Indirect evaluation of estrogenic activity post heterotopic ovarian autograft in rats

Avaliação indireta da atividade estrógênica após transplante heterotópico de ovário em ratas

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ABSTRACT

Purpose: To morphologically evaluate the estrogenic effect on the uterus and vagina of rats submitted to ovarian autografts.

Methods: Twenty Wistar EPM-1 adult rats were bilaterally ovariectomized, followed by ovarian transplants in retroperitoneal regions. The animals were divided in four groups of five animals, according to the day of euthanasia: G4, G7, G14 and G21, corresponding to the 4th, 7th, 14th and 21st day after surgery, respectively. Vaginal smears were collected from the first day of surgery until euthanasia day. After that, the vagina and uterus were removed, fixed in 10% formaldehyde and submitted to histological analysis and stained with hematoxiline and eosine.

Results: All animals showed estrous cycle changes during the experiment. In 4th day, the uterus showed low action of estrogen with small number of mitosis and eosinophils as well as poor development. On the 7th day, the endometrium was atrophic without mitotic signals and presented a small number of eosinophils. On the 14th and 21th days the histological findings were similar, with the presence of mitosis in the endometrial glands and intense leucocyte infiltration with a large number of eosinophils. Morphometric results showed that the endometrial and myometrial thickness as well as the number of eosinophils presented the highest values during the 14th and 21th days of the evaluation. The 7th day group also presented the lowest eosinophil numbers. Vaginal epithelium features were: 4th and 7th day groups presented non-keratinized stratified epithelium with 5 and 2 cell layers, respectively. The 14th and 21st day groups presented non-keratinized stratified epithelium with 14 and 15 cell layers.

Conclusion: Experimental ovarian autografts in the evaluated organs presented maximum estrogen activity after the 21st day of surgery, according to morphological and morphometric data.

Key words: Transplantation. Ovary. Uterus. Vagina. Rats.
Introduction

Currently, ovarian transplantation is subject to intense scientific investigation, since pregnancy and labor after transplantation are fundamental for the good quality of life of patients subjected to cancer chemotherapy. The outcome of ovarian transplantation may vary, depending on the locus elected for grafting, on whether it is orthotopic or heterotopic, on its size (complete or sliced ovary), the type of preservation and the presence of vascular anastomosis, among other factors. The results depend on the number of viable ovarian follicles and on the time of graft survival, but all authors agree that a decrease in the number of follicles is due to previous ischemia of the graft.

The presence of antral follicles happens regardless of the implantation site of the ovary, which is implanted on ectopic sites. The transplanted ovarian graft without vascular anastomosis produces a series of growth factors, like the vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and gonadotropins which, in their turn, lead to angiogenesis in a relatively short, though critical, interval after graft transplantation (48 h). These stimuli seem to be triggered by hypoxia, mainly when the ovary is implanted on ectopic sites. The presence of antral follicles happens regardless of the implantation site of the ovary, which is implanted on ectopic sites. The transplanted ovarian graft without vascular anastomosis produces a series of growth factors, like the vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and gonadotropins which, in their turn, lead to angiogenesis in a relatively short, though critical, interval after graft transplantation (48 h). These stimuli seem to be triggered by hypoxia, mainly when the ovary is implanted on ectopic sites. The presence of antral follicles happens regardless of the implantation site of the ovary, which is implanted on ectopic sites. The transplanted ovarian graft without vascular anastomosis produces a series of growth factors, like the vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and gonadotropins which, in their turn, lead to angiogenesis in a relatively short, though critical, interval after graft transplantation (48 h). These stimuli seem to be triggered by hypoxia, mainly when the ovary is implanted on ectopic sites. The presence of antral follicles happens regardless of the implantation site of the ovary, which is implanted on ectopic sites. The transplanted ovarian graft without vascular anastomosis produces a series of growth factors, like the vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and gonadotropins which, in their turn, lead to angiogenesis in a relatively short, though critical, interval after graft transplantation (48 h). These stimuli seem to be triggered by hypoxia, mainly when the ovary is implanted on ectopic sites. The presence of antral follicles happens regardless of the implantation site of the ovary, which is implanted on ectopic sites. The transplanted ovarian graft without vascular anastomosis produces a series of growth factors, like the vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and gonadotropins which, in their turn, lead to angiogenesis in a relatively short, though critical, interval after graft transplantation (48 h). These stimuli seem to be triggered by hypoxia, mainly when the ovary is implanted on ectopic sites.

The thickness of the endometrium, miometrium and vaginal epithelium was evaluated; the presence of leukocytes in the endometrium was also quantified. Appropriate image capture was made using a light microscopy (AxioLab Standard 10, Carl Zeiss, Jena, Germany) coupled to a video camera (AxionCam, Carl Zeiss, Jena, Germany). Measurement was carried out with image analysis software (AxioVision 4.2). Leukocytes were counted in a total area of 35 mm² of each animal.

Under surgical anesthesia, vagina and uterus were removed and immediately fixed in 10% formaldehyde. Routine histological processes were employed for paraffin inclusion, sectioning and hematoxyline-eosine staining.

Methods

The animals were housed and used upon approval (Protocol no. 1327/2006) of the Committee on Ethics and Research of the São Paulo Federal University (UNIFESP). Twenty virgin, adult (120–180 day-old, 200–300 g b.w) female Wistar EPM-1 rats were used. During the experimental period, the animals were fed with rat chow and water ad libitum.

The animals were weighed and anesthetized with ketamine (70 mg/Kg) and xylazine (10 mg/Kg), and were kept on natural room air breathing during all the procedures. Procedures were made under surgical microscope (DF Vasconcellos®)

Results

Citology

All animals showed an altered estrous cycle during the experimental period. In the G4 and G7 groups, the estrous cycle was irregular predominating the diestrous phase with few observable cells. G4 presented some cells from the superficial layers and G7 a high percentage of basal cells could be seen, besides mucus and numerous neutrophilis. In G4 and G21 a more regular estrous cycle was detected; at the end of the experiment the animals were all at estrus.

Acta Cirúrgica Brasileira - Vol. 23 (4) 2008 - 373
Uterus

In G4 the uterus was lined with a simple cylindrical epithelium; the endometrium contained some eosinophils and euchromatin-rich fibroblasts (Figures 1A and 2A). In G7 the uterus was somewhat atrophic and lined with cubic-to-cylindrical epithelium; the endometrium contained a very high number of fibroblasts presenting small or picnotic nuclei (Figures 1B and 2B). In G14 and G21 the uterus was well developed, with a simple cylindrical epithelium lining and fibroblast-rich propria laminae with a high number of eosinophils (Figures 1C, 1D, 2C and 2D).

FIGURE 1 – Photomicrography showing representative uterus of rats: A = G4, B = G7, C = G14 and D = G21. In A, presence of some endometrial glands (arrows); in B all the endometrial extension (Endom) and the miometrium (Miom) are seen. In C and D the endometrium appears well developed with a high content of endometrial glands. H.E (200X)

FIGURE 2 – Photomicrographs of rat endometrium (groupings are as in the legend of Figure 1). Notice that in A there are some leukocytes (arrows), which are fewer in B. Leukocyte number was dramatically increased in C and D. H.E. (400X)
**Vagina**

In G4, the vagina was lined with a 3–4 cell layer of non-keratinized, stratified squamous epithelium. The lamina propria was constituted of dense connective tissue, rich in cells and blood vessels, and the presence of some leukocytes was noticed permeating the epithelial tissue (Figure 3A). In G7, there was a remarkable reduction of the epithelial tissue thickness, which was then constituted of two cell layers and a lamina propria rich in collagen fibers (Figure 3B). Vaginal morphology in G14 and G21 was well developed; in G14 there was a lining of stratified squamous epithelium, formed by 10–12 cell layers (Figure 3C). In G21 this epithelium was keratinized with 14–16 layers. In both cases, the lamina propria was formed by cell-rich connective tissue (Figure 3D).

![FIGURE 3 – Photomicrography showing rat vagina sections (groupings are as in the legend of Figure 1). In A, notice the stratified squamous epithelium (E) and the lamina propria (LP) rich in cells with small leukocyte infiltration (arrows). In B there is a thin epithelium (E) and a collagen-rich lamina propria (LP). In C, the stratified epithelium (E) appears to be thick and in D a squamous stratified epithelium is seen (E). H.E. (400X)](image)

**Morphometry**

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>G4 (n = 5)</th>
<th>G7 (n = 5)</th>
<th>G14 (n = 5)</th>
<th>G21 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrium (μm)</td>
<td>380.7 ± 102.3</td>
<td>195.3 ± 47.6</td>
<td>510.4 ± 49.7</td>
<td>565.8 ± 57.00</td>
</tr>
<tr>
<td>Myometrium (μm)</td>
<td>343.8 ± 27.7</td>
<td>128.9 ± 12.6</td>
<td>322.7 ± 39.2</td>
<td>360.1 ± 30.2</td>
</tr>
<tr>
<td>Vaginal epithelium (μm)</td>
<td>71.0 ± 11.0</td>
<td>34.5 ± 8.6</td>
<td>107.9 ± 12.1</td>
<td>121.6 ± 5.5</td>
</tr>
<tr>
<td>Eosinophils (cells/mm²)</td>
<td>14.2 ± 3.0</td>
<td>0.4 ± 0.03</td>
<td>32.8 ± 2.8</td>
<td>34.2 ± 4.8</td>
</tr>
</tbody>
</table>

Eosinophils in the endometrium: p<0.001 G4 x G7, G4 x G14, G4 x G21, G7 x G14, G7 x G21; p>0.05 G14 x G21. Vaginal epithelium: p<0.01 G4 x G7, G4 x G14, G4 x G21; p<0.001 G7 x G14, G7 x G21; p>0.05 G14 x G21; Endometrium thickness: p<0.001 G4 x G7, G4 x G14, G4 x G21, G7 x G14, G7 x G21; p>0.05 G14 x G21. Myometrium thickness: p<0.001 G4 x G7, G4 x G14, G4 x G21; p>0.05 G4 x G14 x G21.
that the genitalia showed evidences of estrogen effects even during the absence of antral follicle growth, once the rodent genital tract responds to low levels of sex steroids. This could imply that, in the present study, the hormonal levels until the 7th day were low enough to allow progressive uterine and vaginal atrophy, the remission occurring on the 14th and 21st days after the procedure.

It is important to emphasize that the morphologic and morphometric data had a direct correlation to hormonal levels as observed by Oktay et al. and Callejo. In our experiment, there was a decrease in ovary activity in G4, reaching its lowest value in G7 (elevated levels of FSH and reduced estradiol levels), its activity started to recover in G14, and the function was fully restored after 21 days of the transplant.

Currently, transplant procedures include several strategies to maintain the female reproductive functions, such as the cryopreservation of mature or immature oocytes, of embryos and of ovarian tissue, being the latter a possible alternative for patients who need immediate chemotherapy. This way, the implant of ovarian cortex tissue in diverse sites after chemotherapy can be made.

Intact and fresh ovary transplant have been successfully used in rats, rabbits, sheep, dogs and humans, independent of the implantation site. The loci most used have been the subcutaneous space, the renal capsule and the abdominal cavity, all subjected to temperature and pressure influence.

It is believed that the best place for ovary implantation is at close proximity to an important blood vessel like the renal or the femoral artery; accordingly, the subcutaneous site can be viewed to be the worst place in terms of blood supply to the follicle. Notwithstanding, the results are similar, as far as hormonal levels and vaginal cytology are concerned, when the implant is intra-peritoneal or subcutaneous.

Although transplanted ovaries become revascularized and reenervated, the transplantation procedure, with its inherent ischemia/reperfusion insult, alters not only the process of follicle growth itself, but also the organ morphology. As a consequence, there are different degrees of parenchymatous fibrosis and compensatory hyperplasia of the cell layer in a short and long-term.

The critical time for the full recovery of ovarian function after transplant happens during the first 24 h, when there is a response to the ischemia/reperfusion insult that is the main factor responsible for alterations due to this procedure that finishes with angiogenesis and revascularization. Although the activation of angiogenic factors in transplanted ovaries is stimulated by hypoxia, the transient increase of gonadotrophins in the immediate post-surgical period also contributes to the revascularization process.

Further studies of those organs are necessary, with similar analysis of what was already seen on the transplanted ovarian parenchyma, which shows and increase in angiogenic factors like VEGF and TGF, besides apoptosis levels of cellular proliferation characterization.
Conclusion

Experimental ovarian autograft, under the conditions established for this experiment, maintained ovarian activity in genital organs. Signs of hipoestrogenism from the 7th day after operation, with maximum estrogenic activity to the end of the 21st day, as judged by morphologic and morphometric data were observed.

References


Conflict of interest: none
Financial source: FAPESP

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Received: January 18, 2008
Review: February 14, 2008
Accepted: April 16, 2008

How to cite this article

*Color figures available from www.scielo.br/acb*