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ORIGINAL ARTICLE

Melatonin attenuates diabetes-induced oxidative stress in spleen and suppression of splenocyte proliferation in laboratory mice

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ABSTRACT

Hyperglycaemic condition induced oxidative stress in diabetic individuals caused oxidative damages of internal organs, including immune organ spleen. We studied the effects of low doses of melatonin (25, 50, and 100 μg/100g. B.wt./day) on histoarchitecture, oxidative stress, and splenocyte proliferation in streptozotocin-induced diabetic mice. Melatonin significantly resisted the increase in blood glucose levels and showed a dose-dependent effect on circulatory melatonin, body weight, and relative spleen weight in diabetic mice. Exogenous melatonin suppressed the diabetes-induced lipid peroxidation and increased the activity of the antioxidant enzymes and antioxidant GSH in the spleen tissue of diabetic mice in a dose-dependent manner. Melatonin improved the reactivity of Nrf-2 and HO-1 in the spleen of diabetic mice. Melatonin treatment normalised the splenic cellularity and increased the splenocyte proliferation in a dose-dependent manner. The present study may suggest the dose-dependent effect of melatonin in attenuation of oxidative stress and suppression of splenocyte proliferation in diabetic mice.

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KEYWORDS
Melatonin; oxidative stress; antioxidant enzymes; splenocyte proliferation; diabetes

Introduction

The imbalance between reactive oxygen species (ROS) generation and antioxidants system results in oxidative stress in an organism. The excessive production of ROS caused damages of membrane lipids, DNA, and proteins and induced apoptosis of the cell (Su *et al*. 2019). Oxidative damages lead to a vast number of diseases such as atherosclerosis, inflammatory response, hypertension, heart diseases, neurodegenerative diseases, cancer, diabetes, etc. (Liguori *et al*. 2018). Type 1 diabetes mellitus (T1DM) is an autoimmune disease where the immune system has been directed to destroy the pancreatic β-cells, which secrets insulin. Diabetes is the most prevalent disease affecting the majority of the population across the world. In diabetes, high circulating glucose leads to many health complications and affects many organ & organ systems. Prolonged maintenance of hyperglycaemic conditions accelerates the production of reactive oxygen species (ROS), thereby promoting oxidative stress (Ullah *et al*. 2016). Reports suggested that hyperglycaemic effects mainly occur due to oxidative stress (Das and Sil 2012, Kayama *et al*. 2015). Persistence of a high level of blood glucose in human beings caused malfunction of the immune system (Geerlings and Hopelman 1999).

Melatonin is an indoleamine hormone produced by the pineal gland. Melatonin influenced the physiological activities in living organisms such as sleep improvement (Srinivasan *et al*. 2009, van Geijlswijk *et al*. 2010) and immune modulation (Carrillovico *et al*. 2006). It exerts most of the biological effects through the mediation of its MT1 and MT2 membrane receptors. Further, melatonin readily diffuses through the lipid barrier of the plasma membrane and serves as a broad-spectrum antioxidant (Guerrero and Reiter 2002). Melatonin effectively combats oxidative stress and saves the organism from damage induced by toxic radicals (Lobo *et al*. 2010). In mammals, the spleen is the largest secondary immune organ. Splenic tissue hosts different types of cells (T and B lymphocytes, dendritic cells, and macrophages) related to various immune activity. It works by re-circulating lymphocytes, which can promptly bring out specific T or B lymphocyte-mediated immune reactions (Ebaid *et al*. 2015) and also by clearing circulating apoptotic cells (thus contributing to peripheral immune tolerance) (Cesta 2006). The excessive generation of ROS caused damage to spleen tissues. Melatonin protects an organism from oxidative stress by direct neutralisation of ROS and indirectly by stimulating the antioxidant enzyme activities (Jaworek *et al*. 2017). Reports are suggesting that melatonin provides protection from oxidative stress and modulates the immune function in studied organisms. But limited reports are suggesting the effects of melatonin on diabetes-induced devastating effects on immune organs. Therefore, in the present study, we have attempted to observe the effect of melatonin on oxidative stress and splenocyte proliferation in experimentally induced diabetic mice.

Methodology

All of the experiments with animals and their maintenance have been done according to the institutional practice and with the framework of CPCSEA (Committee for the Purpose

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of Control and Supervision of Experimental Animals) and the Act of Government of India (2007) for the animal welfare.

Animal model

Healthy Swiss albino mice colonies were housed in an ambient standard laboratory condition of light (12 h L:12 h D), temperature (25 ± 2 °C), and humidity (55 ± 5%). Mice were kept in groups of five in polycarbonate cages (43 cm × 27 cm × 14 cm) and fed with mice feed and water *ad libitum*. Healthy male mice of 25 g body weight were selected for the experiment.

Induction of diabetes

Experimental diabetes was induced by intraperitoneal administration of multiple low doses of Streptozotocin (STZ). STZ was dissolved in freshly prepared 0.1 M citrate buffer (pH = 4.5). Mice were kept for fasting for 20 h prior to STZ administration. 50 mg/kg of STZ was given to experimental mice for five consecutive days (Mishra *et al*. 2018). A 10% sucrose solution was given to experimental mice during STZ treatment. The blood glucose level of mice was checked on every 2nd day after last STZ administration. Mice with blood glucose levels above 250 mg/dL were considered as diabetic mice.

Experimental design

Diabetic mice were randomly divided into four groups, with five mice in each group. Diabetic group (DB) of mice were received STZ only. Diabetes + Melatonin 25 (DM25) group of mice have received melatonin (25 μg/100 gm Body wt.) after induction of diabetes. Diabetes + melatonin 50 (DM50) group of mice was given melatonin (50 μg/100 gm Body wt.) after induction of diabetes. Diabetes + melatonin 100 (DM100) group of mice was given (100 μg/100 gm Body wt.) after induction of diabetes. Melatonin supplementation to experimental mice was done for 15 consecutive days. Mice of control (Con) group were received ethanolic saline (0.01% ethanol). Mice were sacrificed after the 15th day of the melatonin treatment. The trunk blood was collected for hormonal analysis, and spleen was dissected out. Bodyweight and spleen weight were recorded. Each spleen was divided into three parts. One part of the spleen was fixed in Bouin's fluid, another part of the spleen was processed for analysis of oxidative stress biomarkers, and the third part was processed for the study of splenocyte proliferation index.

Blood glucose determination

Blood glucose was determined with the help of the ACCU-CHEK Active blood glucose monitoring system.

Determination of circulatory melatonin and insulin level

Circulatory level of melatonin was determined by the Melatonin ELISA kit (IBL International, Hamburg, Germany), and insulin level was determined by mouse-specific insulin ELISA kit (Fine Test, Wuhan, China) following manufactures instructions of the assay. For melatonin, specificity was 99%, recovery was 102.4%, and sensitivity was 1.6 mg/ml. For insulin, specificity was 99.9%, recovery was 94%, and sensitivity was 46.9 pg/ml.

Histology

The spleen of experimental mice was fixed in Bouin's fixative overnight and processed for paraffin block preparation and sectioning. 5 μm thick sections were stretched on Mayer's albumin coated slide. Spleen sections were stained by routine Haematoxylin-Eosin double staining methods. Stained sections were observed in Olympus BX-41 Microscope, and micrographs were taken under 10X objective.

Malondialdehyde (MDA) assay

Malondialdehyde is a product of lipid peroxidation and was measured on the basis of its reaction with thiobarbituric acid (TBA) following the method of Ohkawa *et al*. (1979). 10% homogenate of spleen was prepared in phosphate buffer. 0.1 ml of tissue homogenate was mixed with 3.3 ml of TBA reagent (containing 8% SDS, 20% acetic acid (pH 3.5), 0.8% TBA and 0.8% butylated hydroxyl-toluene). Reaction mixtures were boiled, and the optical density of supernatant was determined at 532 nm. Lipid peroxidation was expressed in nmol TBARS formed/mg protein of experimental tissues.

Superoxide dismutase (SOD) activity assay

Superoxide dismutase (SOD; EC 1.15.1.1) activity in the spleen of experimental mice was determined by following the method of Das *et al*. (2000). 10% homogenates of the spleen tissues were prepared in phosphate-buffered saline (pH = 7.4). 0.1 ml of the homogenate was mixed with 1.4 ml of the reaction mixture (containing 50 mM phosphate buffer (pH 7.4), 20 mM L-methionine, 1% Triton-X-100, 10 mM hydroxylamine hydrochloride, 50 μM EDTA). 50 μM of riboflavin was added to the mixture and exposed to a 20 W fluorescence lamp. 1 ml of Griess reagent was added and optical density was determined at 543 nm. One unit of enzyme activity is defined as the amount of SOD inhibiting 50% of nitrite formation under assay conditions and was expressed as SOD activity in U/g tissue weight.

Catalase (CAT) activity

Catalase (CAT; EC 1.11.1.6) activity in the spleen of experimental mice was determined by following the method of Sinha (1972), modified by Hadwan (2016). 10% homogenate of the spleen was prepared in phosphate buffer (pH = 7.4) and centrifuged. The supernatant was added to a reaction

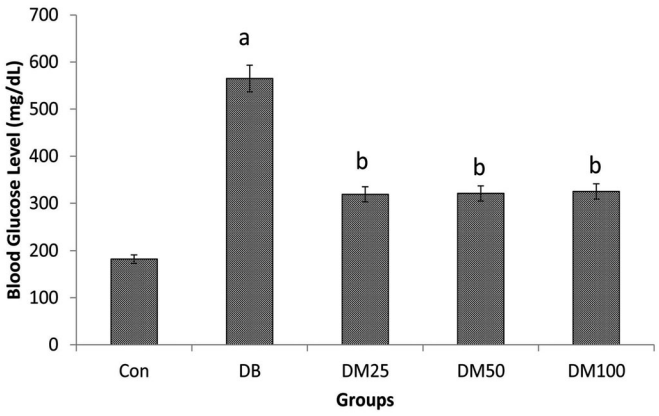

Figure 1. Effect of melatonin treatment on the blood glucose levels in diabetic mice. Histograms represent Mean ± SEM, $n = 5$ for each group. Con = control, DB = diabetic, DM25 = diabetic mice received melatonin 25 μg/mice/day, DM50 = diabetic mice received melatonin 50 μg/mice/day, DM100 = diabetic mice received melatonin 100 μg/mice/day. (a) $p < .01$, Con vs DB; (b) $p < .01$, DB vs DM25, DB vs DM50, DB vs DM100.

mixture containing H_2O_2 and potassium dichromate and boiled in a water bath and centrifuged. The optical density of supernatant was determined at 570 nm and the decrease in the H_2O_2 content was calculated. The activity of CAT was expressed as the amount of H_2O_2 degraded per minute.

Reduced glutathione (GSH) level

Reduced glutathione (GSH) was determined following the methods of Ellman (1959), modified by Gupta *et al.* (2003). 10% homogenate of spleen was prepared in phosphate buffer (pH = 7.4) and mixed with 20% of trichloroacetic acid in 1:1 ratio and centrifuged. 200μl of supernatant was mixed with 1.8 ml of Ellman reagent (containing 1% sodium citrate and 0.04% DTNB in 0.1 M phosphate buffer (pH = 8.0)). The optical density of the mixture was determined at 412 nm. The concentration of reduced glutathione was expressed as μg/g tissue.

Immunohistochemistry of Nrf2 and HO-1

Immunohistochemical study of Nuclear factor erythroid-2-related factor 2 (Nrf2) and Haem oxygenase – 1 (HO-1) in the spleen was done following the modified methods of Singh *et al.* (2017). 5 μm-thick paraffin sections were mounted on 3% gelatin-coated slides. Tissue sections were deparaffinised and rehydrated with alcohol grades. The sections were placed in phosphate-buffered saline (PBS) for 30 min, and endogenous peroxide activity was blocked by 0.3% H_2O_2 in methanol for 30 min at room temperature. Sections were washed thrice with PBS and placed in blocking solution (horse blocking serum, diluted 1:100 in PBS, PK-6200, Vector Laboratories, Burlingame, CA) followed by incubation with primary antibodies (Nrf2; ab31162, Ho-1; ab31163, rabbit polyclonal, Abcam, Cambridge, MA, diluted 1:100) overnight at 4 °C. Sections were washed thrice with PBS and were incubated with biotinylated secondary antibody (Vectastain ABC Universal Kit, PK-6200, Vector Laboratories, Burlingame, CA, dilution 1:1000). Sections were washed thrice with PBS and incubated with preformed AB complex reagent for 30 min. The immune interactions were visualised using the 0.03% peroxidase substrate 3,3-diaminobenzidine (DAB; Sigma–Aldrich Chemicals, St. Louis, MO) and counterstained with Ehrlich's haematoxylin. Sections were dehydrated and mounted with DPX. Microphotographs of the stained sections were taken under 10× objective in Olympus BX-41 Microscope. To test the specificity of the used antibodies, the primary antibody was not added in the control sections. The control sections were incubated with the same dilution of normal serum overnight at 4 °C, and the following morning, the immunohistochemical protocol was followed under the same condition. The intensity of immune reactivity was quantified by Image J software.

Splenocytes proliferation

The spleen of experimental mice was dissected out on chilled PBS and processed for preparation of a single-cell suspension of splenocytes. Erythrocytes of splenic cell suspension were lysed with 1:10 solution of cold 0.5% Tris and 0.84% NH_4Cl (pH 7.2). The cell suspension was washed thrice with chilled PBS. Cell viability was determined by trypan blue exclusion method. Viable cell (which exceeded 95%) number was adjusted to 1×10^7cells/ml in culture medium (RPMI-1640 medium supplemented with Streptomycin (500 μg/ml), Penicillin (5000 U/ml), L-glutamine (2 mM/ml), 0.1% 2-mercaptoethanol (5×10^{-2} mM/ml) and 10% FCS). 100 μl splenocytes suspension was added to the wells of sterile flat bottom 96 well culture plates. Mitogen concanavalin-A (Con A; T-cell mitogen; Sigma-Aldrich Chemicals, St. Louis, MO) solution was prepared at the concentration of 5 μg/ml in the culture medium. 50 μl mitogen solution was added to the wells containing splenocytes suspension and yielded a volume of 150 μl/well (in duplicate). Finally, a volume of 200 μl/well was made by adding complete culture media in each well of the culture plate. The culture plate was incubated in a humidified 5% CO_2 containing chamber at 37 °C for 44 h. 20 μl of MTT solution (3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide, SRL, Mumbai, India, 5 mg/ml in PBS) was added in each well and incubated at 37 °C in 5% CO_2 for additional 4 h. After 4 h 100 μl of acidified propanol (0.04 mol/l HCl in isopropanol) was added in each well and the optical density was determined with a microplate reader (ECIL, India) at 570 nm wavelength (Ahmad and Haldar 2010). Mean OD values for each set of duplicates were used in subsequent statistical analysis. The response was calculated as a percent (%) stimulation index representing the ratio of absorbance of the mitogen-stimulated cultures to control cultures.

Statistical analyses

Statistical analysis of the data was performed with one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) multiple range test. Student *t*-test was performed to test the differences between different doses of melatonin. The differences were considered

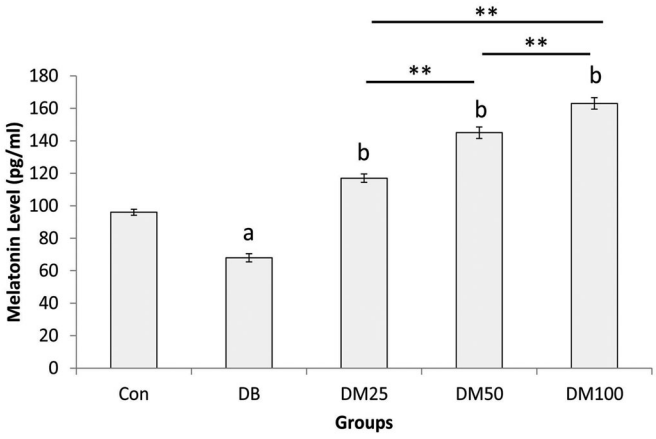

Figure 2. Dose-dependent effect of exogenous melatonin on circulatory melatonin level in diabetic mice. Histograms represent Mean ± SEM, $n = 5$ for each group. Con = control, DB = diabetic, DM25 = diabetic mice received melatonin 25 μg/mice/day, DM50 = diabetic mice received melatonin 50 μg/mice/day, DM100 = diabetic mice received melatonin 100 μg/mice/day. (a) $p < .01$, Con vs DB; (b) $p < .01$, DB vs DM25, DB vs DM50, DB vs DM100. Student *t*-test, $^{**}p < .01$.

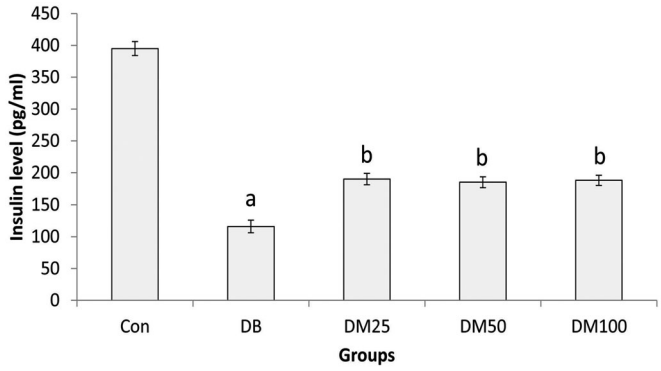

Figure 3. Dose-dependent effect of exogenous melatonin on circulatory insulin level in diabetic mice. Histograms represent Mean ± SEM, $n = 5$ for each group. Con = control, DB = diabetic, DM25 = diabetic mice received melatonin 25 μg/mice/day, DM50 = diabetic mice received melatonin 50 μg/mice/day, DM100 = diabetic mice received melatonin 100 μg/mice/day. (a) $p < .01$, Con vs DB; (b) $p < .01$, DB vs DM25, DB vs DM50, DB vs DM100.

significant when $p < .05$. Statistical Package for the Social Sciences (SPSS) and Microsoft Excel program was used for calculation and graph preparation.

Results

Effect of melatonin on blood glucose level in diabetic mice

Different doses of melatonin have shown effective consequences in lowering the blood glucose level of diabetic mice. Significantly ($p < .01$) decreased blood glucose level was observed after 15 days of treatment in the diabetic mice supplemented with different doses of melatonin as compared to the diabetic group (Figure 1). However, blood glucose level was not dropped near to control level; rather, melatonin maintained a significantly low level of blood glucose in comparison with diabetic mice.

Effect of melatonin on circulatory melatonin and insulin level in diabetic mice

Induction of diabetes significantly ($p < .01$) suppressed the circulatory level of melatonin in diabetic mice. Melatonin supplementation to streptozotocin treated mice significantly ($p < .01$) increased the circulatory melatonin in comparison with diabetic mice (Figure 2). The successive increases in melatonin dose were significantly ($p < .01$) increased the circulatory melatonin level in diabetic mice. Streptozotocin caused the destruction of β-cells in the pancreatic islets. Therefore, a sharp decline in circulatory insulin was noted in the diabetic group of mice. Melatonin supplementation to streptozotocin treated mice significantly ($p < .01$) increased the insulin level but could not restore to normal level (Figure 3). The successive increase in melatonin dose could not bring any dose-dependent change in the circulatory melatonin level.

Effect of melatonin on body weight and spleen weight of diabetic mice

Diabetic caused a significant ($p < .01$) decrease in body weight and spleen weight of experimental mice in comparison with control mice (Figure 4). Melatonin supplementation to diabetic group increased the body and spleen weight. Diabetic mice showed a significant ($p < .01$) decrease in relative spleen weight in comparison with control mice. Different doses of melatonin administered to diabetic mice improved the relative spleen weight in comparison with diabetic mice (Figure 5).

Effect of melatonin on histological observation

Histological observation showed well-differentiated splenic follicles in the cortex and medullar region in the spleen of control mice. Undifferentiated splenic follicles were observed in the spleen of diabetic mice. Fibrous tissue penetration disintegrates the cellularity of splenocytes in the splenic follicles. The melatonin supplementation in all doses prevented diabetes caused deterioration of cellularity in the splenic follicles of experimental mice (Figure 6).

Effect of melatonin on lipid peroxidation (MDA level)

MDA is a reliable marker for lipid peroxidation, was measured in terms of nmol TBARS formed per mg protein of spleen. Development of diabetes in mice significantly ($p < .01$) increased the MDA level in the splenic tissue in comparison with the spleen of control mice. Administration of different doses of melatonin significantly ($p < .01$) decreased the MDA level in diabetic mice, whereas M100 dose was most effective in reducing the MDA level in studied mice (Figure 7).

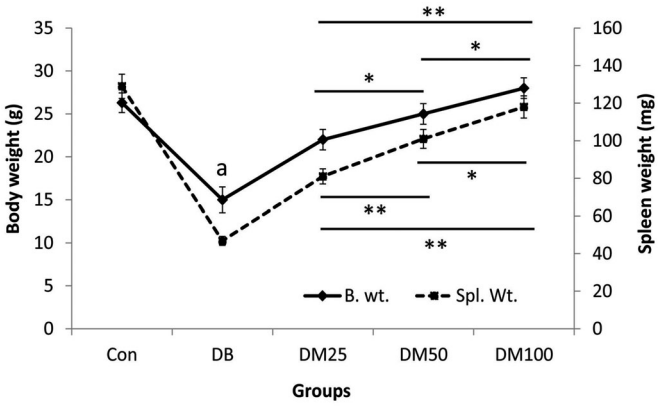

Figure 4. Dose-dependent effect of melatonin treatment on body weight and spleen weight in diabetic mice (Mean ± SEM, $n = 5$ for each group). Con = control, DB = diabetic, DM25 = diabetic mice received melatonin 25 μg/mice/day, DM50 = diabetic mice received melatonin 50 μg/mice/day, DM100 = diabetic mice received melatonin 100 μg/mice/day. (a) $p < .01$, Con vs DB; Student *t*-test, $^{*}p < .05$; $^{**}p < .01$.

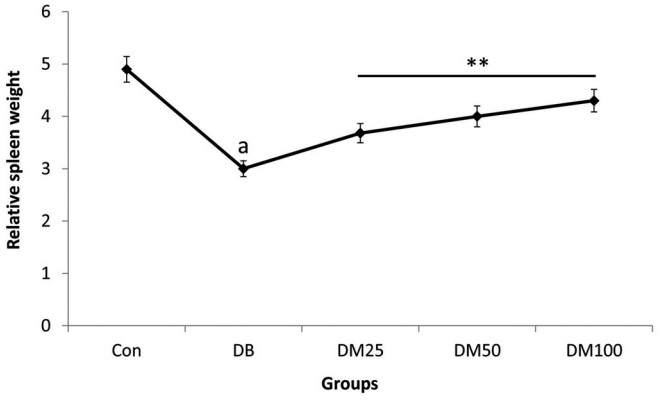

Figure 5. Dose-dependent effect of melatonin treatment on relative spleen weight in diabetic mice (Mean ± SEM, $n = 5$ for each group). Con = control, DB = diabetic, DM25 = diabetic mice received melatonin 25 μg/mice/day, DM50 = diabetic mice received melatonin 50 μg/mice/day, DM100 = diabetic mice received melatonin 100 μg/mice/day. (a) $p < .01$, Con vs DB; Student *t*-test, $^{**}p < .01$.

Effect of melatonin on superoxide dismutase (SOD) enzyme activity

SOD enzyme is an important antioxidant enzyme that responsibly neutralises superoxide anion formed in the living tissues. Significant ($p < .01$) decrease in SOD activity was noted in streptozotocin administered mice in comparison with the spleen of control mice (Figure 8). All the doses of melatonin treatment elevated the SOD enzyme activity significantly ($p < .01$) in comparison with the spleen of the diabetes group of mice. M100 dose of melatonin was most effectively increased the SOD enzyme activity in the spleen of diabetic mice.

Effect of melatonin on catalase enzyme activity

Catalase is an essential antioxidant enzyme that responsibly neutralises the hydrogen peroxide formed in the living tissues. Induction of diabetes significantly ($p < .01$) suppressed the catalase activity in the splenic tissues in comparison with the spleen of control mice. All doses of melatonin-supplemented to diabetic mice significantly ($p < .01$) increased the catalase enzyme activity in comparison with diabetic mice. All the doses of melatonin have increased the catalase enzyme activity. The dose of M100 was most effective in increasing the catalase activity in splenic tissue of diabetic mice (Figure 9).

Effect of melatonin on reduced glutathione level

Glutathione is an important antioxidant and by donating an electron, it reduces hydroperoxides to their corresponding alcohols in living tissues. Streptozotocin treatment significantly ($p < .01$) decreased the reduced glutathione level in the spleen tissue in comparison with the spleen of control mice. All doses of melatonin supplementation to diabetic mice significantly ($p < .01$) increased the reduced glutathione level in comparison with the spleen of the diabetes group. Though all melatonin doses were effective, the dose of M100 was the most effective in increasing the GSH level in the spleen of diabetic mice (Figure 10).

Effect of melatonin on immune reactivity of Nrf2 and HO-1

The nuclear factor erythroid 2-related factor 2 (Nrf2) protein is the key regulator of the expression of antioxidant proteins that provide protection against oxidative radicals. Normal reactivity of the Nrf2 antiserum was noted in the spleen of the control mice (Figure 11). Significant ($p < .01$) decreased reactivity of Nrf2 antiserum was noted in the spleen of the streptozotocin treated mice. Significantly ($p < .01$) strong reactivity of Nrf2 antisera was noted in the spleen of melatonin treated mice. Strongest Nrf2 reactivity was noted in the spleen of the M100 treated diabetic mice group.

Haem oxygenase-1 (HO-1) is one of the genes regulated through Nrf2 in the mammalian system. Reactivity of HO-1 antiserum was noted throughout the spleen in control mice. Significant ($p < .01$) decreased reactivity of the HO-1 antiserum was noted in the spleen of diabetic mice. Significantly ($p < .01$) strong reactivity of HO-1 antiserum was noted in the spleen of melatonin-supplemented mice. HO-1 antiserum reactivity was strongest in the spleen of M100 supplemented mice (Figure 12).

Effect of melatonin on splenocyte stimulation index (%SR)

Splenocyte stimulation index is a measurement of T cell proliferative response. Splenocyte stimulation index was significantly ($p < .01$) decreased in the case of diabetic mice compared to control mice. All doses of the melatonin supplementation to diabetic mice significantly ($p < .01$) increased the splenocyte stimulation index in comparison with the diabetic mice. Successive doses of melatonin significantly ($p < .01$) increased the splenocyte stimulation index. The M100 dose of melatonin maximally increased the stimulation index of splenocytes among all melatonin-supplemented doses to diabetic mice (Figure 13).

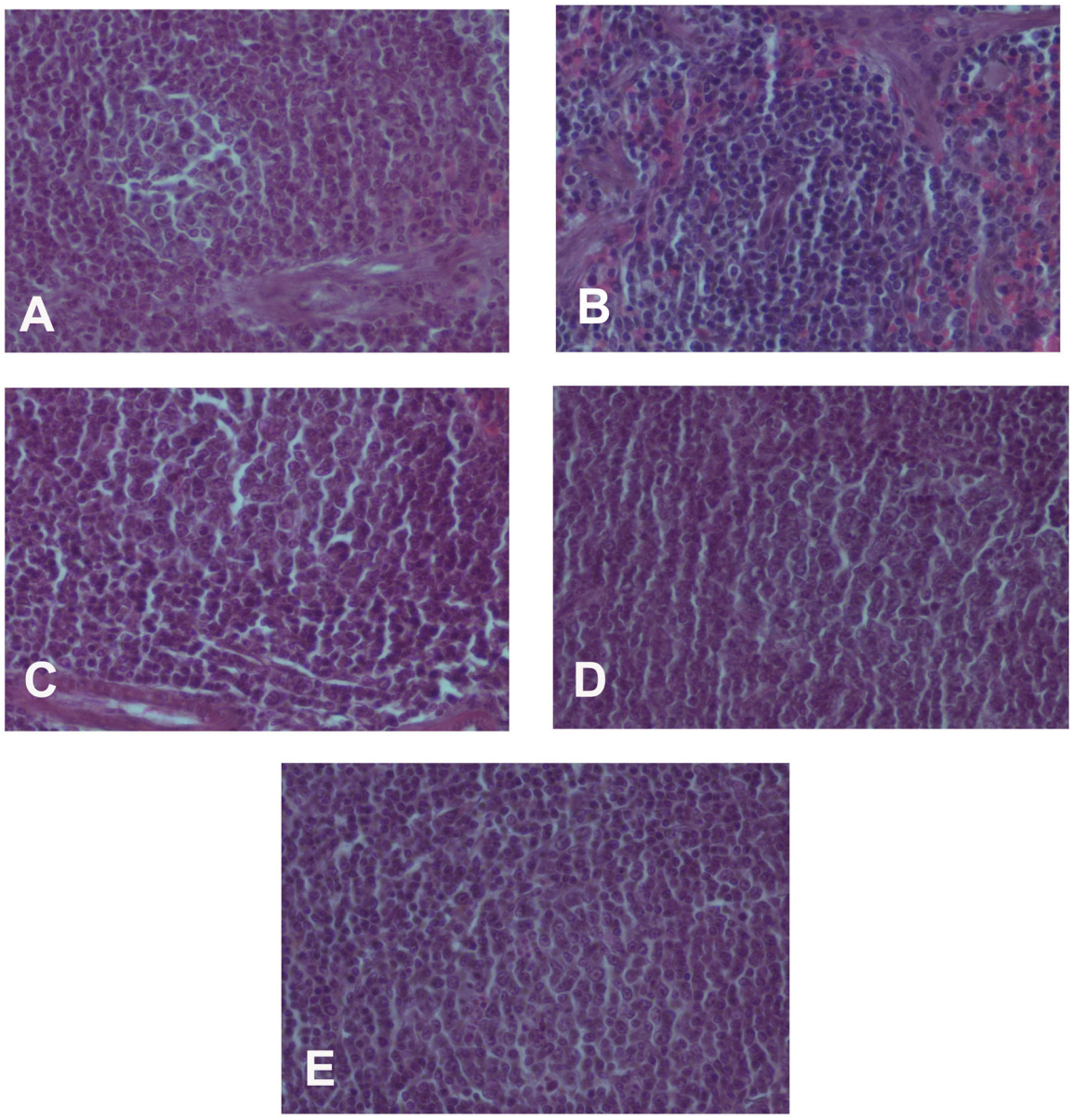

Figure 6. Dose-dependent effect of melatonin treatment on histoarchitecture of spleen in diabetic mice (Mean ± SEM, $n = 5$ for each group). (A) control, (B) diabetic, (C) diabetic mice received melatonin 25 μg/mice/day, (D) diabetic mice received melatonin 50 μg/mice/day, (E) diabetic mice received melatonin 100 μg/mice/day.

Discussion

Type 1 diabetes is a chronic autoimmune disease characterised by the prevalence of hyperglycaemic conditions. Streptozotocin is a toxic substance caused the destruction of pancreatic beta cells, which leads to a failure of insulin production in experimental animals. The absence of insulin results in the persistence of a high blood glucose level in the experimental animal, which reflects the physiological status of type 1 diabetes (King 2012). Experimental mice having blood glucose above 250 mg/dl were considered diabetic mice (Mabley *et al*. 2003). In the present study, streptozotocin administration continuously increased the blood glucose level in mice. All the doses of melatonin showed suppression of the blood glucose levels in mice. Studies suggested that inosine protected multiple low dose streptozotocin-induced hyperglycaemia in dose-dependent manner (Mabley *et al*. 2003).

The circulatory melatonin protects the organisms from various stressful conditions. The induction of diabetes caused suppression of circulatory melatonin levels and caused devastating effects in the experimental mice (Hajam and Rai, 2019). Melatonin supplementation minimises diabetes-induced stress by increasing the circulatory melatonin levels in a dose-dependent manner. Streptozotocin destroyed the pancreatic islets, thereby minimised insulin secretion from the pancreas. The low insulin level causes retardation of glucose uptake by the liver and other cells and leads the persistence of enhanced blood glucose level, pathologically diabetes. Exogenous melatonin improved the streptozotocin caused stress on pancreatic islets but could not restored the circulatory insulin level. Study suggested that melatonin was

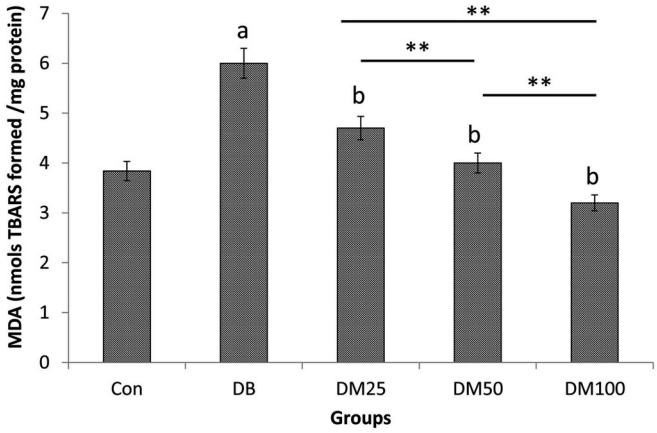

Figure 7. Dose-dependent effect of melatonin on the lipid peroxidation (MDA level) in the spleen of diabetic mice. Histograms represent Mean ± SEM, $n = 5$ for each group. Con = control, DB = diabetic, DM25 = diabetic mice received melatonin 25 μg/mice/day, DM50 = diabetic mice received melatonin 50 μg/mice/day, DM100 = diabetic mice received melatonin 100 μg/mice/day. (a) $p < .01$, Con vs DB; (b) $p < .01$, DB vs DM25, DB vs DM50, DB vs DM100. Student *t*-test, $^{**}p < .01$.

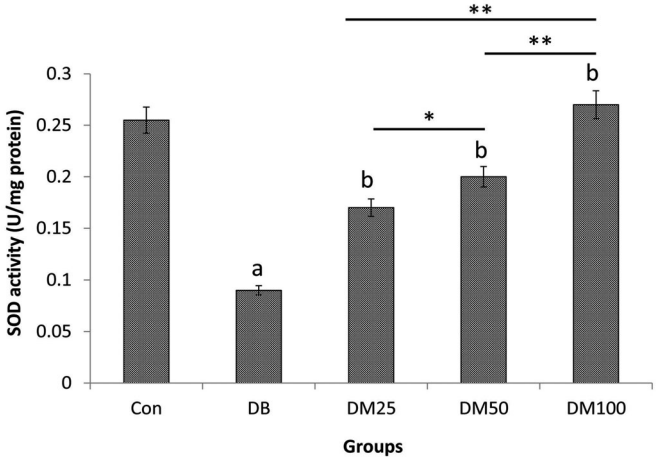

Figure 8. Dose-dependent effect of melatonin on antioxidant enzyme superoxide dismutase (SOD) activity in the spleen of diabetic mice. Histograms represent Mean ± SEM, $n = 5$ for each group. Con = control, DB = diabetic, DM25 = diabetic mice received melatonin 25 μg/mice/day, DM50 = diabetic mice received melatonin 50 μg/mice/day, DM100 = diabetic mice received melatonin 100 μg/mice/day. (a) $p < .01$, Con vs DB; (b) $p < .01$, DB vs DM25, DB vs DM50, DB vs DM100. Student *t*-test, $^{**}p < .01$.

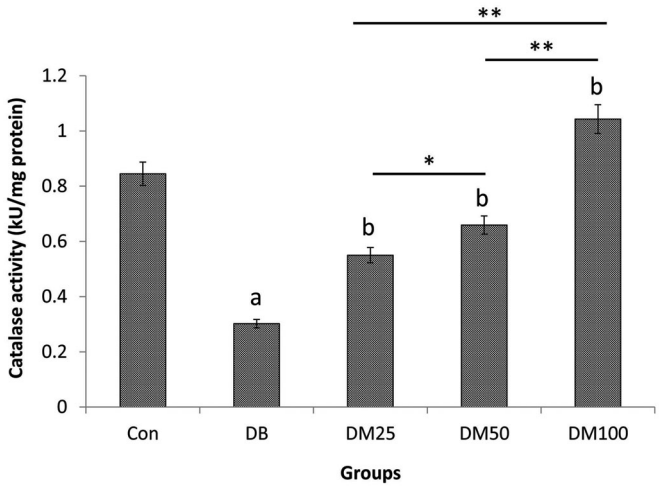

Figure 9. Dose-dependent effect of melatonin on antioxidant enzyme catalase (CAT) activity in the spleen of diabetic mice. Histograms represent Mean ± SEM, $n = 5$ for each group. Con = control, DB = diabetic, DM25 = diabetic mice received melatonin 25 μg/mice/day, DM50 = diabetic mice received melatonin 50 μg/mice/day, DM100 = diabetic mice received melatonin 100 μg/mice/day. (a) $p < .01$, Con vs DB; (b) $p < .01$, DB vs DM25, DB vs DM50, DB vs DM100. Student *t*-test, $^{*}p < .05$; $^{**}p < .01$.

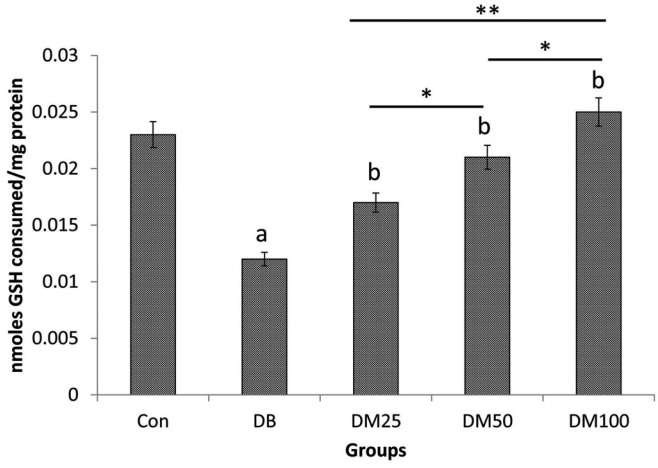

Figure 10. Dose-dependent effect of melatonin on non-enzymatic antioxidant reduced glutathione (GSH) level in the spleen of diabetic mice. Histograms represent Mean ± SEM, $n = 5$ for each group. Con = control, DB = diabetic, DM25 = diabetic mice received melatonin 25 μg/mice/day, DM50 = diabetic mice received melatonin 50 μg/mice/day, DM100 = diabetic mice received melatonin 100 μg/mice/day. (a) $p < .01$, Con vs DB; (b) $p < .01$, DB vs DM25, DB vs DM50, DB vs DM100. Student *t*-test, $^{*}p < .05$; $^{**}p < .01$.

not effectively restored the insulin level but improved the hyperglycaemia induced oxidative stress (Lo *et al*. 2017).

During the progression of diabetes, the increase in blood glucose levels was simultaneous with the rate of deterioration of body weight. Organ weight analysis in toxicological studies sought to be an important endpoint to identify the potentially harmful effects of chemicals. Studies supported the fact that relative organ weight can be the most sensitive indicator to show the impact of an experimental compound, as significant differences in organ weight between treated and control animals (Bailey *et al*. 2004). In the present study, we have noted a decrease in relative spleen weight in diabetic mice. Melatonin supplementation caused dose-dependent effects in the improvement of the relative spleen weight in experimental diabetic mice. The gradual increase in doses of melatonin administration has restored the spleen weight in diabetic mice. Haldar *et al*. (2004) suggested that melatonin treatment increased the spleen weight in *Funambulus pennanti*.

The spleen is an important lymphoid organ responsible for executing immune activity throughout life. The induction of diabetes caused the disintegration of cellularity in splenic follicles in the white pulp area. Melatonin treatment minimised the adverse effects of diabetes and restored the splenic follicular cellularity in studied mice. Ebaid *et al*. (2015) suggested the deterioration of splenic cellularity in diabetic mammals. All the doses of melatonin used in this study were effective in improving the follicle cellularity in the spleen of diabetic mice.

Diabetes caused irregularity in several physiological activities in the living organisms. Experimental diabetes caused unbalanced metabolic activities and increased the lipid

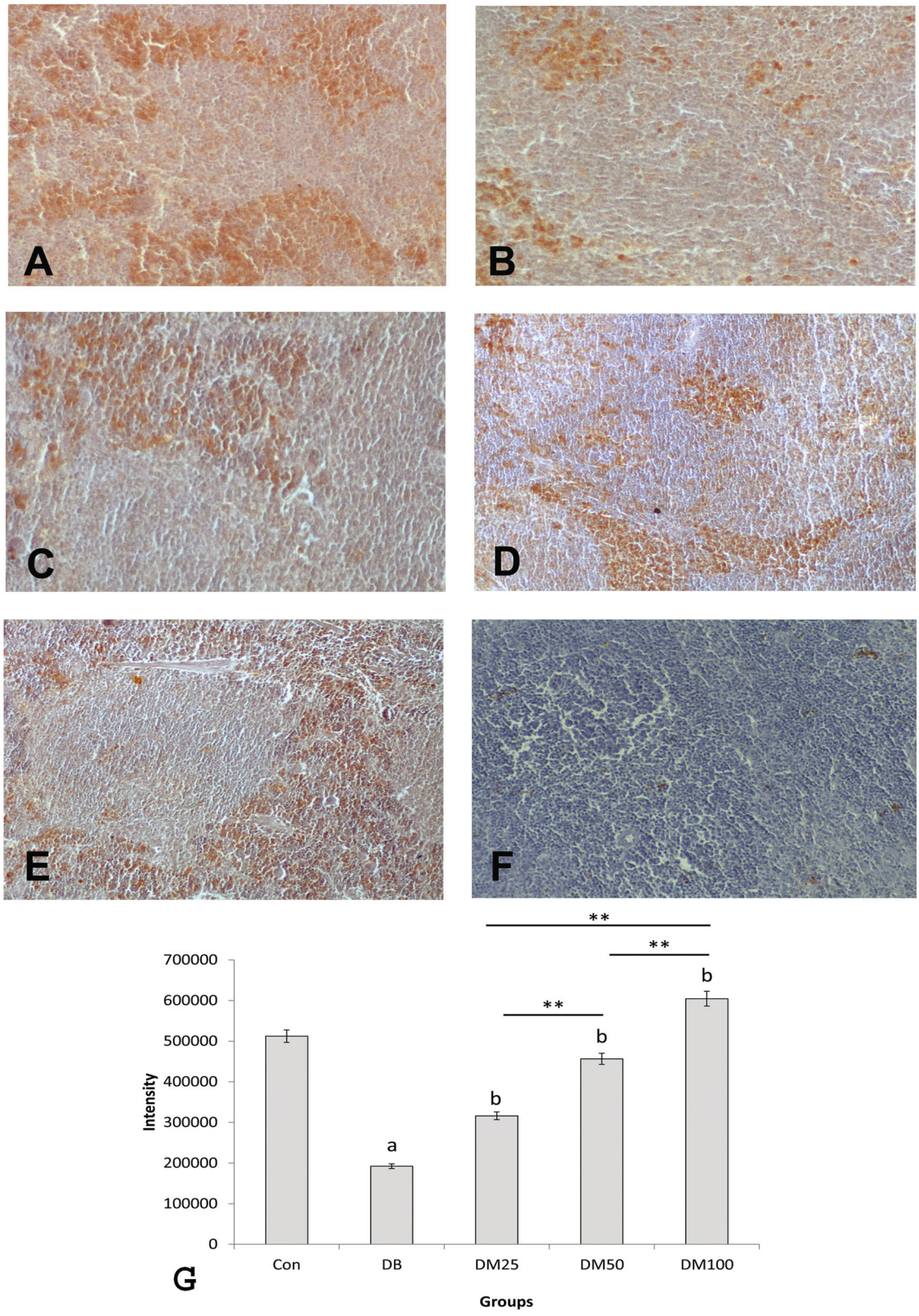

Figure 11. Dose-dependent effect of melatonin treatment on reactivity of Nrf 2 antiserum in spleen of diabetic mice (Mean ± SEM, $n = 5$ for each group). (A) control, (B) diabetic, (C) diabetic mice received melatonin 25 μg/mice/day, (D) diabetic mice received melatonin 50 μg/mice/day, (E) diabetic mice received melatonin 100 μg/mice/day, (F) negative control. (G) Histogram showing intensity of immune reaction. (a) $p < .01$, Con vs DB; (b) $p < .01$, DB vs DM25, DB vs DM50, DB vs DM100. Student *t*-test, $^{**}p < .01$.

peroxidation (MDA level) in the spleen of mice. Increased lipid peroxidation caused damage to cellular membranes in the organism (Ostrea *et al*. 1985). Melatonin supplementation decreased the lipid peroxidation in the spleen of diabetic mice. Melatonin reduced the lipid peroxidation by scavenging the free radicals generated in mice spleen due to diabetic stress. Melatonin neutralises free radicals and forms stable end-products excreted out in urine (Tan *et al*. 2000).

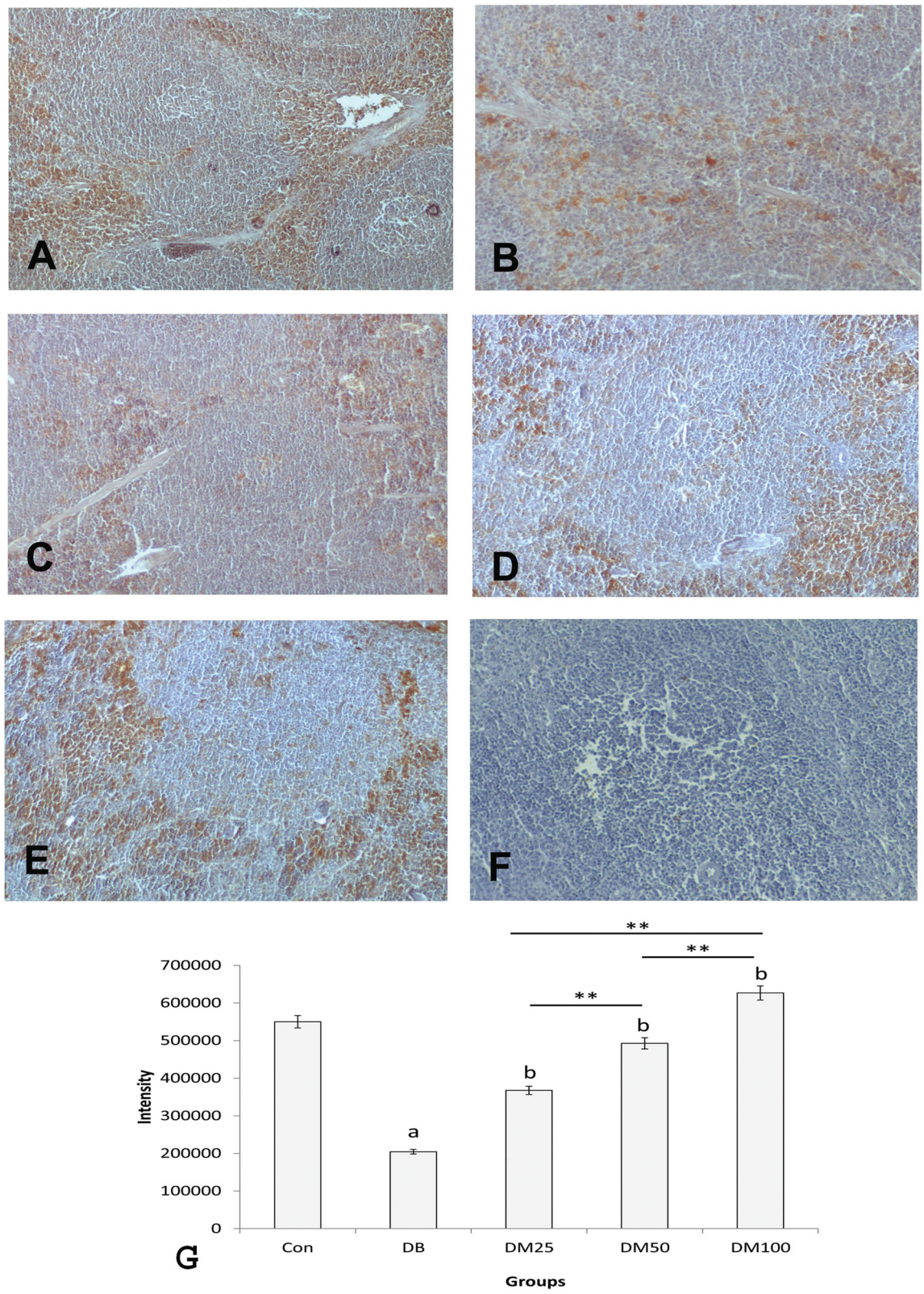

Figure 12. Dose-dependent effect of melatonin treatment on reactivity of HO-1 antiserum in spleen of diabetic mice (Mean ± SEM, $n = 5$ for each group). (A) control, (B) diabetic, (C) diabetic mice received melatonin 25 μg/mice/day, (D) diabetic mice received melatonin 50 μg/mice/day, (E) diabetic mice received melatonin 100 μg/mice/day, (F) negative control. (G) Histogram showing intensity of immune reaction. (a) $p < .01$, Con vs DB; (b) $p < .01$, DB vs DM25, DB vs DM50, DB vs DM100. Student *t*-test, $^{**}p < .01$.

Further, oxidative stress is thought to be the primary factor involved in the pathogenesis of several diseases with macromolecular alterations due to the elevated production of ROS (Esterbauer *et al*. 1991).

Superoxide dismutase (SOD) and catalase (CAT) are antioxidant enzymes which act simultaneously to protect the cells by neutralising reactive oxygen species (Patlevič et al. 2016). SOD catalyses the partitioning of superoxide radical

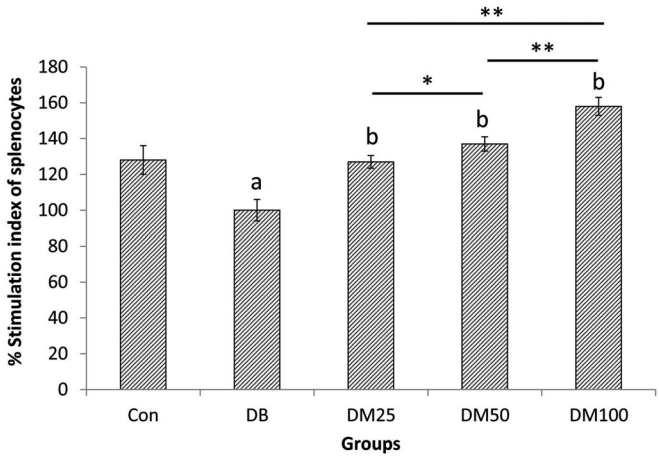

Figure 13. Dose-dependent effect of melatonin on proliferative index of splenocyte in diabetic mice. Histograms represent Mean ± SEM, $n = 5$ for each group. Con = control, DB = diabetic, DM25 = diabetic mice received melatonin 25 μg/mice/day, DM50 = diabetic mice received melatonin 50 μg/mice/day, DM100 = diabetic mice received melatonin 100 μg/mice/day. (a) $p < .01$, Con vs DB; (b) $p < .01$, DB vs DM25, DB vs DM50, DB vs DM100. Student *t*-test, $^{*}p < .05$; $^{**}p < .01$.

into either molecular oxygen or hydrogen peroxide (H_2O_2) (Hayyan *et al*. 2016) and CAT catalyses the decomposition of H_2O_2 to water and oxygen (Chelikani *et al*. 2004). Streptozotocin significantly suppressed the SOD and catalase activity in the spleen of experimental mice. Experimental doses of melatonin caused the restoration of antioxidant enzyme activity differentially in the spleen of diabetic mice. The M100 dose of melatonin most effectively restored the SOD and CAT activity in the spleen of mice. Glutathione, an antioxidant, provides non-enzymatic protection against metabolic oxidants in the living organisms (Ighodaro and Akinloye 2018). Induction of diabetes caused a decrease in the glutathione level in the spleen of mice. Melatonin treatment enhanced the glutathione level and the M100 dose was most effective in counteracting the diabetes-induced suppression of glutathione level in the diabetic mice. Reports suggested the stimulatory action of melatonin on antioxidant enzymes (Hajam *et al*. 2017, 2020). Melatonin stimulates cellular antioxidant enzymes and resists the deleterious effects of oxidative stress-mediated cell damages (Paul *et al*. 2018). Melatonin administration in single, as well as multiple doses, enhances the antioxidant enzyme activity in various organs of rats (Liu and Ng 2000, Ozturk *et al*. 2000).

Nuclear factor erythroid2 (NEF2)-related factor 2 (Nrf 2) is a central regulator of antioxidative stress response in cells (Kwak *et al*. 2002). Studies suggested that elimination of the Nrf 2 gene increased mice susceptibility towards cellular toxicity and diseased conditions associated with oxidative damage (Loboda *et al*. 2016). Haem Oxygenase-1 (HO-1) is a haem degrading enzyme downstream of Nrf2 whose expression enhanced in response to oxidative stress. In the present study, experimental diabetes decreased the Nrf2 and HO-1 reactivity in the spleen of mice. Supplementation of melatonin to the diabetic mice increased the reactivity of Nrf 2 and HO-1 in the splenic tissue. HO-1 has antioxidant, anti-inflammatory, anti-apoptotic, and immunomodulatory effects in vascular cells. Any alteration in Nrf 2 activity potentially hinders the formation of products by HO-1 (Loboda *et al*. 2016).

The spleen is the largest lymphoid organ plays an important role in both innate and adaptive immune responses. The measurement of spleen T cell proliferation indirectly reflects the cell-mediated immune response of an organism. We studied the T-cell proliferation index against Concanavalin A, a T-cell mitogen. Streptozocin treatment suppressed the splenocyte proliferation index in the mice. The impairment of T-cell proliferation has been reported in the diabetic rats (Sakowicz-burkiewicz *et al*. 2006). Melatonin supplementation to diabetic mice improved the T-cell proliferative index in studied mice. The higher dose of melatonin was the most effective and increased the proliferative response of splenic T-cells. Studies have suggested that melatonin can act directly on immune cells to enhance immune function. High-affinity melatonin receptors have been localised on circulating lymphocytes from rodents, chickens, and humans (Drazen and Nelson 2001), and on thymocytes and splenocytes in humans and several rodent and bird species (Pang and Pang 1992, Calvo *et al*. 1995). Exogenous melatonin enhanced both cell-mediated and humoral immune function in many species (Guerrero and Reiter 1992, Maestroni 1993).

Diabetes is considered a substantial global health problem, it is the most prevalent disease, and still, the number of affected is increasing worldwide. Diabetes leads to many health complications and affects many organ & organ systems (Toniolo *et al*. 2019). The high circulating glucose caused oxidative stress and suppressed the antioxidant enzymes in the spleen tissue of the studied mice. Melatonin treatment increased the circulatory melatonin level which improved the antioxidant enzyme activities and minimised the diabetes-induced oxidative stress. Further, melatonin also enhanced the Nrf2 and HO-1 reactivity in the splenic tissues and increased the proliferation index of splenocytes in diabetic mice. The higher dose of melatonin was most effective. The present study may suggest the dose-dependent effect of melatonin in attenuation of diabetes-induced ailments in the spleen of mice.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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