Debasish Bandyopadhyay et al. /JPR:BioMedRx: An International Journal 2013,1(11),997-1004

Research Article
ISSN: 2321-4988

Available online through
www.jpronline.info

Melatonin protects against lead-induced oxidative stress in stomach, duodenum and spleen of male Wistar rats

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Received on:17-08-2013; Revised on:16-09-2013; Accepted on:21-10-2013

ABSTRACT

Environmental or occupational or even accidental exposure to certain heavy metals can cause generation of reactive oxygen species (ROS) which are major causes of various diseases. Our present investigation demonstrates that the oral supplementation of aqueous solution of melatonin has the potential to protect against the oxidative stress in stomach, duodenum and spleen induced by sub-chronic exposure of the experimental rats to the heavy metal , lead (Pb). Rats were intraperitoneally injected with lead acetate (15mg/kg body weight). Another group was pre-treated with melatonin (10 mg / kg, fed orally).The positive control group was fed melatonin (10 mg / kg), and the control animals received vehicle treatment i.p. for 7 consecutive days. The alterations in the activity of the different biomarkers of oxidative stress and the activities of the antioxidant enzymes were studied. Histo-morphological changes in the gastric, duodenal and splenic tissues were studied through H-E staining. Lead caused alterations in all the parameters studied. All these changes were mitigated in all the three tissues when the rats were pre-treated with melatonin. The results indicate that melatonin protects against lead-induced damages in stomach, duodenum and spleen in experimental rats by antioxidant mechanism. Melatonin may have future therapeutic relevance in the prevention of lead-induced gastro-toxicity as well as in splenic toxicity and duodenal toxicity in humans exposed occupationally or environmentally to this toxic heavy metal and may be used for development of new drug with almost no or minimum side-effect.

Key words: Melatonin, stomach, duodenum, spleen, lead, oxidative stress

INTRODUCTION

Lead (Pb), a heavy metal is considered to be an oldest environmental toxin. It is also a recognised occupational toxin.[1] The toxicity of lead (Pb), has been documented in experimental animals and also in human beings.[2] This element is associated with a lot of adverse health effects. Studies reveal that lead exposure causes generation of ROS and alteration of antioxidant defense systems in animals and occupationally exposed workers. Generation of highly reactive oxygen species (ROS), such as superoxide radicals (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH) and lipid peroxides mediated by heavy metal ions are known to damage various cellular components including proteins, membrane lipids and nucleic acids. [3,4] Lead enters the body through multiple routes and through circulation it is carried to various organs and gets deposited mainly in the soft organs and bones.[1]

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N-Acetyl-5-methoxytryptamine commonly known as melatonin is ubiquitous in nature. Melatonin is synthesised and released from the vertebrate pineal gland as well as from some other organs.[5] The indole influences circadian rhythm by acting on the supra-chiasmatic nucleus.[6] Melatonin scavenges ROS and stimulates the endogenous antioxidant system of an organism and thus aids the ability of organisms to resist the oxidative damage caused by radicals and radical related products. Antioxidative enzymes provide a major defence mechanism against free radical damage either by metabolizing them to less reactive species or to non-toxic byproducts.[7,8]

Melatonin has been reported to be present in high concentrations in the gastrointestinal tract, which is considered as a major source of melatonin. Gastro-duodenal ulcer is a very common thing of concern these days not only in India and native countries but also around the globe. Out fast lifestyle and changing food habit is a major contributor to this gastro –duodenal ulcerative damage irrespective of age and socioeconomic status. The gastric lesions develop when the delicate balance between some gastro-protective and aggressive factors is lost. Involvement of free radicals mediated oxidative stress induced

damages leading to destruction of the gastric mucosa has been studied and documented.[8] Studies reveal high levels of melatonin in the bile.[9]. Melatonin receptors have also been reported and characterized in stomach, jejunum, ileum and colon. [10, 11]

Melatonin has been shown to have immune-modulatory activity. Research shows that melatonin can potentially modulate functions of spleen and/or the immune system by regulating the NF-kB DNA binding activity in the therein.[12] Melatonin has been reported to abate lipopolysaccharide induced oxidative stress in duodenum of rabbit.[13] Lead induces oxidative stress through the generation of free radicals and melatonin posses an antioxidant potential. [14, 15] Present investigation is aimed at studying the protective effects of melatonin on lead induced oxidative stress in stomach, duodenum and spleen of rats. Melatonin can be considered as a pharmaceutical agent for future drug development against heavy metal induced gastro toxicity for individuals who get regularly exposed to toxic heavy metals like lead occupationally or environmentally.

MATERIALS AND METHODS

All the chemicals used were of analytical grade unless otherwise specified. Thio Barbituric Acid (Spectro chem.,India) , Folin and Ciocalteu Phenol reagent (SRL, India) , Ethylene Diamine Tera-acetic acid (Qualigens Fine Chemicals, GlaxoIndia Ltd), TRIS-HCl tris(hydroxymethyl)aminomethane (SRL, India), TCA (SRL, India), Dithio bis nitro benzoic acid(DTNB) (SRL, India), GSH (SRL, India), Na_2PO_4 (SRL, India), NaOH (SRL, India), $CuSO_4$ (Merck, India), Na-K-Tartarate (SD Finechem. Ltd, India), $Pb(CH_3COO)_2$.Triton x, H_2O_2(SRL, India).

Experimental animals

Male Wistar rats of body weight 160-180 gm were utilised for the experiments. The animals were maintained and handled as per the guidelines of Institutional Animal Ethics Committee (IAEC) in accordance with the committee for the purpose of control and supervision of experiment on animal (CPCSEA), Ministry of Environment and Forest, Government of India. All the experimental protocols had the approval of Institutional Animal Ethics Committee IAEC [IAEC/PROPOSAL/DB-2/2010, APPROVAL DATE:16/11/2011] of the Department of Physiology, University of Calcutta. Prof. P. K. Samanta, M.Sc. (Vet.), Ph. D., Professor and Veterinary Surgeon and CPCSEA Nominee to Department of Physiology, University of Calcutta, acted as the advisor for animal care and handling.

Pb-induced oxidative stress in stomach, duodenum and spleen *in vivo* and protection with Melatonin

Following acclimatization of the experimental animals to laboratory conditions, the rats of the melatonin and the melatonin + lead acetate group were fed melatonin dissolved in normal drinking water, at a dose of 10 mg kg-1 body weight for 7 consecutive days. An hour after the melatonin was fed, the animals of the lead acetate and the melatonin +lead acetate treated groups were injected with lead acetate solution, i.p., at a dose of 15 mg kg-1 body weight (LD_{50} for lead acetate is 150 mg/ kg bw) for the 7 consecutive days. The animals of the control group received the vehicle only. Each day the body weight of the animals were measured and recorded. At the end of the treatment period, the animals of each group were kept fasted overnight. The body weight of the animals of each group were measured and recorded. The animals were sacrificed through cervical dislocation after subjecting them to mild ether anaesthesia. The stomach, duodenum and spleen were surgically removed after opening the abdominal cavity and immersed in ice-cold 0.9% saline for proper washing, blotted dry and weighed.

Preparation of tissue homogenate

A 10 % tissue homogenate of each of stomach, duodenum and spleen was prepared in two separate buffer solutions, ice cold 0.9% saline,50 mM phosphate buffer(pH 7.4) and 2 mM EDTA buffer (as per the biochemical analysis required), using a Potter Elvehjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30s. and the biochemical analysis was performed.

Measurement of lipid peroxidation level

A portion of the fundic stomach, duodenum and a piece of spleen was homogenized (5%) in ice-cold 0.9% saline (pH 7.0) Potter Elvehjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30 seconds and lipid peroxides in the homogenate were determined as thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust[16] with some modification[17, 18]. Briefly, the homogenate was mixed with thiobarbituric acid–trichloro acetic acid (TBA–TCA) reagent with thorough shaking and heated for 20 min at 80^0C. The samples were then cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant after centrifugation at 1200 g for 10 min at room temperature was measured at 532 nm using a UV–vis spectrophotometer (Bio-Rad, Smartspec Plus, Hercules, CA, USA). Tetraethoxypropane (TEP) was used as standard. Values were expressed as nmoles of TBARS/mg protein.

Measurement of reduced glutathione (GSH)

The level of GSH in the gastric, duodenal and splenic tissues was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlac and Lindsey (1968)[19] with some modifications[20,21].A portion of each tissue was homogenized (10%) separately in 2 mm ice cold ethylenediaminetetraacetic acid (EDTA). The homogenate was mixed with Tris–HCl buffer, pH 9.0, followed by DTNB for color development. The absorbance was measured at 412 nm using a UV–VIS spectrophotometer (BIORAD, Smartspec Plus). Values were expressed as nmoles/mg protein.

Measurement of the activity of superoxide dismutase (SOD; Cu-Zn type)

Copper-zinc superoxide dismutase (SOD1) activity was measured by hematoxylin autoxidation method of Martin *et al*.[22] with some modifications[23]. Briefly, the tissues were homogenized (10%) in ice-cold 50 mM phosphate buffer containing 0.1 mM EDTA, pH 7.4. The homogenate was centrifuged at 12,000 g for 15 min and the supernatant, thus obtained, was collected. Inhibition of hematoxylin auto-oxidation by the cell free supernatant was measured at 560 nm using a UV–Vis spectrophotometer. The enzyme activity was expressed as U / mg of tissue protein.

Measurement of the activity of catalase (CAT)

Catalase was assayed by the method of Beers and Sizer[24] with some modifications[25]. The tissues were homogenized (5%) in ice-cold 50 mM phosphate buffer, pH 7.0. The homogenate was centrifuged in cold at 12,000 g for 12 min. The supernatant, thus obtained, was then collected and the aliquots of the supernatant serving as the source of enzyme were incubated with 0.01 ml of absolute ethanol at 4oC for 30 min, after which 10% Triton X-100 was added to have a final concentration of 1%. The sample, thus obtained, was used to determine the catalase activity by measuring the breakdown of H_2O_2 spectrophotometrically at 240 nm. The enzyme activity was expressed as µmoles H_2O_2 consumed / min / mg protein.

Measurement of tissue protein content

Protein was estimated by the method of Lowry *et al* (1951)[26] using bovine serum albumin (BSA) as the standard with some mdifications[25].

Statistical analysis

Each experiment was repeated at least three times with different sets of male Wistar rats. Data are presented as means ± S.E. Significance of mean values of different parameters between the treatment groups were analyzed using one way analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the treatments. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

RESULTS

Animal behaviour

Lead treated rats became less active and drowsy. During the last 2-3 days of the treatment it was observed that the rats of the lead treated group, became more restless and aggressive. The rats of the Control group and the Melatonin treated group were observed to exhibit a calm and normal behavior. The rats of the Melatonin +lead group were not much aggressive and were active.

Body weights

It was found that the animals of the four groups showed normal growth pattern before and during the period of the work.

Changes in stomach

Treatment of rats with lead significantly altered the lipid peroxidation level (LPO) of the stomach tissue in the rats (76.47% higher vs. control) than the control animals (*P<0.001). Melatonin alone did not significantly alter the lipid peroxidation levels than that of control values. Pre- treatment of rats with melatonin altered the levels of lipid peroxidation (37.78% lower Vs. lead) than that of the control group animals (**P<0.001) [Table 1]. Lead treatment significantly altered the reduced glutathione (GSH) content in the stomach of rats (31.34% lower Vs. control) compared to that in control (*P<0.001). Oral treatment with melatonin alone did not significantly alter the GSH content versus control. Pre- treatment of rats with melatonin increased the GSH content significantly (48.03% higher Vs. lead) than the only lead treated group (**P<0.001) [Table 1]. Lead treatment altered the activity of superoxide dismutase (SOD) values in the stomach of rats (46.46% increase Vs. control) compared to that in control animal (*P<0.001). Oral treatment with melatonin alone however did not significantly alter the SOD activity values versus control. Pre- treatment of rat with melatonin changed SOD activity significantly (31.60% lower Vs. lead) than the lead treated animals (**P<0.001) [Table 1].

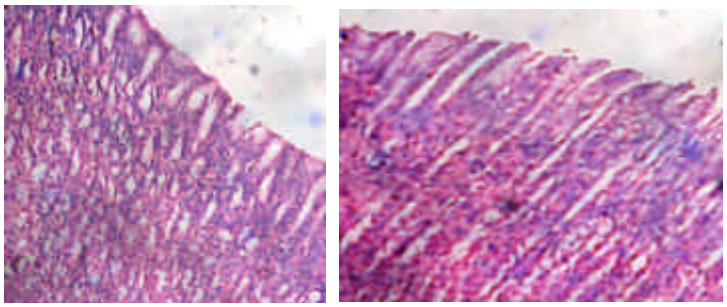
Lead treatment altered the activity of catalase (CAT) significantly (25.54% higher Vs.control) values in the stomach of rats compared to that in control animals (*P<0.001).Oral treatment with Melatonin did not significantly alter the activity of catalase versus control .Pre-treatment of rats with melatonin changed catalase activity significantly (16.83% higher vs. lead) than the lead treated values. (**P<0.001) **[Table 1]**.

Table 1: Table shows the levels of lipid peroxodation, reduced glutathione content as well as the activities of Cu-Zn superoxide dismutase and catalase of the stomach in lead acetate treated and melatonin protected rats.

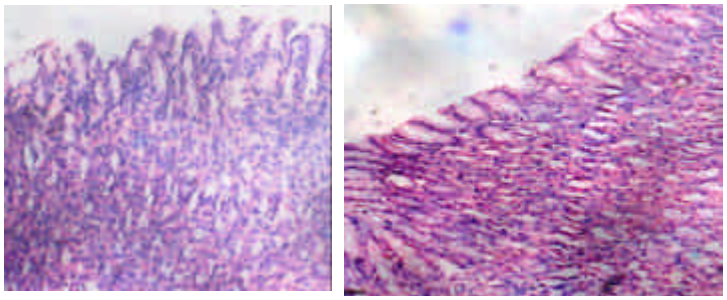
Parameters studied	Control	Melatonin	Lead	Melatonin+Lead
LPO (nmoles of TBARS/mg protein)	0.051±0.008	0.049±0.006	0.090±0.009*	0.056±0.087**
GSH(nmoles/mg protein)	10.37±0.163	10.46±0.154	7.12±0.189*	10.54±0.148**
Cu-Zn SOD activity (units/min/mg protein)	1.076±0.063	1.075±0.066	1.576±0.059*	1.078±0.072**
Catalase activity(µmoles H_2O_2 consumed/min/mg protein)	9.455±0.210	9.567±0.243	11.87±0.266*	9.872±0.206**

Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows. *P<0.001 compared to control; **P< 0.001 compared to lead treated group;

Histological studies showed tissue damage in lead treated stomach of rats. The damage was found to be much lesser in Melatonin+lead treated rats. No significant differences were observed in the sections of the stomach tissues of Melatonin treated and control group rats **[Figure 1]**.



Control **Melatonin**



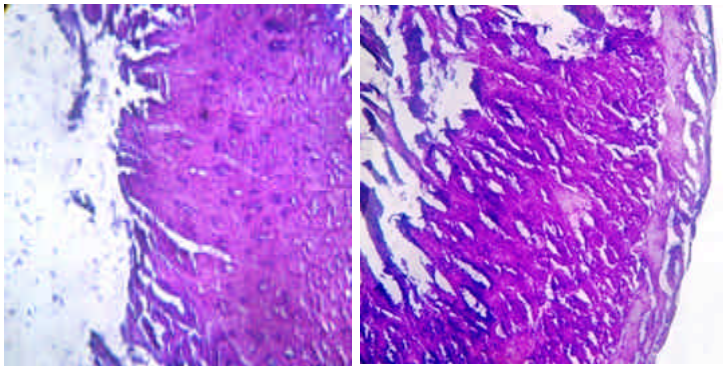
Lead **Melatonin+Lead**

Figure.1. Histopathological studies of gastric tissue [400X]

Changes in duodenum

Treatment of the rat with lead significantly enhanced the lipid peroxidation level in the duodenum of rats (72.86% higher Vs. control) than the control values (*P<0.001). Oral treatment with melatonin alone did not significantly alter the lipid peroxidation levels than that of control values. Pre- treatment of rats with melatonin decreased the levels of lipid peroxidation (40.50% lower Vs. lead) than that of the lead group animals (**P<0.001) **[Table 2]**. Lead treatment significantly altered the GSH values in the duodenum of rats (30.15% lower Vs. control) compared to that in the control rat (*P<0.001). Oral treatment of rat with Melatonin alone did not significantly alter the GSH content when compared with control. Pre- treatment of rats with Melatonin altered the GSH content significantly (44.44% higher Vs. lead) than that observed in the lead treated animals (**P<0.001) **[Table 2]**. Lead treatment significantly increased the SOD activity in the duodenum of rats (34.26% higher vs. control) compared to that in the control group (*P<0.001). Oral treatment of rat with Melatonin alone however did not alter the SOD activity when compared with control. Pretreatment of rats with melatonin decreased the SOD activity significantly (25.37% lower Vs. lead) than the animals of the lead treated group (**P<0.001) **[Table 2]**. Lead treatment significantly increased the catalase activity in the duodenum of rats (72.65% higher Vs. control) compared to that in the control group (*P<0.001). Oral treatment of rats with melatonin alone however did not alter the catalase activity versus control .Pre- treatment of rats with melatonin decreased the catalase activity (35.89% lower Vs. lead) than that observed in lead treated group (**P<0.01) **[Table 2]**.

Histological studies show damage of the integrity of the mucosal layer of duodenal tissue of lead treated rats. Ulcerative changes were observed in the duodenum of the lead treated rats. The damage was protected in Melatonin+lead treated rats. No significant differences were observed in the sections of the duodenum tissues of Melatonin treated and control group rats **[Figure 2]**.



Control **Melatonin**

Lead **Melatonin+Lead**

Figure.2. Histopathological studies of duodenal tissue [200X]

Table 2: Table shows the levels of lipid peroxodation, reduced glutathione content as well as the activities of Cu-Zn superoxide dismutase and catalase of the duodenum in lead acetate treated and melatonin protected rats.

Parameters studied	Control	Melatonin	Lead	Melatonin+Lead
LPO (nmoles of TBARS/mg protein)	0.070±0.006	0.069±0.007	0.121±0.007*	0.072±0.005**
GSH(nmoles/mg protein)	10.05±0.323	10.06±0.354	7.02±0.289*	10.14±0.242**
Cu-Zn SOD activity (units/min/mg protein)	1.474±0.061	1.465±0.066	1.979±0.059*	1.477±0.012**
Catalase activity(µmoles H_2O_2 consumed/min/mg protein)	2.234±0.110	2.321±0.622	3.857±0.124*	2.320±0.706**

Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows. *P<0.001 compared to control; **P< 0.001 compared to lead treated group;

Table 3: Table shows the levels of lipid peroxodation, reduced glutathione content as well as the activities of Cu-Zn superoxide dismutase and catalase of the spleen in lead acetate treated and melatonin protected rats.

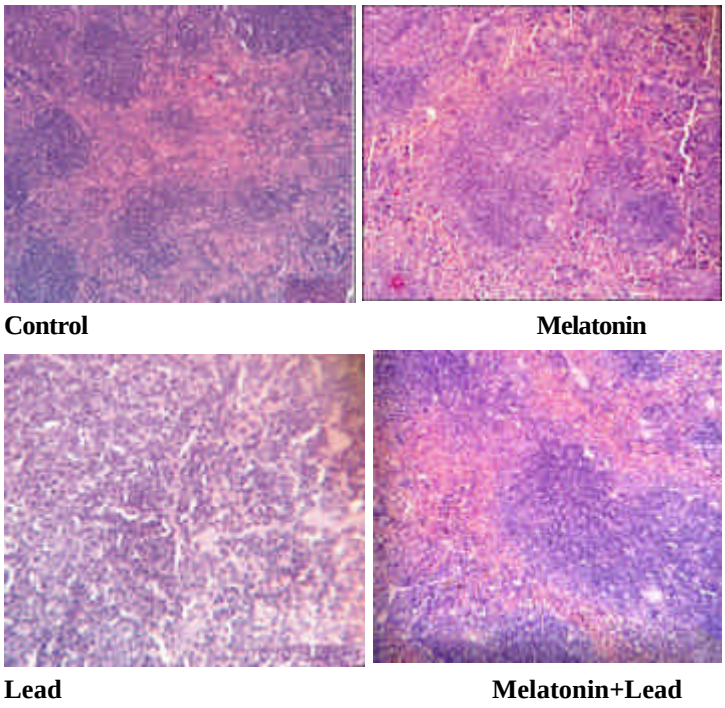
Parameters studied	Control	Melatonin	Lead	Melatonin+Lead
LPO (nmoles of TBARS/mg protein)	0.135±0.068	0.132±0.007	0.201±0.017*	0.138±0.008**
GSH(nmoles/mg protein)	7.05±0.270	7.06±0.243	11.02±0.827*	8.11±0.245**
Cu-Zn SOD activity (units/min/mg protein)	1.412±0.062	1.413±0.057	2.079±0.069*	1.475±0.032**
Catalase activity(µmoles H_2O_2 consumed/min/mg protein)	8.694±0.620	8.592±0.622	10.57±0.734*	8.920±0.806**

Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows. *P<0.001 compared to control; **P< 0.001 compared to lead treated group;

Changes in spleen

Treatment with lead significantly increased the lipid peroxidation level (48.89% higher Vs. control) in the spleen of rats than the control values (*P<0.001). Oral treatment with melatonin alone did not significantly alter the lipid peroxidation level than that of control animals. Pre- treatment of rat with melatonin changed the level of lipid peroxidation (31.34% lower Vs.lead) than that of lead treated group of animals (**P<0.001) [Table 3]. Lead treatment significantly increased the GSH content (56.31% higher Vs. control) in the spleen of rats compared to that control (*P<0.001). Oral treatment of melatonin alone did not significantly alter the level of the GSH. Melatonin with lead changes level of GSH significantly (26.40% higher Vs. lead) than the level of GSH in spleen of lead treated animals (**P<0.001) [Table 3].Lead treatment of rat significantly caused an increase in the SOD activity in the spleen of rats (47.24% higher Vs. control) when compared to that in control group (*P<0.001). Oral treatment of rat with melatonin alone did not significantly alter the SOD activity values versus control. Pre-treatment of rats with melatonin caused a decrease in the activity of SOD (29.05% lower Vs. lead) than in the lead treated groups. (**P<0.001) [Table 3]. Lead treatment significantly increased the Catalase activity in the spleen of rats (21.58% higher Vs. control) compared to that in control (*P<0.001). Oral treatment with Melatonin alone did not significantly alter the CAT activity versus control. Pre-treatment of rats with melatonin increased the CAT activity significantly (15.61% higher Vs lead) than the lead alone treated animals. (**P<0.001) **[Table 3]**.

Histological studies shows marked damage in lead treated spleen of rats. The integrity of the red and white pulp was observed to be distorted in lead treated rats. No such damage was observed in melatonin + lead treated rats. No significant differences were observed in the sections of the splenic tissue of melatonin treated and control group of rats **[Figure 3]**.



Control **Melatonin**

Lead **Melatonin+Lead**

Figure.3. Histopathological studies of splenic tissue [200X]

DISCUSSION

We used young, growing, male, albino rats of Wistar strain for our investigation of lead-induced oxidative stress in stomach, duodenum and spleen and amelioration of the same with melatonin. Melatonin is a potent and popular antioxidant because of its vast, casual and regular presence in different natural foods, and established antioxidant potentiality.[10, 11] Earlier studies showed that lead being a heavy metal, accumulates and subsequently induces oxidative stress in different organs of an organism.[1, 14]

From the results of our study it is seen that intraperitoneal administration of lead to rats caused a significant increase in the level of lipid peroxidation in stomach, duodenum and spleen. Increase in LPO is a well recognised parameter for occurrence of oxidative stress in various models studied till date.[1, 15] Pre-treatment with melatonin at a dose of 10 mg/kg body weight protected the increase in LPO in all the

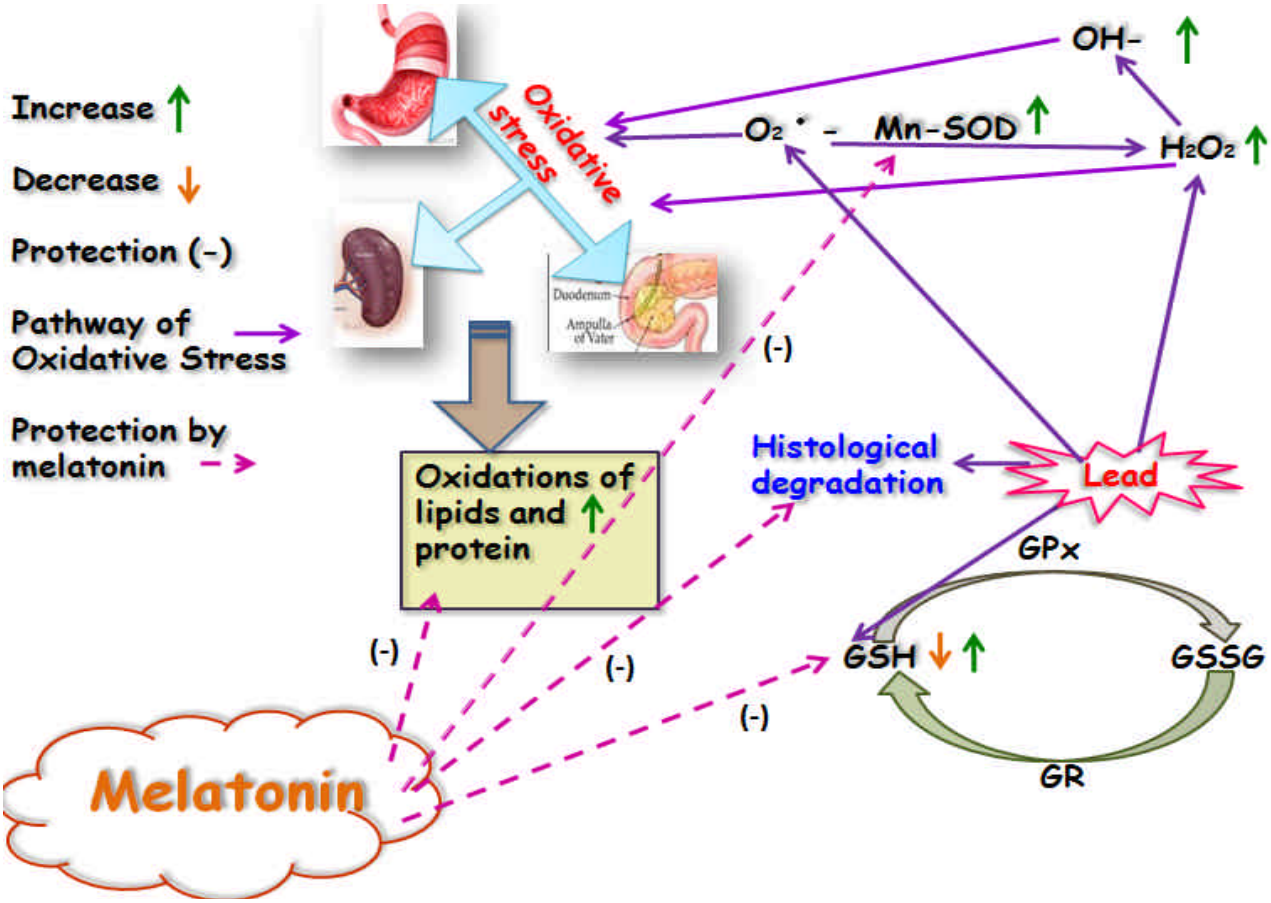
three tissues studied. Only melatonin treated and the control animals showed basal level of lipid peroxidation in the tissues. Earlier also we found a marked increase in level of LPO in various organs in male Wistar rats and amelioration of the same with melatonin.[14, 15]

There was a decrease in the GSH content of stomach and duodenum of lead treated rats. While we observed an increase in the level of GSH in spleen of lead treated rats. There are reports of decreased level of GSH in rat hepatic and cardiac tissues while the animals were treated with heavy metal like cadmium.[21,23] On the other hand it has been observed and reported that lead causes as increase in the level of reduced glutathione *in vivo* and *in vitro* in hepatic as well as cardiac tissues of male rats.[1, 27] Lead induced reduction and depletion in the content of GSH in rat tissues are also there in literature.[28,29] Pre-treatment of the rats with melatonin at a dose of 10 mg/kg body weight protected the level of GSH in all three tissues studied from being altered from the normal level. Only melatonin treated and the control animals showed normal level of GSH in the tissues.

Lead treatment for 7 consecutive days at a dose of 15mg per kg body weight caused a significant increase in the activity of the antioxidant enzymes superoxide dismutase and catalase in stomach, duodenum and spleen of the experimental rats. There has been report of increased activities of these antioxidant enzymes in earlier studies also in situations of lead induced oxidative stress in organs like heart, liver kidneys etc.,[17, 20,30] Melatonin pre-treatment of the rats at a dose of 10 mg/kg body weight maintained the activities of SOD and CAT in all three tissues near normal level. Only melatonin treated and the control animals showed normal activities of SOD and catalase in the tissues.

The histological studies revealed lead-induced oxidative damage in the sections of stomach, duodenum and spleen tissues. The lesser damage in the tissues of the melatonin + lead treated group indicates melatonin's ameliorating role on lead induced oxidative stress mediated damage in the organs.

Our study establishes the fact that melatonin, a very well known ubiquitous antioxidant nutrient has potent ameliorative capability against lead-induced oxidative stress in stomach, duodenum and spleen of experimental rats **[Scheme1]**. Summing up the findings of our present studies with our earlier observations of the protective role of the aqueous solution of melatonin against lead-induced oxidative stress in some of the vital organs, it can be deduced that melatonin possesses a strong protective activity against lead induced oxidative stress situation. The indoleamine acts through its direct as well as indirect antioxidant activity.



Scheme 1. Melatonin protects against lead induced oxidative stress in stomach, duodenum and spleen of experimental rats

ACKNOWLEDGEMENT

Sanatan Mishra is supported from the funds available to Dr. DB from Teacher's Research Grant (BI 92) of University of Calcutta. DG is a Senior Research Fellow (SRF) under INSPIRE program of Department of Science and Technology, Govt. of India. MD is a Woman Scientist under Women Scientists Scheme-A (WOS-A), Department of Science and Technology, Govt. of India. AC is supported from the funds available to her under a UGC Minor Research Grant of Govt. of India. Dr. DB also extends grateful thanks to UGC, Govt. of India, for award of a research project under Centre with Potential for Excellence in a Particular Area (CPEPA) at University of Calcutta

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Source of support: Nil, Conflict of interest: None Declared