The Effect of Progesterone and Exogenous Gonadotropin on Preimplantation Mouse Embryo Development and Implantation

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Abstract: The aim of this study was to evaluate the effects of progesterone and ovarian stimulation on the development and implantation rate of mouse embryos. Two-cell embryos were collected from superovulated mice and cultured in the presence of different concentrations of progesterone (0, 5, 10 and 20 ng/ml). Also other mice were rendered pregnant in unstimulated, unstimulated progesterone-injected, superovulated and superovulated progesterone-injected groups to collect the blastocysts. The number of blastocysts and implantation sites were recorded on the 4th and 7th day of pregnancy, respectively. The diameter and cell number of blastocysts were analyzed in the in vitro and in vivo groups. After 120 h culture, the percentage of hatched blastocyst embryos in control and 5, 10 and 20 ng/ml progesterone-injected groups were 63.9%, 64.2%, 64.2% and 75.6% respectively. There were significant differences between the developmental rates of embryos in the presence of 20 ng/ml progesterone and the control and other concentrations of progesterone-injected groups (P≤0.001). The in vivo blastocyst survival rate (97.68%) and implantation rate (92.06%) in the unstimulated and progesterone-injected groups were higher than in the other groups. Blastocyst cell numbers in the superovulated (128.62 ± 1.30) and superovulated progesterone-injected groups (126.88 ± 1.60) were significantly different from the control (P<0.001). The progesterone injection without ovarian induction improved the embryo survival and implantation rates, but after superovulation it did not ameliorate the negative effects of superovulation on the implantation rate.

Key words: embryo development, implantation, ovarian stimulation, progesterone, survival rate

Introduction

Development of the embryo depends on embryo quality and the uterine microenvironment in which it must develop. The principal hormone controlling the uterine environment is progesterone and its maternal concentra-

ions are closely linked to early embryo development [16, 18, 25].

Progesterone is the hormone of pregnancy and has several critical functions during embryo development and implantation including: endometrial receptivity, embryonic survival during gestation and transformation.

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of the endometrial stromal cells to decidual cells [5, 18, 24]. The anti-inflammatory function of progesterone plays an important role in protecting the embryo from rejection by the maternal immune system [25]. Ovarian hormones, such as progesterone, play a role in the regulation of cytokine production and are involved in growth and differentiation of embryos [22]. Since the physiological effects of progesterone are mediated by progesterone receptors (PR), the expression of PR during the preimplantation stages of pig and mouse embryos was determined [1, 12].

Gonadotropins such as pregnant mare’s serum gonadotropin (PMSG), equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) are commonly administered for superovulation in humans and animals to produce a large number of oocytes. There are controversial reports on the effects of exogenous gonadotropic hormones on oocyte and embryo quality [7, 8, 23, 31], oviductal and uterine environment and embryo implantation [9, 10, 28]. Sato and Marrs [23] showed that oocyte and embryo qualities may be affected by increased amounts of exogenous gonadotropin stimulation. Van der Auwera et al. [29] showed superovulation in the mouse caused delayed embryonic development in vitro and in vivo, increased abnormal blastocyst formation, pronounced fetal growth retardation, and increased numbers of resorption sites. Also, several researchers have shown that ovarian stimulation has deleterious effects on embryo quality as well as uterine milieu and implantation rates of embryos [6, 9, 13, 26, 29]. In contrast, Ziebe et al. [31] demonstrated that the administration of exogenous gonadotropin was not reflected in cleavage capacity or quality assessment of the resulting embryos.

For improving pregnancy rates after ovarian stimulation, progesterone injection has been performed however it is not clear whether progesterone, which plays a key role in establishing and maintaining pregnancy, acts directly or indirectly through the mother’s reproductive tract.

Since embryonic and fetal growth retardation have been described after superovulation in mice, the aim of this study was to determine whether this negative effect is induced by impaired embryo quality, or by abnormal blastocyst formation in the superovulated oviductal or uterine environment. First, in vitro preimplantation embryonic development after superovulation and progesterone administration was observed and compared with controls. Second, in vivo preimplantation embryonic development, implantation rate and embryo quality after superovulation and / or progesterone injections was evaluated in comparison with unsuperovulated control mice.

### Materials and Methods

#### Animals

Adult female (6–10 weeks old) and male (8–12 weeks old) National Medical Research Institute (NMRI) mice were cared for and used according to the guide for the care and use of laboratory animals of Tarbiat Modares University. They were housed under a 12 h light:12 h dark regimen (light on at 7:00), at a temperature of 23 ± 3°C and relative humidity of 44 ± 2%. The animals were used for both the in vitro and in vivo studies.

#### Experimental design

To evaluate the effects of progesterone and superovulation on the development of embryos, our study focused on in vivo and in vitro development. For the in vitro study two-cell mouse embryos were collected and randomly divided into control and different concentrations (5, 10, 20 ng/ml) of progesterone-injected groups.

For the in vivo study the adult female mice were divided into the following groups: A, unstimulated control group; B, unstimulated progesterone-injected group; C, superovulated group and D, superovulated and progesterone-injected group. The mice in all groups were rendered pregnant naturally using fertile-proven adult mice.

#### Reagents and culture media

All reagents were purchased from Sigma Aldrich except for hormones PMSG (Folligon; Intervent) and hCG (Sereno). The medium used for isolation and culture of embryos was T6 medium supplemented with 5 mg/ml bovine serum albumin (BSA).

#### Two-cell embryo collection for in vitro study

The adult mice were superovulated using an i.p. injection of 10 IU PMSG which was followed 48 h later with
another i.p. injection of 10 IU hCG. Then, the superovulated female mice were mated with fertile males. The presence of a vaginal plug on the following day indicated successful mating, and this was designated day 1 of gestation. The pregnant mice were sacrificed by cervical dislocation 48 h after the hCG injection. Their fallopian tubes were dissected and flushed with T6 medium and two-cell embryos with normal morphology (that is equal sized blastomeres with no apparent fragmentation observed under an inverted light microscope) were collected and divided into four groups.

**In vitro culture of embryos and their development assessment**

Groups of approximately 115 embryos were cultured in drops of T6 medium which was supplemented with 5 mg/ml bovine serum albumin (BSA) and different concentrations of progesterone (0, 5, 10 and 20 ng/ml) for 120 h. The embryos were cultured in drops under oil and progesterone was dissolved in the oil to give the same concentration as in the medium [11].

Embryos were observed daily under an inverted microscope, and the number of embryos reaching the 4-cell, 8-cell, morula, blastocyst and hatched blastocyst stage was recorded until 120 h.

The diameters of late blastocysts obtained in vitro were measured by using a calibrated eyepiece.

**Blastocyst staining**

For blastocyst staining, the embryos at expanded and partially hatched stages were chosen and incubated in a 500 µl solution of BSA free T6 with 1% Triton x-100 and 100 µg/ml propidium iodide up to 10 s, or until the trophoectoderm visibly changed color to red and shrank slightly, as monitored visually using a dissecting microscope. Blastocysts were immediately transferred into 500 µl of fixative containing 100% ethanol with 25 µg/ml bisbenzimide and were stored at 4°C overnight. The stained embryos were put into glycerol and mounted on the glass microscope slides [27]. Then cell counting was performed under a fluorescence microscope in 2 or 3 planes (460 nm for blue and red fluorescence, and 560 nm for red only).

**Gonadotropin and progesterone treatment**

For the in vivo study, the adult female NMRI mice were divided into 4 groups.

A, Control (n=19): in this group the mice (unstimulated) were mated with fertile males. The presence of a vaginal plug proved natural pregnancy and this day was considered as day one of pregnancy.

B, Progesterone-injected group (n=15), another group of unstimulated mice: after natural mating as for the previous group, a daily injection of progesterone subcutaneously (1 mg/mouse) was performed [21].

C, Superovulated group (n=19); the adult mice were superovulated with an i.p. injection of 10 IU PMSG followed 48 h later by an i.p. injection of 10 IU hCG to induce ovulation.

D, Superovulated progesterone-injected group (n=17): in this group the mice were superovulated and mated as for the previous group (group C), then a daily progesterone injection was performed as for group B.

**Blastocyst collection for in vivo study**

Pregnant mice (n=17 mice) in each of the four groups were sacrificed by cervical dislocation on day 4 of pregnancy and their blastocysts were evaluated. After dissection and flushing of the uterine horn, their embryos were collected in drops of T6 medium.

**Evaluation of embryo quality**

The embryos collected from each group were observed under an inverted microscope and the numbers of surviving and degenerated embryos were recorded. The diameters of late blastocysts were measured by using a calibrated eyepiece. Blastocyst staining was performed as described above and the total cell numbers of blastocysts were counted in each group.

**Evaluation of implantation sites in vivo**

To evaluate implantation rates in superovulated and unstimulated groups, the pregnant mice (about 15 mice from each group) were sacrificed on day 7 of pregnancy. Their uterine horns were separated and observed under a light microscope. The implantation sites appeared as prominent, dark red spots on the uterine horn. The number of implantation sites was recorded for each group. The ratio of implantation sites to the number of surviving embryos in each group (on day 4 of pregnancy) was considered as the implantation rate.
Statistics

The proportions of embryo development and implantation rate among the groups were compared with the Kruskal-Wallis test. Differences between groups were determined by the Mann-Whitney test. Total cell number and blastocyst diameter were analyzed by analysis of variance test.

Results

The effect of progesterone on two-cell mouse embryo development in vitro

The developmental rates of two cell embryos in media containing different concentrations of progesterone are shown in Table 1. After 48 h culture, the percentage of embryos that reached the morula stage in control and 5, 10 and 20 ng/ml of progesterone-injected groups were 58.6%, 62.6%, 71.6% and 85.2%, respectively. The hatching blastocyst rate after 120 h was 63.9%, 64.2%, 64.2% and 75.6%, respectively. Also the percentages of degenerated embryos at the end of the culture period were 26.3%, 28.6%, 24.2% and 19.3%, respectively. There were significant differences between the developmental rates of embryos in the presence of 20 ng/ml progesterone and the control and the other concentrations of progesterone-injected groups at 72 h of culture ($P \leq 0.001$). However at 120 h of culture there were no significant differences in embryo development among the control and progesterone-injected groups.

Table 1. Effect of different concentrations of progesterone on the development of mouse two-cell embryos in vitro

<table>
<thead>
<tr>
<th>Prog. (ng/ml)</th>
<th>No. of 2 cell</th>
<th>8-cell (24 h)</th>
<th>morula (48 h)</th>
<th>blastocyst (72 h)</th>
<th>blastocyst hatched (96 h)</th>
<th>hatched (120 h)</th>
<th>degenerated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>133</td>
<td>86 (64.6%)</td>
<td>78 (58.6%)</td>
<td>48 (36.1%)</td>
<td>62 (46.6%)</td>
<td>39 (29.3%)</td>
<td>85 (63.9%)</td>
</tr>
<tr>
<td>5</td>
<td>126</td>
<td>83 (65.8%)</td>
<td>79 (62.6%)</td>
<td>50 (39.7%)</td>
<td>59 (46.8%)</td>
<td>38 (30.1%)</td>
<td>81 (64.2%)</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>95&lt;sup&gt;a&lt;/sup&gt; (79.2%)</td>
<td>86&lt;sup&gt;a&lt;/sup&gt; (71.6%)</td>
<td>54 (45%)</td>
<td>59 (46.6%)</td>
<td>40 (33.3%)</td>
<td>77 (64.2%)</td>
</tr>
<tr>
<td>20</td>
<td>115</td>
<td>103&lt;sup&gt;a&lt;/sup&gt;b (89.5%)</td>
<td>98&lt;sup&gt;a&lt;/sup&gt;b (85.2%)</td>
<td>69&lt;sup&gt;a&lt;/sup&gt;b (60%)</td>
<td>52 (45.2%)</td>
<td>48 (41.7%)</td>
<td>87 (75.6%)</td>
</tr>
</tbody>
</table>

Number in parenthesis indicates hours of culture of 2 cell embryos in vitro, Prog: progesterone concentration. a: Significant differences within the same column with control ($P \leq 0.001$), b: Significant differences within the same column with the 5 ng/ml progesterone-injected group ($P \leq 0.001$), c: Significant differences within the same column with the 10 ng/ml progesterone-injected group ($P \leq 0.001$).

The survival rate of embryos in superovulated and progesterone-injected mice in vivo

The mean number of embryos which obtained from unstimulated (control), unstimulated progesterone-injected, superovulated and superovulated progesterone-injected groups were $14.20 \pm 2.60$, $13.14 \pm 2.80$, $21.31 \pm 3.60$ and $21.03 \pm 2.60$, respectively (Table 3). There were significant differences in number of embryos in both superovulated groups with non stimulated-progesterone-injected groups ($P \leq 0.001$). The survival rates of blastocysts in unstimulated, unstimulated progesterone-injected, superovulated and superovulated progesterone-injected groups were 92.25%, 97.68%, 85.17% and 87.25%, respectively. Both superovulated groups had lower survival rates than the control and progesterone-injected groups ($P \leq 0.001$).

The total cell numbers of blastocysts in the unstimulated control group were $75.68 \pm 1.19$ (Fig. 1a), in the unstimulated progesterone-injected group, $128.2 \pm 1.10$ (Fig. 1d), in the superovulated group, $126.31 \pm 2.60$ (Fig. 1e) and in the superovulated progesterone-injected group, $126.88 \pm 1.60$ (Fig. 1f) (Table 4). There were some signs of nuclear fragmentation in blastocyst derived from superovulated mice (Fig. 1e). There were significant differences between all treatment groups and the control ($P \leq 0.001$), and both superovulated groups had higher total cell numbers than the progesterone-injected group ($P \leq 0.005$).

The diameter of blastocysts in the unstimulated were
127.2 ± 2.40 μm, in the unstimulated progesterone-injected group, 112 ± 0.70 μm, in the superovulated group, 128.62 ± 1.30 μm, and in the superovulated progesterone-injected group 128.64 ± 0.70 μm (Table 4). There were no significant differences in diameters of blastocysts among four groups in vivo.

**Implantation rates of embryos in superovulated and progesterone-injected mice**

The implantation rates in the four groups are summarized in Table 5. The implantation rates of embryos were statistically higher in the progesterone-injected group (92.06%) than in the control (80.99%), superovulated (39.72%) and superovulated progesterone-injected (41.2%) groups ($P \leq 0.001$). However, there was no significant difference between the two superovulated groups and the progesterone-injected group. Implantation rates in superovulated groups were significantly lower than in the unstimulated groups ($P \leq 0.001$).
The principal hormone controlling the uterine environment is progesterone, and circulating concentrations of maternal progesterone are closely linked to early embryo development [17].

The results of our in vitro study clearly demonstrate that the culture media supplemented with 20 ng/ml progesterone significantly improved embryo development up to 72 h of culture in comparison with other concentrations of progesterone (5 and 10 ng/ml) and control (P≤0.001); but after 120 h culture of embryos there was no significant difference with the control. Also the results of this study showed that progesterone could not alter mouse embryo quality (diameter and total cell number of blastocysts) in vitro. These results may be due to indirect effects of progesterone via other epithelial or stromal cells on the embryo development [15].

Wilmut et al. [30] reported that rabbit endometrial tissue cultured in the presence of progesterone and estradiol was beneficial to embryo development in vitro, but other researchers have shown that addition of progesterone to the culture system did not affect embryo development or reduce its developmental rates [11, 30].

The results of our in vivo study indicate that ovarian stimulation increased the number of embryos, but the

### Table 3. The number and survival rates of blastocysts collected from superovulated and/or progesterone-injected mice on the 4th day of pregnancy

<table>
<thead>
<tr>
<th>Treatment groups (n)</th>
<th>No. of collected embryos mean ± SD (%)</th>
<th>No. of surviving blastocysts mean ± SD (%)</th>
<th>No. of degenerated blastocysts mean ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (19)</td>
<td>14.2 ± 2.6 (100%)</td>
<td>13.1 ± 1.9 (92.25%)</td>
<td>0.87 ± 0.01 (5.69)</td>
</tr>
<tr>
<td>Progesterone (15)</td>
<td>13.14 ± 2.80 (100%)</td>
<td>13.1 ± 2.10 (97.68%)</td>
<td>0.31 ± 0.01 (2.1%)</td>
</tr>
<tr>
<td>Superovulated (19)</td>
<td>21.31 ± 3.60 (100%)*</td>
<td>18.15 ± 2.90 (85.17%)*</td>
<td>3.16 ± 0.10 (14.8%)*</td>
</tr>
<tr>
<td>Superovulated + Progesterone (17)</td>
<td>21.03 ± 2.60 (100%)*</td>
<td>18.35 ± 1.40 (87.25%)*</td>
<td>2.68 ± 1.20 (12.7%)*</td>
</tr>
</tbody>
</table>

a: significant difference with the control group (*P≤0.01, **P≤0.001), b: significant difference with the progesterone injected group (P≤0.001).

### Table 4. Total cell number and diameter (micrometer) of blastocysts in superovulated and/or progesterone-injected mice in vivo

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Total cell number mean ± SD (n)</th>
<th>Blastocyst diameter mean ± SD (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>87.37 ± 1.40 (8)</td>
<td>127.20 ± 2.40 (20)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>112 ± 0.70 (7)</td>
<td>128.20 ± 1.10 (20)</td>
</tr>
<tr>
<td>Superovulated</td>
<td>126.82 ± 1.30*</td>
<td>126.31 ± 2.60 (19)</td>
</tr>
<tr>
<td>Superovulated + Progesterone</td>
<td>126.88 ± 1.60*</td>
<td>128.64 ± 0.70 (18)</td>
</tr>
</tbody>
</table>

a: significant difference with the control group (P≤0.001), b: significant difference with the progesterone injected group (P≤0.005).

### Table 5. The implantation rates of embryos in superovulated and/or progesterone-injected mice on 7th day of pregnancy

<table>
<thead>
<tr>
<th>Treatment groups (n)</th>
<th>Ratio of surviving embryos/ total embryos</th>
<th>Implantation sites mean ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (13)</td>
<td>13.1 / 14.2</td>
<td>10.61 ± 0.01 (80.99)</td>
</tr>
<tr>
<td>Progesterone (16)</td>
<td>13.1 / 13.41</td>
<td>12.06 ± 0.09 (92.06)*</td>
</tr>
<tr>
<td>Superovulated (14)</td>
<td>18.15 / 21.31</td>
<td>7.21 ± 0.06 (39.72)*</td>
</tr>
<tr>
<td>Superovulated + Progesterone (16)</td>
<td>18.35 / 21.03</td>
<td>7.56 ± 0.06 (41.2)*</td>
</tr>
</tbody>
</table>

a: significant difference with the control group (P≤0.001), b: significant difference with the progesterone-injected group (P≤0.005).
number of embryos was not affected by progesterone injection. Clearly, the action of exogenous gonadotropin produced a large number of oocytes in both superovulated groups and the proportion of embryos was increased.

Examination of degenerated blastocysts in the superovulated groups revealed a significantly higher proportion of degenerated blastocysts than in the control group ($P \leq 0.001$). On the other hand, the survival rate of blastocyst in the progesterone-injected group was significantly higher than the control group and both superovulated groups. These results suggest that progesterone may act directly as a survival factor or indirectly promote the expression of cytokines that are helpful to embryonic survival and development. Martal et al. [19] showed ovarian hormones, such as progesterone, play a role in the regulation of cytokine production. For example granulocyte macrophage colony stimulating factor is a cytokine secreted by the conceptus and endometrium and its synthesis is controlled by progesterone and it is considered as a growth factor for the embryo [20].

In both superovulated groups the degeneration of embryos was increased in comparison with unstimulated groups. Similar studies have shown superfertilization in murine resulted in fewer viable embryos than in controls [8, 9]. This reduction in viability and development of embryos may be caused by an increase in chromosomal abnormalities of the oocyte or embryos in superovulated female mice. Ovarian stimulation produces a cascade of hormonal and physiological events, and oocytes mature in an environment different from that of naturally matured oocytes. We also observed the fragmentation of DNA in blastocysts derived from superovulated mice (Fig. 1e). However, it is also possible that the degeneration of embryos in superovulated mice is due to the incidence of apoptosis.

The present study showed that an injection of 1 mg/mouse progesterone in unstimulated mice significantly improved implantation rates compared to the control and superovulated groups ($P \leq 0.001$). Progesterone is an important hormone of pregnancy and it is essential for endometrial receptivity during implantation. It possibly acts on different pathways: e.g. on the endometrial epithelium, stromal cells and uterine immune system. Lessey et al. showed an increase in growth factor production in the stromal cells in response to progesterone administration [16].

In this study, we also showed that the implantation rates in superovulated groups were significantly lower than in the unstimulated groups. This failure of implantation in superovulated mice was due to abnormal embryos or dysfunction of the endometrium. In agreement with this study, investigations on humans and experimental animals have shown that after superovulation, the implantation rate declined in comparison with unstimulated control groups [2, 3, 14]. Fossum et al. [10] reported a significant decrease in the implantation rates after embryo transfer to ovarian superovulated mice using PMSG and hCG and suggested that this failure was caused by changes in uterine receptivity. Basir et al. [2] concluded that excessively high concentrations of estradiol after superovulation lead to a suboptimal endometrial environment for implantation and this may explain the findings regarding the decreased implantation and pregnancy rates in superovulated female mice.

Other studies in agreement with our results presented here have demonstrated that the quality of embryos was affected by superovulation; for example Champlin AK et al. [4] showed that blastocysts that developed in vivo in superovulated mice have fewer microvilli on their surface.

Overall our results show a progesterone injection without ovarian induction improved embryo survival and implantation rate, but administration of progesterone after superovulation did not ameliorate the negative effects of superovulation on the implantation rate. These results may have potential implications for clinical ovarian stimulation during infertility treatment and embryo implantation and development.

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References


