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The Impact of Ovarian Stimulation and Luteal Phase Support on Embryo Quality and Implantation Process in Mice

Mahmoud Hashemitabar Ph.D., Babak Ghavamizadeh Ph.D., Fatemea Javadnia Ph.D., Esmaiel Sadain M.S.

Department of Anatomy and Embryology, Ahwaz Medical University, Ahwaz, Iran.

Background: The luteal phase defect is a common event following the ovarian stimulation. The aim of the present study was to evaluate the use of human chorionic gonadotropine (hCG) and progesterone hormones to improve the luteal phase defect.

Materials and Methods: 60 mice were superovulated routinely with human menopausal gonadotropin (hMG) (7.5U) and hCG (10U). The mice were mated and divided into 3 groups: 1- control (n=20) 2- hCG treatment (n= 20), and 3-Progesterone treatment (n=20). Each group was divided again into two subgroups. The mice (10 from each group) had no injection in group one and were injected intraperiteneal (IP) by hCG (5U/day) and progesterone (1mg/day) subcutaneously (sc) in groups 2 and 3, respectively for four days. On the day 5, the animals were killed by cervical dislocation and the uterus were flushed to count the number of blastocyst and their quality. The above treatment were carried out for 12 days in the other 10 mice in each group. Similarly group one had no injection and groups 2 and 3 were injected by hCG and progesterone for 12 days respectively by the same manner as mention above. The animals were killed on day 13 and the implanted embryos were counted. The uterus and ovary were processed on days 5 and 13 of pregnancy for histological studies.

Results: The mean number of blastocysts per mouse were: 12.2%, 2.6% and 3% in group 1 to 3, respectively. The nomber of implanted embryos were 29 as: 13 living fetus in one mouse and 16 resorption fetus in the other. The morphology of uterus on day 5 was as follow: no development in the stroma and endometrial gland in control group, the stroma and endometrial gland so developed to form the saw teeth appearance which indicated on receptivity of uterus in hCG treated group similar to progesterone treated group, but without the saw teeth appearance. The continuation of hCG injection maintained the receptivity of uterus; while, the continuation in progesterone caused metaplesia of epithelium. The morphology of ovaries in all three groups showed no changes in corpus luteum size on day 5, and showed the following changes on day 13: increasing the number of primary and secondary follicles in control group; while, reducing the size of corpus luteum in hCG group.

Conclusion: Progesterone did not improve the uterus and implantation rate. The prolonged usage of progesterone can change the morphology of uterus to more abnormal state in conterast to the prolonged usage of hCG.

Key Words:*Implantation, Luteal Phase Defect,Ovarian Stimulation, Embryo, Mice*

Introduction

The process of implantation depends on the quality of embryo and receptivity of the uterus. Both quality of embryo (Ertzeid and Storeng 2001) and receptivity of the uterus (Tavaniotou *et al*., 2002) reduced significantly following the controlled ovarian hyperstimulation (COH) by gonadotropin hormone.

However, the Gonadotropins have been widely used for COH during in vitro fertilization (IVF) procedures. It must be born in mind the negative role of ovarian stimulation on the quality of embryo which reduce the number of blastomers and increase the fragmentation of embryo in preimplantation stage (Van der Auwera and Hooghe 2001). On the other hand, using hMG in an IVF protocols may exert the release of a high level of estrogen in vivo. The estrogen with a positive feedback lead to the early surge of the LH and early luteal phase induction and so proceeding of implantation window to open in an earlier time than in natural cycle. This may be reflected to a defective asynchrony in embryo-endometrial interaction and low level of pregnancy

Corresponding Author*:*
Mahmoud Hashemitabar. Department of Anatomy and Embryology, Ahwaz Medical University, Ahwaz, Iran.
E-mail : Hashemi_tabar@hotmail.com

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Table I. The number and quality of embryos on day 5 of pregnancy in different groups.

Group	No. of positive plug	No. & Mean of embryo/ mouse	4 cells	8 cells	Morula	Blastocyst	Failed Blastocyst
Control	5	61(12.2)	0	0	3(4.9)	46(75.4)	12(19.7)
hCG treated	7	18(2.61)*	0	0	0	9(50)	9(50)
Progesterone treated	5	15(3)*	2(13.3)	4 (26.7)	1(6)	8(54)	0(0)

* significantly different in comparison to control group (P<0.05)
Values in parentheses are percentages

rate (Valbuena *et al.*, 1999). It is not exactly clear whether the COH will cause the unreceptivity of uterus directly, or it is distrupted the estrogen/ progesterone balance indirectly (Zayed *et al*., 2003). The embryo transfer to uterus in an IVF cycle (which is often carried out on the second day after insemination at 4 to 8 cells stage) is usually accompanied by closing of implantation window; therefore, it fails to implant successfully (Paulson *et al*., 1990).

In order to improve the implantation process, it is strongly suggested to use the progestrone in hormone replacement protocols. So, the progesterone improves receptivity of uterus by increasing the proliferation of endometrial stroma cells and coil the endometrial gland consequently to induction of secretary phase to uterus (Ben–Nun *et al*., 1990). Therefore, the study of preparing uterus by progesterone is a critical step in the assisted reproductive technology (ART) to acquire better result of implantation following the embryo transfer. The LH and the hCG act on the corpus luteum by binding to LH receptor on the granolosa cells; thereby, enhancing the progesterone secretion which has a high potential to improve the implantation rate indirectly (Garcia *et al*., 1988).

The positive effect of progesterone and hCG for preparation of uterus has been pointed out in replacement hormone therapy and in the natural cycle. We used progesterone and hCG by the same function as the LH to evaluate the possible positive role of either of these regimens in improving the implentation process following the COH protocol

Materials and Methods

The total number of 60 mice (NMRI) aged 6-8 weeks were superovulated by hMG (7.5 IU) intraperitonaly (IP), and 48 hr later with hCG (10 IU) IP. The mice divided to 3 groups (no=20 for each groups): 1 -control; 2- treated by hCG; 3- treated by progesterone. Then, the mice were mated by a male (NMRI) and checked by vaginal plug on the next day to determine the positive plug for confirmation of pregnancy in each group.

The mice in each group was subdivided equaly into two subgroups: first subgroup were studied on day 5 of pregnancy (n=30) and the second subgroup were studied on day 13 of pregnancy (n=30). The study of luteal phase support on day 5 of pregnancy was carried out as follows: 1- No adminstration in group one, using only from COH regimen as a control group (n=10). 2- Using hCG IP (5 IU/day) from day 1-4 of pregnancy in group two (n=10). 3- Using progesterone administration (sc) (1 mg/day) from day 1-4 in group three (n=10).

The positive plug mice were killed by cervical dislocation on day 5 of pregnancy and the uterine horns were flushed by M2 media to count the number of blastocysts and their quality. The quality of embryos was evaluated according to routine IVF procedures (Trounson and Gardner 2000). To study the luteal phase support on day 13 of pregnancy; the above treatment was continued for another 8 days. So, no adminstration in group one (n=10), hCG adminstration in group two (n=10) and progesterone in group three (n=10), as the same manner of first half of each groups. The positive plug mice were killed on day 13 and the implantated embryos were counted.

The histological sections were carried out on both positive and negative plug mice on days 5 and 13 of pregnancy. The samples were fixed by formalin buffer. Then, samples were process by the routine procedures of dehydration, and clearing, followed by impregnation and paraffin blocks. Finally, the 5µm sections were stained using Hematoxilline and Eosin (H&E).

Statistical Analysis was performed by student t-test to compare the mean of embryos among the different groups by using SPSS software.

Results

Embryo Assessment

Table I presents the results generated from three groups of control, hCG treated and progesterone. The hCG and the progesterone adminstration revealed that a significantly lower number of the blastocysts, were obtained when compared to control group. It also showed a significantly decrease in the quality of embryos in hCG group, but not in progesterone group; whereas, there was a retardation in cleavage rate. The mean number of embryos that reached the blastocyst stage (54%) was significantly lower in progestron group when compared to control group (75% P< 0.05).

The total number of implanted embryos were 29/2 mice in hCG treated group, which was 13 living fetus

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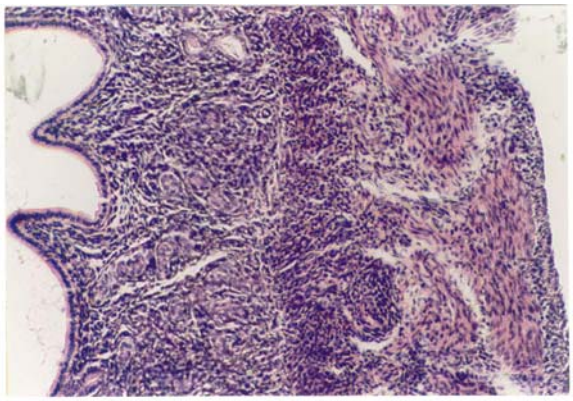

Figure 1. Longitudinal section of uterian horn in control group on day five. Simple columnar epithelium with poor endometrial gland and stroma cells proliferation. H&E(X40).

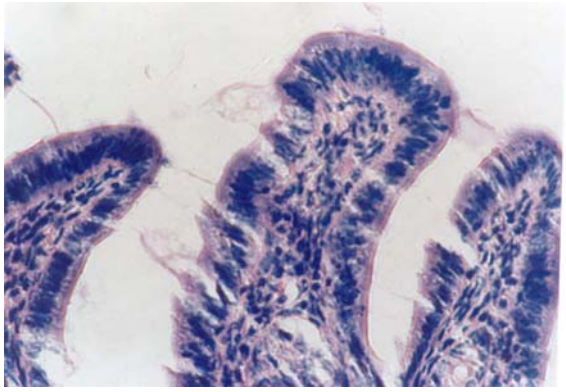

Figure 3. Longitudinal section of uterian horn in progesterone treated group on day five. The irregular columnar epithelium with high endometrial gland and stroma cells proliferation indicated on endometrial improvement. H&E (X400).

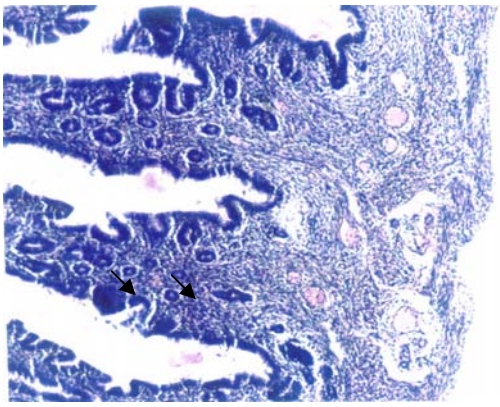

Figure 2. Longitudinal section of uterian horn in hCG treated group on day five. Saw teeth appearance (arrows) with irregular columnar epithelium. High endometrial gland and stroma cells proliferation indicated on more endometrial receptivity. H&E (X100).

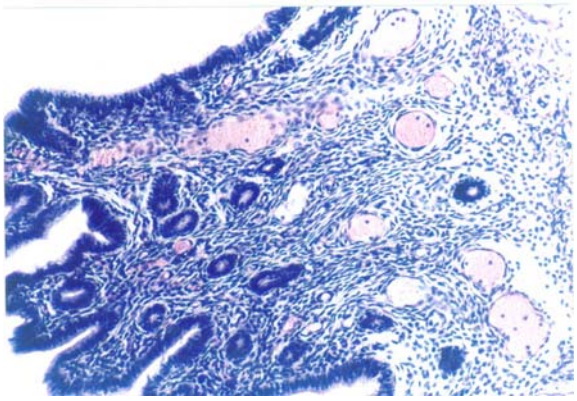

Figure 4. Longitudinal section of uterian horn in hCG treated group on day 13. The endometrial morphology is as day five, as well as edematous reaction in stroma.

in one and 16 resorptions fetus in another mouse.No embryo implantation was observed in the other groups.

Morphological assessment of utrian horn

Following the COH with hMG in control group, the morphology of the endometrium changed to undeveloped state as; a poor storma, poor endometrial glands and a regular simple columnar epithelium (Fig. 1). Treatment with hCG following COH in group two changed the endometrial morphology to a developed state as; increasing the stormal cells proliferation, increasing the number of endometrial glands and changing the epithelium to irregular simple columnar. The most important index in improvement endometrium was establishment of saw-teeth appearance (Fig. 2). The progesterone treatment following the COH in group three improved all the indexes such as: stroma and glandular cells proliferation, but there was no saw teeth appearance (Fig 3). The morphological changes of endometrium on day 13 was as same as day 5 in groups one and two. The continued treatment with hCG improved the receptivity of uterus showed by observation of more arterial profiles in lamina propria layer (Fig. 4). However, continued adminstration of progesterone strongly affected the morphology of endometrium by metaplasia induction and changing the epithelium to stratified layer as well as elimination of stroma cells and lamina propria layer (Fig. 5).

Morphological assessment of ovary

Considering the size of corpus luteum, the morphological study of the ovary on day 5 showed no differences between the three groups. This suggest that using the hCG and progesterone treatment in groups two and three has no considerable effects on the size of corpus luteum in comparison to control group. The morphological changes on day 13 showed that the number of immature follicles increased in

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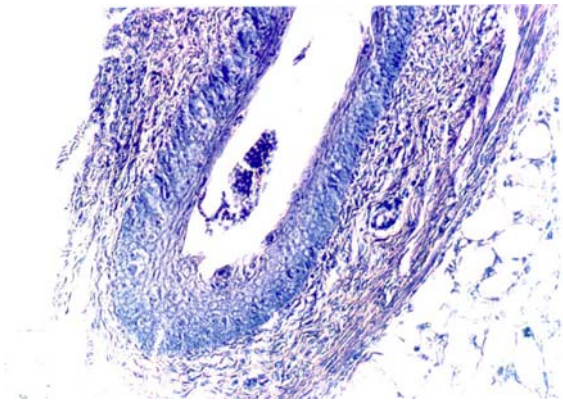

Figure 5. Longitudinal section of uterian horn in progesterone treated group on day 13. Metaplasia of epithelium and loss of stroma cells. H&E (X200).

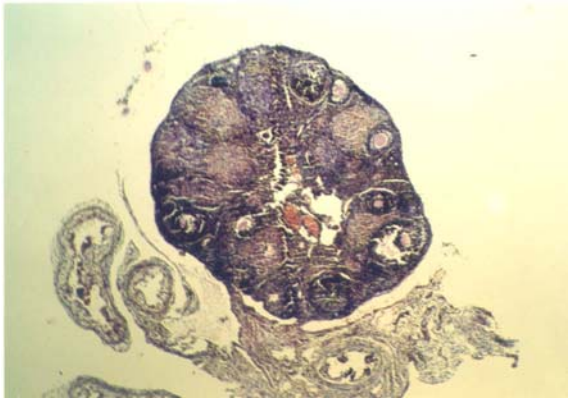

Figure 7. The section of ovary in hCG treated group following COH on day 13. H&E (X40).

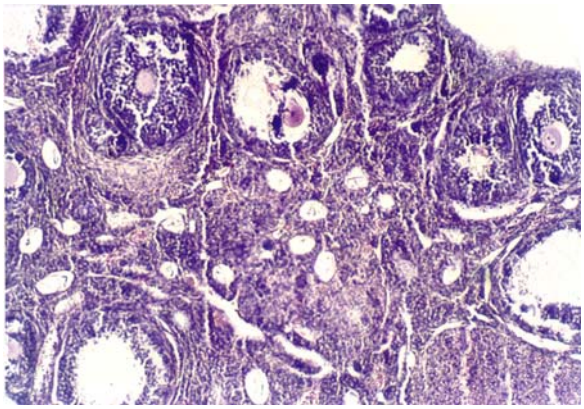

Figure 6. Ovary in control group following the COH on day 13. H&E (X100)

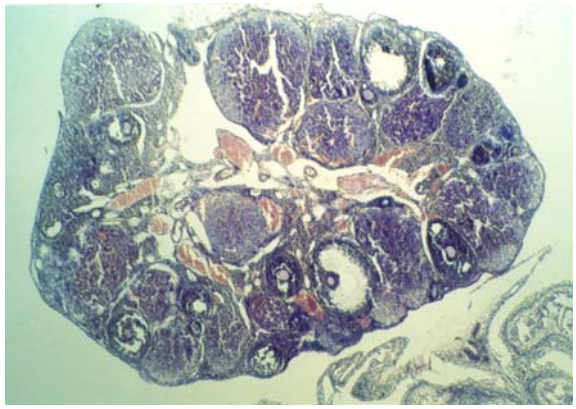

Figure 8. The ovary in progesterone treated group following the COH on day 13. H&E (X40).

control group (fig. 6), while the treatment by hCG and progesterone have no such consequences. The continued adminstration of hCG caused the hypotrophy of ovary and decreased the size of corpus luteum (fig. 7). Continued injection of progesterone has no effect on the size of corpus luteum and the number of primary or secondary follicles (fig. 8).

Discussion

The purpose of embryo evaluation our study was to investigate whether the oversecration of esteroid hormones orhCG may affect the quality of the embryo in oviduct and uterine horn. Ertzeid and Storeng (2001) previously reported that in natural cycle, the number of mice embryo in one cell stage reaching to blastocyst stage was 60% versus 41% in an COH cycle. Auwera Vander *et al*., (1999) also reported a similar result in a case of COH cycle and showed that implantation rate improves following embryo retrieval and in vitro culture until blastocyst stage for transfer to pseudopregnant mice. The results of this study also indicate that manipulation of uterus for superovulation by progestrone or hCG for inhancing the implantation rate may impair the oviduct microenvironment. As in our study, the hCG and progestrone decreased the number of embryo significantly from 12.2% in control group to 2.6% and 3% in hCG and progesterone group respectively. In addition, the number of failed blastocysts increased from 19.7% in control to 50% in hCG treated group. Although, the main goal of using hCG treatment is to improve the implantation rate, but our study showed that it impaired the quality of embryo as well this effect could be due to alteration of oviduct milieu, following the superovulation and hCG therapy which may exerts an abnormal effect on oviductal secretion (growth factor and proteins) necessary for early embryo development (Boatman 1997).

In the case of progestrone treated group, there was a retardation in cleavage division as well as smaller number of embryos compared to controls. Juneja

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(1995) explained that the blocking of pregestrone with monoclonal antibody RU486 in preimplant stage of embryos will cause the cleavage retardation in vivo and in vitro. Certainly, there is a receptor for progestrone transport in the embryo cells which activate the cleavage division, but it is not clear why both the Progestrone (in our study) and Antiprogestrone drugs (in the above study) act with the same manner and cause the retardation in cleavage division. In addition, the results of our study for implanted embryos in hCG treated group indicated that from 5 positive plug mice, 29 embryos implanted per 2 mice. This is a promising result for implantation rate. So, to determine the positive effect of hCG on uterus, the morphological assessment designed on all the positive and negative plug mice. This positive effect on days 5 and 13 was defected as the acceleration of stroma and glandular cells proliferation and consequently the improvement of receptivity of uterus by means of more secretion of endogenous progesterone from theca cells of corpus luteum. But, more recently Ku *et al*., (2002) and Zhou *et al*., (1999) have shown that presence of the LH/hCG receptors on human endometrial cells means that the endometrial cells can directly respond to hCG by increasing the vasodilation action of uterian arteries. Also, by more differentiation of stroma cells to react as decidualization as well as edeomatous reaction and by stimulation of epithelial cells to interact with blastocyst and continuation of pregnancy. Therefore, both direct or indirect effect of hCG in maintenance of corpus luteum confirmed the efficiency of hCG in progression of implantation. This was in contrast to exogenous progesterone which in the short time usage, despite of its positive effect on morphology of uterus, is insufficient to support a successful pregnancy. Also, in long term adminstration there is a metaplesia in epithelium and elimination of stroma cells instead of implantation improvement.

Therefore, in this study the progesterone has no advantages in optimization of implantation process; although, it is a conventional method in preparation of uterus in routine IVF cycle and in hormone replacement method for embryo transfer (Navot *et al*., 1989). Therefore, we suggest more safety effects for hCG instead of progesterone in maintenance of implantation event. Of course, we have to be aware of the serious effect of hCG on the quality of early embryo. Therefore, more investigations are nessessary to solve this controversy between the hCG effect on quality of embryo and its effect on implantation improvement.

Acknowledgement

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Editorial Comments

I read with interest the paper by Hashemitabar *et al.* (2004) reporting "The Impact of Ovarian Stimulation and Luteal Phase Support on Embryo Quality and Implantation Process in Mice" and would like to comment on some of the methodological and clinical aspects of the study. Luteal support is necessary in ovarian stimulation protocols, such as those commonly prescribed for in vitro fertilization and embryo transfer (smith *et al*.1989,Balaish-Allart *et al*.1990). Abnormal luteal function occurs when ovulation induction is induced with gonadotropins or when endogenous gonadotropins are suppressed with a gonadotropin-releasing hormone agonist (GnRH-a) (Olson *et al*.1983, Smitz.*et al*.1992b). Or granulosa cells of the follicles are removed or destroyed during oocytes retrieval, so steroid hormones secretion from the corpus luteom is impaired and luteal phase defect is occurred.

We feel that the paper by Hashemitabar *et al*. (2004) appears to have methodologically problems because the authors concluded that progesterone or hCG administration for luteal phase support have negative effects on implantation and embryo quality in mice. So, some questions are raised in this respect. First, the authors used only induction ovulation without oocytes retrieval and embryo transfer. However, I don't know how the responses of ovaries to gonadotropins was and how many embryos developed or arrested in the mice uterine? In addition, it is impossible to evaluate the effect of progesterone based on the number and embryo quality. The second, GnRH-a was not used for pituitary suppression in this study. As far as we know luteal phase defect has been demonstrated in cycles stimulated by using a protocol which contains GnRH-a, because reduced serum sex hormone level in luteal phase may influence embryo implantation in IVF-ET. In order to improve the clinical pregnancy rate, it is necessary to supply progesterone from the day of oocyte retrieval onwards to the IVF-ET patients.

Systematic review of the literature was performed to determine whether luteal phase support increases reproductive success in IVF cycles. A Meta-analyses were conducted when multiple homogeneous studies addressed a single issue. Luteal supplementation with either hCG or progesterone significantly improved fertility outcomes compared with no treatment (Prittz.2002). Progesterone and hCG have both been used for this purpose, with comparable outcomes (Martinez.2000). Progesterone is the product of choice; however, as it is associated with a lower incidence of ovarian hyperstimulation syndrome (OHSS). Its use is indicated up to the 12th weeks of pregnancy until placenta introduces steroidal hormones (Penzias, 2002).

In this study, the authors evaluated the effects of progesterone, hCG or no treatment on the morphology of endometrium but not on the implantation because we haven't know how many oocytes and embryo developed and how many of them arrested. In the other studies, it has been shown that progesterone administrated is capable of reproducing all the endometrial changes normally seen in the luteal phase of menstrual cycle (Smitz *et al*,1992 , 1993).

On the other hand, I feel that we cannot extend these results to the ART treatment cycles in human. However, GnRH-a protocols necessitate the use of luteal phase support. Some researchers believe that hCG is better but it increased the risk of OHSS. After all, the relationship between progesterone, hCG and endometrium as well as embryo quality is more complex than the conclusion of the work by Hashemitabar *et al*. (2004). However, the use of progesterone or hCG is strongly recommended because without supporting the luteal phase, the outcome of ART cycles are impaired.

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Dr. Mohammad A. Karimzadeh
Department of Obstetrics and Gynecology, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

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