

Research Article

Melatonin protects against cardiac damage induced by a combination of high fat diet and isoproterenol exacerbated oxidative stress in male Wistar rats

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Running title: Protective role of melatonin against cardiac injury induced by oxidative stress.

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ABSTRACT

In the current study, it was found that high fat diet (60% of total kCal) (H) or/and isoproterenol (I) exacerbated oxidative stress and caused myocardial damage. This was indicated by increased levels of LPO, PCO, abnormal mitochondria and altered activities of metabolic as well as antioxidant enzymes in myocardium of rats. Melatonin at different doses (10, 20 and 40 mg/kg) effectively protected against myocardial damage induced by H or/and I and preserved all of these altered parameters. Morphological analyses showed that combination of H and I treatment led to the extensive myofibril disintegration and neutrophil infiltration. Melatonin at the dose of 40 mg/kg almost completely prevented these pathological alterations. The mechanistical studies have uncovered that the protective effects of melatonin on the myocardial damage induced by H and I are attributed to its direct and indirect antioxidative capacity, i.e., it directly scavenges free radicals and also regulates the gene expression of antioxidant enzymes. Collectively, based on the evidences gathered from the current study, it will not be unwise to suggest that melatonin can serve as an ideal therapeutic agent for those cardiovascular diseases caused by oxidative stress.

Key words: antioxidant, high fat diet, isoproterenol, heart, oxidative stress, melatonin.

1. INTRODUCTION

Melatonin, a widely used antioxidant, previously thought to be secreted only from pineal gland in mammals; however, currently, melatonin is found to be synthesized in retina, skin, gastrointestinal tract, kidneys, liver, pancreas, bone marrow, Harderian gland, etc. (1). Interestingly, melatonin is present in many food stuffs and edible plants. Consumption of these food stuffs and plant products significantly elevated the blood melatonin levels in animals and humans (2). Melatonin plays a remarkable role in the synchronisation of circadian rhythm, regulation of seasonal reproduction and enhancement of immune function in vertebrates (3). It

is also a potent and broad spectrum antioxidant (3–5). Melatonin executes its antioxidant activities by up-regulation of the antioxidant enzymes, down-regulation of the pro-oxidant enzymes and directly detoxification of the free radicals (6, 7). Melatonin scavenges a wide range of reactive oxygen species (ROS) and reactive nitrogen species (RNS) via mechanisms involving electron transferring, hydrogen donation, metal chelation and radical adduct, etc. (8, 9). ROS are continuously generated in the living organisms as a result of different physiopathological processes. It requires antioxidant defence mechanism to maintain the particular redox state by quenching and neutralising ROS (10). When this balance is disturbed the excess amount of ROS due to its high reactivity readily react and damage different cellular components including proteins and lipids (11). Such condition is referred as oxidative stress. For example, ROS initiates chain reactions in phospholipids or polyunsaturated fatty acids in the cell membranes, thereby, produces lipid peroxy radicals and lipid hydroperoxides (12). Thus, this chain reaction exhibits unequivocal detrimental effects on cells, tissues and organs. Oxidative stress plays pivotal role in the etiology of many chronic illnesses including neurodegenerative diseases, diabetes, cardiovascular diseases, etc. (13–15). Uncontrolled oxidative stress also results in inflammatory reactions along with the LDL oxidation and ultimately led to form macrophage derived foam cells. These cells start differentiation and proliferation in smooth muscle cells and result in vascular wall injury. The injured extracellular matrix is vulnerable to free radical attack and fastens the plaque rupture of the vessels. It was reported that the advanced oxidative products of protein were closely associated with the development of coronary artery diseases (CAD) (16). Endothelial dysfunction is one of the prime causes along with atherosclerosis for the development of CAD (17). High fat diet potentiates the development of atherosclerosis by not only depositing cholesterol but also causes injury to endothelium by enhancing the production of free radicals and oxidised LDL (18). It was observed that when high fat diet combines with certain pharmacological agents, this combination significantly promotes the severity of CAD in any individual (19). Isoproterenol (I) is one of these agents.

Isoproterenol is clinically used to treat diverse pathological conditions including asthma and bradycardia (20); however, it also cause different cardiac complications. I treatment elevates systolic blood pressure through its cardiac inotropic and chronotropic effects; whereas it is a potent vasodilator and it decreases the diastolic pressure. In general, it elevates the heart rate and cardiac output (21). Palpitations, sinus tachycardia, angina and severe arrhythmias are the potential consequences of I administration. The myocardial damage by I, involves multiple mechanisms. These include increased oxygen consumption, calcium overload, cytosolic cAMP, electrolyte imbalance, altered membrane permeability and cardiac metabolism. All of these finally lead to myocardial oxidative stress and injury (22). I can initiate a cascade reaction which produces quinone and generates superoxide anion and hydrogen peroxide. Superoxide anion promotes release of iron from its storage and produces hydrogen peroxide as a byproduct. Iron along with hydrogen peroxide produce hydroxyl radical through Fenton's reaction (23). The hydroxyl radical is the major culprit of the myocardial injury caused by I (22, 23).

Therefore, in the current study, high fat diet and I were used to induce oxidative myocardial damage to mimic the condition where myocardial infarction may occur during short period of high fat consumption. Melatonin is selected to test whether this potent antioxidant provide protective effects on the myocardial damage induced by the combination of high fat diet and I.

2. MATERIALS AND METHODS

2.1. Reagents.

The primary antibodies against CuZnSOD (SOD1; ab13534), MnSOD (SOD2; ab13534), catalase (ab16731), glutathione peroxidase 1 (GPx 1; ab108427), glutathione reductase (GR;

ab124995) and secondary antibody (ab97048) were purchased from Abcam Biotechnology Company, USA. TNF α , IL-1 β , IL-6, IL-10 and adiponectin ELISA kits were purchased from Ray Biotech, Inc., USA. Isoproterenol bitartrate, oxidised glutathione (GSSG), GR, DL- β -hydroxy butyrate and few other chemicals were purchased from Sigma Aldrich, USA. Melatonin, trichloro acetic acid, 5, 5'-dithiobis-2-nitrobenzoic acid, bovine serum albumin (BSA), nicotinamide adenine dinucleotide phosphate –reduced and oxidized (NADPH and NADP⁺), reduced glutathione (GSH), 5-Bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT), pyrogallol, NAD⁺, α -ketoglutarate, isocitrate, succinate and other important reagents were procured from Sisco Research Laboratories (SRL), Mumbai, India. Thiobarbituric acid (TBA) and other chemicals were purchased from Merck Limited, Delhi, India. Only analytical grade chemicals are used in the assays.

2.2. Animal experiments and composition of diet.

Male Wistar rats, (age 3-4 months) weighing 150-170 gm, were procured from supplier registered under Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The experimental and treatment protocols were approved (IAEC/IV/Proposal/DB-12/2015) by the Institutional Animal Ethics Committee (IAEC) of the Department of Physiology, University of Calcutta.

Following experimental groups were constituted to execute the study after 7 days acclimatization with normal diet and followed by 12 days of treatment -

- i. **Control (C):** Animals in this group were treated with vehicle and fed normal diet.
- ii. **Melatonin treated group (M):** Animals were fed with normal diet along with melatonin (by gavage) at a dose of 10 (M10), 20 (M20) or 40 mg/kg (M40), respectively.
- iii. **High fat diet group (H):** Animals were fed with 60% kCal high fat diet (24).
- iv. **High fat diet+ melatonin group (HM):** Animals were fed high fat diet and melatonin at a dose of 10 (HM10), 20 (HM20) or, 40 mg/kg (HM40), respectively.
- v. **Isoproterenol group (I):** Animals were fed with normal diet and I (subcutaneous injection) at a dose of 25 mg/kg (two injections 24 hours apart) on 11th and 12th day of the study to accelerate myocardial injury (23).
- vi. **Isoproterenol + melatonin group (IM):** Melatonin was given by gavage at a dose of 10 (IM10), 20 (IM20) or 40 mg/kg (IM40), respectively, 30 mins prior to I injection.
- vii. **High fat diet + I group (HI):** Animals were fed with high fat diet and injected with I.
- viii. **High fat diet + I + melatonin group (HIM):** According to the dose of melatonin this group was subdivided into following groups: -
 - a) HIM10- melatonin was fed at a dose of 10mg/kg.
 - b) HIM20- melatonin was fed at a dose of 20mg/kg.
 - c) HIM40- melatonin was fed at a dose of 40mg/kg.

Each group consisted of 6 rats. Rats were kept in animal house under controlled laboratory conditions (12-hour light/dark cycles with relative humidity of 40-60% and temperature of 25 \pm 2 $^{\circ}$ C). Fresh food was given every day for 12 days. Melatonin was fed by gavage at evening for 12 days.

The normal diet consisted of 63.24 gm% carbohydrate (69.54 % of the total energy from carbohydrate), 17.73 gm% protein (19.50% of the total energy from protein), 4.43 gm % fat (10.96% of the total energy from fat) and the rest were from salt, vitamin etc. The high fat diet consisted of 30.17 gm % carbohydrate (21.76% of the total energy from carbohydrate), 25.24 gm % protein (18.21% of the total energy from protein), 36.99 gm % fat (60.03% of the total energy from fat) and the rest were from salt, vitamin etc. HMW Salt mixture and vitamins were added to the diets according to the needs (25).

2.3. Collection of blood and tissues.

Rats were sacrificed after stipulated time period (i.e. on 13th day) by subjecting to mild anaesthetic ether followed by cervical dislocation. Chest cavity was opened through vertical incision and blood was collected by puncturing the heart. Serum was prepared using standard procedure (23). Heart was carefully removed washing in cold saline and soaking properly by blotting paper and stored at -20°C for future biochemical analysis. For histological studies, a ventricular portion of cardiac tissue was fixed by 10% buffered formaldehyde and a small ventricular portion of cardiac tissue was subjected to 3% glutaraldehyde immediately after its collection for scanning electron microscopy (SEM) study.

2.4. Preparation of cytosolic and mitochondrial fractions.

Cardiac tissues were homogenized in ice cold phosphate buffer (50 mM), pH 7.4. The homogenate (10%) was subjected to centrifugation at 2,000 rpm for 10 minutes at 4°C to remove nuclear portion as pellet. The supernatant was collected and centrifuged at 15,000 rpm for 40 min (4°C). Supernatant was used as cytosolic sample and the pellet was re-suspended in sucrose buffer (50 mM Tris HCl, pH 7.8 containing 250 mM sucrose) to obtain mitochondria. Both the samples were stored at -20°C for future biochemical assays.

2.5. Measurement of cardiac injury markers.

The activity of serum glutamate oxaloacetate transaminase was assayed by the method of Reitman and Frankel (26).

The serum activity of LDH 1 was measured by using sodium pyruvate as substrate according to the method of Sritmatter (27) with some modifications by Varcoe (28). The activities of the enzymes were expressed as IU/L.

2.6. Assays related to oxidative stress and antioxidant mechanisms.

2.6.1. Estimation of oxidative stress biomarkers in cardiac tissue.

The method of Buege and Aust (29) with some modifications by Bandyopadhyay *et al.* (30) was applied to estimate the lipid peroxides in cardiac tissue homogenates. The values were expressed as nmoles of TBARS/mg of protein.

The reduced glutathione (GSH) content were estimated by the method of Sedlack and Lindsay (31) with some modifications adopted by Bandyopadhyay *et al.* (30). The values were expressed as nmoles GSH/ mg of protein.

DNP assay of Levine *et al.* (32) was used to estimate the protein carbonyl content in cardiac tissue. The values were expressed as nmoles protein carbonyl/mg of protein.

2.6.2. Measurement of the activities of antioxidant enzymes in cardiac tissue.

Activities of CuZnSOD and MnSOD were measured according to the pyrogallol auto-oxidation method of Marklund and Marklund (33). The enzyme activities were expressed as U/mg of protein.

The activity of CAT was estimated according to the method of Beer and Sizer (34) with some modifications adopted by Chattopadhyay *et al.* (35). The enzyme activity was expressed as U/mg of protein.

GPx activity was determined by the method of Paglia and Valentine (36) with some modification adopted by Chattopadhyay *et al.* (37). The specific activity was expressed as U/mg tissue protein.

GR activity was determined by the method developed by Krohne Erich *et al.* (38). The specific activity of the enzyme was expressed as U/mg of protein.

2.6.3. Western blot analysis of antioxidant enzymes.

Fifty micro grams of protein was loaded for immuno-detection. The method of Laemmli (39) with some modifications (23) were used to obtain the bands and pixel density of the bands were quantified using Image J software (NIH, Bethesda, MD, USA) to ensure any alteration in the levels of proteins following the treatments.

2.6.4. Measurement of the activities of pro-oxidant enzymes.

The activity of xanthine oxidase (XO) was assayed by the conversion of xanthine to uric acid according to the method of Greenlee and Handler (40).

Xanthine dehydrogenase (XDH) activity was measured spectrophotometrically at 340 nm by the reduction of NAD⁺ to NADH according to the method of Strittmatter (27) with some modifications (41). Enzyme activities were expressed as mUnits/mg protein.

2.6.5. Estimation of hydroxyl radical (\bullet OH) in cardiac tissue.

The \bullet OH generated in cardiac tissue was estimated by the method of Bandyopadhyay *et al.* (30). The values were expressed as nmoles of \bullet OH/mg protein (Supplementary material 1).

2.6.6. Auto fluorescence study of amino acids.

The fluorescence emission spectra (from 300 to 450 nm, 5 nm slit width) of tryptophan were measured by excitation at 295 nm (2 nm slit width) (42). Fluorescence emission spectra of di-tyrosine, a product of tyrosine oxidation, were recorded in range 380 to 440 nm (5 nm slit width) at excitation wavelength 325 nm (5 nm slit width) (43) to determine the effects of oxidative damage on these amino acids.

2.7. Assays linked to metabolic enzymes.

2.7.1. Measurement of the activities of the glycolytic enzymes and glucose-6-phosphate dehydrogenase activity.

Phosphofructokinase (PFK I) activity was measured spectrophotometrically from cytosolic sample by oxidation of NADH (44). Hexokinase (HK) activity was determined spectrophotometrically by reduction of NAD to NADH (45). The activity of glucose-6-phosphate dehydrogenase (G6PDH) was assayed spectrophotometrically by reduction of NADP to NADPH (46). The activities of these cytosolic enzymes were expressed as U/mg protein.

2.7.2. Measurement of the activities of pyruvate dehydrogenase and Krebs cycle enzymes.

Pyruvate dehydrogenase (PDH) activity was estimated according to the method of Chretien *et al.* (47) with some modifications. Citrate Synthase activity was spectrophotometrically

analysed by the method of Parvin (48). Isocitrate dehydrogenase (ICDH) activity was measured by the method of Duncan *et al.* (49). The method of Duncan *et al.* (49) was used to determine the activity of α -ketoglutarate dehydrogenase (α -KGDH). The method of Veeger *et al.* (50) with some modifications was used to determine succinate dehydrogenase (SDH) activity spectrophotometrically by recording the reduction of potassium ferricyanide [$K_3Fe(CN)_6$] at 420 nm. The activities of all the enzymes were expressed as U/mg protein and mitochondrial fraction was used for the assays.

2.7.3. Measurement of the activities of respiratory enzymes.

According to the method of Goyal and Srivastava (51) NADH-Cytochrome c oxidoreductase activity was measured spectrophotometrically by following the reduction of oxidized cytochrome C at 565 nm. Cytochrome c oxidase activity was determined according to the method of Goyal and Srivastava (51) by the oxidation of reduced cytochrome c by the enzyme at 550 nm. Enzyme activities were expressed as U/mg protein and mitochondrial sample was used for assays.

2.7.4. Measurement of the activity of enzymes involved in fat and ketone body metabolism.

The activity of 3-ketoacyl-CoA thiolase, the key regulatory enzyme for beta oxidation, was estimated in mitochondrial sample spectrophotometrically by recording the absorption of the enol form at 303 nm (52). β -hydroxybutyrate dehydrogenase (β HBDH) activity was determined in mitochondrial sample by the reduction of NAD^+ to NADH at 340 nm according to the method of Delafield and Doudoroff (53). The activities of both the enzymes were expressed as U/mg protein.

2.7.5. Estimation of lactate dehydrogenase (LDH) activity in cardiac tissue.

LDH activity in the cardiac tissue was determined by monitoring the rate of pyruvate reduction to lactate in presence of NADH with the method of Wroblewski and Ladue (54) with slight modification by Samaga *et al.* (55). The specific activity of LDH was expressed in U/ mg of the protein.

2.7.6. Estimation of lipid fraction in serum.

Triglyceride, total cholesterol, HDL cholesterol and LDL cholesterol were estimated in serum by using commercial kit purchased from Arkray healthcare Pvt. Ltd. (India). Kit instructions were strictly followed to perform the tests and calculations were done by the instruction provided with the kit where Friedewald's equation was used to estimate LDL cholesterol.

2.8. Assays linked to systemic inflammation.

2.8.1. Estimation of different inflammatory markers and adiponectin in serum.

Different inflammatory markers IL-1 β (ELR-IL1b), IL-6 (ELR-IL6), IL-10 (ELR-IL10) and TNF α (ELR- TNF α) and adiponectin (ELR-Adiponectin) were estimated by standard ELISA kits purchased from RayBiotech, Inc. (USA).

2.9. Histological and morphological studies.

2.9.1. Haematoxyllin-eosin (HE) Staining

The fixed cardiac tissues were embedded in paraffin and routine procedure was followed described by Bancroft and Gamble (56). Tissue sections with 5µm thickness were prepared and stained with haematoxyllin-eosin (HE) stain. The changes in tissue morphology were observed through light microscope and captured by camera attached to it. A minimum of 10 readings were made on each section at least 5 samples from each specimen and observations were measured at 500µm intervals with a calibrated eyepiece ocular micrometer.

2.9.2. Scanning electron microscopy.

The fixed cardiac tissues were processed according to the method described by Noronha-Dutra *et al.* (57) and modified by Mukherjee *et al.* (58). After keeping the tissue sections for three days in 3% glutaraldehyde, the sections were dehydrated in graded alcohol and at last in isoamyl alcohol. Then, the tissue sections were observed through scanning electron microscope (Carl Zeiss) and images of the surface of each section were captured. A minimum of 10 readings were made on each section at least 5 samples from each specimen and observations were measured at 500µm intervals with the help of software provided by Carl Zeiss.

Mitochondrial samples were kept overnight at 4°C in 3% glutaraldehyde. The samples were dehydrated by graded alcohol and iso-amyl alcohol. Then, mitochondrial smear was prepared and observed through scanning electron microscope to obtain the images of mitochondria.

2.9.3. Fluorescence study of mitochondrial DNA by confocal imaging.

Mitochondrial smears were obtained on slide. The smears were equilibrated to pH 7.0 using a neutral pH buffer and then stained with DAPI stain (59). The slides were examined under a confocal microscope and images were captured with a digital camera attached to it. Then captured images were analyzed using ImageJ software (ImageJ, NIH) and total fluorescence of each image measured and expressed as mean fluorescence intensity (MFI).

2.10. Estimation of protein.

The protein content of each sample was determined following the method described by Lowry *et al.* (60).

2.11. Statistical analysis.

Data are presented as means ± S.E.M. One way analysis of variances (ANOVA) followed by post hoc test (Tukey's HSD test) was employed to determine significant differences between the mean values of different treatment groups. Each experiment was repeated at least for 3 times and statistical analysis was performed using Graph pad prism version 7.0.5.

3. RESULTS

3.1. Effects of melatonin on myocardial injury.

A significant rise in the activity of SGOT was observed in H or I group when compared to C group. This rise was further significantly elevated when the animals were treated with the

combination of H and I. Melatonin treatment at the different doses significantly inhibited the rise of SGOT activity. It appeared that melatonin exhibited a dose dependent manner to suppress the rise of SGOT activity induced by the H, I or Hand I and the most effective dose is 40 mg/kg (Fig. 1A). Similar results were observed in LDH1 activity (Fig. 1B).

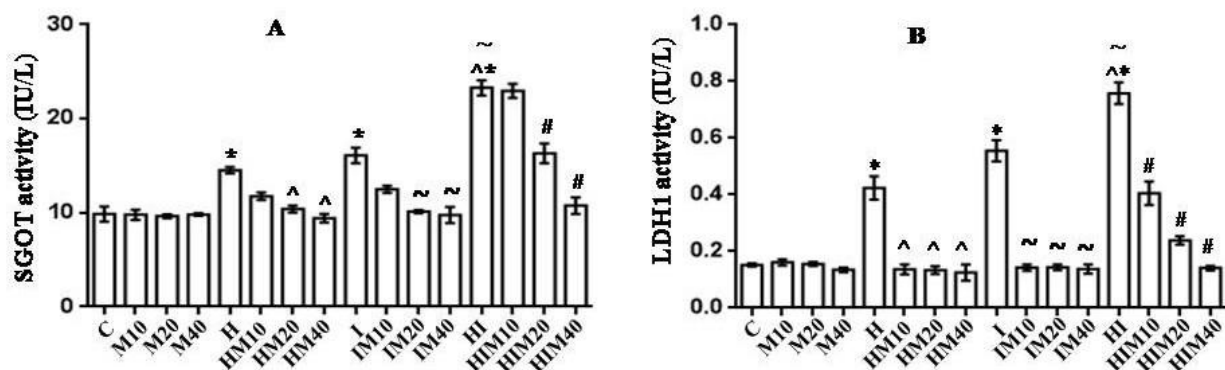


Figure 1: Effects of melatonin on the activities of SGOT (A) and LDH1 (B) in serum of rats treated with H, I or HI, respectively.

C: Control; M10: Melatonin 10mg/kg group; M20: Melatonin 20mg/kg. group; M40: Melatonin 40mg/kg group; H: high fat diet group; HM10: High fat diet + melatonin 10mg/kg group; HM20: High fat diet + melatonin 20mg/kg group; HM40: High fat diet + melatonin 40mg/kg I: Isoproterenol group; IM10: Isoproterenol+ melatonin 10mg/kg group; IM20: Isoproterenol+ melatonin 20mg/kg. group; IM40: Isoproterenol + melatonin 40mg/kg group; HI: High fat diet + isoproterenol group; HIM10: High fat diet + isoproterenol + melatonin 10 mg/kg group; HIM20: High fat diet + isoproterenol + melatonin 20 mg/kg group; HIM40: High fat diet + isoproterenol + melatonin 40 mg/kg group. Values are expressed as means \pm S.E.M (N = 6). *P<0.001 versus C, ^P<0.001 versus H, ~P<0.001 versus I and # P<0.001 versus HI.

3.2. Effects of melatonin on oxidative stress in cardiac tissue.

Significant rises in LPO level (Fig. 2A) and PCO content (Fig. 2B) were observed in heart of animals treated with either H or I. These rises were further elevated in animals treated with the combination of H and I. Melatonin treatment at the different doses significantly inhibited the rises of LPO and PCO with a dose dependent manner. It appeared that the most effective dose is 40 mg/kg. In contrast, the myocardial GSH content was significantly decreased by the treatments of H, I, or H and I with highest decrease in HI group. Melatonin treatment at the different doses significantly increased GSH content. It appeared that the most effective dose was 40 mg/kg and melatonin exhibited a dose dependent manner to increase the GSH content (Fig. 2C).

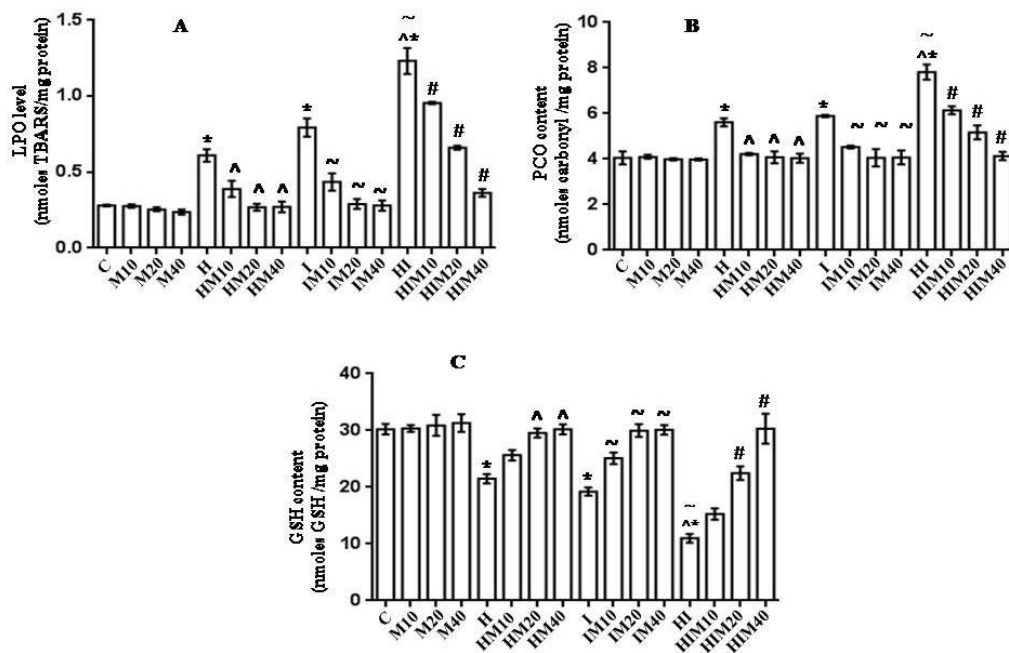


Figure 2: Effects of melatonin on the LPO (A), PCO (B) and GSH (C) levels in myocardial tissue treated with H, I or H I, respectively.

C: Control; M10: Melatonin 10mg/kg. group; M20: Melatonin 20mg/kg group; M40: Melatonin 40mg/kg group; H: High fat diet group; HM10: High fat diet + melatonin 10mg/kg group; HM20: High fat diet + melatonin 20mg/kg group; HM40: High fat diet + melatonin 40mg/kg group; I: Isoproterenol group; IM10: Isoproterenol+ melatonin 10mg/kg group; IM20: Isoproterenol + melatonin 20mg/kg group; IM40: Isoproterenol + melatonin 40mg/kg group; HI: High fat diet + isoproterenol group; HIM10: High fat diet + isoproterenol + melatonin 10 mg/kg group; HIM20: High fat diet + isoproterenol + melatonin 20 mg/kg group; HIM40: High fat diet + isoproterenol + melatonin 40 mg/kg group. Values are expressed as means \pm S.E.M. (N=6). * $P < 0.001$ versus C, ^ $P < 0.001$ versus H, ~ $P < 0.001$ versus I and # $P < 0.001$ versus HI.

3.3. Effects of melatonin on the gene expression and activities in myocardial antioxidant enzymes.

The activities of CuZnSOD (Fig. 3A) and MnSOD (Fig. 3B) were significantly decreased in H group but elevated in I group ($p < 0.001$) compared to the control. The activities of both the enzymes were significantly elevated ($p < 0.001$) in HI group compared to C or H groups. The significant decrease in the gene expression of CuZnSOD (Fig. 4AI) and MnSOD (Fig. 4AII) was noted in H group where the significant elevations of both enzymes were observed in I group. The significant elevations in the gene expression of both SODs in HI group was also observed compared to C or H groups ($p < 0.001$). A significant elevation of catalase (CAT) activity in H group whereas a significant reduction in I group compared to C were observed ($p < 0.001$). The significant ($p < 0.001$) reduction in the activity of CAT (Fig. 3C) was also noted in HI group compared to C or H group. The gene expression of CAT protein (Fig. 4A III) in the myocardial tissue was found to be significantly reduced ($p < 0.001$) in HI group compared to the C or H groups.

The activities of GPx (Fig. 3D) and GR (Fig. 3E) were significantly diminished in H and I group compared to the control ($p < 0.001$). A further significant reduction was also observed in HI group. The gene expression of GPx (Fig. 4A IV) and GR (Fig. 4A V) of myocardial tissue

was significantly low in H and I groups when compared to control ($p < 0.001$). A further significant reduction in GPx and GR protein was also observed in HI group compared to H or I group ($p < 0.001$).

Melatonin at a dose of 40 mg/kg significantly restored the activity and the gene expression of different antioxidant enzymes to control level ($p < 0.001$ versus HI).

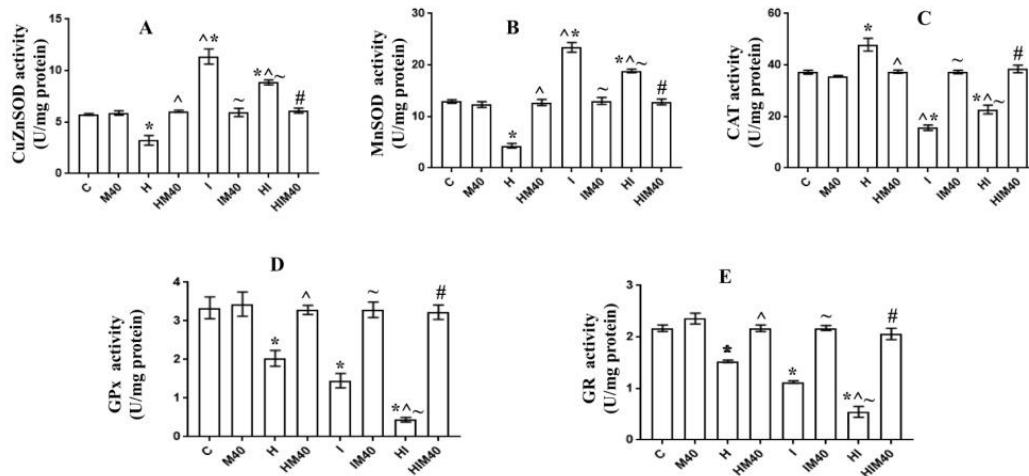


Figure 3: Effects of melatonin on the antioxidant enzymes CuZnSOD (A), MnSOD (B), CAT (C), GPx (D) and GR (E) in myocardial tissue of rats treated with H, I, or HI, respectively.

C: Control; M40: Melatonin 40mg/kg. group; H: High fat diet group; HM40: High fat diet + melatonin 40mg/kg group; I: Isoproterenol group; IM40: Isoproterenol + melatonin 40mg/kg group; HI: High fat diet + isoproterenol group; HIM40: High fat diet + isoproterenol + melatonin 40 mg/kg group. Values are expressed as means \pm S.E.M. ($N = 6$). * $P < 0.001$ versus C, ^ $P < 0.001$ versus H, ~ $P < 0.001$ versus I and # $P < 0.001$ versus HI

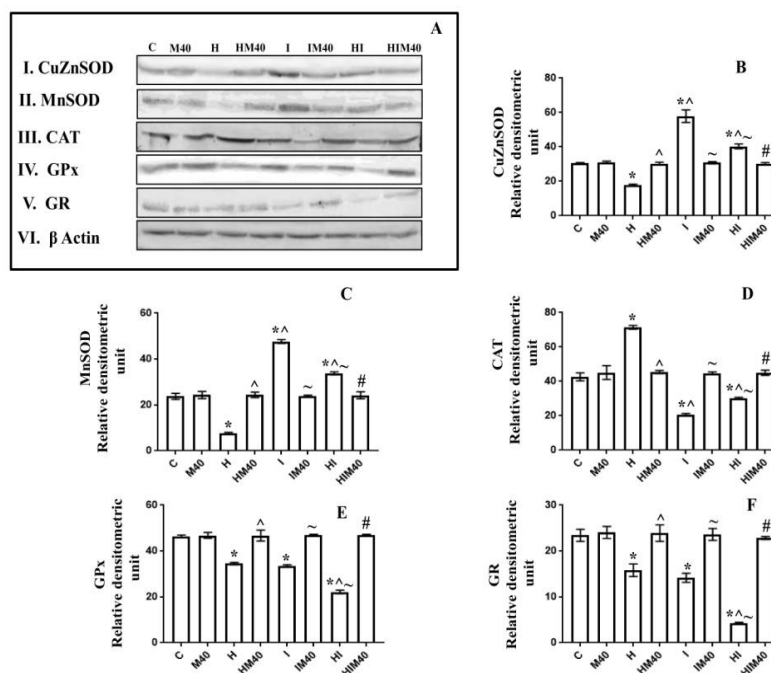


Figure 4: Effects of melatonin on the gene expression of antioxidant enzymes of heart in rats treated with H, I or H I, respectively.

Representation of images of western blot of (A-I) CuZnSOD, (A-II) MnSOD, (A-III) CAT, (A-IV) GPx, (A-V) GR and graphical representation of changes in the relative protein abundance of (B) CuZnSOD, (C) MnSOD, (D) CAT, (E) GPx, (F) GR with β -actin (A-VI) as internal control. C: Control; M40: Melatonin 40mg/kg group; H: High fat diet group; HM40: High fat diet + melatonin 40mg/kg group; I: Isoproterenol group; IM40: Isoproterenol + melatonin 40mg/kg group; HI: High fat diet + Isoproterenol group; HIM40: High fat diet + Isoproterenol + melatonin 40mg/kg group. Values are expressed as means \pm S.E.M. (N= 6). * P <0.001 versus C, ^ P <0.001 versus H, ~ P <0.001 versus I and # P <0.001 versus HI.

3.4. Effects of melatonin on myocardial superoxide generating enzymes.

The activities of XO (Fig.5A), XDH (Fig. 5B) and the ratio of XO/XDH (Fig. 5C), XO + XDH (Fig. 5D), XO/(XO+XDH) (Fig. 5E) were significantly increased with treatments of H, I and H I. The highest increase was found in the rats treated with the combination of HI. Melatonin at the dose of 40 mg/kg completely brought all these alterations back to the control level.

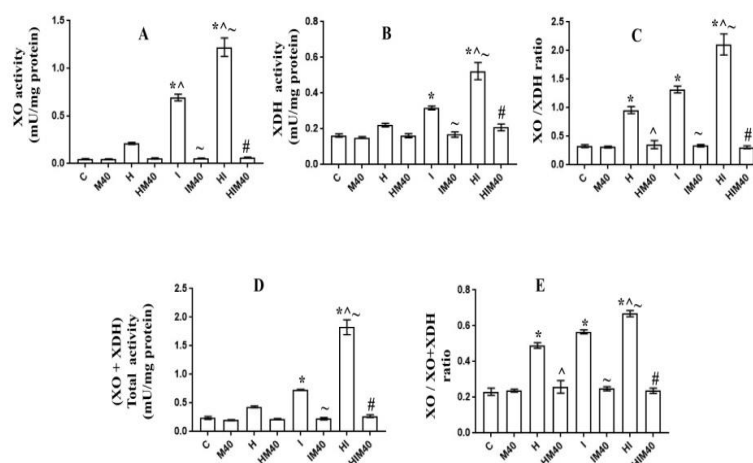


Figure 5: Effects of melatonin on superoxide generating enzymes XO (A), XDH (B), ratio of XO/XDH (C), XO+XDH (D) and ratio of XO/(XO+XDH) (E) in rat myocardium treated with H, I or HI, respectively.

C: Control; M40: Melatonin 40mg/kg group; H: High fat diet group; HM40: High fat diet + melatonin 40mg/kg group; I: Isoproterenol 25mg/kg; IM40: Isoproterenol + melatonin 40mg/kg group; HI: High fat diet+ isoproterenol group; HIM40: High fat diet + isoproterenol + melatonin 40 mg/kg group. Values are expressed as means \pm S.E.M. (N=6). * P <0.001 versus C, ^ P <0.001 versus H, ~ P <0.001 versus I and # P <0.001 versus HI.

3.5. Effects of melatonin on the ROS generation and amino acid oxidation.

Significant elevation of hydroxyl radical was noted in H, I and HI group compared control (Table 1). The highest hydroxyl radical formation was detected in the HI group. Melatonin at the dose of 40 mg/kg almost completely suppressed the hydroxyl radical elevation induced by the H, I and HI, respectively. In consistent with the hydroxyl radical formation, the amino acid oxidation in cytosolic and mitochondrial portions of the myocardial tissue exhibited the similar trend and melatonin effectively prevented these amino acids oxidation.

Table 1: Effect of melatonin treatment on the myocardial levels of hydroxyl radical, and fluorescence intensities of di-tyrosine and tryptophan in the cytosolic and mitochondrial fractions.

Groups	Hydroxyl radical nmoles MSA/mg protein	Mean Fluorescence Intensity (Arbitrary Unit)			
		Mitochondrial fraction		Cytosolic fraction	
		Di- tyrosine	Tryptophan	Di-tyrosine	Tryptophan
C	90.1 ± 0.36	33.4 ± 2.01	373.7 ± 34.54	52.8 ± 0.79	432.7 ± 16.15
M40	84.4 ± 7.63	34.0 ± 0.40	377.0 ± 15.01	53.0 ± 0.63	434.4 ± 37.29
H	154.5 ± 4.58*	46.4 ± 2.81*	247.2 ± 24.85*	64.2 ± 1.79*	266.5 ± 15.70*
HM40	90.2 ± 2.33 [^]	31.2 ± 0.14 [^]	373.6 ± 1.75 [^]	53.7 ± 0.88 [^]	444.6 ± 18.46 [^]
I	179.1 ± 4.95*	53.3 ± 1.93*	195.5 ± 14.09*	67.8 ± 3.37*	249.1 ± 15.62*
IM40	90.5 ± 5.34 [~]	33.9 ± 1.87 [~]	371.1 ± 19.79 [~]	51.8 ± 0.17 [~]	415.1 ± 37.48 [~]
HI	225.8 ± 4.91 ^{*^~}	62.4 ± 0.83 ^{*^}	97.3 ± 5.81 ^{*^~}	82.5 ± 1.61 ^{*^~}	117.7 ± 5.47 ^{*^~}
HIM40	107.1 ± 12.26 [#]	36.1 ± 1.10 [#]	371.8 ± 21.39 [#]	53.7 ± 0.63 [#]	399.2 ± 10.81 [#]

Values are expressed as means ±S.E.M (N =6). *P<0.001 versus C, [^]P<0.001 versus H, [~]P<0.001 versus I and # P<0.001 versus HI.

3.6. Effects of melatonin on the activities of PFK, HK and G6PD in myocardial tissue.

The activities of PFK (Fig. 6A) and HK (Fig. 6B) were significantly increased in H, I and HI groups, respectively when compared to the values of C. The highest elevation was found in the HI group. Melatonin treatment at the dose of 40 mg/kg preserved the normal activities of these enzymes in all the treated groups. In contrast, the activity of the G6PDH was significantly reduced in the myocardial tissue treated with H, I and HI, respectively. The lowest activity was detected in the animals treated with the combination of H and I. Melatonin treatment preserved its normal activity in all the treated groups (Fig. 6C).

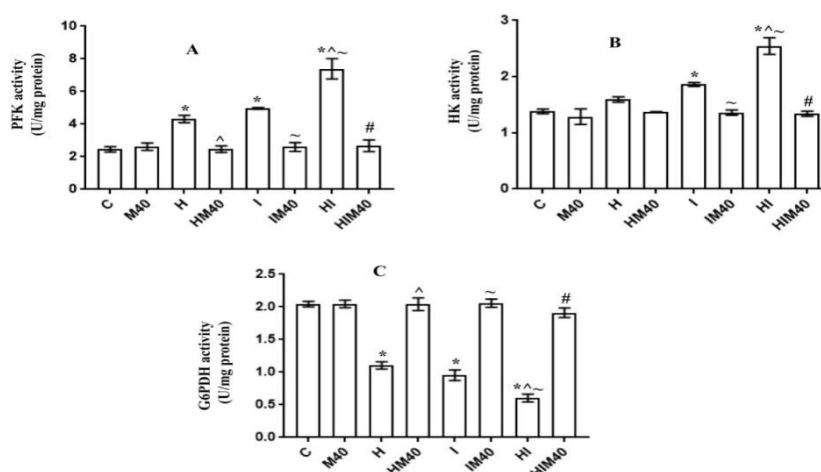


Figure 6: Effects of melatonin on the activities of PFK (A), HK (B) and G6PDH (C) of myocardial tissue treated with H, I or HI, respectively.

C: Control; M40: Melatonin 40mg/kg group; H: High fat diet group; HM40: High fat diet + melatonin 40mg/kg group; I: Isoproterenol group; IM40: Isoproterenol + melatonin 40mg/kg group; HI: High fat diet + Isoproterenol group; HIM40: High fat diet + Isoproterenol + melatonin 40 mg/kg group. Values are expressed as means ±S.E.M. (N=6). *P<0.001 versus C, [^]P<0.001 versus H, [~]P<0.001 versus I and # P<0.001 versus HI.

3.7. Effects of melatonin on the activities of PDH and Krebs cycle enzymes in myocardial tissue.

The activities of PDH and Krebs cycle enzymes were significantly reduced by the treatments of I or H, respectively compared to the control ($P < 0.001$). The lowest activity was found in the combination of HI treatment. Melatonin at the dose of 40 mg/kg effectively preserved the activities of these enzymes in all treated groups ($P < 0.001$) (Table 2).

Table 2: Effect of melatonin on the activities of PDH and citrate synthase, ICDH, α KGDH and SDH in myocardial tissue treated by H, I and HI, respectively.

Groups	Activity of PDH and Krebs cycle enzymes (U/mg protein)				
	PDH	Citrate synthase	ICDH	α KGDH	SDH
C	4.92 \pm 0.34	1.2 \pm 0.02	5.4 \pm 0.52	5.9 \pm 0.36	48.2 \pm 3.34
M40	5.1 \pm 0.19	1.3 \pm 0.06	5.2 \pm 0.18	5.9 \pm 0.54	48.5 \pm 1.79
H	2.7 \pm 0.16*	0.9 \pm 0.05*	3.4 \pm 0.30*	3.2 \pm 0.16*	32.7 \pm 0.97*
HM40	5.1 \pm 0.24 [^]	1.2 \pm 0.02 [^]	5.2 \pm 0.22 [^]	5.8 \pm 0.55 [^]	47.7 \pm 2.82 [^]
I	2.2 \pm 0.24*	0.9 \pm 0.06*	3.1 \pm 0.03*	2.8 \pm 0.16*	29.7 \pm 0.70*
IM40	5.0 \pm 0.13 [~]	1.21 \pm 0.02 [~]	5.0 \pm 0.24 [~]	5.8 \pm 0.14 [~]	47.6 \pm 3.30 [~]
HI	0.8 \pm 0.09 ^{*^~}	0.5 \pm 0.06 ^{*^~}	1.1 \pm 0.11 ^{*^~}	0.6 \pm 0.07 ^{*^~}	13.3 \pm 1.07 ^{*^~}
HIM40	4.8 \pm 0.17 [#]	1.20 \pm 0.06 [#]	4.9 \pm 0.31 [#]	5.9 \pm 0.64 [#]	47.8 \pm 3.66 [#]

Values are expressed as means \pm S.E.M ($N = 6$). * $P < 0.001$ versus C, [^] $P < 0.001$ versus H, [~] $P < 0.001$ versus I and # $P < 0.001$ versus HI.

3.8. Effects of melatonin on the activities of respiratory enzymes in the myocardial tissue.

The result showed that the activities of cytochrome c oxidase and NADH cytochrome c oxidoreductase (Table 3) were significantly reduced in H, I and HI groups compared to C, respectively ($c < 0.001$). The lowest activities of both enzymes were detected in the HI group. Melatonin at the dose of 40 mg/kg effectively preserved the activities of these enzymes in all treated groups ($P < 0.001$).

Table 3: Effect of melatonin treatment on the activities of different respiratory enzymes (cytochrome c oxidase and NADH cytochrome c oxidoreductase) in the myocardial tissue of rat treated with H, I and HI, respectively.

Groups	Activity of respiratory enzymes (U/mg protein)	
	Cytochrome c oxidase	NADH-cytochrome c oxido-reductase
C	1.3 \pm 0.02	3.2 \pm 0.21
M40	1.4 \pm 0.05	3.2 \pm 0.13
H	0.8 \pm 0.05*	2.3 \pm 0.05*
HM40	1.4 \pm 0.04 [^]	3.2 \pm 0.19 [^]
I	0.6 \pm 0.02*	2.0 \pm 0.08*
IM40	1.4 \pm 0.04 [~]	3.2 \pm 0.10 [~]
HI	0.4 \pm 0.02 ^{*^~}	1.1 \pm 0.21 ^{*^~}
HIM40	1.3 \pm 0.04 [#]	3.1 \pm 0.22 [#]

Values are expressed as means \pm S.E.M ($N=6$). * $P < 0.001$ versus C, [^] $P < 0.001$ versus H, [~] $P < 0.001$ versus I and # $P < 0.001$ versus HI.

3.9. Effects of melatonin on the activities of thiolase, β -hydroxy butyrate dehydrogenase and LDH in myocardial tissue.

The activities of thiolase (Fig. 7A), β -hydroxy butyrate dehydrogenase (Fig. 7B) and LDH (Fig. 7C) were significantly elevated in H, I and HI groups compared to the control, respectively, ($p < 0.001$). The highest level was detected in the group of HI. Melatonin at the dose of 40 mg/kg effectively brought activities of these enzymes back to the control level in all treated groups.

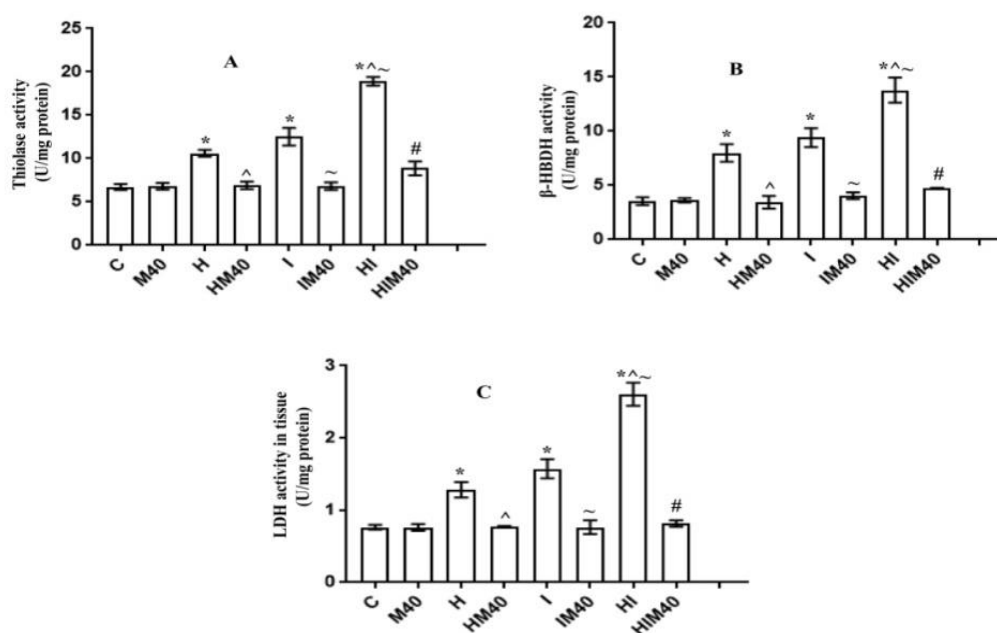


Figure 7: Effects of melatonin on the activities of thiolase (A), β -hydroxy butyrate dehydrogenase (B) and LDH (C) in myocardial tissue treated with H, I and HI, respectively.

C: Control; M40: Melatonin 40mg/kg group; H: high fat diet group; HM40: High fat diet + melatonin 40mg/kg group; I: Isoproterenol group; IM40: Isoproterenol + melatonin 40mg/kg group; HI: High fat diet + isoproterenol group; HIM40: High fat diet + isoproterenol + melatonin 40 mg/kg group. Values are expressed as means \pm S.E.M. (N=6). * $P < 0.001$ versus C, ^ $P < 0.001$ versus H, ~ $P < 0.001$ versus I and # $P < 0.001$ versus HI.

3.10. Effects of melatonin on the lipid profile of rats.

The values of triglycerides, total cholesterol, LDL-c, HDL-c and the ratios of total cholesterol: HDL-c and LDL-c: HDL-c are shown in table 4. The values of all parameters (except of the HDL-c) were significantly increased in the rats treated with H, I and HI compared to the control, respectively. The highest value was observed in the group of HI. Melatonin at the dose of 40 mg/kg significantly suppressed the elevated values induced by the H, I and HI treatment. In contrast, the value of HDL-c was significantly reduced in rats treated with H, I and HI compared to the control, respectively. However; melatonin at the dose of 40 mg/kg brought this value back to the control level.

Table 4: Effects of melatonin on the concentrations of triglyceride, total cholesterol, LDL-c, HDL-c, LDL:HDL and total cholesterol: HDL-c in serum of rats treated with H, I and HI, respectively.

Groups	Lipid profile of serum					
	Triglyceride (mg/dl)	Total Cholesterol (mg/dl)	LDL-c (mg/dl)	HDL-c (mg/dl)	Total Cholesterol: HDL-c	LDL-c: HDL-c
C	54.7 ± 2.17	51.5 ± 2.96	4.1 ± 0.35	36.5 ± 3.06	1.4 ± 0.05	0.2 ± 0.04
M40	54.7 ± 5.88	51.7 ± 0.59	3.7 ± 0.84	41.1 ± 0.75	1.3 ± 0.03	0.1 ± 0.09
H	94.2 ± 3.52*	95.1 ± 4.36*	42.1 ± 7.00*	23.0 ± 0.39*	3.4 ± 0.34*	2.1 ± 0.30*
HM40	60.1 ± 2.59 [^]	54.7 ± 5.52 [^]	9.2 ± 3.24 [^]	35.5 ± 2.80 [^]	1.6 ± 0.15 [^]	0.3 ± 0.09 [^]
I	92.0 ± 8.60*	107.1 ± 5.94*	71.7 ± 7.33*	27.0 ± 1.45*	4.4 ± 0.34*	2.7 ± 0.38*
IM40	61.2 ± 2.01 [~]	54.8 ± 1.29 [~]	5.0 ± 1.271 [~]	37.2 ± 0.33 [~]	1.5 ± 0.03 [~]	0.1 ± 0.03 [~]
HI	123.3 ± 1.99 ^{^*~}	144.4 ± 5.57 ^{^*~}	94.5 ± 9.39 ^{^*~}	22.3 ± 1.24*	6.4 ± 0.59 ^{^*~}	4.4 ± 0.53 ^{^*~}
HIM40	66.9 ± 4.04 [#]	76.7 ± 1.35 [#]	23.6 ± 1.65 [#]	39.6 ± 1.12 [#]	1.9 ± 0.07 [#]	0.6 ± 0.05 [#]

Values are expressed as means ± S.E.M. (N = 6). *P < 0.001 versus C, [^]P < 0.001 versus H, [~]P < 0.001 versus I and # P < 0.001 versus HI.

3.11. Effects of melatonin on serum pro- and anti-inflammatory cytokines.

The concentrations of TNF α , IL-1 β and IL-6 were significantly increased in H, I and HI group compared to control group, respectively (Table 5). The highest value was observed in the HI group. Melatonin at the dose of 40 mg/kg effectively suppressed the elevated values induced by the H, I and HI treatments. In contrast, the value of anti-inflammatory cytokine, IL-10, was significantly reduced in rats treated with H, I and HI. Again, melatonin at the dose of 40 mg/kg significantly elevated the suppressed value. No significant difference has been observed on the value of adiponectin with any treatment.

Table 5: Effect of melatonin on the concentrations of different pro- and anti-inflammatory cytokines (TNF α , IL-1 β , IL-6, IL-10) and Adiponectin in serum of rats treated with H, I and HI, respectively.

Groups	Status of inflammatory markers in serum				
	TNF α (pg/ml)	IL-1 β (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	Adiponectin (ng/ml)
C	3.9 ± 0.65	33.2 ± 0.12	74.1 ± 1.64	4.39 ± 0.44	0.3 ± 0.00
M40	3.9 ± 0.23	27.5 ± 0.13	71.4 ± 0.44	4.6 ± 0.47	0.3 ± 0.00
H	41.2 ± 0.50*	49.9 ± 2.98*	102.4 ± 3.26*	0.6 ± 0.03*	0.2 ± 0.02
HM40	3.9 ± 0.69 [^]	29.50 ± 0.32 [^]	79.3 ± 2.73 [^]	3.7 ± 0.17 [^]	0.3 ± 0.01
I	58.3 ± 5.89 ^{^*}	53.8 ± 0.92*	108.6 ± 4.20*	0.6 ± 0.03*	0.3 ± 0.00
IM40	5.2 ± 0.56 [~]	33.5 ± 2.98 [~]	74.9 ± 3.02 [~]	3.7 ± 0.21 [~]	0.3 ± 0.00
HI	77.9 ± 0.45 ^{^*~}	70.8 ± 3.72 ^{^*~}	137.3 ± 8.69 ^{^*~}	0.6 ± 0.01*	0.3 ± 0.00
HIM40	5.2 ± 0.23 [#]	32.3 ± 4.19 [#]	80.3 ± 2.75 [#]	3.6 ± 0.34 [#]	0.3 ± 0.01

Values are expressed as means ± S.E.M (N=6). *P < 0.001 versus C, [^]P < 0.001 versus H, [~]P < 0.001 versus I and # P < 0.001 versus HI.

3.12. Effects of melatonin on myocardial morphology and mitochondrial structures.

The results showed that H, I or HI treatment caused myocardial morphological alterations. These include the disarrangement of myofibrils, inflammatory cell infiltration and cytoplasmic vacuolisation. The most serious morphological abnormality was observed in the HI group including the remarkable furrowing of the tissue with rugged, uneven surface in the myocardial tissue (Fig. 8). Melatonin at dose 40 mg/kg significantly reduced the morphological abnormality induced by H, I and HI treatments.

The images of the cardiac mitochondria captured through SEM (Fig. 9A) showed H or I treatment damaged the surface topology of mitochondria. The combination of HI treatment further aggravated the damages. Melatonin at the dose of 40 mg/kg effectively prevented mitochondrial damage.

In addition, the fluorescence intensity of DAPI stained mitochondrial DNA was significantly increased in H, I and HI groups compared to the control (Fig. 9B and C) The highest fluorescence intensity was detected in the HI among the groups. Melatonin administration at the dose of 40 mg/kg significantly alleviated the mitochondrial DNA damage.

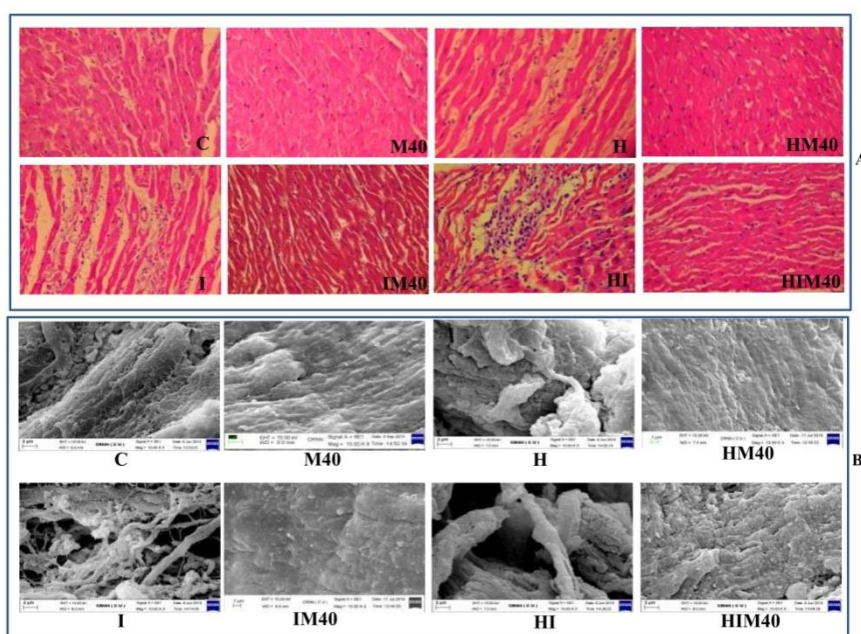


Figure 8: Effects of melatonin on myocardial morphology in rats treated with H, I and HI, respectively.

Panel A represents the photomicrographs of the ventricular sections of cardiac tissue (5 μ m thick paraffin sections) with HE staining and captured at 20X magnification. The sections of C, HM40, IM40 and HIM40 show uniform orientation and intact myofibril, while the sections of H and I show disintegration of myofibril with neutrophil infiltration. The section of HI shows greater extent of disintegration and neutrophil infiltration of the myofibril.

Panel B represents the images of the surface of the cardiac tissue (ventricular section) under SEM, captured at 10KX magnification. The surfaces of cardiac tissue of C, HM40, IM40 and HIM40 show no disintegration or furrowing, while the sections of H and I show furrowing and the most severe disruption and furrowing of the surface were seen in HI group.

C: Control; M40: Melatonin 40 mg/kg group; H: High fat diet group; HM40: High fat diet + melatonin 40mg/kg group; I: Isoproterenol group 25mg/kg; IM40: Isoproterenol+ melatonin 40mg/kg group; HI: High fat diet + isoproterenol group; HIM40: High fat diet + isoproterenol + melatonin 40 mg/kg group.

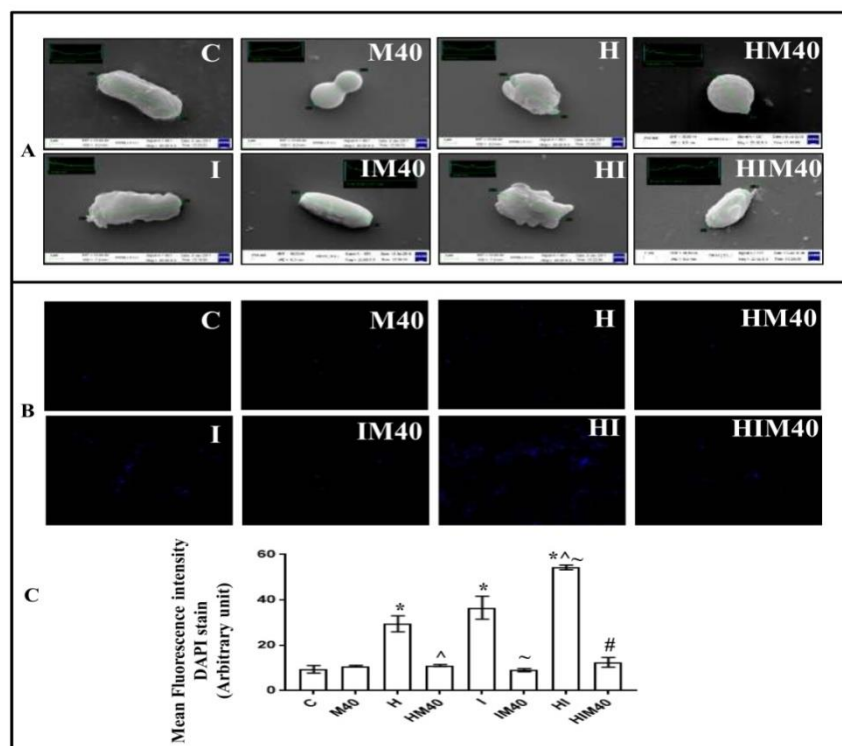


Figure 9: Effects of melatonin on mitochondrial structures and mitochondrial DNA damage in rats treated with H, I and HI, respectively.

Panel A represents the images of mitochondria isolated from cardiac tissue captured at 20KX magnification under SEM. The images of mitochondria of C, HM40, IM40 and HIM40 show no surface damage whereas image of the mitochondria in H, I and HI group shows greater extent of disintegration of the mitochondrial surface. Panel B shows representative images (40X magnification) of DAPI (4, 6-diamidino-2-phenylindole) stained mitochondrial smear. The images were captured by confocal laser scanning microscope. The fluorescence intensity indicated the damage of mitochondrial DNA. Panel C Histogram shows mean fluorescence intensity (MFI) of the DAPI stained mitochondrial smear. C: control; M40: Melatonin 40mg/kg group; H: high fat diet group; HM40: High fat diet + melatonin 40mg/kg group; I: Isoproterenol group; IM40: Isoproterenol + melatonin 40mg/kg group; HI: High fat diet + isoproterenol group; HIM40: High fat diet + isoproterenol + melatonin 40 mg/kg group. Values are expressed as means \pm S.E.M (N= 5). * $P < 0.001$ versus C, ^ $P < 0.001$ versus H, ~ $P < 0.001$ versus I and # $P < 0.001$ versus HI.

4. DISCUSSION

It has been well documented that high fat diet consumption even for a short period increases the risk of myocardial damage (61, 62). In order to mimic the clinical situation in the current study, the rats were fed with high fat diet for 12 days. Notably, to exacerbate the high fat diet induced myocardial damage, I was also injected to these rats, since I facilitates the unpredicted myocardial event (22, 23). The combination of HI treatment will significantly elevate the free radical generation and cause the myocardial oxidative stress, and lead to the myocardial tissue damage. A potent antioxidant, melatonin, was selected to test whether it can prevent the myocardial damage induced by oxidative stress in the current HI animal model.

The results showed that HI indeed caused myocardial damage indicated by the increased serum levels of SGOT and LDH1 which was leaked due to structural alterations of the cardiac

tissue (23, 41). In addition, the morphological abnormalities of myocardial tissue were uncovered by the SEM and histological studies. The mechanistic studies revealed that oxidative stress was the major cause of the myocardial damage since the HI treatment significantly increased the ROS formation in the myocardial tissue, especially the hydroxyl radical. ROS attack lipids to causing LPO (63, 64) and attack protein to increase PCO. Melatonin treatment, especially at the dose of 40 mg/kg, effectively protected all these alterations caused by HI treatment. We found that melatonin not only directly scavenged the ROS, but also upregulated the gene expression and activities of antioxidant enzymes while downregulated the pro-oxidant enzymes. The multiple interactions of melatonin with ROS render it as a most powerful antioxidant among others (8, 9, 65).

Increase in ketone body catalysing enzyme, β -HBDH in HI group implies greater amount of ketone bodies formation. Ketone bodies are metabolically converted into acetyl-CoA for energy production (66). Here we found that the rate of Krebs cycle was immensely slowed down indicating severe energy deficiency in cardiac tissue. Increase in the glycolytic enzymes along with increased LDH activity in myocardium in HI group indicates oxidation of NADH with accumulation of lactate in cardiac tissue. NAD^+ helps to sustain glycolysis as a source of energy but accumulation of lactate is inevitable. Lactate anions can also cause mitochondrial degenerations (67) along with ROS. Currently, melatonin is referred as a mitochondrial targeted antioxidant (68) due to it being synthesized and functioned in mitochondria (68, 69). The profound protective effects of melatonin on mitochondrial function and morphology were observed in our study. Melatonin reduced the mitochondrial surface injury, preserved the function of mitochondrial energy metabolism and stimulated the activities of mitochondrial associated enzymes including cytochrome c oxidase, NADH cytochrome c oxidoreductase, PDH, citrate synthase, ICDH, α KGDH, and SDH, etc.

Another mechanism of HI induced myocardial damage is related to inflammation. The levels of serum proinflammatory cytokines (TNF α , IL-6 and IL-1 β) were significantly increased in the rats in HI group. Pro-inflammatory and anti-inflammatory cytokines are highly responsible for the development of atherosclerosis (70) and different cardiovascular diseases (71). Moreover, according to a study, inflammation and oxidative stress can form a vicious cycle to further damage the myocardial tissue (72). The profound anti-inflammatory effects of melatonin have been well documented in many tissues including myocardium (73–75). In this study, we observed that melatonin treatment reduced the levels of pro-inflammatory cytokines and thus, broken this inflammation-oxidative stress vicious cycle.

Based on the results, it can be concluded that melatonin, a natural antioxidant, may serve as a potential therapeutic agent for heart diseases which are related to oxidative stress and inflammation, particularly in those with abnormal lipid metabolism. Considering its low or non-toxicity, its availability and inexpensiveness melatonin should be recommended for clinical trial to evaluate its protective effects on the patients with heart diseases.

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AUTHORSHIP

Dr. DB and Dr. AC conceived and designed the experiment, revised the manuscript critically and approved it. AG executed the experiment, analyzed the data, prepared figures, drafted the manuscript and edited it. GB and TD contributed in executing the experiments. Dr. PKP contributed in analyzing the data and edited the manuscript. SM contributed in executing the experiments.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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