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Research Article

BENEFICIAL ROLE OF MELATONIN IN THE COMPLETE RECOVERY FROM ISOPROTERENOL-INDUCED CARDIAC INJURY IN RATS

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ABSTRACT

Objective*:* The objective of the present study was to determine the efficacy of the pineal hormone melatonin in the recovery of rat myocardium after Isoproterenol (ISO)-induced ischemic injury.

Methods*:* Myocardial ischemia was generated by subcutaneous injection of ISO (25mg/kg BW) twice at interval of 24hrs. The rats were pre-treated with melatonin at a dose of 10mg/kg BW intraperitoneally (i.p.) 30mins prior to ISO treatment. In case of the recovery groups, melatonin treatment was continued for 2 and 4 days respectively post ISO treatment. ISO-induced cardiac injury was determined by the histopathological changes of the rat myocardium and LDH1 activity. Induction of oxidative stress was determined by the activities of xanthine oxidase, xanthine dehydrogenase, SOD1 and catalase. Activation of stress and apoptotic pathways by ISO were determined by western blotting, DAPI staining and DNA degradation.

Results*:* ISO induced cardiac injury was evident from increased LDH 1 activity and histopathological studies. Changes in the activity of pro and antioxidant enzymes indicated involvement of oxidative stress. ISO also caused activation of the stress and apoptotic pathways and DNA damage. Pre-treatment of rats with melatonin significantly ameliorated many of the damaging effects of ISO but could not bring them back to control levels. However, continuation of melatonin treatment post ISO for 2 and 4 days, completely brought back all the parameters of cardiac injury to control levels indicating improvement in cardiac function.

Conclusion*:* The present study shows that continuation of melatonin treatment post-ISO is capable of completely ameliorating the ISO-induced ischemic injury pointing toward melatonin's efficacy as a cardioprotective agent.

Keywords: Antioxidant, isoproterenol, Melatonin, Myocardial injury, Oxidative stress, Tissue recovery

INTRODUCTION

Cardiovascular diseases are currently one of the major causes of disability and mortality in both economically well-developed as well as developing countries [1]. Ischemic heart disease (IHD) is considered to be one of the particularly debilitating problems of global concern [2]. Myocardial ischemia or IHD occurs when there is a disparity between the oxygen demand and supply to the myocardium usually due coronary artery disease. A reduction in coronary blood flow due to atherosclerotic plaques or vasospasm can be sufficiently prolonged to result in severe damage to the myocardium which leads to cellular injury and eventually to cellular death due to apoptosis and/ or necrosis. Loss of this vital tissue ultimately leads to compromised cardiac function thereby jeopardizing the overall health of the individual and possibly leading to myocardial infarction or heart attack [3, 4].

Recently, generation of free radicals particularly reactive oxygen species have been implicated in the pathogenesis of various cardiovascular disorders including ischemia/reperfusion (I/R), atherosclerosis, hypertension and cardiotoxicity induced by drugs [1].

Isoproterenol (ISO), a synthetic β-adrenergic agonist, produces pathophysiological changes in the rat heart similar to those seen in humans after myocardial infarction. We have provided evidence that ISO-induced myocardial infarction occurs through the production of reactive oxygen species [4]. We have also shown that the ROS mediated cardiomyocyte damage involves a faltering electron transport chain in mitochondria and activation of stress sensitive signaling pathways that regulate gene expression resulting in cellular damage [5].

Melatonin (N-acetyl-5-methoxytryptamine), a tryptophan derivative secreted by the pineal gland, is a highly evolutionarily conserved molecule – present virtually in all organisms. In addition to its important physiological functions in mammals including seasonal reproductive regulation, immune enhancement, regulation of light–dark signal transduction etc, melatonin has widespread antioxidant actions [6, 7]. In our earlier studies, we have demonstrated that melatonin significantly protects against ISO-induced myocardial infarction through its direct and indirect antioxidant capacity. Our studies also indicated that melatonin played a role in the recovery of the damaged myocardium in the post-ischemic period as opposed to natural healing of the heart.

In these studies, we further elucidate melatonin's role in the recovery process of the ISO-treated ischemic heart after withdrawal of ISO-treatment. Our studies reveal that this low molecular weight natural indole helps the rat heart to recover from ISO administered injury through its direct and indirect antioxidant actions as well as by ameliorating the ISO-induced induction of the stress and apoptotic pathways.

Melatonin is a highly conserved natural molecule, present both in plants and animals including humans and its pharmacological doses have been found to be non-toxic [8]. Understanding its role in ameliorating ischemic damage, particularly in helping the myocardium to recover from an ischemic shock, is interesting since it may have the potential for being used as a future therapeutic against myocardial ischemia.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats, weighing 180–220 g were used for the experiment. They were housed in the departmental animal house with food and water ad libitum. The animals were handled as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. All the experimental protocols had approval of the Institutional Animal Ethics Committee (IAEC) of the Department of Physiology, University of Calcutta. Prof. P. K. Samanta, M. Sc. (Vet.), Ph. D., CPCSEA Nominee to IAEC, Department of Physiology, University of Calcutta, monitored care and experiments on animals.

Drugs and chemicals

Melatonin, isoproterenol bitartrate (ISO), eosin, ethidium bromide, DAPI (4, 6-diamidino-2-phenylindole), nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were obtained from Sigma, St. Louis, MO, USA. Hematoxylin, isopropanol, trichloro acetic acid (TCA), perchloric acid (PCA) were obtained from Merck Limited, Delhi, India. Bovine serum albumin (BSA), agarose, bromophenol blue, glycerol were obtained from Sisco Research Laboratories (SRL), Mumbai, India. Apaf-1 (H 324), pP38 (Tyr 182) and actin (I-19) polyclonal antibodies and donkey anti-goat and anti-mouse IgG conjugated with alkaline phosphatase were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA.

Animal Experimental protocol

Five groups of rats, each comprising of 6 animals, were employed in the present study. The dose selection for isoproterenol-bitartrate (ISO) and melatonin was based on previous dose response studies carried out by us **[**4]. All the groups except the control group were given ISO at a dose of 25mg/kg BW subcutaneously twice at difference of 24hrs, and melatonin at 10mg/kg BW intraperitoneally for different durations. There was no mortality of animals during the treatment period. The animal groups were as follows:

Group I: Control group (C)

The rats of this group comprised of the vehicle (0.9% saline) treated control

Group II: Isoproterenol treated group (I)

The rats of this group were treated with ISO only (25mg/kg BW s.c. twice at an interval of 24hrs). The rats were then euthanized by cervical dislocation 48 hrs after start of experiment, and the hearts collected and stored at -80ºC for further analyses. Blood was also collected for isolation of serum.

Group III: ISO + melatonin protected group (I + m)

The rats were pretreated with melatonin at a dose of 10mg/kg BW i.p. 30mins prior to ISO treatment (two doses 24hrs apart). The rats were euthanized with the ISO treated rats and blood and cardiac tissue collected.

Group IV: Melatonin protected group-2 days (Iw +m 2D)

Rats of this group were treated with initially ISO and melatonin was given prior to ISO treatment for 2 days like the I+m group. ISO treatment was then withdrawn and only melatonin treatment continued for 2 more days. The rats were euthanized five days after start of experiment and blood and tissue collected as before.

Group V: Melatonin protected group- 4 days (Iw + m 4D)

In rats of this group, melatonin treatment was continued for 4 days after withdrawal of ISO treatment after 2days. Rats were euthanized seven days after the start of experiment and blood and tissue collected as before.

Measurement of serum LDH1 levels

The cardiac specific Type 1 isoform of lactate dehydrogenase (LDH1) was obtained according to the method of Varcoe et al. [9] by incubating the serum samples at 65°C for 30mins, which destroys all isoforms except LDH1 . The enzyme activity was then obtained by measuring the oxidation of NADH (0.1mM) to NAD^+ at 340nm using 1.0mM sodium pyruvate as the substrate according to the method of Strittmatter [10]. Values were expressed as Units/ml.

Measurement of superoxide dismutase (SOD) and catalase (CAT) activities

The activity of copper-zinc superoxide dismutase [Cu-Zn-SOD (SOD1)], an important cytosolic antioxidant enzyme, was measured by hematoxylin autooxidation method of Martin et al. [11]. Inhibition of hematoxylin autooxidation by the cell free supernatant was measured at 560nm using a UV-VIS spectrophotometer. The enzyme activity was expressed as Units per mg of tissue protein.

Catalase was assayed by the method of Beers and Sizer [12] with some modifications as adopted by Chattopadhyay et al. [13]. The cell free supernatant in 50mM phosphate buffer, pH 7.0, was incubated with 0.01ml of absolute ethanol at 4°C for 30 min, after which 10% Triton X-100 was added. The enzyme preparation, thus obtained, was used to determine catalase activity by measuring the breakdown of H_2O_2 spectrophotometrically at 240nm. The enzyme activity was expressed as μmoles H_2O_2 consumed / mg of tissue protein.

Assessment of superoxide anion free radical (O_2^-) generation by the xanthine oxidase/xanthine dehydrogenase system

Xanthine oxidase (XO) was assayed by measuring the conversion of xanthine to uric acid following the method of Greenlee and Handler [14] with some modifications by Mukherjee et al. [4]. The cell free supernatantwas used for spectrophotometric assay at 295nm using 0.1mM xanthine in 50mM phosphate buffer, pH 7.8, as the substrate. The enzyme activity was expressed as milli Units / mg protein.

Xanthine dehydrogenase (XDH) was assayed by following the reduction of NAD^+ to NADH according to the method of Strittmatter [10] with some modifications [4]. Cell free supernatant was used for enzyme assay using a UV/VIS spectrophotometer at 340nm with 0.3mM xanthine as the substrate (in 50mM phosphate buffer, pH 7.5) and 0.7mM NAD^+ as an electron donor. The enzyme activity was expressed as milli Units / mg protein.

Western Blot Analysis

The whole homogenate from left ventricular tissues was prepared as described earlier [5] using lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1mM sodium orthovanadate, 1 μg/ml each of pepstatin A, leupeptin, and aprotinin. Protein 35 μg for pP38 and 50 μg for Apaf 1 and actin were loaded and resolved in 10% SDS-PAGE for immunodetection.The proteins were transferred to nitrocellulose membranes in an electroblotting apparatus (Mini Trans-Blot, Bio-Rad). The membranes were blocked using 10% non-fat dried milk for 2hrs at room temperature then washed and incubated overnight with the respective primary antibody (1: 2000 dilution). The blots were then washed and incubated for 2hrs with secondary antibody at room temperature. The immunoreactive bands were detected with alkaline phosphatase buffer (pH 9.5) in the presence of NBT and BCIP in the ratio of 2:1. The pixel density of the bands obtained was quantified using ImajeJ software (NIH, Bethesda, MD, USA)

Measurement of total DNA and RNA content of rat cardiac tissue

Total DNA and RNA content of the cardiac tissue were determined using the method of 16Schmidt and Thannhauser with some modifications [15]. AA 10% homogenate of the rat cardiac tissue in 0.25M sucrose was treated with 10% trichloro acetic acid (TCA) and centrifuged at 4°C, 2000rpm for 10mins. The supernatant was discarded and the pellet suspended in 5% TCA. It was then centrifuged again for 5mins (2000rpm, 4°C). The pellet, thus obtained, was re-suspended in cold 8% perchloric acid (PCA) and centrifuged (2000 rpm at 4°C for 5mins). The supernatant was discarded and pellet suspended in cold 2% PCA, and, centrifuged again. The residue obtained was re-suspended in distilled water and 3N NaOH was added to it and mixed well. It was then incubated at 37°C for 1hr. Then, ice-cold 20% PCA was added to it and kept in cold for 10mins. The mixture was then centrifuged in cold at 2500 rpm for 10mins. The supernatant, thus obtained, contained the RNA fraction. The total RNA concentration was determined by recording the absorbance at 270nm and 290nm using a UV-VIS spectrophotometer against distilled water as the blank.

The residue left after decanting the RNA fraction was suspended in cold 2% PCA and centrifuged at 3000rpm in cold for 10mins. The supernatant was discarded and pellet re-suspended in cold 8% PCA. It was incubated at 70°C for 45mins, then cooled to room temperature. The mixture was then centrifuged at 3000rpm at 4°C for 15mins and the supernatant collected. This forms the DNA fraction whose concentration was determined by recording the absorbance at 270nm and 290nm using a UV-VIS spectrophotometer with 8% PCA as blank.

Determination of DNA degradation

Rat cardiac tissue genomic DNA was extracted according to the method of Wu et al. [16] with some modifications. Briefly, tissue samples suspended in phosphate buffered saline (PBS) were dispersed into small pieces and single cells by passing multiple times through a 3 ml syringe. After allowing the large debris to settle for 1 min, the cell suspension was transferred to a 5 ml culture tube and centrifuged at 1200 rpm for 7 mins at 4ºC.The pellet was suspended in lysis buffer (400 mM NaCl, 100 mM TRIS-HCl, pH: 8.5, 5 mM EDTA, 0.2 % SDS, 20 µg/ ml RNAse A, 500 µg/ µl Proteinase K). 3 ml lysate was placed in 100mm petridishes. The petridishes were incubated overnight at 37ºC. After digestion, an equal volume of isopropanol was added to each plate and mixed by gentle rocking. The dishes were left undisturbed at room temperature for atleast 2 hrs until the white DNA precipitate could be seen adhering to the bottom of the dish. The supernatant was poured off gently and the DNA washed twice with ice-cold 70 % ethanol. After washing, the DNA was air-dried for 10 mins then dissolved in appropriate amount of Tris-EDTA (TE) buffer by incubating at 4ºC overnight. The DNA thus obtained gave an average 260/280 absorbance ratio of 2-2.5. The obtained DNA samples were then mixed with 6X loading dye and resolved in an 0.8% agarose gel. The gel was stained with ethidium bromide and DNA bands detected in a Gel-Doc apparatus (Biorad, Hercules CA).

Estimation of proteins

Proteins of the different samples were determined by the method of Lowry et al. [17].

Histological studies

The extirpated hearts were fixed immediately in 10% formalin and embedded in paraffin following routine procedure [18]. Left Ventricular (LV) sections (5 µm thick) were prepared and stained with hematoxylin–eosin (H-E). The tissue sections were examined under an Olympus BX51 (Olympus Corporation, Tokyo, Japan) microscope and images were captured with a digital camera attached to it.

Determination of apoptosis using immunoflourescence microscopy

Formalin fixed paraffin embedded 5uM thick left ventricular sections were processed according to routine procedure. They were then equilibrated to pH 7.0 using a neutral pH buffer. The tissue sections were then stained with DAPI stain according to the method of [19]. The tissue sections were examined under an Olympus BX51 (Olympus Corporation, Tokyo, Japan) immunoflourescence microscope and images were captured with a digital camera attached to it. When stained with DAPI, the DNA appears as blue-white fluorescence under ultraviolet (UV) illumination. The digitized images were analyzed using ImageJ analysis system (ImageJ, NIH software) and total fluorescence of each image measured and expressed as mean fluorescence intensity (MFI).

Statistical evaluation

Each experiment was repeated at least three times with different rats. Data are presented as mean ± S.E.M. Significance was calculated using one-way ANOVA followed by Tukey's multiple comparison test. $P < 0.001$ was taken as significant.

RESULT

Isoproterenol-induced cardiomyopathy and protection by melatonin

Isoproterenol at the dose of 25mg/kg BW s.c. for 2 days caused myocardial infarction as is evident from the distinct ischemic patch visible in the ISO treated rat heart in figure 1.

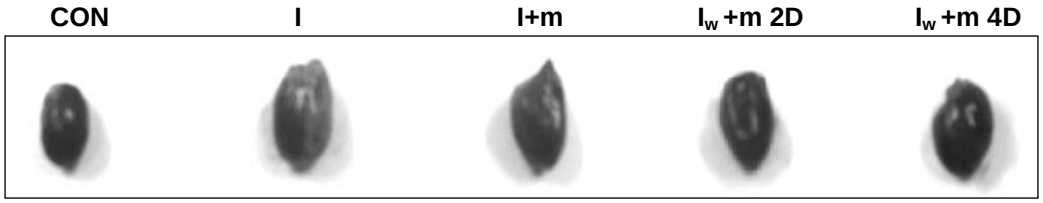


Figure 1

Fig. 1: Representative image showing morphology of rat hearts after treatment with ISO (I) and melatonin (I+m). Melatonin treatment was continued for 2days (I_w+m 2D) and 4days (I_w+m 4D) after discontinuation of ISO treatment. The control (CON) rats were treated with vehicle only.

There was significant enlargement of the heart due to ISO treatment as also evidenced by increases HW/BW ratio (Table 1, *P< 0.001 vs C). Pre-treatment of rats with melatonin reduced the infarct size but was unable to significantly decrease the ISO induced enlargement of the rat heart. Continuation of melatonin treatment for 2 and 4 days post ISO-treatment however, completely ameliorated the ISO-induced cardiac hypertrophy and infarction with maximum effect seen at 4 days post ISO-treatment. The hematoxylin-eosin stained left ventricular sections (Fig. 2) showed considerable damage to the myocardial tissue architecture with ISO treatment leading to myofibril degeneration and mononuclear cell infiltration.

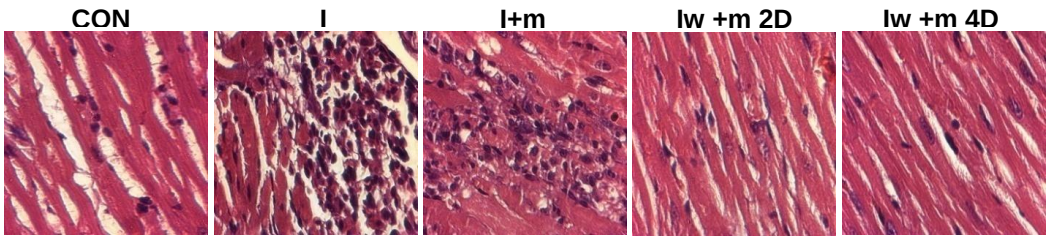


Fig. 2: Representative images (400X magnification) of haematoxylin-eosin stained left ventricular longitudinal sections of rat hearts of Control (CON) ISO (I) treated and Melatonin (m) protected, pre (I+m) and post (I_w+m 2D, I_w+m 4D) ISO treatment.

Pre-treatment of rats with melatonin significantly lowered the ISO-induced changes to the myocardial tissue. However, the recovery from tissue injury was found to be complete when melatonin treatment was continued for another 2 and 4 days after withdrawal of ISO-treatment (Fig 2).

ISO-induced cardiac injury was also evident from the increased activity of the diagnostic enzyme for myocardial damage, the lactate dehydrogenase type1 (LDH1). Melatonin time-dependently reduced the activity of this enzyme to control level after 4 days (Iw+m 4D) (Fig.3; ^P< 0.001 vs I and I+m).

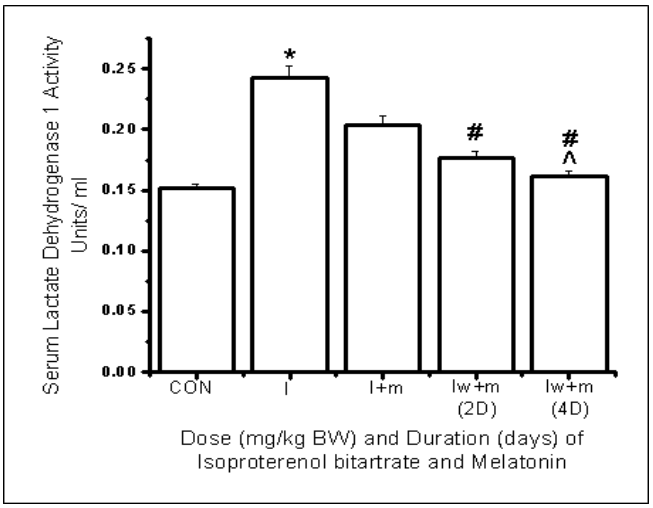


Fig. 3: Protective effect of melatonin against ISO-induced elevation of cardiac specific LDH 1 activity. Values are means ± S.E.M. of six rats in each group; *P < 0.001 vs CON; #P < 0.001 vs I; ^P < 0.001 vs I+m.

Isoproterenol-induced generation of superoxide anion free radical ($O_2^{\bullet -}$) in vivo and protection by melatonin

As evident from table 2, the activities of xanthine oxidase (XO) and xanthine dehydrogenase (XD), XO/XD ratio, the total enzyme activity i.e. XO plus XD and XO/ XO+XD ratio all increased significantly following ISO treatment of rats indicating enhancement in the generation of superoxide anion free radical *in vivo*. All these parameters were time-dependently restored to control levels when the rats were pre-treated with melatonin and then melatonin treatment was continued even after withdrawal of ISO treatment after 48 hrs.

Effect of ISO treatment on cardiac antioxidant enzymes and protection by melatonin-a time-dependant study

Figure 4 reveals that ISO treatment significantly increased the activity of Cu-Zn superoxide dismutase (SOD 1) and decreased the activity of catalase of the cardiac tissue of rats.

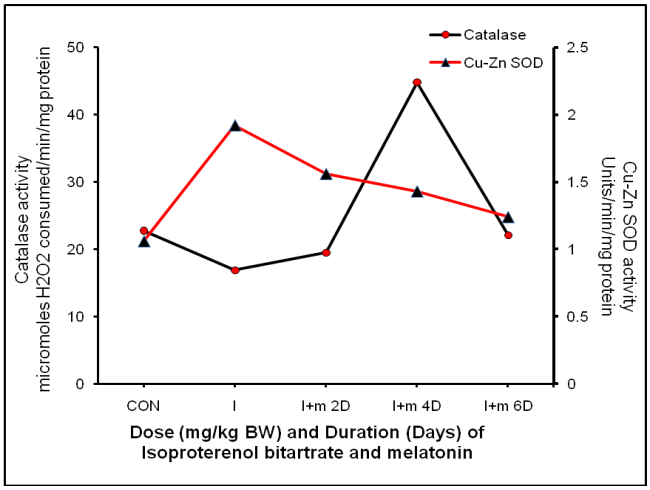


Fig. 4: Protective effect of melatonin against ISO-induced decrease in catalase (primary axis) and increase in Cu-Zn SOD (SOD 1 (secondary axis) activity of rat heart tissue. The rats were treated with ISO (I) at a dose of 25 mg/kg body weight s.c. Melatonin (m) protected rats were treated with 10 mg/kg body weight i.p. 30 min before ISO-treatment, and post ISO treatment period of 2 and 4 days. The control (CON) rats were treated with vehicle only. Values are means ± S.E.M. of six rats in each group; *P < 0.001 vs CON; #P < 0.001 vs I; ^P < 0.001 vs I+m.

This enhancement of SOD 1 activity following ISO treatment was significantly lowered when the rats were pre-treated with melatonin for 2 days. A further reduction in the activity of the enzyme was also observed when melatonin treatment was continued for a period of 2 (Iw+m 2D) and 4 (Iw+m 4D) more days after ISO withdrawal (^P< 0.001 vs I and I+m).

Similarly, pre-treatment of rats with melatonin caused a significant (#P< 0.01 vs I) elevation in catalase activity compared to ISO-treated rats. Interestingly, continuation of melatonin treatment for 2 more days after withdrawal of ISO treatment (Iw+m 2D) caused a more than two fold increase in catalase activity compared to the activity observed in the control rats (P < 0.001 vs C). This elevation of the enzyme activity following withdrawal of the ISO treatment, however, was brought down to the activity observed in the control animals when the rats were continued to be treated with melatonin for another 2 days (i.e., 4 days from ISO withdrawal- Iw+m 4D).

ISO-induced activation of stress proteins- amelioration by melatonin

Figure 5A shows ISO-induced elevation in the levels of apoptotic protease activating factor (Apaf)-1, a protein involved in the activation of the mitochondria related intrinsic apoptotic pathway of the cell. Melatonin pre-treatment for 2 days caused significant decrease in the level of Apaf 1 (P< 0.001 vs I). However, continuation of melatonin treatment lowered the level of this stress protein further with maximum protection seen after 4 days of melatonin treatment (Iw+m 4D; ^P< 0.001 vs I and I+m).

Figure 5B shows activation of p38 MAP kinase protein by ISO treatment (*P< 0.001 vs C. Melatonin time- dependently lowered p38 expression to control levels with Iw +m 4D values being even lower than those of the control animals.

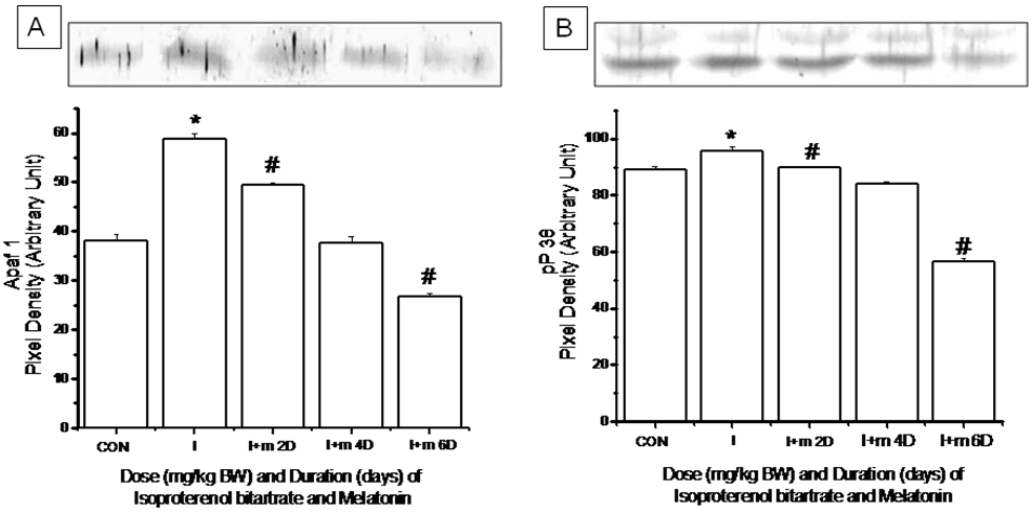
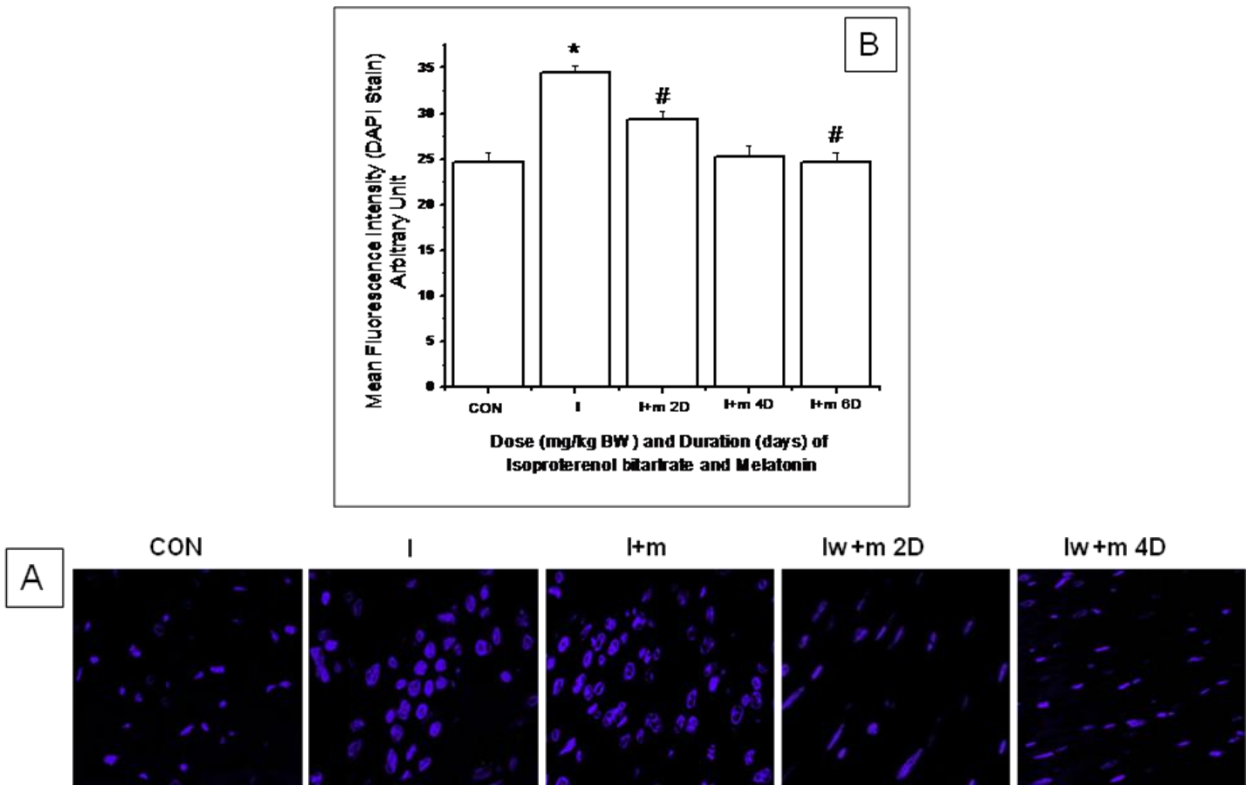


Fig. 5: Western blot analysis of (A) phosphorylated p38 and (B) apoptotic protease activating factor (Apaf)-1 of heart tissue in control (CON), ISO (I) treated and melatonin (m) protected hearts pre (I+m) and post (Iw+m 2D, Iw+m 4D) ISO treatment. The western blot analyses were repeated atleast 3 times. Actin served as loading control. The pixel density of the bands obtained through western blotting was quantified with imageJ software (NIH) and the values (means ± S.E.M) are presented below the blots in the form of bar graphs. *P < 0.001 vs CON; #P < 0.001 vs I; ^P < 0.001 vs I+m.

Isoproterenol-induced changes in rat cardiac tissue total DNA and RNA levels and induction of nuclear apoptosis: protective role of melatonin

Figure 6A and 6B shows the ISO-induced induction of nuclear apoptosis through the DAPI stained cardiac sections. ISO treatment caused an increase in the number of mitotic nuclei as evident from the increased mean fluorescence intensity. Pre-treatment of rats with melatonin for only 2 days could not completely restore the apoptotic changes brought about by ISO to the nuclei of the myocardial cells. But when the treatment of rats with melatonin was continued even after withdrawal of ISO treatment, the structure of the cardiac cell nuclei were restored to that comparable with the structure of the nuclei of the cardiac cells of the control rats. Figure 6C shows ISO-induced DNA fragmentation by agarose gel electrophoresis. Melatonin pretreatment and continuation prevented DNA fragmentation time dependently.



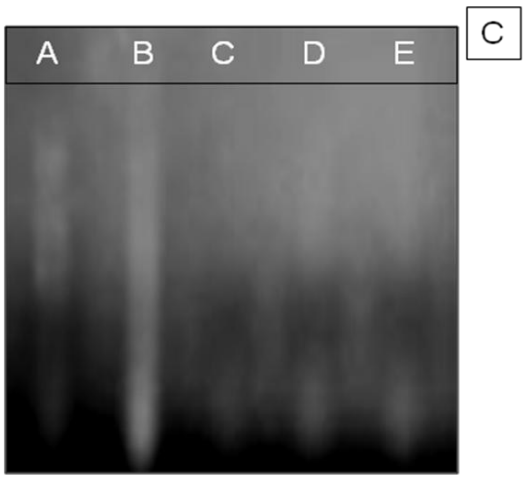


Fig. 6: (A) Representative images (600X magnification) of DAPI (4, 6-diamidino-2-phenylindole) stained sections of left ventricular longitudinal sections of rat hearts of Control (CON), ISO (I) treated and Melatonin (m) protected, pre (I+m) and post (Iw+m 2D, Iw+m 4D) ISO treatment. The images were captured by confocal laser scanning microscope. (B) Histogram showing mean fluorescence intensity (MFI) of the DAPI stained ventricular tissue sections. Values are means ± S.E.M. *P < 0.001 vs CON; #P < 0.001 vs I; ^P < 0.001 vs I+m. (C) Analysis of DNA degradation using agarose gel electrophoresis of genomic DNA from ventricular tissue of rat hearts. From left, control (A), ISO-treated (B) and melatonin pretreated (C) and post treated for 2days (D) and 4 days (E). The extraction of genomic DNA and agarose gel electrophoresis was repeated at least 3 times and best representative blot given.

We also studied the effect of ISO treatment on total DNA and RNA levels of the cardiac cells. As is evident from Fig. 7A and B, treatment of rats with ISO caused a significant increase in the total DNA and RNA content of the cardiac cells. Pre-treatment of rats with melatonin was unable to reduce the ISO-induced increase in DNA and RNA levels. However, on continuation of melatonin treatment for another 2 and 4 days after withdrawal of ISO treatment, the total DNA and RNA levels were restored to control levels (*P < 0.001 vs I).

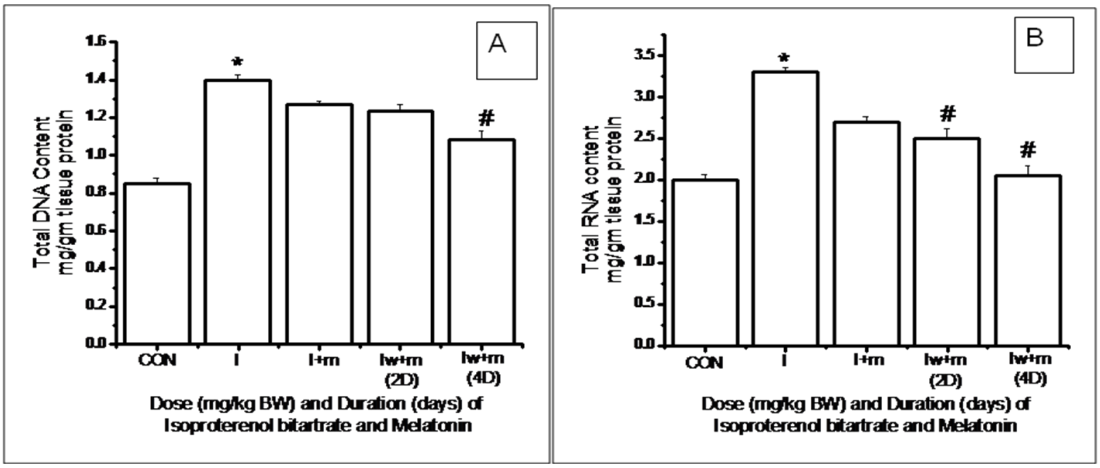


Fig. 7: Protective effect of melatonin against ISO-induced increase in the (A) Total DNA content and (B) Total RNA content. The rats were treated with ISO (I) at a dose of 25 mg/kg body weight s.c. Melatonin (m) protected rats were treated with 10 mg/kg body weight i.p. 30 min before ISO-treatment, and post ISO treatment period. The control (CON) rats were treated with vehicle only. Values are means ± S.E.M. of six rats in each group; *P < 0.001 vs CON; #P < 0.001 vs I; ^P < 0.001 vs I+m.

Table 1: Isoproterenol-induced cardiac hypertrophy is inhibited by melatonin

Parameters studied	Experimental Groups				
	CON	I	I+m	Iw+m 2D	Iw+m 4D
HW	620 ± 18.6	813 ± 28.0*	774 ± 19.3	741 ± 16.8	662 ± 10.5#
BW	229 ± 9.4	223 ± 9.3	220 ± 8.9	224 ± 7.1	217 ± 7.7
HW/BW (mg/g)	2.71± 0.06	3.83 ± 0.06*	3.53 ± 0.03	3.29 ± 0.05#	3.05± 0.04^#

*P< 0.001 vs control, #P < 0.001 versus I, ^P < 0.001 versus I+m; n=6.

Table 2: Isoproterenol-induced generation of superoxide anion free radical ($O_2^{\cdot}$) *in vivo* and protection by melatonin

Parameters studied	Experimental groups				
	CON	I	I+m	Iw+m 2D	Iw+m 4D
XO	1.12 ± 0.07	4.70 ± 0.03*	1.48 ± 0.04#	1.21 ± 0.05#	1.04 ± 0.02#^
XDH	5.69 ± 0.35	9.38 ± 0.38*	5.62 ± 0.08#	5.24 ± 0.14#	5.12 ± 0.16#^
XO+XDH	6.81 ± 0.30	14.08 ± 0.7*	7.08 ± 0.10#	6.43 ± 0.20#	6.16 ± 0.20#^
XO/XDH	0.22 ± 0.010	0.53 ± 0.010*	0.26 ± 0.01#	0.225 ± 0.01#	0.20 ± 0.01#^
XO/XO+XDH	0.17 ± 0.006	0.32 ± 0.016*	0.21 ± 0.006#	0.19 ± 0.004#	0.17 ± 0.01#^

*P< 0.001 vs control, #P < 0.001 versus I, ^P < 0.001 versus I+m; n=6.

DISCUSSION

The studies on the cardio-protective ability of melatonin in animal models as well as in humans have been the subject of intense research during the last decade [20, 21]. The usefulness of melatonin as a cardio-protective antioxidant against various experimental and clinical cardiac situations is getting increasing appreciation.

In our earlier study [4], we demonstrated that ISO caused myocardial ischemia (at a dose of 25mg/ kg BW s.c.) via the induction of oxidative stress. ISO-induced oxidative stress was both by direct generation of ROS as well as by inhibiting the antioxidant defense mechanisms of the myocardial cells. Melatonin (at the dose of 10mg/kg BW i.p.) was capable of ameliorating the ISO-induced stress [4]. ISO-treatment also disturbed the mitochondrial TCA cycle and respiratory chain functions leading to activation of both the mitochondrial and extra mitochondrial stress signaling pathways finally converging on apoptosis. It was found that melatonin treatment, when given simultaneously with ISO for 2 days, was unable to restore cardiac histopathology and heart function and few of the key stress signaling proteins to the levels seen in control animals, thus, raising the possibility of testing a higher dose of melatonin or a longer duration of treatment with melatonin. In our earlier study [5] we have demonstrated that continuation of melatonin treatment significantly improved the status of the myocardium as compared to natural healing of the rat heart in which case hardly any difference was found from the ISO only treated rats. In this present study, we further elucidate this mechanism of recovery by melatonin after inducing ischemia. Herein we demonstrate that melatonin improves cardiac physiology of ISO-treated rats and helps in the complete myocardial recovery mainly through its antioxidant ability as well as by repressing the activation of the stress mediated and apoptotic signaling pathways of the cell.

Photographic images of the rat hearts indicate the development of large infarcts in the ISO treated hearts. This could be due to the induction of apoptosis in the cardiac cells by ISO-treatment which lead to cell death and necrosis. Other studies have also shown an extensive cardiac remodeling and hypertrophy due to induction of oxidative stress [22]. Melatonin pre-treatment at 10mg/kg BW was able to ameliorate the myocardial infarction, but could not significantly reduce the cardiac enlargement. Continuation of melatonin treatment, however, was able to restore the cardiac size to that seen in control animals. Melatonin's ability to reduce cardiac hypertrophy has been shown earlier [18, 22, 23]. However, the details of the mechanism remain to be elucidated.

A significant increase in the activity of the diagnostic enzyme for myocardial injury, lactate dehydrogenase type 1 (LDH 1) (Fig. 3) indicated the induction of myocardial injury. Increase in LDH 1 activity level in serum occurs when the enzyme leaks into the surrounding fluid from damaged cardiac cells. Pre-treatment of the rats with melatonin could not significantly ameliorate this effect. However, continuation of melatonin treatment for 2 (Iw+m 2D) and 4 (Iw+m 4D) more days respectively after withdrawal of ISO restored the activity of this enzyme completely to that seen in control animals indicating melatonin's ability to repair the cardiac tissue. The improvement of cardiac status was also evident from the histopathological studies of the myocardial tissue. The haematoxylin-eosin stained cardiac tissue sections showed significant cellular damage and degeneration following ISO treatment. Pre-treatment of rats with melatonin for only 2 days was unable to restore the cardiac tissue architecture. Continuation of melatonin treatment for another 2 and 4 days however, restored the cardiac tissue morphology in a time-dependant manner. Earlier studies done on spinal cord trauma have shown melatonin to play a role in tissue recovery [24].

The studies showed that ISO caused cardiac damage through the induction of oxidative stress as evident from the ISO-induced generation of $O_2\bullet^-$ anion radical by the activation of the xanthine oxidase (XO)- xanthine dehydrogenase (XDH) pro-oxidant enzyme system (Table 2). Our earlier studies have also shown ISO-induced elevation of $O_2\bullet^-$ level [4].

The increase in the activity of the $O_2\bullet^-$ scavenging enzyme Cu-Zn superoxide dismutase may be a compensatory reaction to increased $O_2\bullet^-$. Many studies indicate over expression of various SODs which confers significant protection against ischemia-reperfusion injury [25]. This increment in activity was found to be partially but significantly restored when the rats were pre-treated with melatonin for 2 days. However, continued melatonin treatment in rats for another 2 and 4 days after discontinuation of the ISO-treatment gradually and significantly restored the SOD 1 activity to control levels indicating again a role of melatonin in the recovery process either by directly scavenging the $O_2\bullet^-$ produced, or by inhibiting the XO-XDH enzyme system or both as also shown by other studies [26]. When $O_2\bullet^-$ levels are high, several enzymes vital to cardiac functions are vulnerable to inactivation by this radical. The decrease in catalase activity after ISO-administration in rats may be due to excessive generation of $O_2\bullet^-$ leading to inactivation of the enzyme [27]. In our present studies, pre-treatment of rats with melatonin for two days significantly restored the catalase activity to almost near control levels. A significant surge in catalase activity was observed when melatonin treatment was continued for 2 more days after discontinuation of ISO-treatment which was found to return to the activity observed in the control rats on continuing melatonin treatment (Iw+m 4D). Melatonin's ability to enhance the synthesis and activity of peroxidase enzymes including catalase have been shown by earlier workers [28, 29, 30]. This surge in catalase activity on melatonin treatment may be due to an adaptive response of the cardiac cells to increased ROS like H_2O_2. Due to the removal of ISO stress after 2 days, the level of accumulated H_2O_2 would have decreased by the 2nd day post-ISO. The melatonin induced activation of the enzyme may have persisted as an adaptive response. However, further studies are required for proper elucidation.

An increase in cytosolic SOD1 levels and decrease in catalase levels indicates a possible accumulation of H_2O_2. H_2O_2 may lead to the generation of free $\bullet$OH radical through the Fenton or Haber-Weis pathways. Nearly, a six-fold rise in endogenous generation of $\bullet$OH following treatment of rats with ISO and amelioration by melatonin have been demonstrated recently [4]. Melatonin's ability to scavenge free $\bullet$OH *in vivo* has also been shown by earlier workers in other models of oxidative stress [31, 2, 32].

Generation of $\bullet$OH may cause DNA damage and apoptosis. Our studies indicate an increase in the levels of stress and apoptotic signal proteins p38 and apaf 1 respectively (Fig 5A and 5B), as well as total DNA and RNA content of the cardiac cells (Fig 7A and 7B), with a concomitant increase in DNA degradation (Fig 6C). ISO-induced myocardial infarction was associated with an increase in the total number of nuclei as was evident from the DAPI stained sections of the myocardial tissue studied through confocal microscopy (Fig 6A and 6B). Cardiac myocytes are terminally differentiated cells. However, DNA synthesis in adult cardiac myocytes after myocardial infarction in rats [33] as well as in humans [34] was described. Further on, observations in humans have provided strong supportive evidence that myocyte cellular hyperplasia [23, 35] and polyploidization may occur under pathological conditions characterized by large and prolonged stress on the myocardium [36]. However, the perennial issue of whether DNA replication leads exclusively to the formation of ploidy or to nuclear mitotic division has been a matter of controversy [34]. Our studies indicate that the activation of DNA synthesis in myocytes might be involved in the myocardial response after infarction as evident from increased MFI of the DAPI stained cells. In our studies, pre-treatment of rats with melatonin for 2 days, was incapable of significantly lowering the total DNA and RNA levels or decrease the number of myocyte nuclei. Similar results were obtained by other workers in case of myocardial infarction as well as coronary artery narrowing induced cardiomyopathy in rats [37, 38]. However, continued melatonin treatment could reduce the total number of nuclei and the total DNA and RNA levels to that observed in control rats indicating again at the role of melatonin in the cardiac recovery process after ischemia.

These observations suggest that melatonin could have a potential clinical application in the treatment of myocardial ischemia, even if the mechanisms(s) underlying this protection remain to be determined [39].

CONCLUSION

The results of the current studies clearly indicate that melatonin not only has the ability to protect the heart against ischemic stress but may also play a critical role in the improvement and maintenance of normal cardiac function even after ischemic episode. This small indole and several of its metabolites are excellent antioxidants [40, 41]. They also reduce the toxicity of different drugs [38, 42]. Moreover, pharmacological doses of melatonin do possess very low or no toxicity [39]. Therefore, it will be worth investigating whether melatonin can be used along with other cardio-protective drugs [43, 44, 45] as a co-therapeutic in the treatment of ischemic heart disease. The available information to date suggests that melatonin may be an ideal candidate for thorough investigation with respect of its cardio-protective ability.

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