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Supplementation Vitrified-thawed Media with Melatonin Do Not Protecting Immature Mouse Testicular Tissue from Vitrified-thawed Induced Injury

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ABSTRACT

The purpose of this study was to test of efficacy of melatonin on the Optimizing of cryopreservation media in the testis tissue samples. Testes from neonate BALB/c mice were vitrified and then thawed under standard condition with or without the addition of 100 μ M melatonin to both of vitrification and thawing solution. After that, Vitrified-thawed whole testes were digested under standard condition and subsequent viability of the cells in the suspension was analyzed using cytotoxicity kit and Apo-BrdU tunnel assay kit. The mean proportion of apoptotic testicular cells in the treated vitrified-thawed testes in comparison to no-treated ones was noted significantly (5.2 ± 0.47 vs. 1.56 ± 0.62 , respectively). Moreover, melatonin cause decreasing the viability of the treated vitrified-thawed testicular cells in compared to no-treated vitrified-thawed testicular cells (4.78 ± 0.46 vs. 8.39 ± 0.76 , respectively). In addition, the mean cytotoxicity of melatonin on the vitrified-thawed testicular cells was 9%. The associated reduction in healthy testicular cells in the treated vitrified-thawed testes suggests that melatonin in doses of 100 μ M don't protected testicular tissue from damaged induced in the process vitrification and thawing. However, further well-designed studies such as dosimetry melatonin and applied another cryoprotectants in the matching with melatonin are essential to offer a final conclusion.

Key words: Vitrification, melatonin, testis, apoptosis

INTRODUCTION

Recently, many researches have been done to optimize of Cryopreservation media (Curaba *et al.*, 2011a, b; Gouk *et al.*, 2011; Abd-Allah, 2011; Sundararaman and Edwin, 2008; Wang *et al.*, 2011). Cryopreservation is a procedure to preserve their fertility after treatment with chemotherapy agents or radiotherapy (Dohle, 2010; Wyns *et al.*, 2010, 2011). Immature testicular tissue harvested from the testis and then immersed in cryoprotectant media and transferred immediately to liquid nitrogen (rapid-freezing) or refrigerator with decrease it's temperature in the base of programs (slow-freezing) (Abrishami *et al.*, 2010; Amorim *et al.*, 2011; Curaba *et al.*, 2011b). In the Slow-freezing (SF) procedure, functional sperm production, percentage of seminiferous tubules containing spermatogonia and survival rate of

spermatogonia after transplantation was diminished (Abrishami *et al.*, 2010; Amorim *et al.*, 2011; Curaba *et al.*, 2011b). Rapid-freezing or vitrification is a better strategy because it avoids ice crystal formation and biologically damaging effect (Abrishami, 2009). Nevertheless, vitrification and thawing induces a damage to cells such as reduce viability, induction apoptosis, loss of integrity of DNA, breakdown of cell membrane, oxygen Free radicals, solution effects, intracellular ice crystal may damage to cells (Abrishami, 2009; Bagchi *et al.*, 2008; Bank and Brockbank, 1987; Fuller, 2004; Pegg, 2002, 2007). Therefore, reduce injury to cells in the process of vitrification and thawing needed. Melatonin is small biological molecule that secreted in the pineal gland and other organs e.g. retina, testis (Fildes *et al.*, 2009; Hardeland *et al.*, 2011; Zawilska *et al.*, 2009). Effects of melatonin are studied in many regulatory functions of cells such as immune response, cell signaling, protects fatty acids from oxidation and nuclear DNA from damage, control tumor growth and inhibit cell proliferation, oncostatic action, antiapoptotic effect on many normal cells, enhances or promotes apoptosis in the tumor cells and significant anti-aging properties (Ahmad and Haldar, 2010; Balao da Silva *et al.*, 2011; Fildes *et al.*, 2009; Hardeland *et al.*, 2011; Martin-Renedo *et al.*, 2008; Millan-Plano *et al.*, 2010; Radogna *et al.*, 2006, 2007, 2008; Reiter *et al.*, 2009; Zhao *et al.*, 2010; Hemadi *et al.*, 2011). *In vitro* incubation of mesenchymal stem cells with melatonin ameliorates survival and paracrine activity (Mias *et al.*, 2008). Addition of melatonin to cryopreservation media of stallion spermatozoa significantly decrease lipid peroxidation and apoptosis and improve survival of cells (Balao da Silva *et al.*, 2011). Melatonin reduces injuries to ram spermatozoa during freeze-thaw process (Succu *et al.*, 2011). Incubation of red deer spermatozoa after thawing with melatonin ameliorates survival and decrease oxidative stress to cells in the dose-dependent manner (Dominguez-Rebolledo *et al.*, 2010). Supplemented melatonin to vitrified-thawed media of murine ovaries ameliorates cumulus-oocyte complexes quality (Hemadi *et al.*, 2009). Because the anti-tumor, proapoptotic and antioxidant mechanisms of melatonin in the tumor and normal cells, present study investigated the effect of melatonin on the vitrification solutions of germ cells in immature testis. We believed that use of melatonin for pretreatment of germ cells may represent a novel and safe strategy to improving vitrification or preserve immature testicular tissue in the prepubertal boy with cancer.

MATERIALS AND METHODS

All experiments were performed in accordance with principles of laboratory animal care. Male 6-old-day BALB/c mouse pups were obtained from physiology research center. Mice were euthanized by excessive doses of ketamine HCl (80 mg kg⁻¹) and xylazine (10 mg kg⁻¹) (Pharmacia and Upiohn, Erlangen, Germany) (Hemadi *et al.*, 2011) in accordance with the protocols approved by the Ahvaz Jundishapur University Medical Science Animal Care and use committee. Every effort was made to minimize the number of animals used and their suffering. Male mice were randomly assigned to one of two experimental groups. After collecting the intact testes from the mice, they were vitrified in the vitrification medium that supplemented with melatonin (group A) and medium without melatonin (group B).

Vitrification procedure: Testis were transferred to vitrification solution 1 (VS 1) containing 0.5 molar sucrose, 7.5% ethylene glycol, 7.5% DMSO and 100 µg mL⁻¹ melatonin. Testis was transferred to vitrification solution 2 (VS 2) After 10 min, containing 0.5 molar sucrose, 15% ethylene glycol, 15% DMSO and 100 µg mL⁻¹ melatonin. samples After 10 min were transferred to VS 3 that containing 0.5 molar sucrose, 15% ethylene glycol, 15% DMSO, 100 µg mL⁻¹ melatonin and 20% FBS. Sample after 10 min were transferred to liquid nitrogen tank.

Thawing procedure: Samples were maintained 30 sec in the room temperature after removal from liquid nitrogen. Samples were hold in water bath 37°C until defreeze then were transferred to thawing solution 1 (T.S.1) in 4°C that containing 0.5 molar sucrose and 100 µg mL⁻¹ melatonin. Samples after 5 min were transferred to thawing solution 2 (T.S.2) in 4°C that containing 0.25 molar sucrose and 100 µg mL⁻¹ melatonin. Samples after 5 min were transferred to thawing solution 3 (T.S.3) in 4°C that containing 0.125 molar sucrose and 100 µg mL⁻¹ melatonin.

Digestion 6 day old mouse testes: The cell digestion done accordance to Milazzo and his colleagues with little changed. Briefly, after removed tunica albogina, 6 day old mouse testes digested in the two steps. In the first step incubated 10 testes in 1 mg mL⁻¹ collagenase type IV and 200-700 µg mL⁻¹ DNaseI for 15 min in 37°C with slow pipetting. After centrifuge with 100×g for 5 min in the second steps discarded supernatant and resuspended cells in 1cc trypsin-EDTA (sigma) and 200 µg mL⁻¹ DNaseI for 5 min in 37°C. Trypsin inactivated with adding 10% FBS to cell suspension (Milazzo *et al.*, 2008).

Cytotoxicity assay: Lactate Dehydrogenase (LDH) enzyme released from the cytosol of damaged cells in the vitrified and thawed testes samples after digestion. The LDH activity was assessed by ELISA reader at 492 nm using the cytotoxicity detection kit according to manufactory company catalog (Roche Applied Science, Sandhofer, Mannheim, Germany).

TUNNEL assay: Apo-BrdU Tunnel Assay Kit was used to detect apoptotic cells and cell cycle (Arends *et al.*, 1990; Bortner *et al.*, 1995). The procedure was performed according to production user's (Invitrogen, Catalog Number A23210). In the brief, 1×10⁶ cells were suspended in 0.5 mL of Phosphate-buffered Saline (PBS). The cells suspension were fixed in 5 cc paraformaldehyde 1% and 5 cc ethanol 70% for 15 and minimum of 30 min on the ice, respectively. Negative and positive controls were provided by kit and fixed in ethanol 70%. Cells incubated in the DNA-labeling solution for 60 min at 37°C in a temperature controlled bath. The samples were shaken every 15 min to keep the cells in suspension. One hundred microliter of antibody staining solution added for each sample by mixing 50 µL of the Alexa Flour 488 dye-labeled anti-BrdU antibody with 95 µL of rinse buffer. Incubated the cells in this solution for 30 min at room temperature and protect from light during the incubation. Added 0.5 mL of propidium iodide/RNase a staining buffer to each sample. The cells were incubated for 30 min at room temperature and protected from light during the incubation. The samples were analyzed by flow cytometry.

Flow cytometry analysis: In the brief, cells suspension analyses were performed using the partec Flow Max. Fluorochrome were excited with 488 lasers. Green and red fluorescence were detected using FL1 and FL3 detectors, respectively. Red fluorescence also was detected using FL2 detectors. For viability, BrdU-FITC/PI, 10000 events were analyzed. FL1 and FL2 fluorescence signals were recorded with logarithmic amplification and FL3 fluorescence signals was recorded with logarithmic linear amplification. Percentage of apoptotic cells were determined by Dot plot of FL1 to FL3.

Statistical analysis: Results of treated group with melatonin and untreated group were compared using Mean-Whitney U-test and SPSS.16 Software. Results are presented as Means±SD and statistical analysis were considered significant at p = 0.008.

RESULTS

Cell cytotoxicity after vitrification: LDH test repeated 3 times for each group. The results of three repeated measurement data with ELISA reader is expressed in Table 1. According to Table 1, the mean of the measured data for freezing-thawing media with and without melatonin supplementation and high control samples are, respectively 2.33, 2.26 and 3.02. The amount of melatonin cytotoxicity is 9% based on the kit manufactory recommendation.

Flow cytometry: Result of flow cytometry assay repeated 5 times for each group. The mean of apoptotic testicular cells in the treated vitrified-thawed testes and no-treated ones were 5.2 ± 0.47 and 1.56 ± 0.62 , respectively (Table 2, 3). The statistically comparison between the mean of apoptotic testicular cells in the mentioned groups was significant ($p = 0.008$). Moreover, the mean of viable cells in the vitrified-thawed media with and without melatonin supplementations were 4.78 ± 0.46 and 8.39 ± 0.76 , respectively (Table 2, 3). The comparison mean of viable cells noted significantly ($p = 0.008$). Percentage of viable, apoptotic and necrotic cells in the freezing-thawing media without melatonin in five times repeated flow cytometry analysis presented in Table 2. Table 3 showed results of five times repeated flow cytometry analysis. Table 3 showed percentage of viable, apoptotic and necrotic cells in the freezing-thawing media supplementation with melatonin. The percentage of viable, apoptotic and necrotic cells are shown in the Fig. 1 for instance. The comparison between the vitrified-thawed media with and without melatonin supplementations, positive control and negative control were shown in the Fig. 2.

Table 1: Supplementation effect on LDH activity

Variable	Repeat			Mean
	1	2	3	
Supplementation with melatonin	2.15	2.05	2.79	2.33
Supplementation without melatonin	2.09	2.20	2.50	2.26
High control	2.84	3.26	2.96	3.02

Table 2: Percentage of viable, apoptotic and necrotic cells in the vitrified-thawed media without melatonin supplementation

Variable	Repeat					Mean±SD
	1	2	3	4	5	
Viable (%)	84.82	84.19	84.21	83.25	82.97	8.39±0.76
Apoptosis (%)	15.12	15.06	15.19	16.12	16.37	1.56±0.62
Necrosis (%)	0.62	0.71	0.57	0.61	0.65	0.63±0.02

Table 3: Percentage of viable, apoptotic and necrotic cells in the vitrified-thawed media with melatonin supplementation

Variable	Repeat					Mean±SD
	1	2	3	4	5	
Viable (%)	51.52	51.47	51.55	52.51	51.36	4.78±0.46
Apoptosis (%)	47.92	47.90	47.92	46.89	47.90	5.20±0.47
Necrosis (%)	0.55	0.62	0.52	0.59	0.73	0.60±0.03

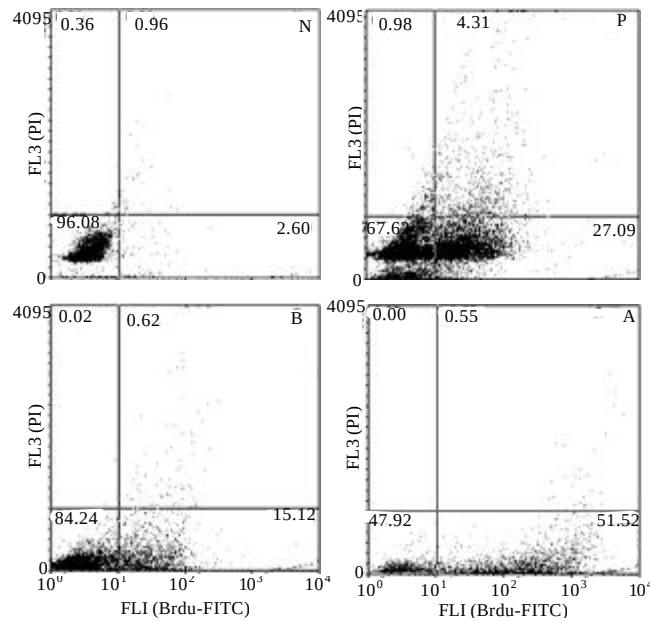


Fig. 1: Flow cytometry study. Cells were analyzed for green fluorescence (FITC) and for red fluorescence (PI) by flow cytometry. Lower left, lower right and upper right quadrant showed percentage of viable, apoptotic and necrosis cells, respectively. Neonate testicular cells that vitrified and thawed with solutions A: Supplemented with melatonin, B: Without melatonin P: Positive control and N: Negative control. Negative and positive control was supplied with Apo-BrdU Alexa flour kit

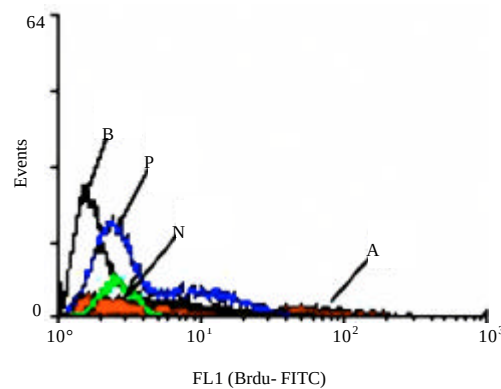


Fig. 2: Flow cytometry study. Neonate testis cells vitrified-thawed when; B: Without melatonin, A: supplemented with melatonin, N: Negative control and P: Positive control

DISCUSSION

The protection from germinal cells after testes cryopreservation have not yet yielded satisfactory results because of damage which may be as a result of free radicals of the tissue. Therefore, this problem could maybe diminished by using agents that may protect tissue during the freezing-warming period (Abrishami, 2009). In the vitrification method, cells subjected to traditional liquid

cryoprotectants (DMSO, glycerol, ethylene glycol and sugars) at high concentrations (Abrishami, 2009; Bagchi *et al.*, 2008; Bank and Brockbank, 1987; Fuller, 2004; Pegg, 2002, 2007). Exposing cells to high concentrations of cryoprotective additives can be injurious and strategies need for reduce their cytotoxicity (Abrishami, 2009; Bagchi *et al.*, 2008; Bank and Brockbank, 1987; Fuller, 2004; Li *et al.*, 2007; Nathanailides *et al.*, 2011; Pegg, 2002, 2007). High concentrations of cryoprotectants, high freezing rate, intracellular ice crystal formation, osmotic shock, solution effects, freeze-dehydration (extracellular ice formation) and scavenging oxygen free radicals are introducing as dangerous agent for the cells within the process of vitrification (Abrishami, 2009; Bagchi *et al.*, 2008; Bank and Brockbank, 1987; Fuller, 2004; Pegg, 2002, 2007). On the other hand, post-thawed induced injuries to vitrified testis tissue are major critical factors. Therefore, as some researchers mentioned, for removing of cryoprotectants, rehydrating of cells, inhibiting of intracellular ice formation and also preventing of scavenging free radicals and oxidative stress to cells, the method of thawing must be essentially optimized (Abrishami, 2009; Bagchi *et al.*, 2008; Bank and Brockbank, 1987; Fuller, 2004; Pegg, 2002, 2007). In this study, it was proposed that addition of $100\text{ }\mu\text{g mL}^{-1}$ melatonin to vitrification and thawing media may benefit for survival rate of cells in the vitrified-thawed testis tissue. Melatonin (N-acetyl-5-methoxytryptamine) was secreted by the pineal gland through the period of dark in the biological rhythm (Fildes *et al.*, 2009; Haldar and Ahmad, 2010; Hardeland *et al.*, 2011; Reiter *et al.*, 2009; Singh *et al.*, 2011). Moreover, melatonin was synthesized from other organs such as retina, testis, gastrointestinal tract, leukocytes and harderian gland (Fildes *et al.*, 2009; Haldar and Ahmad, 2010; Hardeland *et al.*, 2011; Reiter *et al.*, 2009). Melatonin is a pleiotropic molecule that represents an exceptional multiplicity of actions such as immunostimulatory, anti-oxidative, anti-apoptotic, anti-diabetic, alleviated of cryptorchidism, visceral antinociception, hepatocyte proliferation after toxin injury, endocrine function, antibiotic and antiviral properties (Abdelmeguid *et al.*, 2008; Gehad *et al.*, 2008; Fildes *et al.*, 2009; Gunduz, 2001; Haldar and Ahmad, 2010; Hardeland *et al.*, 2011; Masoud and Ghasem, 2010; AL-Rawi, 2007; Abdel-Salam *et al.*, 2006; Reiter *et al.*, 2009; Saalu *et al.*, 2006). Hemadi *et al.* (2011) showed that supplementation thawing solution with $100\text{ }\mu\text{M}$ melatonin was improved the survival rate of vitrified neonate testis. They were deduced that melatonin might improve the recovery of graft by inhibition of apoptosis and also by improving of the cell proliferation (Hemadi *et al.*, 2011). Observations of Hemadi *et al.* (2011) triggered us to adding $100\text{ }\mu\text{M}$ melatonin to vitrification and thawing media during vitrification and warming testicular tissue. However, the current results shows that using melatonin in doses of $100\text{ }\mu\text{M}$ in the vitrification solution and also thawing solution do not ameliorates of recovery of vitrified-thawed testis tissue and the healthy rate of cells in the present experiment it was observed that supplementation of vitrification and thawing solutions with melatonin led to higher cytotoxicity and higher apoptotic cells in the testicular cells treated group in compared with no treated group. The result dedicated from flow cytometry and LDH assay also confirmed cytotoxicity melatonin in the dose $100\text{ }\mu\text{M}$. Using Brdu-FITC in combination with propidium iodide can distinguish between viable, apoptotic and necrotic cells. Comparison between groups treated and no treated showed that supplementation vitrification-thawing media with melatonin induced apoptosis and reduce of viable cells. Also, the data obtained from LDH assay reported cytotoxicity 9% for melatonin. Harms *et al.* (2000) showed that melatonin don't protect neural cell death from caspase-dependent and free radical- independent apoptosis in the neural primary cultures but may protect neural cells from necrosis (Harms *et al.*, 2000). The results of Harms support of results of this article. It's worth mentioning that the differences results reported in the present study with results suggested by

Hemadi *et al.* (2011) may be because of adding melatonin to both vitrification media and thawing media whereas Hemadi *et al.* (2011) only add melatonin to thawing solution. Also may be due to differences that there are in the concentrations and type of cryoprotectant. Using of melatonin in the freeze-thaw solutions' of spermatozoa related to dose. Succu *et al.* (2011) claimed that supplementation freeze-thaw media with 1 mM melatonin ameliorate viability rates and percentage of total motile spermatozoa While Dominguez-Rebolledo *et al.* (2010) suggested that incubation of thawed spermatozoa with 1 mM melatonin ultimate to minimal protection but supplementation post-thawed media with 2.5 and 5 mM was increasingly effective. The results of Succu and Dominguez are in contradiction with results of this article. As for useful properties and dose dependency of melatonin, further studies are essential to say a final conclusion about the efficacy of different dose of melatonin match with different cryoprotectants in the thawing and freezing media. Also, well-designed, studies are needed to show the complex relationship between melatonin, vitrification and the testes.

CONCLUSION

In conclusion, data from this study indicates that melatonin do not protect vitrified-thawed induced injury in immature testis tissue. As a flow cytometry and LDH assay showed that supplementation with melatonin led to increased apoptosis.

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