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Melatonin improves the structure and function of autografted mice ovaries through reducing inflammation: A stereological and biochemical analysis

Maryam Noori Hassanvand, Malek Soleimani Mehranjani*, Elham Shojafar

Department of Biology, Faculty of Science, Arak University, Arak 381-5688138, Iran

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ABSTRACT

Melatonin has anti-oxidant, anti-inflammatory and anti-apoptotic properties. We aimed to investigate the effect of melatonin on the structure and function of mice ovaries following autograft transplantation.

NMRI mice were divided into: control, autografted + saline, autografted + melatonin (20 mg/kg/day i.p. injection for 1 day before until 7 days after transplantation). 28 days post transplantation, ovary compartments were studied stereologically. Follicle apoptosis and the level of progesterone and estradiol were also measured. The inflammation, serum MDA concentration and total antioxidant capacity were also assessed on day 7 post transplantation.

The total volume of the ovary, cortex and medulla ($P < 0.05$) and the number of different types of follicles ($P < 0.001$), the concentration of IL-10, progesterone and estradiol ($P < 0.001$) and TAC ($P < 0.01$) significantly decreased in the autografted + saline group compared to the control. The levels of IL-6 ($P < 0.01$), TNF-α, MDA and the apoptotic rate ($P < 0.001$) increased significantly in the autografted + saline group compared to the control, while the total volume of the ovary, cortex and medulla ($P < 0.05$) and the number of different types of follicles ($P < 0.001$), the concentration of IL-10, progesterone and estradiol ($P < 0.001$) and TAC ($P < 0.01$) significantly increased in the autografted + melatonin group compared to the autografted + saline group. The levels of IL-6 ($P < 0.01$), TNF-α, MDA and the apoptotic rate ($P < 0.001$) decreased significantly in the autografted + melatonine group compared to the autografted + saline group. In the autografted + melatonin group, the localization of CD31-positive cells in the theca layer was similar to the control group.

Melatonin can improve the structure and function of the grafted ovary.

1. Introduction

Ovary tissue transplantation is a promising way to preserve fertility in cancerous patients who undergo chemo/radiotherapy [1,2] However, this method faces problems such as ischemia/reperfusion and its consequences. Ischemia/reperfusion is one of the most common injuries to the ovary tissue [3] due to the reduction of blood flow [4] which increases the production of free oxygen radicals and disturbs the regulation of intracellular calcium, the function of mitochondria [5] and the endothelial system [6] which eventually leads to oxidative stress, exacerbation of inflammation and cell death [5]. All this together, cause reduction in the number of follicles and disorder in the endocrine function of the transplanted ovary [7–9]. Many scientists have tried to reduce the injuries to the transplanted ovary through inhibiting oxidative stress and inflammation [9–11]. Melatonin (*N*-acetyl-5-methoxytryptamine) is a molecule with potent antioxidant [12,13], anti-inflammatory [14–16] and anti-apoptotic [14,17] properties which can reduce the production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and the lipid peroxidation rate [16,18] and can also block the release of inflammatory agents [15]. Previous studies have shown that melatonin plays a positive role in reducing oxidative stress and apoptosis [19,20] following ovarian transplantation ultimately preserving the follicular storage [20,21] and improving the function of the transplanted ovary tissue [20]. Despite the occurrence of inflammation in the transplantated ovary [22,23], and the ability of melatonin to inhibit inflammation [15,16] none of these researches studied the inflammatory indexes in the case of ovary transplantation following melatonin treatments. Therefore, in the present study, for the first time, we evaluated the inflammatory factors and the structure of the autografted ovaries in addition to apoptosis and oxidative stress investigation, after 7 days of melatonin treatment, using stereological techniques.

* Corresponding author.
E-mail address: m-Soleimani@araku.ac.ir (M. Soleimani Mehranjani).

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2. Materials and methods

2.1. Animals and treatment

36 female Naval Medical Research Institute (NMRI) (4–5 weeks old) from the Pasteur Institute of Iran (Tehran, Iran) were purchased and kept in the animal house of Arak University under standard conditions (22 ± 2 °C and 12 h of light/dark cycles (light–dark cycles for 12 h) and free access to enough water and food). All the experimental procedures were carried out according to the ethics committee of Arak medical science University (IR.ARAKU.REC.1397.111). In this experiment NMRI mice were used since previous studies have reported that NMRI mice are suitable for ovary tissue transplantation [8,9,24]; mice were divided into three groups (n = 12): control, autografted + saline (20 mg/kg/day intraperitoneal injection), autografted + melatonin (Melatonin, Sigma, USA) (20 mg/kg/day intraperitoneal injection). Treatment was performed 1 day before until 7 days after transplantation at 6:00 p.m.

2.2. Ovarian autotransplantation

Mice were anesthetized with intraperitoneal injections of anesthetic solution including ketamine (100 mg/kg, ketamine 10%, Alfasan, Woerden, the Netherlands) and xylazine (10 mg/kg, xylazine 2%; Alfasan, Woerden, the Netherlands). Under aspectic conditions, the dorsal areas of each mouse were shaved and bilateral incisions on each side of the spinal column in the dorsal body wall was made inorder to excise the ovaries. Then a 0.5 cm surgical incision was made along the right and left gluteus superficialis muscle fibers of the posterior limb and excised ovaries were autotransplanted into the gluteal muscle. Finally incisions were closed by suture.

2.3. Vaginal cytology

To detect the resumption of cyclic ovarian activity, daily vaginal aspirations using 50 μL saline were carried out starting on day 7 after ovary autotransplantation until the observation of the first cornified epithelial cells and the ovarian cycle (pro-estrous, estrous, metestrous, diestrous) was determined immediately by the light microscope [9].

2.4. Stereological studies

28 days after transplantation, mice were anesthetized with an intraperitoneal injection of ketamine-xylazine mixture and the left and right grafted ovaries were removed and fixed in Bouin's fixative for 24 h and dehydrated in ascending concentrations of ethanol (70–100%) then the samples were blocked in paraffin. To prepare the IUR (isotropic uniform random) sections the isector method was used and 5 and 20-μm thick sections were prepared serially and stained with haematoxylin and eosin (Merck, Darmstadt, Germany) [8,9].

2.5. Estimation of the total volume of ovary, cortex volume and medulla volume

To estimate the total volume of the ovary and the volume of the cortex and medulla the Cavalieri method was used, 12 section per ovary from 5-μm thick sections were randomly selected and using a special probe and a microscope (Olympus, BX41TE) with 4× magnification, the number of points superimposed on the images were counted and the total volume of the ovary was calculated with the following formula:

$$V_{\text{total ovary}} = \sum_{i=1}^{n} p \times a(p) \times t$$

$\sum_{i=1}^{n} p$ is the total number of the counted points superimposed on the images of tissue sections, a(p) is the area associated with the probe points and t is the thickness of the selected sections.

The volume density of the cortex and medulla was calculated according to the following formula:

$$Vv_{cortex/medulla} = \frac{\sum_{i=1}^{n} p_{cortex/medulla}}{\sum_{i=1}^{n} p_{total}}$$

where $\sum_{i=1}^{n} p_{total}$ is the total number of the superimposed points on all selected sections and $\sum_{i=1}^{n} p_{cortex/medulla}$ is the total number of probe points superimposed on the cortex or the medulla. The volume of the cortex or the medulla was estimated by multiplying the volume density of each compartment by the total volume of the ovary [8,9].

2.6. Estimation of the number of follicles

The number of different types of follicles were estimated with the assistance of an optical disector, 12 sections from 20-μm thick sections were randomly selected and studied under a light microscope (Olympus, BX41TE), with 100× magnification. During the movement of the microscopic stage in equal distances, microscopic fields were selected and to measure the movement of the microscopic stage along the z-axis, a microcator (ND221B; Heidenhain, Traunreut, Germany) connected to a computer and a microscope (OlympusBX51, Tokyo, Japan) was used. A specific counting frame was located on the selected perspective [8] and to avoid any possible artifacts, 5 μm from the top and the bottom of the sections were ignored. The follicles with a clear oocyte nuclei inside the counting frame which were not in contact with the exclusion lines were counted and classified into four categories: primordial, primary, preantral and antral [25]. The number of follicles were obtained using the following formula:

$$NV = \frac{\sum_{i=1}^{n} Q}{h \times \sum_{n=1}^{n} P \times {}^{a}/_{f}}$$

where NV is the number of the follicles per unit volume of the ovary (density number), ΣQ is the number of the counted follicles in the disector height. (h), ΣP is the total number of the frames counted and a/f is the area of each counted frame in the tissue sections. The total number of follicles was then calculated by multiplying the density number by the total volume of the ovary [8,9].

2.7. Estimation of the volume of oocyte and its nuclei

The nucleator method was applied to calculate the oocyte volume and its nuclei, 12 sections from 20-μm thick sections were randomly selected and studied under a light microscope (Olympus, BX41TE) with 100× magnification using Motic software. The distance from the centre of the nucleolus to the oocyte membrane and the distance from the centre of the nucleolus to the nuclear membrane were measured respectively [25,26]. The volume of the oocytes and their nuclei was calculated using the following formula [9]:

$$V_n = \frac{4}{3} \bullet L_n^3$$

where $\overline{l_n^3}$ is the distance from the centre of the nucleolus to the oocyte membrane or to the nuclear membrane.

2.8. Calculation of the zona pellucida thickness

To calculate the zona pellucida thickness, 12 sections from 20-μm thick sections were randomly chosen and studied using a microscope (Olympus, BX41TE) with 100× magnification. To measure the

thickness of the zona pellucida a specific probe with three parallel lines was randomly superimposed on the selected follicles and from the site of intersection of the grid lines with the inner membrane, a perpendicular line to tangent with the outer membrane of the zona pellucida was considered and its length was measured with the Motic software (for each ovary an average of 100 to 200 encounters were measured) these measurements have been shown as OI (Orthogonal Intercept) and the Zona pellucida thickness was calculated by [9]:

Harmonic mean layer thickness

$= 8/3\pi \times$ harmonic mean of orthogonal intercepts

where harmonic mean is the number of measurements divided by the sum of the reciprocal of orthogonal intercepts lengths (oi) = no. measurements $/\left[\frac{1}{oi_1} + \frac{1}{oi_2} + \frac{1}{oi_3} + \frac{1}{oi_4} + \ldots\right]$.

2.9. Apoptosis assay

DNA fragmentation was measured by the TUNEL assay (terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick-end labeling) based on the kit protocol (In Situ Cell Detection Kit, Roche, Germany). Briefly 5-μm thick tissue sections were deparaffinized, rehydrated and incubated with hydrogen peroxide (H_2O_2 Merck, 3% Germany) for 10 min, followed by incubation in 20 μg/ml proteinase K for 30 min at 37 °C. Then, sections were incubated with recombinant TdT reaction mixture in a humidified dark chamber at 37 °C for 60 min, after which sections were washed with phosphate-buffered saline (PBS) and then Converter-POD was added and sections were incubated for 30 min at 37 °C. On some slides, the reaction mixture was added without the presence of TdT enzyme as the negative control. The sections were then stained with DAB (Roche, Germany) for 10 min, and after counterstaining with haematoxylin, they were washed with distilled water. Ultimately, the sections were mounted with entellan (Merck, Germany) and were studied under the light microscopy (Olympus, Japan, BX51). Follicles were considered apoptotic if > 10% of their follicular cells were TUNEL-positive [8,9].

2.10. Measurement of inflammatory factors

7 days after transplantation, blood samples were collected and centrifuged (13,000 rpm for 5 min). Then the serum level of TNF-α and IL-6 were measured using TNF-α and IL-6 ELISA kits (cat no.: BMS607/3, eBioscinence, USA) with the sensitivity of 2.97 pg/ml and 7.9 pg/ml respectively. The level of IL-10 was also estimated using an ELISA Kit (cat number: M1000B, Minneapolis, MN, R&D Systems) with the sensitivity of 5.22 pg/ml.

2.11. MDA assay

Using the method described by Buge et al. [27] the Serum concentration of MDA was measured. A reaction mixture containing trichloroacetic acid (Sigma-Aldrich), hydrochloric acid and thiobarbituric acid (Sigma-Aldrich) was added to serum samples and incubated at 100 °C for 10 min. After cooling, samples were centrifuged at 3000 *g* for 10 min at room temperature, and using a spectrophotometer (T80 + ; PG Instruments) the absorbance of the supernatant at 535 nm was measured. MDA concentrations (μM) were determined using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

2.12. Total antioxidant capacity measurement

To measure the total antioxidant capacity, the ferric ion reducing antioxidant power (FRAP) method was used according to the Benzie et al. [28] method. 50 μl of serum sample was incubated with the reaction mixture containing 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ; Sigma-Aldrich), $FeCl_3$ and sodium acetate buffer (Sigma-Aldrich) for 10 min at 37 °C and its absorbance was read at 593 nm using a spectrophotometer (T80 + ; PG Instruments) and the Serum levels of TAC were calculated using an absorbance standard curve. This method depends on the ability of serum in the regeneration of Fe^{3+} ions into Fe^{2+} in the presence of the TPTZ substance [29].

2.13. Hormonal assay

28 days after transplantation, blood samples were collected and centrifuged (13,000 rpm for 5 min). After serum separation, the concentrations of progesterone and estradiol were measured using the progesterone kit (DRG Progesterone ELISA kit, EIA-1561; DRG Instruments GmbH, Marburg, Germany), with a sensitivity of 0.045 ng/ml and a range of 0–40 ng/ml and the estradiol kit (DRG Estradiol ELISA kit, EIA-2693; DRG Instruments GmbH, Marburg, Germany), with a sensitivity of 9.714 pg/ml and the measurement range of 9.7–2000 pg/ml according to the manufacturer's instructions, respectively.

2.14. CD31 assay

28 days post ovary transplantation, immunohistochemical analyse was performed to investigate vascularization in the transplanted tissue. 5 μm thick sections were deparaffinized using three treatment steps with xylene, then rehydrated in alcohol series. Rehydrated slides were incubated with 20 μg/ml proteinase K solution for 30 min at 37 °C, for antigen retrieval. After incubation with 0.3% H_2O_2 solution for 10 min, slides were rinsed in PBS-Tween 20 solution (0.05%) and then were placed in Triton X-100 (0.05%) for 10 min. Incubation with Bovine serum albumin 10% (wt/vol) (Gibco) as a buffer blocker was performed for 1 h at 37 °C. CD31 primary antibody (ab56299, Abcam, USA) was diluted 1:500 in PBS and incubated overnight. After washing with PBS-Tween20 (0.05%) solution, slides were incubated in the secondary antibody (ab6843, Abcam, USA) diluted to 1:100 in PBS for 1 h in dark. Finally after three times of washing (5 min each), slides were detected using fluorescence microscopy (BX51 TRF; Olympus).

2.15. Statistical analysis

Data were analyzed using one-way ANOVA and Tukey's test with SPSS software and the means were considered significantly different at $P < 0.05$.

3. Results

3.1. The total volume of the ovary, the volume of the cortex and medulla

The mean total volume of the ovary, the volume of the cortex and medulla decreased significantly in the autografted + saline group compared to the control group ($P < 0.001$) While, a significant increase in the mentioned parameters was found in the autografted + melatonin group compared to the autografted + saline group ($P < 0.05$) (Table 1).

Table 1
Comparison of the mean total volume of the ovary, volume of the cortex and medulla (mm^3) in different groups of mice, 28 days after heterotopic graft in the gluteus superficialis muscle and treatment with melatonin (20 mg/kg/day).

Groups	Ovary volume	Cortex volume	Medulla volume
Control	1.95 ± 0.63[a]	1.48 ± 0.21[a]	0.47 ± 0.13[a]
Autografted + saline	0.64 ± 0.11[b]	0.52 ± 0.11[b]	0.12 ± 0.01[b]
Autografted + melatonin	1.22 ± 0.16[c]	0.90 ± 0.06[c]	0.32 ± 0.14[c]

Data are means ± SD. The means with different code letter are considered significantly different (one-way ANOVA and Tukey's test, $P < 0.05$).

Table 2
Comparison of the mean number of follicles in different groups of mice, 28 days after heterotopic graft in the gluteus superficialis muscle and treatment with melatonin (20 mg/kg/day).

Groups	Primordial follicles	Primary follicles	Pre-antral follicles	Antral follicles	Total
Control	2031.60 ± 370.71[a]	580.76 ± 41.09[a]	285.74 ± 14.95[a]	116.28 ± 11.71[a]	3014.38 ± 360.83[a]
Autografted + saline	1134.60 ± 74.23[b]	352.87 ± 78.30[b]	128.52 ± 13.83[b]	55.53 ± 6.57[b]	1671.52 ± 57.52[b]
Autografted + melatonin	1635.39 ± 249.28[a]	558.49 ± 55.21[a]	221.00 ± 20.91[c]	99.19 ± 10.49[a]	2514.07 ± 250.36[a]

Data are means ± SD. The means with different code letter are considered significantly different (one-way ANOVA and Tukey's test, $P < 0.05$).

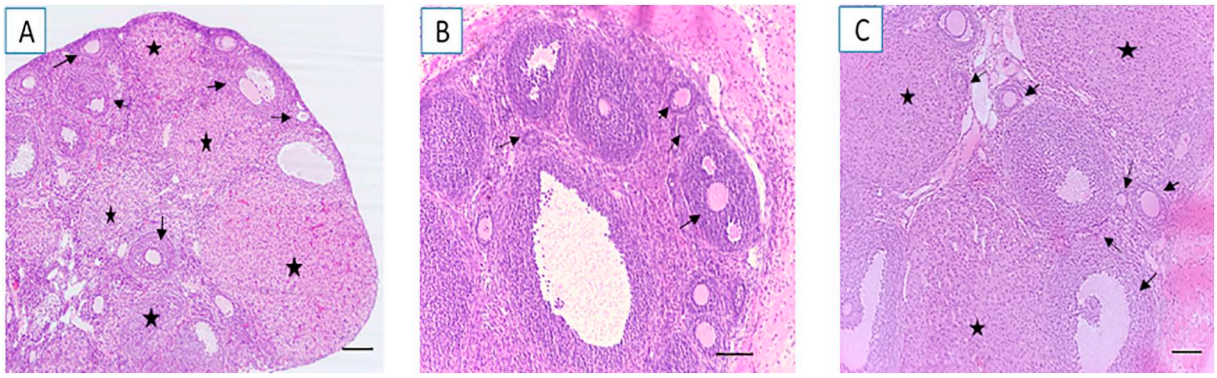

Fig. 1. Microscopic images of ovarian sections in different groups of mice, 28 days after ovary autografting and treatment with melatonin. A) Control group:- indicating the presence of different types of follicles; B) autografted + saline group: fewer follicles(arrows) were observed. C) Autografted + melatonin group: a considerable number of follicles (arrows) in different stage of development were observed. (corpus luteum, *; scale bar = 100 μm).

3.2. The number of different follicles types

A significant decrease in the mean number of different types of follicles was observed in the autografted + saline group compared to the control group ($P < 0.001$), but it significantly increased in the autografted + melatonin group compared to the autografted + saline group ($P < 0.001$). Moreover, the mean number of the primordial, primary and the antral follicles in the autografted + melatonin group reached to the control level ($P > 0.05$) (Table 2, Fig. 1). 83.33% of the ovaries in the autografted + melatonin group and 33.33% in the autografted + saline group were observed to contain the corpus luteum.

3.3. The volume of the oocytes and its nuclei and zona pellucida thickness

The mean volumes of the oocytes and their nuclei in the primordial, primary, pre-antral and antral follicles and also the mean thickness of the zona pellucida of the antral follicles didn't show a significant difference in any of the groups ($p > 0.05$) (Tables 3 and 4).

3.4. Apoptosis assay

The mean percentage of the apoptotic follicles significantly increased in the autografted + saline group compared to the control group ($P < 0.001$), while it showed a significant reduction to the control level in the autografted + melatonin group (Figs. 2 and 3).

3.5. Level of inflammatory factors

A significant increase in the levels of TNF-α and IL-6 and a significant decrease in the level of IL-10 was observed in the autografted groups compared to the control group ($P < 0.001$), meanwhile a significant decrease in the levels of TNF-α ($P < 0.001$) and IL-6 ($P < 0.01$) and a significant increase in the IL-10 level ($P < 0.05$) was observed in the autografted + melatonin group compared to the autografted + saline group (Table 5).

3.6. Malondialdehyde and total antioxidant capacity analysis

The mean concentration of malondialdehyde significantly increased in the autografted + saline group compared to the control group ($P < 0.001$), while this parameter significantly decreased to the control level in the autografted + melatonin group ($P < 0.001$). On the other hand, the mean total antioxidant capacity was significantly lower in the autografted + saline group compared to the control group ($P < 0.01$), while a significant increase to the control level in this parameter was observed in the autografted + melatonin group ($P < 0.01$) (Table 6).

3.7. Hormones assay and estrous cycle

The mean concentrations of progesterone and estradiol significantly decreased in the autografted + saline group compared to the control

Table 3
Comparison of the mean oocyte volume (μm^3) in different types of follicles in different groups of mice, 28 days after the heterotopic graft in the gluteus superficialis muscle and treatment with melatonin (20 mg/kg/day).

Groups	Oocyte volume (μm^3)			
	Primordial follicles	Primary follicles	Pre-antral follicles	Antral follicles
Control	1651.95 ± 436.71[a]	3460.73 ± 371.51[a]	79,947.50 ± 4357.51[a]	141,084.96 ± 4340.59[a]
Autografted + saline	1608.60 ± 313.53[a]	3301.22 ± 240.99[a]	76,427.76 ± 1386.35[a]	135,655.16 ± 6694.90[a]
Autografted + melatonin	1644.11 ± 758.43[a]	3384.97 ± 358.35[a]	78,423.39 ± 2587.93[a]	148,891.09 ± 3765.46[b]

Data are means ± SD. The means with different code letter are considered significantly different (one-way ANOVA and Tukey's test, $P < 0.05$).

Table 4
Comparison of the mean volume of the oocyte nucleus (μm^3) in different types of follicles and the mean zona pellucida thickness (μm) of the antral follicles in different groups of mice, 28 days after heterotopic graft in the gluteus superficialis muscle and treatment with melatonin (20 mg/kg/day).

Groups	Oocyte nucleus volume (μm^3)				Zona pellucida thickness (μm)
	Primordial follicles	Primary follicles	Pre-antral follicles	Antral follicles	Antral follicles
Control	562.08 ± 36.64[a]	743.69 ± 77.30[a]	2742.73 ± 161.81[a]	6643.36 ± 362.67[a]	17.41 ± 0.25[a]
Autografted + saline	531.83 ± 55.34[a]	706.10 ± 34.22[a]	2603.56 ± 72.24[a]	6131.19 ± 297.97[a]	17.06 ± 0.05[a]
Autografted + melatonin	572.11 ± 25.14[a]	758.09 ± 33.91[a]	2656.71 ± 119.71[a]	6261.20 ± 287.56[a]	17.25 ± 0.31[a]

Data are means ± SD. (one-way ANOVA and Tukey's test, $P < 0.05$). The means with different code letter are considered significantly different.

($P < 0.001$), while these parameters significantly increased to the control level in the autografted + melatonin group ($P < 0.001$).

Restoration of the estrous cycle was delayed in the autografted + saline group compared to the control group ($P < 0.001$), however the estrous cycle restored more rapid in the autografted + melatonin group compared to the autografted + saline group ($P < 0.001$). (Table 7).

3.8. CD31 assay

28 days post ovary autografting, the localization of CD31-positive cells in the theca layer of the preantral and antral follicles were observed in the cortex of the control group. In the autografted + saline group, the CD31-positive cells were rarely observed in the theca layer of the antral and preantral follicles while in the melatonin-treated group, the CD31-positive cells were detected in the theca layer of most preantral and antral follicles in cortex (Fig. 4).

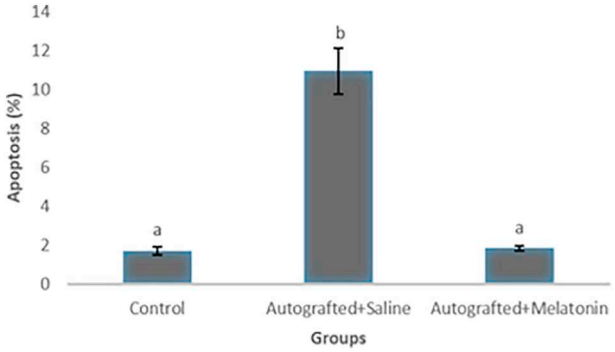

Fig. 3. The mean percentage of apoptotic follicles in different groups of mice, 28 days after the ovary autografting and treatment with melatonin. Values presented as mean ± SD and the different code letters are considered significantly different. The graphs are Mean ± SD (One way ANOVA, Tukey's test $p < 0.05$).

4. Discussion

One of the most important factors in improving the quality of the transplanted ovary tissue is the rapid establishment of the vascular connection, which is essential for the survival of the follicles [3]. Previous studies have shown that selecting a suitable graft site could affect the angiogenesis rate [30] and in this study, the site of the back muscle was selected due to its high rate of angiogenesis which has also been used as a graft site in other studies [8,9].

Previous studies have shown that revascularization occurs between the transplanted tissue and the host 7 days after ovary transplantation [31,32] and during this time, the occurrence of oxidative stress [33] and inflammation [22,23], can damage the transplantated ovary. This is why we chose to carry out melatonin treatment during the first week after ovary transplantation, in order to reduce ischemia/reperfusion injuries. The dose of 20 mg/kg/day of melatonin was selected based on the results of other studies [20,34,35].

Our results revealed a significant increase in the concentrations of pro-inflammatory factors such as TNF-α and IL-6 and a significant decrease in the level of anti-inflammatory factor IL-10 in both autografted groups compared to the control group which is similar to the results achieved by Shojafar et al. [10,11]. Treatment with melatonin reduced the level of TNF-α and IL-6 and increased the level of IL-10 compared to the autografted + saline group. Melatonin can inhibit c-Jun NH2-terminal kinase (JNK), nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear transcription factor kB (NF-kB) activity which reduces the production of pro-inflammatory factors [14,36–39]. Moreover, as a potent antioxidant, melatonin can reduce the production of free radicals which leads to a reduction in the production of pre-inflammatory cytokines [40]. Bruck et al., also indicated that melatonin protects against thioacetamide induced liver damage in rats through reducing inflammation [39]. In addition, Cuzzocrea et al., confirmed on the anti-inflammatory effect of melatonin through the inhibition of pro-

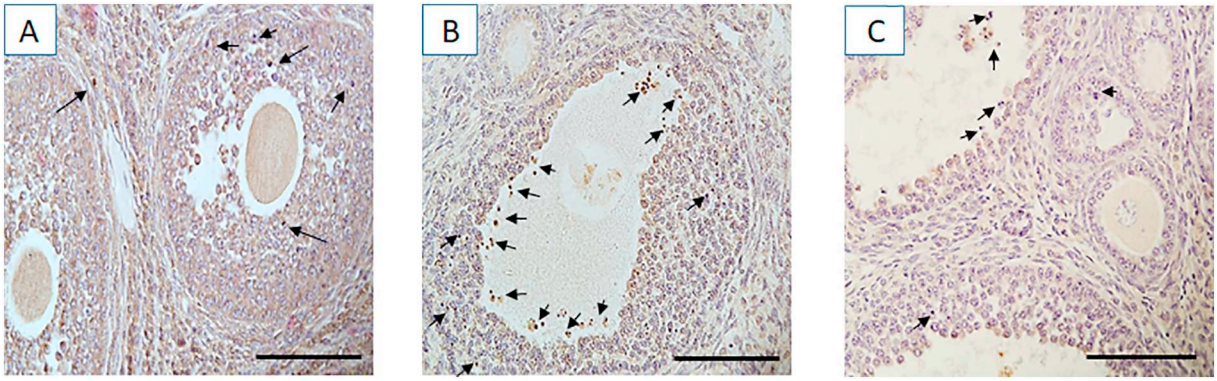

Fig. 2. Evaluation of apoptosis by TUNEL staining in different groups of mice, 28 days after ovary autografting and treatment with melatonin. A) Control group; B) autografted + saline group: a considerable number of apoptotic cells were detected; C) autografted + melatonin group: fewer apoptotic cells were observed compared to the autografted + saline group. Dark brown nucleus indicate TUNEL-positive cells (arrows). Scale bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5
Comparison of the mean level of inflammatory factors in different groups of mice, 7 days after heterotopic graft in the gluteus superficialis muscle and treatment with melatonin (20 mg/kg/day).

Groups	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)
Control	57.75 ± 13.86[a]	201.98 ± 43.88[a]	147.25 ± 29.16[a]
Autografted + saline	783.26 ± 54.81[b]	1262.00 ± 322.61[b]	8.75 ± 4.77[b]
Autografted + melatonin	414.67 ± 2.94[c]	827.04 ± 109.19[c]	32.00 ± 4.94[c]

Data are means ± SD. The means with different code letter are considered significantly different (one-way ANOVA and Tukey's test, $P < 0.05$).

Table 6
Comparison of the mean serum concentration of malondialdehyde and the mean total antioxidant capacity in different groups of mice, 7 and 28 days after heterotopic graft in the gluteus superficialis muscle and treatment with melatonin (20 mg/kg/day).

Groups	MDA(7 days after transplantation)	MDA (28 days after transplantation) μM/L	TAC (7 days after transplantation) μM/g
Control	3.55 ± 0.22[a]	3.09 ± 0.59[a]	0.76 ± 0.04[a]
Autografted + saline	7.60 ± 0.59[b]	4.60 ± 0.59[b]	0.57 ± 0.06[b]
Autografted + melatonin	3.49 ± 0.23[a]	2.45 ± 0.16[a]	0.74 ± 0.12[a]

Data are means ± SD.The means with different superscript letters indicate statistically significant differences (one-way ANOVA and Tukey's test, $P < 0.05$).

Table 7
Comparison of the mean levels of hormones and the mean starting day of the estrous cycle in different groups of mice, 28 days after heterotopic graft in the gluteus superficialis muscle and treatment with melatonin (20 mg/kg/day).

Groups	Progesterone (ng/ml)	Estradiol (pg/ml)	Starting day of estrous cycle
Control	1.40 ± 0.36[a]	44.83 ± 7.60[a]	8 ± 0.89[a]
Autografted + saline	0.32 ± 0.12[b]	26.82 ± 3.45[b]	11 ± 1.41[b]
Autografted + melatonin	1.74 ± 0.51[a]	46.11 ± 5.78[a]	8.16 ± 0.98[a]

Data are means ± SD. The means with different code letter are considered significantly different (one-way ANOVA and Tukey's test, $P < 0.05$).

inflammatory factors releasion [41]. Mauriz et al., also demonstrated the elevation of the anti-inflammatory genes expresion following melatonin treatment [42].

In this study, the percentage of apoptotic follicles increased significantly in the autografted + saline group compared to the control group, which is in accordance with the previous studies [8–10]. Treatment with melatonin can reduce the apoptosis rate of the follicles which has been also reported by other studies [20,21]. Melatonin not only prevents apoptosis through inhibiting the ROS production and reducing the activation of caspase 9 and 3 [43] but also by decreasing the concentration of intracellular calcium [17] which is a key element for the activity of the protease and endonuclease enzymes [44] and therfore protecting the ovarian cells from apoptosis.

Our results also revealed an increase in the MDA concentration and a decrease in the total antioxidant capacity in the autografted + saline group compared to the control group, which has been also indicated by the previous studies [7,9,10]. During the ischemic/reperfusion phase, the cell membrane lipids, are affected by oxidative stress resulting in an increase in the production of malondialdehyde [45], while in the present study, treatment with melatonin caused a reduction in the level of MDA and an increase in the total antioxidant capacity compared to the autografted + saline group. These results have been also confirmed by other researches [12,19]. Melatonin improves the activity of antioxidant enzymes and reduces the level of free radicals and therefore can inhibit lipid peroxidation and protect cellular lipids, proteins and DNA against free radicals injuries [18].

Our results showed that in the autografted + saline group, CD31 expression were detected mainly in the medulla and were rarely found in the theca layer of some pre-antral and antral follicles, which is in accordance with the previous studies [10,11] while in the autografted + melatonin group, the CD31 positive cells in the theca layer of most preantral and antral follicles were detected. In this study, we concluded that during the time of ischemia after ovarian transplantation, the rate of inflammation, especially the TNF-α level, increases, and as previous studies have reported, this increase in the TNF-α level, can suppress angiogenesis [46]. In this case, treatment with melatonin, can reduce the level of TNF-α, thereby it can enhance angiogenesis. It has also been shown that melatonin stimulates the production of IGF-I (as an angiogenesis agent) in cultured humans granulosa cells [47],

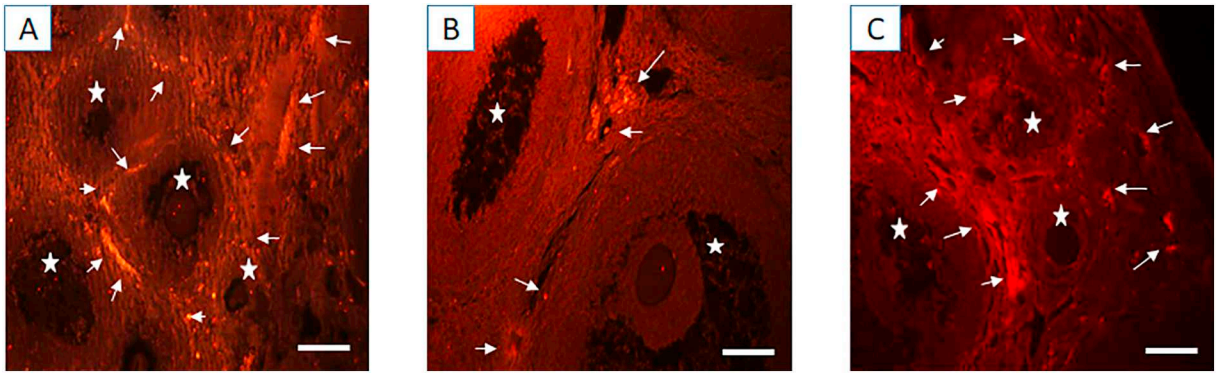

Fig. 4. Immunohistochemical staining of CD31 in different groups of mice, 28 days after ovary autografting and treatment with melatonin. A) Control group: CD31-positive cells were detected in the theca layer of preantral and antral follicles; B) autografted + saline group: localization of CD31-positive cells was scarce in the theca layer of preantral and antral follicles and they were found mainly in the medulla; C) autografted + melatonin group: the CD31 positive cells were roughly localized in the theca layer of preantral and antral follicles compared to the autografted + saline group. Arrows indicate CD31-positive cells and stars show preantral and antral follicles (scale bar = 100 μm).

which confirms the effect of melatonin on angiogenesis.

In the present study, the total volume of the ovary, the volume of the cortex and medulla, and also the number of different types of follicles were significantly lower in the autografted + saline group compared to the control group. This reduction in the mentioned parameters have also been reported by other studies [8–10]. In the early stages after ovary transplantation the absence of blood flow, damages the granulosa and oocyte cells, particularly in the growing follicles, leading to follicle apoptosis [20] and ultimately reduction in the number of follicles, the total volume of the ovary and volume of the cortex. Moreover, the stromal cells which play an important role in normal ovary function, follicular survival and follicular growth, are also destructed in ischemic conditions [3,48] which also cause a decrease in the total volume of the ovary and the volume of the medulla. Meanwhile in the present study, melatonin treatment caused an increase in the above parameters in the autografted + melatonin group which could be due to the ability of melatonin in inhibiting oxidative stress, inflammation and apoptosis.

In the present study, no significant difference was observed in the volume of the oocytes and their nuclei in different types of follicles and also the zona pellucida thickness of the antral follicles in any of the groups.

Our data also showed that the progesterone and estradiol concentrations were significantly lower in the autografted + saline group compared to the control group, which is in accordance with previous studies [8,9]. Reduction in the number of corpus lutea following ovarian transplantation [8–11], and the reduction in the number the growing follicles following ischemia-reperfusion [20] is probably the underlying reason for the reduction in the level of estradiol and progesteron in the autografted + saline group.

In the present study, treatment with melatonin significantly increased the level of estradiol and progesterone. Previous investigations have shown that melatonin increases the number of corpora lutea in the grafted ovary [35], which leads to an increase in the synthesis of progesterone. Some other studies have also reported that the increasing concentration of melatonin in the follicular fluid, is an important factor in preventing follicular atresia [49] with preserves the granulosa cells and enhances the production of steroid hormones. Increasing the number of antral follicles following treatment with melatonin is probably another reason explaining the increase in the concentration of estradiol.

5. Conclusion

According to the results obtained from this study, melatonin can reduce the adverse effects of ischemia-reperfusion on the transplanted ovary tissue and improve the structure and function of the grafted ovaries through reducing oxidative stress and inflammation. Therefore, administration of melatonin in the case of ovary transplantation is recommended.

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