TÜBİTAK

Effects of n-acetylcysteine on ovarian tissue autografted into granulation tissue compared to back muscle in rats

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Background/aim: Ovarian transplantation can preserve fertility in cancer patients. The aim of this study was to compare ovarian transplantation into granulation tissue (GT) versus back muscle (BM) sites and also to investigate the effects of N-acetylcysteine (NAC) as an antioxidant on ovarian survival in a rat autograft model.

Materials and methods: Twenty-eight adult female rats were divided into four equal groups (n = 7 each) as follows: Group 1, ovarian tissue grafted into GT + saline (GT + saline); Group 2, ovarian tissue grafted into GT + NAC (GT + NAC); Group 3, ovarian tissue grafted into BM + saline (BM + saline); and Group 4, ovarian tissue grafted into BM + NAC (BM + NAC). After 28 days, serum concentrations of malondialdehyde (MDA), progesterone, and estradiol (E2) were measured. Ovarian follicle counts were performed from hematoxylin and eosin-stained slides. Furthermore, apoptosis was assessed by terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL).

Results: There were no significant differences found in levels of MDA, progesterone, and E2 among the groups. The percentage of apoptotic granulosa cells was significantly ($P < 0.05$) lower in the ovarian tissue autografted in the GT + NAC group compared to the GT + saline and BM + NAC groups. In addition, the number of blood vessels formed in the ovarian tissue in the GT + NAC group was significantly higher than in the GT + saline and BM + NAC groups ($P < 0.05$ and $P < 0.001$ respectively).

Conclusion: The granulation tissue site is a suitable candidate for ovarian graft in rats. Furthermore, NAC could not restore autografted ovarian functions.

Key words: Ovarian tissue autograft, granulation tissue, N-acetylcysteine, back muscle, rats

1. Introduction

Recent advances in cancer treatment have significantly increased the number of cancer survivors. Cancer treatments such as chemotherapy and radiotherapy threaten reproductive potential, resulting in premature ovarian failure and infertility (1,2).

Ovarian tissue transplantation has been performed to restore fertility among infertile cancer survivors who have undergone chemo/radiotherapy; however, despite these promising findings, there still exist some unresolved limitations that affect the success of transplantation (3,4).

Ischemia/reperfusion (IR) injury during the first few days after transplantation is the most important challenge in ovarian tissue transplantation that leads to oxidative stress, cytokine release, and apoptosis, resulting in massive follicular loss in the transplanted ovary (5–7). Therefore, using any means to reduce ischemic injury during the transplantation period and enhancing angiogenesis in the

ovarian grafts can play a vital role in the success of the ovarian tissue transplantation.

It has been proven that the survival of a grafted ovary is strongly correlated with the neovascularization process. Accordingly, one suggestion is to activate this process after ovarian transplantation, especially during the first few days, because this process can take 2–7 days to complete (8,9).

Selection of the ovarian transplantation site is another important factor involved in the future of graft survival. Therefore, it is important to determine which site is the most suitable for maintaining graft viability and function. The ovary can be transplanted into its original site (orthotopic) or to other sites (heterotopic) (5). Easy access for oocyte collection and noninvasiveness of the surgical technique are the advantages of heterotopic sites that should be considered (10).

Many studies have reported several heterotopic sites for ovarian transplantation including back muscle (BM)

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(11), the retroperitoneum (12), under the kidney capsule (13), subcutaneous sites (8), and granulation tissue (GT) (14). Because of rapid revascularization, ovarian transplantation under the kidney capsule site is common (13). Youm et al. showed that the kidney capsule site, in comparison to BM and subcutaneous sites, is the optimal site for ovarian transplantation in the murine model (15). Soleimani et al. found that ovarian graft into the BM had some advantages compared to the kidney capsule because of good angiogenesis conditions (11). Other investigators found that grafting ovarian tissue into the ovarian bursa is better than grafting under the kidney capsule, which itself is better than a subcutaneous site (16).

Wound-healing GT is a promising site for ovarian transplantation as reported by Israely et al. Their study also showed an improvement in graft vascularization and follicle survival (14). The new blood formation in GT is a crucial component that transports nutrients, inflammatory cells, and oxygen to the wound location (17). Moreover, it has been shown that at the site of surgical operations, tumor angiogenesis and growth are accelerated (18).

There is no report to compare and evaluate the efficacy and influence of the GT site for ovarian transplantation with other heterotopic sites such as the kidney capsule, BM, and subcutaneous sites.

Some researchers have tried to identify factors such as antioxidants (19), gonadotropin hormones (16), and growth factors (20) that can decrease IR injury during postimplantation by enhancing and establishing angiogenesis in the grafted ovary.

N-acetylcysteine (NAC) is an antioxidant and a precursor of glutathione and is used as a mucolytic agent and also as treatment for paracetamol (acetaminophen) overdose (21). Experimental studies have indicated the protective effects of NAC on IR injuries of various organs, such as the liver, lungs, kidneys, and ovary (22–25). Other studies have demonstrated that using NAC in patients with polycystic ovarian syndrome improves sensibility to insulin (26). In addition, it has been proven that NAC can inhibit apoptosis and promote cell survival (27). Mahmoodi et al. showed that NAC improves follicular survival in mice ovarian autografts into the BM by decreasing oxidative stress and apoptosis (25). Amorim et al. also reported that subcutaneous injection of NAC at a high dose improves follicular survival of ovarian tissue grafted into a retroperitoneal position (28).

Considering the easy access to GT for ovarian grafts and the beneficial effects of NAC in experimental studies, the aim of the current study was to investigate the effects of NAC on ovarian autografts and also to compare the effect of graft site (the GT versus the BM) on the survival rate of fresh ovarian autografts in rats.

2. Materials and methods

2.1. Animals
Twenty-eight adult female Wister rats were used in this study. They had unlimited access to food and water and were kept under standard conditions of a daily cycle of 12 h of light and 12 h of darkness.

The animals were randomly divided into four groups (seven rats per group) as follows: Group 1, ovarian tissue autografted into the GT and treated with saline (GT + saline); Group 2, ovarian tissue autografted into the GT and treated with NAC (Zambon S.P.A, Italy) at a dose of 150 mg/kg (GT + NAC); Group 3, ovarian tissue autografted into the BM and treated with saline (BM + saline); Group 4, ovarian tissue autografted into the BM and treated with NAC (BM + NAC). All the groups received injections intraperitoneal from 2 days before surgery until 7 days after grafting. The doses of NAC were selected according to previous studies (25,29). After 28 days, the ovarian tissue was removed from the transplantation site and histological evaluations were performed.

2.2. Ovarian retrieval and transplantation
The animals were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg, ketamine 10% Alfasan, Woerden, the Netherlands) and xylazine (10 mg/kg, xylazine 2%; Alfasan). Before the surgery, the dorsal skin of each rat was shaved and then swabbed with alcohol. Then, under sterile conditions, bilateral incisions were made on each side of the spine column and a bilateral ovariectomy was performed. The ovarian blood vessels were cauterized in order to stop the bleeding. The excised ovaries of each rat were placed in a dish with normal saline, removed out of periovarian fat, and cut in two halves and then immediately autografted either into the BM or into wound-healing GT. The muscle and skin were then sutured.

To prepare wound-healing GT, a 0.5-cm full-thickness dermal incision was made in the back of each rat by scalpel. The incision was closed by 4/0 sutures. Four days later, after removal of the sutures and excessive tissues, ovarian tissue was autografted into the wound and sutured (14).

2.3. Vaginal smear collection
The vaginal smear of each rat was obtained at 0800–0900 hours before surgical procedures with a swab imbibed in physiological solution on a standard slide and was immediately observed under a light microscope (40×) in order to be categorized as preestrous, estrous, or diestrous depending on the cells found in the smears. The ovarian transplantation was performed during the diestrous phase (12,30).

2.4. Histological assessment
After 28 days, rats were reanesthetized with an injection of ketamine and xylazine. The blood and ovarian tissue

were obtained for hormonal analysis and histological assessment, respectively.

The ovarian samples were rapidly fixed in 10% formalin solution (Asia Chemical Co., Tehran, Iran) for 24 h and were completely processed and embedded in paraffin and blocked. Sections of 5-μm slides were stained with hematoxylin and eosin (Merck, Darmstadt, Germany).

In order to evaluate the follicular development and blood vessel density, ovarian follicles and blood vessels were counted in four fields per section per animal at 100× magnification. Follicles were categorized into three groups: immature (follicles including primordial, primary, and preantral), antral, and corpus luteum. Primordial follicles consist of the oocyte surrounded by a single layer of follicular (granulosa) cells. Primary follicles are distinguished by more than one layer of follicular cells surrounding the oocyte. Preantral follicles are follicles containing an oocyte surrounded by follicular cells layers that still lack an antrum. Antral follicles are follicles with only one cavity adjacent to the oocyte (8,12,28). All microscopic assessments were performed at least by two blinded investigators.

2.5. TUNEL assay
Apoptosis was assessed by TUNEL in accordance with the kit manufacturer's manual (In Situ Cell Death Detection Kit; Roche, Berlin, Germany). Briefly, after the samples were deparaffinized and rehydrated, they were washed in phosphate-buffered saline (PBS; Sigma, St. Louis, MO, USA) for 5 min, three times. Then they were exposed to proteinase K (Sigma) for 15–30 min at room temperature. Once again, they were washed in PBS and then the samples were embedded in 0.3% H_2O_2 (Asia Chemical Co.) for 10–15 min at 15–25 °C in the dark. After washing in PBS, the TUNEL reaction mixture was added to the samples in the dark and kept for 1 h at room temperature but was not added to the other sample (i.e. the negative control). Then they were all washed in PBS. Converter-POD (Roche) was added to the samples, followed by incubation in a humidified chamber for 30 min at 37 °C. Once again, they were washed in PBS. Following that, the samples were stained with diaminobenzidine (Sigma) and were then incubated at 15–25 °C for 10 min in the dark, followed by a wash in PBS. Hematoxylin (Merck) was used for nuclear staining. The samples were washed with distilled water, dehydrated, and embedded in ethanol (Merck) and finally in xylene (Asia Chemical Co.). The apoptotic index was determined as the percentage of granulosa cells yielding apoptosis in 1000 cells from the tissue under a microscope in high power fields (400×) (31).

2.6. Hormonal assay
After 28 days, blood samples were obtained and centrifuged (3000 × g, 10 min). Next, serum was analyzed in duplicate with the E2 and progesterone hormones being measured

within assay precision by ELISA kits (Monobind Inc., Lake Forest, CA, USA) in accordance with the manufacturer's instructions.

2.7. Measurement of MDA concentration
MDA is a lipid peroxidation product used as the marker of oxidative stress. It was evaluated in blood serum on the 28th day after transplantation using an ELISA kit (Shanghai Crystal Day Biotech Co. Chain, Shanghai, China) in accordance with the manufacturer's instructions.

2.8. Statistical analysis
Data analyses were conducted using SPSS 16.0, applying one-way analysis of variance (ANOVA) and Tukey's test. P < 0.05 was accepted as statistically significant.

3. Results
3.1. Number of follicles
The mean numbers of immature follicles, antral follicles, and corpus lutea in the NAC-treated groups were higher than in the saline groups. There were no significant differences, however, among all the groups as shown in Table 1 and Figure 1.

3.2. Number of blood vessels
The mean number of blood vessels in ovarian tissue was significantly higher in the GT+NAC group compared to GT+saline and BM+saline groups (P < 0.05 and P < 0.001 respectively). Moreover, the mean number of blood vessels in the GT + NAC group was significantly higher than that in BM + NAC group (P < 0.001), as shown in Table 2 and Figure 2.

3.3. Apoptosis assay
As shown in Figures 3 and 4, the percentage of ovarian apoptosis in the GT + NAC group was significantly lower than that in the GT+saline and BM+NAC groups (P < 0.05).

3.4. Concentration of malondialdehyde
Despite the low level of MDA in the NAC-treated groups, there were no significant differences among groups (Table 1).

3.5. Hormonal assay
Despite the high amount of estrogen and progesterone hormones in the NAC-treated groups, there were no significant differences among the groups. These results are shown in Table 1.

4. Discussion
In the present study, in order to find out the most suitable location for transplantation, rat ovaries were autografted into two different sites, namely the BM and wound-healing GT. Furthermore, we treated the rats with NAC to determine its effects on follicular survival and ovarian function. In this study, bilateral ovariectomy was performed to prevent any ovarian hormonal secretions that help the better follicular development by increasing

Table 1. Comparison of the mean number of different follicles and corpus lutea and the levels of E2, progesterone, and MDA in different groups of rats 28 days after ovarian tissue transplantation. GT + saline group, ovarian graft in granulation tissue, saline-treated; GT + NAC group, ovarian graft in granulation tissue, NAC-treated (150 mg/kg); BM + saline group, ovarian graft in back muscle, saline-treated; BM + NAC group, ovarian graft in back muscle, NAC-treated.

Groups	Immature follicles	Antral follicles	Corpus lutea	E2 (pg/mL)	Progesterone (ng/mL)	MDA (nmol/mL)
GT + saline	5.4 ± 1.14	1.33 ± 0.81	2.83 ± 1.47	48.13 ± 7.64	23.19 ± 4.53	19.5 ± 2.63
GT + NAC	6.8 ± 1.09	1.83 ± 1.47	4.16 ± 2.13	60.77 ± 6.81	27.85 ± 6.1	17.96 ± 3.31
BM + saline	5.6 ± 1.67	1.66 ± 1.03	1.66 ± 0.81	59.31 ± 8.97	23.57 ± 3.51	18.82 ± 2.07
BM + NAC	7.2 ± 1.48	2 ± 0.63	5.83 ± 3.43	66.91 ± 7.61	26.55 ± 6.64	15.11 ± 2.15

All values represent mean ± SD. One-way analysis of variance and Tukey's test showed no statistically significant differences.
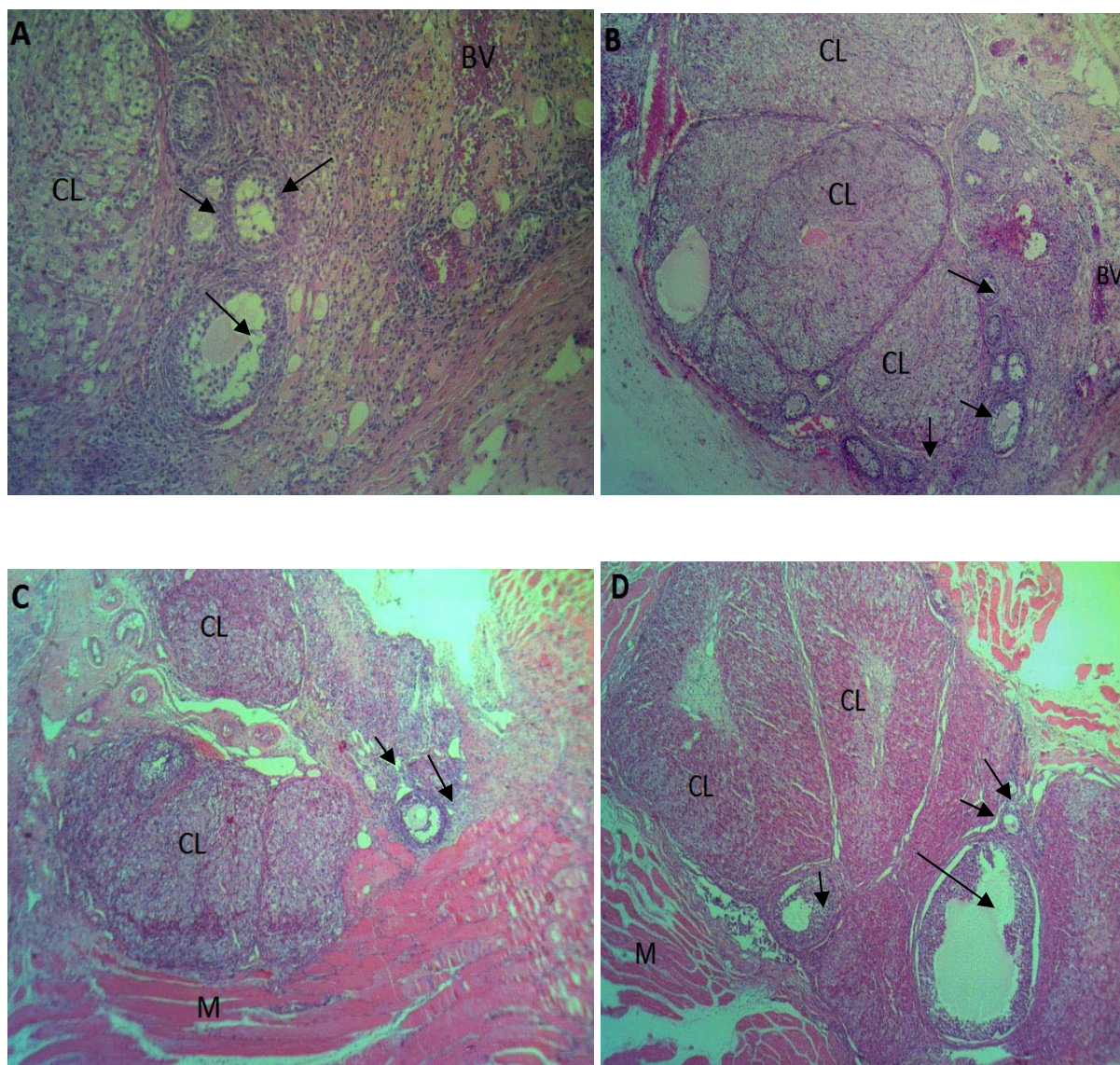
Figure 1. Photomicrographs of rat ovarian tissues (sections stained with H&E) in four groups 28 days after transplantation: A) ovarian graft in granulation tissue, saline-treated; B) ovarian graft in granulation tissue, NAC-treated; C) ovarian graft in back muscle, saline-treated; D) ovarian graft in back muscle, NAC-treated. Arrows showing follicles, CL showing corpus luteum, and M showing muscle. Original magnification: 100×.

Table 2. Comparison of the mean number of vessels in different groups of rats 28 days after ovarian tissue transplantation. GT + saline group, ovarian graft in granulation tissue, saline-treated; GT + NAC group, ovarian graft in granulation tissue, NAC-treated (150 mg/kg); BM + saline group, ovarian graft in back muscle, saline-treated; BM + NAC group, ovarian graft in back muscle, NAC-treated.

Groups	Number of vessels
GT + saline	32.85 ± 5.45[a]
GT + NAC	44.28 ± 5.7[bc]
BM + saline	14.28 ± 1.6
BM + NAC	23.42 ± 1.51[d]

Values are means ± SD.
[a] $P < 0.001$ compared to corresponding value of BM + saline group.
[b] $P < 0.05$ compared to corresponding value of GT + saline group.
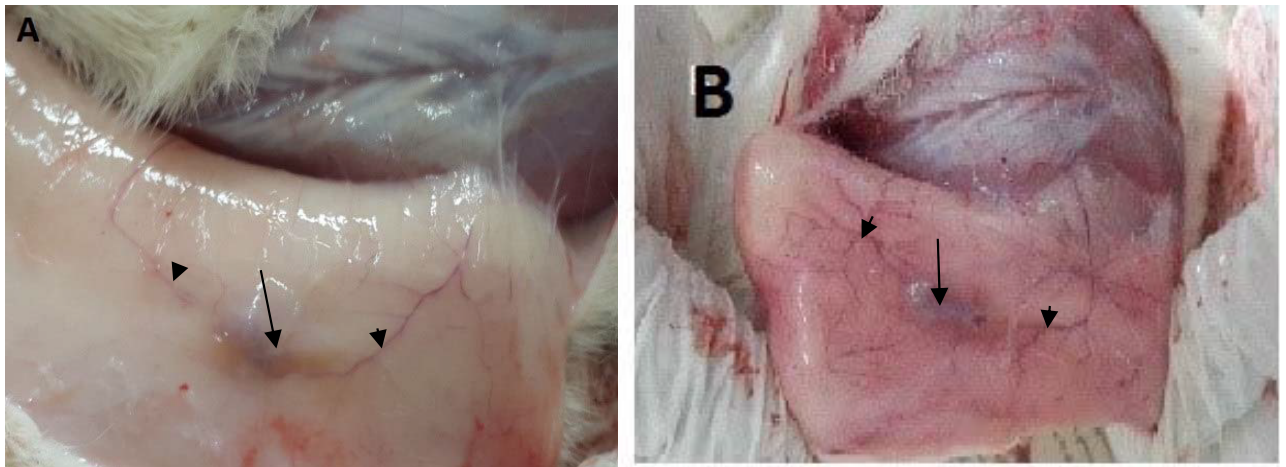[c] $P < 0.001$ compared to corresponding value of BM + NAC group.
[d] $P < 0.05$ compared to corresponding value of BM + saline group.

Figure 2. Macroscopic images of rat ovarian tissues 28 days after transplantation in granulation tissue saline-treated group (A) and NAC-treated group (B). Arrows showing ovary and arrow heads showing neovascularization adjacent to the graft.

gonadotropins (13). The treatment groups received NAC injections from 2 days before graft surgery until 7 days after transplantation to obtain better results. A dose of 150 mg/kg NAC was chosen, which was shown to be the most effective in reducing IR injury (25,29).

In this study, the histological sections clearly showed that neovascularization in ovaries grafted into the GT was higher than in those grafted into BM. Furthermore, we confirmed the better survival of follicles, through preventing apoptosis, in the GT graft site compared to the BM graft site. We also confirmed that NAC helps this process and enhances neovascularization in both graft sites.

Furthermore, in the present study, the recovery rates of the ovarian grafts transplanted into the GT and BM were

100%. In addition, stromal tissue, which has an important role in tissue integrity, was normal.

The site of the ovarian graft affected the recovery of the graft and the number of oocytes retrieved. The kidney capsule graft site has a rich blood supply that promotes the revascularization process, which is vital for recovery of the graft and the support of follicular growth. The main cause of graft and follicle loss in the process of ovarian transplantation is believed to be ischemia after transplantation (32). In mice, ovarian transplantation accounts for approximately 42% of the loss of follicle population (33).

Duration of the ischemia before revascularization and survival of transplanted ovarian tissue depend on several factors, such as the size of the tissue, the graft site, and the
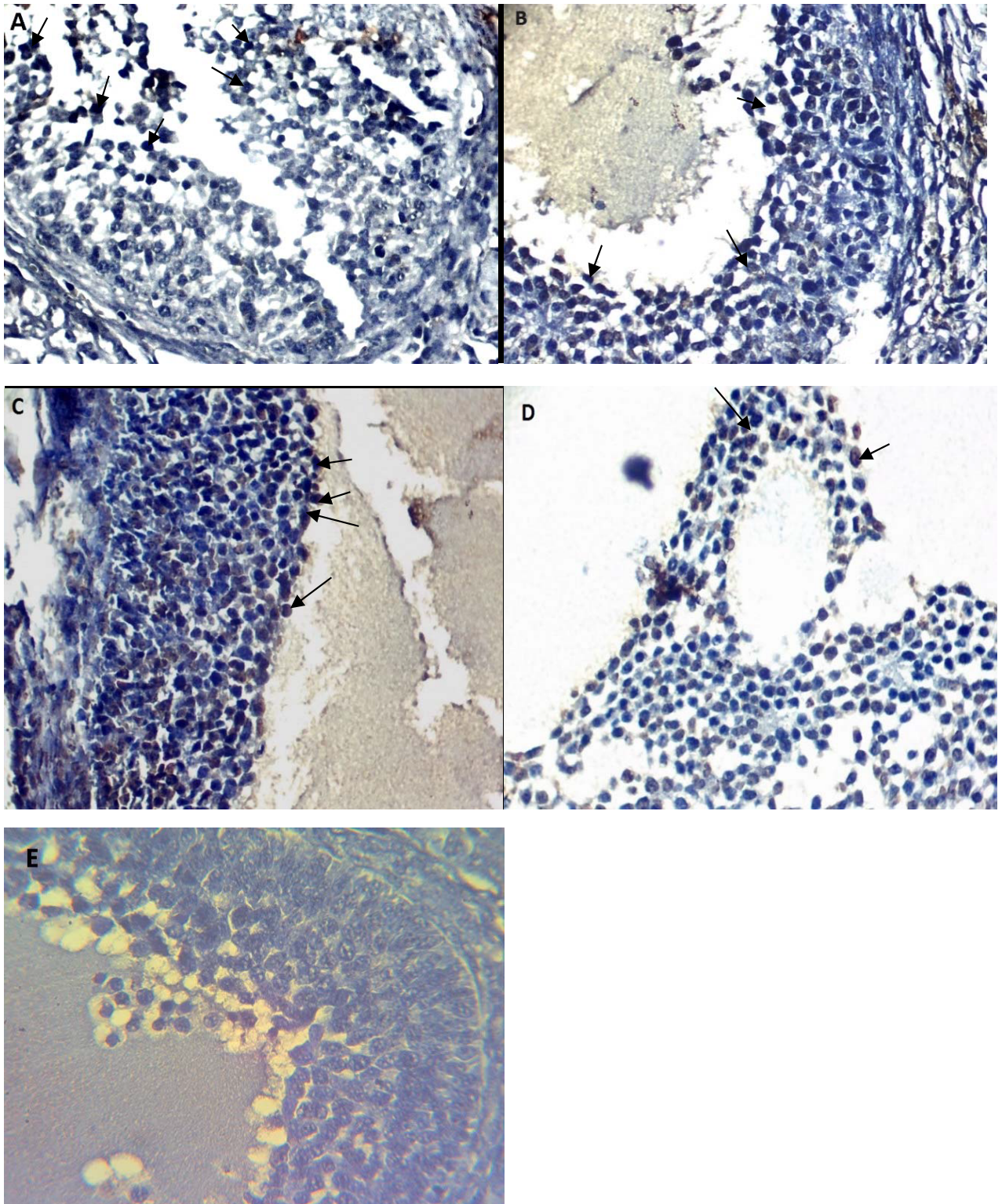
Figure 3. Histological assessment of apoptosis by TUNEL staining in ovarian tissue 28 days after transplantation: A) ovarian graft in granulation tissue, saline-treated; B) ovarian graft in granulation tissue, NAC-treated; C) ovarian graft in back muscle, saline-treated; D) ovarian graft in back muscle, NAC-treated; E) ovarian tissue, negative control TUNEL staining. Arrows indicate TUNEL-positive granulosa cells (dark brown nucleus). Original magnification: 400×.
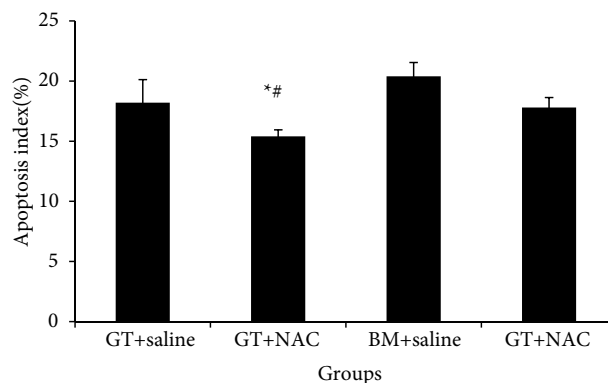
Figure 4. Comparison of apoptosis rate of granulosa cells in different groups of rats 28 days after ovarian transplantation. Values are means ± SD.
*: P < 0.05 vs. GT+saline.
#: P < 0.05 vs. BM+NAC.

presence of angiogenic factors (32). Revascularization of ovaries in rodents occurred within 24–48 h after grafting (9).

Israely et al. showed that subcutaneous grafts have no adequate blood supply and varied vascular integrity (34). Callejo el al. reported that revascularization of transplanted ovarian tissue into the ovarian bursa is better than revascularization in other heterotopic ovarian graft sites (35).

The BM site supports murine ovarian graft survival and has shown its unique capacity to preserve the follicles, which is likely due to rapid vessel formation and better support of blood in the grafted site (11). Soliemani et al. compared the kidney site to the BM site and showed that the BM site provides better support for neovascularization with a specific blood stream generation in the graft site (11). Their results were confirmed by findings suggesting that there are significantly higher numbers of blood vessels in the BM than in the kidney site. Our histological evaluation showed that the number of blood vessels is significantly higher in the GT than in the BM site, and this is probably due to the rich content of angiogenic factors such as vascular endothelial growth factor. Apart from the benefits in terms of better follicular survival and rich neovascularization around the site of grafting in the GT side, graft accessibility is an additional benefit of this site compared to the other sites of grafts.

According to a previous study conducted by Dissen et al., neovascularization after ovarian transplantation occurred within 42 h in rats and during neovascularization the ovarian tissue grafts are susceptible to ischemia and hypoxia (9). Therefore, reducing the ischemia time after transplantation is vital to the successful restoration of ovarian function (15). It could be deduced that GT is a

good site for ovarian transplantation because it provides a supportive neovascularization environment in the graft site.

Our results also demonstrated that the apoptosis rate of ovarian granulosa cells in the wound-healing GT site was lower than in the BM site, and NAC attenuated it in both NAC-treated groups. The beneficial effects of NAC on the reduction of apoptosis rates have been shown by other investigators, as well (25).

Prevention of apoptosis by NAC may be achieved through inhibiting the production of preinflammatory cytokines such as tumor necrosis factor alpha and interleukin-1 beta, inducible nitric oxide synthase, and nitric oxide production, which have important functions in IR injury (36).

Although it was found in this study that NAC had an antiapoptotic effect, it could not reduce the relevance of IR injury after ovarian transplantation in graft sites, such as recovery of ovarian follicles or levels of progesterone and estradiol hormones. These results are inconsistent with those of the previous studies. This could be because the NAC administration method in rats, in terms of duration and given doses, was not adequate for ovary endocrine function recovery. Moreover, severe injuries to the ovary in the first days of ischemia before neovascularization should also be considered. In addition, the inadequate delivery of gonadotropin to grafts by the vascular remodeling that happens in the ovarian graft and the low number of follicles in the ovarian grafts might be the other reasons (33). However, more studies with modified doses, treatment duration, and NAC administration method may improve the obtained results.

Collectively, data from this study indicate that developing follicles in ovarian grafts by NAC, irrespective

of the graft location, do not correct the disturbance of hormonal feedback between the pituitary and ovary induced by grafting.

In conclusion, our study showed that the wound-healing GT is an optimal site and is better in terms of the richness of neovascularization and reduction of apoptosis than the BM site for ovarian tissue grafting in the rat model. Moreover, we confirmed that despite the antiapoptotic effect of NAC, it could not restore autografted ovarian functions.

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