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Zoology

MELATONIN PROTECTS OVARY FROM DIABETES INDUCED OXIDATIVE DAMAGES IN LABORATORY MICE.

Anindita Deb — Molecular Endocrinology Lab, Department of Zoology, Tripura University, Suryamaninagar, Tripura-799022.

Sangita Sutradhar — Molecular Endocrinology Lab, Department of Zoology, Tripura University, Suryamaninagar, Tripura-799022.

Shiv Shankar Singh* — Molecular Endocrinology Lab, Department of Zoology, Tripura University, Suryamaninagar, Tripura-799022. *Corresponding Author

ABSTRACT Diabetes is a combination of different metabolic disorders as a result of insulin deficiency and improper action. Improper neutralization of reactive oxygen species produced during metabolism results in oxidative stress in living organisms. Melatonin is a known antioxidant, neutralizes reactive oxygen species in living organisms. We have evaluated the effectiveness of low doses (25µg, 50µg and 100µg/100g B.wt.) of melatonin on diabetes caused oxidative damages in the ovary of mice. The induction of diabetes increased the lipid peroxidation (MDA level) and decreased the antioxidant enzyme (SOD and CAT) activity, reduced glutathione (GSH) level, Nrf2 and HO-1 reactivity in the ovary of mice. Melatonin supplementation suppressed the MDA level and increased the SOD, and CAT activity, GSH levels, Nrf2 and HO-1 reactivity in the ovary of experimental mice in a dose-dependent pattern. Therefore, the present study may suggest that melatonin ameliorates the adverse effects of diabetes by reducing the oxidative stress in the ovary of experimental mice.

KEYWORDS : Melatonin, diabetes, streptozotocin, oxidative stress, ovary.

INTRODUCTION

Diabetes mellitus (DM), a combination of different metabolic disorders represents one of the extreme threats to human health all over the world. Autoimmune destruction of the pancreatic beta-cell is main cause of the Type 1 diabetes mellitus (T1D). Along with causing various physiological complications, diabetes has also been associated with reproductive impairment both in male and female including children and young individuals.[1-3] In living organisms, reactive oxygen species (ROS) are produced during cellular metabolism that can damage cell structures and alter their functions.[4] Hyperglycaemic condition stimulates free radical production and caused cellular injury. Enzymatic and non-enzymatic antioxidants are usually effective in blocking the harmful effects of ROS in aerobic organisms.[5] The ovary is the primary reproductive organ of the female reproductive system. The two main reproductive functions of ovaries are ovulation and the production of gonadal steroids.

Melatonin (N-acetyl 5- methoxytryptamin), an indolamine is secreted by the pineal gland. Along with free radicals scavenging and immune modulating properties, melatonin also has antihyperglycaemic activity.[6-7] Melatonin plays an important role in reproductive function by affecting the ovarian activity and estrous cyclicity depending on day-night length. Recent studies suggested that melatonin influences diabetes and other metabolic disorders by playing an important role in the regulation of insulin release through both receptor-dependent and independent manner.[8-9] But how low doses of melatonin affect the oxidative damages caused by diabetic stress in ovary was less studied. Therefore, we have made an attempt to study the effect of low doses of melatonin on oxidative damages caused by experimentally induced diabetes in ovary of female laboratory mice.

MATERIALS AND METHODS

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 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 (12L:12D), temperature (25 ± 2°C) and humidity (55±5 %). Mice were kept in groups of five in polycarbonate cages (43cm x 27cm x 14cm) and fed with mice feed and water *ad libitum*. Healthy female mice of 25g body weight were selected for the experiment.

Experimental Design

Diabetes was induced by intraperitoneal administration of multiple low doses of streptozotocin (MLD-STZ) to experimental female mice. The STZ was dissolved in freshly prepared 0.1M citrate buffer (pH = 4.5) and was administered (50mg/kg body weight/mice) intraperitoneal for consecutive 5days.[10]. The experimental mice were divided in five groups and each group contained five mice.

1. Con - Control group of mice received only ethanolic saline (0.01%).
2. DB - Diabetic group of mice
3. DM25 – Diabetic mice received melatonin 25 µg/100 g. B. wt./day
4. DM50 – Diabetic mice received melatonin 50 µg/100 g. B. wt./day
5. DM100 – Diabetic mice received melatonin 100 µg/100 g. B. wt./day

Melatonin was administered for 15 consecutive days. Mice were sacrificed by decapitation after 15th day of melatonin administration. Blood glucose was measured from trunk blood. The ovaries were dissected out. One ovary of each mouse was fixed in boiun's fluid and another ovary was processed for measurement of oxidative stress biomarkers.

Blood glucose Determination

The blood glucose level was determined with the help of ACCU-CHEK Active, blood glucose monitoring system.

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.[11] 10% homogenates of ovaries were prepared in phosphate buffer. 0.1ml 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 532nm. Lipid peroxidation was expressed in nmol TBARS formed/ mg protein of experimental tissues.

Superoxide Dismutase (SOD) Activity Assay

Superoxide dismutase activity in the ovary of experimental mice was determined by following the method of Das et al.[12] 10% homogenates of the ovaries were prepared in phosphate-buffered saline (pH=7.4). 0.1ml of the homogenate was mixed with 1.4ml of reaction mixture (containing 50mM phosphate buffer (pH 7.4), 20mM L-methionine, 1% Triton-X-100, 10mM hydroxylamine hydrochloride, 50µM EDTA). 50µM of riboflavin was added to the mixture and exposed to a 20W fluorescence lamp. 1ml of Griess reagent was added and optical density was determined at 543nm. 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 assay

Catalase activity in the ovary of experimental mice was determined by

following the method of Sinha,[13] modified by Hadwan.[14] 10% homogenate of the ovary was prepared in phosphate buffer (pH=7.4) and then centrifuged. The supernatant was added to a reaction 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 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) assay

Reduced glutathione was determined following the methods of Ellman,[15] modified by Gupta et al.[16] 10% homogenate of ovaries was prepared in phosphate buffer (pH = 7.4) and mixed with 20% of trichloroacetic acid in 1:1 ratio and centrifuged. 200ul of supernatant was mixed with 1.8 ml of Ellman reagent (containing 1% sodium citrate and 0.04% DTNB in 0.1M phosphate buffer (pH = 8.0)). The optical density of mixture was determined at 412nm. The concentration of reduced glutathione was expressed as µg/g tissue.

Immunohistochemistry of Nrf2 and HO-1

Immunohistochemical study of Nrf2 and HO-1 in the ovary was done following the modified procedure of Singh et al.[17] 5µm-thick paraffin sections were mounted on 3% gelatin-coated slides. Sections were deparaffinized 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% H2O2 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, USA, 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 antigens were visualized using the 0.03% peroxidase substrate 3, 3-diaminobenzidine (DAB; Sigma–Aldrich Chemicals, St. Louis, USA) and counterstained with Ehrlich's hematoxylin. Sections were dehydrated and mounted with DPX. Microphotographs of the stained sections were taken under 40X objective in Olympus BX-41 Microscope.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed for the statistical analysis of the data. ANOVA was followed by Tukey's honest significant difference (HSD) multiple range tests. Student t-test was performed to test the differences between different doses of melatonin. The differences were considered significant when $p<0.05$. 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

Streptozotocin treated mice showed very high level of blood glucose in the circulation. All the doses of melatonin showed effective consequences in lowering the blood glucose level in Melatonin administration significantly ($p<0.01$) lowered the blood glucose level in comparison with streptozotocin treated mice. Melatonin supplementation maintained the significant low level of blood glucose in the diabetic mice (Figure 1).

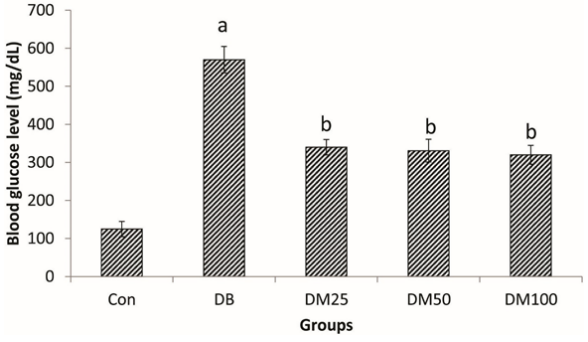


Figure 1. Effect of exogenous melatonin on blood glucose level in streptozotocin induced diabetic mice. Histogram represents Mean ± SEM, n=5 for each group. (a) p<.01, Con vs DB; (b) p<.01, DB vs DM25, DB vs DM50, DB vs DM100.

Effect of melatonin on lipid peroxidation in ovary

MDA is the faithful marker of lipid peroxidation in the living tissues. Lipid peroxidation in the ovary was measured in the terms of nmol TBARS formed/ mg tissue protein (Figure 2). Significant ($p<0.01$) increase in the MDA level in the ovary of mice was noted in comparison with the ovary of control mice. All the doses of melatonin treatment to diabetic mice significantly suppressed the MDA level in comparison with the ovary of diabetes mice. The M100 dose of melatonin was found most effective and significantly suppressed the MDA level among the studied doses of melatonin in diabetic mice.

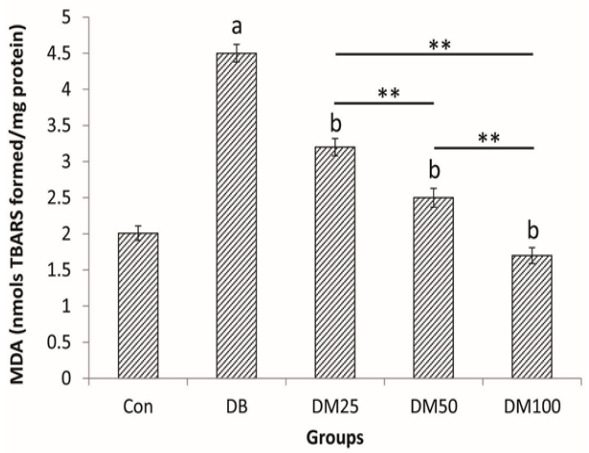


Figure 2. Effect of exogenous melatonin on lipid peroxidation (MDA level) in the ovary of streptozotocin induced diabetic mice. Histogram represents Mean ± SEM, n=5 for each group. (a) p<.01, Con vs DB; (b) p<.01, DB vs DM25, DB vs DM50, DB vs DM100. Student t-test, **p<0.01

Effect of melatonin on superoxide dismutase (SOD) enzyme activity in ovary

SOD enzyme is an important antioxidant enzyme responsible for the neutralization of superoxide anion formed in the living tissues. Significant ($p<0.01$) suppression of SOD activity was noted in streptozotocin administered mice in comparison with the ovary of control mice (Figure 3). All the studied doses of melatonin treatment elevated the SOD enzyme activity significantly than the diabetes group in ovary of mice. Among the given doses of melatonin, M100 dose most effectively increased the SOD enzyme activity in ovary of diabetic mice.

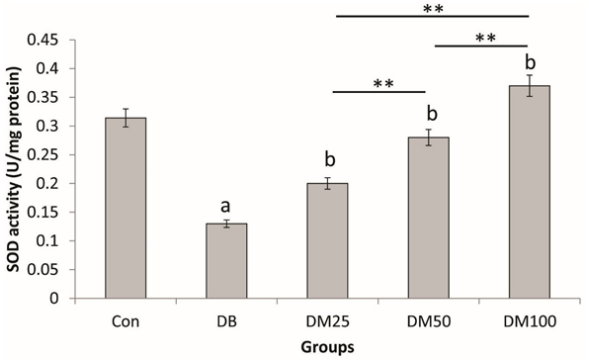


Figure 3. Effect of exogenous melatonin on super oxide dismutase (SOD) activity in the ovary of streptozotocin induced diabetic mice. Histogram represents Mean ± SEM, n=5 for each group. (a) p<.01, Con vs DB; (b) p<.01, DB vs DM25, DB vs DM50, DB vs DM100. Student t-test, **p<0.01.

Effect of melatonin on catalase enzyme activity in ovary

Catalase is an important antioxidant enzyme responsible for the neutralization of hydrogen peroxide formed in the living tissues. Streptozotocin administration significantly suppressed the catalase enzyme activity in the ovary of mice in comparison with the ovary of control mice (Figure 4). All the studied doses of melatonin treatment in diabetic mice significantly increased the catalase enzyme activity than the diabetes group. All the doses of melatonin were able to significantly ($p<0.01$) increased the catalase enzyme activity in the ovary of diabetic mice. The M100 dose was found most effective.

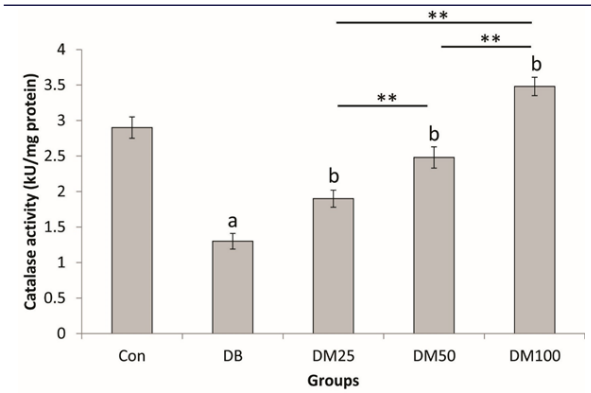


Figure 4. Effect of exogenous melatonin on Catalase (CAT) activity in the ovary of streptozotocin induced diabetic mice. Histogram represents Mean ± SEM, n=5 for each group (a) p<.01, Con vs DB; (b) p<.01, DB vs DM25, DB vs DM50, DB vs DM100. Student t-test, **p<0.01.

Effect of melatonin on reduced glutathione level

Glutathione by donating an electron reduces hydroperoxides to their respective alcohols in living tissues. Streptozotocin administration significantly suppressed the glutathione level in the ovary of mice in comparison with the ovary of control mice (Figure 5). All doses of melatonin administration to diabetic mice significantly increased the glutathione level than the diabetes group. All melatonin doses were significantly increased the GSH level, but the M100 dose was most effectively increased the GSH level in the ovary of diabetic mice.

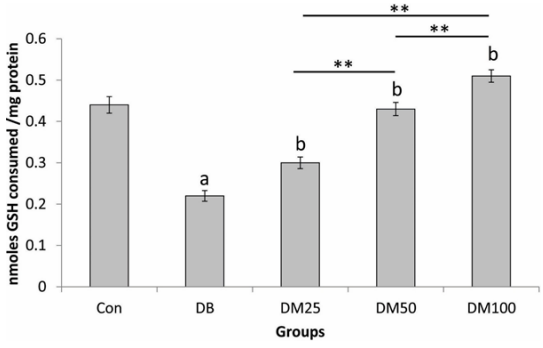
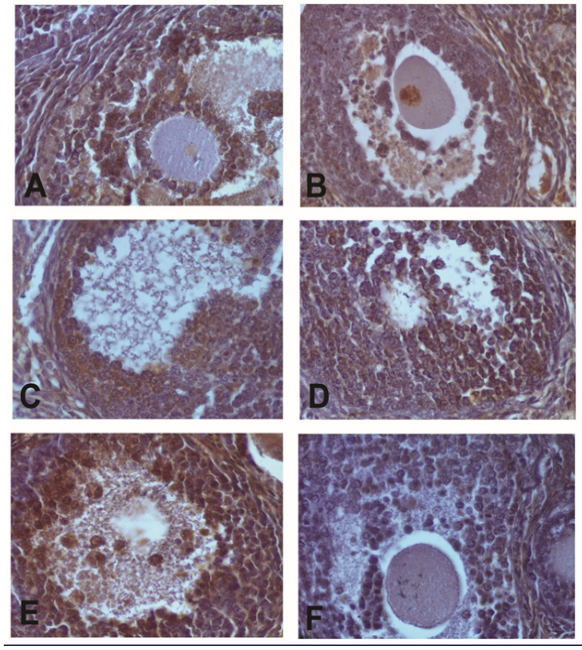


Figure 5. Effect of exogenous melatonin on reduced glutathione (GSH) level in the ovary of streptozotocin induced diabetic mice. Histogram represents Mean ± SEM, n=5 for each group (a) p<.01, Con vs DB; (b) p<.01, DB vs DM25, DB vs DM50, DB vs DM100. Student t-test, **p<0.01.



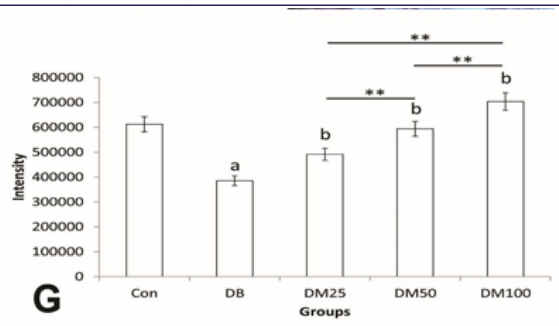


Figure 6. Effect of exogenous melatonin on reactivity of Nrf2 in the ovary of streptozotocin induced diabetic mice (Mean ± SEM, n=5 for each group). (A) control, (B) diabetic, (C) DM25 (D) DM50, (E) DM100, (F) negative control. Histogram showing intensity of immune reaction. (a) p<.01, Con vs DB; (b) p<.01, DB vs DM25, DB vs DM50, DB vs DM100. Student t-test, **p<0.01.

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

The nuclear factor erythroid 2-related factor 2 (Nrf2) proteins regulate the expression of antioxidant proteins that protects against oxidative damages. Normal reactivity of Nrf2 antisera was noted in the cells of ovarian follicles in the control mice (Figure 6). Less reactivity of Nrf2 antiserum was noted in ovarian follicles in the streptozotocin treated mice. Stronger reactivity of Nrf2 antisera was noted in the follicular cells in the ovary of melatonin treated mice. Strongest Nrf2 reactivity was noted in the ovarian follicles of the M100 treated mice group.

In the mammalian system, haem oxygenase-1 (HO-1) is one of the genes regulated through Nrf2. Reactivity of HO-1 antiserum was noted in the follicular cells of the ovary of control mice. Induction of diabetes, significantly (p<.01) decreased the reactivity of the HO-1 antiserum in the ovarian follicles in the mice. Melatonin supplementation to diabetic mice caused significant (p<.01) increased in reactivity of HO-1 antiserum in the ovarian follicles of mice. M100 supplemented diabetic mice showed strongest HO-1 antiserum reactivity in the ovarian follicles of mice (Figure 7).

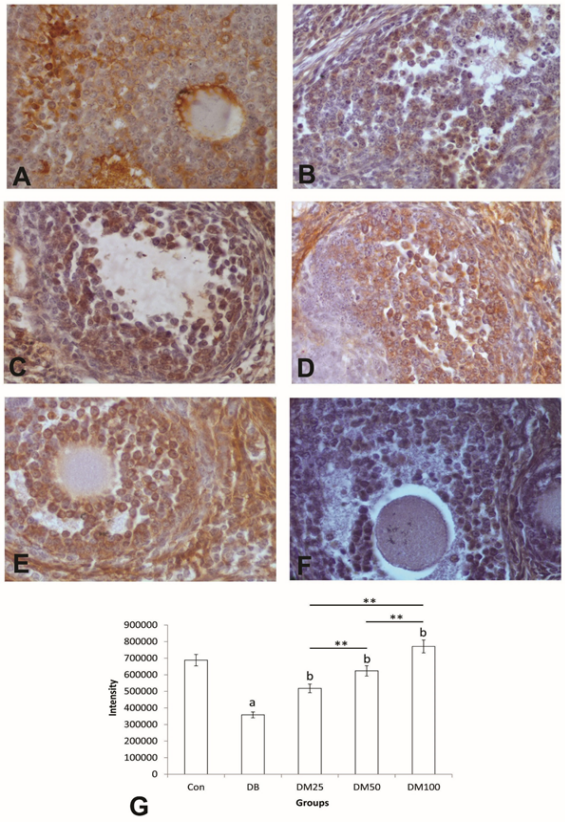


Figure 7. Effect of exogenous melatonin on reactivity of HO-1 in the ovary of streptozotocin induced diabetic mice (Mean ± SEM, n=5 for each group). (A) control, (B) diabetic, (C) DM25 (D)

DM50, (E) DM100, (F) negative control. 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<0.01.

DISCUSSION

Streptozotocin is a toxic substance caused the destruction of pancreatic beta cells and results in the failure of insulin production in experimental animals. Due to the absence of insulin, a high blood glucose level constantly persisted in the experimental animal which reflects the physiological status of type 1 diabetes. Mice having blood glucose above 250mg/dl were considered diabetic.[18] Streptozotocin administration continuously increased the blood glucose level in experimental mice. Melatonin treatment restricted the increase in the blood glucose level in a dose-dependent pattern. The inhibitory effect of melatonin on blood glucose levels was noted from the 10th day of treatment. Earlier studies suggested the dose-dependent protection from multiple low doses streptozotocin-induced hyperglycaemia by the administration of inosine.[18]

Lipid peroxidation is a critical biomarker of oxidative stress in relation to ROS forming malondialdehyde (MDA) and reacts with thiobarbituric acid. More MDA production indicates a larger rate of lipid peroxidation resulting in more cell damage. In streptozotocin treated mice, increased MDA level was measured than the control group. Different doses of melatonin lowered the MDA level in dose dependent manner. Melatonin is a well-known antioxidant and directly neutralizes reactive oxygen species and does not undergo redox cycling. Melatonin minimizes the oxidative stress caused damages in the ovarian follicles and enhances the fertilization rate in human subjects.[19] Melatonin scavenges more free radicals than other classic antioxidants and protects the organism from oxidative damages.[20,21]

Superoxide dismutase (SOD) provides the first line of defence against ROS mediated cell injury by catalysing the dismutation of superoxide radicle to molecular oxygen or peroxide.[32] The low level of SOD indicates a higher rate of oxidative stress. Streptozotocin administration to the mice suppressed the SOD level. Melatonin increased the SOD level according to the melatonin doses. Catalase (CAT) is a regulator of hydrogen peroxide metabolism that can, in excess, cause serious damage to lipids, RNA and DNA. Catalase enzyme neutralizes H_2O_2 by catalytically converting it into water and oxygen.[23] In the case of catalase deficiency, beta cell of pancreas that contains a large number of mitochondria, under-goes oxidative stress by producing excess ROS that leads to β-cells dysfunction and ultimately diabetes.[24] Streptozotocin treatment decreased the CAT activity in the ovary of mice. Melatonin treatment increased the CAT activity in diabetic mice and the M100 dose was most effectively increased the catalase activity in the ovary of mice. Glutathione reductase enzyme is found in the cell that metabolizes peroxide to water and converts glutathione disulfide back into glutathione.[25] Glutathione, an antioxidant is comprised of the second line of defence against metabolic oxidants in the cell and any alteration in their levels will make the cells prone to oxidative stress and hence cell injury.[26] Streptozotocin administration decreased the glutathione level in the ovary 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 ovary of streptozotocin treated mice. Reports suggested the stimulatory action of melatonin on antioxidant enzymes. Melatonin alleviated the doxorubicin intoxication by stimulating the activity of antioxidant enzymes in myocardial cells in rats.[27]

Nrf2 is a key transcription factor that activates expression by binding to the antioxidant response element in the promoter region of many genes.[28] Reports suggested the increased susceptibility of mice towards cellular toxicity and diseased condition associated with oxidative stress after elimination of Nrf2 gene.[29] Haem oxygenase-1 (HO-1) is haem degrading enzyme downstream of Nrf2 signalling cascade. Hyperglycaemic condition reduced the reactivity of Nrf2 and HO-1 antisera in the ovarian follicles whereas stronger reactivity of Nrf2 and HO-1 antisera was noted in the ovarian follicles of melatonin treated mice. Melatonin supplementation increased Nrf2 and HO-1 protein expression and protects splenic tissue from streptozotocin induced oxidative damages in mice,[30] and from LPS induced oxidative damages in spleen of *Funambulus pennanti.*[31] Further alteration in Nrf2 in activity potentially caused hindrance in formation of product by HO-1.[32] Studies also suggested that during experimental sepsis loss of Nrf2 impacts survival while its activation can improve survival.[45]

In the present study, streptozotocin-induced diabetes increased MDA level and decreased antioxidant enzyme activity as well as decreased Nrf2 and HO-1 reactivity in the ovary of mice. Melatonin supplementation minimizes the diabetes-induced oxidative stress and caused restoration of antioxidant enzymes in the ovary of mice. All the studied doses were efficient in amelioration of diabetes-induced changes in the ovary of mice but M100 dose was most effective. The present study suggest that melatonin ameliorates the devastating effects caused by hyperglycaemia and protects the ovary from diabetes-induced oxidative stress in the laboratory mice.

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