Research Article

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Improving of Cryopreservation Quality of Spermatogonial Stem Cells after Freezing-Thawing by Melatonin

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ABSTRACT

Cryopreservation of spermatogonial stem cells (SSCs) is an advantageous method to restore fertility in young boys' cancer survivors. Recently, for reduction of SSCs cryoinjering various antioxidants have been used. The purpose of this study was to evaluate antioxidant effects of melatonin in frozen-thawed SSCs. Spermatogonial stem cells were isolated from testes of neonate mice (3-6 days old) and their purities were measured by flow cytometry with GFRα1. After culturing, the cells were frozen in two groups 1- control and 2- melatonin (100μM) for one month. Finally, the cell viability, colonization rate, intracellular ROS were evaluated after freezing thawing. Melatonin increased the viability, colonization of SSCs, and decreased intracellular ROS. The results of this study show that melatonin with antioxidant effects as a good option can be used for freezing and long-term storage of cells and infertility treatment in the clinic.

KEYWORDS: Cryopreservation; Melatonin; Spermatogonial stem cells

ABBREVIATIONS: SD: Standard Deviation; SSCs: Spermatogonial Stem Cells; GDNF: Glial Cell-Derived Neurotrophic Factor; PLZF: Promyelocytic Leukemia Zinc Finger; GFRα1: GDNF family receptor α1; LIF: Leukemia Inhibitory Factor

INTRODUCTION

Recent success in cancer treatment has increased in prepubertal boys that have cancer and receive high doses of chemotherapy and radiotherapy. It has been reported that 35% of young boy's cancer survivors become infertile in adulthood because spermatogonial stem cells are very sensitive to the toxic effects of cancer treatment [1,2]. In fact, trying to return fertility in these children is one of the most important subjects in clinical and research area. The cryopreservation of SSCs and finally transplantation of SSCs after recovery can initiate spermatogenesis and fertility in these patients [3-5].

Spermatogenesis process with the aim of correct transfer of genetic factors to the next generation depends on the spermatogonial stem cells [6,7]. SSCs as undifferentiated cells have ability of self-renewal, differentiation and transferring genetic information to the next generation and are therefore considered unique cells among human stem cells [8-10].

Cryopreservation as an effective method for infertility preservation is associated with biochemical changes of the cell plasma membrane, increased reactive oxygen species, DNA fragmentation, decreased cell activity and finally reduced fertility of SSCs [11,12]. So far, different antioxidants have been used to reduce the injuries following cryopreservation [13-15]. Despite the introduction of antioxidant agents and useful effects of them, the use of a suitable factor to minimize the freezing complications is still debated.

Melatonin as a hormone has antioxidant, anti-inflammatory, and anti-apoptotic properties in different types of stem cells [16,17]. Several reports have emphasized that melatonin as an effective antioxidant has a key role in increasing antioxidant enzymes and

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scavenging free radicals. In fact, melatonin with this characteristic can protect SSCs against oxidative stress [18,19]. Moreover, melatonin, with its antioxidant properties has an important role in regulating self-renewal and differentiation of stem cells. There is also evidence of the role of melatonin as an appropriate cryoprotectant in male reproductive system [20-22]. Considering the above and the lack of knowledge regarding suitable factors for long-term preservation of SSCs, this study aimed at assessing the antiapoptotic and antioxidant effects of melatonin in improving of Cryopreservation quality of Spermatogonial Stem Cells.

METHODS AND MATERIALS

Isolation and Enrichment of SSCs

In this section, male BALB/c mice (3-6 days old) were used to isolate of SSCs. The protocol of Kanatsu-Shinohara with some modification was used for isolating SSCs. Briefly, under sterile conditions the removed testes were rapidly washed in PBS supplemented with 1% penicillin/streptomycin (Sigma-Aldrich) and then mechanical digestion was performed carefully. The minced samples were put into digestion medium containing 5μg/mL DNase (Sigma-Aldrich.USA), 1mg/mL collagenase type IV (Gibco,CA), and 1mg/mL hyaluronidase (Sigma-Aldrich. USA) and incubated at 37 °C for 20min with 5% CO_2 and then pipetted gently every 5 minutes. Finally, Centrifugation was done at 1,500g for 5min. The second stage of enzymatic digestion was performed using the same method and digestion medium. SSCs obtained from enzymatic digestion were purified using the plating differential technique. In this technique, the cells were transferred to culture dishes coated with Lectin for 2h at 37 °C. After 2h, somatic cells attach to the bottom of the dish and the supernatant containing the purified SSCs is collected. Flow cytometry using GFRα1 marker was used to determine the purity of the collected SSCs. First, 105 cells were incubated in 100μl PBS/FBS and 10μl primary antibody (1:100) at 4 °C for 1 hour. After washing twice with PBS, the cells were incubated in 100μl PBS/FBS and 10μl secondary antibody at 4 °C for 1 hour. FITC goat anti-rabbit IgG H&L (ab6717, Abcam, UK) was used as the secondary antibody for GFRα1. The control cells were not treated with any antibodies. Finally, the cells were kept in a dark room on ice and the purity percentage was determined by flow cytometry.

Culture of SSCs

Purified cells (2×105 cell/cm2) were seeded in culture dishes and culture medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 10ng/Ml Leukemia Inhibitory Factor (LIF; Sigma, Haverhill), 10ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill, NJ), 10ng/mL glial cell line-derived neurotrophic factor (GDNF; Sigma- Aldrich), 100U/mL penicillin (Sigma-Aldrich, Darmstadt), and 100μg/mL streptomycin (Sigma, Germany) was added to cells. Finally, culture dishes were transferred in a humidified 5% CO incubator at 37 °C and the fresh medium was replaced every 2-3 days.

Dosimetry of Melatonin

The methyl thiazol tetrazolium assay (MTT; Sigma-Aldrich) was applied to define the optimum concentration of melatonin. In this technique, cell viability was evaluated in control and treatment groups with 50, 100, and 150μm/ml melatonin after the freezing-thawing process. First, 400μl DMEM and 40μl MTT were added to SSCs, and the mixture was incubated at 37 °C for 4 hours. Then, MTT solution was replaced with 400mL dimethyl sulfoxide freezing solution (DMSO; Sigma). The optical density was measured at 540nm using an Elisa Reader (BioTek Instruments).

Freezing–Thawing Process

Freezing of SSCs was done according to a protocol described by Izadyar et al. After cell harvesting, the cells (0.5mL) was transferred to a 1.8mL freezing vial (Cryovial, Nunc, Denmark) and basal freezing medium, including 10% DMSO and 10% FBS was slowly added to the cells. After complete blending, the cryovials were kept to a -80 °C freezer overnight. Then, the frozen vials were dunked in liquid nitrogen for 1 month. The treated group was frozen in the basic freezing medium with 100μm/ml melatonin (Sigma-Aldrich). For thawing, the cryovials were put at room temperature for 30 seconds and then quickly transferred in a 37 °C water bath for 2 minutes. The thawed cells were transferred to a falcon tube containing DMEM and 10% FBS. Finally, cell centrifugation was done at 1500g for 5 minutes. After removing the supernatant solution, the cell pellet was used to perform the further techniques.

Assessment of Cell Viability

To determine the melatonin effect on cell viability, MTT assay was done on cultured cells after the freezing–thawing process. First, SSCs were seeded in a 96-well plate and 400μl DMEM with 40μl MTT were added to SSCs. Then plate was incubated at 37 °C for 4 hours. After discarding of MTT solution, 400mL DMSO was replaced. The optical density was measured at 540nm using an Elisa Reader (BioTek Instruments).

Colony Assay

To Assessment the melatonin effect on SSCs colonization, SSCs (2 x 105 cell/cm2) were seeded in petri dishes. Cells were cultivated in a DMEM containing 10% FBS, 100U/mL penicillin, 100mg/mL streptomycin, 10ng/mL LIF, and 10mg/mL GDNF in 5% CO2 at 37 °C for 1 week after the freezing-thawing process. The SSC colonies were observed and captured using an Invert microscope. Then, the number and diameter of the colonies were analyzed using the Image J software.

Assessment of Intracellular ROS

The intracellular ROS was measured using 2', 7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) as a fluorescent oxidative probe. First, the cells were incubated with 10μl of 2′, 7′-dichlorofluorescin diacetates at 37 °C for 25 minutes. Next, the fluorescence intensity of DCFH-DA was assessed between 500 and 530nm wavelengths using a BD FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Statistical Analysis

The results were processed using the GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA). One-way ANOVA and Tukey post-hoc test was used for the statistical analysis of the results. P values less than 0.05 were considered statistically significant ($P \leq 0.05$). The data were presented as mean±standard

RESULTS

Evaluation of the Purification of SSCs

Determining the purity of SSCs (2×105 cell/cm2) were done using flow cytometry with undifferentiated marker GFRα1 after enzymatic digestion and differential plating. The results analysis of

flow cytometry displayed that 98.32% of the cells were positive for GFRα1 (Figure 1).

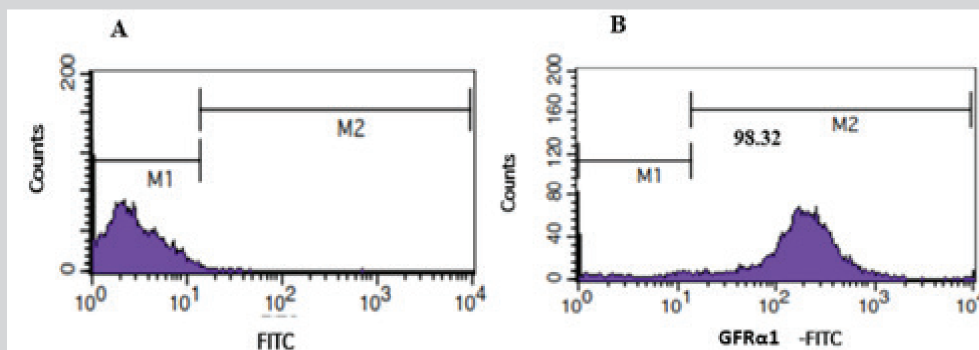

Figure 1: Results of flow cytometry indicating the purity percentage of SSCs with GFRa1 marker. M1: Marker Negative Cells; M2: Marker Positive Cells; A: Control; B: GFRa1 positive.

SSC Morphology and Confirmation of Colonies

The purified SSCs were cultured to reach optimal confluence before freezing process. Confirmation of SSC colonies were performed by Alkaline phosphatase staining. The red color of the colonies demonstrated the stem cell alkaline phosphatase activity in the colonies (Figure 2).

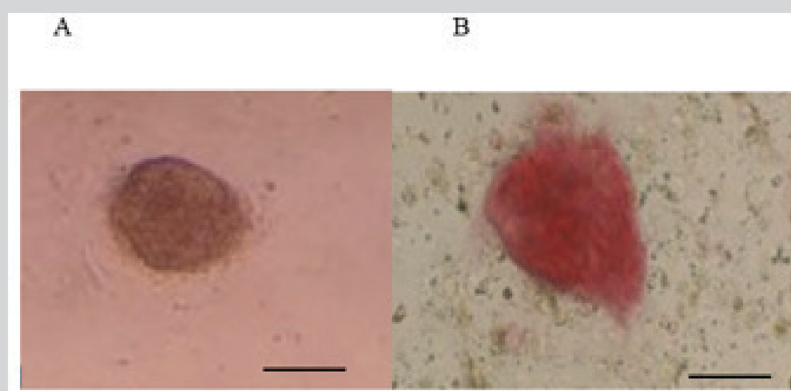

Figure 2: Assessment of SSCs colonization.
A: Colony of SSCs. B: Alkaline phosphatase positive colony Scale bars = 100µm

Dosimetry of Melatonin

The MTT assay was applied to detect the optimum concentration of melatonin one month after cryopreservation. The results demonstrated that 100µM melatonin significantly increased the cell viability (95.83± 0.88) compared to other groups. According to these results, 100µM of melatonin was selected as the optimum concentrations in this study (Figure 3).

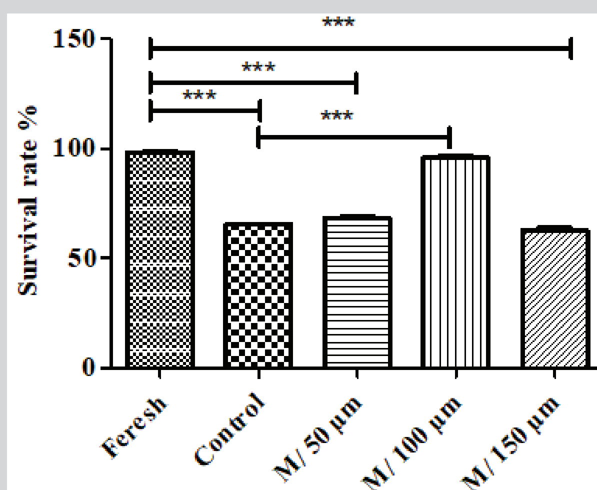

Figure 3: Determining the optimum concentration of melatonin by MTT assay. According to these results, 100nM of melatonin was selected as the optimum concentrations. The experiment was performed in triplicate and repeated three times. The results are reported as mean ± SE, ***$p < 0.001$.

Effect of Melatonin on Viability of SSCs

After freezing- thawing process, the viability of SSCs were evaluated by MTT technique. The results of MTT assay displayed that the freezing-thawing process notably decreased the viability of SSCs in the control group (65.17±0.31) compared to the fresh group. Also, the viability of treated SSCs with melatonin was significantly higher (96.77±0.18) compared to the control group (Figure 4).

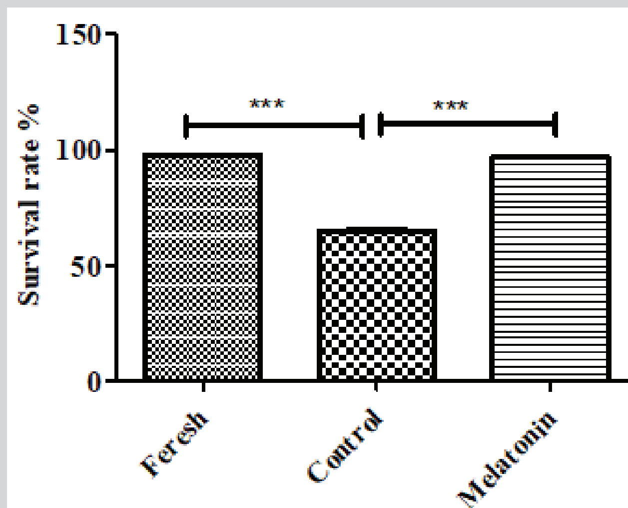

Figure 4: Analysis of the cell viability after the thawing process by using the MTT. Viability percentage in melatonin group was significantly more than other groups and close to the fresh group. The experiment was performed in triplicate and repeated three times (there are 2×105 cell/cm2 of SSCs in each group). The results are reported as mean±SE, ****p*<0.001.

Effect of Melatonin on Intracellular ROS

The level of ROS production was measured in three groups of SSCs including fresh, control and cryopreservation with melatonin. This experiment data analysis indicated that ROS production was notably higher in the control group (48.50±0.28) compared with the other groups. Whereas ROS production decreased significantly in the cryopreservation group with melatonin (24.83±0.12) compared with the control group (Figure 5).

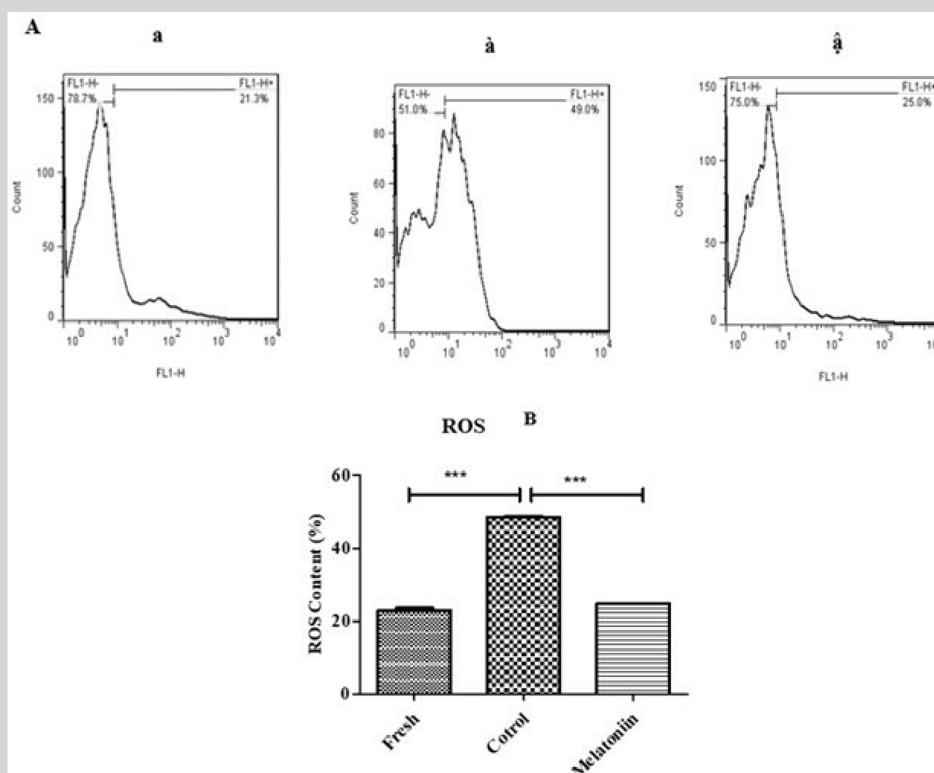

Figure 5: A- Measurement of intracellular ROS after the thawing process by using DCFH-DA staining in different experimental groups (a): fresh, (à): control, (â): melatonin. B- Analysis of intracellular ROS production. Melatonin significantly decreased ROS level. The experiment was performed in triplicate and repeated three times (there are 2×105 cell/cm2 of SSCs in each group). The results are reported as mean±SE, ****p*<0.001.

Colony Assay

Assessment of the melatonin effect on colonization of frozen-thawed SSCs were done by an inverted microscope and Image J software one week after culturing the thawed SSCs (Figure 6). Data analysis showed that melatonin could effectively increase (195.9±6.06) the diameter of SSC colonies in comparison with the control group. The number of colonies was significantly larger in SSCs treated with melatonin (4.66±0.33) compared to the control group (Figure 6).

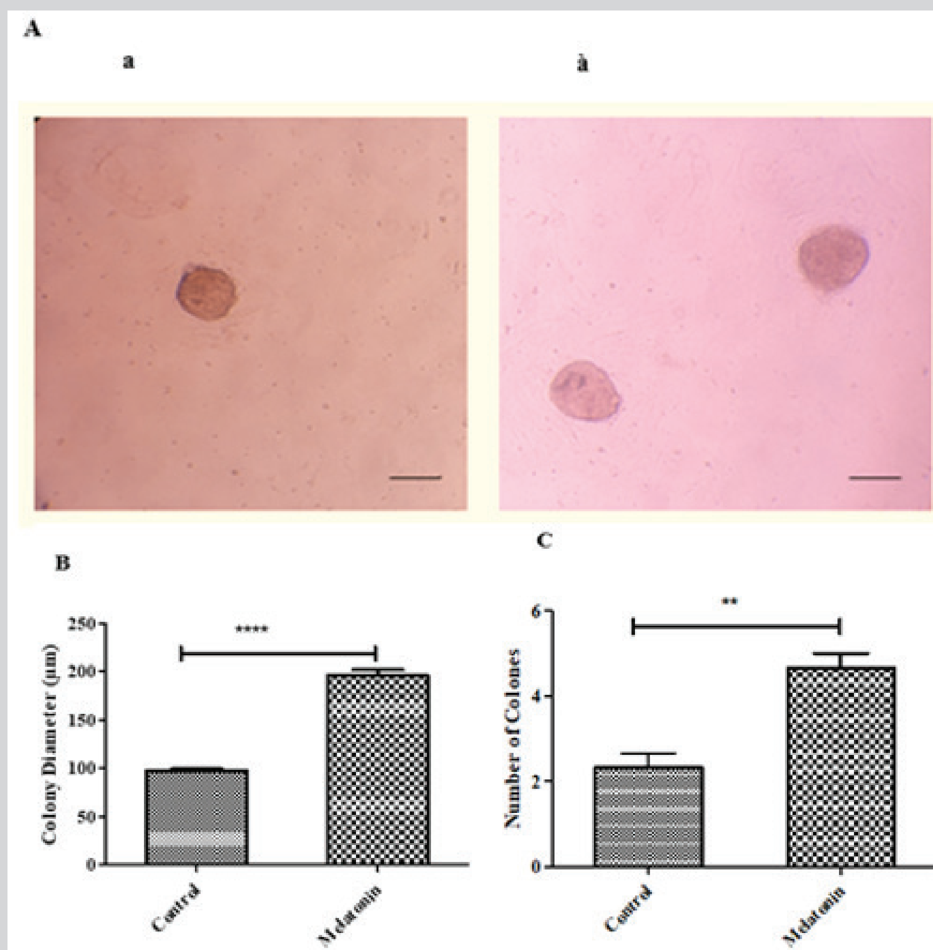

Figure 6: A- Microscopic morphology of SSCs cultured 1 week after the freezing–thawing process. a: control group. à-melatonin group. Scale bars are 100µm. (B, C): Comparison of diameter (B) and number (C) of SSCs colonies. The experiment was performed in triplicate and repeated three times (there are 2×105 cell/cm2 of SSCs in each group). The results are reported as mean±SE. ****$p \le 0.0001$ and **$p \le 0.01$.

DISCUSSION

Infertility occurs in almost one-third of the young boy's cancer survivors due to toxic effects of chemotherapy and radiotherapy drugs [15]. Freezing and long-term preservation of SSCs as an effective method for restoration of fertility is one of the most important subjects in clinical and research field [23,24]. Previous studies showed that normal function of SSCs decreases after the freeze-thaw process due to cryoinjuries. Therefore, it is necessary to add cryoprotectants to the freeze medium of SSCs [25,26]. In this study, melatonin as a cryoprotectant was added in the freeze medium to reduce injuries of cells after the freeze-thaw process.

The results of this study showed that cryopreservation of SSCs with 100 µm melatonin improved cell viability compared to the control group. As reported by other studies, melatonin as a cryoprotectant also increased cell viability after thawing process [27,28]. According to evidence Cryoprotectants that are added to the freezing medium are divided to intracellular and extracellular cryoprotectants. The intracellular cryoprotectants have a low molecular weight and the ability to penetrate the cell membrane. They protect cells against cryoinjuries through these properties. The extracellular cryoprotectants with formation a sheath around the cells prevent cell dehydration during freezing. In fact, cryoprotectants act through two different mechanisms to diminish cryoinjuries [29]. It seems that melatonin enhances the viability of SSCs through formation of a sheath around cells to prevent cell water loss during cryopreservation.

Freezing process increases the intracellular ROS production. An increase in the intracellular ROS led to cell injuries such as lipid peroxidation, protein oxidation and DNA fragmentation. For this reason, in this study melatonin was added to the freeze medium to reduce ROS production. The results showed a marked reduction in intracellular ROS production in melatonin treated SSCs compared to the control group. Many researchers found that melatonin, as a potent antioxidant, could reduce oxidative stress and protect the sperm against cryoinjury through scavenging the produced ROS [30]. It seems that melatonin can eliminate ROS probably through neutralizing toxic free radicals.

In general, we believe that melatonin, as a cryoprotectant, protects SSCs from cryoinjuries. Although the exact mechanism is not clear, two general categories of explanations have been proposed: 1- melatonin may cause changes in the physical condition of the freeze medium and may form a sheath around cells to minimize dehydration, 2- Melatonin may protect cells through a mechanism independent of receptors that facilitates the elimination of free radicals.

According to the results of this study, melatonin may be a suitable candidate for freezing, long-term cell preservation, and infertility treatment in the clinical setting considering its antioxidant and anti-apoptotic properties as well its effects on increased colonization and cell viability of SSCs.

CONCLUSION

Based on the results, it can be deduced that melatonin with antioxidant and anti-apoptosis effects can be used as a useful antioxidant for freezing and long-term storage of cells in the clinic.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

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The present research has been performed in department of anatomy - Tehran University of Medical Sciences, Tehran, Iran.

AUTHOR CONTRIBUTIONS

T. N Designed experiment and Performed study. F.F Performed analyses, edited article, and supervised the research.

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