility**	**Progressive**	**Non progressive**	**Immotile**
Post-thaw	1 (2)	25.71 ± 2.29	38.69 ± 5.46	29.57 ± 3.63	9.12 ± 1.83	61.31 ± 5.45
	2 (2)	24.13 ± 2.63	43.10 ± 6.20	31.10 ± 4.47	12.00 ± 1.73	56.90 ± 6.20
	3 (2)	22.37 ± 0.32	39.38 ± 1.55	30.51 ± 1.59	8.87 ± 0.04	60.62 ± 1.55
	4 (2)	30.79 ± 1.21	45.42 ± 1.37	35.82 ± 1.66	9.60 ± 0.28	54.49 ± 1.28
P values		0.160	0.682	0.367	0.321	0.475
Selected sperm	1 (2)	8.44 ± 1.35	53.34 ± 1.49	40.57 ± 1.03	12.77 ± 0.46	46.66 ± 1.49
	2 (2)	9.88 ± 0.74	77.57 ± 0.29	63.36 ± 2.14	14.24 ± 1.85	22.40 ± 0.28
	3 (2)	6.68 ± 0.39	72.85 ± 2.07	58.93 ± 2.27	13.92 ± 0.20	27.15 ± 2.07
	4 (2)	5.86 ± 0.15	62.73 ± 1.67	52.68 ± 3.26	10.05 ± 1.600	37.53 ± 1.41
P values		0.104	0.083	0.139	0.160	0.083

n; Number of straws from one freezing batch.

Morphology abnormalities, hypo-osmotic swelling test positive and viable spermatozoa

There was no significant difference between bulls after neither PT nor SSp in the different morphology abnormality classes, HOS positive test and viable spermatozoa frequencies (Table 2).

Fertility

Data of fertility (NRR or CNRR) are presented in Table 3. Field fertility of different bulls was varied widely, from low (51.9%) to high (62.1-81.8%) for NRR and from low (57.8%) to high (67.2-98.5%) for CNRR; so that fertility is the highest in the bull 1 and the lowest in the bull 2. Although NRR differences tend toward significance between different bulls (P=0.067), a significant difference in CNRR between bulls was observed (P=0.018).

Our results presented in Table 4 show that CNRR is negatively correlated to the following SSp parameters: total motility, progressive spermatozoa and abaxial implantation frequencies, respectively (r=-0.999, P=0.001; r=-0.990, P=0.010; r=-0.988, P= 0.012). A negative correlation trend was determined between frequency of acrosome abnormality and CNRR (r=-0.931, P=0.069). In contrary, CNRR were positively correlated to immotile spermatozoa frequency (r=+0.995, P=0.005).

Table 2: Percentage of morphology abnormalities. Hos-positive and viable spermatozoa (means ± SE) after post-thaw and selected sperm

	Post-thaw sperm (%)					Selected sperm (%)				
Bulls (n)	**1(2)**	**2(2)**	**3(2)**	**4(2)**	**P**	**1(2)**	**2(2)**	**3(2)**	**4(2)**	**P**
Proximal cytoplasmic droplets	0.00 ± 0.00	0.20 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.392	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.000
Pyriform heads	0.23 ± 0.23	0.44 ± 0.44	0.39 ± 0.39	1.80 ± 0.99	0.344	2.51 ± 0.32	2.81 ± 0.73	1.25 ± 0.87	4.16 ± 2.61	0.608
Strongly folded/coiled tails	0.23 ± 0.23	0.44 ± 0.44	0.39 ± 0.39	1.80 ± 0.99	0.344	0.62 ± 0.26	0.92 ± 0.09	1.21 ± 0.07	2.17 ± 1.01	0.129
Midpiece defects	0.45 ± 0.45	0.88 ± 0.88	0.97 ± 0.19	1.77 ± 1.46	0.809	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.000
Maldeveloped	0.00 ± 0.00	0.44 ± 0.44	0.00 ± 0.00	0.00 ± 0.00	0.392	0.00 ± 0.00	0.67 ± 0.16	0.00 ± 0.00	0.19 ± 0.19	0.116
Craters	0.22 ± 0.22	4.24 ± 1.45	0.00 ± 0.00	1.22 ± 0.72	0.161	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.000
Primary abnormalities	1.13 ± 0.20	6.66 ± 0.56	2.18 ± 0.17	5.36 ± 0.52	0.083	3.13 ± 0.06	4.39 ± 0.66	2.47 ± 0.94	6.52 ± 3.44	0.367
Distal droplets	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.000	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.000
Tailless heads	1.14 ± 0.21	1.50 ± 0.27	1.77 ± 0.99	1.27 ± 0.34	0.908	3.89 ± 2.14	0.26 ± 0.26	1.41 ± 0.13	0.79 ± 0.02	0.083
Simple bent/terminal coiled tails	1.82 ± 0.89	1.26 ± 0.38	2.53 ± 0.96	1.52 ± 0.90	0.682	1.94 ± 0.19	2.9 ± 1.66	3.47 ± 0.8	1.55 ± 1.15	0.682
Small/giant heads	3.45 ± 0.75	6.22 ± 1.30	6.44 ± 0.24	4.40 ± 1.17	0.212	5.87 ± 0.17	7.47 ± 7.47	3.38 ± 0.83	11.54 ± 1.98	0.446
Abaxial implantations	0.24 ± 0.24	0.87 ± 0.46	1.95 ± 0.38	0.20 ± 0.20	0.148	0.18 ± 0.18	1.38 ± 0.14	1.26 ± 0.88	0.59 ± 0.19	0.198
Abnormal acrosomes	12.54 ± 2.58	10.32 ± 1.48	16.46 ± 1.68	13.15 ± 0.27	0.244	7.75 ± 1.83	26.32 ± 3.05	15.98 ± 0.57	14.81 ±2.31	0.112
Secondary abnormalities	19.17 ± 2.46	20.17 ± 0.18	29.13 ± 1.80	20.54 ± 0.00	0.193	25.89 ± 4.62	47.11 ± 7.17	30.41 ± 3.17	42.32 ± 1.26	0.139
Total abnormalities	20.30 ± 2.25	26.83 ± 0.73	31.31 ± 1.63	25.90 ± 0.52	0.104	29.02 ± 4.68	51.5 ± 6.51	32.87 ± 4.1	48.85 ± 4.7	0.129
Hos-positive spermatozoa	36.68 ± 5.68	43.41 ± 2.77	39.38 ± 10.2	52.08 ± 6.25	0.539	64.51 ± 1.88	72.97 ± 0.48	62.71 ± 1.17	61.40 ± 4.32	0.212
Viability	53.49 ± 1.22	40.77 ± 4.72	30.83 ± 8.76	35.86 ± 1.94	0.212	82.37 ± 4.47	73.58 ± 6.77	73.62 ± 6.53	67.62 ± 1.08	0.446

n; Number of straws from one freezing batch.

Table 3: Fertility of different bulls expressed as NRR or CNRR

Bulls	n	Fertility	
		NRR (%)	CNRR (%)
1	22	81.8	98.5
2	27	51.9	57.8
3	29	62.1	67.2
4	32	78.1	82.3
P values		0.067	0.018

n; Number of artificial insemination performed per bull, NRR; Non return rate-56 days, and CNRR; Corrected non return rate-56 days.

Table 4: Relation between fertility (CNRR) and selected sperm parameters

Sperm parameters	CNRR	P values
Total motility	r= -0.999	0.001
Progressive motility	r= -0.990	0.010
Immotile	r= +0.995	0.005
Abaxial implantation	r= -0.988	0.012
Acrosome Abnormality	r= -0.931	0.069

r; Coefficient of Pearson correlation and CNRR; Corrected non return rate-56 days.

Discussion

In the current study, cows were inseminated under difficult Algerian subtropical environment conditions, where the temperature was mostly high and cows received poor quality nutrition; these difficult rearing conditions could decline cow reproduction (25). The objective of current study is to research semen parameters, after post-thaw sperm or after density gradient selected sperm, as an indicator for fertility *in vivo* under farm management conditions.

In our study, no relationship was found between fertility and all semen parameters after PT, which is in agreement with the previous finding (8). Sperm motility is one of the parameters frequently evaluated in artificial insemination laboratories. There is no doubt that motility is an essential test for fertilization, regarding that spermatozoa should interact with oocyte for fertilization. None the less, motility of spermatozoa has been proven to be a poor indicator of fertility in frozen-thawed sperm, and poor relationships were found between fertility and post-thaw motility (9, 10). This finding is consistent with our results, while other authors reported a correlation between fertility and some post-thaw sperm parameters, such us sperm motility (11, 12), morphology, concentration and subjectively motility (13, 14), tail abnormalities and average path velocity (15).

Nevertheless, our results indicated that PT morphology abnormalities were <30% in most of bulls' thawed-semen (3/4), agreeing limited value considered by most of the artificial insemination centers.

In cattle industry, field fertility is assessed by quantifying NRR. Selected bulls have usually an NRR, ranging between 60 and 80% (26). Most of the proven bulls tested in our study exhibited a high fertility (3/4 bulls, 62.1-81.8% for NRR or 67.2-98.5% for CNRR) which can explain lack of the relationship between fertility and PT semen parameters.

In our study, semen parameters after PT or SSp were not different between bulls. Interestingly, some semen parameters after SSp were related to the fertility, expressed as CNRR, but not to those after PT. Thus, CNRR was increased with decrease of SSp total motility, progressive spermatozoa and abaxial implantation frequencies. While CNRR was decreased, as SSp immotile spermatozoa was decreased, underlying that the maximal limit of assessed immotile spermatozoa is 47%. Gillan et al. (13), determined no correlation between fertility and subjectively or CASA- motilities after semen swim-up; while, a positive correlation was found between fertility and total motility-CASA after semen swim-up (15). We clearly demonstrated that spermatozoa after PT were different than those after SSp. After AI, spermatozoa need some modifications to be able to fertilize oocyte, such as capacitation. Indeed, Bovipure is used to clean sperm and select high quality of spermatozoa before fertilization *in vitro* in the bovine reproduction laboratories. There is persuasive evidence that capacitation of those spermatozoa participating in fertilization is actively and progressively coordinated within succeeding regions of the female tract and also coordinated with the time of ovulation. Under a normal sequence of biological events, mating would precede ovulation within particular time (27). Thus, sperm that reach an adequate capacitation state are released and able to move to the fertilization place (28, 29).

Our study reveals that high frequency of total and progressive motility spermatozoa of SSp ap-

pears to be not favorable for fertility, suggesting that high motility shorten lifespan of the sperm; thus, when semen deposited in cervical uterus and undergo capacitation spermatozoa progress up rapidly in oviduct and reach the place of fertilization before ovulation. So that it cannot successfully fertilize the ovum. It seems that the male sperm, carrying Y chromosome, exhibit high motility, but has a shorter lifespan than the female sperm (sperm containing X chromosome) (30). However, Y sperm in the isthmus would achieve capacitation earlier than X sperm, releasing from the oviductal epithelium, and reach the fertilization place long before the ovulation, leading to death for most of these cells and fertilization could not occur in this case. However, delayed AI (≥30 hours) produces a significant deviation of the sex ratio towards the males (72.06%) (31). Further studies should be conducted to investigate relationship between motility and lifespan of sperm. Nevertheless, based on our results as well as those of the previous studies, and considering that cows were often inseminated between 12 and 24 hours, AI with high motility SSp should be delayed to improve fertility and produce male calves.

The results of the present study explain that the test of gradient selected sperm can mimic the conditions of female reproductive tract. Hence, evaluations of semen parameters after SSp reflect better semen quality *in vivo*. It was shown that selected spermatozoa in ram represented a different sperm sub-population, compared to the unselected one which could be related to the fertility *in vivo* (16).

Until now, no study reported relationship of fertility *in vivo* and semen parameters after BoviPure semen separation. However, it was demonstrated that *in vitro* cleavage rates and embryo production appeared to be superior, following BoviPure® compared to Percoll® separation (32, 33). Also, Data from studies performed *in vivo*, on humans, are scarce in comparison with those of studies *in vitro* (34).

Our study was carried out in small farms when cattle are bred under difficult condition; different works reviewed for this research are controversial and further studies merit to investigate the relationship between fertility *in vivo* and semen parameters after BoviPure® separation.

Conclusion

In the current research work, the highest fertility rate was observed in the bull with the lowest total motility, progressive spermatozoa and abaxial implantation as well as high immotile spermatozoa frequencies.

We highlighted that high percentage of progressive spermatozoa motility is an indicator for low fertility, so excess in sperm motility appeared to be unfavorable for fertility. Spermatozoa progress up rapidly in oviduct before the ovulation and they cannot successfully reach and fertilize an oocyte. Moreover, abaxial implantation frequency observed in SSp could be considered as sperm morphology abnormality, leading to fertility decline.

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