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Original Article

Protective effects of melatonin solid lipid nanoparticles on testis histology after testicular trauma in rats

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Abstract

Testicular traumatic injuries occur frequently, which can result in an alteration in spermatogenesis. These injuries can also cause oxidative stress and male infertility. Antioxidant efficiency of melatonin (MLT), known as a potent antioxidant, will be improved if used in a form of solid lipid nanoparticles (MLT-SLN). The aim of the current study is to evaluate the effect of MLT-loaded SLN on traumatic testis in rats. In this study 32 adult male Wistar rats were divided into 4 groups. Group 1 (sham group), right testicle was drawn out from the scrotum and returned without manipulation. Group 2, right testicle was dropped by 25 g sinker for 4 times. Group 3, animals were received a single dose (25 mg/kg) of MLT intraperitoneally after trauma. Group 4, animals were received a single dose of MLT-SLN intraperitoneally after trauma. Under anaesthesia, rats were sacrificed, and their testicles were removed three days after the surgery. After tissue processing, the sample sections were H&E stained. MLT and MLT-SLN could partially repair spermatogenesis by Johnson's criteria but the repairs were significant only in MLT-SLN group ($P = 0.02$). Trauma decreased seminiferous tubule diameter and its epithelium height. MLT could restore epithelium height ($P \leq 0.05$) but its NPs improved both epithelium diameter ($P \leq 0.05$) and thickness ($P \leq 0.001$). The Malondialdehyde increased significantly in trauma group ($P = 0.002$), but decreased in MLT and NPs groups compared to trauma group ($P = 0.098$ and $P = 0.002$ respectively). This decrease was significant only in NPs group. Testicular trauma disturbed spermatogenesis, morphometric, and oxidative parameters. MLT and specially MLT-SLN improved traumatic damages.

Keywords: Melatonin; Nanoparticles; Rats; Testis.

INTRODUCTION

Testicular traumatic injuries occur almost frequently in urban and rural society and account nearly 1% of traumatic patients (1). These injuries can be divided into four types: penetrating, blunt, degloving, and thermal according to the mechanism of the injury (2). Blunt trauma which happens during sports and motor vehicle accidents are more common than others. Important complications of blunt trauma are testicular rupture, fracture, hematoma, hematocele, dislocation, and torsion (2,3).

Treatment depends on the type and extent of injury as well as the time lost after the trauma (4). It is reported that unilateral traumatic problems such as non-descent and torsion, affect both ipsilateral and contralateral testicular function (5).

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These effects include a decrease in spermatogenesis and seminiferous tubule diameters and chronic granulomatous changes. These events suggest infertility due to produced immunity defects in the traumatic patients (4). Male infertility is often due to increased oxidative stress associated with the damage (6). This accounts for about 30-80% of male infertility cases (7). It is known that the malondialdehyde (MDA) concentration is the marker of lipid peroxidation in damaged tissue (8).

Melatonin (N-acetyl-5-methoxytryptamine, MLT), a hormone of mammal's pineal gland which is secreted at night, is undertaken to regulate sleep-wake cycle, seasonal reproduction, and circadian rhythm (9). Although the MLT is a potent antioxidant which detoxifies hydroxyl radical, it stimulates the antioxidative enzyme glutathione peroxidase. MLT protects DNA against oxidative damage by entering into the cell nucleus and increasing the levels of cellular messenger RNA related to antioxidant enzymes (5). MLT has a direct effect on the male reproductive system and it also involves in the synthesis of testosterone from the Leydig cells (10). In addition, MLT stimulates testis growth (11). MLT has been reported to have antitumoral effects on several cancers (12). Although MLT is easily absorbed from the mucosa, it has several serious shortfalls (such as sensitivity to oxidation, high oral metabolism, and low oral bioavailability) limiting its use in the clinics. These drawbacks has led the researchers to focus on designing an efficient and proper drug delivery system alongside with new routes of administration to achieve reliable products to deliver a therapeutic dose (13).

The oil-in-water emulsions, liposomes, micro and nanoparticles based on synthetic polymers or natural macromolecules are particulate drug carriers which have been investigated for many years (14). Lipid nanoparticles are appropriate carriers for both hydrophilic and lipophilic drugs (15). Solid lipid nanoparticles (SLNs) which consist of nanosized solid lipids dispersed in an aqueous medium have all advantages of the traditional systems, without some major disadvantages. One of the main advantages of SLN over polymeric nanoparticles is its ability to be produced by high-pressure homogenization same as parenteral oil-in-water emulsions (16). In a study, animals were treated by MLT-loaded SLN (MLT-SLN) to improve the induced cardio cytotoxicity (12). Their study showed that MLT-SLN had a protective effect by an anti-apoptotic mechanism (12).

To the best of our knowledge, the effect of nanoparticles of MLT on testis histology has not yet been reported. Therefore, the aim of the present study was to prepare smart MLT nanoparticles and investigate their effects on testis histology in an experimental testicular trauma.

MATERIALS AND METHODS

Materials

MLT (Sigma-Aldrich, USA), Compritol 888 (Gattefosse, France), Tween® 80, Span® 80 (Merck Co. Germany), dichloromethane (DCM) and high performance liquid chromatography (HPLC) grade acetonitrile, methanol, and tri-ethylamine (Merck Co. Germany) were used. Distilled water was purified using a Milli-Q system (Millipore, Direct-Q). Highly ordered pyrolytic graphite (HOPG) was purchased from the Nanotechnology Systems Corporation (NATSYCO Co., Tehran, I.R. Iran).

Preparation of melatonin solid lipid nanoparticles

Several formulations were prepared as follows. A mixture of compritol 888 and Span® 80 was heated at 85 °C. Then, MLT, Tween® 80, and deionized (DI) water were homogenized using a high-shear homogenizer (D-91126 Schwabach, Heidolph, Germany). The organic phase was then added dropwise to one third of the preheated aqueous solution (85 °C) containing Tween® 80 sonicated for 5 min (BandelinSonopuls, Berlin, Germany) in order to form a coarse pre-emulsion. After sonication, the mixture was dispersed into the remaining two third of the aqueous surfactant solution which was maintained in an ice bath and was homogenized by a

high-shear homogenizer at 13,000 rpm for 7 min. The formulation was subjected to centrifugation for 20 min (HERMLE, Z36HK, Germany) at 25,000 rpm and the supernatant was removed. The sediment was withdrawn and used for injection in suitable dosage (17).

Physical characterization of the particle size and surface charge

SLNs were characterized in terms of mean particle size, polydispersity index (PI) and zeta potential (ZP) using a Zeta Sizer Nano ZSZS (Malvern Instruments, UK) at 25 °C.

Drug release measurement

The MLT release was evaluated using the dialysis tube technique. To determine the release profile of MLT from the nanoparticles, 5 mL of the prepared MT-SLN dispersion was poured into the dialysis bag (cut-off of 12,500 Da), dropped into 500 mL of the phosphate buffer at a pH of 6.8, and stirred on magnetic stirrer at 60 rpm at 37.0 ± 0.1 °C. Samples were withdrawn at predetermined time intervals of 30 min and 1, 1.5, 2, 3, 4, 6, and 24 h and replaced with fresh medium maintained at the same temperature. The samples (1.5 mL) were withdrawn, centrifuged (30 min at 25,000 rpm), filtered (pore size: 0.22 µm) and assayed by the HPLC method. The amount of MLT was determined by HPLC Agilent 1100 at 244 nm, which was equipped with the Agilent Eclipse XDB-C18 column (5 µm, 4.6 mm × 250 mm). The mobile phase, composed of 0.02 M KH_2PO_4 buffer (pH 4): methanol: tri-ethylamine (70:30:0.1 v/v), was delivered at 0.1 mL/min and the retention time of the drug was 3.8 min.

Transmission electron microscopy

Transmission electron microscopy (TEM) (Hitachi H-7500, Japan) was used for morphological evaluation and operated at 120 kV. Briefly, SLN sample was diluted two times with distilled water.

One drop of the diluted sample was placed on a 200-mesh carbon-coated copper grid, stained with 2% phosphor tungstic acid solution and dried at room temperature.

Determination of melatonin loading

Using the dialysis technique, entrapment efficiency (EE%) was measured by determining the amount of non-entrapped MLT. Four mL of MLT-SLN was put into a dialysis bag made of cellulose acetate (Spectra/PorR membranes, MW cut off 12,000-14,000) which was immersed into 100 mL of distilled water and magnetically stirred at 30 rpm to separate the non-entrapped MLT from NPs. Two mL of samples were withdrawn from the receiver solution and were replaced with the fresh water at specific time intervals. Using UV spectrophotometer at 276 nm, the samples were analyzed for MLT content. The EE %was then calculated through the following equation (13):

EE%= ((total drug - diffused drug)/total drug) × 100…… …(1)

Drug loading (DL%) was calculated by the following equation:

DL% = ((total drug - diffused drug)/examined quantity of NPs) × 100 (2)

Animals

Rats were allowed to acclimatize to the animal room for one week before the study and maintained in a standard condition of 12/12-h light/dark cycle and constant humidity (60%). Food and water were provided to them *ad libitum*. All experiments were approved by the Animal Research Centre Ethics Committee at Mazandaran University of Medical Sciences, Sari, I.R. Iran (Ethics approval code: IR.MAZUMS.REC.94.1147).

Experimental design

In this experimental study, 32 adult male Wistar rats (weighing 170-200 g) were randomly divided into four groups of 8 each. Group 1, sham: right testicle was drawn out from the scrotum and then returned without any manipulation. Group 2, trauma: after firming animals and their testis on a plate, right testicle was dropped by 25 g sinker for 4 times. Group 3, MLT plus trauma: animals received a single dose (25 mg/kg) of MLT intraperitoneally after trauma. MLT was dissolved in 1% ethanol. Group 4, MLT-SLN plus trauma: animals received a single dose of MLT-SLN intraperitoneally after trauma.

Under anaesthesia induced by ketamine (50 mg/kg) and xylazine (5 mg/kg), rats were sacrificed, and both testicles were removed

three days after the surgery. Samples were fixed in 10% formalin. After embedding in paraffin, samples were sectioned in 5 μm and stained with haematoxylin and eosin (H&E) for histological studies,

Histopathology

The tubular diameter and epithelium height of the tubules were assessed by light microscopy (10× optic lens and 40× objective lens). This measurement was performed by using calibrated OLYSIA Soft Imaging System GmbH, version 3.2 (Japan). It was tried to examine only circular and near circular tubules as much as possible. The seminiferous epithelium height was considered from basement membrane to lumen of each tubule.

Spermatogenesis assessment

In order to evaluate the spermatogenesis, Johnsen's score (a grading system for assessment of spermatogenesis) was applied and optical microscopy was used for the purpose of assessment (18). At each tubule, grades 1-10 is measurable as follows: 10, complete spermatogenesis; 9, many spermatozoa are present but disorganized spermatogenesis; 8, only a few spermatozoa are present; 7, no spermatozoa is present but many spermatids are present; 6, only a few spermatids are present; 5, no spermatozoa or spermatids are present but a lot of spermatocytes are present; 4, only a few spermatocytes are present; 3, only spermatogonia is present; 2, no germ cells are present; and 1, no germ cells or Sertoli cells are present. For each animal, 100 seminiferous tubule cross-sections were observed.

Determination of malondialdehyde

0.5 mL organ homogenates, 0.5 mL physiological solution, and 0.5 mL trichloroacetic acid 25% were mixed and centrifuged at 2000 rpm for 20 min. A mixture of 1 mL protein-free supernatant and 0.25 mL thiobarbituric acid 5% was heated at 95 ºC for 1 h. This product was cooled and the intensity of its pink colour was determined at a wavelength of 532 nm. The following equation was applied to calculate the MDA concentration (19):

$$\text{Absorbance coefficient } \varepsilon = 1.56 \times 10^5 \text{ cm}^{-1}\text{mol}^{-1} \quad (3)$$

Data analysis

Descriptive and inferential statistics were implemented to analyze the data. Results were analyzed by One-way ANOVA, followed by a post-hoc Tukey test. Using SPSS version 15, quantitative data of experimental and control groups were evaluated and a $P \leq 0.05$ was considered to be significant.

RESULTS

Nanoparticle characteristics

The particle size, zeta potential, and EE% of the developed SLNs are shown in Table 1. These results demonstrated that all formulations were able to form MT-SLNs, notably in the nano-sized range.

Table 1. Main characteristics of melatonin-loaded solid lipid nanoparticles.

NP	Formulation composition						Characteristics		
	Oil phase		Aqueous phase		Intermediate phase		Size (nm)	PDI	Zeta potential (mV)
	Compritol® 888 (mg)	Span® 80 (mg)	Tween® 80 (mg)	Water (g)	Drug (mg)	Water:ethanol (50:50) g			
F1	200	250	500	89	50	10	50.31 ± 9.31	0.575 ± 0.08	-16.0 ± -1.2
F2	400	500	1000	78	100	20	70.52 ± 11.52	0.463 ± 0.06	-12.3 ± -0.9
F3	200	250	500	88.95	100	10	75.99 ± 10.46	0.522 ± 0.04	-11.3 ±-0.8
F4	400	250	500	88.8	50	10	84.70 ± 13.28	0.390 ± 0.03	-17.4 ±-1.1
F5	400	250	500	88.75	100	10	99.31 ± 14.89	0.500 ± 0.04	-12.3 ±-1.2

NP, nanoparticle; PDI, polydispersity index.

The drug release profile of the MT-SLNs is presented in Figure 1. In this study, the MT-SLNs showed burst release behavior at the initial stage (the first 30 min) followed by a sustained release pattern. The burst release in this profile could be due to the diffusion of the drug located on the surface of SLN and thereafter from the core where MLT was homogeneously distributed in the nanoparticles because of using probe ultrasonication method and incorporation of lipid in structure of nanoparticle (20). For the best formulation, DL% and EE% were found to be 5% and 20%, respectively.

Figure 2 shows the TEM photomicrographs of MT-SLNs in optimal formulation. This figure demonstrated that the nanoparticles had smooth surfaces and were almost spherical in shape.

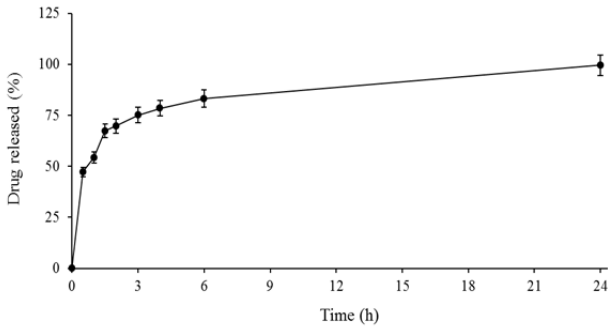

Fig. 1. The release profile of melatonin from related nanoparticles.

Evaluation of spermatogenesis

Spermatogenesis was normal in sham group but trauma caused this normal process to degenerate. The hematoma was observed in traumatic testes. Seminiferous tubules were detached and the distance between the tubules was increased. Interstitial cells were destroyed in the trauma group. MLT or its NPs could not repair these changes significantly as shown in Figure 3. Trauma (6.30 ± 1.1) was led to the degeneration of all lineages of germ cells compared with sham group (9.09 ± 0.79) by Johnson's criteria ($P < 0.001$). A single dose of both MLT (6.64 ± 1.16, $P = 0.51$) and MLT-SLN could partially repair spermatogenesis by Johnson's criteria compare to the trauma group, but these changes were significant only in MLT-SLN group (7± 1.5, $P = 0.02$).

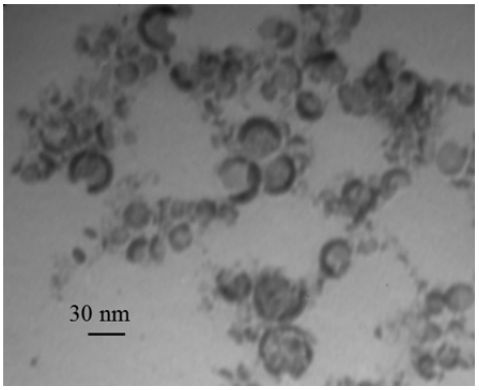

Fig. 2. Transmission electron microscopy micrographs of melatonin loaded on solid lipid nanoparticles.

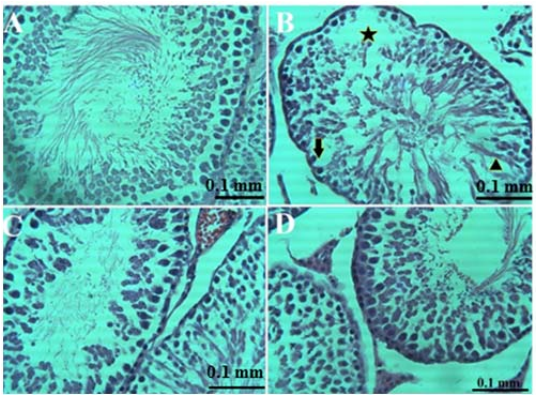

Fig. 3. Hematoxylin and eosin stained sections of testis tissue in the sham and experimental rats. (A) Nearly normal spermatogenesis is seen in the sham group. (B) Spermatogenesis is disturbed in trauma group and some degrees of germ cell detachment and sloughing (star), and vacuolization (arrow) is seen. Karyolitic cells are seen between Sertoli cells, next to the tunica albuginea (arrow head). (C) Melatonin could repair these changes to some extent. (D) Melatonin-loaded solid lipid nanoparticles also improved the spermatogenesis. (H & E. ×400). Scale bar = 100 µm.

Table 2. Testes morphometric parameters in experimental rats. Values are expressed as mean ± SD. * and ┼ indicates significant changes ($P \leq 0.05$ and $P \leq 0.001$ respectively) compared to the trauma group.

Groups	STD (µm)	SHE (µm)
Sham	470.66 ± 67.83┼	126.29 ± 19.81┼
Trauma	377.73 ± 91.15	100.28 ± 16.84
MLT	404.26 ± 70.17	108.83 ± 15.49*
MLT-SLN	426.49 ± 62.87*	111.68 ± 13.44┼

STD, Seminiferous tubule diameter; SHE, seminiferous epithelium height; MLT, melatonin; MLT-SLN, melatonin-loaded solid lipid nanoparticles.

Morphometry

According to Table 2, trauma has decreased the seminiferous tubule diameter and its epithelium height. MLT could restore epithelium height, but it has no effect on its diameter significantly. MLT-SLN has improved both epithelium thickness and seminiferous tubule diameter in traumatic rats.

Malondialdehyde result

The MDA concentration (lipid peroxidation index) was increased significantly in trauma group (7.35 ± 0.5) µg/100 mL compared to the sham group (4.96 ± .93) ($P = 0.002$). It was decreased in MLT (6.06 ± 0.7) and MLT-SLNs groups (4.97 ± 0.61) compared to the trauma group ($P = 0.098$ and $P = 0.002$ respectively). This decrease was significant only in MLT-SLNs group.

DISCUSSION

In this study, grade I injury was induced in right testis by blunt trauma. A standard grading system was described as follows. In Grade I injury, tunica albuginea was intact and intra-testicular haemorrhage was seen. In grade II, hematocele with lacerated tunica albuginea was observed. Multiple lacerations of the tunica with ruptured testis denote grade III. In grade IV injury, the testis was completely destroyed and it was nonviable (5).

Srinivas *et al* induced grade I injury to testis of the rats by dropping 5 g weights 3 times. After 60 days, germ cell maturation was significantly disturbed in both ipsilateral and contralateral testis (5). The results obtained in the present study were in agreement with the results published by Srinivas *et al* (5).

In this study, grade I injury caused a decrease in the diameter of the tubules. The decreased seminiferous tubule diameter indicates germ cell loss whereas an increased diameter may be due to fluid retention as a result of impaired emptying of tubules (21). Several studies stated that the same stress to testis could injure the germ cells and alter the seminiferous tubule morphometric parameters (21-24). Based on Johnsen's criteria, it was shown that MLT and MLT-SLN partially repaired spermatogenesis, which had disturbed by trauma, and MLT-SLN was more effective.

SLN formulation seems to be a desired sustained release drug for a substance with unfavorable kinetics such as MLT. It has been shown that significant plasma levels of MLT were maintained for a longer period of time after oral MLT-SLN administration compared to oral MLT administration (25). Aitken *et al.* reported that the peroxidative damage is the main reason for testicular function deficiency from testicular torsion to diabetes and xenobiotic exposure (26). MLT is a potent antioxidant and a free scavenger. Several models have shown that MLT could be protective against lipid peroxidation (9).

In both *in vitro* and *in vivo* studies, it was demonstrated that MLT-loaded Eudragit® S100 nanocapsule suspensions improved the antioxidant effects of MLT (9). MLT suppresses apoptosis by scavenging free radicals, or by affecting melatonin's receptors within the cells. MLT-SLN showed more antioxidative effects than MLT per se. MLT-SLN may involve a cellular internalization pathway that induces a more effective antiapoptotic process than MLT itself (12). The same effect of MLT is seen on the epithelium thickness and seminiferous tubule diameter. In a comparable study to the present work, Koksal *et al.* tested the effect of 10 mg/kg MLT 10 min prior to

1-h ischemia and indicated that MLT could improve histopathological changes and increase Johnson score. Also, they demonstrated that MLT treatment could restore abnormal sperm rate to normal (27). Similarly, we indicated that MLT and specially MLT-SLN elevated the Johnson score. MDA, total antioxidant status, and total oxidative status levels were not significantly changed in the aforementioned research and MLT had not changed the oxidative factors (27). However, MLT or MLT-SLN could not repair tubular detachment or changes in integration and interstitial cells in the current study. Probably, a longer duration of MLT or MLT-SLN treatment is needed to obtain the same results. Ozkan *et al.* used zinc aspartate pretreatment in a 4-h induced unilateral testicular ischemia/reperfusion injury to assess its effects as an antioxidant on the testis. All histological and biochemical parameters were improved after antioxidant therapy (28). In the present study MLT (as an antioxidant) improved several histological and biochemical parameters. In a research conducted by Yurtçu *et al.*, animals were treated with 17 mg/kg MLT 15 min before testicular torsion/detorsion trauma. MLT improved the MDA and raised the Johnsen's criteria (29).

In 2008, Kurcer *et al.* induced testicular ischemia to experimental animals by clamping of testicular vessels for 1 h. They treated the animals with a single dose of 10 mg/kg MLT, 10 min before ischemia. Ischemia resulted in abnormal sperm rate, but melatonin could restore this change. MDA was unchanged among the group (30). Their study, similar to the findings of Koksal *et al*. (27), was in contrary to the present work, which showed an elevated MDA levels in trauma group and a significant decrease in treatment groups. It may be due to the type of trauma and the dose of MLT which were not the same (10 mg/kg as opposed to 25 mg/kg after trauma). Contralateral testes (left) showed normal tissue structure in the mild induced trauma. It may cause different results in more severe trauma.

CONCLUSION

Testicular mild trauma disturbed the spermatogenesis, morphometric, and oxidative parameters. MLT, as an antioxidant, could repair these changes to some extent. A decreased MDA in MLT-SLN group ascertains that this nanoparticle improves the antioxidative property of MLT such that it could repair the traumatic damage more effectively compared to MLT.

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