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Research Article

EXPLORATION OF CARDIOPROTECTIVE AND ANTIOXIDANT ACTION OF BILIRUBIN IN ISOPRENALINE-INDUCED ISCHEMIA MODEL OF MALE WISTAR ALBINO RATS

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ABSTRACT

Objectives: Ischemic heart disease is one of the leading causes of death worldwide. Bilirubin, an endogenous product of heme catabolism, has remarkable antioxidant properties. This study was planned with an aim to evaluate cardioprotective potential of bilirubin in isoprenaline (ISO)-induced rat myocardial ischemia model.

Methods: In this study, a total of 24 adult male Wistar albino rats (200–250 g) were divided into four groups of six rats each. Groups I, III, and IV, respectively, received distilled water (10 mL/kg bw), bilirubin (40 mg/kg bw), and bilirubin (60 mg/kg bw) intraperitoneally (i.p.) for 21 days. Along with that, Groups III and IV received ISO (85 mg/kg bw) subcutaneously (s.c.) on 20th and 21st day. Group II received ISO (85 mg/kg bwi. p.) on 20th and 21st day. After 24 h of ISO administration, rats were sacrificed and biochemical and histopathological parameters were assessed. These parameters were evaluated: Cardiac biomarkers-lactate dehydrogenase (LDH) and creatine kinase (CK-MB) and antioxidant-glutathione reductase (GR). Isolated heart specimens were processed for light microscopy.

Results: Administration of bilirubin in Groups III and IV significantly prevented ISO-induced elevation of CK-MB and LDH levels as compared to Group II. Furthermore, there is a significant increase in the levels of GR in Groups III and IV compared to Group II. These changes were significantly higher in Group IV (high dose) compared to Group III (low dose). Light microscopic findings of the myocardium in bilirubin-treated group revealed a well preserved normal morphology of cardiac muscle with minimal evidence of myocardial injury as compared to ISO-treated hearts.

Conclusion: Pre-administration with bilirubin is protective against ISO-induced myocardial infarction. This beneficial effect is most likely due to its antioxidant property. This study may trigger interest toward the use of bilirubin as a cardioprotective agent in myocardial infarction.

Keywords: Bilirubin, Antioxidants, Myocardial infarction, Isoprenaline.

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INTRODUCTION

Acute myocardial infarction (AMI) is a most dreaded sequel of ischemic heart diseases (IHD) resulting from myocardial ischemia – an imbalance between myocardial oxygen demand and oxygen supply. Despite emergence of modern medications and interventions, AMI remains leading cause of death in present era. Factors responsible for development of AMI include: Atherosclerosis, emboli, vasospasm, thrombosis, reactive oxygen species (ROS), reduced oxygen carrying capacity, or increased oxygen demand [1].

ROS is chemically reactive free radicals containing oxygen and formed as a natural by-product of aerobic metabolism. To prevent, the ROS-induced oxidative damage human body has developed an antioxidant defense system, which is capable of inhibiting oxidation of other molecules by donating an electron to the free radicals without destabilizing themselves and includes enzymes and free radical scavenging activities to neutralize the ROS.

Studies suggested that during myocardial ischemia, residual oxygen may lead to formation of ROS. The effect of increased oxidative stress leads to the oxidative damage of proteins, lipids, DNA, and enzymes of myocardial cells that involved in energy production. These factors contribute to myocardial cell damage, extensive necrosis, and cellular edema seen with ischemia.

Bilirubin, a product of heme degradation and earlier believed to be non-functional and toxic, in recent years, has shown to exert strong antioxidant activity. Recent researches have showed that people with higher serum levels of bilirubin in their body compared to normal people have less chances of getting coronary artery diseases which may be due to antioxidant activity of bilirubin [2]. It was also found that in Gilbert's syndrome, patients with congenital hyperbilirubinemia, incidence of IHD was low compared to the normal population. With this background, the present study was carried out, to explore effects of bilirubin in myocardial ischemia, induced by isoprenaline (ISO) in male Wistar albino rats.

The aim of the present study was to assess the possible cardioprotective effect of intraperitoneal administration of bilirubin in myocardial infarction. Moreover, if there is any cardioprotective effect then whether it is due to antioxidant effect of bilirubin or not.

METHODS

Drugs

1. ISO: It was purchased in powder form from Sigma Chemical Co. It was dissolved in distilled water (50 mg/mL) and used immediately for subcutaneous (S.C.) administration.
2. Bilirubin: It was purchased in powder form from Sisco chemical Co. Bilirubin powder was dissolved in 0.1 M NaOH to get the concentration of 10 mg/mL and drops of HCL were added to get the pH of 8±1. It was given to the rats through intraperitoneal injections.

Experimental animals

All experiments were performed after prior permission from the Institutional Animal Ethics Committee, Government Medical College, Surat, Gujarat, India.

Wistar albino male rats (200±50 g) were procured from the Central Animal House of Government Medical College, Surat, Gujarat, India. Twenty-four adult male albino rats were housed in clean polypropylene

cages under standard conditions of humidity, temperature, and fed with standard diet and water ad libitum.

Induction of myocardial injury

Myocardial infarction was induced in rats by giving ISO (85 mg/kg) s. c. dissolved in distilled water for two subsequent days, at the interval of 24 h.

Experimental design

There were total four groups in the study. Each group contained six animals. Twenty-four rats were allocated randomly into each group.

- Group 1 (Control Group): Rats were given distilled water 10 mL/kg intraperitoneally for 21 days
- Group 2 (ISO-Treated Group): Rats were given ISO 85 mg/kg S.C. on 20th and 21st day at the interval of 24 h
- Group 3 (Test Group-1): Rats were given bilirubin at 40 mg/kg I.P. for 21 days and ISO 85 mg/kg S.C. on 20th and 21st day at the interval of 24 h
- Group 4 (Test Group-2): Rats were given bilirubin at 60 mg/kg I.P. for 21 days and ISO 85 mg/kg S.C. on 20th and 21st day at the interval of 24 h.

After 24 h of second ISO injection, rats were weighed and then anesthetized with thiopentone sodium and blood was collected by cardiac puncture for biochemical estimation and heart was dissected out for histopathological examination.

Biochemical estimation

Blood was collected by cardiac puncture. The serum was separated by centrifugation at 3000 rpm at 30°C for 10 min. Then, serum myocardial isoenzyme creatine kinase – MB (CK-MB), lactate dehydrogenase (LDH), glutathione reductase (GR), and serum bilirubin levels were estimated using standard kits.

Histopathological study

After blood collection heart was dissected out and was immediately fixed in 10% formalin. Paraffin sections of 5 μm thickness were prepared and stained with hematoxylin and eosin (H and E).

Data analysis

The quantitative variables are presented as mean ± SEM. The statistical analysis was performed using analysis of variance followed by Tukey Kramer's multiple comparison test to compare variables among different groups. $p<0.05$ was considered significant.

RESULTS

Biochemical results: (LDH, CK-MB, and GR)

As shown in Table 1 and Graph 1.

ISO-administered group of animals showed a significant increase in levels of LDH and CK-MB as compared to control group. Treatments with low-dose and high-dose bilirubin significantly decreased levels of LDH and CK-MB compared to ISO-treated group.

ISO-administered group showed a significant decrease in the GR levels as compared to the control group. Both bilirubin-treated groups showed significantly increase GR level as compared to ISO-administered group. Group IV showed significant increase in the GR level as compared to Group III.

Histopathology (light microscopy)

Group I-control group

H- and E-stained sections of heart showed the normal architecture of myocardium while branching and anastomosing cardiac muscle fibers running in different directions. Cardiomyocytes revealed normal vesicular nuclei. Nuclei of connective tissue cells could be noticed in the intestinal tissue between the cardiac muscle fibers (Fig. 1a and b)

Group II-ISO-treated group

H- and E-stained sections of heart from rats treated with ISO alone showed muscle splitting, edema, RBC extravasation with hemorrhage (Fig. 2a and b), inflammatory infiltration, and hyalinization (Fig. 2c).

Fragmentation of cardiac muscle fibers was noted. There was also infiltration with mononuclear cells and some of the blood vessels appeared congested. Extravasation of RBC was seen both perivascular as well as interstitial between the cardiomyocytes.

Group III-bilirubin low-dose group

H- and E-stained sections of hearts from rats of this group showed moderate inflammatory infiltration, minimal edema, and minimal muscle splitting (Fig. 3a and b).

Group IV-bilirubin high-dose group

H- and E-stained sections of hearts from rats of this group showed minimal edema only, no muscle splitting or inflammatory infiltration, no hyalinization (Fig. 4a and b).

DISCUSSION

IHD is still leading cause of mortality despite improvements in prevention and treatment strategies during past few years. Various factors work together to supply myocardium, its needed amount of oxygen and imbalance between myocardial oxygen demand and supply leads to myocardial infarction. Several mechanisms of myocardial tissue injury include: oxygen deprivation and depletion of high energy phosphates, osmotic stress, lysosomal activation, intracellular calcium overload, complement activation, apoptosis, inflammatory cell infiltration, free radical formation, and heat shock protein production [1].

Accumulation of free radical generation is an important etiological mechanism for the development of myocardial infarction, in which there is either increased generation of ROS or inadequate antioxidant defense system. Earlier it was believed that oxidative stress is seen in reperfusion injury only, but recent studies have suggested that small amount of oxygen may still be present in myocardial tissue during ischemia, giving rise to the possibility of generation of ROS even before reperfusion [3]. However, to prevent or decrease the oxygen radicals induced damage, human body has developed antioxidant defense mechanisms, including enzymatic and free radical scavenging activities to neutralize these radicals after they have formed. Some important natural antioxidants are as follows: GR, superoxide dismutase, carotenoids, vitamin C, vitamin E, bilirubin, etc.

Table 1: Mean±SEM values of LDH, CK-MB, GR, and serum bilirubin among different groups

Group	LDH (U/L)	CKMB (U/L)	GR (U/L)	S. bilirubin (mg/dL)
Group I (normal control)	537.83±21.26	603.5±77.71	122.95±1.88	0.10±0.003
Group II (ISO control)	1108.67±36.79*	1167.5±38.46*	68.67±8.95*	0.11±0.02
Group III (low-dose bilirubin)	943.5±54.88^	851.17±11.74^	82.7±3.13^	0.21±0.02$^{\#}$
Group IV (high dose bilirubin)	623.67±61.53^	781.83±10.68^	110.61±5.02^	0.21±0.02$^{\#}$

Data are expressed as Mean±SEM (n=6 for each group). *$p<0.05$ as compared Group I, ^$p<0.05$ as compared to Group II, $^{\#}p<0.05$ as compared to Group I by one-way analysis of variance followed by Tukey Kramer's multiple comparison test. ISO: Isoprenaline, LDH: Lactate dehydrogenase, CK-MB: Creatine kinase-MB

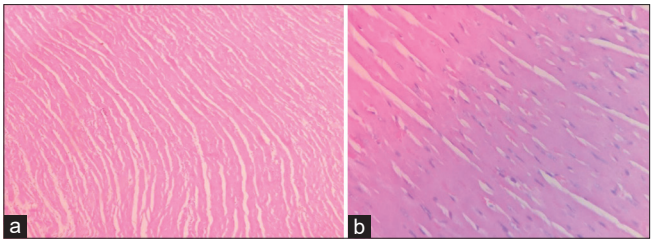


Fig 1: (a) (×10 view): Photomicrograph in ×10 view of normal myocardium of control group. The normal architecture of the myocardium with branching and anastomosing cardiac muscle fibers running in different directions. (b) (×40 view): Photomicrograph in ×40 view of normal myocardium of control group. Branching of cardiac muscles as well as nuclei of cardiac muscle can be seen. Nuclei of connective tissue cells could be noticed in the interstitial tissue between the cardiac muscle fibers

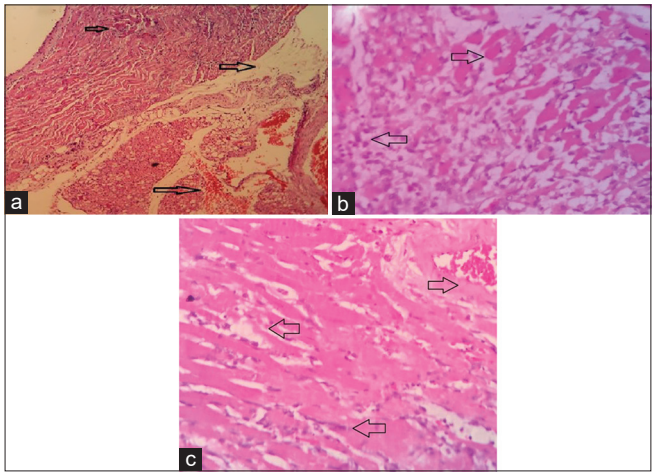


Fig 2: (a) (×10 view): Photomicrograph of a section of the myocardium of an ISO-treated group showing. First arrow: Muscle splitting, Second arrow: Edema, Third arrow: RBC extravasation and hemorrhage. (b) (×40 view): Photomicrograph of a section of the myocardium of an ISO-treated group in ×40 view showing. First arrow: Hyalinization, Second arrow: Inflammatory infiltration. (c) (×40 view): Photomicrograph of a section of the myocardium of an ISO-treated group in 40x view showing. First Arrow: RBC extravasation and hemorrhage, second arrow: muscle splitting and edema, and third arrow: inflammatory infiltration

Bilirubin – an endogenous pigment and ultimate breakdown of heme degradation, was earlier believed to be toxic waste product, but, recent studies, it is found to have antioxidant activity [4]. After the discovery of bilirubin as an antioxidant, there have been plenty of experiments suggesting benefits of antioxidant activity of bilirubin. It has been observed that there is an inverse relationship between bilirubin levels and atherosclerosis risk and it has been supported by meta-analysis which suggests that bilirubin is a protective factor. Higher serum bilirubin is associated with decreased risk for the early familial coronary artery disease. Different forms of bilirubin (albumin-bound, conjugated, and free) were found to have protective effects against peroxyl radicals involved in ischemia-reperfusion injuries, and even protect against peroxidation of low-density lipoproteins, thus providing protection against cardiovascular diseases [5]. Low serum bilirubin concentration was also proposed to be useful as a provisional risk factor of coronary artery calcification based on the finding that an increase of 1μmol/L in serum bilirubin concentration was associated with 14% decrease in the coronary artery calcification scores [6]. It was found that in patients of Gilbert syndrome, in which there is decreased expression of the enzyme UDP-glucuronosyltransferase type A1A, the enzyme almost

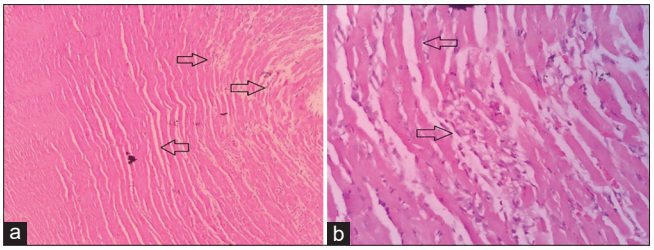


Fig. 3: (a) (×10 view): Photomicrograph of a section of the myocardium of low-dose bilirubin-treated group (40 mg/kg) in ×10 view showing. First arrow: Minimal inflammatory infiltration, Second arrow: Minimal edema and hyalinization, Third Arrow: Moderate muscle splitting. (b) (×40 view): Photomicrograph of a section of the myocardium of low-dose bilirubin-treated group (40 mg/kg) in ×40 view showing. First arrow: Minimal edema and muscle splitting, Second arrow: Minimal inflammatory changes

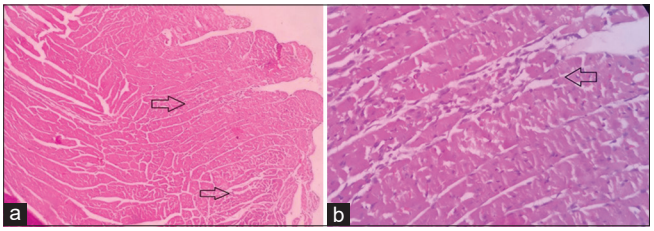
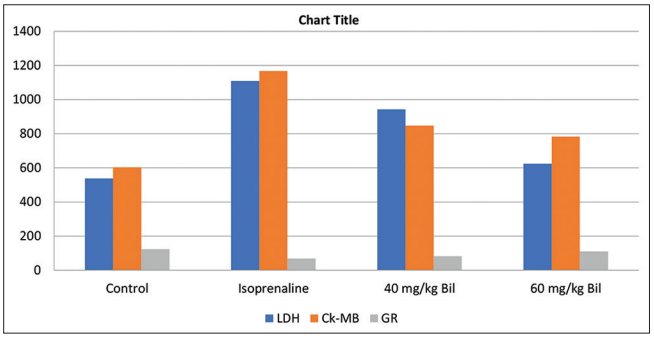


Fig 4: (a) (×10 view): Photomicrograph of a section of the myocardium of high dose bilirubin-treated group (60 mg/kg) in ×10 view showing. First Arrow: Less inflammatory infiltration, second arrow: less edema, no muscle splitting, no hyalinization, no extravasation of RBCs. (b) (×40 view): Photomicrograph of a section of the myocardium of high-dose bilirubin-treated group (60 mg/kg) in ×40 view showing. First arrow: Less inflammatory infiltration, and very minimal changes of myocardial infarction



Graph 1: Histogram comparing the mean values of LDH, CK-MB, and GR between all groups. Values (mean ± SEM) of LDH, CK-MB, GR, and serum bilirubin among different groups: Data are expressed as mean±SEM (n=6 for each group). *p<0.05 as compared Group I, ^p<0.05 as compared to Group II, #p<0.05 as compared to Group I by one-way analysis of variance followed by Tukey Kramer's multiple comparison test

responsible for bilirubin conjugation, and as a result serum unconjugated bilirubin tends to be elevated by 2-3-fold individuals; the prevalence of ischemic heart disease is 2% compared to 12% of the general population [7]. All these experiments suggested possible aid of bilirubin in the prevention of myocardial infarction as ROS and oxidative stress plays a pivot role in pathogenesis of myocardial infarction.

In the experimental animal models, pathological myocardial infarction can be mimicked by many methods. ISO, a sympathomimetic

ß-adrenergic receptor agonist, causes severe stress to the myocardium resulting in infarct such as necrosis of heart muscles and the rat model of ISO-induced myocardial necrosis is accepted as a standardized model to evaluate several cardiac dysfunctions and to study the effects of various natural and synthetic cardioprotective agents.

The myocardium contains an abundant amount of diagnostic marker enzymes such as cardiac troponin (I or T), Ck-MB, LDH, AST, and ALT for myocardial infarction and if a troponin assay is not available, the best alternative is the measurement of CK-MB isoenzyme (CK-MB) in the blood and the serum levels of these marker enzymes reflect the alterations in membrane integrity and/or permeability and are the best way to estimate the extent of infarction.

In the present study, 24 rats were divided into four groups with six rats in each group and ISO-induced myocardial infarction was produced in Groups II, III, and IV while intraperitoneal bilirubin was administered in Groups III and IV in doses of 40 mg/kg and 60 mg/kg, respectively. ISO-administered group of animals showed a significant increase in the levels of LDH and CK-MB as compared to the control group. Treatment with low-dose bilirubin (40 mg/kg) significantly decreased levels of LDH and CK-MB as compared to ISO-administered group. Treatment with high-dose bilirubin (60 mg/kg) also significantly decreased levels of LDH and CK-MB as compared to ISO-administered group. The reduction was more in rats with high dose of bilirubin administration compared to low-dose bilirubin rats.

ISO-administered group showed a significant decrease in the antioxidant GR level as compared to the control group. Both bilirubin-treated groups showed significantly increase GR level as compared to ISO-administered group. Levels of serum bilirubin were increased in Group III and IV. This increase was significant compared to Group I. These results suggest that therapeutic intervention with antioxidant activity may be useful in preventing deleterious cardiac lesions.

Bilirubin-treated group, along with improvement in cardiac markers and antioxidant levels, also showed protection of myocardial injury in histopathological examination. It reduced the extent of myocardial damage and thereby restricted the leakage of these enzymes from myocardium as seen in light microscopy. Both low-dose and high-dose prevent myocardial damage induced by ISO but not complete restoration of myocardial morphology as normal. However, high dose showed less damage than low dose of bilirubin in histopathological examination.

From this observation, it can be suggested that the exogenous bilirubin administration demonstrates concentration-dependent antioxidant activity which might be correlated with beneficial effects observed on myocardium as suggested by histopathological examination and biochemical markers of myocardial damage.

From the present experimental study, it can be concluded that bilirubin preserved the normal histological architecture of the myocardium and prevented elevation of LDH as well as CK-MB following experimental myocardial injury by isoprenaline administration. As bilirubin administration maintained GR level, beneficial effect seems to be related to its antioxidant property. Further, experimentation and exploration may be carried out in similar direction.

AUTHORS' CONTRIBUTIONS

Dr. C. R. Acharya presented the idea for the research and supervised the project. Dr. Asif Barejia and Dr. Ankit Patel carried out the experiment and result analysis. Dr. Vipul Navadiya and Dr. Aashal Shah helped with the statistical data. All authors discussed the results and contributed to the final manuscript.

CONFLICTS OF INTEREST

We have no conflicts of interest to disclose.

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REFERENCES

1. De Zwaan C, Daemen MJ, Hermens WT. Mechanisms of cell death in acute myocardial infarction: Pathophysiological implications for treatment. Neth Heart J 2001;9:30-44. PMID 25696691
2. Novotný L, Vítek L. Inverse relationship between serum bilirubin and atherosclerosis in men: A meta-analysis of published studies. Exp Biol Med (Maywood) 2003;228:568-71. doi: 10.1177/15353702-0322805-29, PMID 12709588
3. Becker LB, Van den Hoek TL, Shao ZH, Li CQ, Schumacker PT. Generation of superoxide in cardiomyocytes during ischemia before reperfusion. Am J Physiol 1999;277:H2240-6. doi: 10.1152/ajpheart.1999.277.6.H2240, PMID 10600842
4. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science 1987;235:1043-6. doi: 10.1126/science.3029864, PMID 3029864
5. Yamaguchi T, Terakado M, Horio F, Aoki K, Tanaka M, Nakajima H. Role of bilirubin as an antioxidant in an ischemia-reperfusion of rat liver and induction of heme oxygenase. Biochem Biophys Res Commun 1996;223:129-35. doi: 10.1006/bbrc.1996.0857, PMID 8660358
6. Tanaka M, Fukui M, Tomiyasu K, Akabame S, Nakano K, Hasegawa G, *et al*. Low serum bilirubin concentration is associated with coronary artery calcification (CAC). Atherosclerosis 2009;206:287-91. doi: 10.1016/j.atherosclerosis.2009.02.010, PMID 19296952
7. Vítek L, Jirsa M, Brodanová M, Kalab M, Marecek Z, Danzig V, *et al*. Gilbert syndrome and ischemic heart disease: A protective effect of elevated bilirubin levels. Atherosclerosis 2002;160:449-56. doi: 10.1016/s0021-9150(01)00601-3, PMID 11849670