Research Article

Protective mechanisms of melatonin on caprine spleen injury induced by cadmium (Cd): an *in vitro* study

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Running title*:* Melatonin protects spleen tissue from cadmium toxicity

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ABSTRACT

Current study explores the potential mechanisms of melatonin on cadmium-induced spleen tissue injury of goat. Spleen tissues were incubated with different concentrations (50, 100, 200, 400 and 600 µM) of cadmium acetate (Cd), respectively and the lipid peroxidation of the tissue was measured. It was found that Cd at the level of 400 µM induced maximum spleen damage among other concentrations. Thus, Cd 400 µM was selected to examine whether melatonin treatment can protect against this damage. The results showed that Cd increased the oxidative stress in the spleen tissue either by elevating pro-oxidant enzymes, or, by suppressing the variety of antioxidant enzymes and thus, to increase the intracellular reactive oxygen species (ROS). Melatonin treatment at the concentrations of 0.25, 0.5 and 1 mM significantly reduced all these alterations, respectively. At the level of cellular organelles, Cd caused mitochondrial morphological and functional injuries. These include mitochondrial surface distortion and inhibitions of glycolytic, Krebs cycle, and respiratory chain enzymes. Melatonin at a concentration of 0.5 mM almost completely preserved Cd induced mitochondrial pathological alterations. Cd pollution is a cause of serious health hazard world wide, particularly in the developing areas and currently, there is no specific remedy for Cd toxicities. The results suggest that melatonin is a promising therapeutic agent to combat Cd-induced oxidative stress and it deserves further investigation clinically.

Keywords: Cadmium, oxidative stress, spleen, melatonin, antioxidant.

1. INTRODUCTION

Cadmium (Cd) is a well known heavy metal exhibiting diverse toxicity in organisms . Its hazardous effects on human health have been proven with the advancement of scientific technologies and methods. Its adverse effects are not only confined to the occupational class of people as originally believed but it actually impacts majority of the population. Thus, it has

been categorized as a type I carcinogen by the International Agency for Research on Cancer in USA (1). Cd contamination causes oxidative tissue damage. Cd *per se* does not generate reactive oxygen species (ROS) at the cellular level due to the fact that it is not redox active metal (2). However, it can replace intracellular iron and thereby elevate the endogenous pool of free irons which promote Fenton reaction (3). Contaminated water, soil and air caused by smelting, mining, fossil fuel burning and cigarette smoking (4) are the prime sources of Cd exposure. Cd pollution in water body impairs the growth of aquatic organisms including *Daphnia magna*, *Carassius auratus* and *Photobacterium phosphoreum* (5–7). If the Cd enters into the body of mammals, including human, through oral, nasal and dermal routes (8) it will bind to metallothionein (9) and albumin (8) from where it is distributed to the entire body. The Cd bound protein complex is primarily transported into the liver (10) but the maximum retention of Cd takes place in the kidney (11) due to its relatively long biological half-life. Currently, its cytotoxic effects in the spleen (12) and thymus (13) have gained attention. Spleen is the site for the destruction of erythrocytes and it has a high accumulation of iron (Fe) with erythrocytic hemolysis (14). Thus the abundant amounts of Fe in spleen makes this organ easy to be attacked by Cd to induce oxidative stress by the induction of Fenton reaction (3). As a mitochondria-rich organ, the spleen plays important role in ATP synthesis. A relationship between ATP synthesis and immunomodulation of spleen is a really interesting topic for research (15). The spleen tissue oxidative stress induced by Cd would cause immunosuppressive and proinflammatory reactions in mammals (16).

Both natural and artificial antioxidants ameliorate the oxidative damage induced by Cd in diverse organisms (17, 18). Melatonin is one of them. Melatonin- a small molecule, originally was believed to be exclusively produced in the pineal gland and its function involves the regulation of circadian rhythm (19). Thereafter, it was found to be a potent antioxidant to protect against oxidative stress in vertebrates (20). Apart from the pineal gland, melatonin was found to be synthesized in almost all organs/tissues (20). The presence of two major enzymes of the melatonin biosynthetic pathway- arylalkylamine-*N*-acetyltransferase (AANAT) and hydroxyindole-O-methyltransferase (HIOMT) in the spleen tissue suggests that spleen has the capacity to synthesize melatonin too (21). Although the amounts of melatonin synthesized in the spleen is relatively low compared to the other sources, its potent role in combating acute oxidative stress cannot be ignored (22). Many studies have proven the protective effects of melatonin on oxidative stress in variety of organs (23), but none of them has explored its antioxidant potentiality against Cd-induced oxidative damage in the spleen of any caprine species. Considering limitation in existing knowledge, the present investigation is set up to test whether melatonin also exhibits protective effects on Cd-induced spleen injury in goat. Thus, different oxidative biomarkers will be measured. In addition, the structural and functional aspects of mitochondria will be evaluated by scanning electron microscopy and different fluorescence staining method.

2. MATERIALS AND METHODS.

2.1. Chemicals and Reagents.

Cadmium acetate [$(CH_3COO)_2Cd.2H_2O$] was purchased from Qualigens Limited, (Mumbai, India). Melatonin, Janus Green B, o-phenylenediamine (OPD) and phenyl methanesulfonate (PMS) were purchased from Sigma-Aldrich Merck (St. Louis, MO, USA). 2′,7′-Dichlorofluorescin diacetate (DCFDA) was purchased from Abcam Biotechnology Company, (Abcam, USA). Succinate, isocitrate, reduced glutathione (GSH), α-ketoglutarate, tricarboxylic acid (TCA) and cytochrome-c were procured from Sisco Research Laboratories (SRL) (Mumbai, India). Thiobarbituric acid (TBA) was purchased from LobaChemie Pvt. Ltd. (Mumbai, India). All the other chemicals and reagents used in this study were of

analytical grade and were procured from SRL (Mumbai, India), Qualigens Limited (Mumbai, India) and Sigma-Aldrich Merck (St. Louis, Missouri; USA).

2.2. Spleen tissue collection.

Spleen tissues were collected from adult male goats with average weight of 35 kg. The tissue samples were procured from Kolkata Municipal Corporation approved slaughterhouses which function under the stringent supervision of Ministry of Food and Civil Supplies and Department of Health, Govt. of West Bengal. The study protocol was approved by the coordinator of the Biochemistry Special Paper under PG (4^{th} Semester) Special Paper Project Program in Biochemistry Special Paper, Department of Physiology, University of Calcutta.

2.3. Experimental Procedure.

2.3.1. Tissue Preparation for in vitro study.

Immediately after collection, the spleen tissues were washed in ice-cold PBS (pH-7.4) followed by perfusion with Hank's Balanced Salt Solution (HBSS). The perfused tissues were then homogenized (20%) in ice-cold PBS (pH-7.4) in a Potter-Elvehjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA), after which the homogenates were passed through a nylon mesh to avoid unwanted cellular debris or clumps and was equally divided into two portions for two separate *in vitro* studies, respectively.

2.3.2. Incubation with Cd and melatonin.

2.3.2.1. Cd dose-response study.

Homogenized sample of spleen tissue was divided into 6 groups (n=4/group) and was incubated in PBS buffer (pH 7.4) (24) containing either vehicle (control) or, different doses of 50 (Cd-1), 100 (Cd-2) , 200 (Cd-3) , 400 (Cd-4) or, 600 μM (Cd-5) , respectively.

2.3.2.2. Cd plus melatonin treatment.

In this study, spleen tissue homogenate was divided in eight groups (n=4/group) and was incubated in PBS buffer (pH 7.4) containing either vehicle (control) or, Cd (400 μM) alone or different concentrations (0.25, 0.5 or 1 mM) of melatonin (Mel) alone, respectively or Cd (400 μM) plus different concentrations of 0.25, 0.5 or 1 mM melatonin, respectively.

2.4. Estimation of different biochemical parameters.

2.4.1. Levels of different intracellular oxidative stress markers.

Lipid peroxidation (LPO) levels in the spleen tissues were estimated as thiobarbituric acid reactive substances (TBARS) and the changes in absorbance were recorded at 532 nm following the method of Buege and Aust (25) with minor modifications (26). The levels of LPO were expressed in terms of nmoles of TBARS/mg protein.

Levels of protein carbonyl content (PCO) in the splenic tissues were estimated using the DNPH assay of Levine *et al.* (27) and the absorbance was recorded at 370 nm. The values were expressed as nmoles/mg tissue protein.

The activities of total tissue lactate dehydrogenase (LDH) in the spleen tissues were determined using the method as suggested by Kjeld (28). Alteration in absorbance was recorded at 340 nm and the activities were expressed in terms of U/mg protein.

2.4.2. Determination of the levels of GSH, GSSG and redox status of the tissue.

Levels of reduced glutathione (GSH) in the tissue samples were measured following the

method of Sedlak and Lindsey (29) with minor modifications (26). The values were expressed as U/mg protein.

The levels of oxidized glutathione (GSSG) were estimated following the technique of Wendell (30). Change in absorbance was recorded at 340 nm and the levels were expressed as U/mg protein.

The redox status of the tissue was assessed by the formulation as suggested by Schafer and Buettner (31).

2.4.3. Estimation of the activities of different antioxidant enzymes.

The pyrogallol auto-oxidation method of Marklund and Marklund was employed to assess the activities of both superoxide dismutases (SOD-1 and SOD-2) (32). Enzymatic activities were expressed as U/mg protein.

The activity of catalase (CAT) was estimated following the method described by Beers and Sizer (33), with minor modifications (24) and change in absorption was recorded at 240 nm. Enzyme activity was expressed in terms of U/mg protein.

The activity of glutathione reductase (GR) was determined as mentioned by Krohne-Ehrich *et al.* (34); while glutathione peroxidase (GPx) activity was estimated following the method of Castro *et al.* (35). Both the enzyme activities were expressed in terms of U/mg protein.

2.4.4. Estimation of the activities of different pro-oxidant enzymes.

The activity of xanthine oxidase (XO) was estimated following the method of Greenlee and Handler (36) with minor modifications (37). Similarly, xanthine dehydrogenase (XDH) activity was measured using the method of Bhattacharjee *et al.* (17). Both the enzyme activities were expressed as milli U/mg protein.

2.4.5. Preparation of mitochondrial and cytosolic fractions from spleen tissue.

Mitochondrial fraction was isolated from the splenic tissue following the method of Hare *et al.* (38) with minor modifications (24). Briefly, incubated tissue samples were centrifuged at 16,000 g for 45 min at 4°C. The supernatant containing cytosolic fraction was used to estimate the activities of different enzymes- hexokinase (HK), aldolase (ALD) and glucose-6-phosphate dehydrogenase (G-6-PDH); whereas the pellet containing mitochondrial fraction was resuspended in 50mM Tris-sucrose buffer (pH 7.8) and was used for estimating the activities of pyruvate dehydrogenase (PDH), citrate synthase (CS), isocitrate dehydrogenase (ICDH), alpha-ketoglutarate dehydrogenase (α-KGDH) and succinate dehydrogenase (SDH). Both the cytosolic and mitochondrial fractions were stored at −20°C for the following biochemical assays.

2.4.6. Estimation of the activities of different glycolytic enzymes and glucose-6-phosphate dehydrogenase.

Activities of G-6-PDH and HK were estimated spectrophotometrically following the method of Noltmann *et al.* (39) and Abdel-Hamid *et al.* (40), respectively. On the other hand, ALD activity was measured as described by Layzer *et al.* (41). Enzymatic activities were expressed as U/mg protein.

2.4.7. Determination of the activities of PDH and Krebs cycle enzymes.

Pyruvate dehydrogenase (PDH) activity was measured spectrophotometrically using the method of Chretien *et al.* (42) with minor modifications (17). In brief, the reduction of NAD^+

to NADH was measured at 340 nm in the presence of substrate (0.5 mM sodium pyruvate in 50 mM phosphate buffer, pH 7.4). The enzyme activity was expressed as U/mg protein.

The activity of citrate synthase (CS) was determined following the method of Shepherd and Garland (43) using DTNB and the changes in absorbance was recorded at 412 nm. Enzyme activity was expressed as U/mg protein.

Activities of ICDH and α-KGDH were estimated following the method of Duncan *et al.* (44) and the changes in absorbance were spectrophotometrically recorded at 340 nm. The enzyme activities were expressed in U/mg protein.

SDH activity was measured using the method of Veeger *et al.* (45) and change in absorption was recorded at 420 nm. The enzyme activity was expressed in U/mg protein.

2.4.8. Determination of the effects of Cd on the activities of PDH and different Krebs cycle enzymes.

In order to determine the nature of inhibitory pattern of Cd on the activities of PDH, ICDH, α-KGDH and SDH, Lineweaver Burk (LB) plot studies were conducted in splenic mitochondria in the presence of gradually increased concentrations of their respective substrates, i.e. sodium pyruvate (0.0625–0.5 mM) for PDH; isocitrate (0.0625–0.5 mM) for ICDH; α-ketoglutarate (0.0625–0.5 mM) for α-KGDH; and succinate (0.5–4 mM) for SDH (Supplementary Fig.1).

2.4.9. Determination of the activities of different respiratory chain enzymes.

The activities of NADH- Cytochrome c oxidoreductase and cytochrome c oxidase were estimated spectrophotometrically at 565 and 550 nm, respectively, following the method of Goyal and Srivastava (46). Enzyme activities were expressed as milli U/mg protein.

2.4.10. Scanning Electron Microscopic (SEM) study for the evaluation of mitochondrial architectures.

Mitochondrial samples were fixed in 2.5% chilled glutaraldehyde for 48 h for SEM study following the method of Mukherjee *et al.* (37) and were analysed by SEM (Zeiss Evo 18 model EDS 8100).

2.4.11. Measurement of the intracellular level of ROS.

The tissue level of ROS were determined by DCFDA staining technique using a BD FACS Aria II flow cytometer, USA. Briefly, samples were incubated with the stable non-fluorescent DCFDA which becomes oxidized to a highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS (47). The data was presented as staggered overlays and fluorescence using FlowJo software (version 10).

2.5. Estimation of protein content.

Protein concentration in the spleen tissue samples was estimated following the method of Lowry *et al.* (48).

2.6. Statistical analysis.

All the values (n = 4/group) were presented as Mean ± S.E.M. One way analysis of variance (ANOVA) followed by a *post hoc* Tukey test was used to determine the statistical significance between different experimental groups. The results were considered statistically significant at the level of $p < 0.05$. Data presentation and all statistical analyses were performed using Graph Pad Prism 6.03, Statistical Package for the Social Sciences (SPSS; version 25.0) and Microsoft Office 2013.

3. RESULTS

3.1. The dose responsive effects of Cd on LPO in spleen tissues.

The dose responsive effects of Cd on LPO were showed in Figure 1. All selected concentrations of Cd caused significant increasese in the levels of LPO in spleen tissue. The highest LPO level was observed in the tissues treated with Cd 400 µM. Thus, this concentration was selected for the rest of the experiments.

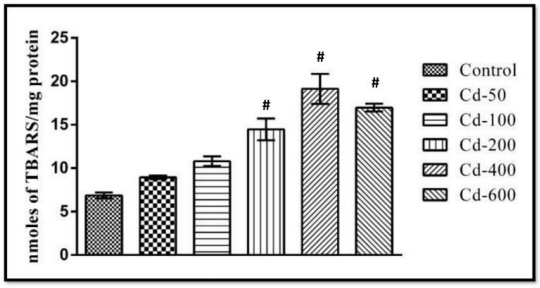

Fig. 1: The dose responsive effects of Cd on LPO.

Levels of LPO in spleen incubated with different concentrations of Cd. The highest LPO level was found in Cd 400 µM treated group. The values are expressed as Mean ± S.E.M. (n = 4); # p < 0.001 versus control.

3.2. Effects of Cd and melatonin on the LPO, PCO and LDH in spleen tissues.

Cd at a concentration of 400 µM significantly increased the levels of LPO, PCO and LDH compared to control (p < 0.001) (Fig. 2). Melatonin alone at different concentrations has no significant influence on LPO, PCO and LDH compared to the control. However, melatonin at a variety of concentrations almost completely inhibited the elevations of LPO, PCO and LDH induced by Cd. It appeared that melatonin at the concentration of 0.50 mM exhibited the best protective effect on Cd induced oxidative biomarkers.

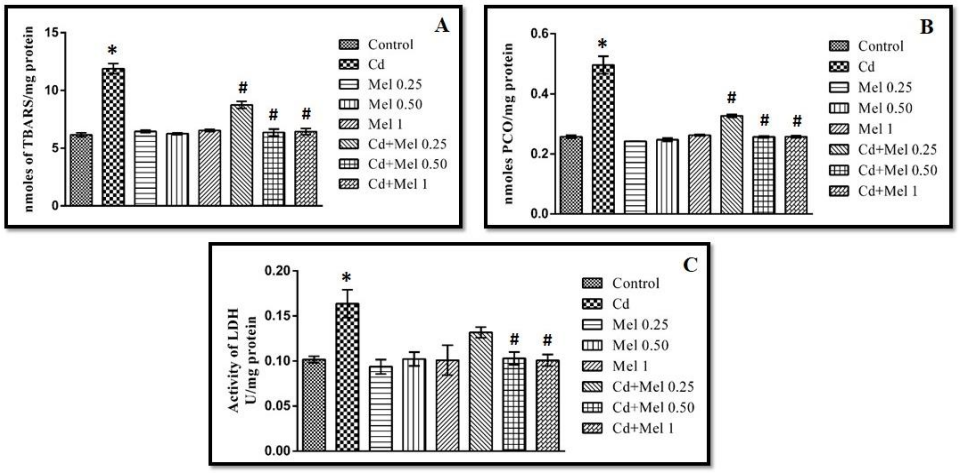

Fig. 2: Effects of Cd and melatonin on the LPO, PCO and LDH in the spleen tissues.

*Ameliorative effect of melatonin (0.25 , 0.50 and 1 mM) on Cd (400 µM) induced alterations in the levels of (A) LPO, (B) PCO and (C) LDH. Cd: Cadmium acetate (400 µM); Mel: Melatonin. The values are expressed as Mean ± S.E.M. (n = 4); # p < 0.01; versus Cd ; * p < 0.01 versus control using one way ANOVA.*

3.3. Effects of Cd and melatonin on the levels of GSH, GSSG and redox potential in spleen tissues.

Cd treatment significantly ($p < 0.001$) reduced the tissue level of GSH compared to the control groups (Fig. 3A). This decrease was almost completely preserved by melatonin treatment at tested concentrations. On the other hand, Cd treatment significantly elevated the level of GSSG and GSSG:2GSH ratio when compared to the control (Fig. 3: B-C). However, treatment with melatonin restored their levels significantly ($p< 0.001$) in a dose-dependent manner with maximum protection being observed in the Cd + Mel 0.50 mM group (Fig. 3: B-C). Melatonin alone did not influence these parameters compared to the control values.

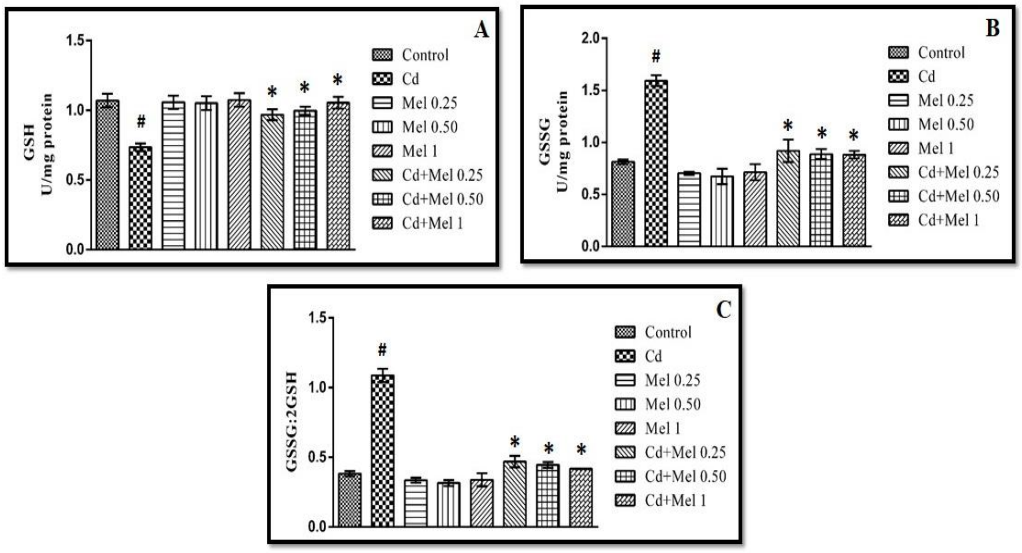

Fig. 3: Effects of Cd and melatonin on the levels of GSH, GSSG and redox potential in spleen tissues.

*The levels of (A) GSH, (B) GSSG and (C) GSSG:2GSH. Cd: Cadmium acetate (400 µM); Mel: Melatonin (mM). The values are expressed as Mean ± S.E.M. (n = 4); # $p < 0.01$; versus control; * $p < 0.01$ versus Cd using one way ANOVA.*

3.4. Effects of Cd and melatonin on the activities of antioxidant enzymes in spleen tissues .

A significant ($p < 0.001$) elevation in the activity of GPx was noted in the spleen tissues of Cd-treated group compared to the control group (Fig. 4A). Melatonin at the tested doses, significantly ($p < 0.01$) reduced elevated GPx activities induced by the Cd. The best protective dose of melatonin was 0.5 mM for this parameter (Fig. 4A). In contrast, Cd treatment caused a significant ($p < 0.001$) reduction in the activities of GR, catalase, Cu-Zn SOD and Mn-SOD, compared to the control values (Fig. 4: B-E). Melatonin significantly ($p < 0.05$) preserved the altered activities of all these enzymes induced by Cd with maximum protection of melatonin at the dose of 0.5 mM. Mel alone did not influence all these parameters compared to the control.

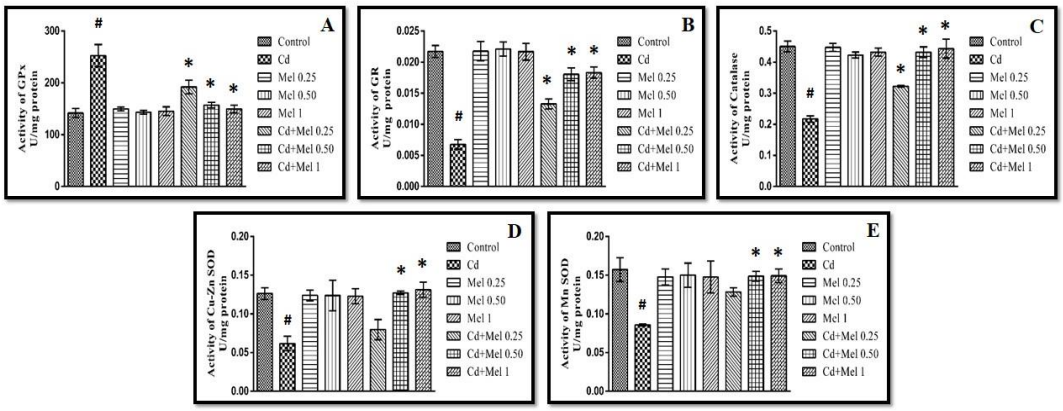

Fig. 4: Effects of Cd and melatonin on the activities of antioxidant enzymes in spleen tissues.

*The levels of (A) GPx, (B) GR, (C) CAT, (D) Cu-Zn SOD, (E) Mn-SOD. Cd: Cadmium acetate (400 µM); Mel: Melatonin (mM). The values are expressed as Mean ± S.E.M. (n = 4); # p < 0.01; versus control; * p < 0.01 versus Cd using one way ANOVA.*

3.5. Effects of Cd and melatonin on the activities of pro-oxidant enzymes in spleen tissues.

Activities of XO and XDH, and their ratio were significantly (p< 0.001) elevated with Cd treatment compared to control values (Fig. 5). These elevations induced by Cd were significantly inhibited with different doses of melatonin treatment with the best protective dose of 0.5 mM. (Fig. 5). Melatonin alone did not influence these parameters compared to the control values.

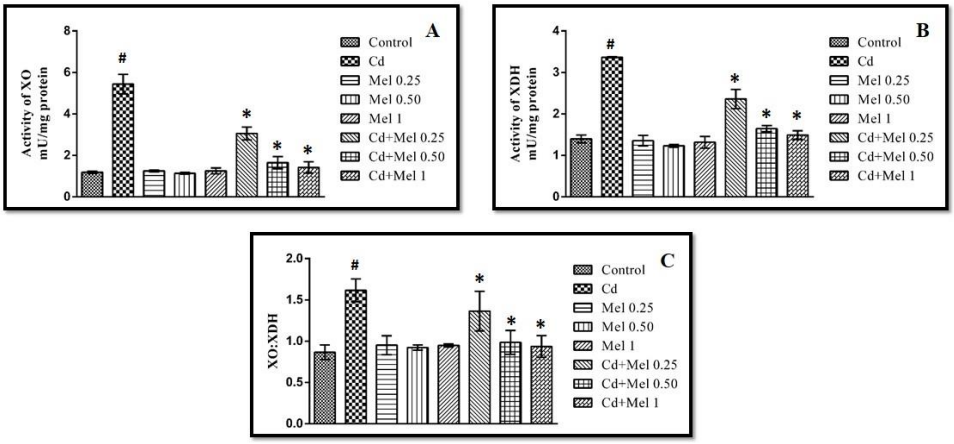

Fig. 5: Effects of Cd and melatonin on the activities of pro-oxidant enzymes in spleen tissues

*The activities of (A) XO, (B) XDH and (C) XO:XDH. Cd: Cadmium acetate (400 µM); Mel: Melatonin (mM). The values are expressed as Mean ± S.E.M. (n = 4); # p < 0.01; versus control; * p < 0.05 versus Cd using one way ANOVA.*

3.6. Effects of Cd and melatonin on the activities of glycolytic enzymes and glucose-6-phosphate dehydrogenase in spleen tissues.

The activities of HK, ALD and G-6-PDH (Fig. 6: A-C) were significantly (p < 0.001) decreased in Cd-treated groups compared to the control groups. These decrease induced by

Cd was almost completely preserved by different doses of melatonin treatments. Melatonin alone did not alter these activities compared to control.

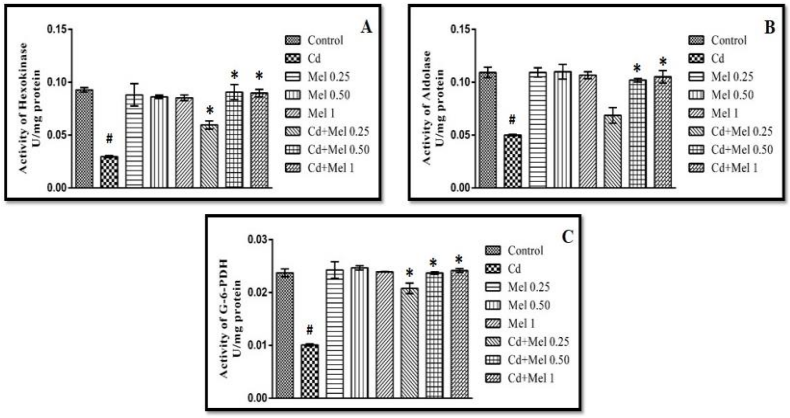

Fig. 6: Effects of Cd and melatonin on the activities of glycolytic enzymes and glucose-6-phosphate dehydrogenase in spleen tissues.

*The activities of (A) Hexokinase, (B) Aldolase, (C) Glucose-6-Phosphate dehydrogenase. Cd: Cadmium acetate (400 µM); Mel: Melatonin (mM). The values are expressed as Mean ± S.E.M. (n = 4); # p < 0.01; versus control; * p < 0.01 versus Cd using one way ANOVA.*

3.7. Effects of Cd and melatonin on the activities of PDH and different Krebs cycle enzymes in spleen tissues.

Cd treatment caused a significant ($p < 0.001$) reduction in the activities of PDH, CS, ICDH, α-KGDH and SDH compared to the control values (Fig. 7). The Cd induced reduction in the activities of PDH, CS, ICDH, α-KGDH and SDH were significantly recovered with different doses of melatonin treatment with the best protective dose of 0.5 mM. In comparison to the control values, no significant alteration in the activities of PDH, CS, ICDH, α-KGDH and SDH was noted in melatonin alone (Mel) treated groups.

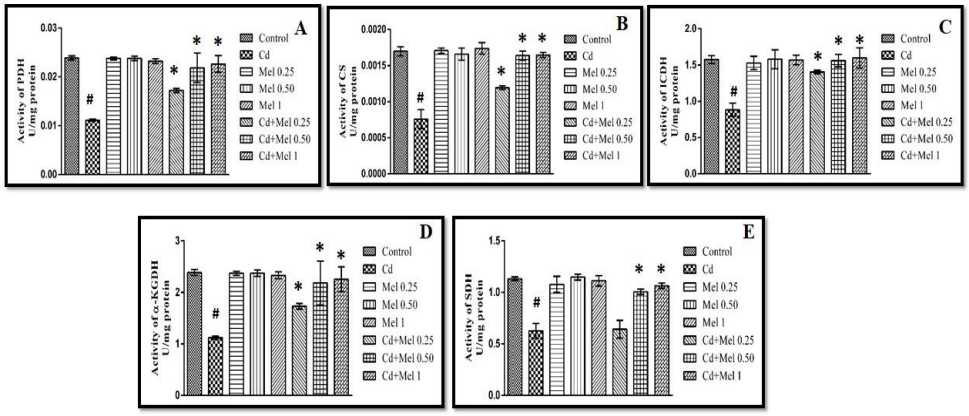

Fig. 7: Effects of Cd and melatonin on the activities of PDH and different Krebs cycle enzymes in spleen tissues.

*The activities of (A) PDH, (B) CS, (C) ICDH, (D) α-KGDH and (E) SDH. Cd: Cadmium acetate (400 µM); Mel: Melatonin (mM). The values are expressed as Mean ± S.E.M. (n = 4); # p < 0.01; versus control; * p < 0.01 versus Cd using one way ANOVA.*

3.8. Effects of Cd on the activities of PDH and Krebs cycle enzymes in spleen tissues: Pattern of inhibition.

As evident from different intercepts in the Y-axis, the inhibitory actions of Cd on the activities of PDH, ICDH, α-KGDH and SDH were found to be of uncompetitive in nature (Supplementary Fig. 1: A-D).

3.9. Effects of Cd and melatonin on the activities of respiratory chain enzymes in spleen tissues.

Cd treatment significantly reduced the activities of cytochrome-c-oxidase and cytochrome-c-oxidoreductase ($p < 0.01$) compared to control; however, these decreases were partially or completely reversed by melatonin treatment at different doses (Fig. 8: A-B). Melatonin treatment alone did not alter the activities of these enzymes compared to control.

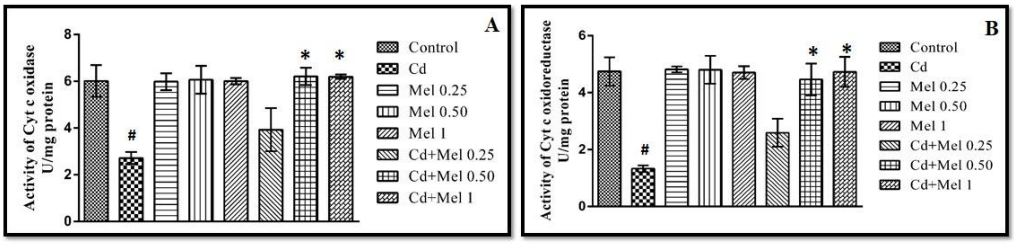

Fig. 8: Effects of Cd and melatonin on the activities of different respiratory chain enzymes in spleen tissues.

*The activities of (A) cytochrome c oxidase and (B) cytochrome c oxidoreductase. Cd: Cadmium acetate (μM); Mel: Melatonin (mM). The values are expressed as Mean ± S.E.M. (n = 4); # p < 0.01; versus control; * p < 0.01 versus Cd using one way ANOVA .*

3.10. Effects of Cd and melatonin on the mitochondrial architecture in spleen tissues.

The mitochondrial architecture was measured by SEM. Cd treatment resulted in mitochondrial surface distortion in the form of major blebs with rough and ruptured surface compared to control. (Fig. 9). Melatonin treatment at a dose of 0.50 mM preserved the altered mitochondrial architecture caused by Cd.

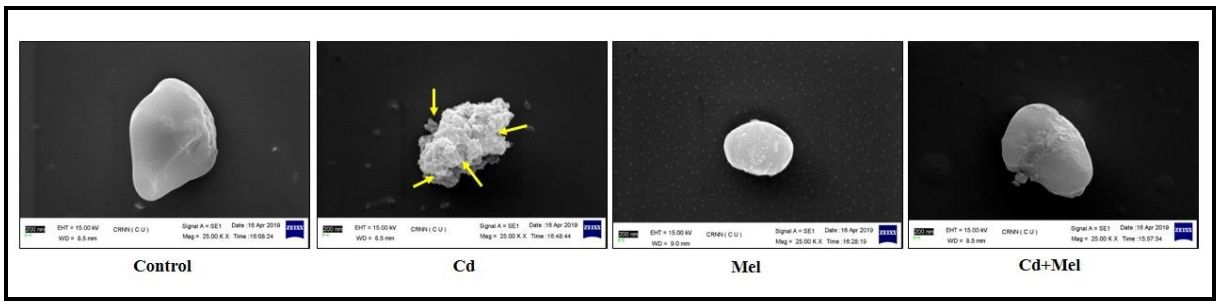

Fig. 9: Effects of Cd and melatonin on the assessment of mitochondrial architecture in spleen tissues.

Representative images of scanning electron micrograph (25000 X magnifications) of splenic mitochondria in control. Cd-treated (Cd) (400 μM); Melatonin alone (Mel)(0.5 mM) and Cd plus melatonin groups. Yellow arrow heads indicate distortion of mitochondrial surface in Cd -treated sample.

3.11. Effects of Cd and melatonin on the levels of intracellular ROS in splenocytes.

The level of ROS in splenocytes was detected using DCFH-DA (2,7-dichlorodi-hydrofluorescein diacetate), which is conveted into the fluorescent chromophore DCF (2,7-dichlorofluorescein) by interaction with ROS. As per a flowcytometric analysis (Fig. 10), after treatment with Cd, the DCF fluorescence increased significantly compared to the control cells ($p < 0.001$). However, DCFH-DA oxidation induced by Cd was significantly suppressed by melatonin treatment at the dose of 0.50 mM compared to Cd treated alone. This was indicated by the significantly decreased fluorescence of DCF.

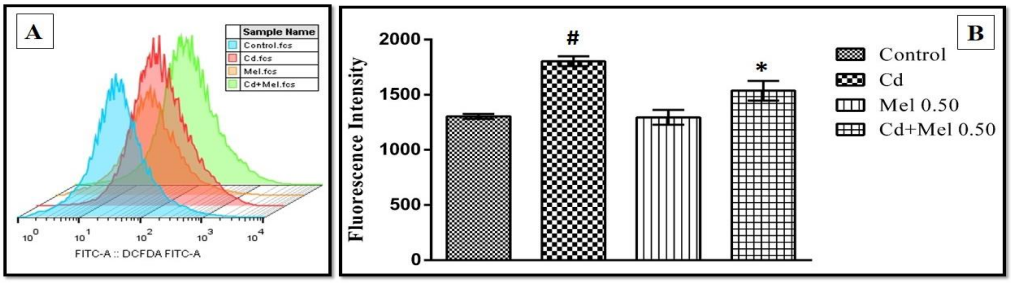

Fig. 10: Effects of Cd and melatonin on the levels of intracellular ROS in splenocytes.

*Staggered overlay presentation of the effects of Mel (melatonin 0.50 mM) on levels of ROS induced by Cd (Cadmium acetate 400 µM) in splenocytes via flowcytometric analysis following (A) DCFDA staining of control, melatonin alone (Mel), Cd alone and Cd plus melatonin (Cd+Mel) and (B) their fluorescence intensities are represented as column diagram showing total cellular ROS levels in the splenocytes. The values are expressed as Mean ± S.E.M.(n = 4); # p<0.001; versus control; * p<0.05 versus Cd using one way ANOVA.*

4. DISCUSSION

To our knowledge, the present study is the first report regarding the protective role of melatonin against Cd-induced oxidative stress in goat splenic tissues. The observations provide additional evidence to support the potent antioxidant and free radical scavenging properties of melatonin in combating Cd-induced oxidative insult in the spleen tissue.

The levels of LPO reflects the status of lipid peroxidation caused by radical-mediated membrane damage, hence, it serves as an important marker of oxidative damage in any tissue (25). In this study, increased levels of LPO in the spleen tissues clearly indicated the increased production of intracellular ROS induced by Cd. This has been observed in other organs/tissues including liver and heart (12, 17). On the other hand, PCO serves as a bio-marker of oxidative protein damage (49) which was also observed in Cd treated spleen tissue. The alterations of LPO and PCO caused by Cd were preserved by melatonin treatment at the different doses with the best protective dose being 0.5 mM. The results are in favour of the free radical scavenging and antioxidant properties of melatonin to protect against Cd-induced splenic injuries, at least, under *in vitro* system (50).

Being a cysteine-rich non-protein thiol compound, GSH is a major intracellular reduced substance (51). In this study, Cd concentration-dependently depleted spleen tissue GSH to form glutathione disulfide (GSSG), another marker of intracellular oxidative stress (37). This reaction is possibly via the formation of a cadmium-glutathione complex (52). Thus, the

glutathione disulfide-glutathione couple (GSSG/2GSH) served as the intracellular redox environment in the splenic tissue subjected to Cd toxicity (31). The significantly elevated GSSG/2GSH ratio under Cd treatment indicated an increase of ROS production in the spleen tissue. Melatonin (0.5mM) treatment suppressed the increase of GSSG/2GSH ratio caused by Cd. The result further supported the antioxidant potentiality of melatonin against Cd-induced oxidative damage.

It is well accepted that antioxidant enzymes such as GPx, GR, SOD and CAT actively participate in combating ROS mediated cellular damage (23). These enzymes function in a sequential manner within the physiological system. GPx catalyses conversion of H_2O_2 to water at the cost of a molecule of GSH (53), while GR reduces GSSG to form GSH. Similarly, SOD dismutes superoxide anion to H_2O_2 (54) thereafter, CAT converts it to water and molecular oxygen (55). In our study, Cd profoundly decreased the activities of GR, SOD and CAT, possibly through down-regulation of these enzymes at protein level (56, 57), thus affecting the antioxidant defence system of the spleen tissue. This decrease in the activities of different antioxidant enzymes might also account for the increased levels of LPO and PCO in the spleen tissue (17). Co-incubation with melatonin (0.5mM) restored their normal cellular level suggesting the indirect antioxidant role of melatonin (58) protecting against Cd-induced oxidative stress in the spleen tissue.

Under normal physiological conditions, XO and XDH exist in the interconvertible forms and act on hypoxanthine or, xanthine to produce superoxide anion and uric acid by using the molecular oxygen (59). In the present study, an increase in the activities of XO and XDH following Cd treatment clearly indicated the accumulation of enormous ROS (60) in the spleen tissues. Interestingly, melatonin (0.5mM) treatment was found to restore the activities of both XO and XDH in Cd-treated tissues.

As to the enzymes involved in cellular respiration Cd caused a profound decrease in the activities of HK and ALD suggesting the inhibitory effect of Cd on glycolytic enzymes (61). The inhibitory effects of Cd were also found on the activities of Krebs cycle enzymes including CS, ICDH, α-KGDH and SDH. These observations demonstrated the adverse effects of Cd on mitochondrial energy metabolism. As a mitochondrial targeted antioxidant (62), melatonin effectively preserved the activities of all these enzymes. This result is not surprising since melatonin can be accumulated at high levels through synthesis and/or extraction in the mitochondria (63, 64).

The final step of ATP synthesis is mediated by the electron transport chain (ETC) which accepts electrons from the Krebs cycle electron donors- NADH and FDH via redox reactions, and couples these reactions with the transfer of protons across the mitochondrial membrane. Such reactions are known to create an electrochemical proton gradient that activates the enzyme- ATP synthase and promotes the production of ATP (65, 66). The mitochondrial complex III is considered as the most crucial site for Cd-induced oxidative damage (67), Cd inhibited activities of Cyt C oxidase and Cyt C oxidoreductase to disrupt the ETC, thus promoting the generation of ROS (68). This adverse effect of Cd was almost completely reversed by melatonin co-treatment. The evidence suggests that the divalent heavy metal ions of Cd by inhibting ETC, particularly the complex III induce the overall ROS production in afflicted tissues. The increased fluorescence of DCF, a common marker of intracellular ROS (69, 70), has confirmed this point. Our result show that melatonin directly scavenged the intracellular ROS induced by Cd indicated by significantly reduced fluorescence. Cd induced oxidative stress not only disrupts the mitochondrial function by inhibiting activities of a

variety of enzymes but also destroys the mitochondrial structures visualized by SEM. These included mitochondrial surface distortion, forming major blebs with rough and ruptured surface. Melatonin at the tested concentrations effectively preserved the intactness of mitochondrial function and structure. These observation are consistant with previous publicated information in other tissues (71).

In conclusion, the present study provided novel and important information regarding the adverse effects of Cd on the spleen tissue of goat, probably also in other species. Cd exerts such harmful effects either by altering the activities of multiple endogenous antioxidant enzymes or, by disrupting the functions of enzymes associated with energy metabolism. The excessive production of ROS induced by Cd made a final push for the oxidative tissue damage (72). These processes primarily occurred in mitochondria since majority of the ROS is generated in this organelle. The SEM analysis further confirmed this notion. Melatonin as a mitochondrial targeted antioxidant exhibited strong protective effects on Cd induced oxidative damage in spleen tissue. In the current study, the investigation was focused on the direct effects of melatonin on the oxidative stress. The indirect effects of melatonin on oxidative stress have been well documented (71, 73, 74). It will be interesting to investigate whether melatonin receptor(s) are involved in deciphering the protective role of melatonin in the concerned tissue under Cd toxicity. Thus, use of luzindole, a melatonin receptor blocker, under similar experimental regimen in future studies will definitely disclose some novel information regarding the indirect protective role of melatonin against Cd induced tissue injury.

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AUTHORSHIP

Dr. DB and Dr. AC contributed to the conception,revised the manuscript critically and approved it. RM and MD carried out the experiments, prepared figures and drafted the manuscript. Dr. PKP contributed in carrying out the experiments, drafted the manuscript and edited it. BB contributed in carrying out the experiments.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

ABBREVIATIONS

Cd- Cadmium
SEM-Scanning Electron Microscopy
ROS-Reactive Oxygen Species
Fe-Iron
ATP-Adenosine Tri-Phosphate
AA-NAT-arylalkylamine-*N*-acetyltransferase
HIOMT-hydroxyindole-O-methyltransferase
OPD- o-phenylenediamine
PMS- phenyl methanesulfonate
DCFDA -2′,7′-Dichlorofluorescin diacetate
GSH- Reduced glutathione
TCA- Trichloroacetic acid
TBA- Thiobarbituric acid
PBS- Phosphate Buffered Saline
HBSS- Hank's Balanced Salt Solution
Mel-Melatonin
LPO- Lipid Peroxidation
TBARS- Thiobarbituric acid reactive substance
PCO- Protein Carbonyl
DNPH- Dinitrophenyl Hydrazine
LDH- Lactate Dehydrogenase
GSSG- Oxidised Glutathione
SOD- Superoxide Dismutase
CAT- Catalase
GR- Glutathione Reductase
GPx- Glutathione Peroxidase
XO- Xanthine Oxidase
XDH- Xanthine Dehydrogenase
HK- Hexokinase
ALD- Aldolase
G-6-PDH- Glucose 6 phosphate dehydrogenase
PDH- Pyruvate dehydrogenase
CS- Citrate Synthase
ICDH- Isocitrate dehydrogenase
α-KGDH- α-Ketoglutarate dehydrogenase
SDH- Succinate dehydrogenase
NAD^+- Nicotinamide-Adenine Dinucleotide (Oxidised)
NADH-Nicotinamide-Adenine Dinucleotide (Reduced)
LB-Lineweaver Burk

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