Embryo-dependent induction of embryo receptivity in the mouse endometrium

K. Wakuda, K. Takakura, K. Nakanishi, N. Kita, H. Shi, M. Hirose and Y. Noda*

Department of Obstetrics and Gynecology, Shiga University of Medical Science, Seto-Tsukinowa-cho, Otsu, Shiga 520-2192, Japan

The effect of intraoviductal embryos on endometrial receptivity was studied by intraendometrial and intrauterine embryo transfer. Five-week-old female ICR mice were mated after superovulation; a vaginal plug confirmed day 1 of pregnancy. On day 4 (90 h after hCG injection), blastocysts were collected and transferred to pseudopregnant female mice and to recipient mice in which the uterotubal junction had been ligated bilaterally on day 1 of pregnancy. Three embryos per uterine horn, a total of six embryos per recipient mouse at days 1–6, were transferred to the endometrium or uterine cavity and implantation and pregnancy rates were calculated. The implantation rate for intraendometrial embryo transfer to recipients of days 3, 5 and 6 was significantly higher for uterotubal junction-ligated mice (72.2, 20.8 and 9.7%, respectively) than for pseudopregnant mice (55.0, 8.3 and 0.0%, respectively). The implantation rate for intrauterine embryo transfer to recipients at days 2, 5 and 6 was significantly higher for uterotubal junction-ligated mice (11.1, 25.0 and 8.3%, respectively) than for pseudopregnant mice (0.0, 3.3 and 0.0%, respectively). Uterotubal junction-ligated mice achieved implantation and bore neonates by intrauterine embryo transfer on days 2 and 6, whereas no implantation was achieved in pseudopregnant mice. The difference in implantation rate could not be explained by a difference in progesterone concentration between the groups. The distribution of proliferating cells in the endometrium was also studied immunohistochemically by use of antiproliferating cell nuclear antigen (PCNA) antibody in the recipient mice. PCNA-positive cells were more abundant in uterotubal junction-ligated mice and demonstrated a marked extension from the epithelium to the stroma over time, in contrast to those in pseudopregnant mice. These findings indicate that an intraoviductal embryo exerts a biological effect by sending a signal to the endometrial epithelium and stroma, thus facilitating endometrial receptivity to the embryo and improving the rate of implantation.

Introduction

Implantation occurs and pregnancy is achieved only when endometrial receptivity is synchronized with the developmental stage of embryos (Psychoyos, 1986; Harper, 1992) by a process in which two entities with different genetic backgrounds exchange signals and influence one another (Chang, 1950; Weitlauf, 1994). The limited period of endometrial embryo receptivity during which implantation can be achieved is called the ‘implantation window’ (Harper, 1992).

Several embryo-dependent factors have been identified that are involved in implantation (Godkin et al., 1984; Spinks and O’Neill, 1987; Vallet et al., 1987; Harper et al., 1989; Weitlauf, 1989). The application of improved molecular biology techniques (Yokoi et al., 1993) has revealed the presence of relevant cytokines, growth factors, receptors, and newly discovered factors in the embryo and the endometrium (Rappolee et al., 1988; Werb, 1990; Bhatt et al., 1991; Arceci et al., 1992; Stewart et al., 1992; De et al., 1993; Haimovici and Anderson, 1993).

The presence of the embryo itself is thought to play an important role in successful implantation (Spinks and O’Neill, 1987; Godkin et al., 1984; Vallet et al., 1987; Carlone and Rider, 1993). Shiotani et al. (1993) noted the interaction between embryo and endometrium and clarified the biological effect of the embryo on the endometrium. The endometrial adheresiveness to the embryo, which is induced by the presence of an intraoviductal embryo, was lost on the day after the embryo was absent from the uterine cavity, but was maintained until day 7 when an embryo was present. Thus, the presence of embryos in the oviduct or uterine cavity may promote a functional transformation in the receptivity of the endometrial epithelium to the embryo. However, the nature and action of the putative embryonic signals involved in endometrial receptivity are still unknown.

*Correspondence.
Revised manuscript received 19 October 1998.
The aim of this study was to determine the biological effect exerted on the endometrium by the embryo. The effects of intraoviductal embryos on endometrial epithelium and stroma in vivo were investigated with respect to rates of implantation and pregnancy in embryo transfer experiments.

Materials and Methods

Animals

Four-week-old ICR mice, obtained from Charles River Japan Inc., were reared in an incubation room (temperature, 23 ± 2°C; humidity, 50 ± 10%; ventilation, 15–18 h⁻¹; illumination, 12 h; luminous intensity, 250 lux) for at least 1 week before the experiments.

Chemicals

Pregnant mares’ serum gonadotrophin (PMSG; Teikoku Zoki Co., Tokyo), hCG (Teikoku Zoki Co., Tokyo), PBS (Nissui Pharmaceutical Co., Tokyo), BSA (Sigma Chemical Co., St Louis, MO), alpha modification of Eagle’s medium (αMEM; Dainippon Pharmaceutical Co., Osaka), pentobarbital sodium (Nembutal; Abbott Labs, North Chicago, IL), anti-PCNA antibody (PC10; Dako, Glostrup) and an immunostaining ABC Kit (Vectastain Elite ABC Kit; Vector Labs, Burlingame, CA) were used in this study.

Collection of early stage embryos

Superovulation was induced in 5-week-old female ICR mice by the intraperitoneal administration of 5 iu PMSG, followed 48 h later by 5 iu hCG. The mice were mated and those showing vaginal plugs the next day were denoted as day 1 mice. Blastocysts were recovered on day 4 (90 h after hCG injection) by flushing the uterine cavity with Dulbecco’s PBS + 0.3% (w/v) BSA. Embryos with normal morphology were immersed in αMEM + 0.3% (w/v) BSA incubation medium and immediately transferred to the uterine cavity of recipient animals using a glass micropipette.

Recipient mice

Four groups of recipients, 8- to 10-week-old female ICR mice, were prepared in this study. Female mice that had vaginal plugs on the day after mating were denoted as day 1 recipient mice. Group 1 recipients were uterotubal junction-ligated pregnant mice in which the uterotubal junction had been ligated on day 1 (Shiotani et al., 1993), after mating with a normal 10- to 12-week-old male mouse with previous mating experience. Group 2 recipients were pseudopregnant mice that had been mated with a vasoligated male mouse, without ligation of the uterotubal junction. Group 3 recipients were uterotubal junction-ligated non-pregnant mice in which the uterotubal junction had been ligated before mating with a normal male mouse with previous mating experience. Group 4 recipients were uterotubal junction-ligated pseudopregnant mice in which the uterotubal junction had been ligated on day 1 of pseudopregnancy, after mating with a vasoligated male mouse.

Embryo transfer

Embryos were transferred according to the method developed by Beatty (1951), McLaren and Michie (1956), and Goto et al. (1992). Recipient mice were anaesthetized by intraperitoneal administration of 30 mg pentobarbital sodium g⁻¹ and the uterine horns were exteriorized through a dorsal incision. Three embryos from day 4 of pregnancy were transferred to each uterine horn in two groups of recipients on days 1–6 by intraendometrial or intrauterine transfer (see below), resulting in six embryos per recipient. Implantation and pregnancy rates of the two groups were subsequently compared.

Transfer to the uterine cavity; intrauterine transfer

With the aid of a stereoscopic microscope, a 27-gauge injection needle was used to puncture the top of the uterine horn near the uterotubal junction, providing access to the uterine cavity. After removal of the needle, a glass micropipette was inserted through the hole, and three embryos were transferred from the micropipette into the uterine cavity with several microlitres of incubation medium. After confirmation that no embryos remained in the pipette, the uterine horn was replaced in the abdominal cavity and the incision was closed (Beatty, 1951; McLaren and Michie, 1956). The presence and stage of development of the embryos was determined under a dissection microscope after laparotomy on day 11.

Transfer to the endometrium; intraendometrial transfer

Under a stereoscopic microscope, a 27-gauge injection needle was inserted near the uterotubal junction parallel to the uterine axis, giving access to the anti-mesometrial endometrium. After removal of the needle, a glass micropipette was inserted through the hole, and three embryos were transferred to the endometrium with several microlitres of incubation medium. After confirmation that no embryos remained in the pipette, the uterine horn was replaced in the abdominal cavity and the incision was closed (Goto et al., 1992). After the transfer, the growth of the embryos was examined on day 11, as in the intrauterine transfer group.

Feasibility of intraendometrial embryo transfer

Blastocysts were transferred intraendometrially into pseudopregnant mice on days 3 or 4 using the method...
described earlier in an experiment to confirm the presence of transferred embryos in the endometrium. Immediately after the transfer, mice were killed by cervical dislocation and the uteri were resected. After fixation in 10% (v/v) neutral formalin for 5 h, 3 mm transverse sections were obtained from the transfer site of each uterine horn and embedded in paraffin wax. Three millimeter sections were cut sequentially from each block of paraffin wax and mounted on poly-L-lysine-coated glass slides. The slides were stained with haematoxylin and eosin, dehydrated, cleared, and covered with coverslips.

Dye (3 ml of Parker blue ink) was injected intraendometrially into pseudopregnant mice on days 1–6 to quantify the feasibility of intraendometrial embryo transfer. Mice were killed immediately by cervical dislocation and the uteri were resected. The uterine lumen was observed to determine whether the dye had leaked out.

**Observation of intraoviductal embryos in uterotubal junction-ligated mice**

After ligation of the uterotubal junction on day 1, embryos were collected from the oviducts every day from day 2 to day 10, and on day 17. PBS + 0.3% (w/v) BSA solution was perfused into the oviducts from the fimbrial end, and embryos were recovered from the uterine end. The number of embryos and the stage of development were determined by examination under a stereoscopic microscope.

**Intrauterine and intraendometrial embryo transfer to hCG-injected pseudopregnant mice**

After 5 iu hCG had been administered intraperitoneally to pseudopregnant mice on days 3 and 5, the animals were used as recipients for intrauterine and intraendometrial embryo transfer on day 6. Three embryos from day 4 of pregnancy were transferred to each uterine horn of five hCG-injected pseudopregnant mice. After embryo transfer, embryonic growth was observed by laparotomy on day 11.

**Immunohistochemical study of endometrium using anti-PCNA antibody**

Two groups of female mice were used in this experiment: pseudopregnant mice of days 1–6, and uterotubal junction-ligated pregnant mice of days 1–6. In each group, the uteri were removed and 3 mm transverse sections were obtained from the centre of each uterine horn, fixed in 10% (v/v) neutral formalin for 5 h, and then embedded in paraffin wax. From each block of paraffin wax, 3 mm sections were cut and mounted on poly-L-lysine-coated glass slides. Paraffin wax was removed from the sections and they were hydrated and then incubated for 20 h at 4°C with anti-PCNA antibody as a primary antibody. Anti-PCNA antibody-positive cells (García et al., 1989; Gelb et al., 1992) were detected via the modified avidin–biotin complex (ABC) method of Fung et al. (1992) using an immunostaining ABC kit. Normal mouse serum was used as a negative control. PCNA antibody solution was diluted 1:50 to a final concentration of 9.6 mg ml⁻¹. ABC complex was visualized by treating the slides for 8 min with freshly prepared 0.05 mg dianisobenzidine tetrahydrochloride (DAB) ml⁻¹ in 0.1 mol Tris buffer ¹ containing 0.01% (v/v) Triton-X100 and 0.03% (w/v) H₂O₂. Slides were counterstained with haematoxylin, dehydrated, cleared, and covered with coverslips.

**Determination of serum concentrations of progesterone**

Five mice from each group of uterotubal junction-ligated, pseudopregnant, or hCG-injected pseudopregnant mice were anaesthetized with chloroform and blood was collected from the abdominal aorta. Serum concentrations of progesterone in all three groups were determined using a commercial radioimmunoassay kit (DPC Co., Chiba). The intra- and interassay coefficients of variation were 5.4 and 6.4%, respectively.

**Statistical analyses**

Fisher’s exact test was used to compare the implantation and pregnancy rates among the groups. Serum progesterone concentrations were compared by the Mann–Whitney U test. Differences were considered significant when P < 0.05.

**Results**

**Intraendometrial embryo transfer**

In all four groups, the implantation and pregnancy rates on days 3 and 4 were significantly higher than those on days 1, 2, 5 and 6 (P < 0.05) (Table 1).

The implantation rates for uterotubal junction-ligated pregnant mice (group 1) in which embryo transfer was performed on days 3, 5 and 6 of pregnancy were significantly higher than those for any other group on the corresponding days (P < 0.05) (Table 1). When intraendometrial embryo transfer was used, implantation was achieved (16.7–20.8%) as early as day 2, whereas intrauterine transfer failed to achieve implantation on day 2, except for in the uterotubal junction-ligated pregnant mice (group 1) (Tables 1 and 2).

**Intrauterine embryo transfer**

As in the intraendometrial embryo transfer groups, the implantation and pregnancy rates on day 3 and 4 were significantly higher than those on days 1, 2, 5 and 6 (P < 0.05) (Table 2). The implantation rates for uterotubal junction-ligated pregnant mice (group 1) in which embryo transfer was performed on days 2, 5 and 6 of pregnancy were significantly higher than those for any other group on the corresponding days (P < 0.05) (Table 2).
Table 1. Intraendometrial embryo transfer in pseudopregnant and uterotubal junction-ligated mice

(a)

<table>
<thead>
<tr>
<th>Day of embryo transfer</th>
<th>Uterotubal junction-ligated pregnant mice (group 1)</th>
<th>Pseudopregnant mice (group 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of recipient mice</td>
<td>Number of embryos transferred</td>
</tr>
<tr>
<td>Day 1</td>
<td>12</td>
<td>72</td>
</tr>
<tr>
<td>Day 2</td>
<td>12</td>
<td>72</td>
</tr>
<tr>
<td>Day 3</td>
<td>12</td>
<td>72</td>
</tr>
<tr>
<td>Day 4</td>
<td>12</td>
<td>72</td>
</tr>
<tr>
<td>Day 5</td>
<td>12</td>
<td>72</td>
</tr>
<tr>
<td>Day 6</td>
<td>12</td>
<td>72</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Day of embryo transfer</th>
<th>Uterotubal junction-ligated non-pregnant mice (group 3)</th>
<th>Uterotubal junction-ligated pseudopregnant mice (group 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of recipient mice</td>
<td>Number of embryos transferred</td>
</tr>
<tr>
<td>Day 1</td>
<td>12</td>
<td>72</td>
</tr>
<tr>
<td>Day 2</td>
<td>12</td>
<td>72</td>
</tr>
<tr>
<td>Day 3</td>
<td>12</td>
<td>72</td>
</tr>
<tr>
<td>Day 4</td>
<td>12</td>
<td>72</td>
</tr>
<tr>
<td>Day 5</td>
<td>12</td>
<td>72</td>
</tr>
<tr>
<td>Day 6</td>
<td>12</td>
<td>72</td>
</tr>
</tbody>
</table>

*Uterotubal junction ligated on day 1, after mating with normal male mouse.
*Mated with vasectomized male mouse without ligation of uterotubal junction.
*Uterotubal junction ligated before mating with normal male mouse.
*The day of vaginal plug formation is designated as day 1.
*Significant difference between the uterotubal junction-ligated pregnant group and other groups (P < 0.05).

Intrauterine embryo transfer to uterotubal junction-ligated pregnant mice (group 1) on days 2 and 6 of pregnancy achieved successful implantation, resulting in neonates. However, intrauterine embryo transfer in the other groups on the corresponding days failed to result in implantation.

Feasibility of intraendometrial embryo transfer

Almost all embryos transferred to the endometrium were localized in the endometrial stroma (Fig. 1). The feasibility of intraendometrial transfer was quantified using the intraendometrial dye injection experiment. The rates of leakage of the dye into the uterine lumen immediately after intraendometrial injection of dye were 25.0% (5 of 20 horns), 15.0% (3 of 20 horns), 5.0% (1 of 20 horns), 0.0% (0 of 20 horns), 0.0% (0 of 20 horns), and 0.0% (0 of 20 horns) for days 1–6, respectively.

Intraovuductal blastogenesis in uterotubal junction-ligated mice

The number and developmental stage of embryos recovered from oviducts in uterotubal junction-ligated mice are summarized (Table 3). On day 1, an average of 8.8 fertilized ova was recovered by intratubal flushing. The number of embryos recovered gradually decreased on subsequent days. Hatched blastocysts were found in oviducts of uterotubal junction-ligated mice as late as days 6 and 7.

A two-cell embryo present in the oviduct on day 2, morulas and blastocysts present on day 4, and hatched blastocysts present on days 6 and 7 are shown (Fig. 2). The hatched blastocyst present on day 7 appeared to be slightly degenerated (Fig. 2d), but adhered to the plastic dish.

Serum concentrations of progesterone

Serum progesterone concentrations showed no significant difference between pseudopregnant mice and uterotubal junction-ligated mice from day 1 to day 5 (Table 4). On day 6, serum progesterone concentrations were significantly higher in uterotubal junction-ligated mice than in pseudopregnant mice (P < 0.05).

After administration of hCG to pseudopregnant mice on days 3 and 5, serum progesterone concentrations increased.
markedly on days 5 and 6 to 66.8 ± 3.8 and 58.5 ± 1.6 nmol l⁻¹, respectively.

**Immunohistochemical study of endometrium using anti-PCNA antibody**

*Uterotubal junction-ligated pregnant mice (group 1).* On days 1 and 2, PCNA-positive cells were found sporadically in both the luminal and glandular epithelium. On day 3, some of the interstitial cells around the glands became PCNA-positive. On day 4, about half the interstitial cells around the glands were PCNA-positive and on day 5, most of the interstitial cells were positive, whereas the epithelial cells were unstained. On days 6 and 7, almost all the interstitial cells that showed large nuclei and morphological characteristics of decidual cells were positive for PCNA.

*Pseudopregnant mice (group 2).* On days 1 and 2, PCNA-positive cells were found sporadically in both the luminal and glandular epithelium, as in uterotubal junction-ligated mice. However, on day 3, PCNA-positive interstitial cells occurred less frequently than in uterotubal junction-ligated mice. Even on days 4 and 5, there was no increase in PCNA-positive cells in the epithelial or interstitial cells. On days 6 and 7, the luminal epithelium was PCNA-positive, but almost all of the glandular epithelium and interstitial cells remained negative. These results were in marked contrast to those in uterotubal junction-ligated pregnant mice (Fig. 3).

**Intraendometrial and intrauterine embryo transfer to hCG-injected pseudopregnant mice**

Intraendometrial and intrauterine embryo transfer were performed in hCG-injected pseudopregnant mice. A total of 72 embryos were transferred to 12 recipient mice in each transfer group on day 6. Implantation was not achieved by either method of embryo transfer unless an embryo was present in the oviduct, despite high concentrations of serum progesterone induced by the administration of hCG.

**Discussion**

Intraendometrial embryo transfer performed on days 3, 5 and 6 of pregnancy in uterotubal junction-ligated pregnant mice (group 1) resulted in a significantly higher implantation rate than in any other group. Similarly, for intrauterine embryo transfer performed on days 2, 5 and 6 of pregnancy,
uterotubal junction-ligated pregnant mice had a significantly higher implantation rate than any other group. Progesterone concentrations did not differ significantly between uterotubal junction-ligated pregnant mice (group 1) and pseudopregnant mice (group 2) until day 5, but the difference was highly significant on day 6. Excretion of nidatory oestrogen at the luteal phase is important for implantation, as is progesterone concentration in the preceding phase (Psychoyos, 1986; Yoshinaga, 1988; Kapur et al., 1992). It has also been reported that high progesterone concentrations are advantageous for implantation (Lejeune et al., 1985) and that the concentration of progesterone gradually increases after pregnancy is achieved, even in rodents (Wagner, 1976; Sato et al., 1984). In the present study, intraendometrial and intrauterine embryo transfer was performed after administration of 5IU hCG to pseudopregnant mice on days 3 and 5 and using day 6 mice as recipients, to clarify the contribution of progesterone to the higher implantation rate in the uterotubal junction-ligated pregnant group. Progesterone concentrations in hCG-injected pseudopregnant mice were 45.2, 66.8 and 58.5 nmol l⁻¹ on days 4, 5 and 6, respectively. Implantation was not successfully achieved in these animals, despite progesterone concentrations that were higher than those in pseudopregnant mice. These findings indicate that differences in progesterone concentrations were not responsible for the observed differences in implantation rate after intraendometrial embryo transfer on days 3, 5 and 6, or after intrauterine embryo transfer on days 2, 5 and 6 in uterotubal junction-ligated pregnant mice, compared with the other groups. Four groups of recipient mice were used for intraendometrial and intrauterine embryo transfer: uterotubal junction-ligated pregnant mice (group 1), pseudopregnant mice (group 2), uterotubal junction-ligated non-pregnant mice (group 3), and uterotubal junction-ligated pseudopregnant mice (group 4). Only in group 1 recipients were embryos present in the oviduct. Morphological study of intraoviductal blastogenesis in uterotubal junction-ligated mice revealed that embryos

![Fig. 1. Day 4 blastocyst 4 h after intraendometrial transfer to day 4 pseudopregnant mouse. Sections are stained with haematoxylin and eosin. e, embryo; l, uterine lumen; m, muscular layer. Scale bars represent (a) 500 μm, (b) 100 μm.](image)

<table>
<thead>
<tr>
<th>Day of embryo recoverya</th>
<th>Number of miceb</th>
<th>Stage of embryo development</th>
<th>Number of embryos recovered (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>5</td>
<td>One cell</td>
<td>8.8 ± 0.7</td>
</tr>
<tr>
<td>Day 2</td>
<td>5</td>
<td>Two cell</td>
<td>8.0 ± 1.0</td>
</tr>
<tr>
<td>Day 3</td>
<td>5</td>
<td>Morula</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td>Day 4</td>
<td>5</td>
<td>Morula</td>
<td>8.4 ± 0.37</td>
</tr>
<tr>
<td>Day 5</td>
<td>5</td>
<td>Blastocyst</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td>Day 6</td>
<td>5</td>
<td>Morula</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>Day 7</td>
<td>5</td>
<td>Blastocyst</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>Day 8</td>
<td>5</td>
<td>Hatched blastocyst</td>
<td>5.4 ± 0.8</td>
</tr>
<tr>
<td>Day 9</td>
<td>5</td>
<td>Hatched blastocyst</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td>Day 10</td>
<td>5</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Day 17</td>
<td>5</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

'aThe day of vaginal plug formation is designated as day 1.
'bUterotubal junction ligated on day 1, after mating with normal male mouse.
### Table 4. Serum progesterone concentrations in uterotubal junction-ligated pseudopregnant mice and hCG-injected pseudopregnant mice

<table>
<thead>
<tr>
<th>Day of progesterone determination</th>
<th>Number of mice</th>
<th>Uterotubal junction-ligated pregnant mice</th>
<th>Pseudopregnant mice</th>
<th>hCG-injected pseudopregnant mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>5</td>
<td>7.6 ± 2.5</td>
<td>5.7 ± 0.3</td>
<td>nd</td>
</tr>
<tr>
<td>Day 2</td>
<td>5</td>
<td>17.8 ± 2.5</td>
<td>21.3 ± 1.6</td>
<td>nd</td>
</tr>
<tr>
<td>Day 3</td>
<td>5</td>
<td>24.8 ± 1.6</td>
<td>25.4 ± 1.3</td>
<td>nd</td>
</tr>
<tr>
<td>Day 4</td>
<td>5</td>
<td>49.0 ± 1.6</td>
<td>42.0 ± 3.2</td>
<td>45.2 ± 2.2</td>
</tr>
<tr>
<td>Day 5</td>
<td>5</td>
<td>54.7 ± 5.1</td>
<td>42.6 ± 2.2</td>
<td>66.8 ± 3.8'</td>
</tr>
<tr>
<td>Day 6</td>
<td>5</td>
<td>45.8 ± 4.1'</td>
<td>29.3 ± 0.6</td>
<td>58.5 ± 1.6'</td>
</tr>
</tbody>
</table>

*Serum progesterone values are expressed as mean ± SEM.

The day of vaginal plug formation is designated as day 1.

Uterotubal junction ligated on day 1, after mating with normal male mouse.

Mated with vasectomized male mouse.

Mated with vasectomized male mouse and injected with hCG on days 3 and 5.

Significantly different from the pseudopregnant group (P < 0.05).

nd, not determined.

---

**Fig. 2.** Intraovuductal blastogenesis in uterotubal junction-ligated mice. (a) Two-cell embryos on day 2; (b) morulas and blastocysts on day 4; (c) hatched blastocysts on day 6; (d) hatched blastocyst on day 7. Scale bars represent 50 μm.

Continued to grow to hatched blastocyst stage on day 7, even in the oviduct. These findings indicate that intraovuductal embryos are viable for a significant period of time and that they make the intrauterine environment more advantageous for implantation.

Implantation and pregnancy rates in group 3 and group 4 recipients were comparable to those in group 2 pseudopregnant recipients. This indicated that the improved implantation and pregnancy rates in group 1 uterotubal junction-ligated pregnant mice were not the result of ligation.
Fig. 3. Immunohistochemical localization of proliferating cell nuclear antigen-positive cells in the mouse uterus during the peri-implantation period. (A–G) Uterine sections from a pregnant mouse in which the uterotubal junction had been ligated on day 1. (a–g) Uterine sections from a pseudopregnant mouse. Matching upper and lower case letters (A)(a) to (G)(g) correspond to days 1 to 7 of pregnancy, respectively. L, uterine lumen; S, endometrial stroma. Scale bar represents 50 μm.
of the uterotubal junction or intrauterine exposure to semen or spermatozoa.

In intraendometrial embryo transfer experiments, implantation rate depends at least in part on the skill of the operator performing the transfer technique. Attempts to confirm the feasibility of intraendometrial embryo transfer by dye injection experiments showed that in the present study embryo transfer was consistently successful.

In groups 1 and 2, the distribution and intensity of PCNA-positive cells in the endometrium were investigated as a measure of cell proliferation (Celis and Celis, 1985) to clarify whether the difference in implantation rate reflected a difference in some cellular biological function in the endometrial stroma. Herken (1983) studied the cell kinetics of the early gestation mouse uterus and reported that luminal and glandular epithelia proliferated on days 1 and 2, and then ceased proliferation on day 3. On day 4, stromal and vascular endothelial cells proliferated instead of epithelial cells. These findings are consistent with the present study. It has been reported that mechanical injury to the endometrium in pseudopregnant mice induces proliferation of interstitial cells and facilitates formation of deciduomas (Ohta et al., 1994). In the present study, PCNA-positive cells were more abundant in the endometrium of uterotubal junction-ligated pregnant mice than in pseudopregnant mice from days 3 to 7. Furthermore, almost all the interstitial cells showed morphological characteristics of decidual cells and were positive for PCNA on days 6 and 7 in uterotubal junction-ligated pregnant mice. These results were in marked contrast to those in pseudopregnant mice. These findings indicate that proliferation of endometrial interstitial cells, as measured by PCNA-positive cells, was greater when embryos were present in the oviduct than when they were absent. Schlafke and Enders (1975) suggested that implantation resembles the processes of inflammation and wound healing. Therefore, it is proposed that during implantation recipient cells proliferate vigorously and cause a reorganization in the relationships among cells, perhaps inducing embryo receptivity.

The findings of the present study indicate that, in intraendometrial or intrauterine embryo transfer in uterotubal junction-ligated mice, the presence of an intraoviductal embryo results in a significant improvement in implantation rate. This is true in pseudopregnant mice even on days when implantation either cannot be achieved or occurs only at a very low rate in the absence of an intraoviductal embryo. This proposal is supported by the results of the investigation of serum progesterone concentrations, and of embryo transfer to hCG-injected pseudopregnant mice. It is also supported by observations of continuous intraoviductal blastogenesis until day 7 in uterotubal junction-ligated mice and the presence of PCNA-positive cells in the endometrium.

Neonates were obtained after transfer of embryos into the endometrium of pseudopregnant mice on day 2, when implantation could not be achieved by conventional intrauterine transfer (Goto et al., 1992). The success of this procedure may be due to the fact that the embryo is prevented from becoming detached in the uterine cavity at a stage at which the endometrial epithelium is not receptive to the embryo. In addition, the procedure may promote blastogenesis in the endometrium, thus providing an optimum environment for embryonic growth. Furthermore, in the present experiment, successful implantation and neonates were obtained after intrauterine embryo transfer on day 2 of pregnancy, once an embryo was present in the oviduct. On day 2, the endometrial epithelium of both pregnant and pseudopregnant mice is not adhesive (Shiotani et al., 1993). Therefore, successful implantation after intrauterine embryo transfer on day 2 in uterotubal junction-ligated pregnant mice is probably not attributable to an intraoviductal embryo that affects the adhesiveness of the endometrial epithelium, but to embryo-derived factors that facilitate blastogenesis, support the growth and viability of the embryo, and provide a suitable environment for implantation in the endometrial stroma.

The fertilized egg produces various substances, including prostaglandins (Kennedy, 1983), hCG, steroids (Huff and Erik-Nes, 1966), growth factors (Rappolee et al., 1988; Werb, 1990), and proteins, which may be involved in the transfer of information among cells (Arceci, 1992; Pampfer et al., 1992). These substances may serve as signals that act on the embryo itself or on the endometrium and may be involved in implantation. O'Neill (1995) reported on changes in the activities of endogenous inhibitors of platelet-activating factor (PAF) during implantation, highlighting the importance of PAF in the process of implantation. Harper et al. (1989) reported that the early pregnancy factor (Morton et al., 1974) was detectable after administration of PAF to mice and rabbits. PAF may also play an immunomodulatory role in implantation (Harper et al., 1989). From studies in the mouse delayed implantation model, Paria et al. (1993a) suggested a pivotal role for oestrogen in the activation of dormant embryos which determines the window of implantation. They also studied changes in activated embryos and found higher expression of epidermal growth factor receptors in activated compared with dormant embryos (Paria et al., 1993b).

However, the embryo-dependent signal that induces endometrial receptivity has not yet been elucidated. This may be due to the fact that the signal is present in small quantities for only a short period of time or because several interacting signals are involved.

The presence of an intraoviductal embryo results in an increase in implantation rate in pseudopregnant mice on days when implantation either cannot be achieved or occurs at a very low rate in the absence of an intraoviductal embryo. This is the first study in vivo which demonstrates that the presence of an intraoviductal embryo stimulates the endometrial epithelium and stroma for implantation and exerts some biological action that maintains implantation. These putative embryo-dependent factors should be determined and characterized in future studies.

This study was supported in part by grants-in-aid from the Ministry of Education, Science and Culture, Japan (No. 04557073 and No. 07671777). The authors thank Y. Masuda for his kind cooperation in preparing the animals.