Ischemic Heart Disease (IHD), a major health problem of global concern, occurs when there is a disbalance between the oxygen demand and supply to the myocardium. It is one of the leading causes of death throughout the world. Many experimental models exist for studying the initiation and progression of IHD in the laboratory. One such model is using the β-adrenergic agonist isoproterenol (ISO) in experimental rats. Studies have shown that the pathophysiological changes that take place in rat heart following ISO-induced myocardial infarction mimic the changes taking place after myocardial infarction in humans [7].

In the first chapter of our study, ISO was injected subcutaneously twice at an interval of 24 hr at different doses to induce myocardial damage as is evident from the histological studies of the myocardial tissue as well as from studies on the biomarkers of cardiac injury. Our studies indicate the involvement of oxidative stress in the ISO-induced cardiac damage as evident from the changes observed in the levels of biomarkers of oxidative stress and generation of reactive oxygen species in vivo, namely, free hydroxyl radicals. We have also indirectly assessed the in vivo generation of superoxide anion free radical. The activities and protein expression levels of the key antioxidant enzymes were also found to alter due to ISO treatment. ISO treatment was also found to severely affect cardiac contractility and ventricular function.

Use of antioxidant therapy against IHD is an area of extensive research. The pineal hormone melatonin is a small, highly conserved, amphiphilic indole molecule present virtually in all organisms and known to have no morphophysiologic barrier within the cell. Melatonin has several important physiological functions in mammals.
including seasonal reproductive regulation, immune enhancement and regulation of light-dark signal transduction along with the capacity to influence some aspects of aging [1-2]. The role of melatonin and its metabolites as antioxidants is also well documented [2, 4, 8]. The cardioprotective ability of melatonin is also being explored in various models of oxidative stress [1, 2]. In our study, we provide evidence that melatonin, injected intraperitoneally, 30mins prior to ISO-injection dose dependently ameliorated all the ISO-induced effects in the rat heart. The studies further reveal that this low molecular weight natural indole provides protection to the rat heart through its direct as well as indirect antioxidant mechanisms. Moreover, we also provide evidence that melatonin improves cardiac function in ISO-induced ischemic heart.
RESULTS

(I) Dose-response studies for isoproterenol bitartrate (ISO)

Induction of myocardial ischemia in rats by ISO:
Figure 1 reveals a dose-dependent increase in the activity of SGOT following treatment of rats with ISO which indicates myocardial tissue damage. At 50 mg/kg BW, s.c, the level of activity of SGOT increased to a maximal value (*p < 0.001 vs. control). However, administration of ISO at 25 mg/kg BW, s.c. did not cause mortality of rats during the treatment period.

![Fig.1. Effect of ISO on serum glutamate oxaloacetate transaminase activity. Rats were given subcutaneous injections of increasing doses of ISO (indicated in the fig. as I). Control (CON) animals were treated similarly with vehicle only. Values are mean ± SEM of 8 rats in each group; *p < 0.001 vs. CON.](image)

Figure 2 reveals the ISO-induced damage to the rat cardiac tissue morphology. Hematoxylin and eosin (H & E) staining of the left ventricular tissue sections of the ISO-treated rat hearts at 20X magnification show myodegeneration as characterized by a loss of cardiac myofibers and a mononuclear cell infiltration. These histopathological changes show a clear dose-dependent effect with maximum damage being observed at the dose of 50mg/kg BW s.c.
Induction of oxidative stress by ISO:

To examine whether administration of ISO induces oxidative stress, we have measured two important biomarkers, viz., lipid peroxidation and reduced glutathione level of rat heart. Treatment of rats with different doses of ISO elicited a dose-dependent increase in the level of lipid peroxidation (LPO) measured as Thio Barbituric Acid Reactive Substances (TBARS) in the cardiac tissue [Fig 3 A] \( p < 0.001 \) vs. control at the dose 50 mg / kg BW s.c.]. Treatment of rats with ISO also caused a highly significant decrease in the reduced glutathione (GSH) level of the rat heart tissue [Fig3B].
Effect of ISO on cardiac antioxidant enzymes:
To determine the effect of ISO on the activities of the cardiac antioxidant enzymes, we measured the activities of Cu-Zn-SOD and catalase. The results presented in figure 4 A reveals that ISO at the doses of 12.5, 25.0 and 50.0 mg / kg BW, s.c. significantly increased dose-dependently the activity of Cu-Zn SOD in the rat cardiac tissue. Figure 4 B demonstrate that ISO also reduces catalase activity, another important antioxidant enzyme, in a dose-dependent manner with the maximum inhibition of the enzyme activity at 50 mg / kg BW, s.c. (*p < 0.001 vs. control).

From the dose-response studies, it was revealed that the minimum effective dose of ISO at which the changes observed were statistically significant, was 25mg/kg BW, s.c. administered twice at an interval of 24 hrs. At this dose of ISO, there was no mortality of rats during the
treatment period. Thus, subsequent experiments were carried out with this dose of ISO.

(II) Dose-response studies for melatonin (MEL)

(a) Induction of myocardial ischemia by ISO and protection by melatonin (MEL):

Figure 5 documents that pre-treatment of rats with melatonin dose-dependently prevented the rise in serum GOT level following ISO treatment at a dose of 25 mg / kg BW, s.c., twice at an interval of 24 hrs.

Figure 6 reveals the histopathological changes to the cardiac tissue due to ISO treatment at a dose of 25mg/kg BW s.c. twice at an interval of 24 hr, are dose-dependently prevented by melatonin, the best protection being afforded at the dose of 40mg/kg BW i.p.
(b) Induction of oxidative stress by ISO and protection by melatonin (MEL):

Figure 7 reveals that pre-treatment of rats with melatonin dose-dependently prevented the ISO-induced elevation in the level of LPO of the cardiac tissue \((p < 0.001 \text{ vs. control})\). Further, a dose-dependent restoration of the GSH level of the cardiac tissue by melatonin pre-treatment of the rats is also clearly evident from the data presented in the figure 7 B. At 40 mg/kg (i.p.) dose of melatonin, GSH level was almost near control values.

Fig. 7. (A) Protective effect of melatonin against ISO-induced increase in lipid peroxidation level. (B) Protective effect of melatonin against ISO-induced decrease in the levels of GSH of rat heart. Rats were treated with ISO [represented as I in the figure] and increasing doses of melatonin (m). Control (CON) animals were treated with vehicle only. Values are mean ± SEM of 8 rats in each group; \(^*p < 0.001 \text{ vs. CON.}#p < 0.001 \text{ vs I 25.}
(C) Effect of ISO on the antioxidant enzymes and protection by melatonin (MEL)

Figure 8 A reveals that the enhancement of Cu-Zn SOD (also known as SOD1) activity of the cardiac tissue by ISO treatment of rats at a dose of 25 mg/kg BW s.c., was restored to the activity observed in control animals by pre-treatment of rats with melatonin, also in a dose-dependent manner. Moreover, figure 8 B shows a highly significant decrease of catalase activity of rat cardiac tissue following treatment of the animals with ISO at a dose of 25 mg/kg BW, s.c. This decrease in activity of catalase was restored to near normal by pre-treatment of rats with melatonin in a dose-dependent manner.

From the dose-response studies, the dose of 10 mg/kg BW (i.p.) was found to be the minimum effective dose of melatonin at which it was able to significantly ameliorate the ISO-induced alterations to both the tissue morphological and biochemical parameters of the cardiac tissue.
of rats. Hence, subsequent experiments were carried out with this dose of melatonin.

(III) Studies with minimum effective dose of ISO and melatonin (MEL)

(a) Induction of myocardial ischemia by ISO and protection by melatonin:

Figure 9 documents the ISO-induced myocardial injury as is evidenced by the increase in the activities (*p < 0.001 versus control) of serum glutamate oxaloacetate transaminase, lactate dehydrogenase and lactate dehydrogenase type 1 isoform. The enzymes are considered as important biomarkers of myocardial injury. Pre-treatment of rats with melatonin at the minimum effective dose of 10 mg/kg BW (i.p.) was found to ameliorate the ISO-induced elevation in the activities of these enzymes to near control levels (#p < 0.001 vs ISO).

![Figure 9](image_url)

**Fig. 9.** Protective effect of melatonin against ISO-induced increase in the activities of (A) serum glutamate oxaloacetate transaminase, (B) total serum lactate dehydrogenase and (C) lactate dehydrogenase type 1 isoform. The rats were treated with ISO [represented in the figures as I] at a dose of 25 mg/kg BW (s.c.) twice at an interval of 24 hr. Melatonin (MEL) [represented in the figures as m] protected rats were treated with 10 mg/kg BW (i.p.) 30 min before ISO treatment [l+m].
The control (CON) rats were treated with vehicle only. Positive control rats were treated with melatonin only. Values are means ± S.E.M. of eight rats in each group; *p < 0.001 vs CON; #p < 0.001 vs l.

(b) Isoproterenol (ISO)-induced oxidative stress in rat heart and protection by melatonin:

Figure 10A demonstrates ISO-induced increase in lipid peroxidation level (25% increase versus control, $P < 0.001$) of the rat cardiac tissue. This is significantly lowered ($P < 0.001$ versus ISO) on pre-treatment of rats with melatonin. The results presented in the figure 10 B indicates a highly significant decrease in the rat cardiac tissue reduced glutathione levels due to ISO treatment (37% decrease versus control, (*p < 0.001) which is significantly ameliorated by pre-treatment of rats with melatonin (#p < 0.001 versus ISO).
Fig. 10. (A) Protective effect of melatonin against ISO-induced increase in lipid peroxidation (LPO) level of rat cardiac tissue. The rats were treated with ISO [represented in the figures as I] at a dose of 25 mg/kg BW (s.c.) twice at an interval of 24 hr. Melatonin (MEL) [represented in the figures as m] protected rats were treated with 10 mg/kg BW (i.p.) 30 min before ISO treatment [I+m]. The control (CON) rats were treated with vehicle only. Positive control rats were treated with melatonin only. Values are means ± S.E.M. of eight rats in each group; *p < 0.001 versus CON; #p < 0.001 versus I;

Fig. 10. (B) Protective effect of melatonin against ISO-induced decrease in reduced glutathione (GSH) level of rat cardiac tissue. Values are means ± S.E.M. of eight rats in each group; *p < 0.001 versus CON; #p < 0.001 versus I. Positive control rats were treated with melatonin only.

To determine the effect of ISO on the activities and the levels of the cardiac antioxidant enzymes and the role of melatonin in ameliorating the ISO-induced effects, we measured the activities and the levels of Cu-Zn-SOD [SOD 1], Mn-SOD [SOD 2], catalase (CAT) and glutathione peroxidase (GPx).

Figure 11A and 11B show the ISO-induced elevation in the activity and the level respectively of Cu-Zn SOD, an important antioxidant enzyme. There occurred a significant reduction in the activity and a complete restoration of the protein level of the enzyme when the rats were pre-treated with melatonin.
**Fig. 11.** (A) Protective effect of melatonin against ISO-induced increase in Cu-Zn SOD activity of rat heart tissue in control (CON), melatonin only (m), ISO-treated (I) and melatonin protected (l+m) rats. Values are mean ± SEM of 8 rats in each group; *P < 0.001 vs. CON. #P < 0.001 vs. I. (B) Representative result of Western blot analysis for determining the level of Cu-Zn SOD (lanes from left) of rat heart tissue in control (CON), ISO-treated (I) and melatonin (m) protected rats. The Western blot analysis was repeated at least three times. Actin served as loading control. The pixel density of bands obtained through Western blotting was quantified with image J software (NIH, USA) and the values (mean ± SEM) were presented below in the form of a bar graph. *p < 0.001 vs CON; #p < 0.001 vs I.

Figure 12 A reveals a highly significant increase in the activity of Mn-SOD in the rats treated with the same dose of ISO. The activity of Mn-SOD comes back to near control values when the rats were pre-treated with melatonin. That this enhancement of Mn-SOD activity is due to elevation in the level of Mn-SOD protein is evident from the results presented in figure 12 B. The Mn-SOD levels are significantly elevated following treatment of rats with
ISO. However, this increment is significantly reduced, though not reaching the control levels, when the rats were pre-treated with melatonin

Fig 12. (A) Effect of ISO on Mn-SOD activity of rat heart tissue. The rats were treated with ISO (I) at a dose of 25 mg/kg (s.c.). Melatonin (m) protected rats were treated with 10 mg/kg (i.p.) 30 min before ISO treatment. The control (CON) rats were treated with vehicle only. Values are mean ± SEM of 8 rats in each group; *p < 0.001 vs. CON; #p < 0.001 vs I.

(B) Representative result of Western blot analysis for determining the level of Mn-SOD (lanes from left) of heart tissue in control (CON), ISO-treated (I) and melatonin (m) protected rats. The Western blot analysis was repeated at least three times. Actin served as loading control. The pixel density of bands obtained through Western blotting was quantified with image J software (NIH, USA) and the values (mean ± SEM) are presented below in the form of a bar graph. *p < 0.001 vs CON; #p < 0.001 vs I.

Figure 13 A demonstrates ISO (25 mg/kg BW, s.c.)-induced highly significant reduction (*p < 0.001 vs Con) in catalase activity, another important antioxidant enzyme. The catalase activity was significantly restored when the rats were pre-treated with melatonin (10mg/kg BW, i.p.)
This decreased activity of catalase following ISO treatment of rats is supported further by a reduced level of the enzyme protein as is evident from western-blot analysis. The enzyme level was significantly elevated in the rats pre-treated with melatonin (Fig. 13 B).

Fig.13. (A) Protective effect of melatonin against ISO-induced decrease in catalase activity of rat heart tissue. The rats were treated with ISO (I) and melatonin (m). The control rats were treated with vehicle only. Positive control rats were treated with melatonin only. Values are mean ± SEM of 8 rats in each group; *p < 0.001 vs CON. #p < 0.001 vs I. (B) Representative result of Western blot analysis is for determining the level of catalase (lanes from left) of heart tissue in control (CON), ISO-treated (I) and melatonin (m) protected rats. The Western blot analysis was repeated at least three times. Actin served as loading control. The pixel density of bands obtained through Western blotting was quantified with image J software (NIH, USA) and the values (Mean ± SEM) were presented above in the form of a bar graph.*p < 0.001 vs CON; #p < 0.001 vs I.
Figure 14 shows that treatment of rats with ISO significantly reduced the activity of GPx in the cardiac tissue (24% versus control, P < 0.001). However, the rats when pre-treated with melatonin exhibited a near normal activity of GPx of the cardiac tissue. The melatonin only treated group of rats which constituted the positive control group showed a slight statistically insignificant enhancement of glutathione peroxidase (GPx) activity as compared to control rats.

Figures 15 A and B reveal that ISO-induced myocardial oxidative stress is associated with a reduction (40%) in the level of the enzymes GR and GST which play an essential role in the metabolism of GSH in the cardiac tissue of rats. Pre-treatment of the rats with melatonin at a dose of 10 mg /kg (i.p.) significantly restored the level of these enzymes.
Fig 15. Western blot analysis of the levels of (A) glutathione reductase (GR) and (B) glutathione-S-transferase (GST) of heart tissue of rats in control (CON), ISO-treated (I) and melatonin (m) protected rats. The Western blot analysis was repeated at least three times. Actin served as loading control. The pixel density of bands obtained through Western blotting was quantified with imageJ software (NIH, USA) and the values (Mean ± SEM) were presented above in the form of a bar graph. *p < 0.001 vs CON; #p < 0.001 vs I.

Isoproterenol-induced generation of reactive oxygen species (ROS) and protection by melatonin:
We examined whether ISO administration to rats have caused the generation of ROS. The results presented in the figure 16 A-E clearly indicate that there was an enhancement in the generation of $O_2^*$ in vivo following treatment of rats with ISO. The activities of xanthine oxidase (XO), xanthine dehydrogenase (XD), total enzyme activity, i.e., XO plus XD, XO-XD ratio and XO / XO+XD ratio all increased significantly following ISO treatment of rats. All these changes in the enzyme activity were restored to normal or near normal levels when the rats were pre-treated with melatonin indicating melatonin’s ability to neutralize free radicals in vivo. The enzyme activity in the rats treated with melatonin only (positive control group) showed no change compared to that of the control rats.
Fig. 16. Protective effect of melatonin against ISO-induced increase in the activities of (A) xanthine oxidase and (B) xanthine dehydrogenase in control (CON), melatonin only (m), ISO-treated (l) and melatonin protected (l+m) rats. Values are mean ± SEM of 8 rats in each group. *p < 0.001 vs CON, #p < 0.001 vs l. (C) Total enzyme activity (XO + XDH), (D) xanthine oxidase / xanthine dehydrogenase (XO/XDH) ratio, (E) xanthine oxidase / xanthine dehydrogenase (XO/XO+XDH) ratio.
Figure 17 illustrates the effect of melatonin on the scavenging of *OH generated in vivo following treatment of rats with ISO. Treatment of rats with ISO caused nearly a six-fold increase of endogenous generation of *OH. Pretreatment of rats with melatonin decreased the ISO-induced *OH formation to near basal level.

(c) Protective effect of melatonin against isoproterenol-induced changes in the rat cardiac tissue morphology:
Administration of isoproterenol to rats caused significant damage to the cardiac tissue morphology which is evident from the results presented in figure 18. The figure 18A shows H-E stained left ventricular tissue sections of rat heart at 200X magnification. Considerable damage to the cardiac muscle fibres along with significant infiltration of neutrophils can be observed in the ISO-treated tissue sections. Pre-treatment of rats with melatonin at a dose of 10mg/kg BW, (i.p.) for 2 days though partially prevented the ISO-induced damage to the myocardium, was unable to completely ameliorate the ISO-effect. Figure 18B shows the same H-E stained sections at 400X magnification.

The ISO-induced damage to the rat cardiac tissue morphology and inability of melatonin to completely ameliorate the damage at the given dose (i.e. 10 mg/kg, (i.p.) is also evident from figure 19 which reveals that treatment of
rats with ISO caused a significant reduction in the level of alpha-actinin, an important structural protein of myocardial tissue. However, this protein was not restored to the levels observed in the control rats when they were pre-treated with melatonin.

![Representative images (200X magnification) of haematoxylin-eosin stained left ventricular longitudinal sections of rat hearts of Control (CON), ISO (I) treated and melatonin protected (I+m) rats. (B) Similar images captured at 400X magnification.](image)

![Western blot analysis of the level of alpha-actinin (lanes from left) of rat heart tissue in control (CON), ISO (I)-treated and melatonin (m) protected rats. The Western](image)
blot analysis was repeated at least three times. Actin served as loading control. The pixel density of bands obtained through Western blotting was quantified with image J software (NIH, USA) and the values (mean ± SEM) were presented below in the form of a bar graph. \( p < 0.001 \) vs. CON.

The tissue morphological damage to the rat heart due to ISO treatment was further confirmed by the observation that there was a loss of collagen from the intercellular space compared to control group of rats and the results are presented in the figure 20(A and B). This loss of collagen was found to be partially prevented from occurring when the animals were pre-treated with melatonin. The results indicate that melatonin has the ability to provide protection to the myocardial tissue against ISO-induced damage. However, the given dose may be insufficient to completely protect the cardiac tissue from ISO-induced damage.
Fig. 20. (A) Representative images (600X magnification) of Sirius red stained left ventricular longitudinal sections of rat hearts of Control (CON), ISO (I) treated and Melatonin (I+m) protected rats. Red colour stretches indicate the tissue collagen. (B) The similar images captured by confocal laser scanning microscope for quantification of fibrosis. Arrow heads indicate collagen fibers. (C) Histogram showing % collagen volume in the ventricular tissues.

Values are means ± S.E.M. Percent collagen from 3 images from each of 3 rats of each group; *p < 0.001 vs CON; #p < 0.001 vs I.

Figure 21 shows the changes brought about to the rat cardiac endo and myocardium following ISO treatment through scanning electron microscopy (SEM). The cardiac tissue sections of the ISO-treated rats showed a perforated endocardium having cells with convoluted cell membranes. These cells, which were markedly contracted, with pronounced nuclear bulges, also had large membrane blebs covering the cell surface. A few cells appeared to be separating from each other, and a few polymorphonuclear neutrophils (PMNs) were present adhering to the endocardial cells. These ISO-induced changes in the rat heart endocardium were found to be partially protected when the rats were pre-treated with melatonin.

Fig. 21. Scanning electron micrograph (X6000) of endocardial cells of rat hearts of Control (CON), ISO (I) treated and Melatonin (I+m) protected rats. Arrow heads indicate perforated tissue structure and membrane blebbings.
Isoproterenol-induced changes in the hemodynamic parameters of rat heart and protection by melatonin:

As shown in table 1, the systolic blood pressure was significantly ($p < 0.001$ vs Con, n=6) decreased in ISO-treated rats ($P_{\text{max}}$, 72.10 ± 0.52 mm Hg) compared to those of rats of the control group ($P_{\text{max}}$, 116.60 ± 1.60 mm Hg). The cardiac output (CO) was significantly ($p < 0.001$ vs Con, n=6) reduced in ISO-treated rats. Pre-treatment of rats with melatonin for 2 days significantly ($p < 0.001$ vs Con, n=6) increased the cardiac output. The parameters of systolic ($dP/dt_{\text{max}}$) as well as diastolic function ($dP/dt_{\text{min}}$) were significantly reduced by ISO compared to control rats which were restored significantly by melatonin. These data indicate that pre-treatment of rats with melatonin significantly restored the ISO-induced alterations of hemodynamic parameters ($##p < 0.001$ vs ISO).

Table 1. Hemodynamic parameters in control hearts and those treated with isoproterenol (ISO) with or without melatonin (MEL)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>CONTROL</th>
<th>MELATONIN</th>
<th>ISO</th>
<th>ISO+MEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate (bpm)</td>
<td>367 ± 3.00</td>
<td>375 ± 2.00</td>
<td>348 ±1.00 #</td>
<td>393 ± 2.00 ##</td>
</tr>
<tr>
<td>$P_{\text{max}}$ (mmHg)</td>
<td>116.60 ± 1.60</td>
<td>125.60 ± 1.80</td>
<td>72.10 ± 0.52 #</td>
<td>116 ±1.50 ##</td>
</tr>
<tr>
<td>$P_{\text{min}}$ (mmHg)</td>
<td>20.10 ± 1.50</td>
<td>19.10 ± 2.0</td>
<td>18.745 ± 0.23</td>
<td>10.00 ± 0.40</td>
</tr>
<tr>
<td>CO (uL/min)</td>
<td>45971 ± 744</td>
<td>42652 ± 450</td>
<td>18416 ± 211 #</td>
<td>26775 ± 470 ##</td>
</tr>
<tr>
<td>$dP/dt_{\text{max}}$ (mmHg/s)</td>
<td>6975 ± 225</td>
<td>7250 ± 230</td>
<td>1913 ± 20 #</td>
<td>4158 ± 91 ##</td>
</tr>
<tr>
<td>$dP/dt_{\text{min}}$ (mmHg/s)</td>
<td>-6019 ± 21 #</td>
<td>-5913 ± 210</td>
<td>-1510 ±14 #</td>
<td>-4248 ± 64 ##</td>
</tr>
</tbody>
</table>
The therapeutic effect of melatonin has been well documented in various pathophysiological conditions including cardiovascular diseases [78]. Here, in our work, we have demonstrated that melatonin not only protects the heart from myocardial injury but also improves ventricular function in the ISO-induced ischemic rats. Besides, we have further provided evidence that melatonin improves cardiac physiology of ISO-treated rats mainly due to its antioxidant ability.

Isoproterenol (ISO) when administered at high doses causes myocardial ischemia and infarction via beta adrenergic pathway [79]. In "Chapter 1" of the present study, ISO (Isoproterenol bitartrate) was administered at the doses of 12.5, 25.0 and 50.0 mg/kg BW, (s.c.) twice at an interval of 24 hr to induce myocardial ischemia in rats. A significant dose-dependant increase of SGOT level in the ISO-treated rats indicated the development of myocardial ischemia in the rat heart. Serum GOT is one of the diagnostic enzymes of clinical importance for the detection of myocardial infarction. When the rats were pre-treated with melatonin, the activity of this enzyme was restored to near control levels. This restoration of SGOT activity by melatonin also showed a clear dose-dependant pattern when the rats were pre-treated with this indole at the doses of 5.0, 10.0, 20.0 and 40.0 mg/kg BW (i.p.), respectively.

That treatment of rats with ISO induces oxidative stress is clearly evident from a highly significant increase in the levels of cardiac tissue lipid peroxidation (LPO) and also a highly significant decrease in the GSH content of the cardiac tissue. The alterations in these two parameters, recognized as the primary bio-markers of oxidative stress, were found to be ameliorated dose-dependently by melatonin. Lipid peroxidation may be due to the oxidation of ISO to semiquinones which react with oxygen to produce $O_2^-$ and $H_2O_2$ [80]. Catecholamines readily form chelate complexes with metal ions such as iron, copper and manganese, which strongly catalyze oxidation of catecholamines [80]. Copper and iron are mobilized in the coronary flow following myocardial ischemia [81]. Both these ions are present in the
coronary flow fraction in a redox active form that supports free radical-mediated deleterious reactions [81]. Another study revealed that catecholamines undergo cyclization to aminochromes. This process can occur enzymatically or through autoxidation and involves the formation of free radicals. Aminochromes are highly reactive molecules that can cause oxidation of protein sulfhydryl groups and deamination catalysis among other deleterious effects [5]. Melatonin may reduce LPO levels by interfering with any of the steps in catecholamine metabolism or by scavenging the free radicals generated due to redox-active transition metals like copper or iron. Melatonin may also reduce the level of LPO by detoxifying the transition metals that are reported to be mobilized following myocardial ischemia [81]. The dose-dependant restoration of the tissue GSH levels by melatonin may be the outcome of an alteration in the glutathione metabolizing pathway.

We further studied the ISO-induced damage to rat cardiac tissue morphology by H and E staining of left ventricular sections. The cardiac tissue sections show a clear dose-dependant deterioration of the myocardial tissue morphology due to ISO treatment resulting in damage to the myofibrils as characterized by a loss of cardiac myofibers and a mononuclear cell infiltration. The ISO-induced cardiac tissue damage was found to be dose-dependently prevented when the rats were pre-treated with melatonin.

We also studied the dose-dependent effect of ISO on the activity of the antioxidant enzymes, like Cu-Zn SOD and catalase. The activity of the cytosolic Cu-Zn SOD was found to increase with ISO administration while the catalase activity was found to decrease with increasing doses of ISO. Many studies indicate over expression of various SODs which confers significant protection against ischaemia-reperfusion injury [2]. However, when $O_2^*$ levels are high, several enzymes vital to cardiac function, are vulnerable to inactivation by this free radical. The decrease in catalase activity after ISO administration may be due to excessive generation of $O_2^*$ leading to the inactivation of the enzyme. Superoxide anion free radical ($O_2^*$) is small enough to gain access to the hemes of catalase and might convert the resting enzyme to ferro-oxy state (compound III) which is known to be
inactive [82]. When the rats were pre-treated with melatonin, this small indole was found to lower the SOD activity and increase the catalase activity dose-dependently.

From our dose-dependent studies, we found the dose of 25 mg/kg BW (s.c.) as the minimum effective dose for ISO while in case of melatonin the dose of 10 mg/kg BW (i.p.) was used as the minimum effective dose. Further studies were carried out using these minimum effective doses of ISO and melatonin respectively.

As was evident from our dose-dependent studies, ISO administration to rats, at a dose of 25 mg/kg BW (s.c.) caused myocardial infarction shown by a significant increase in SGOT activity which was brought back to near control levels by pre-treatment of the rats with melatonin at the dose of 10 mg/kg BW (i.p.). In order to further confirm the ISO-induced cardiac ischemic injury, we measured the activity of a more specific marker enzyme, serum Lactate Dehydrogenase (LDH) and its Type 1 isoform (LDH1). The activities of both of these enzymes were found to be significantly increased due to ISO treatment. Pre-treatment of rats with melatonin was found to reduce the levels of activity of LDH and LDH1 to near control values indicating strongly its ability to protect the heart against isoproterenol induced injury.

The fact that the ISO-induced ischemic myocardial injury was induced by oxidative stress was evident from our dose-response studies which showed a clear increase in the level of LPO and a decrease in tissue GSH content. Using the minimum effective doses of both ISO and melatonin, we again demonstrated that ISO causes a significant rise in the tissue LPO levels and a decrease in tissue GSH content. Both these effects were ameliorated when the rats were pre-treated with melatonin. We also observed a reduction in the protein level of the two key enzymes of the glutathione synthesizing pathway, glutathione reductase (GR) and glutathione-S-transferase (GST), following ISO treatment. The protein levels of both these enzymes were found to be restored to control levels when the animals were pre-treated with melatonin. This indicates that melatonin raises the GSH level in vivo, in the face of oxidative challenge, due to increased biosynthesis. Melatonin has also been shown to restore the tissue GSH levels in various models of
oxidative stress, perhaps, through its stimulatory effect on GSH synthesis [83].

We further studied the effect of ISO on the key antioxidant enzymes of the cardiac tissue and melatonin’s ability to protect them. The activity and the protein levels of both Cu-Zn SOD and Mn-SOD showed significant increase when compared to control rats following ISO treatment. The increase in Cu-Zn SOD (the cytosolic enzyme) as well as Mn-SOD (the mitochondrial enzyme) activity in ISO-treated animals may probably be an adaptive response towards oxidative stress. A decreased activity of catalase and glutathione peroxidase (GPx) following ISO-treatment of rats, as observed, is expected to further aggravate the situation of oxidative stress. Interestingly, melatonin at the low pharmacological dose of 10mg/kg, i.p. [minimum effective dose], restored the activities of the key antioxidant enzymes to that observed in the control rats. The increased SOD and a decreased catalase protein level, as evident from the western blot analysis, demonstrate that increased and decreased activity of the key antioxidant enzymes (i.e. SOD and catalase) are the result of altered protein expression following treatment of rats with ISO. Once again, pre-treatment of rats with melatonin restored the antioxidant enzyme protein level to near normal. These observations strongly support the notion that melatonin protects tissues and organs against oxidative stress through its indirect antioxidant mechanism(s).

In our current studies, we have assessed the generation of O$_2^*$ in vivo in rat heart tissue following treatment with ISO by determining the activities of xanthine oxidase (XO) and xanthine dehydrogenase (XDH). Our results clearly reveal that following ISO treatment of rats, the activities of XO and XDH are highly significantly increased compared to the control rats with a concomitant increase in the XO plus XDH, XO / XDH ratio, XO / XO+XDH ratio. This strongly indicates that metabolic reactions involving these two enzymes do serve as the source of this ROS. Earlier workers have also indicated the involvement of XO in free radical production [56]. Moreover, our studies further demonstrate nearly a six-fold rise in the endogenous generation of hydroxyl radical ('OH) following treatment of rats with ISO. Pre-treatment of rats with melatonin was shown to reduce the endogenous level of 'OH to
basal level. This clearly documents melatonin's ability to directly neutralize \(^{1}OH\) in vivo. Melatonin's ability to scavenge free hydroxyl radical \(^{1}OH\) in vivo has also been shown by earlier workers in different models of oxidative stress [20, 46, 25, 55].

We also studied the protein expression level of one of the important structural proteins of the cardiac tissue of rat, the alpha-actinin, by western blot analysis. Treatment of rats with ISO significantly reduced the level of alpha-actinin when compared to the levels observed in the control rats. However, melatonin, at the otherwise minimum effective dose, did not restore the level of this protein to that observed in the control rats. The reason for this may be that for complete restoration, the dose of melatonin may be insufficient or the time required for restoration of this protein may be longer than the time period for which the experiments were carried out. This damage to one of the myocardial structural proteins was further evident from our histological studies of the cardiac tissue sections. Haematoxylin-eosin stained cardiac tissue sections showed significant cellular damage and degeneration following ISO treatment. Pre-treatment of rats with melatonin for only two days was unable to completely restore the cardiac tissue morphology. Additionally, the depletion of collagen in the cardiac tissue following ISO treatment of rats was also found to be partially but significantly ameliorated by pre-treatment of the animals with melatonin for two days. This observation was further supported from our studies of the cardiac tissue sections with confocal microscopy. Besides, the left ventricular tissue morphological studies through scanning electron microscopy (SEM) also revealed severe tissue injury at the infarcted site following ISO treatment of rats. This tissue injury was also found to be partially but significantly restored when the rats were pre-treated with melatonin. The results indicate the ability of melatonin to serve not only as a protective antioxidant but also point toward its role in promoting recovery of tissue injury resulting from oxidative onslaught. For complete recovery from ISO-induced tissue injury, either a higher dose of melatonin or a longer duration of melatonin treatment or both may be required. Continuation of
treatment of rats with melatonin after the withdrawal of ISO treatment was shown to have greater benefit, and, the results are presented in Chapter-II.

Melatonin protects the isolated rat heart from ischemia/reperfusion injury (I/R) by scavenging \(^\cdot\)OH, significantly improving left ventricular function and duration of ventricular tachycardia or ventricular fibrillation [46]. The results of another study have shown a spectacular protection against I/R injuries (on arrhythmias as well as on infarct size) in rats pre-treated with melatonin [46]. This observation suggests that melatonin could have a potential clinical application in the treatment of myocardial ischemia, even if the mechanism(s) underlying this protection remain to be determined [84, 85]. Night-time melatonin synthesis is reduced in patients with coronary artery disease [86]. Whether a decreased melatonin level may be a predisposing factor for coronary artery disease, or whether the occurrence of coronary artery disease decreases melatonin synthesis remains to be determined [87].

Oxidative mutilation of essential bio-macromolecules involved in cardiac metabolism and cardiac contractility leads to diminished cardiac function [7]. Isoproterenol, at the present dose (25 mg /kg BW, s.c.; two injections 24 hr apart) caused a significant increase in heart rate and systolic pressure with peripheral vasodilation. The ISO eventually leads to diminished cardiac function. Our experiments, however, demonstrated that pre-treatment of rats with melatonin restored the cardiac function to that observed in the control rats. This improvement of cardiac function in rats by melatonin may be of future therapeutic importance.