Research Article

Adrenaline induced disruption of endogenous melatonergic system, antioxidant and inflammatory responses in the gastrointestinal tissues of male Wistar rat: an *in vitro* study

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ABSTRACT

The current study aimed to demonstrate the potentially adverse effects of adrenaline, an endogenous stressor, on the melatonergic system, oxidative status, antioxidative responses and inflammatory markers in different parts of gastrointestinal tract of Wistar rat. These included stomach, duodenum and colon and they were incubated with different concentrations (2.5, 5.0 and 10.0 µg/mL) of adrenaline for 1h, respectively. The levels of melatonin, gene expressions of arylalkylamine N-acetyltransferase (AANAT) and melatonin receptor 1 (MT1) as well as other stress-induced parameters including NF-kB expression, levels of cAMP, calcium, malondialdehyde, protein carbonyl content, reduced glutathione, nitrate, superoxide dismutase, catalase, glutathione peroxidase and glutathione *S*-transferase, tumour necrosis factor-α, IL-1β, IL6 and IL10 were systemically measured in these tissues. An adrenaline dose-dependent decrease in level of melatonin, AANAT, MT1 and NF-kB in these tissues were observed. In contrast, the profound increases in the levels of cAMP, calcium and all oxidative stress markers, inflammatory cytokines (except IL10), and activities of antioxidant enzymes (except superoxide dismutase) were observed after adrenaline treatment. A maximum effect was found in tissues treated with 5 µg/mL of adrenaline. The Correlation studies between melatonin level and other parameters (any two at a time) indicated a potentially physiological interplay between adrenaline stress and melatonin tissue levels. Collectively, the results provided the novel data on the adverse effects of adrenaline on the endogenous melatonergic system, antioxidant and inflammatory responses in the gastrointestinal tissues of rats.

Keywords: adrenaline, gastrointestinal tract, oxidative stress, melatonin, AANAT, MT1, antioxidant, inflammation

1. INTRODUCTION

Apart from differently exogenous oxidative stress, the endogenous sources are also responsible for the overproduction of reactive oxygen species (ROS) (1). Under stressful

conditions, catecholamines, particularly, adrenaline is released from sympathetic nervous system and adrenal medulla to serve a pivotal role in preparing the organism to fight or flight by modulating numerous physiological functions (2, 3). However, excessive release of adrenaline, due to its pro-oxidant and auto-oxidation properties, generates enormous ROS that may induce detrimental effects on cells and tissues. These include loss of DNA integrity (4), cardiotoxicity (5), hepatotoxicity (6), cancer (7, 8) and age-related disorders (9). It was reported that exogenous administration of adrenaline promoted a redox cycle and enhanced intra-cellular production of ROS (10). Notably, an already elevated release of adrenaline under acute stress could be further increased by chronic stressful conditions (11). The adverse effects of excessive adrenaline on mammalian gastrointestinal tissue have not been extensively studied although the adrenaline induced mucosal erosion in rat was reported by Raugstad *et al*. (12).

To protect against oxidative stress, the GI tract possesses its own defense machinery comprising of various hormones, enzymatic and non-enzymatic antioxidants (13). Among them melatonin (N-acetyl-5-methoxy tryptamine), a low molecular weight indolamine, seems to be the most potent free radical scavenger and antioxidant (14). It regulates numerous important physiological functions in a variety of tissues (15, 16) including the GI tract (17-19). Melatonin, due to its lipophilic and hydrophilic natures, not only directly scavenges cytotoxic free radicals, but also stimulates the activities of different antioxidant enzymes (20), ultimately reducing the level of oxidative stress in cells and tissues (21, 22).

Except of pineal gland, melatonin is also synthesized in the mucosal enterochromaffin cells of the GI tract (23, 24), where AANAT serves as the key rate limiting enzyme for melatonin biosynthesis (25). It is well documented that adrenaline can mediate the message of photic information to the pinealocytes and thus, it indirectly regulates the AANAT activity and thereby, affects melatonin synthesis in vertebrates (26). However, the potential relationships between the melatonergic system and adrenaline induced oxidative stress in GI tract of vertebrates has never been reported.

Considering limitations in the existing knowledge, the current study attempts to gather basic but important information on the responses of endogenous melatonergic, antioxidant and inflammatory systems subjected to adrenaline stress in the gastrointestinal tissue of male Wistar rat. The profiles of melatonin, AANAT, MT1, cAMP, calcium, different proinflammatory cytokines and indices of oxidative status in stomach, duodenum and colon will be measured. The selected statistical tools will be used to clarify the potential association between melatonin level and oxidative stress or inflammatory responses induced by the adrenaline in GI tract.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents.

The primary antibodies, AANAT (ab3506), SOD-1 (ab16831), CAT (ab16731), GPx (ab108427), NF-kB p65 (ab16502) and Goat anti-rabbit IgG alkaline phosphatise (ALP)-conjugated secondary antibody (ab97048) were purchased from Abcam Biotechnology Company, Abcam, USA. Primary antibodies of β-actin (sc-130657) and MT1 (orb234902) were procured from Santa Cruz Biotechnology, Inc. and Biorbyt, respectively. 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), ethylene diamine tetra acetic acid (EDTA) were purchased from Sisco Research Laboratories (SRL), Mumbai, India. All other chemicals and reagents of highest available purity grade were procured from Sigma-Aldrich Co., St. Louis, MO, USA.

2.2. Experimental designs.

This animal study has been approved by Institutional Animal Ethical Committee (IAEC), Department of Physiology, University of Calcutta; Approval Number: IAEC-IV/Proposal/DB-06/2014/dt. 13/03/2014].

2.2.1. Culture of gastrointestinal tissues.

Immediately after sacrifice of the Wistar rats, the GI tract was surgically extirpated and desired portions of gastrointestinal tissue (i.e. stomach, duodenum and colon) were thoroughly washed in ice cold PBS (pH 7.4) for complete removal of food/faecal. Then, the tissues were transferred to sterilized pectri plates containing RPMI 1640 culture medium in which they were cut into small pieces (each piece weing~25 mg). The isolated tissue samples were distributed equally in individual wells of 24-well culture plates. Each well contained ~100 mg gastrointestinal tissue immersed in 1 mL of RPMI 1640 culture medium (pH 7.5) with BSA (1 mg/mL), penicillin G (100 U/mL), streptomycin (100 µg/mL) and ascorbic acid (0.1 mg/mL). The samples were incubated at 20±1°C for 1 h (27) and then different concentrations of adrenaline bitartrate [2.5µg/mL (AD-1), 5µg/mL (AD-2) or, 10 (AD-3) µg/mL)] were added into the culture medium, respectively and cultured for an additional 1 h. Each treatment was replicated for at least 5 times.

2.2.2. Preparation of tissue homogenates.

After incubation, gastrointestinal tissues were collected in separating micro-centrifuge tubes, respectively, and washed thrice with ice cold PBS (pH 7.4). The samples were homogenized with homogenizing buffer [100 mM Tris-HCl buffer (pH 7.2–7.4), containing 0.1 mM PMSF, 250 mM sucrose and 1% leupeptinhemisulfate] using a Potter-Elvenjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30 sec at 4°C. The homogenates were centrifuged at 1500 x g for 10 min at 4°C (19). The supernatant was collected as the crude enzyme extract and stored at -80°C for Western blot analysis of gene expression and for other biochemical assays.

2.3 . Measurement of gastrointestinal melatonin concentration.

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

2.4. Electrophoresis and Western blot analysis.

Gastrointestinal tissue homogenates were prepared for Western blot analysis following the method as described in earlier studies (18, 27, 28). In brief, the samples were subjected to SDS–PAGE (12%) following the method of Laemmli (29). Each well was loaded with sixty

microgram proteins for immunodetection of AANAT, MT1, NF-kB, superoxide dismutase-1 (SOD1), catalase (CAT) and *β*-actin. After completion of the running, the gel was transferred to nitrocellulose membrane (Pall Corporation, USA), the immunoblot was then incubated in blocking solution (5% bovine serum albumin) for 1 h followed by washing trice in TBS-T (15 min each). The blot was then incubated with respective primary antibodies (1:2000) for overnight at 4°C. The bolt was washed thrice in TBS-T and incubated with secondary antibody (Goat anti-rabbit IgG ALP conjugate 1:3000) for 2 h at 4°C. Finally, the membrane was washed again and incubated in BCIP/NBT substrate. Individual band intensity of respective immunoblots was normalized by the intensity of *β*-actin (NIH, Bethesda, MD, USA) and quantification was performed by densitometry using ImageJ software and expressed in relative densitometric units (18, 28).

2.5. Estimation of the tissue levels of cAMP and calcium (Ca^{2+}).

The levels of cAMP and Ca^{2+} in the gastrointestinal tissue homogenates were estimated by cAMP ELISA assay kit (581001; Cayman Chemicals, MI, USA) and calcium estimation test kit (732; Bayer Diagnostics Pvt. Ltd., India), respectively, following the manufacturer's instructions.

2.6. Estimation of the levels of oxidative products.

2.6.1. Malondialdehyde (MDA).

The levels of MDA were estimated following the TBARS (thiobarbituric acid reactive substances) assay (30) with minor modifications Briefly, gastrointestinal tissue homogenates were centrifuged at 3000 × g for 15 min at 4°C and the supernatant (1 mL) along with TBA reagent (20% TCA, 0.5% TBA and 2.5 N HCl; 2 mL) was heated for 20 min in a boiling water bath and then allowed to cool down. The solution was centrifuged at 500 × g for10 min and the supernatant was collected and its absorbance was recorded at 532 nm. MDA level was expressed as nM/mg protein using an extinction coefficient of 1.56×10^5/Mcm.

2.6.2. Protein carbonyl (PCO) content.

The PCO content in the gastrointestinal tissue was estimated by DNPH assay according to the method of Levine *et al*. (31). The absorbance was measured at 370 nm and the level of PCO was expressed as nM/mg protein.

2.6.3. Nitric oxide (NO).

The level of NO in the tissue samples were estimated according to the method of Green *et al*. (32). Briefly, 0.4 mL of enzyme sample was added to Griess reagent (1% sulfanilamide and 0.1% N-1-naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated in dark for 10 min at room temperature. Finally, the absorbance was recorded at 540 nm. The concentration of NO in the sample was determined from sodium nitrite standard curve and level was expressed as µM/mg protein.

2.7. Assays of activities of antioxidant enzymes.

2.7.1. Superoxide dismutase (SOD).

The activity of SOD was determined according to the method described by Ewing and Janero (33). The absorbance was recorded at 560 nm using a micro-plate reader (Bio-Rad, USA). Briefly, 25-µl enzyme 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.

2.7.2. Catalase (CAT).

The method described by Aebi (34) 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. In order to validate the assay, tissue enzyme samples were treated with a known inhibitor (sodium azide) of CAT activity (35).

2.7.3. Glutathione peroxidase (GPx).

GPx activity was estimated by the method described by Castro *et al*. (36). 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.

2.7.4. Glutathione S-transferase (GST).

The method described by Habig *et al.* (37) was used to estimate the activity of GST in the tissue homogenates, where 1-chloro-2, 4-dinitrobenzen (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.

2.7.5. Glutathione reductase (GR).

Activity of GR was estimated following the method described by Pinto and Bartley (38) 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 340nm. In order to validate the assay, NADPH was oxidized in absence of glutathione.

2.8. Estimation of reduced glutathione (GSH).

GSH level was estimated based on the method described by Ellman (39). Briefly, 100 µL of enzyme 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 the use of GSH and was expressed as µM/mg protein.

2.9. Determination of the levels of tissue proinflammatory cytokines.

The tissue levels of IL-1β (ELR-IL1b), IL-6 (ELR-IL6), IL-10 (ELR-IL10) and TNFα (ELR-TNFα) were estimated following the manufacturer's (RayBiotech, Norcross, GA) instructions.

2.10. Estimation of protein.

The concentration of proteins in the gastrointestinal samples were estimated by the method of Lowry *et al*. (40) using the bovine serum albumin as the standard.

2.11. Statistical analysis.

The data of AANAT were expressed as mean ± SEM (n=5). Shapiro-Wilks test was performed to ensure normal distribution of the data sets. After all data sets passed the normality test ($p < 0.01$), one-way analysis of variance (ANOVA) was used to analyse each variable of the data among the experimental groups. When F values were indicated significance, means were compared by a *post-hoc* Duncan's multiple range test (DMRT) between groups and $p < 0.05$ was set up as significance (41).

A correlation coefficient analysis was used to identify any possible correlation between melatonin levels and other variables (any two variables at a time) in the same experimental group. Additionally, a linear regression analysis was also performed in the same manner to express the dependence of a response variable on an independent (predictor) variable. Statistical analysis and data presentation were carried out using Statistical Package for the Social Sciences (SPSS), Inc., Statistics 21.0 and Graph Pad Prism 6.03 software.

3. RESULTS

3.1. Levels of melatonin in the gastrointestinal tissues.

Melatonin levels, irrespective of the regions of gastrointestinal tract, were found to be significantly decreased in a dose dependent manner in all adrenaline treated groups when compared to the control values ($p<0.001$), where a maximum reduction was noted in the group treated with highest dose (10 µg/mL) of adrenaline (AD-3) (Fig. 1A).

3.2. Relative abundance of AANAT protein in the gastrointestinal tissues.

Parallel to the concentrations of melatonin, a dose dependent decrease in the relative abundance of AANAT protein was observed in the stomach, duodenum and colon tissues treated with adrenaline compared to the control levels ($p<0.001$) (Fig. 1, B-E). Notably, irrespective of the regions of gastrointestinal tract, maximum decrease in the AANAT protein abundance was noted in the tissues treated with highest dose of adrenaline (AD-3).

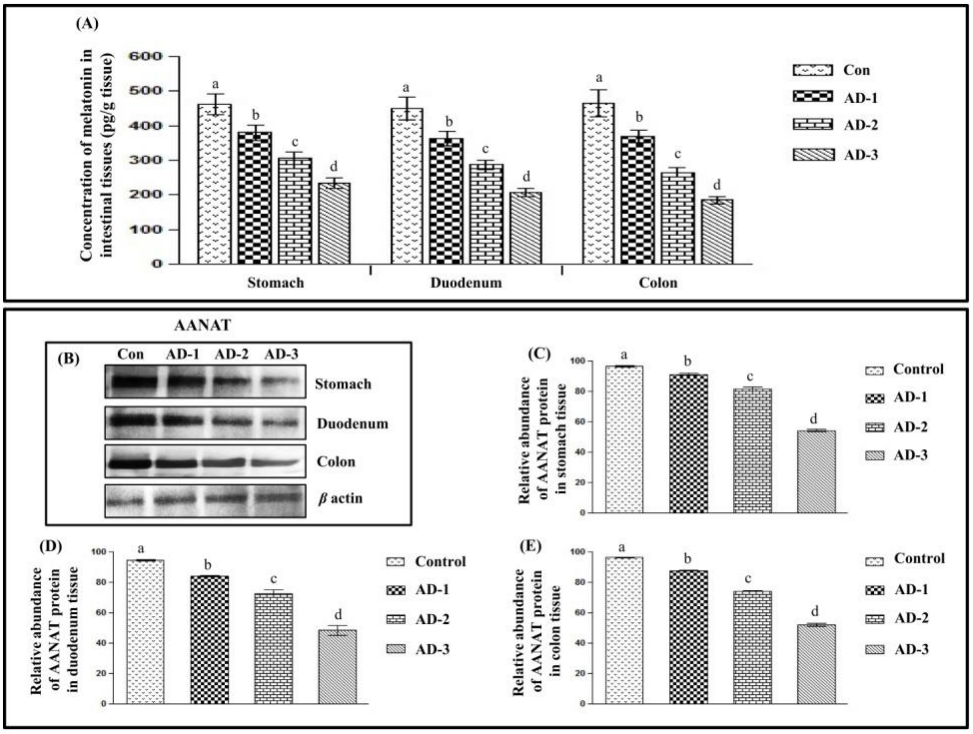

Figure 1. Effects of adrenaline on the melatonin level and AANAT expression in stomach, duodenum and colon of rats.

Histograms representing values (Mean ± SEM, in vertical bars) (n = 5) of (A) concentrations of melatonin and (B-E) expression of AANAT (~23 kDa) protein and their relative abundances (compared to the expression of β-actin as shown in the lower panel) in the (C) stomach, (D) duodenum and (E) colon tissues of Wistar rat incubated with different doses of adrenaline [2.5 (AD-1), 5.0 (AD-2) and 10.0 (AD-3) µg/mL]. The different scripts on the error bars indicate significant differences (different scripts vs each other) ($p< 0.05$) in the values of a particular variable between the experimental groups following one-way ANOVA and a post-hoc Duncan's multiple range test.

3.3. Relative abundance of MT1 and NF-kB proteins in the gastrointestinal tissues.

Relative abundance of MT1 protein, irrespective of the regions of gastrointestinal tract, was significantly ($p<0.001$) reduced in an adrenaline dose-dependent manner in all treated groups compared to the level in control tissues (Fig. 2, A-D). Maximum reduction in the MT1 protein abundance was found in tissues incubated with the highest dose of adrenaline (AD-3) (Fig. 2, A-

D). In contrast, a dose dependent increase in the relative abundance of NF-kB protein was noted in all adrenaline incubated groups compared to the values in control tissues ($p<0.001$), where a maximum increase was found in the tissues treated with highest dose of adrenaline (AD-3) (Fig. 2, E-H).

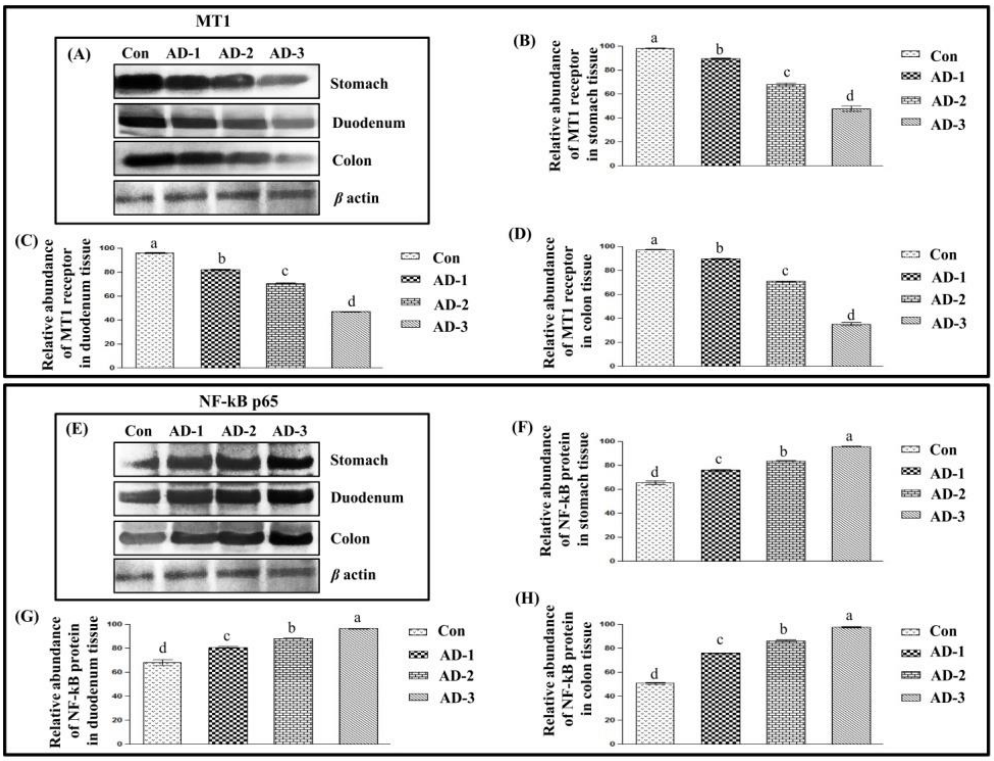

Figure 2. Effects of adrenaline on the expressions of MT1 and NF-kB p65 proteins in the stomach, duodenum and colon tissues.

Diagrammatic representation of the immunoblots and values (Mean ± SEM, in vertical bars) (n = 5) of their relative abundances (compared to the expression of β-actin as shown in each lower panel) of (A-D) MT1 and (E-H) NF-kB p65 proteins in the stomach, duodenum and colon tissues, respectively with different doses [2.5 (AD-1), 5.0 (AD-2) and 10.0 (AD-3) µg/mL] of adrenaline. The different scripts on the error bars indicate significant differences (different scripts vs each other) (p< 0.05) in the values of a particular variable between the experimental groups following one-way ANOVA and a post-hoc Duncan's multiple range test.

3.4. Levels of cAMP and Ca^{2+}.

A dose dependent increase in the intra-cellular levels of both cAMP (Fig. 3A) and Ca^{2+} (Fig. 3B) were noted in the stomach, duodenum and colon tissues following incubation with adrenaline compared to the control levels ($p<0.001$) (Fig. 1B). Notably, maximum levels of cAMP were noted in the AD-2 group of duodenum and AD-1 group of colon tissues (Fig. 3A). Similarly, maximum Ca^{2+} levels were noted in the AD-2 group of stomach, but AD-1 groups in duodenum and colon tissues (Fig. 3B).

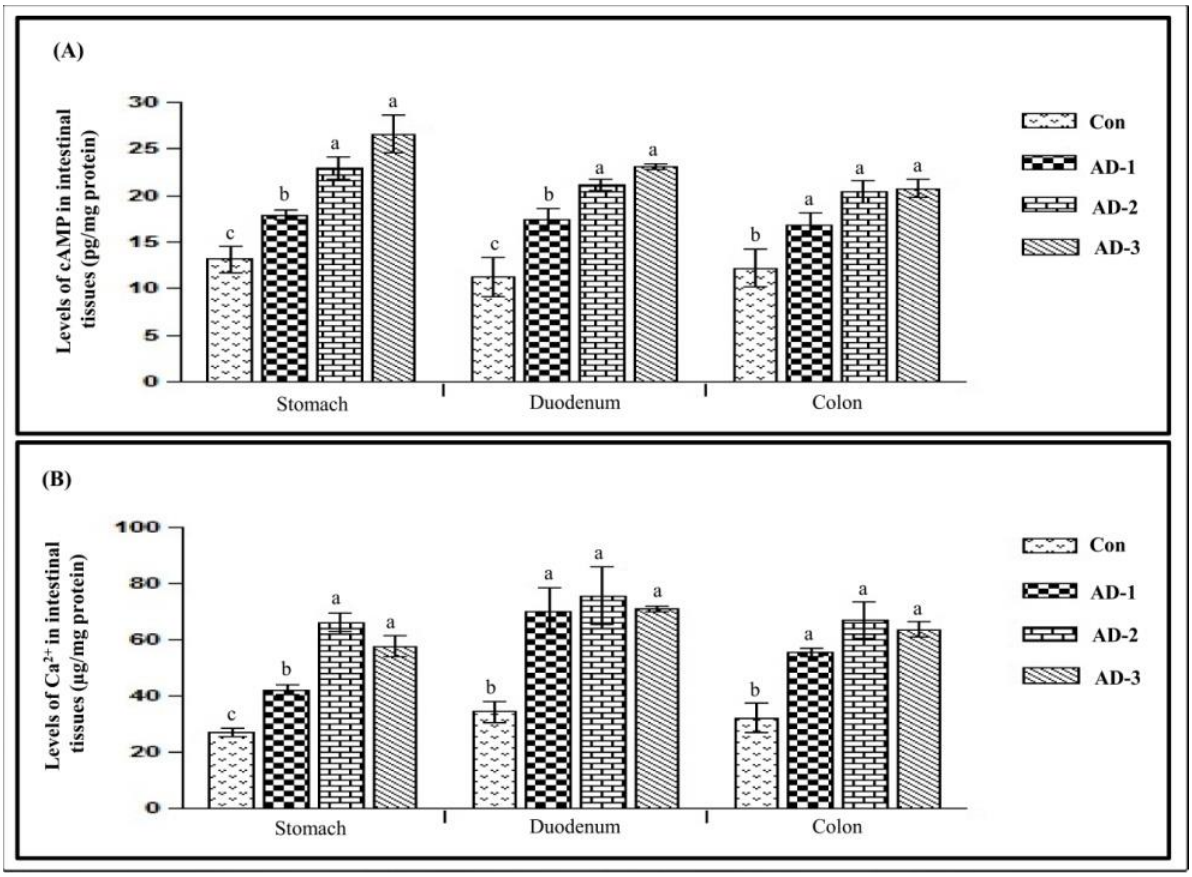

Figure 3. Effects of adrenaline on the intracellular levels of cAMP and Ca^{2+} in the stomach, duodenum and colon tissues.

Histograms representing values (Mean ± SEM, in vertical bars) (n = 5) of the intracellular levels of (A) cAMP and (B) calcium (Ca^{2+}) in the stomach, duodenum and colon tissues of Wistar rat incubated with different doses [2.5 (AD-1), 5.0 (AD-2) and 10.0 (AD-3) µg/mL] of adrenaline. The different scripts on the error bars indicate significant differences (different scripts vs each other) ($p < 0.05$) in the values of a particular variable between the experimental groups following one-way ANOVA and a post-hoc Duncan's multiple range test.

3.5. Levels of oxidative stress markers.

Levels of MDA (Fig. 4A) and PCO content (Fig. 4B) were significantly increased in a dose dependent manner in all adrenaline treated groups compared to the control values ($p<0.001$), where a maximum increase was noted in the groups treated with highest dose (10 µg/mL) of adrenaline (AD-3). Notably, a similar response to adrenaline treatment was found in the levels of NO. The maximum NO levels were identified in stomach of the AD-2 group, but in duodenum and colon tissues in AD-1 group (Fig. 4C). In contrast, the GSH levels, were significantly ($p<0.001$) decreased in a dose dependent manner in all adrenaline treated groups, where maximum decrease was noted in the AD-2 groups (Fig. 4D).

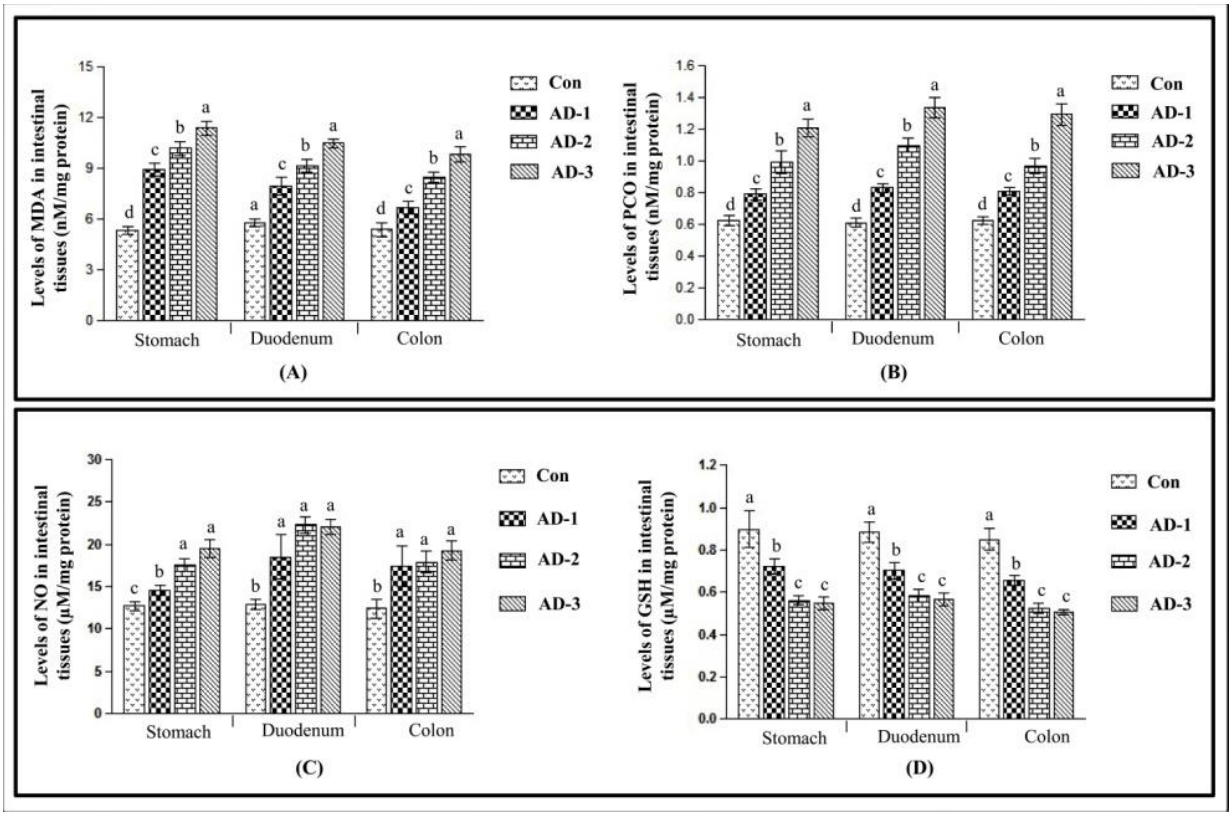

Figure 4. Effects of adrenaline on the levels of MDA, PCO, NO and GSH in the stomach, duodenum and colon tissues.

Histograms represent values (Mean ± SEM, in vertical bars) (n = 5) of the levels of (A) MDA, (B) PCO, (C) NO and (D) GSH in the stomach, duodenum and colon tissues, respectively, with different doses [2.5 (AD-1), 5.0 (AD-2) and 10.0 (AD-3) μg/mL] of adrenaline. Different scripts on the error bars indicate significant differences (different scripts vs each other) ($p < 0.05$) in the values of a particular variable between the experimental groups following one-way ANOVA and a post-hoc Duncan's multiple range test.

3.6. Protein expressions of antioxidant enzymes.

Relative abundance of SOD-1 protein, irrespective of the regions of gastrointestinal tract, was significantly decreased in a dose dependent manner in all adrenaline treated groups compared to their levels in the control tissues (Fig. 5, A-D) ($p<0.001$). Maximum reduction in the SOD-1 protein abundance was found in AD-2 adrenaline treated groups (Fig.5, A-D). In contrast, the expressions of CAT and GPx proteins were dose dependently ($p<0.001$) increased in all adrenaline treated groups compared to the control values (Fig. 5, E-H and I-L), where a maximum increase was found in the AD-2 adrenaline treated groups.

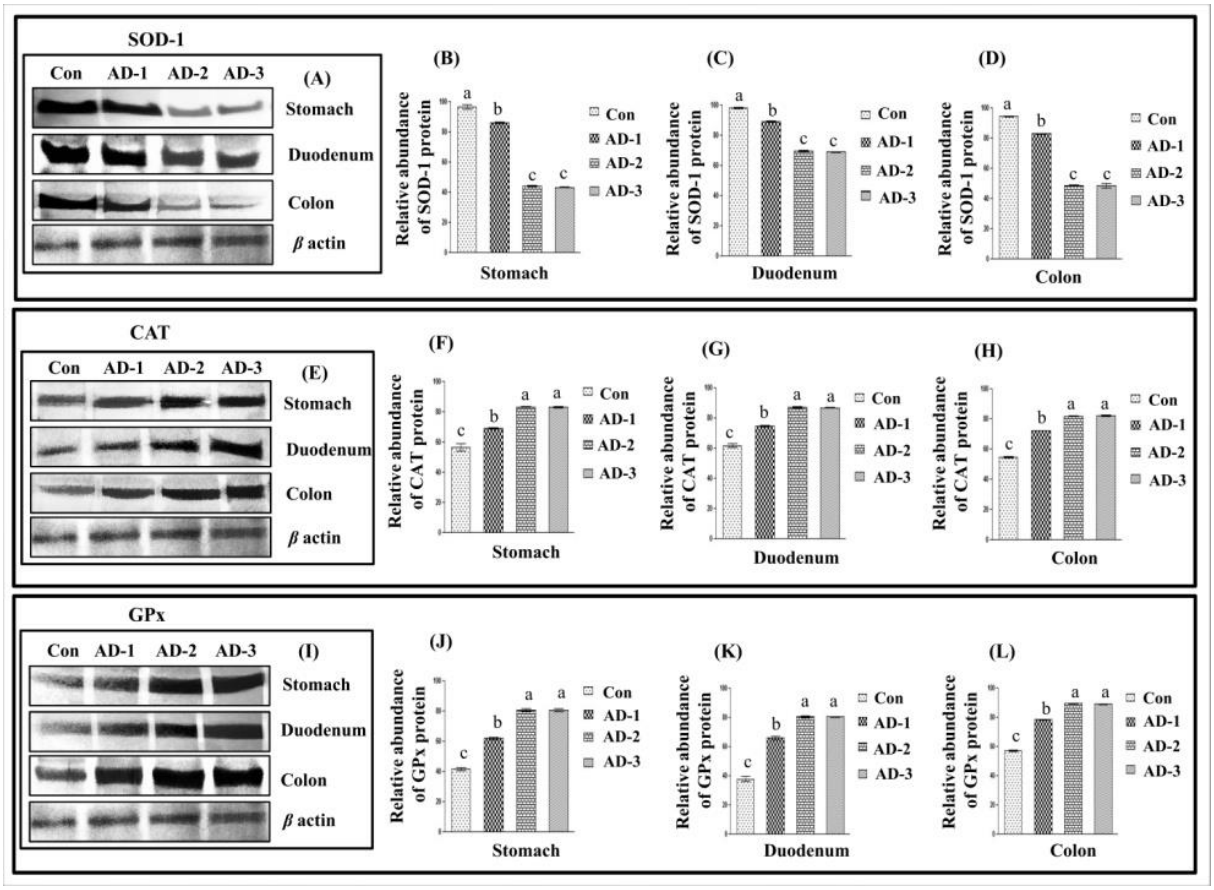

Figure 5. Effects of adrenaline on the protein expressions of SOD-1, CAT and GPx.

Diagrammatic representation of the immunoblots and values (Mean ± SEM, in vertical bars) (n = 5) of their relative abundances (compared to the expression of β-actin as shown in each lower panel) of (A-D) SOD-1, (E-H) CAT and GPx (I-L) proteins in the stomach, duodenum and colon tissues, respectively, with different doses [2.5 (AD-1), 5.0 (AD-2) and 10.0 (AD-3) µg/mL] of adrenaline. The different scripts on the error bars indicate significant differences (different scripts vs each other) (p< 0.05) in the values of a particular variable between the experimental groups following one-way ANOVA and a post-hoc Duncan's multiple range test.

3.7. Activities of antioxidant enzymes.

The activities of SOD (Fig. 6A) and GR (Fig. 6E), displayed a dose dependent decrease in all adrenaline treated groups compared to the control values ($p<0.001$), where maximum reduction was noted in AD-2 groups. In contrast, the activities of CAT, GPx and GST exhibited a dose dependent increase in all adrenaline treated groups compared to their respective control values [CAT (Fig. 6B), GPx (Fig. 6C) and GST (Fig. 6D)] ($p<0.001$). Notably, maximum increase in the CAT, GPx and GST activities was noted in all AD-2 groups.

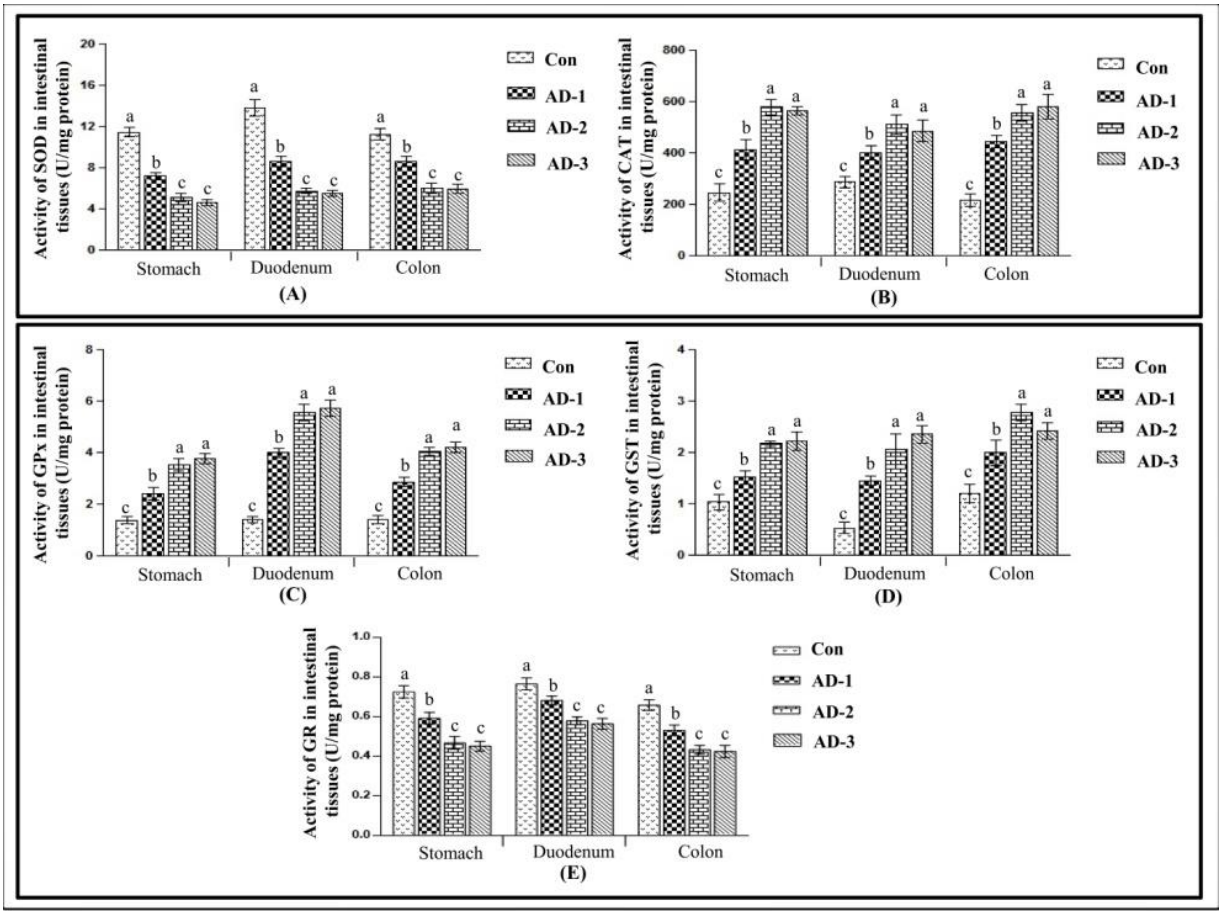

Figure 6. Effects of the adrenaline on the activities of SOD, CAT, GPx, GST and GR.

Histograms represent values (Mean ± SEM, in vertical bars) (n = 5) of the activities of different antioxidant enzymes- (A) SOD, (B) CAT, (C) GPx, (D) glutathione S- transferase (GST) and (E) GR in the stomach, duodenum and colon tissues, respectively, with different doses [2.5 (AD-1), 5.0 (AD-2) and 10.0 (AD-3) µg/mL] of adrenaline. The different scripts on the error bars indicate significant differences (different scripts vs each other)($p < 0.05$) in the values of a particular variable between the experimental groups following one-way ANOVA and a post-hoc Duncan's multiple range test.

3.8. Levels of inflammatory cytokines.

A dose dependent increase in the intra-cellular levels of IL-1β (Fig. 7A), IL-6 (Fig. 7B) and TNFα (Fig. 7C) were found in the stomach, duodenum and colon tissues treated with adrenaline compared to the control levels ($p<0.001$). Notably, maximum increase in the levels of IL-1β and TNFα were found in the AD-3 adrenaline treated groups and the IL-6 was in AD-2 (Fig. 7). In contrast, the IL-10 levels (Fig. 7D) were significantly decreased in a dose dependent manner in all adrenaline treated groups compared to their control levels ($p<0.001$), where a maximum reduction was noted in the AD-3 adrenaline treated groups.

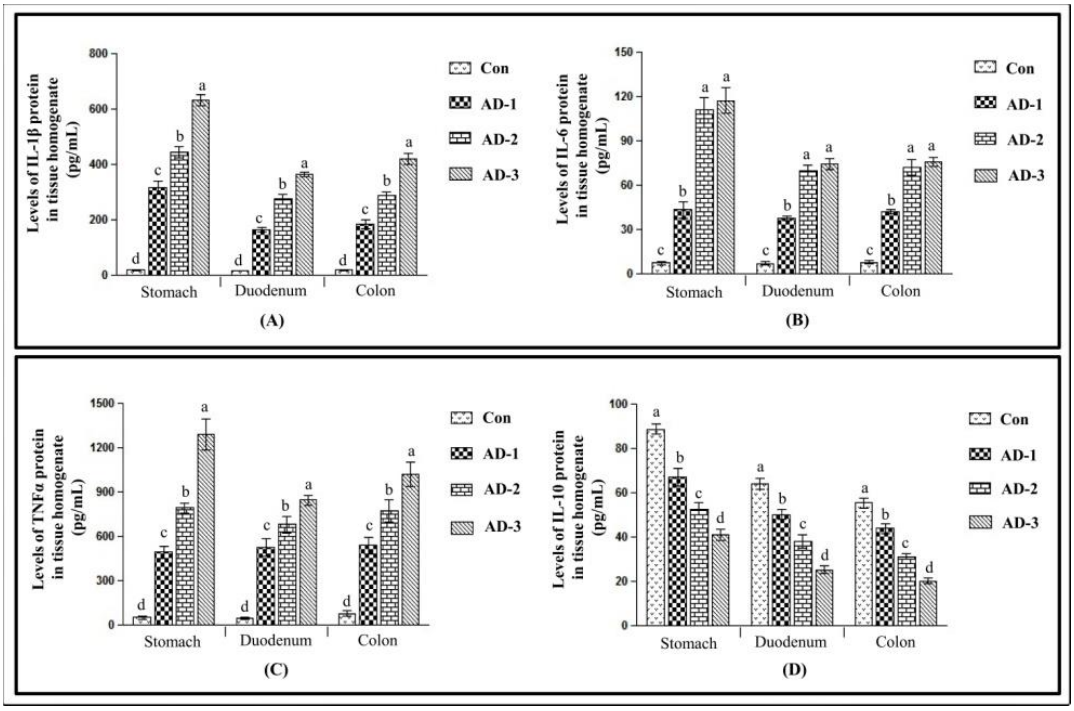

Figure 7. Effects of adrenaline on inflammatory cytokines.
Histograms represent values (Mean ± SEM, in vertical bars)(n = 5) of proinflammatory cytokines-(A) IL-1β, (B) IL-6, (C) TNFα and (D) IL-10 in the stomach, duodenum and colon tissues, respectively, treated with different doses [2.5 (AD-1), 5.0 (AD-2) and 10.0 (AD-3) µg/mL] of adrenaline. Different scripts on the error bars indicate significant differences (different scripts vs each other) ($p < 0.05$) in the values of a particular variable between the experimental groups following one-way ANOVA and a post-hoc Duncan's multiple range test.

3.9. Correlation analyses between melatonin level and expression of AANAT, MT1, NF-kB and antioxidant enzymes, respectively.

Correlation coefficient analysis of data revealed a significant positive correlation between melatonin level and relative abundance of AANAT, MT1, and antioxidant enzymes (SOD-1, CAT and GPx) in the stomach, duodenum and colon tissues, respectively (Table 1). In contrast, a negative correlation between melatonin level and expression of NF-kB was observed in all studied tissues (Table 1). The observations of the correlation also fitted well with the results of regression analysis (Fig. 8).

Table 1. Correlation analyses between melatonin levels and other parameters.

Studied tissues	*Variables*	AANAT	MT1	NF-kB	SOD-1	CAT	GPx
Stomach	**Melatonin**	0.805	0.853	-0.872	0.814	-0.841	-0.819
Duodenum	**Melatonin**	0.834	0.862	-0.864	0.836	-0.823	-0.805
Colon	**Melatonin**	0.866	0.850	-0.871	0.850	-0.848	-0.825

Values of "r" revealed from simple correlation coefficient analysis between the melatonin level and the levels of different variables of melatonergic system, NF-kB and antioxidant enzymes (any two variables at a time) in the different regions of GI tract, $p<0.001$.

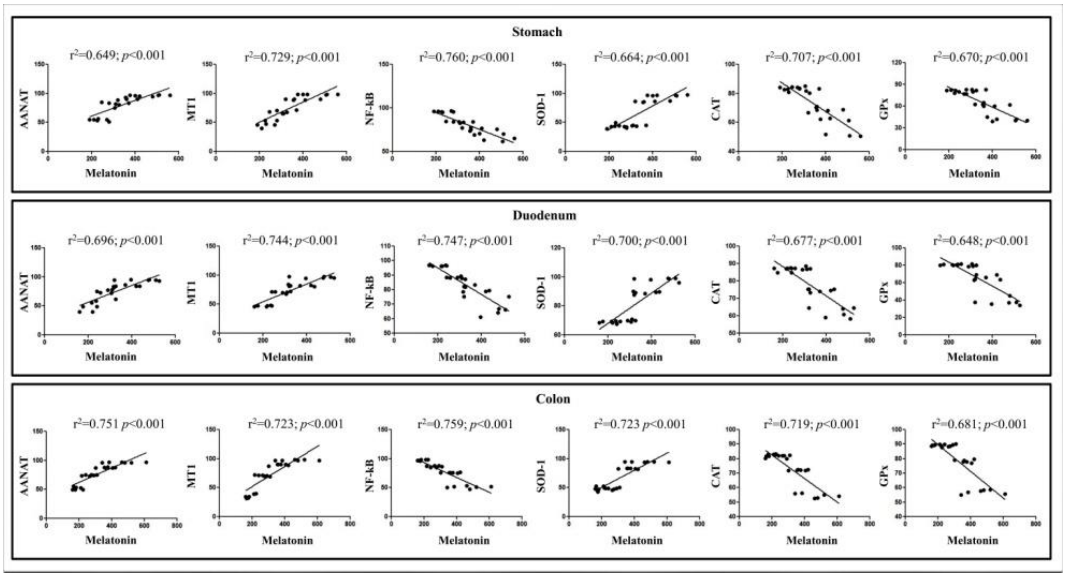

Figure 8. The results of single regression analyses of melatonin levels against other parameters.

Scatter plot showing the single regression analysis between concentrations of melatonin, and protein levels of AANAT, MT1, NF-kB and different antioxidant enzymes in the stomach, duodenum and colon tissues, respectively, with different doses [2.5 (AD-1), 5.0 (AD-2) and 10.0 (AD-3) µg/mL] of adrenaline. r^2 denotes goodness-of-fit and p-values indicate the significant level. Different abbreviations used are same as in Figures 1, 2 and 5.

3.10. Correlation between melatonin level and oxidative stress markers and activities of antioxidant enzymes.

As depicted from the correlation coefficient analysis, a significantly ($p<0.01$) negative correlation, was observed between melatonin level and each of the oxidative stress markers (MDA, PCO and NO) and activities of antioxidant enzymes (CAT, GPx and GST), but a strong positive correlation with the levels of GSH and activities of SOD (Table 2). The data of the correlation analysis were supported by the results of regression analysis (Fig. 9).

Table 2. Correlation analyses between melatonin levels and oxidative markers and activities of antioxidant enzymes.

Studied tissues	*Variables*	MDA	PCO	GSH	NO	SOD	CAT	GPx	GST	GR
Stomach	**Melatonin**	-0.755	-0.799	0.559	-0.799	0.768	-0.706	-0.806	-0.736	0.684
Duodenum	**Melatonin**	-0.811	-0.789	0.670	-0.665	0.802	-0.586	-0.759	-0.771	0.633
Colon	**Melatonin**	-0.798	-0.798	0.808	-0.504	0.672	-0.766	-0.805	-0.571	0.720

Values of "r" revealed from simple correlation coefficient analysis between melatonin level and the levels/activities of different variables of oxidative stress markers and antioxidant enzymes (any two variables at a time) in the tissue homogenates of different regions of gastrointestinal tract of Wistar rat, p<0.01.

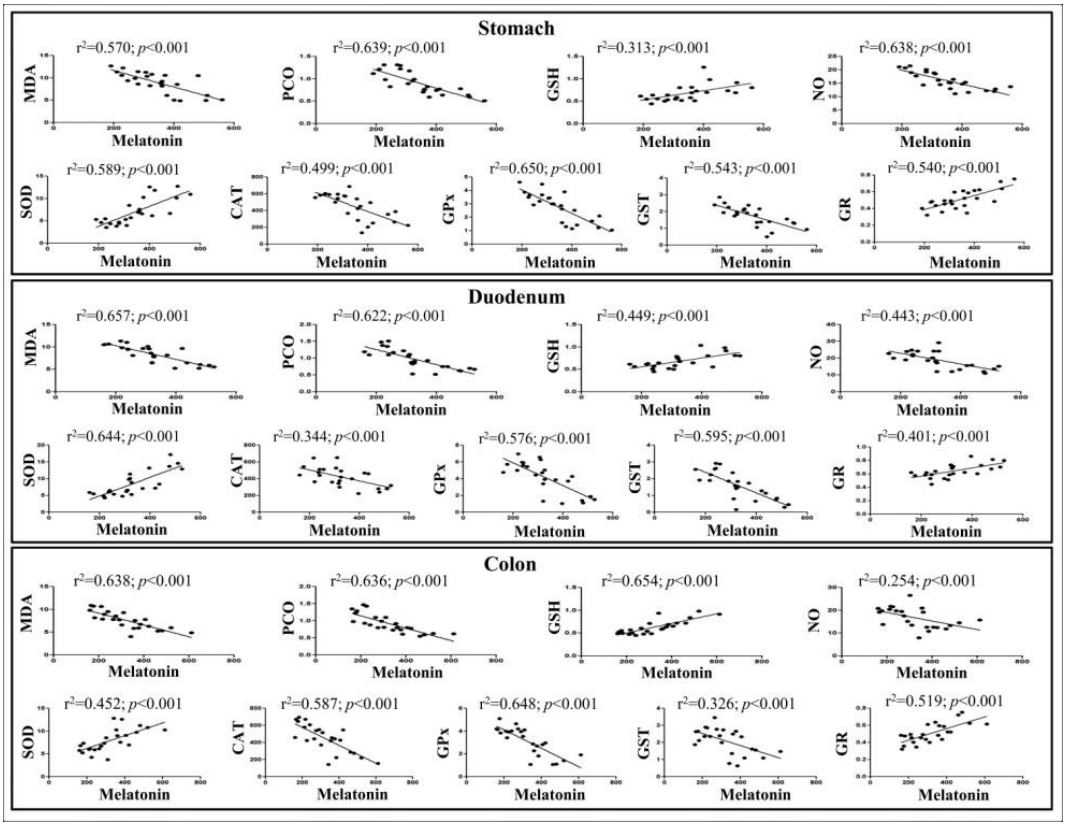

Figure 9. The results of single regression analyses of melatonin levels against other oxidative parameters.

Scatter plot showing the single regression analysis between concentrations of melatonin and the levels of MDA, PCO, GSH, NO as well as activities of antioxidative enzymes in the stomach, duodenum and colon tissues, respectively, with different doses [2.5 (AD-1), 5.0 (AD-2) and 10.0 (AD-3) µg/mL] of adrenaline. r^2 denotes goodness-of-fit and p-values indicate the significant level. Different abbreviations used are same as in Figures 1, 4 and 6.

3.11. Correlation between melatonin level and inflammatory cytokines:

Correlation coefficient analysis of data exhibited a significant negative correlation between the melatonin level and levels of proinflammatory cytokine IL-1β, IL-6 and TNFα in the stomach, duodenum and colon tissues, respectively ($p<0.001$). In contrast, the melatonin level with IL-10 exhibited a strong positive correlation ($p<0.001$) (Table 3). The data of the correlation coefficient analysis were supported by the results of regression analysis (Fig. 10).

Table 3. Correlation analyses between melatonin levels and proinflammatory cytokines.

Studied tissues	*Variables*	IL-1β	IL-6	IL-10	TNF-α
Stomach	**Melatonin**	-0.822	-0.754	0.765	-0.824
Duodenum	**Melatonin**	-0.864	-0.843	0.799	-0.779
Colon	**Melatonin**	-0.849	-0.842	0.864	-0.828

Values of "r" revealed from simple correlation coefficient analysis between the melatonin level and the levels of different variables of proinflammatory cytokines (any two variables at a time) in the different regions of GI tract, respectively, p<0.001.

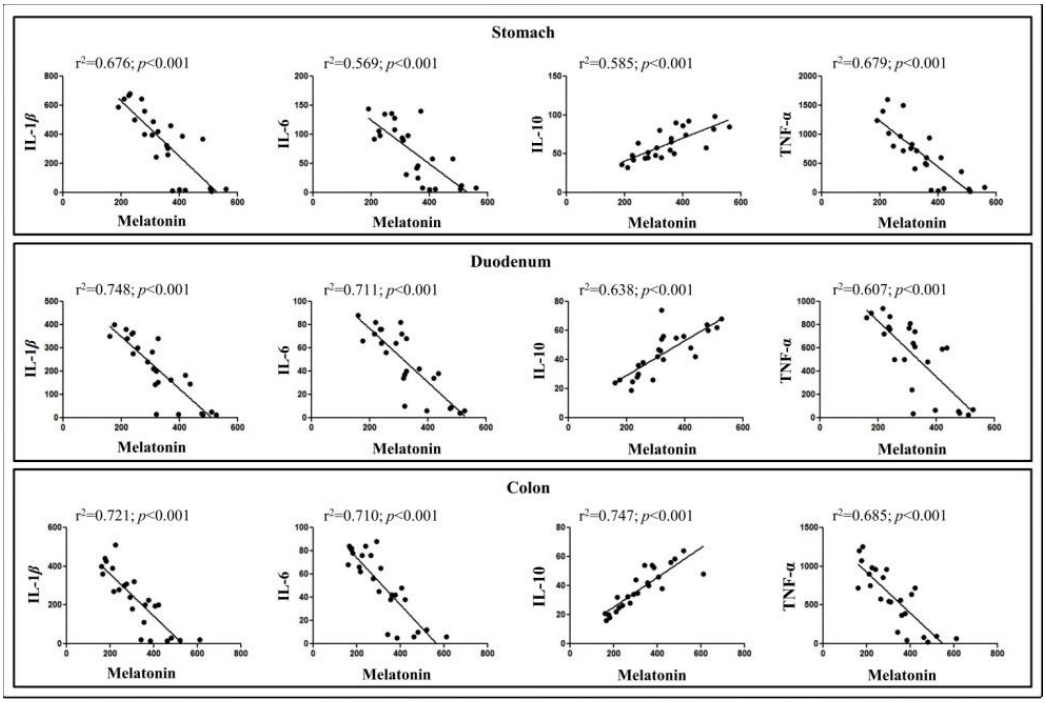

Figure 10. The results of single regression analyses of melatonin levels against proinflammatory cytokines.

Scatter plot showing the single regression analysis between melatonin level and levels of different proinflammatory cytokines IL-1β, IL-6, IL-10 and TNF-α in the stomach, duodenum and colon tissues, respectively, with different doses [2.5 (AD-1), 5.0 (AD-2) and 10.0 (AD-3) µg/mL] of adrenaline. r^2 denotes goodness-of-fit and p-values indicate the significant level. Different abbreviations used are same as in Figures 1 and 7.

4. DISCUSSION

To the best of our knowledge, the current study is the first report evaluating the effects of adrenaline induced oxidative stress on the melatonergic system, antioxidant enzymes and proinflammatory cytokines in GI tract. The results have revealed that excessive adrenaline exhibits the profound adverse effects on GI tract by disturbing the physiological balance of melatonergic system and thereafter the antioxidant enzyme as well as the proinflammatory system.

MDA level indicates lipid peroxidation and membrane damage caused by free radical attack and, thus it commonly serves as a potent marker of oxidative stress (42). In this it was observed that MDA production was significantly elevated by adrenaline treatment, indicating an ROS attack and oxidative stress in the GI tissue. Adrenaline is known to generate ROS 913, 43, 44) to provoke cellular damage through lipid peroxidation 945, 46). This is consistent with the previous studies in which adrenaline caused the elevation of MDA in rat [6] and humans (47).

PCO is another oxidative parameter related to protein damage and its alteration is associated with diverse pathophysiological conditions (48). Adrenaline also caused a dose-dependent increase of PCO in stomach, duodenum and colon tissues, respectively. The result suggests that functionally active proteins undergone spontaneous oxidative damage by the free radical attack probably due to adrenaline auto-oxidation (49), as observed in the heart and liver tissues of rat (6). A similar response in the levels of NO was also noted in all adrenaline treated gastrointestinal tissues which was reported in the hepatic and cardiac tissues previously (6). It is well established that elevated levels of $O_2^{\cdot -}$ and NO form peroxynitrite which is capable to cause oxidative tissue injury (50). Thus, the elevated NO level might be additional cause for the oxidative stress in the GI tissues. In contrast, a dose dependent decrease in the levels of GSH was also noted in the GI tissues after adrenaline treatment. GSH serves as a potent free radical scavenger and protects cell from oxidative stress (51). It is obvious that the GSH has been exhausted after adrenaline treatment, which is similar to the observations in the cardiac tissues of rat (60. An increased level of GSH induced by adrenaline treatment in the hepatic tissue of rat has also been reported (6). It appears that this difference may be organ specificity in animals.

Importantly, it was first observed that the level of melatonin, a potent antioxidant, in the GI tract was significantly reduced by the adrenaline treatment. Melatonin has been identified to synthesize in GI tract in a large quantity. Its production in GI tract is several hundred-folds higher than produced in the pineal gland (16, 23, 24, 26). Its synthetic enzymes have been found in the GI tissues (19, 24, 23, 25). The melatonin in GI tract is to regulate the physiological activities (14-16, 18-19, 21, 26) and locally protect GI tissue from the free radical damage (14-15, 18-19, 22).The mechanism study indicated that the gene expression of the melatonin synthetic enzyme, AANAT and its membrane receptor, MT1 was significantly down regulated by adrenaline. The decreased melatonin production is always associated with oxidative damage in tissues and organs (14, 19-20). The pro-oxidation of adrenaline can cause DNA damage and impact the integrity of DNA (4, 52). Thus, adrenaline auto-oxidation may disrupt the AANAT and MT1 gene expressions; ultimately reduce the synthesis of melatonin. This assumption should remain as an interesting topic of future research. On the other hand, NF-kB levels were found to be increased following incubation with adrenaline. Possibly, elevation of intracellular oxidative stress upregulates the gene expression of NF-kB as evidenced in an earlier study in rat (53).
Notably, intra-cellular calcium and cAMP mediate important functions in the melatonin biosynthesis pathway. Our study revealed that the levels of intra-cellular cAMP and calcium were increased following adrenaline treatment. Adrenaline is known to increase the influx of intra-cellular levels of Ca^{2+} (54); while by stimulating adenylate cyclase, it increases the level of cAMP (55). However, increased cAMP level was unable to enhance the level of AANAT protein and melatonin synthesis in the GI tissues. The underlying mechanism of such response remained unknown and thereby warrants further investigation.

Since adrenaline can regulate activities of different antioxidant enzymes in the gastric tissue (13), it is necessary to evaluate both the gene expressions and activities of these enzymes. Surprisingly, activities of CAT, GPx and GST were increased with adrenaline treatment in the stomach, duodenum and colon, while SOD activity was decreased. Molecular analysis further confirmed that the gene expression of these enzymes showed the same results as their activities after adrenaline treatment. Physiologically, these antioxidative enzymes work together to detoxify the ROS. For example, SOD converts superoxide anion to hydrogen peroxide (H_2O_2) (56), in turn, CAT catalyzes H_2O_2, to water and molecular oxygen, alternatively GPx metabolizes H_2O_2 to water at the expense of GSH (57) and the GR reduces the oxidized

glutathione to GSH again to complete a cycle. The alterative activities of these enzymes in adrenaline treated GI tissues can be considered as the responses of the tissues to the adrenaline-induced oxidative stress. The similar observations have been reported previously (53, 58).

Another important aspect is the impacts of the adrenaline on the proinflammatory cytokines, TNFα, IL1β, IL6 and IL10. Adrenaline is known to modulate the immune system by acting on the adrenoceptors in mammal (59). In the study, the levels of TNFα, IL1β and IL6 were significantly increased in the adrenaline-treated GI tissues which were similar to the previous publications (58-62). All changes may relate to the alternated melatonergic system induced by adrenaline. The correlation coefficient analysis in deed revealed correlation between the melatonin level and the levels/activities of antioxidant enzymes and proinflammatory cytokines in the GI tissues treated with adrenaline. Many studies have documented the influences of melatonin on the gene expressions and activities of antioxidant enzymes [6, 14, 15, 20] as well as proinflammatory cytokines (22, 53, 60, 62). Generally, melatonin stimulates the activities of antioxidants and suppresses the proinflammatory cytokines and thus, provides protective effects on the oxidative stress in different organs and tissues (20, 63). Moreover, suppressed melatonin level and upregulation of NF-kB expression may also account for the increase in proinflammatory levels (53).

In conclusion, the current study provided novel information regarding the adrenaline induced oxidative damage in GI tissues. It mainly suppresses the melatonergic system by downregulation of the expression of AANAT and MT1, thus, reducing the melatonin levels as well as its activity to binding to its receptor. Since melatonin is the master regulator for the antioxidant enzymes and inflammatory response, low melatonin level induced by the adrenaline inevitably resulted in the alterations of these parameters observed in the studies. The correlation coefficient analysis strongly supports this conclusion. The association between melatonin level and any of the parameter is statistically significant (Table 1-3). The results of the current study accumulate information on the mechanisms as to how adrenaline causes GI tract damage and also provide valuable data to instruct prevention and treatment of excessive adrenaline toxicity clinically. It seems that melatonin is the suitable molecule for this purpose.

AUTHORSHIP

Dr. DB and Dr. AC conceived and designed the experiment, revised the manuscript critically and approved it. Dr. PKP contributed to conception and executed the experiment, analyzed the data, prepared figures, drafted the manuscript and edited it. BB contributed in executing the experiments and drafting of manuscript. Dr. AKG contributed in executing the experiment.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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