

The Study of Estrous Cycle Phases Female Rats With the Use of N-Acetylcysteine in Ovarian Graft

Estudo do Ciclo Estral de Ratas com Uso de N-Acetilcisteína no Enxerto Ovariano

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Abstract

Ovarian graft may be the target of the biochemical effects of oxidative stress caused at the time of transplantation. In order to evaluate the effect of N-acetylcysteine on the ovarian graft, regarding the estrous cycle preservation, 50 female and virgin EPM-1 Wistar rats, weighing up to 250g, originating from CEDEME of UNIFESP, were kept in adequate sanitary conditions, receiving their own food and water. Daily vaginal smears were performed to identify the estrous phase for 8 days. The animals were randomly distributed into 05 groups: 1st Group (GTx), saline was administered subcutaneously, 2nd (NAC 150mg/Kg), 3rd (NAC 300mg / Kg), 4th (NAC 600mg / Kg) and 5th (NAC 1200mg / Kg), that were administered NAC subcutaneously on the abdominal face, 60 minutes before left unilateral ovarian transplantation in retroperitoneum and contralateral oophorectomy for purposes of histomorphological analysis, with colpocytological evaluation. Euthanasia was performed by means of anesthetic lethal dose in half of the animals on the 4th postoperative day, with a single vaginal smear collection and euthanasia on the rest of the animals, between the 14th and 16th days, after the material was collected in order to define the estrus phase. It was evaluated in the graft that the animals exhibited in all groups return of estrous cycle in the later phase of the post-transplant, with better definition of regular cycle in the highest dosages of N-acetylcysteine. N-acetylcysteine induced the return of the estrous cycle in the rats' ovarian graft, mainly in the highest dosage, proving its effectiveness in revascularization of the tissue after ischemia and reperfusion.

Keywords: Acetylcysteine. Reperfusion. Histocompatibility Antigens. Menstrual Cycle.

Resumo

O enxerto ovariano pode ser alvo dos efeitos bioquímicos do stress oxidativo causado no momento do transplante. Com o objetivo de avaliar o efeito da N-acetilcisteína no enxerto ovariano, quanto à preservação do ciclo estral, foram utilizados 50 ratos EPM-1 Wistar, fêmeas e virgens, pesando até 250g, originários do CEDEME da UNIFESP, mantidos em adequadas condições sanitárias, recebendo ração própria e água. Realizados esfregaços vaginais diários para identificação da fase estral durante 08 dias. Os animais foram distribuídos aleatoriamente em 05 grupos: 1º Grupo (GTx), administrada solução salina via subcutânea, 2º (NAC 150mg/Kg), 3º (NAC 300mg/Kg), 4º (NAC 600mg/Kg) e 5º (NAC 1200mg/Kg), aos quais foi administrada NAC por via subcutânea em face abdominal, 60 minutos antes do transplante unilateral esquerdo do ovário em retroperitônio e à ooforectomia contra-lateral para fins de análise histomorfológica, com avaliação colpocitológica. A eutanásia foi realizada por meio da dose letal do anestésico em metade dos animais no 4º dia de pós-operatório, realizado única coleta de esfregaço vaginal e a eutanásia no restante dos animais, entre o 14º e 16º dia, após a coleta do material para definição da fase estro. Foi avaliado no enxerto que os animais apresentaram em todos os grupos retorno de ciclo estral na fase mais tardia do pós-transplante, com melhor definição de ciclo regular nas dosagens mais elevadas de N-acetilcisteína. A N-acetilcisteína induziu o retorno do ciclo estral no enxerto ovariano de ratas, principalmente na maior dosagem comprovando sua eficácia na revascularização do tecido após isquemia e reperfusão.

Palavras-chave: Acetilcisteína. Reperfusion. Transplante. Ciclo Ovariano.

1 Introduction

In an attempt to preserve the endocrine function, ovarian transplantation is a good alternative for those women of reproductive age who have undergone cytotoxic treatments^{1,2}. Radiotherapy and chemotherapy may affect the ovarian function, even when the ovaries are not treated^{3,4}.

According to Marcondes et al.⁵, the female rats' estrous cycle is determined in four phases, proestrus, estrus, metestrus and diestrus, from which the cellular types were defined, respectively, observed in the rats' vaginal smear. Due to ischemia and reperfusion injury at the time of transplantation,

there are already studies showing loss of up to 50% of ovarian follicles after transplantation⁶.

Several experimental studies have demonstrated the drugs efficacy use to prevent or attenuate ischemia and reperfusion injuries, such as the use of superoxide dismutase, catalase, mannitol, allopurinol, vitamin E and N-acetylcysteine⁷⁻⁹.

Ischemia and reperfusion injury caused at the time of transplantation are also associated with inflammation, cytokine production and increased expression of adhesion molecules by hypoxic endothelial cells recruiting polymorphonuclear leukocytes to the reperfused tissue^{10,11}. Therefore, the oxidative stress that occurred at the time of transplantation results in an

increase in free radicals and a decrease in proliferative activity as a result.

Ovarian transplantation for its functionality only requires that the implant site be close to a large vascular network, so that neoangiogenesis occurs, constituting the avascular heterotopic transplant, a technique already well known by literature and with satisfactory results¹².

Study with ovarian autologous implantation in the main motor showed viability with regular cycle return, obtaining better morpho functional preservation with the slices implantation¹³.

N-acetylcysteine, in turn, is considered a precursor of GSH due to its chemical model providing radical sulfhydryl reactive oxygen species and has already been studied with good perspective in some studies to prevent IR injury¹⁴⁻¹⁶.

The objective of this experiment is to evaluate the quality of the ovarian graft with the use of N-acetylcysteine, in terms of preservation of the female rats' estrous cycle.

2 Material and Methods

After the evaluation and approval by the Ethics Committee in Research, in animal experimentation, of the Federal University of the State of São Paulo (UNIFESP), with CEP no. 1737/11 for use with animals, 50 rats, EPM- 1 Wistar lineage, females, virgins, aged between 03 and 04 months were used, weighing about 250 grams. The animals were supplied by Centro de Desenvolvimento de Modelos de Animais de Experimentação (CEDEME) of UNIFESP-EPM, kept grouped in appropriate boxes with controlled temperature and light, and water and food ad libitum.

The sample was randomly distributed in four groups of 10 animals each, one control group and the groups of the experiment according to the applied dose of N-acetylcysteine per mg/kg, respectively, NAC 150, NAC 300, NAC 600, NAC 1200. All animals were submitted to daily vaginal smears, 08 days before transplantation to determine diestrus, a phase established to perform transplantation.

The animals in both groups were anesthetized with ketamine hydrochloride at a dose of 60mg.kg⁻¹ and xylazine hydrochloride at a dose of 10mg.kg⁻¹ by intramuscular use on the lateral face of the right rear paw. With the adequate anesthetic plane, epilation and antisepsis of the abdominal region were performed, followed by subcutaneous NAC administration, half applied to each hypochondrium, in the median abdomen line.

After 60 minutes of absorption, laparotomy of the abdominal-pelvic cavity was performed in the median line and fat dissection was performed to identify the uterine and ovary horns, followed by the ovaries bilateral removal at the junction of the uterine horns. The right ovary was immediately placed in 10% buffered formaldehyde solution for the pathologies exclusion. The left ovary, also intact, was immediately reimplanted in the retroperitoneum by means of

a stitch with nylon 6-0 wire, close to the great vessels, without vascular anastomosis.

According to the postoperative periods, the 4th and 14th days, the animals were euthanized with lethal dose of anesthetic. In the post-transplant period, 5 animals from each group were randomly subjected to the single collection of vaginal smears on the 4th postoperative day, in order to define the estrous phase and then subjected to euthanasia. The remaining animals from each group were collected daily on the following days, from the 10th day, until the estrus phase or proestrus phase of the estrous cycle was detected, being considered the 14th post-transplant day of euthanasia.

3 Results and Discussion

In the groups tested, whether on the 4th or 14th day of post-transplantation, it was observed that the colpocytological examination reveals the presence of a new cycle beginning with the passage through the diestrus phase.

The proestrus presented predominance of nucleated and anucleated epithelial cells, followed by estrus, characterized by the presence of cornified or keratinized cells. In the metaestrus, in addition to the cells in the two previous phases, the presence of leukocytes was observed.

The smears on the 14th day show the presence of a new diestrus, characterized by the scarcity of cellular elements and a great concentration of leukocytes and mucus, confirming the return of the estrous cycle after transplantation.

The four stages of the estrous cycle cited by Marcondes (2002) were observed in both groups. The results obtained in the present study show that the use of N-acetylcysteine, at the highest dosage - 1200mg/kg, contributed to the return of cyclicity, which was confirmed in the later period of post-transplantation. A table is followed bellow, with the stages of estrus in the groups of females rats used, Control and dosages of N-acetylcysteine, with analysis of the swabs of the estrus cycle on day 4 and on day 14 post-transplant days.

Table 1 - Stages of the estrous cycle of vaginal smear on the 4th and 14th day of post-transplantation

| Group of female Rats | Estrus stages - 4 th Day | | Final Cycle - 14 th Day | |
|----------------------|-------------------------------------|-----------------|------------------------------------|-----------|
| | Phase D | Phase P, E or M | R Cycle | I Cycle |
| GTx | 66.7% (2) | 33.3% (1) | 33.3% (2) | 66.7% (4) |
| NAC 150 | 75% (3) | 25% (1) | 50% (3) | 50% (3) |
| NAC 300 | 100% (4) | 0% (0) | 50% (3) | 50% (3) |
| NAC 600 | 33.3% (1) | 66.7% (2) | 83.3% (5) | 16.7% (1) |
| NAC 1200 | 40% (2) | 60% (3) | 100% (5) | 0% (0) |

*Independence test (Fisher's Exact Test). Test Hypotheses: Ho:there is independence between group and variable. HA:there is association between group and variable. Value p=0.745 (4th DAY). Value p=0.149 (14th DAY).

Source: Research data.

In order to maintain the gonads functionality and their physiology, heterotrophic ovarian autologous implant has been proposed in many studies. However, the relevant

question to consider ovarian autotransplantation as a promising alternative is to ensure the reduction of the harmful effects caused by ischemia and reperfusion.

There are several strategies in the transplantation of avascular heterotopic ovary developed with the objective of minimizing the loss of number of follicles, due to the of I/R injury, since about half of the primordial follicles are lost in initial ischemia, persisting until the beginning of neoangiogenesis¹⁷.

Study with ovarian transplantation in inguinal region in rats, both with avascular autologous implant, showed to be promising in order to preserve the ovarians vitality¹⁸.

In view of the fact that the surgical removal of an organ itself results in injury due to ischemia, studies also showed that the influx of oxygen into the ischemic tissue would lead to a series of biochemical and cellular alterations mediated mainly by the formation of oxygen free radicals¹⁹. Therefore, ischemia and reperfusion injury are the main cause of massive failure of ovarian follicles during transplantation²⁰.

An analysis of the estrous cycle of rats after the autologous ovarian transplant will contribute as an initial step to evaluate the return of graft functionality. Because of the short duration of their estrous cycle, female rats constitute a good model for the study of the alterations that occur during the reproductive cycle²¹.

According to studies using NAC, in different posologies and showing that it is effective on the I/R injury in organs such as kidney, liver, lung, fasciocutaneous flap and ovary^{22,23}, NAC was chosen for the present study.

In the present study, in all doses, the animals presented a regular cycle, showing a preservation of the follicles in relation to the ischemia involved in the transplant process, with total cycle regularity in the group where higher dose of NAC was administered

N-acetylcysteine is targeted at mitochondria, in which the respiratory chain is processed, and has therefore been used in numerous clinical and experimental studies in order to make clear its role in modulating the activity of reactive oxygen species²⁴.

Damous et al (2009), studying the vaginal epithelium in the post-transplant period, evidenced on the 4th and 7th day a non-keratinized squamous epithelium, and on the 14th and 21st day a squamous and keratinized epithelium, with maintenance of the estrogenic activity and at the end of 21 days in maximum activity²⁵.

The research with remote ischemic preconditioning corroborates the current research to attenuate the ischemia and reperfusion injury, ensuring the return of gland functionality, since it shows a tendency to restart early in the estrus cycle²⁶.

Petroianu et al.²⁷ during a 10-day evaluation period between 3 and 6 months of heterotrophic transplantation, reported inadequate ovarian function in the transplanted group, in animals that remained in irregular cycles or diestrus. According to Weissman et al. animals subjected to

heterotrophic transplantation in subcutaneous tissue began their estrous cycle on the 9th PO Day²⁸. In the current study, estrus was identified in groups with higher levels of NAC and in the later period with greater ease.

In the colpocytological evaluation of smears in the transplanted rats, the diestrus corresponding to the initial action of estradiol on the organism did not present significance in relation to the Control group on the 4th day, however, it showed in the next phases of the estrous cycle a significant presence in the dosages 600 and 1200 mg/kg.

N-acetylcysteine has shown a beneficial effect on the cyclicity preservation of the transplanted female rats, revealing an attenuation in the ischemia injury and reperfusion of the ovarian graft. Studies with later postoperative period should contribute to confirm this effect in the long term.

The results showed that in the later phase of post-transplantation, all groups presented an estrous cycle with the use of NAC, as well as the massive presence of regular cycle at the highest dosage administered.

4 Conclusion

The use of N-acetylcysteine in higher dosage provided for the return of regular estrous cycle in the ovarian graft of female rates, mainly at the highest dosage observed in later period of post-transplantation, proving its efficacy in the tissues revascularization after ischemia and reperfusion

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