Diagnostic Accuracy of Transvaginal Sonography in Infertile Patients with Endometrial Polyps
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Abstract

Background: To evaluate the diagnostic accuracy of transvaginal sonography (TVS) in infertile patients and compare its results with hysteroscopy, as the gold standard.

Materials and Methods: A total of 679 infertile women who underwent both TVS and diagnostic hysteroscopy were retrospectively investigated. TVS was performed in the mid-follicular phase (days 5-8) of their cycles. Sensitivity, specificity, and positive and negative predictive values were calculated for TVS.

Results: Hysteroscopy diagnosed endometrial polyps in 197 out of 679 cases (24.5%). TVS confirmed the hysteroscopy findings in 174 of 197 (88.3%) cases. The sensitivity, specificity, and positive and negative predictive values of TVS compared to hysteroscopy in the detection of endometrial polyps were 88.3%, 91.9%, 81.6% and 90.8%, respectively.

Conclusion: TVS is both a cost-effective and non-invasive method for the diagnosis of intrauterine lesions such as polyps. When used in conjunction with a saline infusion, it can be a proper alternative for diagnostic hysteroscopy that saves time and enables the surgeon to perform the operative hysteroscopy procedure with greater accuracy.

Keywords: Endometrial Polyp, Diagnosis, Infertility

Introduction
The uterine cavity provides an environment for successful implantation and placentation. It is well known that structural abnormalities within the uterine cavity such as mullerian anomalies, fibroids, polyps, and synechiae play an important role in subinfertility, implantation failures and recurrent abortions (1, 2). Assessment of the uterine cavity has been performed by hysterosalpingography and hysteroscopy, but the introduction of transvaginal sonography (TVS) in the late 1980s has enabled physicians to evaluate the uterine cavity in greater detail. Recently, sonohysterography (normal saline infusion sonography) has become increasingly popular in the investigation and treatment of infertility (3, 4).

Hysteroscopy provides a three-dimensional visualization of the endometrium and is the preferred imaging study in 50%-70% of all infertility evaluations (3-7). Some studies have shown that hysteroscopy failed to diagnose uterine abnormalities which were disclosed by further diagnostic examinations such as sonohysterography and histology (8, 9).

The goal of this retrospective study was to evaluate the diagnostic validity of TVS in the detection of uterine cavity lesions in infertile patients, when compared with hysteroscopy as the gold standard.

Materials and Methods
We performed a retrospective study of infertile women who had undergone both TVS and diagnostic hysteroscopy during the period from October 2007 to October 2008. Of 755 patients identified, 76 cases were excluded from the study and 679 patients were evaluated. This study was performed at Royan Institute’s Infertility Clinic and Reproductive Biomedicine Research Center and approved by the Royan Research Center Ethics Committee. The patients were healthy infertile women ages 20-45 with a history of primary or secondary infertility of one year or greater.

TVS was performed during the mid-follicular phase (days 5-8) of the patients’ cycles (after ces-
sation of bleeding) and prior to diagnostic hysteroscopy in order to evaluate the size and shape of the uterus, thickness of the endometrial stripe or the endometrial echo-texture and presence of intrauterine lesions such as submucosal myomas and polyps. All sonographic examinations were done using an Aloka α-10 color doppler with a trans-vaginal 6 MHz probe by an expert radiologist. Polypoid lesions were considered as either round or oval localized echogenic lesions in the endometrial cavity.

Exclusion criteria were as follows: all patients with inaccurate visualization of the endometrium due to improper timing of the sonographic evaluation, a heterogenic or echogenic endometrium which resulted from either a hyperplasia or clot and cervical polyp. In cases that had normal endometrial findings on TVS, a hysteroscopy was not scheduled due to ethical considerations.

The indications for hysteroscopy were: failed in vitro fertilization (IVF) or failed intra uterine insemination (IUI), an observed focal localized endometrial pathology such as a polyp submucosal myoma, irregular endometrium and synechiae, or mullerian anomalies observed during hysterosalpingography or TVS.

Diagnostic hysteroscopy was performed under general anesthesia using a Storz 4mm hysteroscope (Karl. Storz , GmbH and Co. Tuttlingen, Germany) by an expert gynecologist. Distention of the cavity was obtained with glycine serum. The numbers, sizes and locations of endometrial polyps were investigated. All women with clinically significant abnormalities at hysteroscopy underwent immediate operative hysteroscopy and specimens were sent for histological examination. The sensitivity, specificity, positive and negative predictive values of TVS were calculated and compared with hysteroscopic findings, as the gold standard.

**Results**

Of 679 patients who underwent hysteroscopy, endometrial polyps were diagnosed in 197 (24.5%) patients.

TVS confirmed the presence of endometrial polyps in 174 out of 197 cases (88.3%), however misdiagnosed 23 cases of polyps as normal endometrium.

In 482 women, no polyps were detected during hysteroscopy. TVS was agreement with 443 (91.9%) of hysteroscopy and in 39 cases sonographic finding was suggestive of polyp (Table 1 and Table 2). Hysteroscopy was considered as gold standard.

**Table 1: TVS and hysteroscopic findings of 679 patients**

<table>
<thead>
<tr>
<th>Hysteroscopy</th>
<th>TVs</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>174</td>
<td></td>
<td>39</td>
<td>213</td>
</tr>
<tr>
<td>Negative</td>
<td>23</td>
<td></td>
<td>443</td>
<td>466</td>
</tr>
<tr>
<td>Total</td>
<td>197</td>
<td></td>
<td>482</td>
<td>679</td>
</tr>
</tbody>
</table>

**Table 2: Diagnostic accuracy of TVS compared with hysteroscopy as the gold standard for diagnosing endometrial polyps**

<table>
<thead>
<tr>
<th>Examination</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVs</td>
<td>88.3%</td>
<td>91.9%</td>
<td>81.6%</td>
<td>95%</td>
</tr>
</tbody>
</table>

Of the 482 hysteroscopy cases who were negative for polyps, additional findings included: normal endometrium (257/482), short septum (82/482), long uterine septum (35/482), leiomyomas (24/482), intrauterine adhesions (17/482), endometrial hyperplasia (15/482), unicornuate uterus (9/482), and didelphys (2/482).

**Discussion**

Several studies have demonstrated that benign intrauterine lesions such as polyps, septa and leiomyomas could be causative factors for subfertility (9-10). TVS is a non-invasive modality and has diagnostic value in the evaluation of uterus and endometrial abnormalities (10-12). The aim of the present study has been to evaluate the diagnostic accuracy of TVS when performed prior to hysteroscopy with the goal to reduce the number of diagnostic hysteroscopies that would be scheduled for infertility evaluations.

By using hysteroscopy as a gold standard, we determined that TVS had a sensitivity of 88.3%, specificity of 91.9%, positive predictive value of 81.6%, negative predictive value of 95%, and validity of 90.8% in the detection of endometrial polyps in our setting. Loverro et al. (9) and Soares et al. (13) reported that TVS had a sensitivity and specificity as high as 75-85% and 90-100%, respectively for detection of endometrial polyps.

Likewise, the positive predictive value (PPV) of TVS for detecting polyps in our setting was higher than reported by Soares et al. (13).

Of the 197 patients who were positive for endometrial polyps as determined by TVS and hysteroscopy (true positive group), 113 of these had the following pathologies: polyps (101/113), proliferative endometrium (11/113), and endometrial hyperplasia (1/113).

From 39 patients who had a positive TVs and negative hysteroscopy (false positive group),
there were 14 patients whose pathological reports were as follows: endometrial polyps (3/14) and proliferative endometrium (11/14). The true negative group consisted of 443 patients whose findings were negative for polyps by both TVS and hysteroscopy. In this group, biopsies were done in 16 cases; all of which confirmed the absence of polyp.

There were 23 cases whose TVS results showed no endometrial polyps however they had positive findings per hysteroscopy (false negative group). Biopsies were done on 19 patients from this group, whose pathological findings were positive for either endometrial polyps (9/19) or proliferative endometrium (10/19).

Although the most accurate diagnosis is based on pathological confirmation, goal of the present study is determining agreement between transvaginal sonographic detection and direct optic visualization which has been taken during hysteroscopy.

In our study biopsies were not performed on all cases, therefore we used hysteroscopy as the gold standard for determining the presence of endometrial polyps. However, in some cases, our data showed a discrepancy between hysteroscopy and pathology results. This finding has demonstrated that hysteroscopy should not be considered as the gold standard, but it is the best method available for gynecologist. Additional complementary studies will be needed to determine hysteroscopic accuracy in comparison to pathology findings.

Although this method provides a three-dimensional, direct visualization of the uterus however its diagnostic value is dependent upon developed facilities, expert personnel and the patients' level of tolerance.

Indeed, diagnostic hysteroscopy is more invasive and less cost-effective when compared to TVS in the detection of intrauterine lesions. Moreover, this method is associated with complications such as perforations, emboli, endometritis and risks associated with anesthesia.

TVS is a noninvasive method with multiple capacities such as doppler assessment, 4D evaluation and recently saline infusion transvaginal sonography (sonohystrograhy) that can obviate diagnostic hysteroscopy (8, 14-17) (Fig 1).

It may provide a specific diagnosis which would enable the surgeon to proceed directly to operative hysteroscopy. This method is cost-effective, less complicated and less time consuming. TVS can be a proper alternative to diagnostic hysteroscopy by saving time and enabling the surgeon to perform the procedure more accurately (Fig 2).

Fig 1: Transvaginal sonography of an echogenic focal lesion in the deep portion of the endometrial cavity.

Fig 2: Hysterosalpingography shows a clearer image of a large echogenic polyp in the endometrial cavity.

**Conclusion**

We conclude that TVS, as a routine procedure prior to hysteroscopy, would enable physicians to detect localized endometrial lesions in greater detail.

**Acknowledgements**

There is no conflict of interest in this article.

**References**

4. Widrich T, Bradley LD, Mitchinson AR, Collins RL. Comparison of saline infusion sonography with office hysteroscopy for the evaluation of the endometrium.
Comparison of ART Outcomes between Two COH Protocols: Gonal-F versus Gonal-F Plus HMG


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Abstract

Background: The purpose of this prospective, randomized study was to compare ovarian response as well as oocytes, embryo yields and pregnancy rates in women who underwent ovulation induction for intracytoplasmic sperm injection (ICSI) with recombinant human FSH (rFSH) alone or in combination with human menopausal gonadotropin (HMG).

Materials and Methods: Out of 300 patients in assisted reproductive technique (ART) cycles who underwent down regulation with GnRH analogue in a long protocol, 64 patients received 150 IU/d of r FSH until day six when they were randomly allocated into two study groups: group A, who received rFSH alone (n=32) and group B, (n= 32) who received rFSH and HMG.

Results: The total number of ampoules of rFSH, the numbers of oocytes retrieved, embryos and serum concentrations of luteinizing hormone (LH) on the day of hCG administration were similar in both treatment groups. However, the numbers of follicles ≥15mm, serum concentrations of progesterone and estradiol on the day of hCG administration were significantly higher in group B when compared to group A. Although the number of high quality embryos (grades A and B) were significantly better in group B, the number of pregnancies and live birth rates were similar in both groups.

Conclusion: The study shows that the addition of LH to rFSH in pituitary – suppressed women undergoing ART improves some parameters of ovarian response, but doesn’t improve overall pregnancy rates.

Keywords: Recombinant, FSH, HMG, Pregnancy Rate, Ovulation Induction

Introduction

Assisted reproductive techniques (ART), most commonly in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), have evolved greatly during the past two decades both in the technical and medical aspects, such as controlled ovarian hyperstimulation (COH).

The role of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in ovarian follicular growth and maturation have been known for a long time. Today, it is clear that LH plays a basic role in the final stages of follicle maturity. In response to FSH, the antral follicle reaches a stage in which granulosa cells become sensitive to LH stimulation and LH is capable of influencing both the theca and granulosa cells. This effect depends predominantly on the concentration of LH. Moreover, as LH receptors appear, the dependence of follicular growth and maturation on FSH decreases (1, 2).

Therefore LH may also play a fundamental role in the final maturation of oocytes in ICSI cycles. On the other hand, if follicles are affected by high improper LH concentrations an adverse effect may be seen (3, 4). Studies worldwide have used recombinant LH in order to show its effect on follicular maturation. However since recombinant LH is not currently available in Iran, this study is based on the addition of human menopausal gonadotropin (HMG), with equal content of 75 IU of LH and FSH on the seventh day of ovulation stimulation in order to show the effects of LH on patients treated with human FSH in cycles down-regulated with a GnRH agonist in the long protocol.

Materials and Methods

Patient selection

This controlled, double-blind randomized trial was conducted from June 2006–June 2007 at the Infertil-
ity Department of Vali-e-Asr Hospital as a gynecology resident thesis after being approved by the medical university research committee. From among 300 patients who were in their ART cycles, 64 were chosen and after obtaining written consent they were allocated to one of two groups by simple random sampling, using a random numbers table. Patients, ages 20-35 years with a body mass index (BMI) range of 18-30 Kg/m² were included if they had no underlying medical illnesses and no contraindications for pregnancy. The couples had normal karyotypes with the primary cause of their infertility as either tubal or male factor. Patients diagnosed with polycystic ovarian syndrome (PCOS) and those with FSH levels higher than 12 IU/L were also excluded.

Data collection was done via questionnaires completed by clinic staff and laboratory analyses.

**Study design**
For all patients, baseline FSH and LH values were measured in their previous cycles. All patients underwent pituitary down regulation receiving a once daily subcutaneous dose of 0.2cc Buserelin (Suprefact, Hoechst, AG-Germany), a short-acting GnRH analog from the 21st day of their cycles with oral contraceptive pills (OCP) pretreatment. After ceasing OCP and at least 12 days of pituitary desensitization, all patients received recombinant FSH (Gonal-F, Serono, Switzerland) at a fixed dose of 150 IU/d for the first six days. Thereafter, they were randomly allocated into two groups of 32 patients each.

Group A continued the given dose of treatment if they had 2-3 follicles ≥ 10 mm. On alternating days, patients underwent sonography until they had at least two follicles ≥ 18 mm and at least two other follicles with a diameter > 16 mm when they received 10000 IU hCG. If their response was insufficient, on the seventh day they received 1-2 additional ampoules (75-150 IU) of Gonal-F.

Group B received the same treatment as group A until day seven, when instead of 1-2 ampoules of Gonal-F, they were administered one Gonal-F and one HMG (Merional, IBSA Switzerland). If the response was insufficient, patients received an additional 1-2 ampoules of HMG until at least 2 follicles ≥ 18 mm were observed.

After treatment completion serum progesterone, estradiol and LH levels were measured followed by an intramuscular injection of 10000 IU of hCG. Oocyte pickup was performed 34 to 36 hours following hCG administration. Oocyte maturation was assessed with the criteria described by Veeck (5). After the ICSI procedure, embryos were scored according to the morphologic appearance of their blastomers blastomeres and fragmentation (6). Embryo transfer was performed on day three of ovum pickup with no more than 3 embryos being transferred per patient. In all patients, the luteal phase was supported by Cyclogest (Actover, Alpharma, England) a vaginal progesterone at a dose of 400mg/Bid, which started from the day of oocyte retrieval. In cases where chemical pregnancy was detected two weeks following embryo transfer, clinical pregnancy was confirmed with ultrasound examination six weeks thereafter.

**Statistical analysis**
Results were expressed as mean ± standard deviation. Student’s t test was used to evaluate the differences between both groups. Logistic regression model was used to assess the simultaneous effect of variables on ovary response. P-value <0.05 was considered statistically significant. Data were analyzed using SPSS software version 15.

**Results**
Out of 64 patients in this study who responded to ovulation stimulation, 32 received rFSH alone and 32 received a combination of rFSH and HMG. In total, two patients in each group were excluded from the study and the remainder received ovum pickup and embryo transfer. Both groups had similar demographics and basic characteristics (Table 1). Table 2 display a comparison of variables between the two groups.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Group A rFSH (n=30)</th>
<th>Group B rFSH + HMG (n=30)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>27.66 ± 4.3</td>
<td>28.6 ± 3.97</td>
<td>0.387</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.32 ± 3</td>
<td>23.31 ± 4.2</td>
<td>0.415</td>
</tr>
<tr>
<td>Length of infertility (years)</td>
<td>5.75 ± 3.20</td>
<td>6.43 ± 3.40</td>
<td>0.426</td>
</tr>
<tr>
<td>Basic LH (mIU/ml)</td>
<td>1.37 ± 0.28</td>
<td>1.30 ± 0.27</td>
<td>0.366</td>
</tr>
<tr>
<td>Basic FSH (mIU/ml)</td>
<td>5.70 ± 1.47</td>
<td>4.92 ± 1.69</td>
<td>0.065</td>
</tr>
<tr>
<td>Basic Estradiol (pg/ml)</td>
<td>27.56 ± 9.26</td>
<td>29.03 ± 8.77</td>
<td>0.531</td>
</tr>
</tbody>
</table>

Note: Numbers are Mean ± SD
In order to assess the simultaneous effect of the variables on ovarian response, a logistic regression model was used. According to the results, on the day of hCG administration, a significant difference between both groups in the serum levels of progesterone (p<0.001) and estradiol (p= 0.037), the number of follicles 15mm (p= 0.040) and number of grade B embryos (p= 0.003) existed.

Discussion
The results of this study are in favor of using an exogenous LH supplementation during COH in ART cycles which can be either in the form of rLH or the LH component in HMG. In the present study, patients in group B of the treatment protocol (those who received HMG supplement to provide LH) were superior to group A regarding the number of metaphase II oocytes, levels of LH, progesterone and estradiol on the day of hCG administration in addition to the numbers of grade A and B embryos. However both groups were similar in pregnancy rates and the rate of live births. These findings are close to the results of a systematic review performed by Mochtar et al. (7).

In their study on eleven trials involving 2396 women who had used a GnRH agonist, there was no evidence of a statistical difference in the live birth rate reported in two trials (OR=1.51, 95% CI=(0.79 - 2.87)) and none of a statistical difference in clinical pregnancy rates reported in seven trials (OR=1.15, 95% CI=(0.91 - 1.45)). Successful ART cycles depend both on ovarian stimulation and pituitary suppression in cycles treated by GnRH agonists. GnRH agonists do not cause complete elimination of LH. In order to have the maximum estradiol response, in most cases enough LH levels to occupy less than 1% of LH receptor, can be beneficial. Hence, the residual levels of LH (1-10 IU/d) seems to be able to produce maximum theca cell stimulation (8).

A study on oocyte donors has shown that in patients with LH level of less than 1 IU/d due to profound pituitary suppression, adding LH to treatment protocol in the form of HMG could improve the oocytes in terms of both quality and quantity (9). In the present study however, Tesarik et al. implemented long acting GnRH which could cause a longer and stronger suppression of the pituitary whereas in the present study we used the daily short acting subcutaneous form.

De Placido et al. in their study have shown that the immune reactive LH level is not related to a possible need for LH in the process of a follicular response to ovulation induction. Although there is a relation between immune reactive and bioactive LH, the differences are also notable (10). In the present study, patients were homogenous in their baseline LH levels, nevertheless they responded differently to exogenous LH. Several other studies conducted in recent years have indicated the positive impact of adding LH in ovarian folliculogenesis. In a study, using a dose of 150 IU of rLH, O’Dea et al. were able to produce a serum LH concentration of 1.2 IU/d, lower concentrations

### Table 2: Results of stimulation in both groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group A rFSH (n=30)</th>
<th>Group B rFSH + HMG (n=30)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol (day of hCG) (pg/ml)</td>
<td>502.2 ± 129.3</td>
<td>522 ± 196.5</td>
<td>0.64</td>
</tr>
<tr>
<td>Follicles 15mm (No.)</td>
<td>9.1 ± 3.6</td>
<td>10 ± 4.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Oocytes (No.)</td>
<td>7 ± 3.2</td>
<td>8.3 ± 3.6</td>
<td>0.15</td>
</tr>
<tr>
<td>Gonal-F ampoules used (No.)</td>
<td>20.6 ± 6.2</td>
<td>20.5 ± 4.8</td>
<td>0.92</td>
</tr>
<tr>
<td>Total number of embryos</td>
<td>4.2 ± 1.9</td>
<td>5.2 ± 2.1</td>
<td>0.08</td>
</tr>
<tr>
<td>Endometrial thickness(mm) (day of hCG)</td>
<td>9.53 ± 1.9</td>
<td>10 ± 1.2</td>
<td>0.21</td>
</tr>
<tr>
<td>Chemical pregnancy (No.)</td>
<td>6</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Clinical pregnancy (No.)</td>
<td>6</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Live birth (No.)</td>
<td>6</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Progesterone (day of hCG) (ng/ml)</td>
<td>0.72 ± 0.27</td>
<td>1.7 ± 0.28</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>LH (day of hCG) (mIU/ml)</td>
<td>1.45 ± 0.28</td>
<td>1.7 ± 0.29</td>
<td>0.001</td>
</tr>
<tr>
<td>Metaphase II oocyte (No.)</td>
<td>5.36 ± 2.57</td>
<td>6.76 ± 2.95</td>
<td>0.05</td>
</tr>
<tr>
<td>Grade A embryos (No.)</td>
<td>2.46 ± 1</td>
<td>3.6 ± 1.52</td>
<td>0.001</td>
</tr>
<tr>
<td>Grade B embryos (No.)</td>
<td>0.6 ± 0.6</td>
<td>2 ± 1.1</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Note: Numbers are Mean ± SD
of which resulted in decreased success rate of IVF. They also showed that LH has a threshold window (11) and it exerts its beneficial effects in higher levels compared to those which induce atresia in smaller follicles (12).

In another study conducted by researchers at Serono, Swiss company in 1998, a dose of 75 IU/d of rLH was implemented. They observed that on the day of hCG administration, an improvement in follicular maturation as well as increased estradiol and progesterone levels were seen in most cases (13). In our study the hormonal profile showed comparable results.

Nowadays, a number of new protocols including LH have been proposed. There is no doubt about the positive impact of LH on ART cycles, both by improving the quality of oocytes and its positive impact on endometrial receptivity due to higher levels of estrogen (14). Most studies have recommended using LH in poor responders (15) but many others are suggesting it should be also included in protocols for normoresponders (16).

rLH is still not available in many countries. Therefore according to the present work, instead of rLH, starting HMG from the seventh day of stimulation can be a suitable substitute. Finally despite the present knowledge about LH effects, it remains to be elucidated which patients would benefit the most from this practice and therefore more studies are needed to show the importance of LH in ovarian response.

Conclusion

Based on our findings, adding LH either as rLH or HMG to FSH in women under pituitary suppression during their ART cycles would most probably result in improvement of a number of ovarian response parameters; however it does not have considerable impact on pregnancy rates.

Acknowledgements

The authors wish to thank all academic members and staff at the Vali-e-Asr Reproductive Health Research Center for their sincere cooperation. This article is the result of the postgraduate thesis of Dr. Mandana Saberi. There is no conflict of interest in this article.

References

Original Article

Evaluation of Embryos Derived from in vitro Fertilized Oocytes Reconstructed by Meiosis-II Chromosome Transplantation from Aged Mice to Ooplasms of Young Mice

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Abstract

Background: To assess embryos derived by the transfer of meiosis-II chromosomes (M-II-t) from aged mice oocytes into ooplasms from younger mice to overcome the problem of age-related decline in female fertility.

Materials and Methods: The developmental capacity, karyotype, and ultrastructure of reconstructed oocytes derived from meiosis-II chromosome transplantation from aged mice into the ooplasms of young mice by piezo-micromanipulation were assessed.

Results: The survival rate of enucleated young oocytes was 54% and the percent of fertilized reconstructed oocytes was 23%. The rate of embryo development to the two-cell stage after cultivation was 40%. Since 82.4% of the analyzed embryos derived from reconstructed oocytes had condensed nuclei, it was not possible to analyze their chromosomal integrity. However, 17.6% of analyzable reconstructed old oocyte derived embryos (old-ODEs), had normal diploid sets of chromosomes. Major structural differences were not observed between young, old, and M-II-t derived two-cell embryos.

Conclusion: Our findings suggested that ooplasms from younger mice may overcome age-associated problems in older mice.

Keywords: Aging, Chromosome, Nuclear Transfer, Oocyte, Ultrastructure

Introduction

Age-related decline in female fertility is a common phenomenon in older women (1). Maternal age is shown to affect oocyte quality and early development (2) which may relate to aneuploidy in oocytes and embryos (3, 4). Segregation of chromosomes appears to be controlled by the meiotic spindle, but its components are largely supplemented by the ooplasm. Recently, attempts have been made to transfer a young oocyte cytoplasm into an old oocyte (5) or to transfer mitochondria and microtubules into an old oocyte cytoplasm to overcome these problems (6-9). Germinal vesicle (GV) transplantation is proposed as an approach for improving the oocyte quality of aged women (10, 11), assuming that cytoplasmic factor(s) in the ooplasm derived from younger women could reduce the incidence of oocyte abnormalities of older women. The placement of an aged GV nucleus into a younger ooplasm using micromanipulation and electrofusion reduces the occurrence of aneuploidy (11-13). In mice, it has been demonstrated that nuclear transplantation can be accomplished efficiently, and this technique appears not to impair subsequent oocyte maturation or increase the incidence of chromosomal abnormalities (10, 14). Maturation, fertilization, preimplantation and full term development in mice have been established using GV transplantation (15, 16). However, it is unclear whether transfer of meiosis-II chromosome transplantation (M-II-t) from aged mice to cytoplasts derived from oocytes of young mice could improve embryo development. The M-II-t technique enables nearly full genetic contribution of both parents to future embryos; it appears to be more flexible and efficient in boosting the implantation rate than oocyte cytoplasmic transfers (17). In the present study we examine, by M-II-t,
the developmental capacity, chromosomal integrity, karyotype, and ultrastructure of reconstructed oocytes derived from aged mice.

Materials and Methods

Gamete collection
All animal care and surgical interventions were undertaken in strict accordance with the approval of the Royan Institutional Review Board and Institutional Ethics Committee. Female mice (NMRI strain, Razi Institute, Tehran, Iran) of various ages (old: 7-9 months; young: 6-8 weeks) were superovulated by a single i.p. injection with 7.5 IU pregnant mare serum gonadotrophin (PMSG, Intervet, Netherlands) followed 48 hours later by 7.5 IU human chorionic gonadotrophin (hCG, Intervet, Netherlands).

The oviduct was punctured 14-16 hours after hCG to obtain metaphase II (MII) stage oocytes. MII oocytes were harvested in KSOM medium supplemented with bovine serum albumin (BSA, 4 mg/ml, Sigma, A3311). Cumulus cells were removed by briefly exposing MII oocytes to medium containing 300 IU/ml hyaluronidase (Sigma). The denuded oocytes were washed three times in KSOM medium; then transferred and cultured in 20 μl drops of KSOM, under mineral oil, in 5% CO2 and 95% air at 37 °C before micromanipulation.

Spermatozoa were obtained from the caudal epididymis of mature male mice (2-6 months) of the same strain and kept in KSOM medium for at least 1 hour for capacitation prior to injection.

Enucleation of MII oocytes
Two microdrops, each consisting of 3 μl KSOM-micromanipulation medium containing 3% sucrose, 20% fetal calf serum (FCS) and 7.5 μg/ml cytochalasin B (CB, Sigma, C6762) were placed on the bottom of a microinjection petri dish and covered with light mineral oil. Approximately 20 cumulus-free MII oocytes were washed three times in KSOM medium; then transferred and cultured in 20 μl drops of KSOM, under mineral oil, in 5% CO2 and 95% air at 37 °C before micromanipulation. Spermatozoa were obtained from the caudal epididymis of mature male mice (2-6 months) of the same strain and kept in KSOM medium for at least 1 hour for capacitation prior to injection.

Nuclear transplantation and piezo-intra cytoplasmic sperm injection (ICSI)
The donor was obtained by aspiration of karyoplast containing MII meiosis apparatus or spindle from an old oocyte. The oocyte reconstruction was done by transferring an old oocyte donor MII meiosis apparatus into the cytoplasm of a recipient younger oocyte through the slit made by enucleation. Simultaneously, the reconstituted oocytes were injected with a single dissected sperm head by the aid of a piezo actuator, as previously reported (19). Briefly, a single spermatozoon was aspirated into an injection pipette of ~5 μm inner diameter. The sperm head and tail were separated by applying either a single or a few piezo pulses to the neck region. The head alone was injected. The fertilized eggs were cultured in KSOM. Therefore, there were three experimental groups including: embryos derived from young oocytes, old oocytes, and reconstructed old oocytes.

Embryos were collected approximately 24 hours after injection and their survival and developmental rates were evaluated.

Cytogenetic analysis
The zona-free zygotes derived from reconstructed oocytes were fixed for chromosome analysis. Zygotes were treated with 0.1 μg/ml colcemid 40 hours after fertilization. The embryos were then transferred into a 1% hypotonic trisodium citrate solution including 5mg/ml BSA for 15 minutes; swollen cells were transferred onto a clean slide and fixative I (methanol: acetic acid, 3:1, v/v) was added for 5 minutes. Fixative II (methanol: acetic acid, 1:1, v/v) and fixative III (methanol: acetic acid, 1:3, v/v) were subsequently added for at least 5 minutes and the slide air dried. For aging, the slides were maintained at 4 ºC for 24 hours and 28 hours at room temperature. Finally, fixed blastomeres were stained with Giemsa in order to score the chromosomes.

Transmission electron microscopy
Embryos of old, young and reconstructed old oocytes were fixed for at least 1 hour in 2.5% glutaraldehyde (Sigma, USA) in 0.1 M cacodylate (Sigma, USA) buffer (pH 7.2), individually em-
bedded in 1% agarose, post-fixed for 1 hour in 1% aqueous osmium tetroxide, and dehydrated through a graded ethanol and isoamyl acetate series. Finally, the specimens were embedded in araldite (Sigma, USA). Samples were cut from pole to pole, alternating thin (70 nm) and thick (0.5 μ) sections. Thick sections were stained with toluidine blue and examined with a light microscope, whereas thin sections were stained with uranylacetate followed by lead citrate and observed under an electron microscope (Zeiss EM 900, Germany).

**Results**

**Fertilization and embryo development**
The survival rate of enucleated young oocytes was 54% (215/398). Of the reconstructed oocytes, 23% (50/215) were successfully fertilized after piezo/ICSI. Observation of the second polar body and pronucleus was conducted under an inverted microscope six hours after insemination. None of the unfertilized reconstructed oocytes had visible pronuclei. Therefore, we judged that they were not activated by piezo stimuli. The rate of embryo development in reconstructed oocytes to the two-cell stage (Fig 1A) was 40% (20/50). We did not assess further developmental rate of embryos due to a two-cell block in this strain of mice. The in vivo retrieved zygotes also did not pass the two-cell block as well. The perivitelline space was obvious around the blastomeres.

**Cytogenetics of the fertilized reconstructed oocytes**
One-cell stage embryos derived from reconstructed oocytes and old oocytes were processed for cytogenetic assessment. Normal karyotype was found in 93.3% (28/30) of the old-oocyte derived embryos (old-ODEs). Of the 6 (17.6%) analyzable reconstructed oocytes, all had a normal diploid set of chromosomes (Fig 3). However, 82.4% (28/34) of the analyzed embryos derived from reconstructed oocytes had a condensed nuclei and it was not possible to analyze them for karyotyping (Fig 1B and C). All embryos derived from young oocytes had normal karyotype.

**Ultrastructure of two-cell embryos**
We studied 15 two-cell embryos: 5 old-ODEs, 5 young ODEs and 5 reconstructed old-ODEs) by TEM (Fig 2). In all groups, by low magnification, the blastomers were uniformly covered with zona pellucida (Fig 2A, B, C). With higher magnification, the blastomers were connected with the gap junction and covered with long, thin microvilli. At low magnification, the nuclei were located centrally in the blastomeres. Ultrastructural examination was focused on the distinguishing features of the surface, cytoplasm, and nuclei of blastomeres in order to compare ultrastructural features in various groups.

![Fig 1: Embryo development and karyotype of a reconstructed ODE. (A) A two-cell stage embryo produced by piezo injection of a mature reconstructed oocyte. (B) Normal karyotype embryo. (C) Condensed nucleus of an embryo.](image)
blastomeres in all embryos. As the contact points among blastomeres extended, electron-dense areas, possibly developing junctions, also appeared (Fig 2).

**Cytoplasm**

Lipid droplet vesicles containing flocculent material were seen in all groups. Occasionally cortical granules were found in the embryos. Cytoplasmic annulate lamellae were usually seen in the perinuclear area and terminated frequently in SER. Fibrogranular substances, aggregations of granules and round fibers, were observed. Phagosomes and lysosomes were rarely seen. Mitochondria were ovoid with a vaculated and/or hooded extremity.

The mitochondria matrix was dense. Mitochondrial cristae were mainly peripheral. One or more dark granules were also observed within some mitochondria. Intermediate filaments were observed as bundles. The Golgi apparatus with swollen cisternae were found in the nuclear region of all embryos. Abundant SER was observed in all embryos that usually was vesicular or slightly elongated, many of which were in contact with mitochondria and vacuoles. Polysomes were observed. Multivesicular and residual bodies were found in all embryos.

**Nucleus**

Pores were present in the nuclear envelope of all embryos. Small clumps of heterochromatin were observed which, in some nuclei, tended to be peripheral. The nucleoplasm was somewhat granular. Nucleoli, with or without a less electron-dense center was surrounded by granular components or with a vaculated center, were observed in all embryos.

**Comparison of old-ODEs, young ODEs, and reconstructed old-ODEs**

The two-cell embryos appeared similar under phase contrast. A slight difference in perivitelline space was more noticeable in the old-ODEs. Ultrastructural study of the embryos showed the perivitelline spaces were more expanded and irregular in the old-ODEs than young and reconstructed old-ODEs. The cell organelles were homogeneously distributed throughout the cytoplasm in young and reconstructed old-ODEs, however in the old-ODEs, their density decreased peripherally. In the old-ODEs, mitochondria were seldom observed at the periphery of the cell but were more abundant around the nuclear envelope. Multivesicular and residual bodies were more abundant in old and reconstructed old-ODEs. Rough endoplasmic reticulum was seldom observed in the three groups. There were more lipid clusters and small droplet lipid in the old and reconstructed ODEs.

**Discussion**

Our primary finding is that although MII oocytes which were transferred from young to aged oocytes could undergo maturation and early cleavage; the young mice ooplasm could not possibly reduce age-associated problems of MII chromosomes from aged mice. Cui et al. also have reported that the transfer of GVVs to ooplasm of young mice could not rescue age-associated chromosome mis-alignment in the meiosis of oocytes from aged mice (20). These observations may be related to the fact that the nuclear compartment plays a predominant role in the etiology of age-related meiotic defects. Researchers have shown that the behavior of chromosomal alignment and dispersal over the metaphase spindle is predominantly determined by the GV, not the ooplasm, in mice (20, 21). These observations are contrary to those reported in humans. It is reported that when the GV from oocytes of older women are transferred into enucleated immature oocytes of younger women, a normal (12, 13, 22) or abnormal (12) second meiotic metaphase chromosome complement is observed in the reconstructed oocytes. These findings in humans seem to support the idea that a young ooplasm does have a rescuing role in GV from older women. However, because the numbers of reconstructed oocytes examined are few in our study, it is hard to draw any definite conclusions. Whether there is a species difference of young ooplasm effect on aged GVVs between mice and humans remains to be determined.

Moreover, it has been shown that transferring chromosomes from an in vitro matured oocyte into the cytoplasm of an enucleated oocyte matured in vivo (M-II-t) can improve subsequent embryonic development (23, 24). In vitro fertilization (IVF) of mouse oocytes reconstructed by M-II-t can result in live births, although the success rate of electrofusion of the karyoplast and cytoplast pairs from different oocytes and the fertilization rate of the fused oocytes is low (25). Chromosomal analysis of oocytes reconstructed with M-II-t followed by in vitro maturation, has been thoroughly studied in mice (25). Recently, Mitsui and Yoshizawa (26) reported that mice embryos produced by M-II-t have normal development and the incidence of chromosomal abnormalities of the M-II-t groups is similar to in vitro fertilization (IVF) derived embryos as previously reported (27). Additionally, Mitsui et al. reported that reconstructed oocytes which consisted of aged-karyoplasts and young-cytoplasts showed significantly improved embryonic development and development to term as compared with oocytes reconstructed from young karyoplasts and aged-cytoplasts (28). Generally speaking, in our study, at the ultrastructural level there is no significant difference between groups except the perivitelline spaces in the old-ODEs are more expanded and irregular than young and reconstructed old-ODEs.

**Conclusion**

In summary, our results suggest that M-II-t from aged mice transferred to cytoplasts derived from MII oocytes of young mice may improve age-associated problems of oocytes.
Acknowledgements
During the performance of this project we lost the late Dr. Saeid Kazemi Ashtiani. We would like to dedicate this manuscript to him. This work was supported by Royan Institute. There is no conflict of interest in this study.

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Differential Effect of Medium on the Ratio of ICM/TE of Bovine Embryos in a Co-culture System

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Abstract

Background: This study was undertaken to investigate the efficiency of two different embryo somatic cell co-culture conditions, tissue culture medium 199 (TCM199)–vero cells and Menezo B2 (B2)-vero cells, for the in vitro developmental quantity and quality of bovine embryos.

Materials and Methods: Bovine oocytes were allowed to mature and subsequently undergo fertilization in vitro. Their presumptive zygotes were cultured in either TCM199 or B2 culture media in conjunction with vero cells for up to nine days. The culture media were refreshed every two days and the proportion of embryos that cleaved and further developed to the morula and blastocyst (early, expand and hatched) stages were recorded. Hatched blastocysts underwent differential staining in order to determine the numbers of inner cell mass (ICM) and tropho ectoderm (TE) and total cell number (TCN).

Results: Of the two groups, no significant difference was seen between the proportions of the presumptive zygotes cleaved, those which developed to 8-16 cells, morula and reached days 7or 8 blastocyst stage or hatched. However, the values for TCN and TE of the TCM199-vero embryos were significantly greater than those of B2-vero embryos. The values for ICM/TCN and ICM/TE were significantly greater in the B2-vero group versus the TCM199-vero group.

Conclusion: Both TCM199 and B2 culture media in conjunction with vero cells were of the same efficiency when used for in vitro development of bovine presumptive zygotes. However, TCM199 was superior in providing embryos with more embryo cell numbers, whereas B2 medium was superior in providing embryos with greater ICM/TE and ICM/TCN ratios.

Keywords: In Vitro Fertilization, Embryo Culture Techniques, Co-culture

Introduction

In vitro embryo production (IVP) is a reproductive biotechnology with great potential for both medical and agricultural purposes. However the overall credibility of the IVP system, as measured by the percentages of embryos produced per the numbers of in vitro cultured oocytes as well as the percentages of live offspring from the numbers of embryos transferred have lagged behind those of in vivo counterparts (1-3). Although the exact mechanism(s) of this discrepancy is not completely understood, it seems that the culture condition in which the early embryos develop has a remarkable effect on the developmental competence of the cultured embryos (4, 5).

In vivo, cleavage stage embryos develop within the oviduct which contains a highly dynamic environment due to the contraction of the oviduct and differential secretions in various parts of this structure (6). In vitro, the basis of the embryo culture medium is designed considering the compositional analysis of the oviductal fluid. However, the absence of the oviductal cells adversely affects the pattern of embryo interaction with its surrounding environment; a situation which seems to be one important reason for culture medium inefficiency. Therefore, some studies have proposed the co-culture of embryos...
with oviductal epithelial cells to overcome this problem (7-9).

Given the difficulties regarding isolation of oviductal epithelial cells, in addition to experimental inconsistency and possible infectious hazards, numerous studies have attempted to substitute oviductal epithelial cells with different types of somatic cells, including cumulus cells (10, 11), granulosa cells (12), and buffalo rat liver cells (13, 14). Among the somatic cell used for coculture system, the African green monkey (Cercopithecus aethiops) kidney epithelial cells or vero cell lineage is of interest due to a number of advantages, including a common embryonic origin (mesoderm) with the early embryo, lack of heterogeneity and ease of proliferation in the culture condition (15, 16).

However, different types of culture medium have differential effects on both the profile and extent of trophic factors secreted by somatic cells cultured in vitro (10, 13). Accordingly, despite the large number of studies performed on the usefulness of somatic cells for in vitro embryonic development, there is no conclusive report about the effect of culture medium-somatic cell interactions on the efficiency of in vitro embryo production. These studies are essential to address the most suitable embryo culture medium for matched somatic cells.

Tissue culture medium 199 (TCM199 - Gibco) and B2 medium (CCD, Paris, France) are the two commercially available culture media routinely used for in vitro culture of mammalian embryos (17, 18). Therefore, the aim of this study is to compare the efficiency of TCM199 or B2 media with vero cells on preimplantation development of bovine embryos.

Materials and Methods

Unless otherwise specified, chemicals and media were obtained from Sigma (St. Louis, MO, USA) and Gibco (Life Technologies, Rockville, MD, USA) companies, respectively.

This study received the approval of the Ethical Committee of Royan institute.

Oocyte collection, in vitro maturation and in vitro fertilization

These procedures were carried out as described previously (19). In brief, ovaries were obtained from cattle and transported in warm saline (35°C) to the laboratory. After thorough washing, the contents of transparent antral follicles (2 to 8 mm in diameter) were aspirated using an 18 gauge needle attached to a vacuum pump (80 mmHg pressure) and deposited into 10 ml conical tubes for sedimentation. The aspirant was then depleted into 12 cm petri dishes and with a stereo microscope, cumulus oocyte complexes (COCs) which contained homogenous cytoplasms and at least three surrounding cumulus cells were collected. COCs were thoroughly washed in 200 μl droplets of Hepes buffered TCM199 plus 10% fetal calf serum (FCS) and once in 100 μl droplets of maturation medium. Subsequently, groups of 8-10 COCs were cultured in 50μl droplets of maturation medium in the presence of vero cell monolayers. The maturation medium was composed of TCM199 supplemented with 10% FCS, 10μg/ml luteinizing hormone (LH), 10μg/ml follicle stimulating hormone (FSH) and 1μg/ml 17-βestradiol. COCs were incubated at 39°C, 5% CO₂ and maximum humidity for 24 hours. Following in vitro maturation, COCs with expanded and modified cumulus cells were selected and washed three times in fertilization medium. Groups of 40-50 COCs were then allocated in 200 μl droplets of fertilization medium. Two 0.25 ml straws which contained the semen of two bulls with proven fertility were quickly thawed at 37°C and the contents of the straws were centrifuged at 1200 rpm for 10 minutes. Motile spermatozoa were obtained by the wim-up procedure as described by Parrish et al. (20). Washed and prepared spermatozoa were then loaded into the fertilization droplets at a final concentration of 1×10⁶/ml. After co-incubation for 18-20 hours at 39°C in 5% CO₂, oocytes were removed from the spermatozoa and cumulus cells, and washed twice with in vitro culture medium (IVC) to prepare them for in vitro culturing.

Vero cell preparation, in vitro culture and experimental design

Three frozen cryovials of an established vero cell line were obtained from Rowny Institute (www.royaninstitute.org) and used for the entire study as described elsewhere (19). In brief, after the cryovials were thawed, the washed and centrifuged cells at a concentration of 1×10⁶/ml were cultured in 3 cm² culture dishes (Falcon) which contained DMEM medium supplemented with 10% FCS at a temperature of 38.5°C and 5% CO₂ in humidified air. The culture medium was refreshed each two days. The confluent dishes were trypsinised (0.25% trypsin), and the detached and singled cells were either sub-cultured (to sustain the reserve cell source) or used for monolayer preparation. For the latter purpose, cells were diluted in the appropriate amount of

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either B2 or TCM199 plus 10% FCS to make a final concentration of $2 \times 10^5$/ml. Embryos were cultured in micro droplets (50 μl), covered with mineral oil and incubated at 38.5°C, and 5% CO₂ in humidified air. After 24 hours, the medium was replaced with fresh medium and preincubated for at least 2 hours prior to embryo culturing. For in vitro culture, presumptive zygotes were randomly allocated in TCM199-vero or B2-vero culture conditions in 5% CO₂, 5% O₂, and humidified air. Embryos were refreshed into new dishes each two days where, concurrently the numbers of embryos that cleaved and developed into 8-16 cell, morula, blastocyst and hatched blastocysts stages were recorded.

**Differential embryo staining for quality assessment**

In order to determine the total cell number (TCN) and the sole number of cells allocated in the sites of inner mass (ICM) and trophectoderm (TE), the hatched blastocysts in both groups were assigned to differential staining as described by Hosseini et al. (21) with minor modifications. In brief, hatched blastocysts were incubated in 500 μl of 1% triton X-100 and 100 μg/ml propidium iodide (solution 1) for up to 30 seconds, depending on the size of the embryos, and then immediately transferred into a 500 μl solution of 100% ethanol plus 25 μg/ml Hoechst 33258 (solution 2). Care was taken to carry the minimum amount of solution 1 when the embryos were transferred into solution 2. The embryos were extensively washed in solution 2 to remove any trace amounts of solution 1. Samples were then stored in solution at 4°C overnight. Fixed and stained embryos were subsequently mounted onto a glass slide in one drop of glycerol, gently flattened with a cover slip and visualized for cell counting on a fluorescence microscope (excitation filter 460 nm for blue and 560 nm for red). TE cells were visualized as blue and ICM as pink to red. TCN was calculated by counting the numbers of both ICM and TE.

**Statistical analysis**

The analysis of variance (ANOVA) procedure was used for data analysis. The mean of treatments were compared with Duncan’s multiple range test at a 0.05% probability level. Chi-square test was also used for comparison between different treatments with regards to differential staining.

**Results**

From 450 abattoir-derived ovaries, 3500 COCs were selected for in vitro maturation. There were 3028 oocytes subjected to in vitro fertilization which were further cultured up to nine days. Table 1 indicates the overall results of in vitro embryo development of the presumptive zygotes cultured upon the monolayer of vero cells in either TCM199 or B2 medium. As depicted in this table, the cleavage rates of the TCM199 and B2 groups were 76.9% and 81.2%, respectively which were insignificantly in favor of the B2 group (p>0.05). The percentages of embryos that attained the 8-16 cell stage were 54.1% for TCM199 and 67.1% for B2 culture systems, again insignificantly in favor of the B2 medium (p<0.05). The ratios of TCM199 and B2 groups of embryos that further developed into morula (42.5% vs. 39.3%) and days 7 (23.8% vs. 25.7%), 8 (30.3% vs. 29.0%) and 9 (31.7% vs. 27.7%) blastocysts were not statistically significant (p> 0.05).

The overall percentages of hatching were 40.3% and 45.7% for the TCM199 and B2 embryo culture media which were not significant (p>0.05). Differential staining of the embryos (Table 1 and Fig 1) indicated that the TCN of blastocysts that developed in the TCM199 group (338.5) were significantly greater than the B2 group (263), (p<0.05). Moreover, although the ICM rates of both groups were not significantly different; the TE mean number of the TCM199 group was 266.75 which were significantly greater than the B2 group (189.5).

**Table 1: In vitro developmental competence of bovine presumptive zygotes cultured in TCM199-vero cells and B2-vero cells**

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Oocyte (n)</th>
<th>Cleaved</th>
<th>8-16 cells</th>
<th>Morula</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Day 9</th>
<th>Hatched</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM199-vero cells</td>
<td>1528</td>
<td>76.9</td>
<td>54.1</td>
<td>42.5</td>
<td>23.8</td>
<td>30.3</td>
<td>31.7</td>
<td>40.3</td>
</tr>
<tr>
<td>B2-vero cells</td>
<td>1500</td>
<td>81.2</td>
<td>67.1</td>
<td>39.3</td>
<td>25.7</td>
<td>29.0</td>
<td>27.7</td>
<td>45.7</td>
</tr>
</tbody>
</table>

There was no significant difference (p≤0.05) between the proportions of different bovine preimplantation embryos cultured in TCM199-vero cells or B2-vero.
Consequently, the ratios of ICM/TCN and ICM/TE in the TCM199 group were 18.83% and 23.89% which were significantly lower than the related rates of the B2 group (27.9% and 38.78%, respectively).

![Fig 1: Assessment of the cell numbers of hatched blastocysts developed in TCM199-vero cell and B2-vero cell culture conditions. Different letters within the same column shows significant differences among the groups (p≤0.05).](image)

Table 2: Quality assessment of hatched blastocysts developed in TCM199-vero cell and B2-vero cell culture conditions.

<table>
<thead>
<tr>
<th></th>
<th>TCM199-vero cells</th>
<th>B2-vero cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICM/TCN (%)</td>
<td>18.83 b</td>
<td>27.9 a</td>
</tr>
<tr>
<td>ICM/TE (%)</td>
<td>23.89 b</td>
<td>38.78 a</td>
</tr>
</tbody>
</table>

TCN, ICM and TE represents the total cell number, inner cell mass and trophoectoderm respectively. (a, b) different letters within the same row shows significant differences among the groups (p≤0.05).

Discussion

Embryo somatic cell co-culture, a conventional state-of-the art assisted reproduction technique, involves the placement of the embryos on a layer of cells. These cells have been preferentially extracted from the genital tract to create a more natural environment for embryonic development in vitro, thereby enhancing their further competency for both in vitro and in vivo development (19, 22). With regards to human assisted reproduction clinics, this unique approach not only has been traditionally devised and applied, however, more recently there is a new trend to utilize autologous or even heterologous co-culture systems, particularly for "poor prognosis" patients (23). The results of this study indicated that when cultured over a vero cell monolayer, the overall percentages of embryo development were similar, irrespective of the type of media used. In a similar experimental design, Farin and co-workers assessed the developmental potential of embryos cultured in either TCM199 or B2 cultured medium using buffalo rat live cells (BRL) (17). In contrast with our results, they determined that the B2-BRL culture condition was significantly more advantageous when compared to TCM199. Therefore, two rationales exist for this discrepancy: a) the type of cells established for co-culturing in these two studies were different; BRL (17) and vero cells (our study), and b) all cultures in the Farin et al. study were left undisturbed until day eight, whereas in our study the embryos were replated onto newly prepared dishes every two days. Thus, based on available reports in addition to our previous experiences, somatic cells in a continuous culture consume available nutrient elements and deplete the media of a wide range of substrates necessary for normal embryo development (19). Moreover, it is reported that stressful conditions such as trypsinisation and medium refreshment stimulate vero cells to release proteins beneficial for embryo development. Therefore, the presence of vero cells and the method of embryo culture employed in this study may have some advantages of B2 medium over TCM199.

Differential staining of the embryos indicated that the TCN of blastocysts which developed in the TCM199 group (338.5) was significantly greater than the B2 group (263), (p<0.05). Moreover, although the ICM rates of both groups were not significantly different; the TE mean number of the TCM199 group (266.7) was significantly greater than the B2 group (189.5). Consequently, the ratios of ICM/TCN and ICM/TE in the TCM199 group (18.83% and 23.89%, respectively) were significantly lower than the related rates of the B2 group (27.9% and 38.8%, respectively). TCN have been suggested as an important criterion to determine embryonic capacity for post-implantation embryo development (24). Accordingly, the results of Table 2 clearly indicate that development in TCM199 significantly increased the proliferation rate of the blastomeres of the embryos when compared with B2 medium.

As shown in table 2, while the TE number of the blastocysts is greatly higher in TCM199 media relative to B2 media, the ICM numbers of the two groups are not significantly different. ICM is the sole source of cell that participates
in the embryonic tissue formation while TE participates in placentation, it is unclear if having a larger ratio of TE to ICM is favorable for future embryonic and fetal development or not. Although the beneficial effects of embryo somatic cell co-culture have been reported (19), the exact mechanism by which feeder cells support embryo development is not completely understood. It is possible that somatic helper cells may either remove potentially toxic factors that are present or produced in the culture medium, or secrete a wide range of trophic factors such as leukemia inhibitory factor (LIF) and growth factors.

Conclusion
In conclusion, the results of this study indicate that the use of vero cells as helper cells does not show a significant difference in the total number (proportion) of cleaved embryos that develope to both the advanced morula stages and blastocysts in either B2 or TCM media.

The results indicate that the number of cells per embryo at the blastocyst stage had a greater TCN for embryos developed in TCM199 medium relative to B2 medium. However, the ratio of ICM/TE and ICM/TCN of the embryos grown in B2 medium were significantly greater that those grown in TCM199 medium. The results of this study indicate that further studies are required in order to research the observed effects on post-implantation development.

Acknowledgements
This study was funded by a grant from the Islamic Azad University, Marvdasht Branch. The authors would like to express their gratitude to Dr. H Gourabi, president of Royan Institute, for the instrumental and technical supports. There is no conflict of interest in this article.

References


Flow Cytometry: A New Approach for Indirect Assessment of Sperm Protamine Deficiency

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Abstract

Background: Flow cytometry (FCM) has been extensively used to study mammalian sperm in the areas of clinical andrology and reproductive toxicology. FCM provides a powerful advantage over microscopy technique in terms of rapid, accurate and reproducible technology for the quantification of various cell characteristics, including chromatin status. During spermiogenesis, histones are replaced by protamines resulting in a very condensed structure of sperm chromatin. Infertile men have an increased sperm histone: protamine ratio than fertile counterparts. Chromomycin A3 (CMA3) staining represents a useful tool for assessing the packaging quality of sperm chromatin and allows indirect visualization of protamine deficiency. Routinely, fluorescence microscope is used for evaluation of protamine deficiency by CMA3. Considering the advantages of FCM and increasing use of CMA3 in assessment of protamine deficiency in the literature and its possible use as a diagnostic test, the aim of this study is to standardize this procedure for routine laboratory analysis.

Materials and Methods:
Semen samples were collected from 85 infertile men who referred to Isfahan Fertility and Infertility Center. A portion of semen sample was used for routine semen analysis according to WHO criteria and the remainder were evaluated to standardize CMA3 staining procedure for fixation, the number of sperm and duration of exposure to CMA3. The results were compared with standard fluorescent microscopic procedure. Percentage CMA3 positive sperm were compared between flow cytometry and standard fluorescent microscopic procedure.

Results:
Our results show that fixation, the number of sperm and duration of exposure to CMA3 can affect on FCM outcomes. In addition we show that the samples can be fixed, stained with CMA3, stores and then assessed for FCM.

Conclusion:
The optimal conditions for FCM assessment of CMA3 are: fixation, concentration of 0.25 mg/ml, sperm density of 2 million/ml and exposure for 60 minutes.

Keywords: Chromomycin A3, Flow Cytometry, Protamine, Andrology, Microscopy Technique

Introduction

Considering the advances in the field of assisted reproduction technology (ART) and implementation of new sperm selection procedures based on sperm DNA integrity, have increased emphasis on sperm chromatin quality. In intracytoplasmic sperm injection (ICSI), an apparently "normal" sperm is selected and used for insemination. Therefore, during this process, the most of natural selection barriers are bypassed and therefore, increasing the possible risk of genetic abnormalities that can have consequence including failed fertilization and embryo development. These consequences become more marked in semen samples with poor quality (1-3). Several factors such as: sperm parameters, acrosome, and chromatin structure have assessed by microscopy as a potential factor to predictor fertility. However, until now, no sole laboratory test on its own can assess fertility potential. As stated by Evenson et al. 1999 disadvantages of microscopy technique are intra-observer variations, low number of spermatozoa analyzed leading resulting in low statistical value (4, 5). Recently computer-interfaced flow cytometry (FCM) has entered the andrology laboratory and several studies had used from this technique for evaluation of chromatin structure (6), acrosomal status (7), spermatogenetic defects and etc (4, 8). The advantage of FCM includes; rapid, accurate, objectivity, reproducible and power statistical analysis over microscopy.
techniques. In addition, FCM can sort cells based on different cellular characteristic which may have application in assisted reproduction techniques such as sperm sexing (4, 9, 10). Therefore, FCM can be used in clinical setting in order to evaluate fertilization potential in andrology laboratories. Integrity of sperm chromatin structure has a paramount effect on ICSI outcome. Therefore, multiple assays have been developed to measure sperm chromosomal aberrations, abnormal chromatin packaging and chromatin structural integrity by using FCM (11). The chromatin structure of the sperm is very different from that of somatic cells. During spermiogenesis, histones are replaced first by transition proteins followed by protamines resulting in a very condensed structure of sperm DNA which is further stabilized by formation of disulfide bridges between some of the thiol group (-SH) of protamines during epididymal transition. Subsequently, non covalent bonds are formed between free thiol groups by prostatic Zn²⁺ (12-14). It has been shown that the protamine content has been altered in infertile men compare to fertile individuals (15). Therefore alteration of chromatin structure can result in abnormal packaging which makes chromatin susceptible to sperm to DNA damage (12, 16).

During routine ICSI, sperm is selected on the basis of morphology and motility which does not guarantee selection of sperm with normal protamine content. Considering the fact that protamine deficiency and sperm DNA damage are related events and result in poor fertilization, analysis of protamine content of semen samples can be of paramount importance in patient management and for assessment of new sperm selection procedures. The methods used for evaluation of chromatin condensation include: aniline blue staining, SDS+EDTA test, SDS analysis and chromomycin A3 (CMA3) for evaluation of excessive histones, ability of sperm to nuclear decondensation, chromatin stability and indirect measurement of protamine deficiency, respectively (17,18).

CMA3 is a glycosidic antineoplastic antibiotic isolated from the bacterium Streptomyces griseus and reversibly binds to guanine-cytosine (G-C) base pairs in the minor groove of DNA. Therefore, this fluorochrome competes with protamine for binding to DNA, which is dependent on magnesium. Therefore, CMA3 identifies spermatozoa with defective packaging and indirectly evaluates protamine deficiency (19, 20). CMA3 has been widely used by researchers for assessment of etiology of infertility. There are some reports on CMA3 and its relation to sperm fertilization ability in in vitro fertilization (IVF) and ICSI, suggesting that sperm protamine deficiency is associated with fertilization failure (21-23). Recently, CMA3 staining has also implemented for evaluation of bovine sperm (24). Routinely, fluorescence microscope is used for evaluation of protamine deficiency by CMA3. Considering the advantages of FCM and increased usage of CMA3 in assessment of protamine deficiency in the literature, and it's possible use in routine andrology units, the aim of this study is to standardize this procedure for routine laboratory assessment of protamine deficiency.

Materials and Methods
This study received the approval of the Institutional Review Board of Isfahan Fertility and Infertility Center and Royan Institute. Informed consent forms were signed by all patients. All chemicals were obtained from Merck (Germany, Darmstadt), unless otherwise stated.

Sperm Analysis and Sperm Processing
Semen samples were collected from 85 infertile men who referred to Isfahan Fertility and Infertility Center. All semen samples were collected by masturbation into sterile containers after 3-4 days of sexual abstinence and were delivered to the laboratory within 45 min after ejaculation. A portion of semen was used for routine semen analysis according to WHO criteria (25) and the remainder was washed twice in Dulbecco's Ca²⁺-Mg free phosphate buffered saline (PBS) (pH 7.4). A Semen Analysis Chamber was used for assessment of sperm counts.

Experimental designs
The 85 semen samples were used for the below experimental designs. The number of semen samples used for each experiments are indicated in the parenthesis. Some samples were common between the experiments.
1. To evaluate effect fixation on CMA3 outcomes by FCM and to compare the results with fluorescence microscope analysis (N= 20, Fig 2)
2. To evaluate the effect of number of sperm exposed to fixed volume CMA3 solution on CMA3 outcomes by FCM (N=33, Fig 3A)
3. To evaluate the effect of number of sperm exposed to CMA3 solution on CMA3 outcomes by fluorescence microscopy (N=33, Fig 3B)
4. To define the lowest number of sperm required for FCM (N=10, Fig 3C)
5. To evaluate the effect of duration of exposure to CMA3 on FCM outcome in fixed and unfixed samples (N= 37, Fig 4).
6. To evaluate the time of assessment of CMA3 by FCM post staining (N=21)

**Microscopic analysis of CMA3 staining**
CMA3 staining was carried out according Bianchi et al. (17) or Iranpour et al. (20). Briefly, semen samples were washed in PBS free Ca²⁺ and Mg²⁺ and were fixed (1:1) in Carnoy’s solution at 4°C for 5 minutes. Smears were treated for 20 minutes with 100 μl of CMA3 (Sigma, St Louis, MO, USA) solution. The slides were then rinsed in PBS buffer and mounted. Microscopic analysis of the slides was performed on a fluorescent microscope with the appropriate filters (460-470 nm). On each slide 200 sperm cells were evaluated. Evaluation of CMA3 staining was carried out by distinguishing between spermatozoa with bright yellow staining (CMA3 positive) and spermatozoa with dull yellow staining (CMA3 negative). All microscopic CMA3 analysis was carried out by a trained individual (17, 20).

**FCM analysis of CMA3**
The flow cytometry-based CMA3 staining assay was adapted from the slide-based method (17). Semen samples were washed with PBS and diluted to appropriate concentration according to experimental design. 1ml of each samples were centrifuged (200 g, 5 minute) and used directly for staining or fixed with Carnoy’s solution for 5 minutes at -4°C and then stained. For staining, the samples were centrifuged and the pellets were stained with 200 μL of 0.25 mg/ml CMA3 solution at room temperature. The time of exposure to CMA3 is given for each experimental design. CMA3 solution was prepared as for fluorescence microscopy. Then, samples were washed twice with PBS and assessed by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA) using an argon laser with an excitation wave length of 488 nm. Fluorescence from Chromomycin A3 stained sperm was collected in fluorescence detector-2 (FL-2) with a 585/42 nm band pass filter. A minimum of 10000 sperm were examined for each assay and analyzed using WinMDI 2.9 software. A positive control was obtained by pre-incubating the spermatozoa with 200 mmol dithiothreitol, a disulphide reducing agent, at 37°C for 10 minutes.

**Evaluation of fixation on CMA3 outcomes by fluorescence microscope and FCM**
In order to determine if fixation affects the outcome, twenty different ejaculate were divided into three equal portions. In each portion was washed with PBS and diluted to 2 million per ml. One portion was fixed while the second portion was used without fixation, then cells were exposed to CMA3 solution for 60 minute and prepared for FCM according to the above procedure. The third portion was used for fluorescence microscope analysis. The results were compared between the three groups.

**Evaluation of number of sperm exposed to fixed volume of CMA3 solution on CMA3 outcomes by fluorescence microscopic and FCM**
In order to evaluate the effect of number sperm on CMA3 analysis, semen samples from thirty-three infertile individuals were washed, diluted to 2, 4, 8, and 16 million/ml, fixed, centrifuged, exposed to 200 μL of CMA3 solution, for 60 minutes, washed and then assessed by FCM. Concomitantly, in an attempt, to determine whether the number of sperm affects on the CMA3 results by fluorescence microscopic, slides were prepared from the above samples with sperm concentrations of 2 and 20 million/ml. In order to check the procedural validity for semen samples with very low the number of sperm, ten samples were diluted to 2, 1.5, 1 and 0.5 million/ml. Following fixation and centrifugation, instead of adding 200 μl of CMA3, the volume of CMA3 was reduced proportionally to the number of sperm (200, 150, 100 and 50 μl for 2, 1.5, 1, 0.5 million sperm, respectively) to keep the ratio of sperm density to CMA3 concentration. Following exposure to CMA3 for 60 minutes, sperms were washed and the cells were assessed by flow cytometry.

**Evaluation of duration of exposure to CMA3 on FCM outcome in fixed and unfixed samples**
In this study, the effect of the incubation time of CMA3 solution on the percentage of CMA3 positivity was investigated. Therefore thirteen semen samples, each separately diluted to 2 million/ml and 6 aliquots of 1ml volume were prepared. Each aliquot was fixed and stained with CMA3 solution as above mentioned, for 20, 40, 60, 120 and 180 minutes and then washed and assessed by flow cytometry. Similar experiment was repeated on twenty- four unfixed semen samples.

**Evaluation of time of assessment of CMA3 by FCM post staining**
Since immediate assessment of CMA3 by flow cytometry is not always possible, the aim of this section was to evaluate whether the results of fixed or unfixed and stained samples could be assessed 24 hours later. Therefore, seven semen samples were
fixed, stained with CMA3 for 60 minutes, washed and analyzed immediately or stored for 24 hours at 4°C and then analyzed. Concomitant with the above studies, fluorescence microscope analysis was also performed.

Statistical Analysis
A Kolmogorov-Smirnov Z test was used to assess the normal distribution of data. Coefficients of correlation and Student t tests were carried out using the Statistical Package for the Social Studies (SPSS 11.5, Chicago, IL) software to compare results between different groups. CMA3 values are expressed as mean ± standard error of mean (SEM) and P-value lower than 0.05 was considered as statistically significant.

Results
Fig 1 shows the Dot plot of CMA3 staining for spermatozoa. The cells gated in the R1 region were analyzed and debris was excluded from the analysis.

Effect of treatment with DTT
The above semen sample was treated with dithiothreitol (DTT), a disulphide reducing agent. Sperm samples were used unfixed and then assessed for CMA3 positivity. The results were compared to the control semen sample. The percent of CMA3 positivity increased from 1% to 88% following treatment with DTT. Fig 1B and 1C showing disulphide reducing agent increases CMA3 positivity.

Effect of Carnoy’s fixative on FCM outcome
Fig 2 shows the mean percentages of CMA3 positivity of unfixed and fixed samples by FCM were 43.77 ± 5.00 and 25.03 ± 4.02, respectively. The mean of CMA3 positivity for fluorescence microscope analysis was 44.15 ± 3.1. The mean differences between fixed and unfixed samples for FCM were significantly different (p<0.01), while the difference between the unfixed sample with those of the fluorescence microscopic sample were insignificant (p=0.94), which suggested that the percentage of CMA3 positivity was reduced following fixation of the samples in FCM. The general trend of CMA3 positivity in the majority of samples was lower in the fixed sample relative to unfixed samples in FCM. However, in some samples, the values of CMA3 in the unfixed samples, assessed by FCM, were not in accordance with fluorescence microscopy results. Therefore, we assessed the coefficient of correlations between these three procedures. The results revealed a significant correlation between fixed with unfixed FCM (r = 0.632, p=0.003) and between fixed FCM and fluorescence microscopy (r = 0.336, p=0.017). However, no significant correlation was observed between unfixed FCM and fluorescence microscopy (r = 0.009, p=0.969).

Fig 1: FCM analysis of %CMA3 positive spermatozoa obtained from infertile patients. A: Dot plot of spermatozoa. The cells gated in R1 region were analyzed; debris was excluded from the analysis. B: %CMA3 positivity in semen sample that was not treated with DTT. C: %CMA3 positivity in semen sample that was treated with DTT.

Fig 2: Comparison of percentage of CMA3 positivity by fluorescence microscopy (dark column), fixed (white column) and unfixed (gray column) samples assessed by FCM. The mean value of CMA3 positivity in fixed samples by FCM was significantly different from the other two groups (p<0.01). Bars indicate standard error.
Effect of number of sperm exposed to CMA3 solution on CMA3 outcomes by fluorescence microscopic and FCM

Fig 3A shows that the percentage of CMA3 positivity decreased with increased number of sperm exposed to fixed volume of CMA3. The mean CMA3 values of each group is significantly different from the others (p<0.05). In addition, the result of fluorescence microscopic evaluation showed no significant difference between low and high density (Fig 3B). Furthermore, the results of figure 3C show no significant difference between the mean CMA3 values when the ratio of number of sperm to volume of CMA3 were maintained. The only significant difference was observed between the mean of CMA3 value for 0.5 million relative to 2 million sperm exposed to CMA3.

Effect of duration of exposure to CMA3 solution on FCM outcome

Fig 4 represents the percentage of CMA3 positivity as a tri-phase pattern in the fixed sample. The percentage of CMA3 positivity increased gradually with time; however it reached a steady state during 40 - 60 minutes with a subsequent increase. Unlike the fixed samples, the percentage CMA3 positivity increases with time in the unfixed sample.

Fig 3: A. Show that number of sperm exposed to fixed volume of CMA3 effects the CMA3 outcome. The mean value of each group is significantly different from the other group at p<0.05. B. The percentage of CMA3 positivity in fluorescence microscopy for slides prepared with 2 and 20 million/ml were not significant different at P< 0.05. C. Show the mean value of CMA3 positivity in FCM procedure when maintaining the number of sperm to volume of CMA3 solution during CMA3 staining. Only the last group (0.5 million sperm in 50μL CMA3) was significantly different (p< 0.05) from the first group (2 million sperm in 200 μL CMA3 solution).

Effect of the time of assessment of CMA3 by FCM post staining

The results showed no significant difference in FCM analysis between the samples that were stained, washed and assessed immediately or after 24 hours (19.41 ± 7.9 vs 21.58 ± 7.5, p=0.539). Similar results were obtained with another fourteen semen samples that were unixed, stained and read immediately or read 24 hours later (34.98 ± 4.8 vs 33.70 ± 3.13 p=0.769). Concomitant with the above studies, fluorescence microscope analysis was also performed. The results showed no significant difference between the samples that were read immediately or 24 hours later (47.42 ± 6.07 vs 40.80 ± 6.30 p=0.449).

Discussion

The importance of sperm chromatin packaging on male infertility has been well demonstrated from transgenes and knockout models for protamine. Proper chromatin packaging, facilitates sperm transport, protects DNA from chemical and physical damage, results in proper gene re-programming post fertilization, and leads to syn-
chronization of the cell cycle between the oocyte in MII phase and sperm in G1 (26, 27). Several factors related to sperm nuclear packaging have been identified as clinically significant, including the replacement of nuclear histones with a proper ratio of protamine 1 to protamine 2 (P1 / P2 ratio), histone to protamine ratio and the extent of DNA damage in the mature sperm (28, 29). Therefore, measurement of protamine deficiency of human spermatozoa is of particular biomedical interest for diagnosis of male infertility (18). Protamine deficiency can be assessed directly by urea polyacrylamide gel electrophoresis and indirectly by CMA3 staining (21, 30). The latter technique involves fluorescence microscopy and, due to simplicity, it has been widely used by andrologists. However, single cell cytophotometric determinations prove to be time consuming and results can be affected by inter and intra observer variations.

FCM have provided an alternative to the single cell cytophotometric method and have been implemented for sperm since 1970 (31). An as stated by Cordelli et al. "FCM is an automated approach able to measure the amount of one or more fluorescent stains associated with cells in an unbiased manner, offering unmatched properties of precision, sensitivity, accuracy, rapidity and multi-parametric analysis on a statistically relevant number of cells" (2). However, standardization of FCM for growing availability of fluorescent probes is of paramount importance. Therefore, the aim of this study is to standardize CMA3 staining by FCM for the routine andrology laboratory.

The results of the present study show that CMA3 staining can be carried out by FCM, in the presence or absence of fixative. Comparison of the same samples fixed with carnoy’s solution and unfixed show that fixation reduces CMA3 positivity in FCM (Fig 2). On possible hypothesis for this difference is the lower ability of CMA3 to enter the chromatin in a fixed sample and attach to unprotaminated DNA. Indeed treatment of samples with DTT, a disulfide reducing agent which helps to remove protamine and expose the DNA to CMA3, significantly increases CMA3 positivity (Fig 1).

Considering the mean value of unfixed FCM and fluorescence microscopy were similar (not statistically different); however in some samples the CMA3 values assessed by fluorescence microscopy, were not in accordance with the FCM results especially when assessed unfixed. Therefore, we assessed the coefficient of correlations between these three procedures. The results revealed a strong significant positive correlation between fixed with unfixed in FCM and a weak significant correlation between fixed samples by FCM with fluorescence microscopy, while no significant correlation was obtained between unfixed samples with FCM and fluorescence microscopy. These results may account for lower credibility of the microscopic procedure. This difference may be accounted by instrumental precision and more uniformity of staining in the FCM (in the tube rather than slides) in addition to variations such as inter and intra assay variation.

The effect of sperm number exposed to fixed volume of CMA3 was also assessed. Unlike the fluorescence microscope procedure, the results show a significant decrease in percentages of CMA3 positivity with increased number of sperm exposed to fixed volume of CMA3 in FCM (Fig 3A), thus suggesting that a fixed number of spermatozoa should be used during CMA3 assessment in order to compare results within or between experiments. The reason for this observation is that with increase number of sperm, higher CMA3 binding sites are available and therefore, the number of sperm to fixed volume of CMA3 must be maintained during CMA3 assessment. Following this observation, we evaluated effect number of sperm on percentages of CMA3 positivity in the fluorescence microscope procedure. The result showed that the number of spermatozoa fixed on each slide did not affect the results of CMA3 staining (Fig 3B).

In order to evaluate the lowest number of sperm required for assessment of CMA3 positivity by FCM, both number of sperm and the volume of CMA3 solution were reduced proportionally to maintain the final concentration of CMA3. The results revealed that the lowest sperm number required for FCM was 1 million sperm (Fig 3C) to assess CMA3 value in oligozoospermic samples. Lower sperm concentration may affect the validity of results.

The other aim of this study was optimization of duration of exposure to CMA3. The results showed that, when samples were fixed, the percentage of CMA3 positivity increased gradually but reached a steady state between 40-60 minutes and subsequently increased, while in unfixed samples the percentage of CMA3 positivity increased gradually with time (Fig 4). Although it is difficult to explain the difference observed between fixed and unfixed sample, but one possible explanation may be due to the fact that in the fixed sample protamine cannot be easily displaced and chromatin saturation by CMA3 is reached with a time.
point, while in the unfixed samples breakage of disulfide bridges may take place due to auto-oxidation. Therefore, one may propose to fix samples and expose them to CMA3 for 60 minutes so that CMA3 reaches a steady state and the results are not affected by variation in exposure time. Conversely, samples could be assessed unfixed but it is important to note small variations in time may affect CMA3 positivity in FCM.

Conclusion
Considering the value of CMA3 assessment in the management of infertility and research and the use of FCM in two previous studies based on the slide method, we propose FCM is a suitable, precise and accurate method for assessment of CMA3 staining, however it should be standardize. Therefore, we advise researchers to consider the following points during CMA3 assessment by FCM: 1) use fixed samples, 2) use fixed number of sperm per ml (2 million), 3) expose samples for 60 minutes to CMA3 solution and 4) samples can be fixed, stained, washed and assessed later. The factors which affect FCM and may not affect the slide method are: sperm concentration and duration of exposure to CMA3.

Acknowledgements
The authors express their gratitude to the Royan Institute for its financial support, as well as the staff of Isfahan Fertility and Infertility Center. There is no conflict of interest in this study.

References

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Effects of Hydro-alcoholic Extract of Red Dried Stigmas of Crocus sativus L. Flowers (saffron) on the Levels of Pituitary-ovary Hormones and Folliculogenesis in Rats


Biology Department, Islamic Azad University Kazeroun Branch, Kazeroun, Iran

Abstract

Background: In the present study the effects of a hydro-alcoholic saffron (Crocus sativus) extract on pituitary-ovary axis and folliculogenesis were investigated. The aim was to study the possible role of saffron in female fertility and thereby, show its usefulness in treating infertility and reproductive disorders in females.

Materials and Methods: The study consisted of 50 adult female Sprague dawley rats that were divided into five groups of ten: control, sham and three experimental groups. The experimental groups received intraperitoneal injections of 1, 2 and 4 dg/kg body weight (B.W) extract, respectively over a ten day period. The control group was untreated and the sham group received only distilled water. After 10 days, blood samples were taken from all groups in order to measure the serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH) estradiol and progesterone hormones. Ovaries were removed, sectioned and examined by light microscope. The results were analyzed by ANOVA and TUKEY tests.

Results: Statistical analysis of the results showed a significance increase (p ≤ 0.05) in the levels of FSH and estradiol in the experimental groups that received 2 and 4 dg/kg B.W extract with respect to the control group; while the level of LH hormone only rose in the experimental group that was administered the maximum dose (4 dg/kg). In addition, the results indicated that administration of 2 and 4 dg/kg extract had a significant effect on ovarian weight. Histological studies of the ovarian sections showed that administration of 2 and 4 dg/kg extracts enhanced folliculogenesis and increased the numbers of secondary follicles in the ovary.

Conclusion: According to the results of this and other studies, the hydro-alcoholic extract of saffron may enhance pituitary-ovary axis activities, boost the levels of FSH, LH and estradiol in addition to stimulate folliculogenesis in adult female rats.

Keywords: Saffron, Gonadotropins, Estradiol, Progesterone, Rat
tion of Parkinson’s disease (9). If crocin is administered orally in either a single dose or multiple doses, it will not be absorbed, but rather it mostly will be lost through the digestive tract. Therefore, the digestive tract is an important place for crocin hydrolyses (10). Crocin (a glycosidic form of crostin) is absorbed as crocetin after being hydrolysed and is found free or conjugated with glucoronid (mono and diglucoronid) in plasma (11). Traditionally, people have believed that saffron is essential for the reproductive organs and, therefore, necessary for a healthy, complete pregnancy. Thus far, there have been no research studies concerning the effects of a hydro-alcoholic extract of saffron on the female reproductive system and ovarian function. Because the main components of saffron such as crocetin and saffranal (11) have a wide range of various beneficial biological activities, often with no toxic side effects, it is possible that saffron extract may enhance ovarian function. In the present study, the possible effects of a hydro-alcoholic saffron extract on LH, FSH, estradiol and progesterone levels, and ovarian histological changes have been investigated. The results of this study can be useful in treating infertility in reproductive and endocrine centers.

Materials and Methods

Animal groups

Study was approved by Ethics Committee for animals at the Islamic Azad University, Kazeroun Branch. In the present study, fifty adult female Sprague dawley rats, aged ten weeks, weighing about 70 ± 10 g each, were obtained from the animal house at Islamic Azad University, Kazeroun. They were kept at 22 ± 2°C on a 12 hours light/12 hours dark cycle, and fed adequate amounts of water and a standard dry diet. Animals were kept in polycarbonate cages with laced steel roofs. It is the size 15 × 25 × 40 cm. The cage floors were covered with wood powder and cleaned three times each week. During the experiment, all cages were washed three times with water and detergent.

Rats were divided into five groups of ten as follows: control group (no intervention, other than dry food and water), and the sham group who received the same treatment as the control group, with the exception of 1 ml distilled water that was injected into each rat. Three experimental groups which received adequate food and water in addition to a saffron hydro-alcoholic extract on LH, FSH, estradiol and progesterone levels, and ovarian histological changes have been investigated. The results of this study can be useful in treating infertility in reproductive and endocrine centers.

III were given the maximum dose of 4dg/kg B.W for 10 days (Table 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Food and water</th>
<th>Injection (ip)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No limit</td>
<td>nothing</td>
</tr>
<tr>
<td>Sham</td>
<td>No limit</td>
<td>1 ml distilled water</td>
</tr>
<tr>
<td>Experimental group I</td>
<td>No limit</td>
<td>1 dg/kg B.W</td>
</tr>
<tr>
<td>Experimental group II</td>
<td>No limit</td>
<td>2 dg/kg B.W</td>
</tr>
<tr>
<td>Experimental group III</td>
<td>No limit</td>
<td>4 dg/kg B.W</td>
</tr>
</tbody>
</table>

Preparation and administration of the hydro-alcoholic extract of dried red stigmas of Crocus sativus L. flowers (saffron)

High quality saffron1 (100 g) was finely ground into a powder and subsequently placed in a glass flask with 50 ml of 96% alcohol. After 72 hours, the mixture was filtered twice and concentrated by evaporation at 40°C (8). The ratio of saffron to its extract was approximately 48%.

Synchronization of animals’ reproductive cycles

Prior to beginning the experiment, the reproductive cycles of the rats were synchronized by the following method. A few days before the extract was administered, 100 μg estradiol valerate dissolved in 2 ml olive oil was injected intra-muscularly. All rats, after a 24 hour period, received intra-muscular injections of 50 μg progesterone which had been dissolved in 2 ml olive oil. A few hours later, vaginal smears were prepared (12). Examination of vaginal smears showed that all the animals were in the estrous stage of strous cycle.

Administration of the extract

Each day, for 10 days; 2, 4 and 8 g of the saffron extract were weighed, separately placed in separate beakers and readily dissolved in distilled water. Then, 20 ml distilled water was added to each beaker and mixed well. These mixtures were injected intraperitoneally with the use of insulin syringes into each of the experimental groups, respectively. After 10 days, the animals in each group were weighed, anesthetized by ether, and blood samples were taken from their hearts. Blood samples were centrifuged for 15 minutes at 4000 rpm and the separated serum samples were frozen at -20°C and kept for later testing. In addition, ovaries were carefully removed and fixed in a formalin solution at a pH of 7.0. Thin sections were prepared and, after staining with hematoxilin-eosin, ovarian sections were studied with a
light microscope. Concentrations of LH, FSH and estrogen and progesterone were measured by radio immunoassay (RIA). In order to determine the differences between experimental and control groups, the results were analyzed by the ANOVA, TUKEY, LSD and Dunnet tests. Results were considered significant at $p \leq 0.05$.

**Results**

Results of hormone assays among the various groups are shown in Tables 2 and 3. Statistical analyses of the results revealed an increase in ovarian weight in experimental groups that received 2 and 4 dg/kg at the end of the tenth day (Table 3). In addition, the concentration of LH showed a significant rise in experimental group II, which received the medium dose (4dg/kg). Furthermore, the serum levels of FSH and estradiol in experimental groups II and III increased significantly. No significant difference was observed in the concentrations of progesterone among the various groups (Table 2). Histological studies showed no important changes in ovarian tissues, such as: ovarian capsule, stroma tissue, cortex, ovarian medulla, follicles and corpus luteum, in any of the experimental groups. Statistical analyses of the data that resulted from follicle counting revealed no significant differences in primary follicles among the experimental and control groups, but the average number of secondary follicles in experimental groups II and III were significantly different (Figs 1-3). Also, the average number of graffian follicles and corpus luteum revealed no significant differences among the experimental and control groups (Table 3 and Figs 2, 3). There was no significant difference between experimental group I and controls in terms of hormonal and histological changes.

![Fig 1: Ovary tissue in control group (H&E staining; ×4) (This figure has also been printed in full-color at the end of this issue)](image)

**Table 2: Mean ovarian weight and serum hormone levels in the experimental groups in contrast to the control and sham groups at the end of the experimental period**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ovarian weight (mg)</th>
<th>LH (mIU/mL)</th>
<th>FSH (mIU/mL)</th>
<th>Estradiol (pg/mL)</th>
<th>Progesterone (ng/mL)</th>
</tr>
</thead>
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<tr>
<td>Control</td>
<td>108 ± 3.96</td>
<td>0.13 ± 0.005</td>
<td>0.136 ± 0.005</td>
<td>40.63 ± 1.10</td>
<td>35.08 ± 1.72</td>
</tr>
<tr>
<td>Sham</td>
<td>102 ± 4.8</td>
<td>0.12 ± 0.004</td>
<td>0.137 ± 0.008</td>
<td>40.25 ± 1.13</td>
<td>35.17 ± 2.26</td>
</tr>
<tr>
<td>0.1 g/kg B.W</td>
<td>93 ± 20.02</td>
<td>0.14 ± 0.005</td>
<td>0.15± ± 0.005</td>
<td>38.88 ± 0.56</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>0.2 g/kg B.W</td>
<td>137 ± 5.83</td>
<td>0.13 ± 0.002</td>
<td>0.19 ± 0.004</td>
<td>49.14 ± 0.99</td>
<td>34.25 ± 1.66</td>
</tr>
<tr>
<td>0.4 g/kg B.W</td>
<td>185 ± 8.33</td>
<td>0.16 ± 0.005</td>
<td>0.18 ± 0.005</td>
<td>51.11 ± 1.02</td>
<td>33.52 ± 1.9</td>
</tr>
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</table>

*Indicates significant difference ($p<0.05$) between control and experimental groups.

**Table 3: Changes in means between primary, secondary, graffian follicles and corpus luteum in all groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ovarian weight and hormone concentrations</th>
<th>Primary follicle</th>
<th>Secondary follicle</th>
<th>Graffian follicle</th>
<th>Corpus luteum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.5 ± 0.29</td>
<td>1.09 ± 0.15</td>
<td>0.6 ± 0.09</td>
<td>3.8 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>6.3 ± 0.45</td>
<td>1.03 ± 0.15</td>
<td>0.68 ± 0.11</td>
<td>4.3 ± 0.22</td>
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</tr>
<tr>
<td>0.1 g/kg B.W</td>
<td>6.8 ± 0.48</td>
<td>0.73 ± 0.12</td>
<td>0.58 ± 0.08</td>
<td>3.8 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>0.2 g/kg B.W</td>
<td>6.4 ± 0.3</td>
<td>*1.97 ± 0.35</td>
<td>0.7 ± 0.09</td>
<td>4.7 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>0.4 g/kg B.W</td>
<td>7 ± 0.29</td>
<td>*1.03 ± 0.31</td>
<td>0.6 ± 0.1</td>
<td>3.7 ± 0.22</td>
<td></td>
</tr>
</tbody>
</table>

*Indicates significant difference ($p<0.05$) between control and experimental groups.

B,W: Body weight

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Discussion

There is little information about the effect of saffron extracts on reproductive processes. In contrast to their numerous biological effects, the components of saffron have an unknown metabolism. Hence, understanding the precise mechanism of their functions requires numerous studies. In the present study, the effect of alcoholic extract of saffron on ovarian weight, LH, FSH, estradiol, and progesterone hormones, as well as the numbers of primary and secondary follicles, graffian and corpus luteum were investigated.

The results indicated that the administration of 2 and 4 dg/kg saffron extract caused a significant boost in ovarian weight. Previous studies have shown that the compounds present in saffron extracts enhance oxygen diffusion in tissues (8). Murasawa and his colleagues have shown that ovarian weight increases by rising in the numbers of small and medium follicles (secondary follicles). These follicles can be a considerable source of ovarian weight (13, 14). Thus, different compounds of saffron extracts can increase ovarian weight by enhancing oxygen diffusion to granulosa cells and decreasing atresia in small and medium follicles. This finding was confirmed by the significant rise in secondary follicles in the experimental groups II and III (Table 3).

According to table 2, the serum level of LH in the experimental group which received the maximum dose of 4 dg/kg increased significantly when compared to the control and sham groups. Similar effects were seen in the serum concentrations of FSH in experimental groups II and III. It is probable that GnRH and gonadotropic hormones are released by crocin activation of noradrenergic nerves in the locus ceruleus and with saffranal by stimulating the activities of serotonergic nerves, both of which are associated with the hypothalamus (5). Despite an FSH boost in the group that received a dose of 2dg/kg, the level of LH did not change significantly. This may indicate that the hypothalamus as well as the pituitary gland and ovaries are stimulated by the extract. Recent studies have revealed that crocin, crocetin and dimethyl crocetin can influence H1 histone, reduce the interaction between H1 histone and DNA, and increase gene transcription (15). Such influence can increase transcription of α and β subunits of FSH. On the other hand, recent studies have indicated that the positive effects of estradiol on gonadotropin secretion are alpha dependent and occur at the pituitary level (16). Considering the possible effect of crocetin on enhancing the activities of ovarian noradrenergic nerves (5), the role of these nerves in increasing FSH receptors in primary follicles and the rise in estradiol synthesis; one can attribute the increase in FSH concentration in experimental groups II and III to the positive feed-back effects of estradiol at the pituitary level. Recent studies have also shown that the reduced basal level of cyclic adenosin monophosphate response element binding protein (CREB) phosphorylation may sensitize the pituitary to GnRH, as there would be more CREB phosphorylation sites available to respond to the gonadotropin releasing hormone (GnRH) signal (17). A possible effect of crocin can cause an
increase in the sensitivities of gonadotrop cells to GnRH. Therefore, during gonadotrop stimulation by GnRH, these cells will respond vigorously to the stimulating agent, thus producing more gonadotropin (18). As shown in Table 1, the serum concentration of estradiol rose significantly in the groups administered the medium and maximum doses of extract. There was no significant difference in the concentration of progesterone among the various groups, while the serum level of LH significantly increased in the experimental group that received the maximum dose. During the experiment all animals were in the estrous phase, therefore the concentration boost of LH in experimental group III could not be responsible for the elevation of progesterone in this group (Table 2). Similarly, the lack of a significant difference in the numbers of corpus luteum among the various groups also confirmed this finding. Histological studies of the ovarian sections showed that administration of saffron extract had no effect on the ovaries in the experimental groups. The number of secondary follicles in experimental groups that were administered 2 and 4 dg/kg extract increased significantly (Table 3), while the numbers of primary graffian follicles and corpus luteum were not significantly different among the various groups. Hence, this study has shown an enhanced folliculogenesis by saffron extract at the steps affected by gonadotropin, thereby enhancing the growth and differentiation of primary follicles and increasing secondary follicles. It has been reported that FSH stimulated gap junction formation and turnover in rat ovarian granulosa cells and increased connexin43 (Cx43) gene expression in these cells (19). Cx43 gene is expressed in granulosa cells and plays a crucial role in the development of germ cells. Postnatal folliculogenesis in Cx43-deficient ovaries do not proceed beyond the primary follicle stage (20). In addition, estradiol stimulates the proliferation of granulosa cells, protects against apoptosis, and modulates granulosa cell differentiation by enhancing the ability of FSH to induce expression of LH receptors (13). Therefore, in accordance with the increased concentrations of estradiol and FSH hormones in the experimental groups that received 2 and 4 dg/kg extract, the rise in the number of secondary follicles was not unexpected. Recent studies have shown that high concentrations of carotenoids have the ability to stimulate Cx43 formation among cells (21). Thus, the presence of carotenoids in saffron extract can also promote differentiation of primary follicles through increasing the expression of Cx43. Furthermore, the probable effect of crocin which is present in saffron extract can be a factor in increased norepinephrine which in turn can affect increased FSH receptors in granulosa cells and enhance the growth and differentiation of primary follicles (22). For this reason, in the present study, the possible factors for promotion of folliculogenesis and elevation in the number of secondary follicles are a result of the probable effects of saffron extract on ovarian tissues to stimulate cx43 gap junction formation and changes in the ratio of Bcl/Bax proteins (atresia inhibition of follicles). Also saffron extract caused the further stimulation of ovarian noradrenergic nerve activity and its effects on the levels of pituitary-ovary hormones and folliculogenesis increased.

Conclusion
The findings of this study show that 2 and 4 dg/kg hydro-alcoholic saffron extract can promote the pituitary-ovary axis activities at all levels, cause an elevation in the serum concentrations of LH, FSH and estradiol hormones, as well as increase the mean numbers of secondary follicles and eventually ovarian weight. It is probable that these effects are the results of active compounds such as crocin, crocetin and saffranal, all of which are present in the extract. It can be suggested that one might use saffron to enhance fertility and treat infertility in females, although further studies are still required.

Acknowledgements
We wish to express our gratitude to the officials and staff of Kazeroun Islamic Azad University and to the laboratory personnel and technicians at Shiraz Namazi Hospital. Our thanks are also due to H. Khajehi for linguistic copy editing. There is no conflict of interest in this study.

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4. Hosseinzadeh H, Khosravan V. Anticonvulsant effects of aqueous and ethanolic extracts of Crocus sativus L.
A Successful Induction of Lactation in Surrogate Pregnancy with Metoclopramide and Review of Lactation Induction

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Abstract

In surrogate pregnancies genetic parents have little opportunity for early bonding with their infants, either prenatally (in utero) or during the immediate postnatal period. Procedures commonly used to induce lactation include both pharmacologic and nonpharmacologic methods, often in combination. Studies reporting induced lactation are sparse, due to the rarity of augmented lactation. Here we report a case of lactation induction following a surrogate pregnancy. Other methods that can be used to augment lactation are described below. We used metoclopramide in this case due to the success rates reported in previous studies and case reports. Additionally, it is a well tolerated and safe agent.

Keywords: Surrogate, Pregnancy, Lactation, Metoclopramide

Introduction

Infertility is emotionally painful and draining, particularly if the couple has been trying for a number of years with several failed infertility treatment cycles. Choosing adoption or surrogacy can be a positive experience for some couples (1). However, this route is not ideal due to the remorse of not being able to experience pregnancy, give birth or enjoy the bond and closeness experienced with breastfeeding. In this way, the neonate is deprived of the advantages of breastfeeding, which include improved nutritional, emotional and cognitive development (2, 3) as well as vaccination against infections and allergies (4). Induction of lactation is a good option to overcome this problem, unfortunately not enough milk is produced to exclusively breastfeed the infant, but women find satisfaction in this rigorous process because of the maternal-infant bonding it promotes (5). Procedures commonly used to induce lactation include both pharmacologic and non-pharmacologic methods, often in combination (6). Few studies exist regarding induced lactation, because the practice of augmented lactation is uncommon. Bryant, in a recent review of induced lactation, reported no large studies or randomized controlled trials regarding the use of domperidone and metoclopramide in induced lactation (7). In March 2001, the first case of a mother breastfeeding her baby born via a surrogate host was reported (1). Other recent studies have reported the use of metoclopramide as a galactagogue (7-9). Use of this drug may be warranted when risks versus benefits are considered. Controversy exists, however, surrounding the use of domperidone and metoclopramide for induced lactation. We present the process of lactation induction with metoclopramide in combination with nipple stimulation for an Iranian woman who was the commissioning mother in a surrogate pregnancy.

Case report

A 36 year-old woman presented with a 14 year history of secondary infertility from Polycystic ovarian syndrome (PCOS) and repeated assisted reproductive technology (ART) failures (two tubal ectopic pregnancies, and two, eight week pregnancies without fetal heart) in Royan Institute, where she requested the use of a surrogate host in this centre. She underwent ovulation induction using a standard protocol. Following 17 oocyte retrievals and intra cytoplasmic sperm injection (ICSI) performed with the use of her husband’s spermatozoa, there were six embryos which underwent a preimplantation genetic diagnosis (PGD) program due to the patient’s history of abortions and repeated ART failures. Two high quality male embryos were transferred to the surrogate host who was her sister-in-law, which resulted in a singleton pregnancy. Routine prenatal care was performed for the surrogate host. At 28 weeks of pregnancy, according to Biervliet et al. oral metoclopramide therapy
at a dose of 10 mg twice daily was initiated in the commissioning mother (1). The commissioning mother was followed by phone contact to assess for any potential adverse drug effects. After a one week period, due to the absence of adverse drug effects, the dosage was increased to eight tablets daily and continued until one week prior to the expected delivery date. The treatment was well tolerated with no metoclopramide side effects reported. In addition to metoclopramide therapy, mechanical stimulation of the nipples was performed. Following cesarean section of the surrogate host on June 16, 2009, the commissioning mother was unable to feed her baby due to absorbed nipples. However, after encouragement and continued twice daily metoclopramide therapy combined with nipple stimulation by an electric breast pump; lactation was successfully induced. The commissioning mother was able to feed her baby and thus enjoy the close contact of breast-feeding, which seemed to satisfy both the mother and baby. After two months, the metoclopramide dosage was decreased to one-half tablet daily. She successfully continued breast-feeding until three months, although milk production and secretion were present, it was inadequate for the infant and therefore, the feedings were supplemented with formula.

**Induced Lactation**

Normal lactation is maintained by a balance of various hormones in combination with frequent and regular stimulation of the breasts. Estrogen and progesterone prepare the breast by proliferation of both the ductal and alveolar systems during pregnancy, in anticipation of the time when lactation will start (7). Since prolactin is the essential hormone during lactation, therefore attempts to induce lactation are largely based on increasing prolactin levels. Induction of lactation is the process by which a nonpregnant woman who is not currently breastfeeding another child is stimulated to lactate (6, 7). Initially, augmentation of lactation has been used to provide nourishment for infants of mothers who are either unable to breast-feed or who died during childbirth. Currently this process is used for adoptive mothers desiring to breastfeed their adopted infants. Several methods (pharmacological and non-pharmacological) have been implemented to augment lactation (6).

**Non-pharmacological methods**

Nipple stimulation, by hand or electric breast pump, mimics the sucking of a newborn infant and the suggested time period to commence nipple stimulation techniques is at least two months prior to the arrival of the adopted infant (10-12). Other non-pharmacological methods such as dietary aids and breast massage with warm compresses, remain controversial (6).

**Pharmacological methods**

Galactogogues are medications that aid in initiating and maintaining adequate milk production. The most common agents are listed in Table 1. Metoclopramide antagonizes the release of dopamine, which in turn inhibits the effect of prolactin-inhibitory factor (PIF) on the pituitary. Metoclopramide blocks the effects of dopamine, which consequently increases prolactin production and therefore milk production. Prolactin may reach 3-8 times the normal levels within one hour of metoclopramide administration and remain elevated for up to eight hours (13). This drug is also being evaluated in commissioning mothers who desire to breastfeed after their infants are delivered by surrogate mothers (1). Lastly, metoclopramide has been successfully used by adopting mothers to stimulate milk production (7-9). The general dose of metoclopramide for induction of lactation is 10-15 mg, three times daily for a period of 7-15 days. Because metoclopramide crosses the blood-brain barrier, its use can be limited by significant central nervous system (CNS) side effects (13). Sedation is the most common side effect, occurring in approximately 10% of users. Depression occurs less frequently. Extra-pyramidal side effects, such as tardive dyskinesia (1%), can occur and are more common in women and children (9, 13). Although the medical literature reports a low incidence of depression, it is a widely held belief among women seeking to induce lactation that depression is a very significant and common side effect (7). Metoclopramide remains the galactagogue of choice due to its documented record of efficacy and safety in women and infants (9). Domperidone, like metoclopramide, is a dopamine antagonist which crosses the blood brain barrier and enters the breast milk to a lesser extent than metoclopramide; thus decreasing the risk of toxicity to both mother and infant, making it an attractive alternative (7-9, 11, 14). Traditional antipsychotics, sulpiride (6-9, 15) and chlorpromazine (7, 9, 16), have been evaluated however adverse events limit their use. Human growth hormone (9), thyrotrophin-releasing hormone (17), and oxytocin (1, 9) have also been studied. Finally, herbal medications have traditionally been used as galactagogues in some cultures. For example, fenugreek and blessed thistle have not been adequately studied, as objective data have been obtained from women already lactating (7-9). Numerous other herbs such as milk thistle, fennel, alfalfa, oats, and marshmallow root are reported to
aid in lactation, however research is scant (7). Most women who succeed in inducing lactation require supplementation in order to provide an adequate amount of nutrition for their infants. Since frequent nursing encourages milk production, it is desirable to keep the infant at the breast for all feelings. The best way to achieve both goals is to use a supplemental feeding device worn at the breast that delivers formula (or stored breast milk). Two common devices are the SNS by Medela and the Lact-Aid Nursing Training system (7). The mother who chooses to augment lactation will need more support and encouragement from her partner, family and health clinician in her efforts to induce lactation. Emotional and psychological factors can influence prolactin and oxytocin secretions, therefore self-confidence and a strong desire to succeed are important factors of success (16, 18).

### Discussion

A case of lactation induction after a surrogate pregnancy is reported. Other methods that can be used for augmenting lactation are described above. We have used metoclopramide in this case because of the success rates reported in both previous studies and case reports. Additionally, it is a well tolerated and safe agent. Most recommendations in the area of induced lactation are based on anecdotal experience because the few studies that exist are either small, short-term studies or case reports (1,10,19,20). Placebo-controlled trials of metoclopramide have demonstrated doses of 10 to 15 mg three times a day to be effective in increasing prolactin and milk production in women with lactation problems (7). Another placebo-controlled study of domperidone, 10 mg three times a day, reported increases in prolactin levels

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mechanism of Action</th>
<th>Recommended Dosage</th>
<th>Adverse Effects</th>
<th>Hale's Lactation Risk Category*</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metoclopramide</td>
<td>Blockage of pituitary dopamine receptors: crosses blood-brain barrier</td>
<td>Oral: 10-15 mg, 3 times per day</td>
<td>Well tolerated. Diarrhea, sedation, depression, tremor, bradykinesia</td>
<td>L2</td>
<td>Biervliet et al., 2001; Bryant, 2006; Gabay, 2002; Hale, 2006</td>
</tr>
<tr>
<td>Domperidone**</td>
<td>Peripheral dopamine antagonist; minimally crosses blood-brain barrier</td>
<td>Oral: 10-20 mg, 3-4 times per day</td>
<td>Well tolerated. Dry mouth, skin rash or itching, headache, gastrointestinal disturbance</td>
<td>L1</td>
<td>Bryant, 2006; da Silva &amp; Knoppert, 2004; Gabay, 2002; Hale, 2006; Riordan, 2005</td>
</tr>
<tr>
<td>Sulpiride</td>
<td>Selective dopamine antagonist</td>
<td>Oral: 50 mg, 2 times per day</td>
<td>Tremor, bradykinesia, acute dystonic reactions, sedation</td>
<td>L2</td>
<td>Emery, 1996; Gabay, 2002; Hale, 2006</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>Central nervous system tranquilizer; blocks dopamine receptors</td>
<td>Oral: 25 mg, 4 times per day reported; use not recommended because of associated adverse reactions</td>
<td>Sedation, lethargy, tremor, bradykinesia, weight gain</td>
<td>L3</td>
<td>Gabay, 2002; Hale, 2006; Nemba, 1994</td>
</tr>
<tr>
<td>Thyrotrophic releasing hormone</td>
<td>Direct stimulation of adenohypophyseal lactotrophs</td>
<td>Nasal spray: 1mg 4 times daily</td>
<td>Well tolerated. No clinical signs of hyperthyroidism</td>
<td>-</td>
<td>Peters et al., 1991</td>
</tr>
<tr>
<td>Fenugreek</td>
<td>Herbal supplement; reputation as a galactagogue, but mechanism of action unknown</td>
<td>Oral: 2-3 capsules, 3 times per day; variable</td>
<td>Maple syrup odor in urine and sweat, diarrhea, hypoglycemia, dyspnea</td>
<td>L3</td>
<td>Bryant, 2006; Hale, 2006</td>
</tr>
<tr>
<td>Blessed Thistle</td>
<td>Herbal supplement; reputation as a galactagogue, but no data support its use</td>
<td>Unknown</td>
<td>None</td>
<td>L3</td>
<td>Bryant, 2006; Hale, 2006</td>
</tr>
</tbody>
</table>

*Hale's Lactation Risk Categories are as follows: L1 (Safest) = The drug is not orally bioavailable in the infant, or no increase in adverse effects to the infant have been noted in controlled studies or observed with a large number of breastfeeding mothers; L2 (Safer) = A limited number of studies of this drug have shown no increase in adverse effects to the infant and/or little evidence exists of risk to the breastfeeding infant; L3 (Moderately Safe) = No controlled studies by breastfeeding women exist, but risk of untoward effects to infant is possible. Therefore a risk benefit assessment should be made (6).
and milk production in women who were pumping milk for their newborns in the neonatal intensive care unit (NICU) (7). In a comparison study, single doses of 5 and 10 mg of metoclopramide and 10 mg of domperidone were administered to nonpregnant women. Prolactin levels were then measured at various time intervals. The results of this study showed that nulliparous women had a greater response (percentage of elevation above baseline) to both medications, with the greatest response to metoclopramide (10 mg). Multiparous women had similar responses to all medication doses (21). Several studies previously evaluated the efficacy of medications on lactation in puerperal women or adoptive mothers. Our case was the second commissioning mother that succeeded in breast-feeding, the first case was reported by Biervliet et al. An important reason for the increased success in commissioning mothers when compared with adoptive mothers is that surrogate mothers have a longer preparation period and galactogogue treatment can be started sooner after delivery. However, in adoptive mothers, the timing of the adoption is rarely specific and it is frequently a great challenge for a mother to initiate preparation for lactation induction in an adequate amount of time.

For many mothers, the primary goal of lactation induction is not milk production but rather establishing an emotional bond with their infants. The purpose of most adoptive and commissioning mothers interested in lactation induction is to achieve the enhanced mother-infant bonding that breastfeeding promotes, rather than the nutritional benefits it brings. Therefore, it is necessary for both clinicians and nurses to give them sympathetic counseling and adequate information to enable them to achieve high success rates (1,6). The success rates for lactation induction amongst adoptive mothers have ranged from 50-90% (1).

Few studies exist on lactation induction, and those that are available are small and primarily have studied women who completed their pregnancies. Additionally, even less is known about the mechanisms of action that herbal supplements use to increase lactation. In our case, metoclopramide therapy appears to be safe, well tolerated and effective in induced lactation in a surrogate pregnancy. Hence, we suggest that this preparation can be used for all commissioning mothers. However, a need for more well-designed studies in order to consider the risks versus benefits of this drug exists.

Acknowledgements
There is no conflict of interest in this article.

References
Heterotopic Pregnancy after Assisted Reproductive Techniques: Case Reports

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Abstract

Heterotopic pregnancy (HP) is the simultaneous occurrence of intra- and extrauterine pregnancies. It is a rare event in spontaneous pregnancies with an incidence of less than 1:15000, however the frequency has increased to 1% in accordance with the widespread use of assisted reproductive techniques (ART).

The pre-operative diagnosis of this condition is generally difficult and it would be life threatening for mother and the intrauterine pregnancy. In this report, we present three cases of HP following ART. All cases underwent a standard long protocol and embryo transfers (ET) were performed 48 hours after oocyte retrievals. Finally, two viable intrauterine gestations ended in miscarriages and the third resulted in the delivery of a healthy infant.

The possibility of a heterotopic pregnancy following tubal pathologies, endometriosis and pelvic adhesions should be considered by gynecologists during IVF procedures. Moreover by implantation of a single embryo, the incidence of this risky condition will be dramatically reduced.

Keywords: Ectopic Pregnancy, Assisted Reproductive Techniques, In vitro Fertilization, Embryo Transfer

Introduction

Heterotopic pregnancy (HP) is the co-existence of intra- and extrauterine gestations (1). Although extremely rare in a spontaneous pregnancy with an estimated rate from 1:15000 to 1:30000, nowadays its incidence has increased to 1:100 with the widespread use of assisted reproductive techniques (ART) or ovulation induction (2).

HP is a life threatening condition for both the mother and the fetus (3). The probabilities of spontaneous abortions are higher in combined pregnancies than intrauterine-only pregnancies (4, 5). These complicated pregnancies are diagnostic and therapeutic challenges to infertility practitioners (3, 6). Serum serial β hCG concentrations and ultrasound evidences diagnosis of an intrauterine pregnancy might obscure typical signs of an ectopic pregnancy (EP) (1). This delay in diagnosis leads to maternal complications such as tubal rupture and hypovolemic shock which might be fatal (7, 8). Therefore, confirmation of an intrauterine pregnancy is not a reliable indicator to dismiss an extrauterine pregnancy.

With respect to the high incidence of HP following in vitro fertilization/embryo transfers (IVF/ET) and also the possibility of a missed EP diagnosis with increased remarkable consequences, special attention to HP issue is essential in all IVF cycles in order to preserve a viable intrauterine pregnancy and avoid maternal mortality through timely intervention.

In this report, we present three cases of HP following ART.

Case Reports

Case 1:

A 33 year-old woman, nulligravida 0, married for the second time, was admitted to our IVF unit with a 13 year history of primary infertility. Past medical history indicated a history of appendectomy and her history was negative for pelvic inflammatory disease (PID), previous EP, venereal disease or intrauterine device usage.

A recent ultrasound demonstrated an endometrioma cyst with an approximate diameter of 21 mm in the left ovary accompanied by some perianexal adhesions and a submucosal myoma which measured 13×9 mm in the posterior wall of the uterus. For the first time, the patient underwent an agonist long protocol. On the 10th day of stimulation, five mature oocytes were collected by vaginal ul-
Trasound monitoring. Four oocytes were fertilized after insemination and 2, four-cell two, 4-cell stage embryos were transferred into the uterus using labotect catheter. The luteal phase was supported by daily intramuscular injections of progesterone for two weeks. Initial vaginal sonographic examination performed 32 days after ET demonstrated one intrauterine gestational sac with a fetal pole and heart beat at seven weeks of gestation. The patient was hospitalized six days later (38 days after ET), with the chief complaint of low abdominal pain which began two hours prior to her admission. She was hypotensive with a hemoglobin concentration of 5 g/dl and a plasma βhCG level at this stage of 6000 IU/l. A repeat ultrasound scan confirmed a viable intrauterine gestation (eight weeks gestation) and an echogenic mass in the right tube which indicated an EP.

An emergency laparatomy was performed and the ruptured right tubal pregnancy removed. The intrauterine pregnancy continued until a transvaginal ultrasound two months following surgery revealed a missed abortion of an intrauterine pregnancy.

**Case 2:**
A 37 year-old woman, nulligravida 0, with an 11 year history of primary infertility, underwent an IVF procedure at our infertility center. She had a prior failed intrauterine insemination (IUI) attempt two years prior to her admission. During the previous treatment a laparoscopic myomectomy had performed with the removal of a number of adhesions in the left ovary.

The patient underwent a standard long protocol. Oocyte retrieval was performed on day 15 of the cycle which resulted in the recovery of five mature oocytes. Subsequently, four oocytes were fertilized. At 48 hours after ovum pickup three embryos (two, 4-cell and one, 5-cell) were transferred to the uterus transcervically with the use of a labotect catheter. Luteal support was performed by intramuscular administration of daily progesterone for two weeks.

The first transvaginal scan performed 24 days after ET demonstrated a gestational sac in the uterine cavity (5-6 weeks gestation). Additional findings were the existence of enlarged ovaries and pelvic-free fluid which suggested an ectopic pregnancy. Therefore, serial βhCG levels and ultrasound monitoring were suggested.

One week later, the patient was referred to the hospital with severe lower abdominal pain. The second vaginal sonography revealed enlarged ovaries with small amounts of fluid and blood in the cul-de-sac. A suspicious mass was observed between the uterus and right ovary. These findings indicated an ectopic pregnancy together with an intrauterine gestational sac of six weeks.

Laparoscopy with right salpingectomy was performed the following day. At this stage, the luteal phase was supported with both intramuscular and intravaginal progesterone administration. Two weeks after surgery, transvaginal sonography revealed a gestational sac without a heart beat and with two weeks growth retardation, which resulted in a miscarriage.

**Case 3:**
A 25 year-old woman, nulligravida 0, with a four year history of primary infertility and diagnosis of unexplained infertility underwent a second IVF procedure at our institution. A standard long agonist protocol was initiated and ultimately two embryos were transferred into the uterus. The first ultrasound was performed 32 days after ET which demonstrated a viable intrauterine pregnancy of 6.5 weeks gestation. Additional findings demonstrated the existence of an echogenic mass measuring 48×25 mm with free fluid in the cul-de-sac and peri-left ovary, which suggested the possibility of HP.

After two days, she presented to the hospital with complaints of acute abdominal pain and peritoneal irritation. After admission, severe vaginal bleeding commenced. A repeat transvaginal ultrasound revealed a ruptured left fimbria pregnancy with massive free fluid in the cul-de-sac and a seven week intrauterine pregnancy with a normal heart rate was confirmed.

An emergent laparatomy with left salpingectomy was performed and she had a non-complicated post-operative course. The intrauterine pregnancy proceeded uneventfully and she had a cesarean delivery at 34 weeks of gestation due to a premature rupture of the membranes and breech presentation which resulted in the delivery of a healthy 1850 g male infant.

**Discussion**
HP is an uncommon obstetric event in spontaneous pregnancies estimated at approximately one per 15000-30000 pregnancies (2). Many predisposing factors including: pelvic inflammatory disease, salpingitis, endometriosis, pelvic surgery, uterine malformations and a history of ectopic pregnancy all lead to an increased incidence of this hazardous phenomenon (1, 2, 5, 9).

Currently, addressed to the extensive use of ART methods such as IVF and ET which have the potential for multiple pregnancies, the frequency of
this condition has increased as high as 1% (2). The use of ovarian hyperstimulation drugs, multiple ETs, and the techniques of ET, along with previous tubal damage predisposes patients to the development of a combined pregnancy (1,2,5). According to studies, the main risk factor of HP following IVF is tubal infertility (10,11). In a retrospective review that evaluated the risk factors of HP following IVF, ten out of twelve patients had a previous history of salpingitis and one had tubal surgery due to a previous EP (5). The risk of a second EP is 2-15 times higher in women with a prior history of EP (12). It has also been suggested that unilateral salpingectomy may increase the risk of an EP on the opposite side (11).

According to a study by Strandell et al. although there is no consensus that fibroids may be a risk factor for EP following ART, however the myometrial scar may disrupt normal uterine contractions which prevents intrauterine implantation (11). Therefore, further studies need to be performed. It is reported that HP are directly relevant to the number of transferred embryos during IVF/ET treatment (2,13-15). Therefore a reduction in the number of transferred embryos could contribute to a decrease in the occurrence of HP (8). Both the technique and timing of ET have been implicated as risk factors for HP. Yovich et al. have noted a significantly higher rate of extrauterine pregnancies when embryos were implanted nearer to the uterine fundus rather than mid-cavity (16). According to Lesny et al., a difficult ET leads to junctional zone contractions which enhance the risk of EP. The incidence rates of EP exceed 1.5-10 times when ET is performed under difficult rather than easy conditions, in particular if ET is performed on day two compared to day three following oocyte collection (12).

Early detection of HP remains difficult in the absence of a tubal rupture (1). A normal rise in serum β-hCG levels and ultrasonography diagnosis of intrauterine pregnancy might obscure the abnormal pattern typically observed in EP. When ultrasonography reveals an intrauterine pregnancy the possibility of EP is generally ignored (1). Furthermore, the presence of ovarian hyperstimulation syndrome (OHSS) during ART might mask the EP signs in HP cases thus preventing a definitive diagnosis prior to surgery (17). Over half of all HP are recognized during emergency surgeries following tubal rupture (2).

Treatment of HP is complicated because of the co-existing intrauterine pregnancy and should be the least invasive as possible in order to achieve a good outcome for the intrauterine pregnancy. Laparoscopic surgery (salpingostomy or salpingectomy) is the most effective treatment in a woman diagnosed with tubal pregnancy and its safety is demonstrated during pregnancy (18,19). Laparotomy is generally performed for life threatening cases or for occasions that can not be treated by laparoscopy (8).

As seen in these cases, tubal damage, pelvic adhesions, a history of pelvic surgeries and the high number of transferred embryos possibly are risk factors of a combined pregnancy occurrence. Ultimately, addressing recent studies that suggest a single ET would avoid multiple pregnancies without significant effect on pregnancy outcome (20,21) therefore, special attention to this high-risk group is highly recommended before every IVF procedure in order to lessen the HP rate as a result of a single embryo replacement.

Acknowledgment

There is no conflict of interest in this article.

References

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