VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 70, No. 2, pp. 95–102

ISSN 0015–5659
www.fm.viamedica.pl

Assessment of morphological and functional changes in neonate vitrified testis grafts after host treatment with melatonin

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[Received 4 January 2011; Accepted 5 April 2011]

This study was conducted to assess the effect of melatonin on the structure of testis and spermatogenesis dynamics in neonate vitrified testis grafts. Neonate vitrified testes, candidates for transplantation heterotopically to experiment or control groups, were warmed in thawing media which had or did not have a supplement of 100 µM melatonin, respectively. Following transplantation, melatonin (20 mg/ /kg/day) or saline solution was intraperitoneally injected into the treated and the non-treated groups, respectively. The initiating spermatogenesis, spermatogonia survival, and structure of tissue in the testis graft were examined. Cell apoptosis (TUNEL assay) and proliferation (Brdu assay) in germ cells were determined.
Histological studies revealed the dynamic of the spermatogenesis process in the vitrified testis graft. However, dilation of the lumen accompanied by a disorganised epithelium in the non-treated group was higher than in the treated group. Furthermore, the proportion of apoptotic germ cells together with a reduced proportion of proliferated germ cells was higher in the non-treated group than in the treated group. Overall, the number of seminiferous tubules in the testes grafts of both groups remained steady. However, the non-treated testes grafts contained more damaged seminiferous tubules than the treated ones. The thickness of the seminiferous tubules was greater in the melatonin treated group than in the non-treated group. In fact, the thickness of germinal epithelium was significantly higher in the treated group than in the non-treated group. The study may show a positive effect from melatonin resulting in more grafts restoring puberty. Furthermore, the associated increase in the healthy number of seminiferous tubules suggests that melatonin may have a preventative ischaemia/antioxidant role and in fact may be useful to initiate the spermatogenesis process. (Folia Morphol 2011; 70, 2: 95–102)

Key words: melatonin, testis allograft, vitrification, spermatogenesis

INTRODUCTION

Previously it was reported that ovaries of host mice transplanted subcutaneously with melatonin treatment preserved endocrine functions as well as promoted the quality of the follicle in the ovarian graft [9–11]. This technique can be used to mini-

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mise the impaired function of the testis graft as well. Similar to ovarian tissue, testicular banking offers the possibility of fertility and endocrine function conservation if the testis is successfully transplanted [14, 26]. Important progress in minimising the unwanted side effects of transplantation has been achieved by constantly modifying and optimising the revascularisation of the graft. However, revascularisation more than 48 hours after grafting [12] is considered a major obstacle for tissue transplantation due to the loss of a huge part of the tissue during the initial ischaemia [22]. In addition, it was shown that freezing and thawing tissue before transplanting adds additional metabolic stress [8], which indicates that tissue loss is partly due to reactive oxygen species generated during the hypoxic condition due to some clinical disorders such as ischaemia and reperfusion [2]. Therefore, proper steps should be taken to choose a new avenue based on using antioxidants that can be applied to minimise lipid peroxidation in transplant tissue. Melatonin is produced essentially by the pineal gland and is supposed to have free radical scavenger properties [16]. The effects of melatonin on the testis organ and sex hormone synthesis have also been tested in experiments on animals [1]. Since melatonin receptors were found in the reproductive systems of different species, it seems reasonable to assume that melatonin may exert its antioxidant effects via direct interaction with the steroidogenic cells of the reproductive tissue [4]. To our knowledge, there has been no research regarding the effects of melatonin regimen to evaluate putative antioxidant activity of melatonin during transplantation. However, a changed neuroendocrine gonadal axis in males was reported in the experimental study [19]. Seethalakshmi et al. [20] showed that the weight of the testis, sperm count, and motility are declined in grafted testes. Moreover, Cameron et al. [5] reported that the tubule wall thickness, germ cell depletion, and sertoli cell vacuolisation increased in testicular grafts.

Enhanced oxidative stress and changes in antioxidant capacity play critical roles in the pathogenesis of testis transplantation [21, 23]. Although the underlying mechanisms of the alterations associated with testis transplantation are presently not well understood, hypoxia can lead to increased oxidative stress due to the enhancement of the production of several reducing glucoses (during glycolysis and the polyol pathway) [3, 15].

Ultimately, the aim of the present study was to assess the effects of melatonin on mouse testicular tissue and to determine its effects on vitrified-transplanted neonatal testes.

MATERIAL AND METHODS

Drugs

Melatonin (Helsinn Advanced Synthesis SA, Biasca and Sigma Chemicals, Buchs, Switzerland) was used for intraperitoneal injection.

Animals

The experiments were carried out in accordance with the Tehran University Guide for the Care and Use of Laboratory Animals. Six-day-old male (CBA × C57Bl/6) F1 hybrid mice (n = 162) were used as testis donors and 10–12-week-old males of the same strain were used as testis recipients (n = 108) (Pasture Institute of Iran). Recipient mice were randomly assigned either to control (group I) or treated groups (group II). Group I received daily physiological saline and Group II received melatonin (20 mg/kg/day), intraperitoneally. Each group was randomly further distributed into nine subgroups (n = 6) that were sacrificed at weeks 1–9.

Testis vitrification

The cryoprotectant solution was 40% (v/v) ethylene glycol (Sigma, USA) containing 0.9% normal saline and 0.5 M raffinose. The vitrification solution (VS) was diluted with DPBS to make 12.5%, 25%, and 50% (v/v) solutions. Intact testes of six-day-old mice were exposed sequentially to 12.5% and 25% VS at room temperature for 5 min. Subsequently, they were exposed to 50% VS for 15 min at 4°C. Then the testes were transferred to 1.8 mL cryogenic vials (Nunc, Roskilde, Denmark) containing 100% VS and placed directly into liquid nitrogen at –196°C. Before transplanting, cryogenic vials were warmed up in 4°C water until the VS became liquid; 1 mL of cooled 50% VS was added into each cryogenic vial. The testes were removed from the cryovials and placed sequentially in solutions containing 50% and 25% VS concentrations at 4°C for 10 min and finally were transferred successively in 12.5% VS and DPBS at room temperature for 10 min.

Melatonin treatment *in vitro*

The thawed testes were further incubated for 30 min in αMEM plus 10% FBS, with or without 100 μM additive melatonin before being transplanted subcutaneously onto the backs of the castrated recipient mice of the treatment (group II) or non-treatment (group I) groups, respectively.

Transplantation procedure

The host mice were sedated by intraperitoneal injection of ketamine-xylazine mixture (Ketamine 80 mg/kg and Xylazine 10 mg/kg body weight; Pharmacia & Upjohn, Erlangen, Germany). Testectomy was performed and the intact vitrified-thawed testes were subsequently inserted bilaterally into the subcutaneous site on the back of the recipient mice. The time interval between removal and transplantation was less than 1 min.

Melatonin treatment *in vivo*

Melatonin at 20 mg/kg/day was injected intraperitoneally into the mice once a day at 18:00 h (1 h before initiation of a 12 h dark phase) throughout the examined days after transplantation.

Histology staining

Samples were placed overnight in 4% buffered formaldehyde (37% formaldehyde, Merck). Fixed tissues were embedded in paraffin blocks and the whole testis was sectioned serially at 4-μm thickness.

Assessment of apoptosis using TUNEL assay

Apoptosis was assessed by terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate-biotin nick-end labelling (In Situ Cell Death Detection Kit, Roche, Manheim, Germany), according to the manufacturer's instructions. Briefly, after deparaffinisation and rehydration, sections were incubated in 3% H_2O_2 (Merck) for 10 min to neutralise endogenous peroxidases and then digested by 20 mg/mL proteinase K in 10 mM Tris-HCl (Roche) for 30 min at room temperature. The sections were then incubated with TdT for 1 h at 37°C followed by incubation with anti digoxigenin peroxidase antibodies. Control tissue samples were incubated using PBS for the primary antibody. Apoptotic cells were observed by staining with 3, 3-diaminobenzidine (DAB) solutions (Sigma, Aldrich, USA). The samples were counterstained with haematoxylin, rinsed with distilled water, dehydrated, mounted with entellan (Merck, Germany), and observed with a light microscope.

Assessment of proliferative capacity in vitrified--thawed testis graft, using *in vivo* BrdU assay

For tagging of the proliferating cells, 5-bromo--2-deoxyuridine (BrdU; 100 mg/kg body weight; Sigma Aldrich, USA) was infused into the peritoneal cavity 2 h before sacrificing the host mice. Tissue samples were stained for Brdu (Brdu Cell Proliferation Kit; Chemicon). Briefly, the sections were dewaxed, rehydrated, and rinsed in deionised water. Endogenous peroxidase activity was quenched with a 10-min incubation in 3% (v/v) H_2O_2 at room temperature. After that, the samples were incubated with horseradish peroxide (HRP)-conjugated mouse monoclonal anti-Brdu antibody for 1 h at room temperature and rinsed with PBS. Then, the samples were incubated with peroxidase-conjugated goat anti-mouse IgG for 1 h at room temperature. Control samples were incubated using PBS for the primary antibody. Proliferated cells were observed by staining with DAB substrate. The samples were counterstained with haematoxylin, rinsed with distilled water, dehydrated, mounted with entellan, and observed with a light microscope.

Morphometric analysis of the grafts

Histological were assessed by light microscopy. The examined sections (approximately every 10th section) that were photographed were scored using NIH Image software. Additionally, the following parameters were determined for each graft: general graft shape (fibrosis, fatty tissue, presence of seminiferous tubules), seminiferous tubule, germinal epithelium thickness and most advanced germ cell type present in each tubule. The tubular thickness was also determined on micrographs using the NIH Image software. One section per graft was used and, if present, at least 50 tubule cross-sections were evaluated. All the transplant samples were looked at and assessed blindly by the same observer.

Statistical analysis

All data were expressed as mean ± S.E.M. The paired sample t-test was employed to compare the average testis weights of the melatonin-treated *vs* non-treated groups, and to compare the average number of grafts recovered from each group. Quantitative results obtained from the two groups were assessed for statistical differences by one-way analysis of variance (ANOVA). Multiple comparison tests (Tukey test) were employed to compare data from all groups. $P \leq 0.05$ was considered as significant.

RESULTS

Survival of the testis graft

Nine weeks after vitrified tissue transplanting, 22.2% (8/36) of non-treated grafts and 25% (9/36) of grafts which had been treated with melatonin, were recovered. However, this difference was not

Table 1. Graft weights [mg], number of surviving grafts, mean percentage of damaged seminiferous tubules, thickness of the seminiferous tubules [μm], and thickness of the germinal epithelium [μm] in both treated(T)/no-treated (N) groups, 1–9 weeks after grafting of testis and treatment of the host mice with melatonin (20 mg/kg/day); values are mean ± SD

Weeks	Graft weight [mg]		Graft survive (N)		Damaged tubules (%)		Seminiferous tubules [μm]		Germinal epithelium [μm]	
	T	N	T	N	T	N	T	N	T	N
1	20.6 ± 2.5	19.8 ± 3.5	(36/29)	(36/24)	51 ± 0.3	71 ± 0.3	65.3 ± 2.9	55.3 ± 5.9	5.6 ± 0.8	5.4 ± 0.9
2	25.4 ± 3.9	21.4 ± 2.9	(36/25)	(36/20)	50 ± 2	69 ± 4	96.7 ± 3.9	76.7 ± 7.4	20.4 ± 1.3	14.9 ± 3.2
3	29.7 ± 3.3	25.9 ± 3.7	(36/26)	(36/20)	49 ± 4	66 ± 3	128.7 ± 5.7	99.3 ± 9.5	27.9± 3.5	19.8 ± 3.9
4	37.7 ± 4.5	31.7 ± 5.1	(36/21)	(36/19)	48 ± 6	69 ± 5	168.7 ± 6.4	139.5 ± 7.9	37.5 ± 5.3	29.3 ± 5.4
5	38.4 ± 3.7	32.1 ± 2.5	(36/13)	(36/12)	45 ± 5	61 ± 7	209.9 ± 9.7	168.3 ± 9.1	57.5 ± 3.5	41.9 ± 3.9
6	49.9 ± 3.9	42.9 ± 3.6	(36/17)	(36/11)	32 ± 6	59 ± 5	287.5 ± 7.5	219.8 ± 11.4	63.4 ± 9.5	47.3 ± 5.7
7	55.4 ± 4.6	45.4 ± 4.9	(36/19)	(36/13)	31 ± 7	56 ± 8	309.3 ± 13.6	274 ±13.9	68.4 ± 11.5	51.7 ± 5.3
8	59.4 ± 7.5	51.7 ± 3.5	(36/10)	(36/9)	34 ± 8	57 ± 5	279.7 ± 19.7	204 ± 19.3	64.3 ±13.5	45.5 ± 7.8
9	65.7 ± 3.5	52.4 ± 6.5	(36/9)	(36/8)	38 ± 5	56 ± 6	297.3 ±13.3	192.1 ± 11.8	62.4 ± 9.8	41.9 ± 6.9

statistical significantly between the control and treated groups (Table 1).

General observations and weight of the testis graft

The places of transplantation were swollen and became obvious within 9 weeks. However, more frequently, recovered grafts showed a high degree of abundant fat tissue (Fig. 1). In general, after 9 weeks of transplantation, all recovered grafts in the treated group grew larger in comparison to their weight before grafting (14–15 mg before grafting *vs* 55–61 mg after grafting) (Table 1). Albeit, during 1–8 weeks of transplantation the difference in mean weight of the grafts was not statistically significant between the non-treated and treated groups, whereas after 9 weeks, the mean weight of grafts in the treated group (65.7 ± 3.5 mg) became greater than those (52.4 ± 6.5 mg) from the non-treated group.

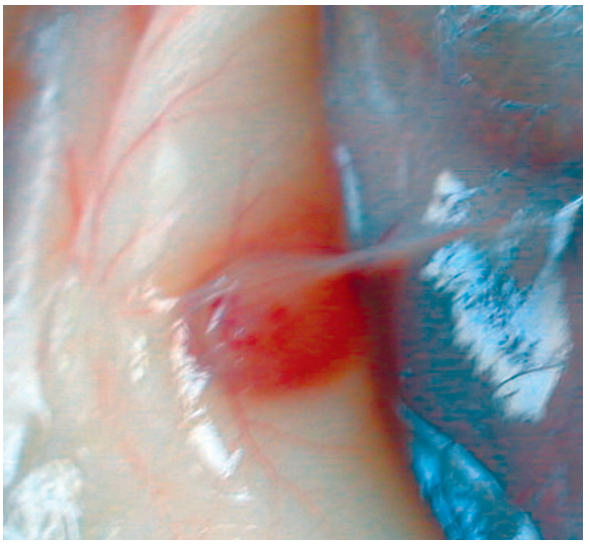

Figure 1. Representative micrograph of subcutaneous site of testis graft in host mice. Note the presence of the revascularisation surrounding the transplant location to provide enough blood to the graft.

Histological analysis of the testis graft

Cryopreservation affects neither the number of surviving germ cells nor their capacity to colonise the seminiferous tubules (Fig. 2B). However, in general, histological analysis of testis grafts showed various degrees of spermatogenesis process in the seminiferous tubules, displaying from total atrophy to full spermatogenesis progression of the seminiferous tubules (Fig. 2A, E). The majority of these atrophic tubules contained only A-spermatogonia cells and no other type of germ cells were presented (Fig. 2E). Moreover, the kinetics of spermatogenic progression were more marked in the treated group than in the non-treated group.

In both groups the spermatocytes were the most advanced germ cells observed after one week of transplantation, which originally contained primitive gonocytes as the only sort of germ cell (Fig. 2C). Furthermore, the complete kinetics of spermatogenic progression was observed in the neonatal mouse testis grafts within 9 weeks (Fig. 2D). A dilation of the lu-

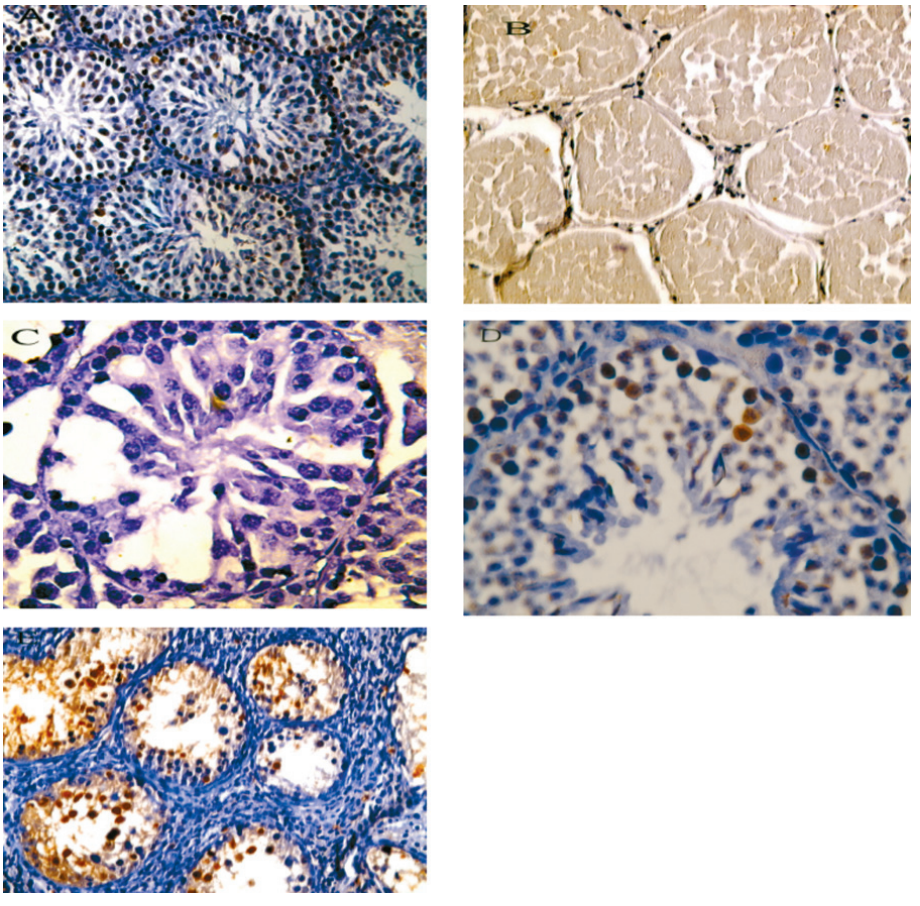

Figure 2. Representative TUNEL-stained sections of vitrified-thawed testis graft from melatonin-treated and non-treated groups during the examined weeks; **A.** TUNEL staining of testis graft sections from the treated group showing a seminiferous tubule with normal morphology and low apoptosis germ cells at nine weeks after transplantation; **B.** TUNEL staining of vitrified-thawed neonate testis (six-day-old mice) showing a seminiferous cord with normal morphology and low apoptosis primordial germ cells (PGC) before transplantation; **C.** TUNEL staining of testis graft sections from the non-treatment group showing a seminiferous tubule with abnormal morphology and high apoptotic cells (brown cells) at 9 weeks after transplantation; **D.** TUNEL staining of testis graft sections from the treated group at 9 weeks after transplantation. Note that the regions contain elongated spermatid in healthy seminiferous tubule; **E.** TUNEL staining of testis graft sections from the non-treated group showing that atrophied tubules containing just A-spermatogonia cells and did not have any type of healthy germ cells at 9 weeks after transplantation; magnification 400 × (B, C, D) and magnification 200 × (A, E).

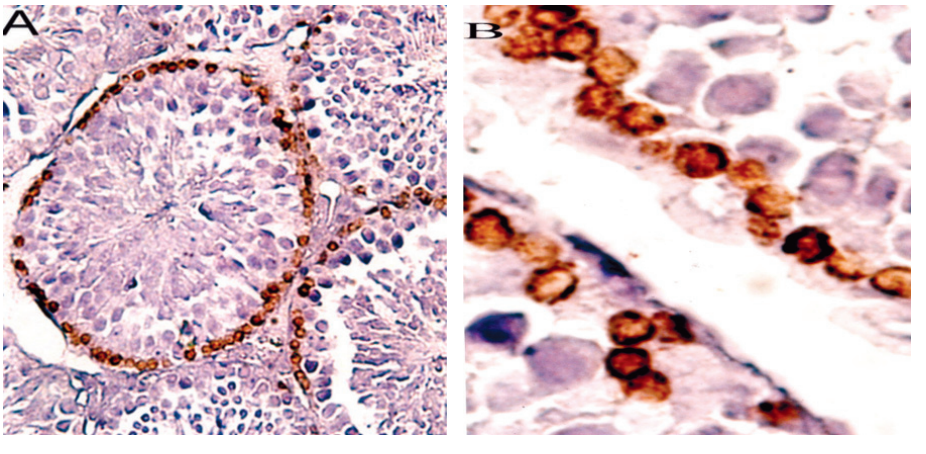

Figure 3. A. BrdU staining of testis graft sections from the melatonin-treated group, confirming the presence of cell proliferation at 9 weeks after transplantation; **B.** BrdU-staining of germ cells in the seminiferous tubule; magnification 400 × (A) and 1000 × (B).

Table 2. Relative percentage of TUNEL and BRDU positive per tubule in both treated and non-treated groups 1–9 weeks after grafting of testes and treatment of the host mice with melatonin (20 mg/kg/day); values are mean ± SD

Weeks	TUNEL positive (%)		BRDU positive (%)	
	Treated group	Non-treated group	Treated group	Non-treated group
1	57 ± 3.2	74± 4.6	20.5± 2.9	13.7 ± 3.1
2	49 ± 3.7	61 ± 5.3	29.3 ± 3.7	18.5 ± 2.9
3	39.7± 3.9	57.3 ± 2.8	31.5 ± 3.9	20 ± 2.3
4	33.5± 5.5	49.7 ± 5.3	40.9 ± 4.3	29.4 + 5.1
5	28.7± 4.9	37.9 ± 7.3	57.9 ± 5.6	41.2 ± 4.9
6	23.5± 3.3	35.7 ± 4.7	69.7 ± 3.7	49.6 ± 5.9
7	21.7± 4.5	39.9 ± 7.9	74.8 ± 5.9	53.6 ± 6.8
8	29.3± 4.7	43.9 ± 5.7	72.6 ± 7.5	51.6 ± 4.9
9	39.5± 6.5	51.3 ± 9.3	69.8 ± 5.9	49.7 ± 6.2

men accompanied by a disorganised epithelium and premature sloughing of postmeiotic germ cells was higher in the non-treated group (Fig. 2E). In some testis grafts, especially in those that had a well spermatogenic progression, a morphologically normal appearance of interstitial space with interstitial cells and peritubular cells was observed (Fig. 2A). However, melatonin administration could be improve the light microscopic morphology of interstitial tissue of the testis grafts.

Seminiferous tubules of the testis grafts

An outline of damaged tubule number, thickness of the seminiferous tubule, and germinal epithelium illustrated in histological sections of testis is shown in Table 1. Overall, the mean proportion of the number of damaged seminiferous tubules throughout the grafting days in each histological section of testis graft was lower in the treated group (42%) than in the non-treated group (62.6%). The mean proportion of the thickness of seminiferous tubules throughout the grafting days was larger in the treated group in comparison to the non-treated group (204.8 μm *vs* 158.8 μm; $p > 0.001$). In fact, the mean proportion of the thickness of the germinal epithelium was significantly different between the controls (33 μm) and the treated-grafted testis (45.2 μm; $p > 0.05$).

TUNEL-positive apoptotic changes and cell proliferative activity of the testis grafts

An outline of the relative percentage of cell apoptosis (TUNEL assay) and proliferation (Brdu assay) in germ cells of the testis is shown in Table 2. As indicated by the persistence Brdu-positive cells in frozen-grafted tissue (Fig. 3), spermatogonial cells were able to survive after cryopreservation and grafting for 9 weeks. However, our data from TUNEL-positive apoptotic cells revealed a significant loss of spermatogonia after freezing and grafting of testis (Fig. 2A–E). Overall, their number was lower in the treated group when compared with the non-treated group (35.7% *vs* 50% per tubule respectively, $p \geq 0.001$). Indeed, 51.8% of spermatogonia continued to proliferate after freezing and grafting in the treated group, compared to (36.4%, $p \geq 0.001$) in the non-treated group. Most of the apoptotic cells were located along the base of the seminiferous tubules, which were identified as pachytene spermatocytes (Fig. 2C).

DISCUSSION

The conservation of male gonad and gamete by transplantation would spread out the applicability of this merits model. The successful freezing of spermatozoa that was collected from grafts presents an opening approach towards the practical application of this method [18]. However, the method of testis transplantation should be optimised and made more accurate before being performed on humans.

Our results confirmed that the germ cells in vitrified and grafted testis tissue might help in survival and retention of their capacity to initiate spermatogenesis. Indeed, this observation suggests that using melatonin just after thawing vitrified testis tissue and then continuing melatonin treatments dur-

ing transplantation is a feasible approach to better maintain the potential of spermatogenesis kinetics, and may serve as a promising method for graft preservation.

However, detachment of the cells or fluid accumulation was seen in all of the grafts, but this problem was more prominently observed in the grafts that were collected from non-treated hosts. Yu et al. [27] reported that fluid accumulation in the seminiferous tubules after grafting leads to degeneration of the graft. Therefore, melatonin may protect against this fluid accumulation.

This observation is in accordance with Li et al. [13], who reported that melatonin, based on its low molecular weight and high tissue penetration, provides superior antioxidant substance, especially in solid tissues.

Our observations indicate that the first progression of spermatogenesis up to the level of spermatocytes one week after transplantation was obviously detected more in melatonin-treated grafts. There was also a reduction in the number of seminiferous tubules which only had A-spermatogonia in the treated grafts.

The present study also showed that cryopreservation affects neither the number of surviving germ cells nor their capacity to colonise the seminiferous tubules. However, since cryopreservation, as performed here, may have a negative impact on graft development, further studies should be carried out with a focus on processing the cryopreservation media containing melatonin to optimise the outcome.

The melatonin-treated grafts showed a fast increase in graft weight between 8 and 9 weeks after grafting, and even the differentiation of spermatogonia and the progression of spermatogenesis up to the level of spermatid were significantly higher than the grafted control samples.

Melatonin, as a well-known direct free radical scavenger or a stimulator of several anti-oxidative enzymatic systems, reduces the ability of reactive oxygen species (ROS) to damage membrane polyunsaturated fatty acids [17]. In accordance with the above-mentioned observations, we showed that melatonin significantly reduces the index cell apoptosis while significantly enhancing the activity of cell proliferation. Thus, the efficacy of melatonin's dual function as both a direct ROS scavenger and an enhancer of proliferation cells of activity is a notable and important point. NF-kappaB is central for the overall immune response through its ability to activate gene coding for regulators of apoptosis and cell proliferation [7]. The defining character activation of iNOS after I/R enhances mucosal apoptosis in the rat small intestine [24] and the inhibition of iNOS ameliorate apoptosis after I/R [24]. Melatonin may contribute to the down-regulation of NF-kappaB and subsequently cause a decrease in iNOS expression [27]. Therefore, we expected that melatonin would subsequently ameliorate apoptosis.

There may be several reasons for the limited development of cryopreserved testis tissue after grafting. In order to allow cryoprotectant to penetrate the tissue, small tissue fragments were prepared. This procedure resulted in a limited number of seminiferous tubules in each preserved tissue fragment and may have increased the variability between fragments. It is also possible that residual cryoprotectant that remained in the frozen--thawed tissue after washing led to tissue toxicity. Some damage to the tissue after freezing and thawing cannot be avoided. As such, the frozen--thawed testis tissue may take longer to become revascularised after grafting, and to initiate spermatogenesis, compared with fresh tissue. This may explain why no spermatozoa were observed in the allograft that was cryopreserved before transplantation in the present study. This observation is in agreement with a previous report that cryopreservation delayed the initiation of spermatogenesis in grafted testis tissue from rhesus monkeys [24]. Accordingly, Frederickx et al. [6] reported that the spermatogonial stem cells may lose their functional capacity through cryopreservation regardless of the suitable viability of the cell suspension observed after thawing. In a recent study, Wyns et al. [25] did not observe any changes in histological morphology in thawed testis tissue compared with fresh grafts. Immunohistological study before grafting, as evaluated in the present study, or pre-grafting tissue morphology could not reveal the later difference in the effect of cryoprotectants on germ cell differentiation. Therefore, previous studies and the present experiment confirm that transplantation is the only functional assay to evaluate the developmental potential of testis organ *in vivo* so far.

CONCLUSIONS

In conclusion, the data from this study indicates that melatonin might improve the recovery of graft and cellular function, reduce histological apoptosis, and increase survival after reperfusion. As a pathophysiological explanation, melatonin significantly reduced

apoptosis, while inducing proliferation of the cells. These results suggest that melatonin may represent a novel therapeutic approach in the prevention of ischaemia reperfusion injury after testis transplantation.

ACKNOWLEDEGEMENTS

This study was supported by a research grant from Tehran University of Medical Sciences, Tehran, Iran (grant no. 5589).

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