Research Article

Amelioration of adrenaline induced oxidative gastrointestinal damages in rat by melatonin through SIRT1-NFκB and PGC1α-AMPKα cascades

Palash Kumar Pal[a^], Swaimanti Sarkar[a^], Sanatan Mishra[a, b], Sreya Chattopadhyay[a], Aindrila Chattopadhyay[b], Debasish Bandyopadhyay[a*]

[a]Oxidative Stress and Free Radical Biology Laboratory, Department of Physiology, University of Calcutta, 92, APC Road, Kolkata-700009
[b]Department of Physiology, Vidyasagar College, 39, Sankar Ghosh Lane, Kolkata-700006
^ Authors have equal contribution
* Correspondence: debasish63@gmail.com; dbphys@caluniv.ac.in, Tel: +91 9433072066

Running Title: Melatonin prevents adrenaline induced intestinal damage

ABSTRACT

Adrenaline at high pharmacological doses may lead to oxidative damages in diverse organs including gut. In this study, we attempt to elucidate the potentially protective effects of melatonin on gastrointestinal (GI) tissue damages induced by adrenaline. Rats were injected (s.c.) with different doses (0.125, 0.25 and 0.50 mg/kg) of adrenaline bitartrate (AD) for 15 days with or without melatonin (2.5, 5 and 10 mg/kg; orally). The results showed that adrenaline caused massive histological and ultra-structural GI injuries and melatonin (20 mg/kg) effectively protected these injuries. The protective mechanisms are related to the antioxidant and anti-inflammatory activities of melatonin indicated by increased glutathione levels and antioxidant enzymes as well as decreased oxidative stress markers and pro-inflammatory cytokines in GI tissues. The signal pathways of melatonin include up-regulating expression of Nrf2, SIRT1 and Bcl2, while down-regulating NFκB, TNFα and Bax. Melatonin also targeted mitochondrial energy homeostasis and biogenesis by up-regulating expression of PGC1α, AMPKα and SOD2 and reduced leakage of cytochrome c. The SIRT1-NFκB and PGC1α-AMPKα signal transduction pathways seem to play the central roles involving in melatonin's protective effects on gastric damages induced by the high doses of adrenaline.

Keywords: adrenaline, oxidative stress, tissue damage, antioxidant, gastrointestinal tract, melatonin.

_____

1. INTRODUCTION

Prolonged high level of catecholamines induced by a constant stress causes hormonal disruptions in mammals (1). Adrenaline (epinephrine), one of the catecholamines, has been extensively used as emergency medication (3-6). However, chronic use of or, exposure to high pharmacological doses of adrenaline lead to oxidative tissue damage (7-10) due to its redox property and ability to undergo rapid auto-oxidation (11). Several studies have documented its hepatotoxic (8), cardiotoxic (7) and DNA damaging activities (12). It also potentiates cellular apoptosis (13) and initiates carcinogenesis (14, 15). Raugstad *et al*. (16)

originally reported mucosal erosive effect in GI tract with adrenaline treatment in rats. Since then, only one recent *in vitro* study confirmed this observation which showed a disruption of endogenous melatonergic system and cellular antioxidant defence system caused by adrenaline treatment (9). Thus, it became necessary to explore the molecular events underlying the deleterious effects of adrenaline on GI tract.

Melatonin, an important chronobiological molecule and a potent antioxidant, exerts diverse biological roles in mammalian physiology (17, 18). Among the extra-pineal sources of melatonin in mammals, the gut resident enterochromaffin cells produce 400 times higher melatonin than that in the pineal gland (19). The rate-limiting enzymes for melatonin synthesis including arylalkylamine-N-acetyltransferse (AANAT) and hydroxyindole-O-methyltransferase (HIOMT) are confirmed to be present in the mammalian GI tract (9, 20). This molecule plays defensive role against varied gastric injuries due to its potent antioxidant capacity (17, 21, 22). Some studies have indicated that adrenaline associated GI damage may involve alteration of endogenous melatonergic system (9, 23). However, the protective effects of melatonin against adrenaline induced oxidative GI injuries are not available till date.

Thus, the present study is perhaps the first attempt to explore the underlying molecular mechanisms regarding the potentially protective effects of melatonin on adrenaline induced GI damages. This study is carried out in intact animals (rats). The results from this study hopefully will provide valuable information for clinical use of melatonin for this purpose.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents.

1-Chloro-2,4-dinitrobenzene (CDNB), BCIP (5-bromo-4-chloro-3 indole phosphate), NBT (Nitro blue tetrazolium), trichloroacetic acid (TCA), phenylmethyl sulphonyl fluoride (PMSF), sodium azide (NaN_3), 5-methylphenazinium methyl sulphate (PMS), 5, 5´-dithiobis (2-nitrobenzoate) (DTNB), o-phenylenediamine (OPD) and ethylene diamine tetra acetic acid (EDTA) were purchased from Sisco Research Laboratories (SRL), Mumbai, India. All other chemicals and reagents of the highest available purity grade were procured from Sigma-Aldrich Co., St. Louis, MO, USA.

2.2. Animals.

Male Wistar rats, weighing 180 ± 20 g, were procured from Saha Enterprise (Kolkata, West Bengal, India; Regd. No. 1828/PO/BT/S15/ CPCSEA) and acclimatized for 5 days under controlled laboratory conditions (light:dark=12h/12h and temperature 20-26ºC). Standard diet and water were accessed by animals *ad libitum*. Daily food consumption was checked for all animals during the entire experimental period to ensure that all animals were healthy. This animal study was approved by Institutional Animal Ethical Committee (IAEC), Department of Physiology, University of Calcutta; Approval Number: IAEC-V/DB-1(Palash Pal)/2019 dated 07.08.2019]. Handling and care of animals werein accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Govt. of India.

2.3. Adrenaline treatment.

Male Wistar rats (n=24), were randomly divided into 4 groups. Control group were injected (s.c.) with vehicle, while rats in the other groups were injected (s.c.) with 0.125

(AD1), 0.25 (AD2) and 0.50 (AD3) mg/kg of adrenaline bitartrate (AD), respectively for 15 days at 12:30 h.

2.4. Melatonin and adrenaline co-treatment.

Male Wistar rats (n=48) were divided into 8 groups. Control group were injected (s.c.) with vehicle, while rats in the second group was injected (s.c.) with 0.25 mg/kg of AD. The 3rd – 5th groups were orally (gavage) given different doses of melatonin (2.5, 5.0, 10.0 mg/kg, respectively). Rats in the 6th- 8th groups were given different doses of melatonin daily (2.5, 5.0, 10.0 mg/ kg, respectively) 30 min prior to AD injection. The treatment was carried out for 15 consecutive days. Melatonin solution (40 mg/mL) was prepared daily in ethanol and saline (0.9%) mixture (1:9) in amber container to avoid its light induced degradation. Animals were treated with melatonin at 12:00 h corresponding to the highest concentration of daily melatonin in the stomach (24), as melatonin levels in the different gastrointestinal tissues is known to have no significant diurnal variation (24). AD treatment was performed at 12:30 h.

2.5. Collection and preparation of samples.

Animals were fasted overnight and sacrificed on the next day at 12:00 h (after 24 hours of last melatonin administration) by cervical dislocation with mild ether anaesthesia. The abdominal cavity was immediately opened and the desired regions of the gastrointestinal tissue (i.e. stomach, duodenum and colon) were extirpated and thoroughly washed in ice cold PBS (pH 7.4) to remove food/faecal matters completely. A small portion of different tissues was immediately fixed in paraformaldehyde for histological studies. Rest of the tissues were collected separately and stored at -80 °C until further analysis. Tissues were homogenized (10%) in homogenizing buffer [100 mM Tris-HCl buffer (pH 7.2–7.4), containing 0.1 mM PMSF, 250 mM sucrose and 1% leupeptin hemisulfate] and centrifuged at 1500 x g for 10 min at 4°C. The supernatant was collected as the source of crude enzyme and stored at -80°C for biochemical and Western blot analysis. In order to collect mitochondria, the tissue homogenates were subjected to an initial centrifugation at 5000 x g for10 min and the supernatant was further centrifuged at 12000 x g for 15 min at 4 ^{o}C. Pellets were re-suspended in an appropriate buffer and subjected to western blot analysis.

2.6. Measurement of melatonin concentration in GI tissues.

Concentration of melatonin in the gastrointestinal tissue homogenates were measured following the manufacturer's instructions of a rat MT (Melatonin) ELISA kit (ER1169; Wuhan Fine Biotech Co., Ltd., Wuhan, China).

2.7. Western blot analysis.

Levels of targeted proteins (Table 1) in the tissue homogenates and mitochondrial samples were evaluated by Western blot (9, 25). In brief, 60 mg protein were loaded in each well and subjected to SDS–PAGE (12%) with the method of Laemmli (26). The dilution used for primary and secondary antibodies were 1:2000 and 1:3000, respectively. Bax/Bcl2 ratios were also calculated. Sixty microgram protein was loaded in each well for immuno-detection. Following completion of the run, the gel was transferred to nitrocellulose membrane (Pall Corporation, USA) and the immuno-blot was incubated in blocking solution (5% bovine serum albumin) for 1 h. Then the immuno-blots were washed thrice in TBS-T and incubated

with specific primary antibody (1:2000 dilution) for overnight at 4°C. Following wash (thrice), the blot was incubated with secondary antibody (Goat anti-rabbit IgG ALP conjugate; 1:3000 dilution) for 2 h at 4°C, washed thrice and then finally incubated in BCIP/NBT substrate. Band intensity of each immuno-blot was normalized by the intensity of β-actin (NIH, Bethesda, MD, USA) and quantified through densitometric analysis using ImageJ software and expressed in relative densitometric units (9). Details of antibodies are mentioned in Table-1.

Table 1. Details of the primary and secondary antibodies used in the present study.

Antibody	Abbreviation	Product No.	Company
Arylalkylamine *N*-acetyl transferase	AANAT	ab3506	Abcam
Nuclear factor kappa beta	NFκB	ab16502	Abcam
Melatonin receptor 1a	MT1a	orb234902	Biorbyt
Nuclear factor erythroid 2-related factor 2	Nrf-2	ab31163	Abcam
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	PGC-1α	ab54481	Abcam
Sirtuin 1	SIRT 1	sc-15404	Santa-Cruz
Bax	Bax	ab53154	Abcam
Bcl2	Bcl2	ab196495	Abcam
Cytochrome C	Cyt C	orb251517	Biorbyt
Caspase 9	Caspase 9	ab52298	Abcam
Caspase 3	Caspase 3	ab4051	Abcam
5′AMP-activated protein kinase alpha	AMPKα	ab133448	Abcam
Superoxide dismutase 2	SOD 2	ab135334	Abcam
Goat anti-rabbit IgG AP	Goat anti-rabbit IgG AP	ab97048	Abcam
Beta- actin	β-actin	sc-130657	Santa-Cruz

2.8. Measurements of oxidative stress markers.

Tissue level of lipid peroxidation (LPO) was estimated in the form of TBARS (thiobarbituric acid reactive substances) (27). Protein carbonyl (PCO) content was measured by DNPH assay with the method of Levine *et al*. (28). GSH level was estimated based on the method described by Ellman (29). Briefly, 100 µL of sample were added to 100 µL of perchloric acid (5%) and centrifuged at 800 × g for 10 min at 4°C. Then, 2 mL of reaction mixture [containing (100 µL) supernatant, 1.88 ml of 0.1 mol/l potassium phosphate buffer (pH 8.0) and 0.02 mL 4% DTNB] were added to each solution and were incubated at RT for 3 min. Finally, the absorbance was recorded at 412 nm for 5 min at a regular interval of 60 s. The level of GSH was calculated from the standard graph prepared with GSH and was expressed as µM/mg protein.

2.9. Activities of antioxidant enzymes.

Superoxide dismutase (SOD) activity was determined according to the method described by Ewing and Janero (30). Activities of catalase (CAT) and glutathione peroxidase (GPx) were estimated following the method of Aebi (31) and Castro *et al*. (32), respectively. Glutathione *S*-transferase (GST) and glutathione reductase (GR) activities were determined

by the method of Habig *et al.* (33), and Pinto and Bartley (34), respectively. The detailed assay methods of different enzymatic and non-enzymatic antioxidants are described below.

2.9.1. Superoxide dismutase (SOD).

The activity of SOD was determined according to the method described by Ewing and Janero (30). The absorbance was recorded at 560 nm using a micro-plate reader (Bio-Rad, USA). Briefly, 25 µL sample was added to 200 µL of 50 mM phosphate buffer (pH 7.4), containing 0.1 mM EDTA, 62 µM NBT, and 98 µM NADH into a microtiter well. The reaction was initiated by the addition of 25 µL of 33 µM PMS in 50 mM phosphate buffer containing 0.1 mM EDTA (pH 7.4). Enzyme activity was expressed as U/mg protein. The serial dilutions of substrates (NADH and PMS) were used for the validation of the assay. Negligible alterations in the absorbance of the test samples were noted.

2.9.2. Catalase (CAT).

The method described by Aebi (31) was used to measure the activity of CAT in the tissue homogenates. Briefly, 40 µL of sample was added to H_2O_2 phosphate buffer (2 mM) and absorbance was recorded at 240 nm for 90 sec at 15 sec intervals using an UV-Vis spectrophotometer. Enzyme activity was expressed as U/mg protein.

2.9.3. Glutathione peroxidase (GPx).

GPx activity was estimated by the method described by Castro *et al.* (32). Briefly, 100 µL enzyme of sample was mixed with 1 mL of OPD in phosphate citrate buffer (pH 5.0). Then 0.9 mL of H_2O_2 (0.013%) was added to each solution and incubated at room temperature for 30 min. The absorbance was determined at 492 nm and enzyme activity was expressed in U/mg protein. In order to validate the assay, serial dilutions of the substrate (OPD) were used.

2.9.4. Glutathione S-transferase (GST).

The method of Habig *et al.* (33) was used to estimate the activity of GST in the tissues, where 1-chloro-2, 4-dinitrobenzene (CDNB, 1 mM/L) and GSH (2.4 mM/L) were used as substrate. The absorbance was recorded at 340 nm for 5 min at a regular interval of 60 s. GST activity was expressed as U/mg protein. Serial dilutions of GSH and CDNB (substrates) were used to measure their optimum concentrations for the highest activity of GST.

2.9.5. Glutathione reductase (GR).

Activity of GR was estimated by the method of Pinto and Bartley (34) based on the glutathione dependent oxidation of NADPH. Briefly, 50 µL of tissue homogenate were mixed with 950 µL reaction mixture [containing 0.15 mM NADPH, 0.5 mM glutathione, 3 mM $MgCl_2$ in 50 mM Tris (pH 7.5)] and the change in absorbance was recorded at 340 nm. In order to validate the assay, NADPH was oxidized in absence of glutathione as the background.

2.10. Tissue levels of pro- and anti-inflammatory cytokines.

Levels of pro-inflammatory (IL-1β, IL-6 and TNFα) and anti-inflammatory (IL-10) cytokines in the tissues were estimated following the manufacturer's (RayBiotech, Norcross,

GA) instructions. In brief, 100 µL samples were added to each well and incubated for 2.5 h at room temperature (RT), then, the solution was discarded and wells were washed 4 times in washing buffer. Then, 100 µL of biotinylated antibody were added to each well and again incubated for 1 h at RT. After discarding the solution, wells were again washed in the same manner. 100 µL of streptavidin solution were added to each well and incubated for 45 min at RT. Again, the solution was discarded and the wells were washed, then 100 µL of TMB substrate reagent were added to each well and incubated for 30 min at RT under complete dark condition. Finally, 50 µL of stop solution was added to each well and the reading was immediately recorded at 450 nm. All reagents were prepared strictly following the manufacturer's instructions.

2.11. Histology and scanning electron microscopy (SEM).

Paraformaldehyde (4%) fixed gastrointestinal tissues were embedded in paraffin and sliced to 5µm thick sections which were further deparaffinised and stained with Haematoxylin-Eosin (HE) to identify tissue morphology (35, 36). Similar deparaffinised slides were used to stain the cell nuclei with 4′, 6′- diamino-2-phenylindole (DAPI; 1µg/ml) for the morphological assessment of the apoptotic cells following the method of Mohankumar *et al*. (37). Stomach, duodenum and colon tissue sections were subjected to SEM analysis following the technique of Cheema and Scofield (38) with some modifications (39) and observed under a scanning electron microscope (SEM; Zeiss Evo 18 model EDS8100) available at CRNN, University of Calcutta, India.

2.12. Estimation of protein.

The concentration of protein in the tissue homogenate was estimated following the method of Lowry *et al*. (40).

2.13. Statistical analysis.

Data of each variable was expressed as mean ± standard error of mean (SEM) (n=6). Shapiro–Wilks test was used to find out whether the data sets were normally distributed or not. Since all the data sets passed the normality test ($p<0.01$), one-way analysis of variance (ANOVA) followed by a *post-hoc* Duncan's multiple range test (DMRT) was performed to evaluate the significance in the mean values of different parameters between the treated groups, $p<0.05$ as threshold of significance (41). In case of Western Blot analysis, the band intensities of the proteins were normalized by the intensity of *β*-actin (internal control) and expressed as relative densitometric values (RDV) using ImageJ software. Statistical analysis and data presentation were carried out using Statistical Package for the Social Sciences (SPSS), Inc., Statistics version 21.0 and GraphPad Prism version 6.03 (GraphPad Software).

3. RESULTS

3.1. Effects of melatonin on AD induced alterations in the levels of oxidative stress markers in GI tissues.

In the first set of experiment, AD treatment significantly ($p<0.001$) increased the levels of LPO and PCO in a dose dependent manner, but decreased the levels of GSH in the stomach, duodenum and colon tissues when compared to control, where maximum damage was noted in the rats treated with AD at a dose of 0.25 mg/kg (Figure 1);- hence, this dose was selected

for further study. Oral administration of melatonin (5 mg/kg) significantly ($p<0.001$) reduced the levels of LPO, PCO and increased GSH content in all gastrointestinal tissues selected in AD+M group compared to AD alone group (Figure 2).
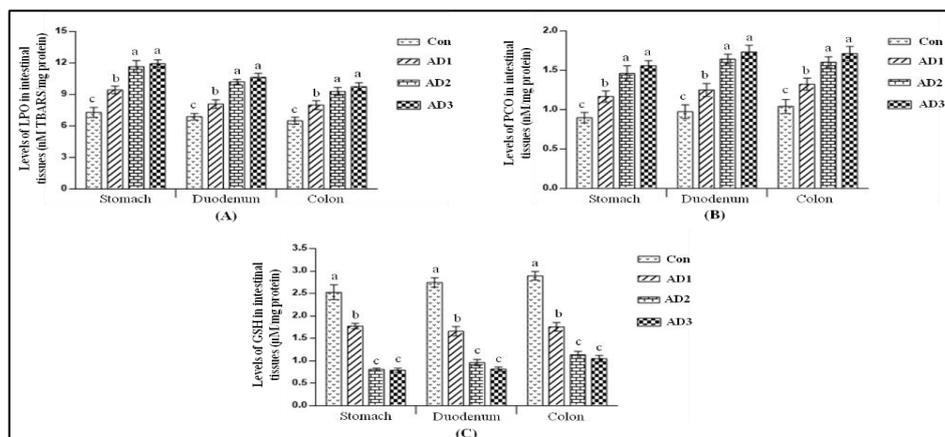
Fig. 1. Effects of adrenaline treatment on the levels of oxidative damage in GI tissues.

Diagrammatic representation of the levels of (A) lipid peroxidation (LPO), (B) protein carbonyl content (PCO) and (C) reduced glutathione (GSH). Con: control, *AD1: adrenaline 0.125, AD2: 0.25 and AD3: 0.5 mg/kg, respectively. Different letters on the error bars indicated significant difference vs each other (p<0.001; n=6/group).*
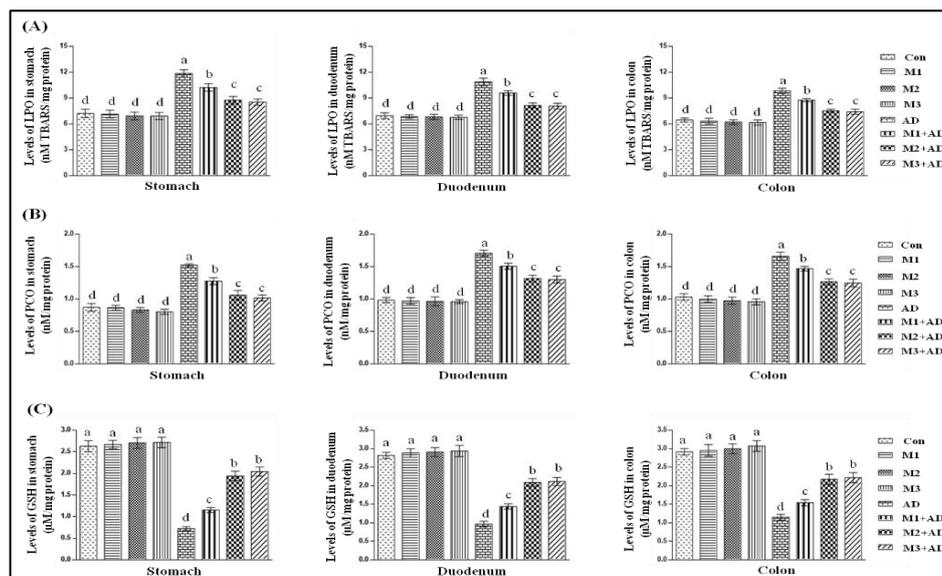
Fig. 2. Effects of melatonin on the adrenaline induced alterations in the levels of different oxidative stress markers in the GI tissue.

Diagrammatic representation of the levels of (A) lipid peroxidation (LPO), (B) protein carbonyl content (PCO) and (C) reduced glutathione (GSH).M1: melatonin 2.5, M2: 5 and M3: 10 mg/kg., respectively. AD: adrenaline 0.25 mg/kg. Different letters on the error bars indicated significant difference vs each other (p<0.001; n=6/group).

3.2. Effects of melatonin on AD induced morphological and ultra-structural alterations in GI tissues.

GI tissues had normal cellular architectures in control and melatonin alone groups (Figure 3). However, AD (0.25 mg/kg) treatment distorted the gastric mucosae and lamina propria in

the stomach tissue and caused desquamation of the villi tips, accumulation of neutrophils and damage in the lamina propria layer, thus reducing the thickness of the mucosa layer of the villi in duodenum and colon (Figure 3). Melatonin (5 mg/kg) pre-treatment preserved the gastric mucosal cells with morphological alterations caused by AD. Moreover, no sign of neutrophil accumulation or, desquamation of the villi or, damage in the lamina propria layer was noted following melatonin pre-treatment.
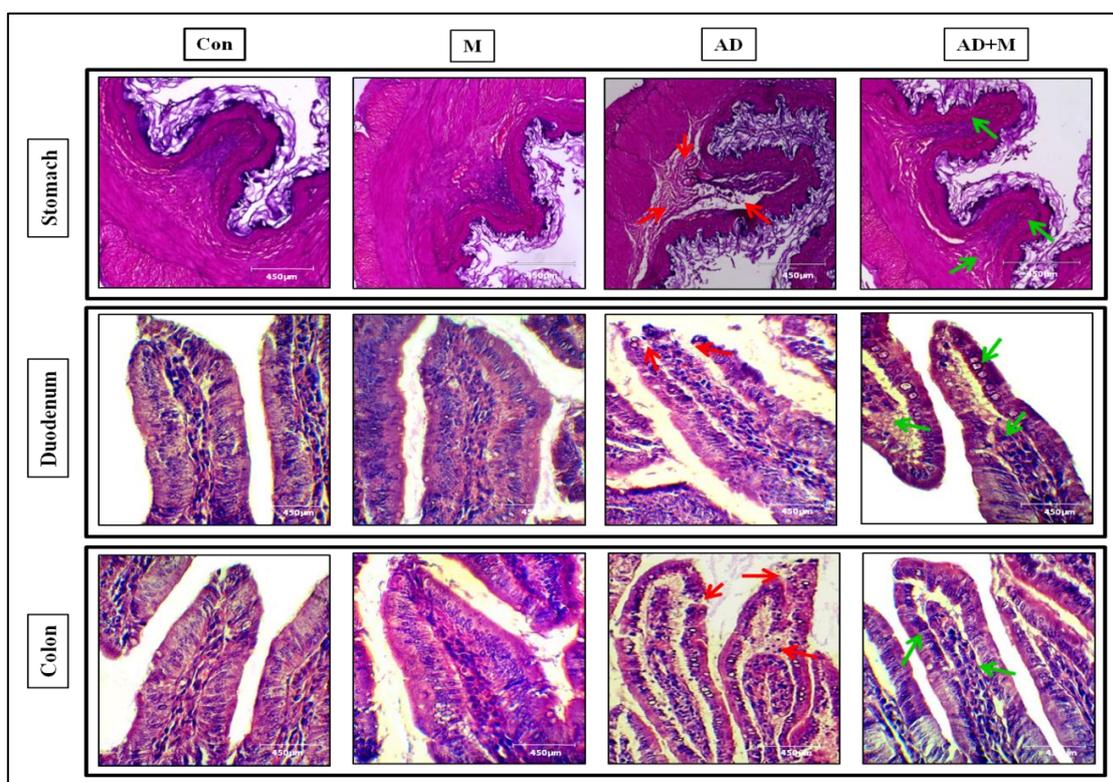
Fig. 3. Effects of melatonin on the morphological alterations induced by AD.
Representative images of HE staining of the stomach, duodenum and colon tissues of rats. All the tissues exhibited normal cellular architecture in the control and melatonin treated groups. AD treatment caused profound cellular distortions (red arrows) in the mucosa and lamina propria along with desquamation of the epithelial lining. Melatonin pre-treatment prevented these alterations in the concerned tissues (green arrows). Con: control, M: melatonin 5 mg/kg, AD: adrenaline 0.25 mg/kg. AD + M: adrenaline + melatonin.

The GI tissues exhibited normal sub-cellular structures in the control and melatonin alone groups under the SEM observation (Figure 4). However, AD (0.25 mg/kg) treatment caused stomach mucosal injury, and apical cellular erosion of the microvilli in the duodenum and colon tissues (Figure 4). Moreover, breakage of the villi tip was widely present in the duodenum and mucosal surface in the colon tissues, leading to loss of tissue integrity. Melatonin treatment protected the AD induced GI tissue damages and there was no erosion in the lamina propria as well as villus tip; thus, epithelial cells were found to be almost intact in all three GI tissues with melatonin pretreatment (Figure 4). The results are consistent with DAPI staining which showed apoptotic changes in GI tissues - treated with AD. These changes included enhancement in the fluorescence along with frequent chromatin condensation in the nuclei of the epithelial cell distributed at the tip of the microvilli and lamina propria and partially denuded epithelium of villi. All changes were also prevented by melatonin pre-treatment (Figure. 5).
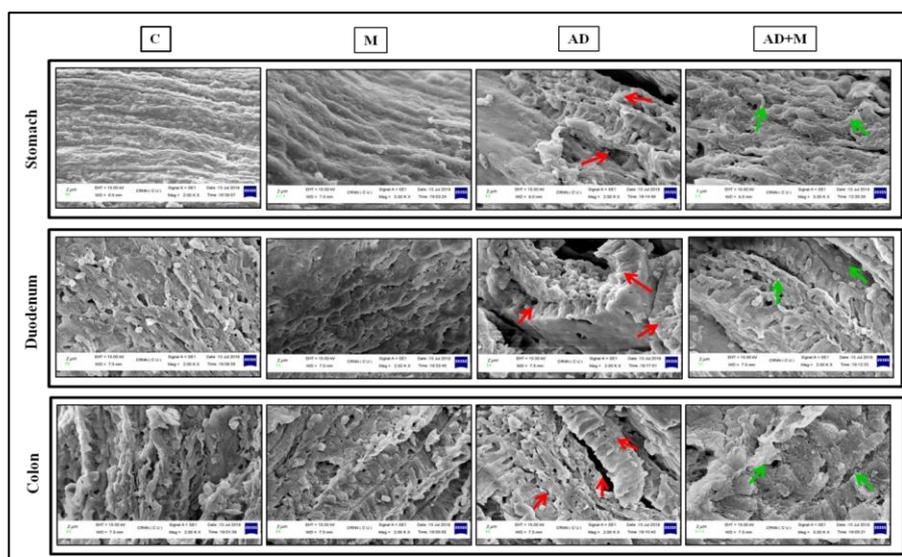
Fig. 4. Effects of melatonin on the ultrastructure of GI tissue treated with AD.

Representative images of scanning electron microscopic (SEM) study of the stomach, duodenum and colon tissues of rats. All the tissues exhibited normal cellular architecture in the control and melatonin treated groups. AD treatment caused structural damages (red arrows) in the mucosa and lamina propria of the gastrointestinal tissues. Melatonin pre-treatment prevented these alterations (green arrows) in the concerned tissues. Con: control, M: melatonin 5 mg/kg, AD: adrenaline 0.25 mg/kg. AD + M: adrenaline + melatonin (0.25 mg/kg).
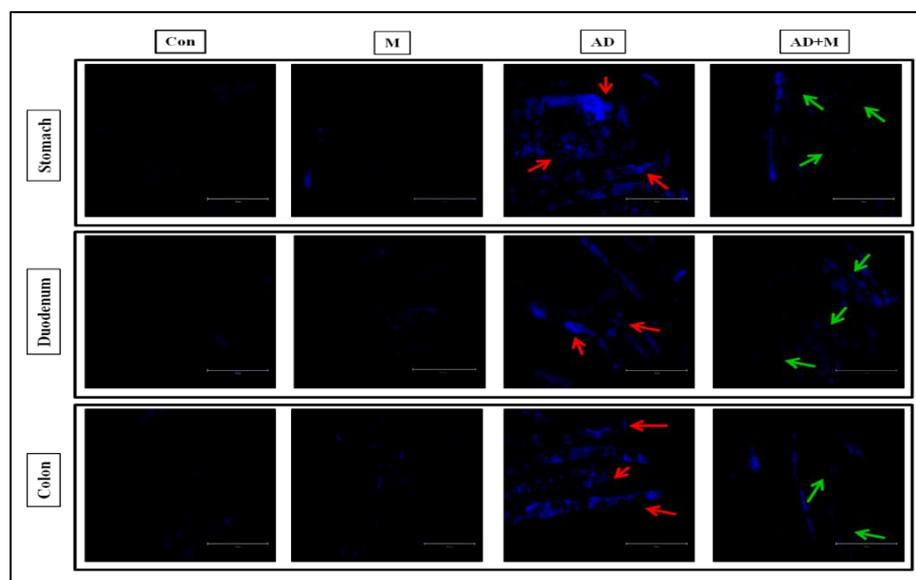
Fig. 5. Effects of melatonin on apoptosis in GI tissues caused by the AD oxidative stress.

Representative fluorescence photomicrographs of DAPI staining of the stomach, duodenum and colon tissues of rats treated with melatonin and adrenaline. In the control or melatonin alone group, there was no sign of apoptosis-specific morphological changes. AD treatment caused partial denudation of the epithelial lining of the villi and enhanced fluorescence in the nuclei of the epithelial cell distributed at the tip of the microvilli and lamina propria (red arrows). Melatonin pre-treatment prevented these apoptotic changes in the concerned tissues (green arrows). Con: control, M: melatonin 5 mg/kg, AD: adrenaline 0.25 mg/kg, AD + M: adrenaline + melatonin.

3.3. Effects of exogenous melatonin on endogenous melatonin synthesis in the GI tissues under AD-induced oxidative stress.

Concentrations of melatonin and relative abundance of AANAT and MT1 (Figure 6) in stomach, duodenum and colon tissues were significantly ($p<0.001$) decreased in AD (0.25 mg/kg) treatment but increased in melatonin alone group compared to control. However, melatonin (5 mg/kg) pre-treatment significantly ($p<0.001$) preserved the levels of melatonin which was depleted by AD treatment. In addition, the expression of *AANAT* and *MT1* was up-regulated in GI tissues of AD+M group compared to AD (Figure 6).
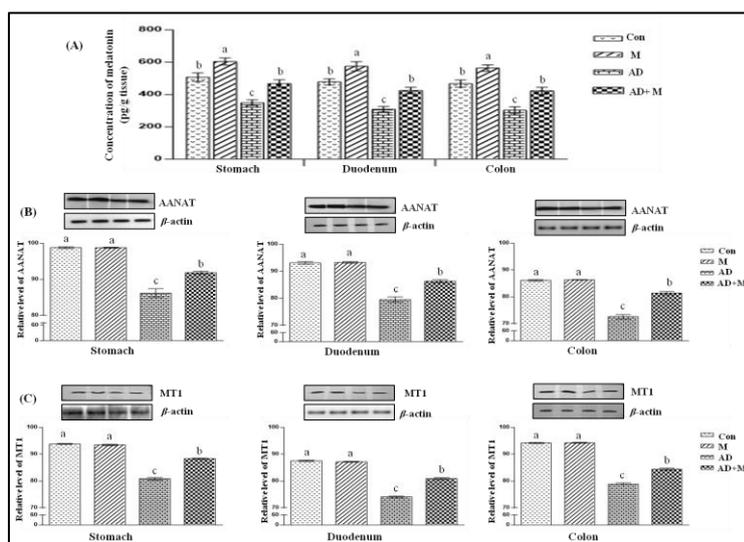
Fig. 6. Effects of exogenous melatonin on the endogenous melatonergic system in GI tissues under AD induced oxidative stress.
Diagrammatic representation of the (A) melatonin tissue levels, and immunoblots and alterations in the relative densities of (B) arylalkylamine N-acetyl transferase (AANAT) and (C) melatonin receptor 1 (MT1) proteins. Different letters on the error bars indicated significant differences vs each other (p<0.001; n=6/group), M: melatonin 5 mg/kg, AD: adrenaline 0.25 mg/kg, AD + M: adrenaline + melatonin.

3.4 Effects of melatonin on antioxidant enzymes of GI tissues under AD-induced oxidative stress.

Activities of SOD and GR in the stomach, duodenum and colon tissues were significantly ($p<0.001$) decreased, while activities of CAT, GPx and GST were significantly ($p<0.001$) increased in AD group compared to control (Figure 7). Melatonin alone did not change these parameters significantly. However, melatonin co-treatment with AD significantly ($p<0.001$) preserved the activities of SOD, GR, CAT, GPx and GST which were depleted by AD treatment (Figure 7).

3.5. Effects of melatonin on the levels of different cytokines under AD induced stress.

Levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) in stomach, duodenum and colon tissues were significantly ($p<0.001$) increased in AD (0.25 mg/kg) group, while level of anti-inflammatory cytokine (IL-10) was decreased compared to control (Fig. 8). However, exogenous melatonin (5 mg/kg) significantly ($p<0.001$) reduced the pro-inflammatory cytokines and enhanced anti-inflammatory cytokine IL-10 in AD+M group compared to AD group (Figure. 8).
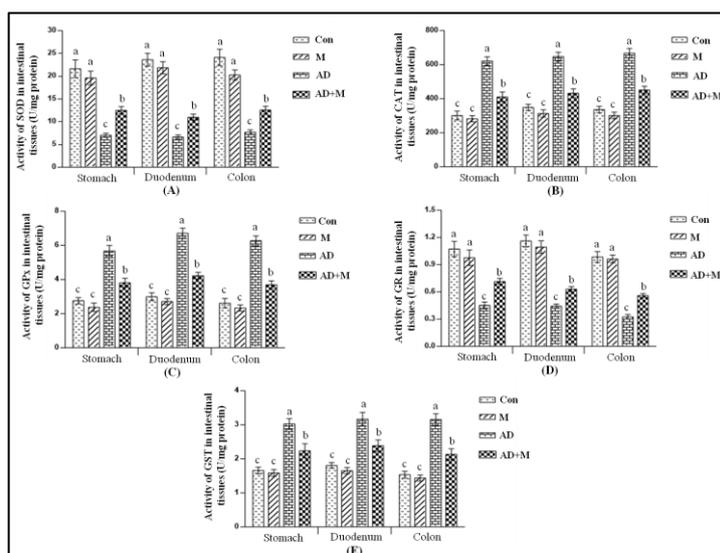
Fig. 7. Effects of melatonin on the activities of antioxidant enzymes in GI tissues under the AD induced oxidative stress.

Diagrammatic representation of the activities of (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) glutathione peroxide (GPx), (D) glutathione reductase (GR) and (E) glutathione-S-transferase (GST). Different letters on the error bars indicated significant differences vs each other (p<0.001; n=6/group). Con: control, M: melatonin 5 mg/kg, AD: adrenaline 0.25 mg/kg, AD + M: adrenaline + melatonin.
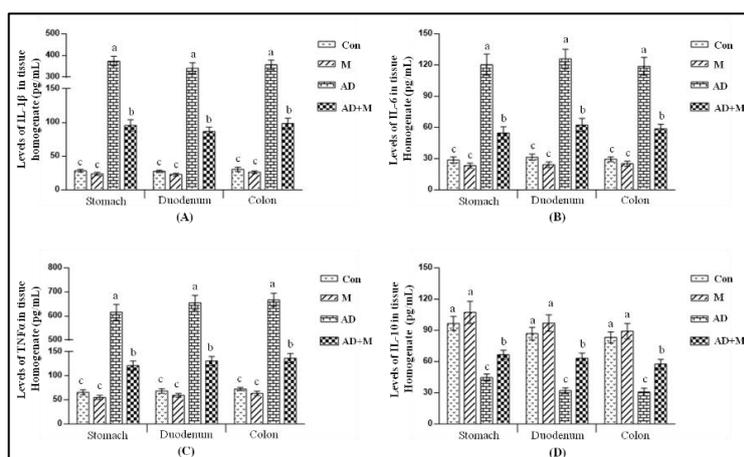
Fig. 8. Effects of melatonin on the cytokines in GI tissues under AD induced oxidative stress.

Diagrammatic representation of the tissue levels of (A) IL-1β, (B) IL-6, (C) tumour necrosis factor alpha (TNFα) and (D) IL-10. Different letters on the error bars indicated significant differences vs each other (p<0.001; n=6/group) Con: control, M: melatonin 5 mg/kg, AD: adrenaline 0.25 mg/kg, AD + M: adrenaline + melatonin.

3.6. Effects of melatonin on NFκB, Nrf2, Caspase 9, Caspase 3, Bcl-2 and Bax proteins in GI tissues under AD induced oxidative stress.

AD (0.25 mg/kg) treatment caused significant ($p<0.001$) increase in the levels of NFκB, caspases (caspase 3 and 9) and Bax proteins, but decreased Nrf2 and Bcl2 levels in the stomach, duodenum and colon tissues when compared to control (Fig. 9), thus an increase in

the Bax/Bcl2 ratio was observed (Figure 9). However, melatonin (5 mg/kg) treatment significantly ($p<0.001$) reduced the levels of NFκB, caspases (caspase 3 and 9), Bax and Bax/Bcl2 ratio, but increased the levels of Nrf2 and Bcl2in all tissues treated by AD (Figure 9).
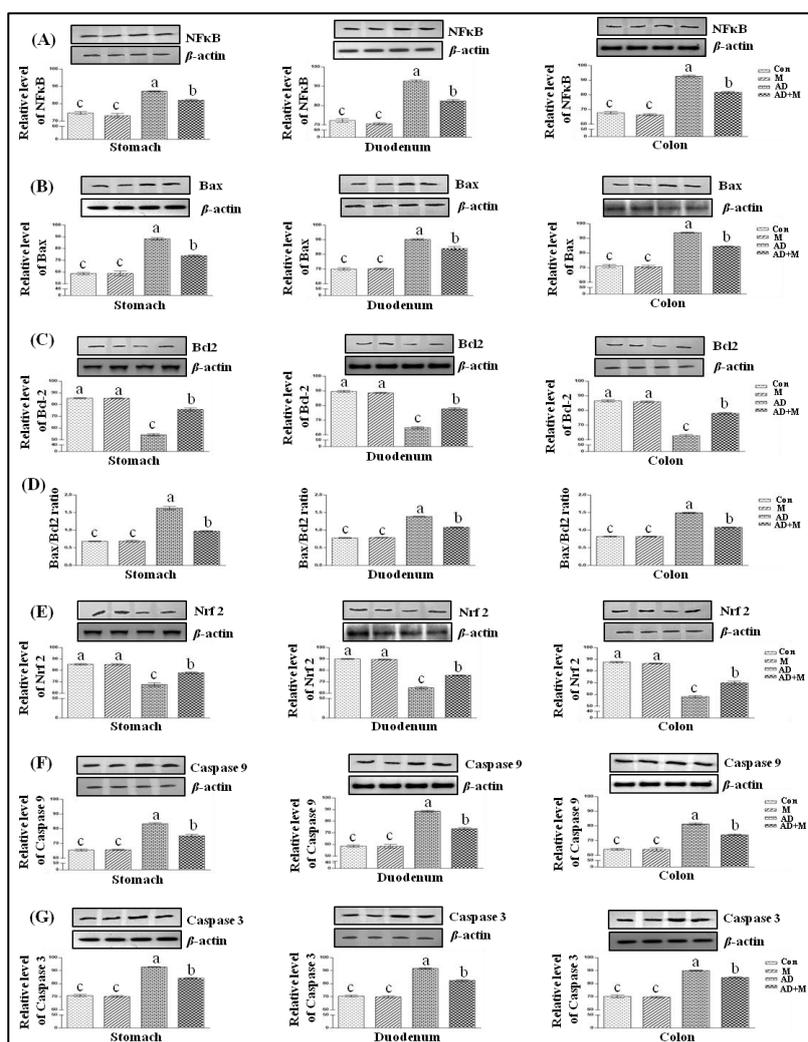
Fig. 9. Effects of melatonin on the expression of the elements involved in apoptotic pathway in GI tissues under AD induced oxidative stress.

Diagrammatic representation of the immunoblots and alterations in the relative densities of (A) NFκB, (B) Bax, (C) Bcl2, (D) Bax/Bcl2 ratio, (E) Nuclear factor erythroid 2-related factor 2 (Nrf2), (F) Caspase 9 and (G) Caspase 3. Different letters on the error bars indicated significant differences vs each other (p<0.001; n=6/group) Con: control, M: melatonin 5 mg/kg, AD: adrenaline 0.25 mg/kg, AD + M: adrenaline + melatonin.

3.7. Effect of melatonin on cellular energy homeostasis, cytochrome c (Cyt C) leakage, and regulation of SOD2, PGC1α, AMPKα and SIRT1 and proteins under AD induced oxidative stress.

Mitochondrial levels of Cyt C, SOD2, PGC1α, AMPKα, and SIRT1 in the stomach, duodenum and colon tissues were significantly reduced and cytosolic level of Cyt C was increased in AD (0.25 mg/kg) group compared to control ($p<0.001$). Melatonin (5 mg/kg) pre-treatment significantly reversed these alterations induced by AD stress ($p<0.001$) (Figure 10).
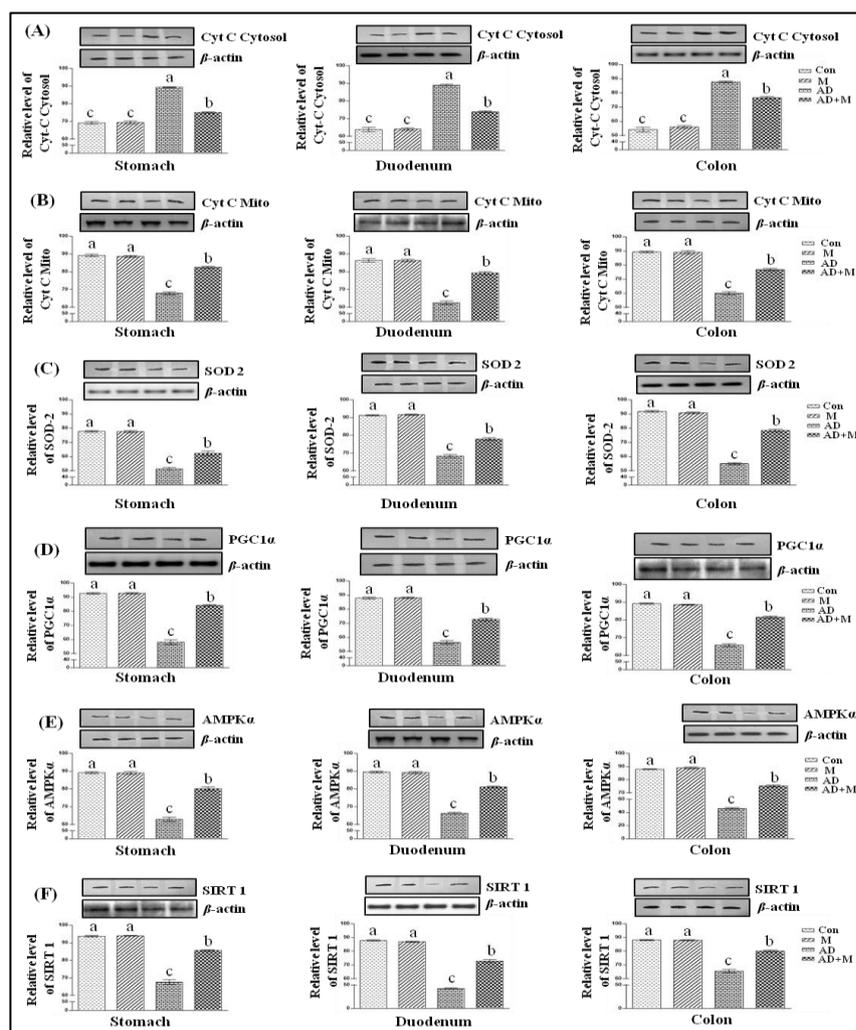
Fig. 10. Effects of melatonin on the expression of the elements involved in mitochondrial homeostasis in GI tissues under AD induced oxidative stress.

(A) Cyt C in cytosol, (B) Cyt C in mitochondria, (C) mitochondrial superoxide dismutase 2 (SOD 2), (D)Peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC1α), (E) 5′AMP-activated protein kinase alpha (AMPKα), and (F) Sirtuin 1 (SIRT1) Different small letters on the error bars indicated significant differences vs each other (p<0.001; n=6). Cyt C: cytochrome C, Mito: mitochondria, Con: control, M: melatonin 5 mg/kg, AD: adrenaline 0.25 mg/kg, AD + M: adrenaline + melatonin.

4. DISCUSSION

Increased levels of LPO in GI tissues might be a consequence of oxidative injury caused by high dose of adrenaline (42). Several animal studies have confirmed these similar observations (8, 9, 13, 43, 44). The PCO is a common marker of protein oxidative damage (8, 9, 45, 46). The increased tissue levels of PCO indicate structural and functional damage of proteins linked to the auto-oxidation of adrenaline. AD can also reduce glutathione (GSH) levels, a potent antioxidant (47) and this further suggests that adrenaline can exhaust endogenous GSH pool, probably by generating large quantity of ROS. The high level of ROS caused by AD has been reported in the intestinal (9) and cardiac (8) tissues of rat. Interestingly, melatonin pre-treatment reduced LPO and PCO levels and increased GSH levels in the rats treated with adrenaline. The results show that melatonin has the capacity to

protect against the GI tissues from adrenaline induced oxidative stress (8, 9). In the current study, it is observed that the significant distortions are present in the mucosa and epithelial lining of GI tissues after AD administration. The observations are consistent with the previous report which also showed mucosal erosion caused by adrenaline (16). In this study, we first report that melatonin administration (5 mg/kg) effectively protected the GI tissues from the oxidative damages induced by adrenaline.

Little is known regarding the response of the endogenous melatonergic system to adrenaline stress in GI tissues. Our results showed that the melatonergic system including its synthetic enzyme- AANAT, melatonin and melatonin receptor 1 (MT1) are ubiquitously present in GI tissues. Adrenaline treatment down-regulated the expression of *AANAT* and *MT1* and led to declined melatonin levels in these tissues. In addition to its auto-oxidation mediated indirect blocking of the melatonergic system (9), adrenaline might also directly interact with *AANAT* and *MT1* genes to induce DNA damage (10, 12, 48). However, pre-treatment of melatonin efficiently increased the expressions of *AANAT* and *MT1* and enhanced the melatonin levels in stomach, duodenum and colon tissues indicating restoration of endogenous melatonergic system which was impeded by adrenaline.

Adrenaline is known to impact the activities of antioxidant enzymes in GI tissue (42). This is confirmed in the present study with increased activities of CAT, GPx and GST, and decreased activities of SOD and GR with adrenaline treatment. These alterations might be the response of the GI tissues to the increasing ROS induced by adrenaline auto-oxidation (8, 9, 46) as also noted in other tissues (9, 44). Melatonin protected oxidative tissue damages via modulation of antioxidant enzyme activities (21, 49).

Adrenaline is capable of influencing immune responses, especially inflammatory reactions (13, 50). The elevated levels of proinflammatory cytokines of IL1β, IL6 and TNFα and reduced level of anti-inflammatory cytokine IL10 observed in the study indicated a progressive inflammation in the GI tissues caused by adrenaline. The observation is similar to those reported in the plasma as well as other diverse tissues of rat (13, 50, 51). Since induction of TNFα involves NFκB, therefore, the levels of NFκB and other apoptosis associated caspases were also investigated. NFκB level is considered as a key mediator in the progression of oxidative stress (52, 53). A parallel increase in the levels of TNFα, NFκB, caspase 9 and caspase 3 indicates the initial up-regulation of NFκB by adrenaline (9) that triggers expression of TNFα (9, 54) and then, followed by up-regulations of caspase 9, caspase 3 and apoptosis (13). The enhanced NFκB expression and reduced melatonin level might also account for the enhancement of the pro-inflammatory mediators (49). The increased expression of Bax protein and decreased Bcl2 levels resulted in an elevation of Bax/Bcl2 ratio, which indicated adrenaline induced intracellular apoptotic pathway (55) in the GI tissues similar to human coronary artery epithelial cells (13, 51). Our results showed that melatonin has the capacity to reduce the pro-inflammatory mediators, TNFα, NFκB, caspase 9, caspase 3 and Bax, and increase IL10 and Bcl2 levels. All of these may contribute to the anti-inflammatory, anti-apoptotic and immunomodulatory functions of melatonin against adrenaline induced GI tissue damages.

Nuclear factor erythroid 2-related factor 2 (Nrf2), an important transcription factor involved in protecting cells from oxidative stress was downregulated, while NFκB levels were upregulated in GI tissues following the adrenaline treatment. This supported the rationale that Nrf2 is the upstream element of NFκB related inflammatory responses (54, 56). The interaction of Nrf2 with response element in the promoter region of antioxidant genes up-regulates these antioxidant enzymes under adrenaline induced oxidative stress (54, 56). Melatonin pre-treatment increased Nrf2 levels and suppressed NFκB associated inflammatory cascades (54, 56). Additionally, adrenaline also downregulated silent information regulator 1 (SIRT1), a potent cellular energy sensor, whereas melatonin reversed the negative effect of

adrenaline as observed in our study and also by others (57). The upregulation of SIRT1 by melatonin may also account for the decrease in NFκB level, since SIRT1 is known to directly inhibit NFκB by deacetylating its RelA/p65 subunit (57, 58).

PGC1α and AMPKα are the master regulators of mitochondrial biogenesis and energy homeostasis, respectively (59). Their expressions are down-regulated by adrenaline in GI tissues, indicating the impairments of mitochondrial biogenesis and energy metabolism. However, we have observed that these disturbances caused by adrenaline are also rescued by melatonin which is consistent with the previous findings (59, 60). Judging from the increase in MT1 and PGC1α expressions it seems that the upregulation of PGC1α is mediated by MT1, another pathway of melatonin protecting tissues from oxidative damages.

SOD2 protein in the GI tissues may also involve MT1 under adrenaline induced oxidative stress (9, 21). It should be known that upregulation of SIRT1 by melatonin might be another possible mechanism to increase SOD2 levels (61). Since cytochrome c leakage is an indicator of mitochondrial membrane potential collapse (62), increase in cytosolic cytochrome c and concomitant decrease in the mitochondrial cytochrome c following adrenaline administration suggested opening of mitochondrial permeability pore and cell apoptosis (62). The increased cytochrome c leakage is also responsible for activation of caspase 9 and caspase 3 in the GI tissues as visualized in hepatoma Hepa cells (63, 64). However, melatonin prevented cytochrome C leakage, playing a critical role in the restoration of mitochondrial membrane potential (63, 64).

In light of the important evidence gathered in this study, it appears that protective effects of melatonin on adrenaline induced oxidative stress in GI tissues were primarily via upregulation of the endogenous melatonergic system, and other intracellular signalling pathways. These include modulation of caspase 9 and 3 mediated cellular apoptotic pathway, NFκB-SIRT1 and Bax-Bcl2 dependent pathways. In addition, melatonin preserves cellular energy homeostasis and mitochondrial biogenesis through PGC1α-AMPKα pathway. These described mechanisms of melatonin to protect against GI damages induced by the adrenaline toxicity are illustrated in the Figure 11.
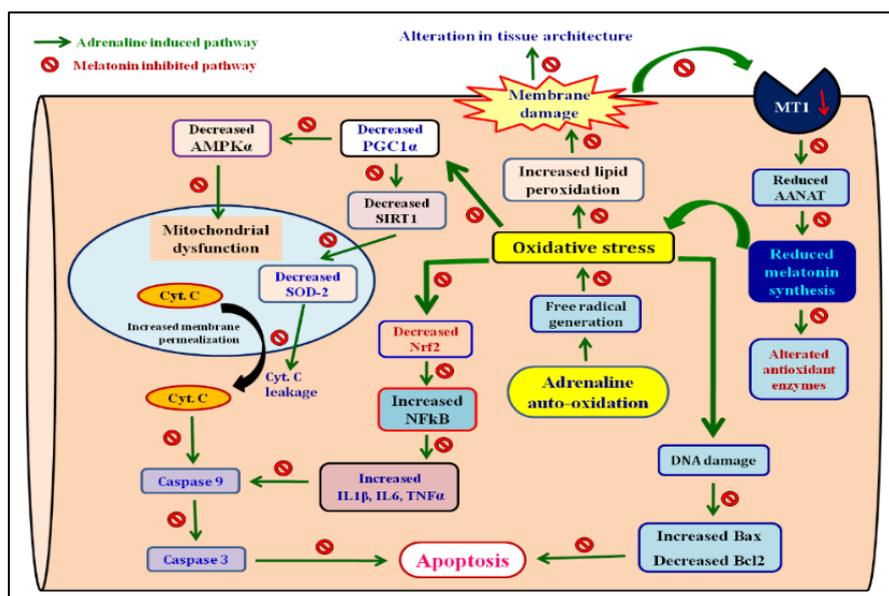
Fig. 11. Pathways involved in the protective roles of melatonin against adrenaline induced damages in the gastrointestinal tract.

Green arrows indicated adrenaline auto-oxidation induced activation of different intracellular signalling pathways. Red stop symbols indicated protective effects of melatonin through inhibition of the intracellular signalling pathways triggered by adrenaline. Red

arrow indicated decrease in the level of MT1. Black arrow indicated leakage of Cyt C from the mitochondria to the cytosol. AANAT- Arylalkylamine N-acetyl transferase; MT1- Melatonin receptor 1; NFκB- Nuclear factor kappa beta; Nrf2- Nuclear factor erythroid 2-related factor 2; TNFα- Tumor necrosis factor alpha; PGC1α- Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SIRT1- Sirtuin 1; Cyt C- Cytochrome C; AMPKα- 5′AMP-activated protein kinase alpha; SOD2- Superoxide dismutase 2.

ABBREVIATIONS

AANAT- Arylalkylamine N-acetyl transferase
AMPKα- 5′AMP-activated protein kinase alpha
CAT- Catalase
GPx- glutathione peroxidise
GR- Glutathione reductase
GSH- Reduced glutathion
GST- Glutatione-S-transferase
LPO- Lipid peroxidation
MT1- Melatonin receptor 1
NFκB- Nuclear factor kappa beta
Nrf2- Nuclear factor erythroid 2-related factor 2
PCO- Protein carbonyl content
PGC1α- Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
SIRT1- Sirtuin 1
Cyt C- Cytochrome C
SOD- Superoxide dismutase
TNFα- Tumor necrosis factor alpha

ACKNOWLEDGEMENTS

AUTHOR'S CONTRIBUTIONS

Prof. DB and Dr. AC conceived and designed the experiment, revised the manuscript critically and approved it. Dr. PKP contributed to conception and designing as well as executed the experiment, analyzed the data, prepared figures, drafted the manuscript and edited it. SS contributed in executing the experiments, drafting and editing of the manuscript. Dr. SM contributed in executing the experiments. Dr. SC permitted use of some of the equipments available with her and also critically read the manuscript with important editing at many points.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

REFERENCES

1. Ranabir S, Reetu K (2011) Stress and hormones. *Indian J. Endocr. Metab*. **15** (1): 18. DOI: 10.4103/2230-8210.77573.
2. Gavrilovic LJ, Stojiljkovic V, Kasapovic J, Snežana P, Ana TP, Snežana B, Slađana D (2012) Chronic physical stress changes gene expression of catecholamine biosynthetic enzymes in the adrenal medulla of adult rats. *Acta Vet-Beograd*. **62** (2-3): 151–169. DOI: 10.2478/acve-2014-0027.
3. Kemp SF, Lockey RF, Simons FE (2008) World Allergy Organization ad hoc Committee on epinephrine in anaphylaxis. Epinephrine: the drug of choice for anaphylaxis. A statement of the World Allergy Organization. *Allergy* **63** (8): 1061-1070. DOI: 10.1111/j.1398-9995.2008.01733.x.
4. Kinsella SM, Tuckey JP (2001) Perioperative bradycardia and asystole: relationship to vasovagal syncope and the Bezold–Jarisch reflex. *Br. J. Anaesth*. **86** (6): 859-868. DOI: 10.1093/bja/86.6.859.
5. Numa AH, Williams GD, Dakin CJ (2001) The effect of nebulized epinephrine on respiratory mechanics and gas exchange in bronchiolitis. *Am. J. Resp. Crit. Care* **164** (1): 86-91. DOI: 10.1164/ajrccm.164.1.2008090.
6. Reiter PD, Roth J, Wathen B, LaVelle J, Ridall LA (2018) Low-dose epinephrine boluses for acute hypotension in the PICU. *Pediatr. Crit. Care Med*. **19** (4): 281-286. DOI: 10.1097/PCC. 0000000000001448.
7. Costa VM, Silva R, Ferreira LM, Branco PS, Carvalho F, Bastos ML, Carvalho RA, Carvalho M, Remião F (2007) Oxidation process of adrenaline in freshly isolated rat cardiomyocytes: formation of adrenochrome, quinoproteins, and GSH adduct. *Chem. Res. Toxicol*. **20** (8): 1183-1191. DOI: 10.1021/tx7000916.
8. Rudra S, Mukherjee D, Dutta M, Ghosh AK, Dey M, Basu A, Pattari SK, Chattopadhyay A, Bandyopadhyay D (2014) Orally administered melatonin protects against adrenaline-induced oxidative stress in rat liver and heart: Involvement of antioxidant mechanism (s). *J. Pharm. Res*. **8**: 303-320. http://jprsolutions.info/files/final-file-56bff9adee9ad8. 28733292.pdf.
9. Pal PK, Bhattacharjee B, Ghosh AK, Chattopadhyay A, Bandyopadhyay D (2018) Adrenaline induced disruption of endogenous melatoninergic system, antioxidant and inflammatory responses in the gastrointestinal tissues of male Wistar rat: an *in vitro* study. *Melatonin Res*. **1** (1): 109-131. DOI: https://doi.org/https://doi.org/10.32794/ mr11250007.
10. Djelić N, Radaković M, Borozan S, Dimirijević-Srećković V, Pajović N, Vejnović B, Borozan N, Bankoglu EE, Stopper H, Stanimirović Z (2019) Oxidative stress and DNA damage in peripheral blood mononuclear cells from normal, obese, prediabetic and diabetic persons exposed to adrenaline *in vitro*. *Mutat. Res-Gen. Tox. En*. **843**: 81-89. https://doi.org/10.1016/j.mrgentox.2019.01.013.
11. Korać J, Stanković DM, Stanić M, Bajuk-Bogdanović D, Žižić M, Pristov JB, Grgurić-Šipka S, Popović-Bijelić A, Spasojević I (2018) Coordinate and redox interactions of epinephrine with ferric and ferrous iron at physiological pH. *Sci. Rep*. **8** (1): 3530. DOI: 10.1038/s41598-018-21940-7.

12. Flint MS, Baum A, Chambers WH, Jenkins FJ (2007) Induction of DNA damage, alteration of DNA repair and transcriptional activation by stress hormones. *Psychoneuroendocrinol*. **32** (5): 470–479. DOI: 10.1016/j.psyneuen.2007.02.013.

13. Romeo F, Li D, Shi M, Mehta JL (2000) Carvedilol prevents epinephrine-induced apoptosis in human coronary artery endothelial cells: modulation of Fas/Fas ligand and caspase-3 pathway. *Cardiovasc. Res*. **45** (3): 788-794. DOI: 10.1016/s0008-6363(99) 00369-7.

14. Liu X, Wu WK, Yu L, Sung JJ, Srivastava G, Zhang ST, Cho CH (2008) Epinephrine stimulates esophageal squamous-cell carcinoma cell proliferation via beta adrenoceptor dependent transactivation of extracellular signal-regulated kinase/cyclooxygenase-2 pathway. *Cell. Biochem*. **105** (1): 53-60. DOI: 10.1002/jcb.21802.

15. Yao H, Duan Z, Wang M, Awonuga AO, Rappolee D, Xie Y (2009) Adrenaline induces chemoresistance in HT-29 colon adenocarcinoma cells. *Cancer Genet. Cytogenet*. **190** (2): 81-87. DOI: 10.1016/j.cancergencyto.2008.12.009.

16. Raugstad TS, Svanes K, Ulven A, Molster A (1979) Interaction between acute gastric ulcer and epinephrine-induced mucosal erosions in the rat: the significance of gastric acid secretion. *Digestion* **19**: 70–72. DOI: 10.1159/000198325.

17. Tan DX, Chen LD, Poeggeler B, Manchester LC, Reiter RJ (1993) Melatonin: a potent, endogenous hydroxyl radical scavenger. *Endocr. J*. **1**: 57–60. DOI: 10.1111/j.1600-079x.1993.tb00498.x.

18. Periasamy S, Lee BF, Hsu DZ, Liu MY (2019) Melatonin aggravated oxaliplatin-mimicking sinusoidal obstruction syndrome: role of platelet aggregation and oxidative stress. *OBM Neurobiol*. **3** (3): 22. DOI: 10.21926/obm.neurobiol.1903033.

19. Huether G (1993) The contribution of extrapineal sites of melatonin synthesis to circulating melatonin levels in higher vertebrates. *Experientia* **49** (8): 665–670. DOI: 10.1007/bf01923948.

20. Pal PK, Bhattacharjee B, Chattopadhyay A, Bandyopadhyay D (2019a) Pleiotropic roles of melatonin against oxidative stress mediated tissue injury in the gastrointestinal tract: An overview. *Melatonin Res*. **2** (2): 158-184. DOI: 10.32794/mr11250027.

21. Rodriguez C, Mayo JC, Sainz RM, Antolin I, Herrera F, Martin V, Reiter RJ (2004) Regulation of antioxidant enzymes: a significant role for melatonin. *J. Pineal Res*. **36** (1):1–9. https://doi.org/10.1046/ j.1600-079X.2003.00092.

22. Pal PK, Sarkar S, Chattopadhyay A, Bandyopadhyay D (2019b) Enterochromaffin cells as the source of melatonin: key findings and functional relevance in mammals. *Melatonin Res*. **2**(4):61-82. DOI: 10.32794/mr11250041.

23. Chowdhury I, Maitra SK (2012) Melatonin in the promotion of health. In: Melatonin time line: from discovery to therapy. Watson RR (Ed.) Boca Raton (FL): Taylor and Francis. Pp. 1-60.

24. Bubenik GA, Niles LP, Pang SF, Pentney PJ (1993) Diurnal variation and binding characteristics of melatonin in the mouse brain and gastrointestinal tissues. *Comp. Biochem. Physiol.* **104**: 221–224. DOI: 10.1016/0742-8413(93)90027-i.

25. Bandyopadhyay D, Ghosh G, Bandyopadhyay A, Reiter RJ (2004) Melatonin protects against piroxicam-induced gastric ulceration. *J. Pineal Res*. **36** (3): 195-203. https://doi.org/ 10.1111/j.1600-079X.2004.00118.x.

26. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685. DOI: 10.1038/227680a0.

27. Draper HH, Hadley M (1990) Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol*. **186**: 421-431. https://doi.org/10.1016/00766879 (90)86135-I.

28. Levine RL, Williams JA, Stadtman ER, Shacter E (1994) Carbonyl assays for determination of oxidatively modified. *Methods Enzymol*. **233**: 346-357. https://doi.org/10.1016 /S0076-6879(94)33040-9.

29. Ellman GL (1959) Tissue sulfhydryl groups. *Arch Biochem. Biophys.* **82**: 7077. DOI: 10.1016/0003-9861(59)90090-6.

30. Ewing JF, Janero DR (1995) Microplate superoxide dismutase assay employing a non-enzymatic superoxide generator. *Anal. Biochem.* **232** (2): 243-248. DOI: 10.1006/abio.1995. 0014.

31. Aebi H (1984) Catalase *in vitro*. *Methods Enzymol*. **105**: 121-126. https://doi.org/10.1016/ S0076-6879(84)05016-3.

32. Castro R, Piazzon MC, Noya M, Leiro JM, Lamas J (2008) Isolation and molecular cloning of a fish myeloperoxidase. *Mol. Immunol*. **45** (2): 428-437. DOI: 10.1016/j.molimm.2007.05.028.

33. Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. *J. Biol. Chem*. **249** (22): 7130-7139.

34. Pinto RE, Bartley W (1969) The effect of age and sex on glutathione reductase and glutathione peroxidase activities and on aerobic glutathione oxidation in rat liver homogenates. *Biochem. J.* **112** (1):109-115. DOI: 10.1042/bj1150865a.

35. Roy SG, De P, Mukherjee D, Chander V, Konar A, Bandyopadhyay D, Bandyopadhyay A (2009) Excess of glucocorticoid induces cardiac dysfunction via activating angiotensin II pathway. *Cell Physiol. Biochem*. **24** (1-2): 1-10.DOI: 10.1159/000227803.

36. Pal PK, Hasan KN, Maitra SK (2016) Gut melatonin response to microbial infection in carp *Catla catla*. *Fish Physiol. Biochem*. **42** (2): 579-592. DOI: 10.1007/s10695-015-0161-7.

37. MohanKumar K, killingsworth CR, McLwaim RB, Timpa JG, Jagadeeswaran R, Namachivayam K, Kurundkar AR, Kelly DR, Garzon SA, Maheshwari A (2014) Intestinal epithelial apoptosis initiates gut mucosal injury during extracorporeal membrane oxygenation in the newborn piglet. *Lab. Invest.* **94**: 150-160. DOI: 10.1038/labinvest.2013.149.

38. Cheema KJ, Scofield AM (1982) Scanning electron microscopy of the intestines of rats infected with *Nippostrongylus brasiliensis. Int. J. Parasitol.* **12**: 199-205. DOI: 10.1016/0020-7519(82)90017-0.

39. Mukherjee D, Ghosh AK, Bandyopadhyay A, Basu A, Datta S, Pattari SK, Reiter RJ, Bandyopadhyay D (2012) Melatonin protects against isoproterenol-induced alterations in cardiac mitochondrial energy-metabolizing enzymes, apoptotic proteins, and assists in complete recovery from myocardial injury in rats. *J. Pineal Res.* **53**: 166–179. DOI:10.1111/j.1600-079X.2012.00984.x

40. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.

41. Zar JH (1999) Biostatistical analysis, 4th Edn. Prentice Hall, Upper Saddle River.

42. Parlakpinar H, Orum MH, Sagir M (2013) Pathophysiology of myocardial ischemia reperfusion injury: a review. *Med. Sci.* **2** (4): 935-954. DOI: 10.5455/medscience.2013.02.8082.

43. Yildirim A, Sahin YN, Suleyman H, Yilmaz A, Yildirim S (2007) The role of prednisolone and epinephrine on gastric tissue and erythrocyte antioxidant status in adrenalectomized rats. *J. Physiol. Pharmacol*. **58**: 105-116.

44. Radaković M, Borozan S, Djelić N, Ivanović S, Miladinović DC, Ristanić M, Spremo-Potparević B, Stanimirović Z (2018) Nitroso-oxidative stress, acute phase response, and cytogenetic damage in Wistar rats treated with adrenaline. *Oxid. Med. Cell Longev*. 2018. Article ID 1805354. https://doi.org/10.1155/2018/1805354.

45. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R (2003) Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta* **329** (1-2): 23-38. https://doi.org/ 10.1016 /S0009-8981(03)00003-2.

46. Luiz F, Costa Rosa BP, Danilo AS, Yara C, Rui C (1992) Effect of epinephrine on glucose metabolism and hydrogen peroxide content in incubated rat macrophages. *Biochem. Pharmacol*. **44**: 2235-2241. https://doi.org/10.1016/0006-2952(92)90352-J.

47. Ugochukw UNH, Babady NE, Cobourne M, Gasset SR (2003) The effect of *Gongronema latifolium* extracts on serum lipid profile and oxidative stress in hepatocytes of diabetic rats. *J. Biosci*. **28** (1): 1-5. DOI: 10.1007/bf02970124.

48. Dobrzynska MM, Baumgartner A, Anderson D (2004) Antioxidants modulate thyroid hormone and noradrenaline-induced DNA damage in human sperm. *Mutagenesis* **19** (4): 325-330. https://doi.org/10.1093/mutage/geh037.

49. Guo Y, Sun J, Li T, Zhang Q, Bu S, Wang Q, Lai DM (2017) Melatonin ameliorates restrain stress-induced oxidative stress and apoptosis in testicular cells via NFkβ/iNOS and Nrf2/HO-1 signaling pathway. *Sci. Rep*. **7** (1): 9599. DOI: 10.1038/s41598-017-09943-2.

50. DeRijk RH, Boelen A, Tilders FJ, Frank B (1994) Induction of plasma interleukin-6 by circulating adrenaline in the rat. *Psychoneuroendocrinology* **19** (2): 155-163. DOI: 10.1016/0306-4530(94)90005-1.

51. Chen S, Liu GL, Li MM, Liu R, Liu H (2017) Effects of epinephrine on inflammation-related gene expressions in cultured rat cardiomyocytes. *Transl. Perioper. Pain Med.* **2** (1): 13-19.

52. Ferreira ZS, Fernandes PA, Duma D, Assreuy J, Avellar MC, Markus RP (2005) Corticosterone modulates noradrenaline-induced melatonin synthesis through inhibition of nuclear factor kappa B. *J. Pineal Res*. **38** (3): 182-188. DOI: 10.1111/j.1600-079X.2004.00191.x.

53. Das N, Mandala A, Naaz S, Giri, Jain M, Bandyopadhyay D, Reiter RJ, Roy SS (2017) Melatonin protects against lipid-induced mitochondrial dysfunction in hepatocytes and inhibits stellate cell activation during hepatic fibrosis in mice. *J. Pineal Res*. **62** (4): e12404. DOI: 10.1111/jpi.12404.

54. Negi G, Kumar A, Sharma SS (2011) Melatonin modulates neuroinflammation and oxidative stress in experimental diabetic neuropathy: effects on NF-κB and Nrf2 cascades. *J. Pineal Res*. **50** (2): 124-131. DOI: 10.1111/j.1600-079X.2010.00821.x.

55. Yi G, Li L, Luo M, He X, Zou Z, Gu Z, Su L (2017) Heat stress induces intestinal injury through lysosome- and mitochondria-dependent pathway *in vivo* and *in vitro*. *Oncotarget* **8** (25): 40741-40755. DOI: 10.18632/oncotarget.16580.

56. Li W, Khor TO, Xu C, Shen G, Jeong WS, Yu S, Kong A (2008) Activation of Nrf2-antioxidant signaling attenuates NFκB-inflammatory response and elicits apoptosis. *Biochem. Pharmacol*. **76** (11): 1485-1489. DOI: 10.1016/j.bcp.2008.07.017.

57. Yang Y, Jiang S, Dong Y, Fan C, Zhao L, Yang X, Li J, Di S, Yue L, Liang G, Reiter RJ, Qu Y (2015) Melatonin prevents cell death and mitochondrial dysfunction via a SIRT 1-dependent mechanism during ischemic-stroke in mice. *J. Pineal Res*. **58** (1): 61-70. DOI: 10.1111/jpi.12193.

58. Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW (2004) Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J*. **23** (12): 2369-2380. DOI: 10.1038/sj.emboj.7600244.

59. Liu D, Ma Z, Di S, Yang Y, Yang J, Xu L, Reiter RJ, Qiao S, Yuan J (2018) AMPK/PGC1α activation by melatonin attenuates acute doxorubicin cardiotoxicity via alleviating mitochondrial oxidative damage and apoptosis. *Free Radic. Biol. Med*. **129**: 59-72. DOI: 10.1016/j.freeradbiomed.2018.08.032.

60. Guo P, Pi H, Xu S, Zhang L, Li Y, Li M, Cao Z, Tian L, Xie J, Li R, He M, Lu Y, Liu C, Duan W, Yu Z, Zhou Z (2014) Melatonin improves mitochondrial function by promoting MT1/SIRT1/PGC-1 alpha-dependent mitochondrial biogenesis in cadmium-induced hepatotoxicity *in vitro*. *Toxicol. Sci*. **142** (1): 182-195. DOI: 10.1093/toxsci/kfu164.

61. Wang SJ, Zhao XH, Chen W, Bo N, Wang XJ, Chi ZF, Wu W (2015) Sirtuin 1 activation enhances the PGC-1α/mitochondrial antioxidant system pathway in status epilepticus. *Mol. Med. Rep*. **11** (1): 521-526. DOI: 10.3892/mmr.2014.2724.

62. Kasahara E, Lin LR, Ho YS, Reddy VN (2005) Manganese superoxide dismutase protects against oxidation-induced apoptosis in mouse retinal pigment epithelium: implications for age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci*. **46** (9): 3426-3434. DOI: 10.1167/iovs.05-0344.

63. Epperly MW, Sikora CA, DeFilippi SJ, Gretton JA, Zhan Q, Kufe DW, Greenberger JS (2002) Manganese superoxide dismutase (SOD2) inhibits radiation-induced apoptosis by stabilization of the mitochondrial membrane. *Radiat. Res*. **157** (5): 568-577. DOI: 10.1667/0033-7587(2002)157[0568:msdsir] 2.0.co;2.

64. Reiners Jr JJ, Caruso JA, Mathieu P, Chelladurai B, Kessel D (2002) Release of cytochrome c and activation of pro-caspase-9 following lysosomal photodamage involves Bid cleavage. *Cell Death Differ*. **9** (9): 934-944. DOI: 10.1038/sj.cdd.4401048.