

THE CARDIOPROTECTIVE POTENTIAL OF MELATONIN AFTER HETEROTOPIC CARDIAC TRANSPLANTATION IN MALE RATS

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Abstract

Global myocardial ischemia reperfusion (I/R), is often obligatory during cardiac surgery, induce an inflammatory response in the transplanted heart. Inflammatory reactions in the graft have a pivotal influence on acute as well as long term graft function. This study was undertaken to investigate the potential role of melatonin in amelioration of I/R injury in the transplanted heart in rat model. The rats were randomized into 3 groups. Group 1 sham group, rat underwent the same anesthetic and surgical procedure as the control group except for heterotopic heart transplantation, Group 2 control group, rats underwent heterotopic heart transplantation and subjected to global ischemia for 30 min and reperfusion for 2 hours, Group 3 donor and recipient rats received melatonin 10 mg/kg i.p. 30 min before the transplant procedure. The heterotopic heart transplantation is done using the cuff technique in the neck (the aorta of the donor is anastomosed with the carotid artery of the recipient and the pulmonary artery of the donor to the jugular vein of the recipient). At the end of experiment (2 hr of reperfusion), blood samples were collected from the heart for measurement of plasma level of cardiac troponin I (cTn I). The heart were harvested, the apical side was fixed in 10% formalin for histological examination and the basal side was homogenized for the measurement of tissue tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) and intracellular adhesion molecule-1 (ICAM-1). Compared with the sham group, levels of myocardial TNF- α & IL-1 β , and ICAM-1; plasma cTn I were increased ($p < 0.05$), Histologically, all induced untreated rats showed significant myocardial injury ($P < 0.05$) melatonin significantly counteract the increase in myocardium level of TNF- α , IL-1, & ICAM-1 ($P < 0.05$). Histological analysis revealed that melatonin markedly reduced ($P < 0.05$) the severity of heart injury in the rats underwent transplant procedure. The results of the present study reveal that melatonin has a promising cardioprotective effect against global I/R injury in the grafted heart via interfering with inflammatory reactions which induced by I/R injury .

Key Words: heterotopic heart transplantation (HHT), ischemia/ reperfusion injury (I/R), TNF- α , IL-1 β , ICAM-1, cTn I, Melatonin .

Introduction

Heart transplantation is a widely accepted therapy for most patients under 65 years of age with advanced heart failure who remain symptomatic with the expectation of high intermediate term mortality, despite optimal heart failure medications [1]. Heterotopic heart transplant (HHT), in which the recipient heart and its innervation remain intact and the donor heart is placed in the right hemithorax with the donor and recipient left ventricles functioning in parallel [2]. HHT models applied in animal experiments studying anastomotic techniques, immunosuppressive therapies, and immunopathology of graft rejection [3]. Global myocardial I/R, is obligatory during cardiac surgery, induces an inflammatory response in the heart [4]. Inflammatory reactions in the graft have a pivotal influence on acute as well as long-term graft function [5]. I/R injury refers to the tissue damage which occurs when blood supply returns to tissue after a period of ischemia and is associated with trauma, stroke, myocardial infarction, solid organ transplantation, thrombolytic therapy, and coronary angioplasty [6]. I/R injury results in local and systemic inflammation if severe enough, the inflammatory response after I/R may result in the systemic inflammatory response syndrome or multiple organ dysfunction syndrome (MODS) [7]. I/R injury is the result of ATP depletion during prolonged hypoxia. Further tissue damage results from the reperfusion of the tissue [5], which is associated with formation of reactive oxygen intermediates, platelet and neutrophil activation, endothelial cell injury, increased vascular permeability, cytokine activity and complement activation [8]. Increase of the intracellular calcium concentration also enhances the activation of phospholipases as well as proteases and caspases which execute programmed cell death (apoptosis) [9]. Adaptive cellular responses activate the innate immune system with its TLR and the complement. This results in a profound inflammatory reaction with immune cells infiltration [5]. The damage is mediated by various cytokines, chemokines, adhesion molecules, and compounds of the extracellular matrix. The expression of these factors is regulated by specific transcription factors with NF- κ B being one of the key modulators of inflammation [5].

TNF- α and IL-1 β is a potent pro-inflammatory cytokines, plays an important role in the pathogenesis of a of I/R injury. The isolated rat myocardium synthesizes and releases TNF- α in response to ischemia and reperfusion which directly

correlates with the post ischemic deterioration in myocardial mechanical performance and the amount of cellular necrosis [10]. IL-1 β and TNF- α directly suppress myocardial function as shown by studies on human serum of patients with acute septic shock marked by reversible myocardial depression. Using immunoabsorption, removal of both TNF- α and IL-1 β (but not either alone) from these sera resulted in the elimination of serum myocardial depressant activity [11]. Adhesion molecules have been shown to play an important role in the pathogenesis of tissue injury from atherosclerosis, autoimmune diseases, transplant rejection, and I/R injury [12]. An increase in the levels of soluble forms of VCAM-1 and ICAM-1 reflecting endothelial activation and damage [13]. The plasma levels of adhesion molecules indicate the intensity of endothelial activation during reperfusion of ischemic myocardium [14]. Troponins are proteins that regulate skeletal and cardiac muscle contraction. cTnI, the subunit which inhibits cross-bridging of actin and myosin at sub-activating concentrations of Ca [15]. Has been found to be a very sensitive serum marker of physical or metabolic myocardial injury, myocardial ischemia, or necrosis with a cardiac specificity of 100% [16].

Melatonin (N-acetyl-5-methoxytryptamine) is a naturally occurring hormone derived from the amino acid tryptophan and produced mainly by the pineal gland in the brain as well as in the retina and GIT [17], melatonin is a pleiotropic compound exerts complex physiological and pharmacological effects on multiple systems and organs [18]. It has a potent free radical scavenger for multiple ROSs and RNSs, and synergistic actions with classic antioxidants & upregulate the activity of antioxidant enzymes [19-20]. Studies suggesting an anti-inflammatory & antiapoptotic effect [21]. Melatonin has a highly favorable effect in reducing tissue damage that follows transient episodes of hypoxia and reoxygenation, numerous reports have confirmed the ability of melatonin to ameliorate cardiac damage resulting from I/R injury [22]. In addition to melatonin, several of its metabolites have been shown to detoxify radicals themselves. This powerful pyramid scheme of radical scavenging has been named "the antioxidant cascade of melatonin" [23]. Melatonin down-regulated the expression of NF- κ B, iNOS, and caspase-3 [21], and has the ability to reduce the expression of the adhesion molecules, P-selectin and ICAM-1 and reduces the recruitment of PMN cells into the inflammatory site [22], also suppress the formation of pro-inflammatory cytokines such as TNF α , IL-1 β , IL-6 [24].

HHT Using The Cuff Technique

Animals were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine, placed in the supine position with their limbs immobilized, and the skin of the operative region sterilized. HHT was performed by a modified cuff technique previously described [26]

Donor operation

A midline abdominal incision was performed within the rat. The viscera were slightly retracted to the left side, and 1ml heparinized saline (100U/ml) was injected through the (IVC). Bleeding was prevented by light compression using a cotton stick. Then, a bilateral thoracotomy along the anterior axillary line to the inferior margin of clavicle was performed, and the anterior chest wall was opened with a rigid scissor. Small piece of ice were put into thoracic cavity to arrest the heart. Blunt dissection of the aorta and pulmonary artery was performed; both were cut as distal as possible. All the vessels, except the aorta and pulmonary artery, were ligated with a 4-0 silk suture. Then the heart was gently pulled upward by the silk sutures, and stored in cold lactated Ringer's solution.

Recipient operation

A longitudinal incision from the right mandibular angle to the middle point of the right clavicle was made. The right submaxillary gland was removed to expose the right external jugular vein. The right common carotid artery was exposed by cutting the right sternocleidomastoid muscle and mobilized to the bifurcation of the internal and external carotid artery. The proximal portion of the carotid artery was occluded with bulldog clamp and the distal portion of it was ligated at the level of the bifurcation with 6-0 silk. The carotid artery was then incised between the clamp and the distal tied end, and the proximal end was irrigated with heparinized saline (100 U/ml). The carotid artery was then passed through the IV cuff (IV cannula, gage 18); the proximal end of the carotid artery was everted over the cuff and wrapped with a circumferential ligature of 6-0 silk. The right external jugular vein was prepared in the same way. The donor heart was placed in the right neck of the recipient. The arterial cuff was inserted into donor aorta and fixed with a preset-ligature (6-0 silk). An 18 gage IV cuff was inserted into the donor pulmonary artery and also fixed with 6-0 silk ligature. The clamp on the external jugular vein was unclamped, followed by unclamping of the carotid

artery. The heart rapidly turned from pale to red.

Preparation of Samples

Blood Sampling for measurement of plasma cTnI.

At the end of experiment, about 2 mls of blood were collected from the heart. The blood sample was placed in a tube containing disodium EDTA (22 mg/ml) as anticoagulant and mixed thoroughly then centrifuged at 3000 rpm for 15 min. Then it was used for determination plasma cTnI according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits (Life Diagnostics, USA).

Tissue Preparation for TNF- α , IL-1 β and ICAM-1 Measurement

The basal side of the heart tissues was rinsed with ice cold saline to remove any red blood cells or clots, then homogenized with a high intensity ultrasonic liquid processor in 1:10 (w/v) phosphate buffered saline that contained 1% Triton X-100 and a protease inhibitor cocktail. The homogenate was centrifuged at 2,500 g for 20 min at 4°C. The supernatant was collected for determination of TNF- α , IL-1 β and ICAM-1 according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits (RayBio, USA)

Tissue Sampling for Histopathology

At the end of the experiment, the transplanted hearts were excised and apical side of heart tissues fixed in 10% formalin and embedded in paraffin the sections were stained with hematoxylin and eosin (H&E) after fixation. Evaluation scores were performed by an investigator who was blinded to the experimental treatment groups. The following morphological criteria were used to assess the histopathological damage: score 0, no damage; score 1 (mild), interstitial edema and focal necrosis; score 2 (moderate), diffuse myocardial cell swelling and necrosis; score 3 (severe), necrosis with the presence of contraction bands, neutrophil infiltration and the capillaries were compressed; and score 4 (highly severe), widespread necrosis with the presence of contraction bands, neutrophil infiltration, capillaries compressing and hemorrhage

Statistical Analysis

Statistical analyses were performed by using SPSS 17.0 for windows Inc. An expert advice was consulted for tests used. Data were expressed as mean \pm SEM. Analysis of Variance (ANOVA) was used for the multiple comparisons among all groups followed by post-hoc tests using LSD method.

Pearson correlation coefficient was used to assess the associations between two variables of study parameters. Spearman correlation coefficient was used for non-parametric correlations. The histopathological heart changes are a non-normally distributed variable measured on an ordinal level of measurement; therefore non-parametric tests were used to assess the statistical significance involving this variable. The statistical significance of difference in total score between more than 2 groups was assessed by Kruskal-Wallis test, while Mann-Whitney U test was used for the difference between 2 groups. In all tests, $P < 0.05$ was considered to be statistically significant.

Results

Effect on Proinflammatory markers (TNF- α , IL-1 β , ICAM-1)

At the end of the experiment, the levels of cardiac TNF- α , IL-1 β and ICAM-1 were significantly ($P < 0.05$) increased in control group as compared with sham group. The levels of cardiac TNF- α , IL-1 β and ICAM-1 of Melatonin treated group were significantly ($p < 0.05$) lower than that of control group. The values of cardiac TNF- α , IL-1 β and ICAM-1 are showed in table 1 and figures 1, 2 and 3.

Effect on plasmalevel of cardiac troponin I (cTnI)

At the end of the experiment; the level plasma of (cTnI) was significantly increased ($P < 0.05$) in control group as compared with sham group. The plasma level of (cTnI) of Melatonin treated group was significantly ($p < 0.05$) lower than that of control group. The values of plasma levels of (cTnI) are showed in table 2 and figure 4.

Histopathological Findings

A cross section of sham rat's heart showed the normal cardiac structure; no interstitial edema and focal necrosis, no diffuse myocardial cell swelling and necrosis; no contraction bands, no neutrophil infiltration, no capillaries compressing and no hemorrhage. (83.3%) of rats in this group showed normal heart appearance and (16.7%) showed mild injury as shown in table 3 & figure 5. There was statistically significant difference between control group (II) and sham group (I) ($P < 0.05$) and the total severity scores of the control group showed severe myocardial injury (50%). And (33%) showed highly severe injury and (16.6%) showed moderate injury as shown in. Treatment of rats with melatonin improved cardiac injury significantly ($P < 0.05$) as compared with control group and the total severity scores mean of this group showed 33.3% of

the group had no damage, 50% had mild cardiac injury and 16.7% had moderate cardiac injury.

Discussion

Ischemia Reperfusion Injury is an inevitable problem in organ transplantation. It is associated with primary allograft dysfunction, contributing to acute and chronic rejection postoperatively [27]. Acute inflammatory reaction plays an important role in I/R injury through leukocyte activation and expression of adhesion molecules and cytokines [28]. Inflammatory cytokines, such as TNF- α , IL-1 β and IL-6 were shown to play key roles in the pathophysiology of ischemia and reperfusion injury [29]. It was found that the improvement in post-I/R functional recovery was parallel to reduction of ICAM-1, MPO activity, PMN accumulation and decreased cytokines TNF- α , IL-1 β [30]. Infiltrating neutrophils also play a crucial role for the production of TNF- α in I/RI(10). Furthermore, the rapid release of TNF- α in reperfused myocardium contributed to the transcriptional activation of ICAM-1 and triggers a cascade of NF- κ B activation and ICAM-1 induction in response to postischemic reperfusion [10].

Effect of Global Myocardial I/R on TNF- α , IL-1 β

In the present study a significant increase in inflammatory cytokine (TNF- α , IL-1 β) level ($P < 0.05$) was found in the I/R rats as compared with sham group. Ischemia plays a crucial role in local TNF release from isolated rat myocardium, it is synthesized during ischemia and released upon reperfusion from the myocardium itself as reported [10]. [31] Demonstrated that I/R increases cardiac TNF- α levels and TNF- α bioactivity in an isolated, crystalloid-perfused model of myocardial I/R injury. [32] stated that Compared with sham-treated animals, I/R in vehicle-treated animals resulted in increased mRNA levels of TNF- α & IL-1 β . In rodent models of myocardial infarction, within the first hours to 1 day, there are robust upregulations of intramyocardial cytokines including TNF- α and IL-1 β , moreover, TNF- α IL-1 β increased the production of superoxide anion, which reacts with NO to form peroxynitrite and in turn desensitizes myofilament to calcium, leading to myocardial contractile failure [33]. The results in the present study are in agreement with that reported [30] who showed that TNF- α , IL-1 β levels in the myocardium increased markedly after I/R injury.

Effect of Global Myocardial I/R on ICAM-1

Neutrophils play a central role in inflammatory response to reperfusion injury. The binding of

leukocytes to endothelial cells is a prerequisite for tissue injury [34]. ICAM-1 is known to be a major ligand on endothelial cells for adhesion of activated leukocytes subsequent passage of leukocytes into the myocardium [35]. In the present study, a significant increase in ICAM-1 level ($P < 0.05$) was found in the I/R rats as compared with sham group. It showed that the basal level of ICAM-1 mRNA was low in the heart and was significantly upregulated by I/R [36]. Stated that immunohistochemical analysis for ICAM-1 showed a significant increase in ICAM-1 staining in the hearts obtained from the rats subjected to regional myocardial I/R, compared with the hearts obtained from sham group rats [30]. In an isolated blood-perfused rat heart model, the effect of monoclonal antibodies against LFA-1 and ICAM-1 on the recovery of myocardial metabolic function resulted in improvement in postischemic cardiac function, myocardial tissue edema and myocardial energy status, those results suggest that a LFA-1/ICAM-1 dependent neutrophil-endothelial cell interaction plays a critical role in myocardial reperfusion injury following global ischemia [34].

Effect of Global Myocardial I/R on Myocardial Injury Marker (cTnI)

In the present study, a significant increase in plasma level of cTn I ($P < 0.05$) was found in the I/R rats as compared with sham group. Cardiac troponin I has already been shown to be a highly specific marker of acute myocardial infarction and of reperfusion after thrombolytic therapy. Stated that cardiac troponin I is an early marker of ischemic injury and cTn I concentration increases as the ischemic period increases. Early cTn I release appears to correlate with the extent of ischemic injury in rats undergoing buffer perfusion, while CK-MB and LD concentrations did not show a similar evolution [37]. The results in the present study are in agreement with that reported, their results showed that serum levels of cTn I increased markedly after I/R injury [38].

Effect of Global Myocardial I/R on Heart Parenchyma

There was statistically significant difference between induced untreated group and normal sham group ($P < 0.05$). The score of the control group shows severe myocardial injury 50% of the control group had severe myocardial injury, 33.3% had highly severe injury, and 16.7% had moderate injury. C. Stated that on histopathological examination, heart tissues from I/R rats (30 min/

2 hours) showed widespread myocardial structure disorder, diffused cloudy swelling, hyperemia, sinusoidal distension and PMNs infiltration with focal vacuolar degeneration as compared with sham group [39]. According, (after 30 min of ischemia and 24 hr of reperfusion) pathological features of the infarct area became apparent with widespread tissue necrosis, the presence of contraction bands, PMNs infiltration, capillary compressing and abundant signs of hemorrhage [40]. In this study, I/R causes significant increase ($P < 0.05$) in myocardial IL-1 β and TNF- α , a well-known pro-inflammatory cytokines that cause cell adhesion, activation, and transmigration of PMNs into cardiac tissues and their oxidative burst, which results in excessive ROS production and myocardial damage, another mechanism is that TNF- α can cause direct myocardial tissue damage by direct cytotoxicity, induction of dysfunction and/or apoptosis.

The Effect of Melatonin on Study Parameters

Effect of Melatonin on Inflammatory Markers (IL-1 β , TNF- α and ICAM-1)

The present study shows that the effect of melatonin administration before the induction of ischemia caused significant lowering ($P < 0.05$) in myocardial level of IL-1 β , TNF- α and ICAM-1. These findings can be explained by the fact that melatonin possesses powerful antioxidant and free radical scavenger activity [19]. so it inhibits the formation of ROS which stimulates the production of inflammatory cytokine. [41] founded that melatonin significantly reduced mRNA level of inflammatory mediators (TNF- α , IL-1 β & IL-6 and mRNA level of ICAM-1) induced in the heart by acute exercise and that the protective effect of melatonin was related with inhibition of (NF- κ B) activation. [42] found that melatonin mitigated the tendency of increasing serum TNF- α levels after cardiopulmonary bypass surgery, prominent differences were detected between the control group and the melatonin treated rats. Exogenous melatonin protected liver from intestinal IR injury by inhibiting the production of free radicals, reducing the concentration of TNF- α in systemic circulation, and suppressing the expression of ICAM-1 in liver as reported [43]. Melatonin treatment down-regulates serum levels of systemic inflammatory response syndrome indicators (TNF- α , IL-1 β , IL-6 and ICAM-1) and decreased MPO activity but up-regulates serum levels of IL-10 during heatstroke in a rat model of heatstroke-associated MODS [44].

Effect of Melatonin on Myocardial Injury Marker cTnI

The present study shows that the effect of melatonin administration before the induction of ischemia caused significant lowering ($P < 0.05$) in plasma level of cTnI. Previous studies demonstrated that melatonin protects the heart against many types of injuries, induced by isoproterenol [45], doxorubicin [46] Induced cardiotoxicity and I/R injury [47]. Showed that serum levels of cTnT and cTnI were significantly reduced in the ISO + melatonin group compared with the ISO group. There were no significant differences between the ISO + melatonin and control (saline) groups in terms of the mean cTnT and cTnI values [45], Found significant reduction in serum troponin I in melatonin-treated group as compared with doxorubicin injected group [46].

Effect of Melatonin on Heart Parenchyma

Treatment of rats with melatonin ameliorates the heart injury significantly ($P < 0.05$) as compared with induced untreated group. The score of the control group shows severe myocardial injury while the score of melatonin treated group shows mild injury. [47] demonstrated that the light microscopic study established the existence of several indications of I/R injuries such as severe edema in cardiac myofibrils, increased vascularity and increased inflammatory infiltration in I/R group. But these findings were minimal in melatonin treated groups. In rat model of isoproterenol myocardial injury. Found acute extensive myofibrillary degeneration, infiltration of neutrophils and interstitial edema (marked myocardial injury) in rat receiving isoproterenol only. Nonetheless in rat receiving melatonin + isoproterenol, small multifocal degeneration with slight degree of inflammatory process (mild myocardial injury). Furthermore, there were no histological changes in the myocardial tissues of two rats in the ISO + melatonin group [45]. Previous studies revealed the cardioprotective effect of melatonin in I/R injury through improving the hemodynamic parameters [48], lowering the activity of MPO enzyme [47], reduction of infarcted size [25], decreased apoptosis [21,48], and protection against oxidative stress [47,48]. Melatonin had been used in many studies in I/R rat model in different organs and proved to have ameliorative effect [22].

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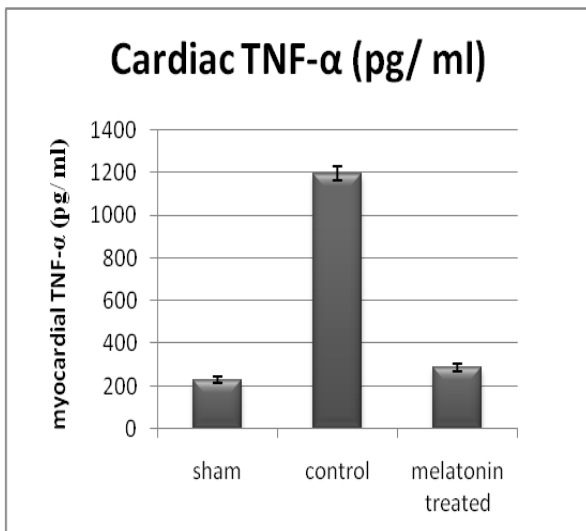
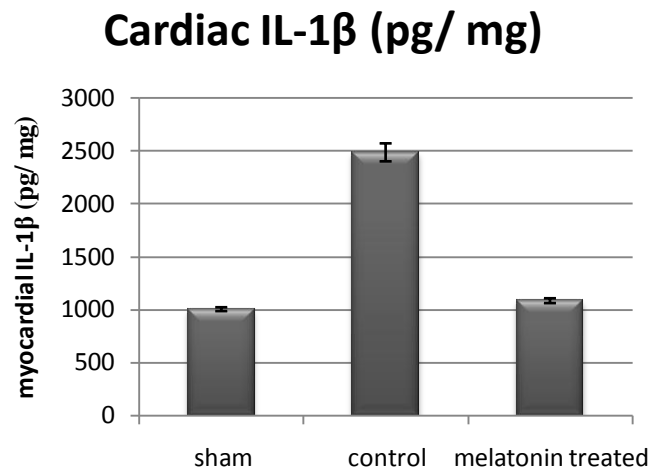
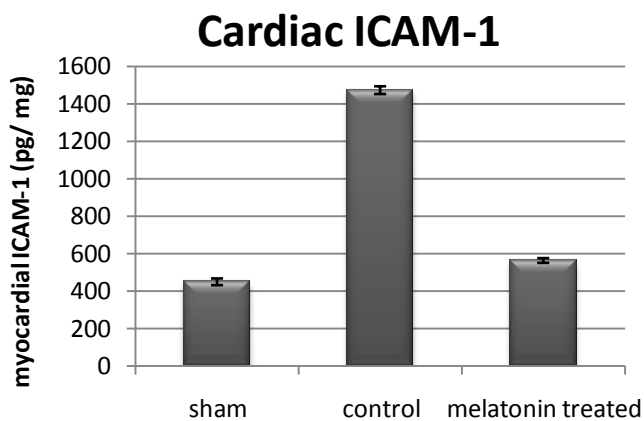
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Table 1. Cardiac TNF- α , IL-1 β and ICAM-1 levels (pg/mg) of the three experimental groups at the end of the experiment

Group	TNF- α	IL-1 β	ICAM-1
Sham	229.4 \pm 16.77	1006 \pm 18.24	451.07 \pm 17.92
Control	1193.4 \pm 33.77*	2489.5 \pm 85.17*	1472.98 \pm 20.65*
Melatonin treated	289.63 \pm 17.6	1086.13 \pm 22.92 †	564.72 \pm 12.63 †

The data expressed as mean \pm SEM (N = 6 in each group). * P < 0.05 vs. sham group, † P < 0.05 vs. control group

**Figure 1.** The mean of cardiac TNF- α level in (pg/mg) in the three experimental groups at the the end of the experiment.**Figure 2.** The mean of cardiac IL-1 β level (pg/mg) in (pg/mg) in the three experimental groups at the end of the experiment.**Figure 3.** The mean of cardiac ICAM-1 level (pg/mg) in the experimental groups at the end of experiment.**Table 2.** Plasma level of (cTnI) (ng/mg) of the three experimental groups at the end of the experiment.

Group	cTn I (ng/ mg)
Sham	0.83 \pm 0.04
Control	8.17 \pm 0.24*
Melatonin treated	2.5 \pm 0.18 †

The data expressed as mean \pm SEM (N = 6 in each group). * P < 0.05 vs. sham group, † P < 0.05 vs. control group

Table 3. The differences in histopathological scoring of abnormal heart changes among the three experimental groups.

Histopathological score	Study groups					
	Sham		control		melatonin treated	
	N	%	N	%	N	%
Score 0	5	83.3	0	0	3	50
Score 1	1	16.7	0	0	2	33.3
Score 2	0	0	1	16.7	1	16.7
Score 3	0	0	3	50	0	0
Score 4	0	0	2	33.3	0	0
Total	6	100	6	100	6	100

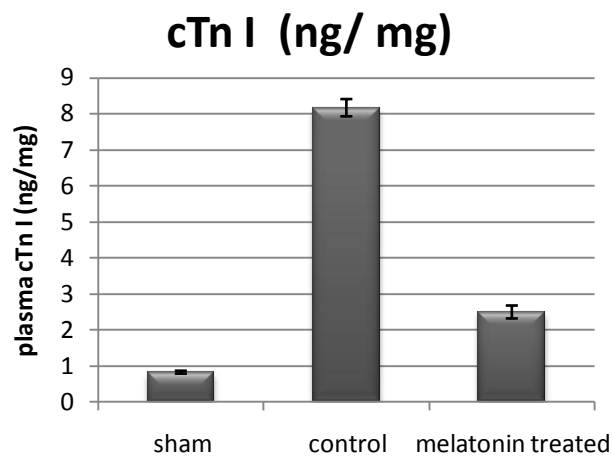


Figure 4. The mean of plasma (cTnI) level (ng/mg) in the three experimental groups at the end of the experiment.

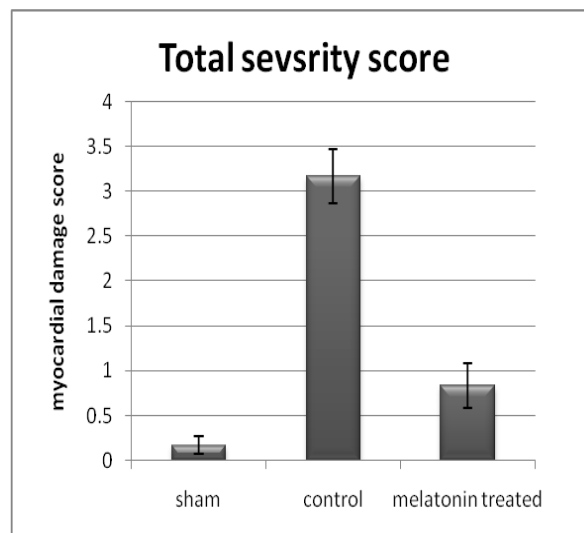


Figure 5. Error bar chart shows difference in mean \pm SEM values of total severity scores in the three experimental groups.

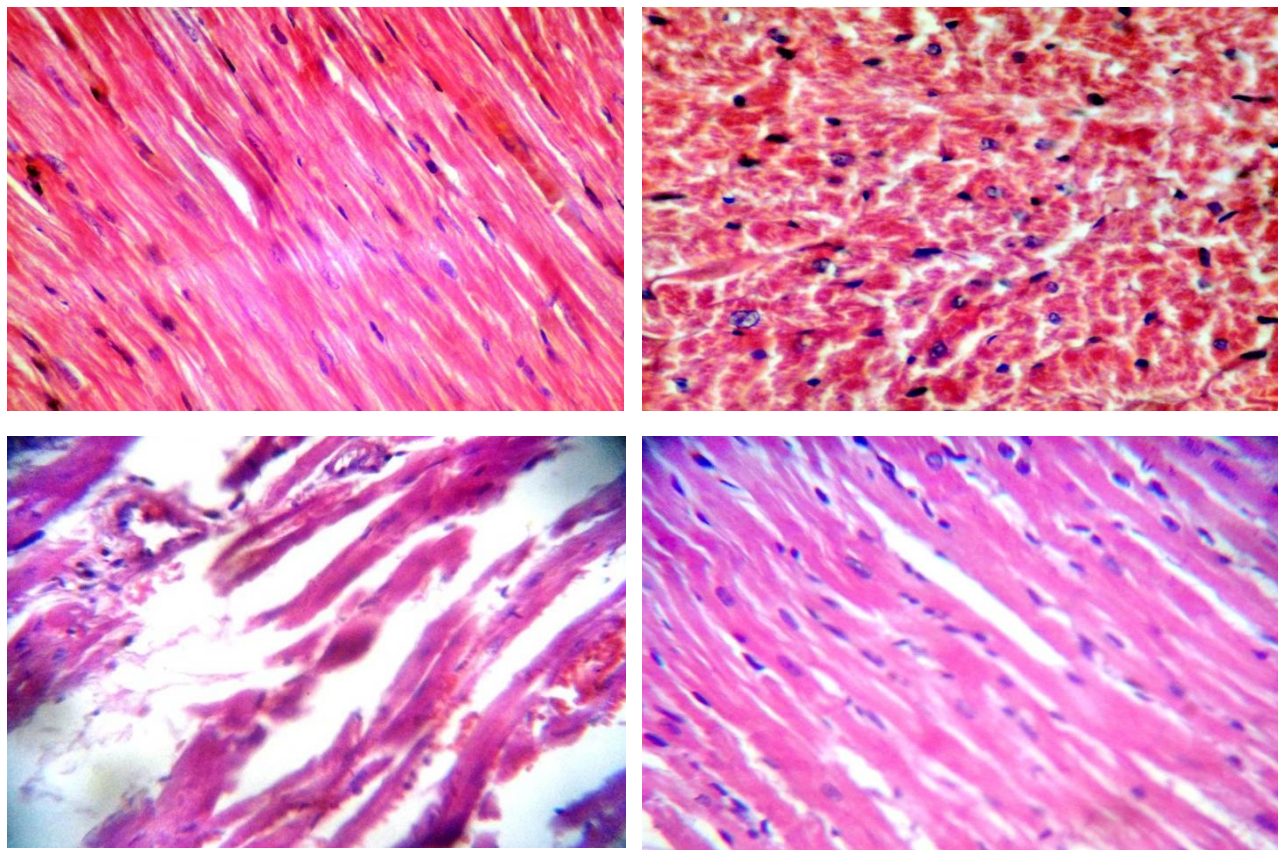


Figure 6. Photomicrograph represent the histopathological changes in rats. A: section of rat heart shows the normal architecture; B: cardiac section showed interstitial edema & neutrophil infiltration; C: cardiac section showed extensive necrosis, contraction bands and hemorrhage; D cardiac section in melatonin treated group. Sections stained with H&E (X40).