ENDOCRINE, MORPHOLOGICAL AND GESTATIONAL ASPECTS OF INTACT AND SLICED AUTOGYNOUS OR HOMOYNOUS OVARIAN ORHTOTOPIC
TRANSPLANTATION WITHOUT VASCULAR PEDICLE

Objective: To assess the rate of natural pregnancy and to determine the endocrine and histological (morphofunctional) aspects of rabbit ovaries after being submitted to bilateral oophorectomy and orthotopic allogenic or autologous intact and sliced ovarian transplantation without a vascular pedicle. Methods: Fifty-six female New Zealand White and California rabbits were studied. The ovaries were removed and orthotopically transplanted or reimplanted without vascular anastomoses: Group 1 (n = 8), sham-operated, only laparotomy and closure; Group 2A (n = 8), with bilateral intact ovaries reimplanted; Group 2B (n = 8), with bilateral sliced ovaries orthotopically reimplanted; Group 2C (n = 8), with an intact ovary reimplanted on one side and a sliced ovary on the other side; Group 3A (n = 8), with bilateral orthotopic intact ovaries transplants; Group 3B (n = 8), with bilateral sliced ovaries orthotopically transplanted; and Group 3C (n = 8), with an intact ovary transplanted on one side and a sliced ovary on the other side. Three months later, the animals were paired with males for copulation. The estradiol, progestrone, FSH and LH levels were assessed nine months after surgery. The morphologic aspect of the ovaries was studied, and the number of pregnancies and litters were also determined. Chi-square test compared the number of successful pregnancies and the number of litters between groups. One-way ANOVA and the Tukey-Kramer tests compared the hormonal levels. The significance level adopted was p < 0.05. Results: Pregnancies occurred in seven rabbits (87.5%) of Group 1, in 37.5% of rabbits in Groups 2A and 3A, in 50% of Groups 2B, 2C and 3B and in 62.5% of Group 3C. Hormone levels and histology confirmed the vitality of all ovaries. Conclusions: Intact or sliced orthotopic allogenic and autologous ovarian transplantation without a vascular pedicle is efficacious in rabbits, and their fertility and hormonal functions are preserved.

Keywords: Ovary/transplantation; Ovary/anatomy & physiology; Cyclosporine/therapeutic use; Fertilization

RESUMO

Objetivo: Avaliar a fertilização bem como aspectos endócrinos e histológicos do ovário após seu reimplante ou transplante ortotópico, sem anastomose vascular. Métodos: Foram utilizadas 56 coelhas da raça Nova Zelândia, variedade branca, e Califórnia distribuídas em: Grupo 1 (n = 8), controle, apenas laparotomia e laparorrafia; Grupo 2A (n = 8), com reimplante ortotópico de ovários íntegros; Grupo 2B (n = 8) com reimplante ortotópico de ovários fatiados; Grupo 2C (n = 8), com reimplantes ovarianos de um lado íntegro, e, do outro lado, fatiado; Grupo 3A (n = 8), com transplante ortotópico de ovários íntegros; Grupo 3B (n = 8), com transplante ortotópico de ovários fatiados; Grupo 3C (n = 8), com transplantes ovarianos de um lado íntegro e, do outro lado, fatiado. A partir do terceiro mês pós-operatório, cada coelha foi colocada para cópula. Dosaram-se o estradiol, a progesterna, o FSH e o LH no nono mês pós-operatório. Estudaram-se as morfologias macro e microscópicas dos ovários, tubas e útero de todos os animais. Os números de gestações e de filhotes foram avaliados por meio do teste do χ² e as dosagens hormonais foram comparadas pelo one-way ANOVA, seguido pelo teste de Tukey-Kramer. O nível de significância adotado foi de p < 0.05. Resultados: No Grupo 1, sete (87,5%) coelhas engravidaram entre o segundo e terceiro meses após início da cópula. No Grupo 2, as gestações ocorreram entre o quinto e o oitavo meses pós-operatórios e, no Grupo 3, entre o quarto e o oitavo meses pós-operatórios. A percentagem de gravidez observada foi de 37,5% no Grupo 2A, 50% no Grupo 2B e 2C, 37,5% no Grupo 3A, 50% no Grupo 3B e 62,5% no Grupo 3C. Os níveis hormonais e o estudo morfofuncional dos ovários, tubas e úteros não apresentaram alterações. Conclusões: O reimplante ou transplante ovariano homógeno ortotópico sem pedículo vascular é eficaz para a manutenção de níveis normais de hormônios ovarianos e permitiu a fertilização natural.

Descritores: Ovário/transplante; Ovário/anatomia & histologia; Ciclosporina/uso terapêutico; Fertilização

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INTRODUCTION

The ovaries of many women who had cancer lose their function, even when not part of the disease, due to treatment with radiotherapy and chemotherapy\(^1,2\). Oophorectomy is also performed during pelvic surgeries, even when not mandatory. Early menopause ensues with functional disorders, such as sexual dysfunction, disturbed lipoprotein profile, as well as a greater risk of osteoporosis and cardiovascular diseases\(^{1,4}\).

A physiological alternative for maintaining hormonal function in women who must undergo procedures causing the loss of their normal ovaries is removing and preserving the ovaries, in order to be reimplemented after the end of the treatment that would injure them. Several experimental techniques of ovarian auto-implantation have been studied\(^{5,6}\). However, because of the multiple varieties of animal species and methods studied for the preservation of ovarian function, the information on the efficacy of hormonal preservation is conflicting\(^7,9\).

The use of cryopreserved ovarian tissue reimplanted in heterotopic position is a more physiological alternative for maintaining hormonal function\(^9,11\). Thus, most studies on the preservation of ovarian tissue are on autogenous transplantation. Homogenous transplantation, orthotopic or heterotopic were seldom investigated, especially because of difficulties for attaining immunosuppression\(^12\).

Cyclosporine (CyA) is a fungus-derived peptide, utilized for the treatment of patients receiving organ transplants. Its immunosuppressive activity is based on inhibition of the cell-mediated immune response and on decreased production of antibody-dependent T lymphocytes\(^{13}\). On the other hand, CyA causes endocrine dysfunction\(^7\), and disturbed gonadal function was described in rats and in humans\(^7,8\). This effect in rats is likely due to an interaction of CyA with prolactin in a common lymphocytic receptor\(^{13}\).

OBJECTIVE

The objective of this study was to ascertain the occurrence of natural pregnancy in rabbits and to evaluate morphologic and endocrine aspects of reimplemented or transplanted intact or sliced homogenous ovaries, fixated in orthotopic position, without vascular anastomosis.

METHODS

This study was performed complying to the recommendations of the international animal protection union and of the brazilian code on animal experiments (1988)\(^{14,15}\) and was approved by the Animal Experimentation Ethics Committee of the Universidade Federal de Minas Gerais (UFMG).

Fifty-six New Zealand White (n = 28) and California (n = 28) female rabbits (Oryctogalus cuniculus) and ten knowingly fertile New Zealand White male rabbits were studied. The animals were kept in individual cages, with free rabbit chow and water. They remained 20 days for adaptation and observation of health status.

At the start of the experiment, all rabbits were four months old, sexually mature and weighing between 2,380 g and 2,740 g. They fasted for 12 hours prior to surgery.

The female does were randomly assigned to one of three groups, half for each race:

- **Group 1 (n = 8)**: sham-operated animals – midline laparotomy and closure.
- **Group 2: reimplantation of both ovaries**
  - **Subgroup 2A (n = 8):** intact bilaterally
  - **Subgroup 2B (n = 8):** sliced bilaterally
  - **Subgroup 2C (n = 8):** intact on one side and sliced on the other

- **Group 3: bilateral homogenous ovarian transplantation**
  - **Subgroup 3A (n = 8):** intact bilaterally
  - **Subgroup 3B (n = 8):** sliced bilaterally
  - **Subgroup 3C (n = 8):** intact on one side and sliced on the other

Antimicrobial prophylaxis was undertaken with cepahdroxyl 50 mg/kg diluted in 10 ml milk, 30 minutes before surgery for all animals.

Anesthetic induction was achieved by the intramuscular gluteal injection of 50 mg/kg 5% ketamine chloride. An additional one quarter of the dose was given when necessary. The heart rate, respiratory rate and voluntary movements of the rabbits were monitored throughout the entire anesthesia time, to detect any possible complications.

Group 1 animals underwent a midline infra-umbilical laparotomy, when the uterus, the Fallopian tubes and the ovaries were identified. After that, the abdomen was closed by running suture in two planes, with 2-0 polyglactin for the aponeurosis, and with 3-0 nylon for the skin.

In Group 2 animals, midline infra-umbilical laparotomy and bilateral oophorectomy were performed, and the integrity of the Fallopian tubes was carefully preserved. The ovaries were immediately orthotopically reimplemented without vascular anastomoses and held in place by a 5-0 nylon knot, as follows:

- **Subgroup 2A:** bilateral orthotopic reimplantation of the intact ovaries;
- **Subgroup 2B:** the ovaries were sliced longitudinally, by scalpel, in three 2 mm thick sections and were immediately reimplemented, orthotopically;
- **Subgroup 2C:** the ovaries were orthotopically reimplemented, intact on one side and sliced on the other.
In Group 3, in addition to antimicrobial prophylaxis, the females were weighed and received 10 mg/kg of a CyA solution one hour prior to the surgery. Both drugs were diluted into 10 ml of milk and given through a 12-Fr orogastric catheter. A pair of does – one California and one New Zealand White – was simultaneously operated. After midline infra-umbilical laparotomy, bilateral total oophorectomy was performed, with preservation of the Fallopian tubes integrity. Both ovaries of each doe were removed. Then, the ovaries of one doe were orthotopically transplanted into the other doe and vice-versa, so that, each animal was donor and recipient at the same time. All ovarian implants were performed without vascular reconstruction and were fixed by a single 5-0 nylon knot, as follows:

- Subgroup 3A: intact ovaries transplanted bilaterally;
- Subgroup 3B: the ovaries were longitudinally sliced, by scalpel, into three 2-mm thick sections, after which they were transplanted;
- Subgroup 3C: one intact and one sliced ovary were randomly transplanted in opposite sides.

The animal’s abdomen was closed by running suture in two planes, with 2-0 polyglactin for the aponeurosis and 3-0 nylon for the skin. After surgery and throughout the entire follow-up period, the does received chow and filtered water, in individual trays, ad libitum, and were kept in individual cages, with appropriate hygienic, ventilation and light conditions.

In the first three post-operative days, the animals were given 50 mg/kg cephalosporin diluted in 10 ml of milk, through orogastric catheter, as antimicrobial prophylaxis. The Group 3 does were daily given 10 mg/kg CyA diluted in 10 ml milk, by a 12-Fr orogastric tube, during nine months. The animals were weekly weighed for CyA dose adjustment. This procedure was made easier by the use of rabbit contention boxes.

Three months after surgery, the does of all groups were placed daily with a different knowingly fertile buck, for mating during six months. The doe was kept unaccompanied in the cage until the end of the pregnancy. The number of pregnancies and litters in each group, as well as any gestational complication observed, were recorded.

Nine months after surgery, blood was drawn to measure the estradiol, progesterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels. Blood samples were processed as done routinely, in specific machines. The gonadal and pituitary hormones were assayed by immunofluorimetry.

Upon completion of the follow-up period, all does were killed by a lethal dose of inhaled ether, after deep ketamine anesthesia (50 mg/kg). The thoracic and abdominal cavities and the organs included were carefully studied after a midline thoracolaparotomy. The uterus, ovaries and Fallopian tubes of all does were removed and processed for histology.

The χ² test with Yates correction for small samples was used to compare the number of successful pregnancies and the litter size, and, the one-way analysis of variance (one-way ANOVA) followed by the Tukey-Kramer test, to compare hormone levels (estradiol, progesterone, FSH and LH). Statistical significance was established at p < 0.05.

**RESULTS**

All animals had spontaneous recovery from surgery and survived unremarkably throughout the nine experimental months. Their weight uniformly increased.

Table 1 shows hormone levels of the groups at the end of the experiment. Ovarian hormones were detected in all reimplanted or transplanted does. The FSH, LH and progesterone levels were not different between subgroups, except for the estradiol level, which was higher in Group 3C than in all other subgroups (p < 0.05). Hormone levels were not different between the two rabbit races studied. Hormone levels were preserved even in the reimplanted or transplanted does which did not become pregnant.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Estradiol (pg/ml)</th>
<th>Progesterone (pg/ml)</th>
<th>FSH (UI/l)*</th>
<th>LH (UI/l)**</th>
<th>Number of does that got pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,801 ± 762</td>
<td>104,200 ± 2,348</td>
<td>0.13 ± 0.05</td>
<td>0.16 ± 0.07</td>
<td>7</td>
</tr>
<tr>
<td>2A</td>
<td>2,500 ± 950</td>
<td>109,300 ± 5,500</td>
<td>0.30 ± 0.20</td>
<td>0.30 ± 0.20</td>
<td>3</td>
</tr>
<tr>
<td>2B</td>
<td>2,860 ± 700</td>
<td>105,400 ± 3,500</td>
<td>0.20 ± 0.10</td>
<td>0.20 ± 0.10</td>
<td>4</td>
</tr>
<tr>
<td>2C</td>
<td>2,950 ± 850</td>
<td>106,100 ± 3,300</td>
<td>0.30 ± 0.20</td>
<td>0.20 ± 0.10</td>
<td>4</td>
</tr>
<tr>
<td>3A</td>
<td>4,063 ± 927</td>
<td>102,906 ± 962</td>
<td>0.13 ± 0.04</td>
<td>0.18 ± 0.07</td>
<td>3</td>
</tr>
<tr>
<td>3B</td>
<td>3,844 ± 821</td>
<td>103,172 ± 1,938</td>
<td>0.14 ± 0.07</td>
<td>0.18 ± 0.07</td>
<td>4</td>
</tr>
<tr>
<td>3C</td>
<td>4,510 ± 1,246</td>
<td>103,713 ± 2,413</td>
<td>0.13 ± 0.04</td>
<td>0.16 ± 0.07</td>
<td>5</td>
</tr>
</tbody>
</table>

1 = Control Group; 2A = intact ovarian reimplantation; 2B = sliced ovarian reimplantation; 2C = intact ovarian reimplantation on one side and sliced in the other side; 3A = intact ovarian transplantation; 3B = sliced ovarian transplantation; 3C = intact ovarian transplantation on one side and sliced in the other side; *FSH = follicle-stimulating hormone; **LH = luteinizing hormone; ***difference between Subgroup 3C and the other subgroups (p < 0.05, test de Tukey-Kramer).
The abdominal cavity looked normal in Group 1, at autopsy. In two rabbits there were adhesions between the Fallopian tube and the ovary. No other anatomical changes were found in the uterus and Fallopian tubes.

Macroscopically, the ovaries of Group 2 (reimplanted) and Group 3 (transplanted) does which became pregnant had preserved outer aspect, with no fibrosis or inflammatory reaction. However, out of the Group 2 does which did not become pregnant, two showed hydrosalpinx, and two had multiple adhesions involving the ovaries, the Fallopian tubes and intestines. In two of the Group 3 does (one New Zealand white and one California), there were adhesions between the Fallopian tube and the ovary, as well as between the ovary and adjacent intestinal loops. The uterus looked normal in all animals. The rest of the abdominal cavity remained unchanged. There was no gross change in the remaining abdominal organs.

Histologically, the ovaries of control, reimplanted or transplanted animals, were preserved and not different among themselves. There were no differences between transplanted, intact or sliced ovaries as well. Ovarian viability was confirmed by the demonstration of satisfactory angiogenesis, several follicles in different maturing stages, as well as corpus luteum and follicular cysts in different proportions. No signs of ischemia or necrosis were found. In one Group 3 New Zealand white doe, multifocal hemorrhage close to the capsule and subcapsular calcification were identified in one of the ovaries.

Tubal histology of Group 1 does was normal, with well developed microvilli, normal tropism and absence of inflammatory or degenerative changes. In four does of Group 2 which did not become pregnant, there were histological changes, with hydrosalpinx. In one doe of Group 3 which did not become pregnant, a mononuclear inflammatory spot in the mesosalpinx and tubal hypotrophy were found. Another doe which did not become pregnant showed foreign body reaction in the mesosalpinx, mild chronic salpingitis and hydrosalpinx. In all other animals, tubal histoarchitecture was normal.

Uterine histoarchitecture of all does was normal. The endometrium showed several phases of proliferation, consistent with the estral phase, indicating appropriate hormone production.

Regarding fertility, Table 2 shows the number of pregnancies occurred, the average litter size and the beginning of deliveries in the three groups studied. There was no difference in the time between mating and birth of the litter between the two races studied (p > 0.05).

There was no difference between Group 1 and each of the subgroups regarding the number of pregnancies

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of gestation</th>
<th>%</th>
<th>Beginning of deliveries (month)</th>
<th>Mean number of does’s litter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>87.5</td>
<td>3º</td>
<td>2.1</td>
</tr>
<tr>
<td>2A</td>
<td>3</td>
<td>37.5</td>
<td>5º</td>
<td>2</td>
</tr>
<tr>
<td>2B</td>
<td>4</td>
<td>50</td>
<td>5º</td>
<td>2.5</td>
</tr>
<tr>
<td>2C</td>
<td>4</td>
<td>50</td>
<td>5º</td>
<td>1.7</td>
</tr>
<tr>
<td>3A</td>
<td>3</td>
<td>37.5</td>
<td>4º</td>
<td>2</td>
</tr>
<tr>
<td>3B</td>
<td>4</td>
<td>50</td>
<td>5º</td>
<td>2</td>
</tr>
<tr>
<td>3C</td>
<td>5</td>
<td>62.5</td>
<td>4º</td>
<td>1.8</td>
</tr>
</tbody>
</table>

DISCUSSION

Ovarian transplantation in patients with ovarian failure is analogous to transplantation of islets of Langerhans for diabetes mellitus or of dopaminergic neurons for Parkinson’s disease. All these disorders may be treated with pharmacological supplementation. However, tissue therapy maintains the physiological activity of hormonal and neuronal secretion(16). The feasibility and advantages of tissue therapies are gradually being known.

Rabbits were chosen as experimental animals because of their good reproductive capacity and of the simple surgical procedure with no need for special materials.

The present study shows that transplanted ovaries without vascular anastomoses in female rabbits are viable and maintain their endocrine function in all cases. There were no adverse effects due to the lack of early vascularization, because none of the animals showed signs of ovarian ischemia. These findings contradict some authors who consider surgical revascularization of ovarian grafts fundamental(16-17). However, they agree with others who also considered unnecessary to have vascular pedicle anastomosis in order to maintain ovarian viability and function. The intense neovascularization around the ovary seems to be enough for maintaining its vitality(18-19). According to the literature, the ovarian tissue is a rich source of angiogenic factors, which stimulate prompt migration of endothelial cells after transplantation, leading to re-establishment of blood circulation(18-23). Disson et al.(24) implanted ovarian cortex adjacent to the jugular vein and observed graft revascularization 48 hours after the procedure. This phenomenon is concordant with the increased expression of specific angiogenic agents, especially that of the endothelium-derived growth factor. These authors have also suggested that the secretion of gonadotropins plays a major role in the vascular
response. Among the pituitary gonadotropins, FSH is likely the most important one, because it stimulates mitosis and inhibits apoptosis of granulosa cells(16).

The transplanted ovarian tissue vitality is also corroborated by the display of maintained endocrine function, identified by presence of blood estradiol and progesterone, as well as by their interaction with the pituitary hormones (FSH and LH). Although hormone levels were similar among the groups, the transplanted animals had higher levels of estradiol. A possible explanation might be that Group 1 does were in the pre-ovulatory phase at the time their blood was drawn—a time when estradiol levels are lower. No other information that could help to understand this finding could be retrieved from the data of the present study or from the literature.

Daily CyA was effective to prevent rejection of the transplanted ovary. This was presumed because there were no signs indicating rejection either in the ovary or in the peri-ovarian vascular mesh. Moreover, there were no histological or functional evidences suggestive of CyA-induced changes of follicular formation or in ovulatory phenomena. Although some authors recommend the use of higher CyA doses (15 mg/kg/day)(21-22), the 10 mg/kg/day dose utilized in the present study, according to specific line of research(25-28), assured the ovarian vitality and function, without possible inconveniences caused by high dose immunosuppression. Even though some authors stated that CyA had anti-ovulatory properties, likely due to the interaction with prolactin in the same lymphocytic receptor, as well as it impaired fertility and disturbed fetal growth, no ovulation inhibitory effect of CyA was detected in the present study(21). The FSH levels were not significantly different among does of Groups 1, 2 and 3. This observation demonstrates that the reimplanted or transplanted ovaries maintained a normal production of estradiol within the normal range, because, by a feedback mechanism, any decrease in its production stimulates FSH release FSH(29).

Basal LH secretion is suppressed by a combination of progesterone secreted by the corpus luteum and estradiol, by the antral follicles(30). As the levels of LH were kept within the normal range in the reimplanted and transplanted animals, appropriate hormone production by these ovaries is evident.

The progesterone, FSH and LH levels were within the normal range in the recipients of ovarian transplants. It was surprising, however, to find high levels of estradiol in this group. According to Gore-Langton(30), CyA increases the plasma levels of estradiol and decreases its concentration in the ovary. The plasma increase could be explained by a direct effect of CyA on the granulosa cells, changing aromatase activity. The direct stimulatory effect of CyA on adrenal mediators may amplify this influence(30).

Macroscopic and microscopic exams did not reveal differences of ovarian preservation whether they were intact or sliced when implanted. On the other hand, in a previous study in female rats(27-29), those reimplanted with sliced ovaries showed better hormonal outcomes. Such difference was not found in the female rabbit. Sliced ovarian implants may be more efficacious than intact ones. The discordant outcomes in the two species that were presented in this study and the apparent lack of subsidies from the literature on this issue call for further investigation as an attempt to find answers to this problem.

The non-occurrence of pregnancies in some does may be attributed to tubal-ovarian adhesions to adjacent organs, such as the omentum, intestines and peritoneum. Although there were no records of pregnancies in some does, the endocrine ovarian function was also maintained. It is pertinent to suspect the occurrence of non-identified pregnancies interrupted by abortion, throughout the follow-up period. Abortions are quite frequent in animals and the literature shows a large variety of causes, including genetic, anatomical, hormonal and infectious disturbances, as well as environmental influences and when the organism is injured, like by CyA. Nonetheless, this study shows that CyA did not interfere in the fertility of the animals investigated.

CONCLUSIONS

In conclusion, natural fertilization was successfully recorded in female rabbits subjected to orthotopic reimplantation or transplantation of autogenous or homogenous ovaries without vascular anastomosis. Such ovaries preserved their endocrine function and morphology. CyA was effective in maintaining ovarian vitality and function throughout the entire study period.

REFERENCES


