ORIGINAL ARTICLE
TRANSPLANTATION

N-acetylcysteine improves morphologic and functional aspects of ovarian grafts in rats[1]

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ABSTRACT

PURPOSE: To evaluate morphological and functional aspects of the ovarian graft in transplanted rats treated with NAC.

METHODS: Female Wistar rats, virgin, 3 to 4 months old, weighing 200-250 grams were used in experiments. The rats have been kept in proper sanitary conditions, receiving food and water ad libitum. Five groups (n=10, each) were constituted: 4 groups treated subcutaneously with NAC, at doses of 150, 300, 600 and 1200 mg/kg (NAC150, NAC300, NAC600 and NAC1200, respectively), one hour of before the ovarian transplantation and control group (GTx) – treated with physiological solution and submitted to ovarian transplantation. The rats were anesthetized and submitted to autologous left ovarian transplantation, without anastomosis in retroperitoneum, and contralateral oophorectomy. During follow-up of 4 or 15 days, the estrous cycle was evaluated by vaginal smears to determine cycle regularity. At the end of 4th or 15th days, rats were re-anesthetized and blood and graft were obtained to estradiol analysis and morphological assessment. Data were analysed by One Way Analysis of Variance (ANOVA) or ANOVA on ranks complemented by Student-Newman-Keuls test.

RESULTS: At 4th day, viable follicles in the graft did not altered by NAC treatments. The NAC300 and NAC600 groups showed increasing in follicle atresia (p=0.012) compared to GTx and NAC1200 group. At 15th day, 50% of GTx, NAC150, and NAC300 rats showed regular oestrous cycle; 83% of NAC600 and 100% of NAC1200 rats returned to regular cycle. NAC1200 group showed increasing in primordial follicle compared to GTx, NAC150 or NAC300 (p=0.011). NAC did not interfere in estradiol levels after 4 or 15 days of transplantation.

CONCLUSION: In autologous ovarian transplantation, high dose of NAC promotes graft viability with recovery of estrous cycle.

Key-words: Transplantation. Autologous. Acetylcysteine. Histology. Estrogen. Ovarium. Rats.

INTRODUCTION

Life expectation of adult women, who survived adolescence or infancy cancer treatment has increased, thanks to early diagnostic and treatment in oncologic centers[1,2]. Early menopause induced by cancer treatment is often followed by osteoporosis, early cardiovascular diseases and infertility, besides a prolonged hormonal reposition treatment requirement (TRH)[1].

There are several alternatives aiming to reduce the adverse effects of chemotherapy and radiotherapy on ovaries, such as the transposition (temporary removal) of ovaries, also known as oophorectomy, the use of antagonistic drugs to Gonadotropin Releasing Hormones (GnRH) together with chemotherapy and new apoptosis antagonistic drugs[3]. For those patients, who need immediate oncologic treatment, a promising option is the oophorectomy, the cryopreservation of ovaries and its posterior re-implantation, after the end of treatment[1]. For the preservation of treated women fertility, several techniques may be used: some already widely used, such as cryopreservation of embryos, mature or premature follicles, others are still experimental, such as the transplantation of ovarian tissue[3,4].

Ovarian transplantation has been studied in several experiments, both on animals and humans, and has been described as a promising alternative for those patients, who will be submitted to oncologic treatment[5,6]. Even thought, until now, it has been described a short survival time of the transplanted ovarian, with loss of more than 50% of the ovarian follicles after transplantation[5]. Several factors are involved in this problem, but ischemia seems to play an important role in this process.[1,5]

Some studies have been performed, in search for strategies for the attenuation of the ischemia and reperfusion lesion (I/R): angiogenic factors, which stimulate new vessels formation, antioxidating substances, such as E vitamin, GnRH analogous and ischemic preconditioning[7-10].

Some previous works have shown the positive effect of N-Acetylcysteine (NAC) at 150 mg/kg dose on various organs. NAC is a low molecular weight thiol, it is derived from cysteine and acts removing reacting species of oxygen, besides improving the action of nitric oxide, acting on the regulation of microcirculation[11-13]. Usta et al.[14] showed NAC improved tissue recovering in ovaries, which had been submitted to torsion and distortion. Some other studies use NAC in polycystic ovarian syndrome patients, resulting in improved sensibility to insuline[15,16].

Considering the positive effects of NAC in clinical or experimental studies, this work aims to evaluate the post transplant effect of NAC on ovarian graft in rats treated with N-Acetylcysteine.

METHODS

This project was approved by the board of the Research Ethics Committee, UNIFESP, under protocol 0907/2010. Procedures have been performed at the laboratory of experimental microsurgery of UNIFESP.

Female Wistar rats, virgins, age 3 to 4 months, weight 200 to 250 grams have been used. All animals came from the Center for Experimental Models Developing for Biology and Medicine, UNIFESP. Animals were kept in proper sanitary conditions, having their environmental temperature and humidity checked, water and food always available along all experiment.

Daily, vaginal smears were performed during 8 days before of transplantation procedures to evaluate the estral cycle. Females with regular cycle were selected, and ovarian transplantations were performed during diestrum. After transplantation, vaginal smears were performed daily and euthanasia was performed during estrum or proestrum stages. During follow-up of four or 15 days, vaginal smears were performed to determine cycle regularity return.

One hour before ovarian transplantation, animals were randomly allocated into five groups, with 10 animals each, according to the treatment. Four groups were treated subcutaneously - along the linea alba - with NAC, at doses of 150mg/kg, 300mg/kg, 600mg/kg or 1200mg/kg - NAC150, NAC300, NAC600 and NAC1200 groups, respectively. One group was treated with 0.9% Sodium chloride solution and was considered control, named GTx.

The autologous left ovarian transplantation was performed in anesthetized rat (Xilazine, 15 mg/kg and Ketamine, 60 mg/kg). The anaesthesia was applied on the lateral side of the left hind leg, intramuscularly.

After medial laparotomy, the left ovarian was carefully removed, through a ligation of the pedicle, and on the junction of the left horn, using 6-0 size nylon. The left ovarian was washed using 0.9% Sodium chloride solution and uterine tubes and periovarian fat were removed. To ovarian transplantation, the left ovarian was fixed in retroperitoneal position, close to the large vessels and, without vascular anastomosis. The right ovarian was removed and anatomical-pathological evaluation was conducted to exclude previous existing disorders.

At the end of 4th or 15th days, rats were re-anesthetized and blood and graft were obtained to estradiol analysis and morphological assessment, respectively.

The ovarian graft was fixed in 10% formalin and embedded to paraffin to histological procedures. Five micra slides were stained with haematoxylin-eosin and assessed by blinded investigator.

For the evaluation of follicular development, ovarian follicles were counted and classified in developing follicles, independently of their stages and atretic follicles, which were subdivided according to the degree of maturity into immature follicles (including primordial, preantral follicles) and antral follicles (with only one bulky antrum) besides the corpora lutea[17]. Measurements were obtained with the image analysis program AxionVision REL 4.6, Karl Zeiss. Counting was always performed in 4 fields per animal at 10x magnification.

Data are presented as mean±SED or median (interquartile range). The comparison among groups in the same time was analyzed by One Way Analysis of Variance (ANOVA) or ANOVA on ranks, when appropriated, complemented by Student-Newman-Keuls test. Comparison between studied times was analyzed by Mann-Whitney test. Differences were considered for $p<0.05$.

RESULTS

After 4 or 14-16 days of follow-up, serological levels of estradiol were similar among groups ($p=0.051$ and $p=0.51$, respectively).

At 4th day after transplantation, ovarian graft morphology indicated that NAC treatment promoted high numbers of atretic follicles in NAC300 and NAC600 groups (Figure 1). Primordial follicle, preantral follicle, antral follicle and functioning and degenerated corpora lutea were similar among groups.

At 14th day after transplantation, ovarian graft morphology indicated that NAC treatment promoted high numbers of primordial follicles in NAC600 and NAC1200 groups (Figure 2). Preantral follicle, antral follicle, atretic follicle, functioning and degenerated corpora lutea were similar among groups.

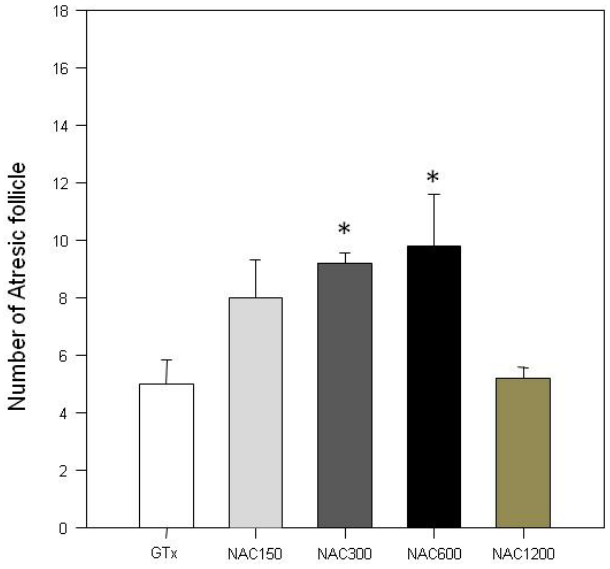

FIGURE 1 – Number of atretic follicle on 4th-day of autologous ovarian transplantation in rats. Rats previously treated with NAC at dosis of 300 and 600 mg/kg (NAC300 and NAC600, respectively) augmented the number of atretic follicle in the graft. One way analysis of variance complemented by Student-Newman-Keuls test; *, $p<0.05$ vs control.

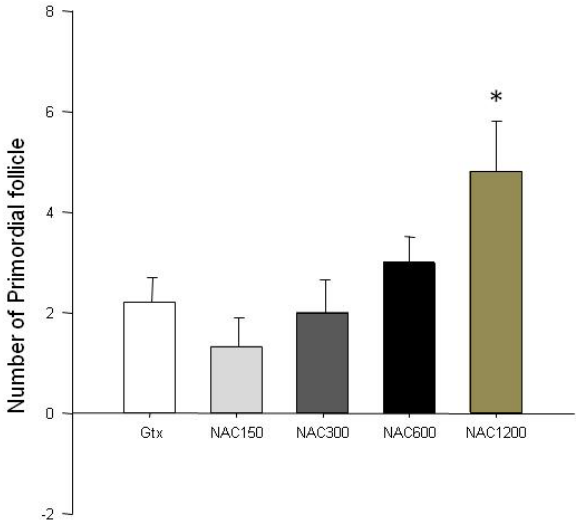

FIGURE 2 – Number of primordial follicle on 14th-day of autologous ovarian transplantation in rats. Rats previously treated with NAC at dosis of 600 and 1200mg/kg (NAC600 and NAC1200, respectively) showed elevation in number of primordial follicle in graft. One way analysis of variance complemented by Student-Newman-Keuls test; *, $p<0.05$ vs control.

TABLE 1 – Follicles and Corpora Lutea after ovarian transplantation in groups treated with NAC in different doses.

		Groups				
		GTx	**NAC150**	**NAC300**	**NAC600**	**NAC1200**
Primordial Follicles	**4th day**	2.2 ± 0.66	1.00 ± 0.32	2.80 ± 0.37	1.00 ± 0.55	1.80 ± 0.58
	14th day	2.20 ± 0.49	1.33 ± 0.56	1.77 ± 0.63	3.00 ± 0.52*	4.16 ± 0.97*#
Preantral Follicles	**4th day**	5.20 ± 1.20	4.40 ± 1.17	5.00± 1.00	3.60 ± 0.81	5.00 ± 1.26
	14th day	7.40 ± 1.29	6.67 + 0.99	7.20 ± 2.89	6.17 ± 1.68	8.20 ± 1.43
Antral Follicles	**4th day**	0.40 ± 0.24	0.40 ± 0.24	0.60 ± 0.24	0.60 ± 0.26	0.40 ± 0.24
	14th day	1.42 ± 0.51*	1.33 ± 0.67	3.40 ± 1.44	1.17 ± 0.50	2.00 ± 0.42*
Atretic Follicles	**4th day**	5.00 ± 0.84	8.00 ± 1.30	9.20 ± 0.37#	9.80 ± 1.80#	5.20 ± 0.37
	14th day	9.60 ± 1.17*	8.67 ± 1.84	6.80 ± 1.36	6.50 ± 0.85	4.00 ± 0.55
Functioning Corpora Lutea	**4th day**	1.20 ± 0.58	0.20 ± 0.20	1.20 ± 0.37	1.20 ± 0.37	2.40 ± 0.68
	14th day	1.40 ± 0.60	1.70 ± 0.71*	2.20 + 0.49	2.00 + 0.45	3.00 + 0.71
Degenerated Corpora Lutea	**4th day**	1.40 + 0.40	1.40 + 0.24	1.60 + 0.40	1.80 ± 0.58	2.00 + 0.32
	14th day	0.60 ± 0.40	0.50 ± 0.22*	0.20 ± 0.20*	0.50 ± 0.22*	0.40 ± 0.24*

Mean±EPM; test-t between 4th and 14th days of follow-up *, $p<0.05$ vs 4th day; One way analysis of variance complemented by post-hoc test among groups, compared to control (GTx); #, $p<0.05$ vs GTx.

The comparison between two intervals showed that high number of atretic follicle and antral follicle in GTx group in 14th day. NAC promoted a better preservation of viable follicles on latest time (14th day after surgery), showing significant results on those groups NAC300 and NAC600 (Table 1; Figure 3).

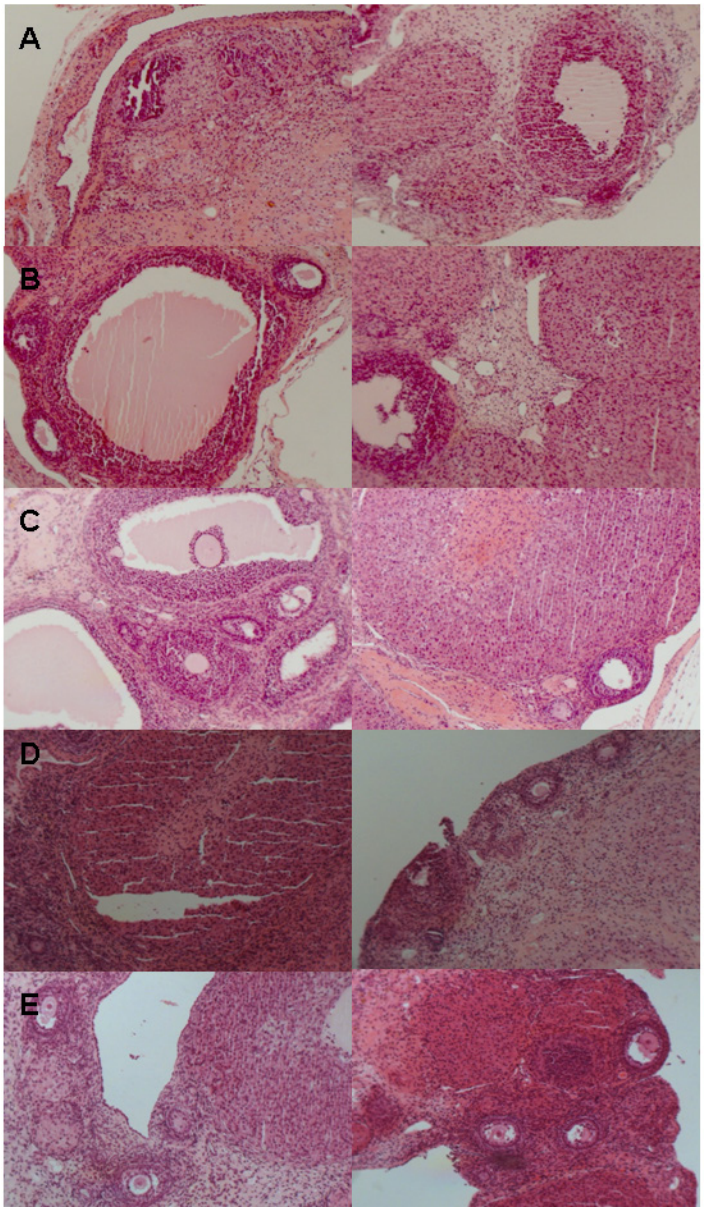

FIGURE 3 - Photomicrographs of ovarian grafts of the groups at 14 days : GTx (A), NAC150 (B), NAC300 (C), NAC600(D), NAC1200 (E). HE – 10 X. F=Follicle CL=Corpus luteum

Comparing the response of the animals at 4 and 14 days after surgery, the animals, which had been treated with NAC1200 showed a better tissue preservation on later stage, with a significant higher number of premature follicles (p=0.018). In all treatment groups at day 14 after transplantation there was an increase in the number of functional corpora lutea, but this was significant just in the group treated with NAC150 (p=0.02). NAC 600 group showed a tendency instead (p=0.074). For what concerns to the degradation of the transplants, NAC treatment promoted a better preservation of the corpus luteum, reducing the degenerative process at the later analysis stage, on day 14.

DISCUSSION

Experimental ovarian transplantation has been performed for some years: autologous, heterologous, with or without vascular pedicle, both in humans and laboratory animals. This operation has been performed in different sites of implantation, and the implants have been functional, both functionally and morphologically[1,3,18].

At transplantation, half of the primordial follicles are lost, persisting until the stage of neoangiogenesis[5]. Many studies have tried to preserve the ovarian tissue from I/R lesion, using substances such as E vitamin, ascorbic acid, and GnRH analogous, or physical strategies including the ischemic preconditioning[2,8-10].

Some work have already used NAC at different doses on several organs, such as kidney, liver, lungs, fasciocutaneous flap, intestine and ovarian, showing good results on I/R lesions[12-14, 18-22].

So in order to mitigate the damage and I/R as the first study in ovarian transplantation, it was decided to give NAC subcutaneously, in order to have a better control over the given volumes and at different doses to determine the relation between the doses and the effect on the ovarian tissue.

There are further applications of NAC, besides its mucolytic activity, in the prevention of obstructive chronic lung disease, in the prevention of kidney complications during contrast procedures, in the reduction of viral flu and pulmonary fibrosis. Furthermore, NAC gave good results on fertility of patients with clomifen-resisting polycystic ovarian[23]. Recent studies have also suggested the use of NAC as chemiopreservative against cancer, auxiliary in the eradication of Helicobacter pylori, in the hearing loss caused by gentamycin in kidney dialysis patients, HIV infection, cardiac diseases, smoke and epileptic[22]. NAC used for long time (5-6 weeks) improved the sensibility to insulin, testosterone levels and lipidic profile in women affected by polycystic ovarian syndrome[24].

A study realized on polycystic ovarian syndrome patients, which compared the effect of metformin to those of the association metformin + NAC for short times (5 days), showed NAC is effective in inducing ovulation, even if the number of mature follicles was not significant, as it was confirmed in our experience[23, 25].

Oktay et al.[26] observed the levels of estradiol in the xenotransplantation and found signals of a estrogenic effect in the genitalia, even if there was a lower number or absence of growth

of antral follicles. This observation confirms, genital organs of rodents are sensitive to low levels of sexual steroids[2,26], data corroborates our results, as we detected low levels of estrogen in all transplanted groups, despite the histology showed graft functionality in the later phase. Callejo et al.[27], analyzing the longevity of the grafts and estradiol levels, suggest, estradiol and FSH are reliable markers of the transplant functionality. The same authors, analyzing intraperitoneal and subcutaneous implantation of ovarian transplantations, found high estradiol levels and low FSH levels in the intraperitoneal transplantation of ovarians after 30 days[27]. Li et al.[28] also describe an elevation of estradiol levels studying follicular growth and oocyte development after transplantation in muscle of spayed male mice.

D'Acampora et al.[17] analysed the transplantation in female rats and perceived, on the 7th day the recovery of the follicular production, which persisted until the 14th day, with the presence of follicles in 75% of the ovaries. Israely et al.[4] implanted grafted ovarian transplants in granulation tissues and showed better vascularization and an higher number of viable follicles, which may serve as a model to reduce the ischemic period and to extended the functionality of the transplant. In this same study NAC was important on the latest stage, protecting the follicles from being atretic, with an anti-apoptosis effect.

Damous et al.[7] showed a tendency to the increase of the follicles number at 48 hours using PCI-R. It is important to stress the direct correlation existing between follicle size and its susceptibility to damages caused by ischemia: antral follicle has an higher tendency to degeneration than primordial follicle. According to Damous et al.[7], PCI-R showed positive effects at the 5th day after surgery, with the presence of ovarian follicles at different development stages and corpus luteum, even if an intense inflammation infiltrate was present. D'Acampora et al.[17] showed similar findings.

Fabbri et al.[18] studied ovarian tissue from a woman, who suffered breast cancer at the age of 26: after freezing, defrosting and 32 weeks growth, the authors could observe the synergy between NAC and FSH, with improvement on growth and preservation of the pre-antral follicle. On follicular growth, after some days of development, the follicle shows some signals of atresy. In vitro studies have produced the complete development from primordial follicle to ovulatory mature follicle just in mice. In humans, the same studies have only produced the maturation from pre-antral to antral stage and from primordial to primary and secondary follicles[25]. The return to gonad functionality on long term ovarian transplantation depends on the survival and growth of the follicles, which itself depends, from the capacity to ovulate[29].

CONCLUSION

In autologous ovarian transplantation, high dose of NAC promotes graft viability with recovery of estrous cycle.

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