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Original Article

Cardioprotective effect of indirubin in experimentally induced myocardial infarction in wistar rats

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Abstract: Recently, there has been as enhanced interest on global level to recognize the potent antioxidant compounds which are pharmacologically active with less or no side effects. Thus, the current investigation was intended to scrutinize the protective effect of indirubin on the cardiac marker, such as, enzymes, LDH isoenzyme, cardiac troponin-T (cTnT), antioxidant enzymes marker and lipid peroxidation (LPO) in response of isoproterenol (ISO)-induced myocardial infarction (MI) in Wistar rats. The experimental animals were categorized into following groups: Group I received saline; Group II received Indirubin (10 mg/kg); Group III received ISO (100 mg/kg) and Group IV received ISO + indirubin (10 mg/kg) for continuous 10 days. The ISO induced MI injury was confirmed via enhanced level of enzymes markers viz., creatine kinase-MB, creatine kinase, lactate dehydrogensae, troponin-T, alanine transaminase (ALT) and aspartate transaminase (AST) in the rats serum. The enhanced expression of LDH (1 and 2) isoenzyme bands were also observed in the ISO induced MI rats. We have also estimated the level of LPO in the heart and plasma, which was found to be significantly ($P<0.05$) improved. Moreover, the marker of enzymatic antioxidant enzymes viz., glutathione reductase (GRx), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and superoxide dismutase (SOD) in the heart, and the level of non-enzymatic antioxidant marker viz., vitamin (C, E) in heart and serum were found to be considerably ($P<0.05$) reduced in the ISO induced MI in Wistar rats. Whereas, the ISO control Wistar rats showed significant ($P<0.05$) increase in the uric acid level in the plasma. The Indirubin treated rats confirmed the significant protective effect via modulation of all biological and antioxidant parameters tested. The result of the investigation was further found in agreement of the histopathological studies of the indirubin treated rats which clearly showed recovery from the myocardial infarction. Thus, on the basis of that, it has been suggested that indirubin showed protection of myocardial tissues against the ISO persuaded oxidative stress.

Keywords: Cardiac troponin-T, indirubin, myocardial infarction

Introduction

Myocardial infarction (MI) is commonly referred as ischemic heart disease which is observed owing to sudden constant restriction of the myocardial blood delivery. This further progressed to the development of the myocardium necrosis and confirmed via alteration of several patho-physiological and biochemical parameters viz., hyperlipidemia, hyperglycemia and lipid peroxidation, etc. The isoproternol (ISO) is recognized as a synthetic catecholamine and when applied in the large doses, it induces MI [1, 2]. The ISO induced cardiac injury found to be aggravated in response of production of free radicals (cytotoxic mediators) via catecholamines auto-oxidation. Thus, the excessive generation of free radicals may results in the loss of membrane integrity and increase permeability. The continuous generation of free radical may assault the polyunsaturated fatty acids (PUFAs) in the membrane because of the presence of peroxyl radicals. These free radicals will also assault the adjacent fatty acids in the membrane and causes the induction of chain reaction of LPO. The production of the end product such as LPO is also considered to be very harmful and accountable for the organ and tissue injury [3, 4]. Thus, in the present study, for the estimation of damage to the cardiac tissues, we have examined the expression of various cardiac enzymes in the serum viz., CK, CK-MB, LDH, AST and ALT. cTnT is a contractile protein which is commonly present in the

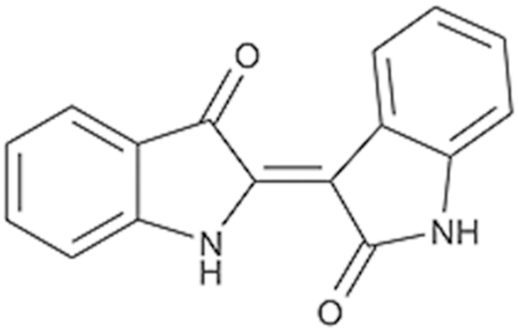

Figure 1. Structure of Indirubin.

serum, and its concentration in the free form suggested the incidence of myocardial necrosis, thus its level will also be quantified. The estimation of the LDH isoenzymes is essential for the highest specificity for cardiac damage [5, 6]. As discussed above, the increased level of LPO causes generation of lipid hydroperoxides (LOOH) and thiobarbituric acid reactive substances (TBARS) during the preliminary phase of tissue generation which is prone to the oxidative injury. It also establishes vibrant connection among antioxidant and reactive oxygen species (ROS). It has been reported that, various cells present in the body will be able to scavenge the free radicals efficiently via antioxidant mechanism mediating with free radical scavenging enzymes viz., CAT, GPx, GST, SOD, and GRx. These enzymes are considered as a primary line of defence for the cellular protection against oxidative damage And the capacity of these enzymes were compromised due to the enhanced lipid peroxidation [7, 8].

Experimental

Induction of myocardial infarction (MI) in experimental rats: ISO was used for the induction of MI in the experimental rats (80-100 g, male). The ISO solution was prepared by dissolving in the normal saline. It was administered at the dose of 100 mg/kg subcutaneously at a time period of 24 h (2 days) to cause the MI [1, 2].

Methods

Animal

Swiss Albino Wistar rats (80-100 g, male) were used in the current experimental study. The animals were procured form the departmental animal house and stored in the single cage with excellent ventilation. The rats were stored in the favorable condition viz., 12 h light/dark, temperature 22 ± 5°C and relative humidity of 60 ± 5%. The rats were feeded with the food and water *ab libitum*, before the experimentation. The entire experimental procedure was adopted according to the instruction for the Care and Use of the Laboratory Animals and duly approved by the Institutional Animal Ethical Committee.

Experimental study

The pilot experiment was performed using indirubin (5 and 10 mg/kg) to estimate its effect at numerous dose in ISO-treated rats (**Figure 1**). Following 10 days of experiment, we have observed that, pre-treated rats with indirubin causes significant ($P<0.05$) inhibition of the increased level of LDH, CK-MB and CK in the ISO control rats. The indirubin at the dose of 10 mg/kg confirmed the maximum inhibitory effect as compared to the indirubin 5 mg/kg. Therefore, we have selected the higher dose for the study.

For the experimental study, we have divided the rats into the following groups and each group contains eight rats. Group I: received vehicle, Group II: received indirubin (10 mg/kg), Group III: received ISO (100 mg/kg), Group IV: received ISO + indirubin (10 mg/kg). The all group rats received the pre-determined treatment for time period of 10 days.

After 12 hr, we administered the second dose of the ISO, except for normal control and normal control treated with indirubin. The rats of the entire groups were anesthetized and sacrificed via cervical decapitation. The blood samples of the rats of all group were collected, and the blood components were divided through the centrifuge. The heart tissue was removed instantly with quick rinsing with the normal saline (ice-chilled). The sample of the heart tissue was weight and homogenized in centrifuge using the Tris-HCl buffer (pH=7) and was utilized for the determination of the different biochemical markers.

Estimation of cardiac marker enzymes

The cardiac enzymes viz., CK-MB and CK and hepatic marker viz., AST, ALT and LDH were scrutinized according to following the guidelines of the available commercial kits.

Table 1. Effect of Indirubin on the level of cardiac marker enzymes in normal and ISO induced myocardial infracted rats

S.NO	Groups	Biochemical Parameters				
		AST (IU/l)	LDH (IU/l)	ALT (IU/l)	CK (IU/l)	CK-MB (IU/l)
1	Normal Control	35.21 ± 4.32	80.12 ± 8.76	24.11 ± 2.43	160.23 ± 12.34	80.87 ± 8.65
2	Normal Control + Indirubin (10 mg/kg)	34.98 ± 3.45	78.65 ± 9.32	23.89 ± 3.21	158.35 ± 10.23	81.23 ± 9.41
3	ISO (100 mg/kg)	57.32 ± 4.32*	158.21 ± 12.32*	44.21 ± 3.65*	275 ± 15.43*	190.32 ± 12.43*
4	ISO + Indirubin (10 mg/kg)	38.21 ± 3.65#	90.32 ± 8.32#	28.32 ± 4.32#	178.3 ± 13.76#	99.94 ± 10.36#

The data presented as the mean ± standard error of the mean; *P<0.05, vs. control group; #P<0.05, vs. ISO group.

Assay of cTnT

The cTnT level was estimated in the serum of the all group rats using the available commercial kits.

Determination of assay of lipid peroxidation and antioxidant markers

The lipid peroxidation level such as, TBARS was determined using the method of Yogi (Yogi, 1987). The LPO was estimated according to the reported method of Jiang et al. 1992. The CAT, SOD, GST, GSH and GPx were determined in accordance with the reported method with minor modification of Kumar et al., 2013, Ahmed et al., 2014, Verma et al., 2014 respectively. The GSH, vitamin C and E level were determined in heart and plasma by the minor modification of the reported method of Ellman, 1959 [9]. The protein content (PC) in the heart was also estimated. The uric acid level was also estimated using the commercial available kit.

Histopathological examination

The heart tissues were quickly detached and instantly cleaned using saline and fixed in formaldehyde (40% solution). Following fixing, the heart samples were further processed via embedding into paraffin. Subsequently, the heart sample was sliced and marked with haematoxylin and eosin (H&E) for further visualization using the light microscope (40×).

Results

The results presented in **Table 1** explained the effect of indirubin on the level of the CK-MB, LDH, AST, CK and ALT in the all group rats. The ISO induced rats confirmed the significant (P<0.05) increase in serum enzymes activities as compared to the normal rats. Previous treatment of the rats with the indirubin (10 mg/kg) for the time period of 10 days causes significant (P<0.05) reduction in the serum enzymes activities of ISO-induced rats as compared to the ISO induced group rats.

As shown in **Figure 2**, it was found that ISO treated rats showed significant increase in the (P<0.05) level of cTnT in serum as compared with the normal rats. Moreover, the ISO rats received indirubin (10 mg/kg) confirmed significant (P<0.05) decline in the cTnT level as compared to ISO rats.

The **Figure 3** showed the expression of serum LDH level in the all group rats. The administration of ISO showed elevated concentration of LDH (isoenzyme) bands as compared to the normal rats. The ISO control rats treated with indirubin (10 mg/kg) showed decline in both LDH isoenzymes. Additionally, the ISO control rats confirmed the significant increase in the level of LOOH and TBRAS in the heart and plasma as compared to the normal group rats. The ISO induced rats treated with the indirubin showed the significant (P<0.05) decline in the level of LOOH and TBRAS in the heart and plasma as comparised to the ISO control rats (**Table 2**).

As shown in **Table 3**, the level of enzymic antioxidants activities viz., CAT, SOD, GRx, GST and GPx in the heart of all group rats were quantified. The ISO treated rats showed significant (P<0.05) decline in the level of the antioxidants activity of the heart as compared to the normal rats. Whereas, the ISO induced rats treated with the indirubin (10 mg/kg) showed (P<0.05) improvement in the antioxidant enzymes activities as compared to the ISO control rats.

The ISO treated rats showed considerable (P<0.05) reduction in the levels of vitamin C, E and GSH in the heart and plasma and significant (P<0.05) increase in the level of uric acid

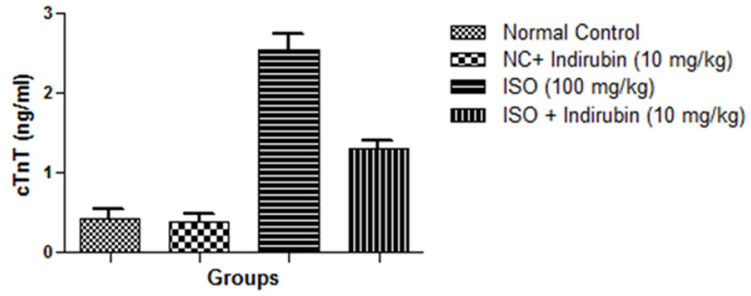

Figure 2. Effect of indirubin on the levels of cardiac troponin-T (cTnT) in serum of normal and ISO-induced myocardial infarcted rats Group I: normal control; Group II: normal control + indirubin (10 mg/kg); Group III: ISO control (100 mg/kg); Group IV: ISO + indirubin (10 mg/kg).

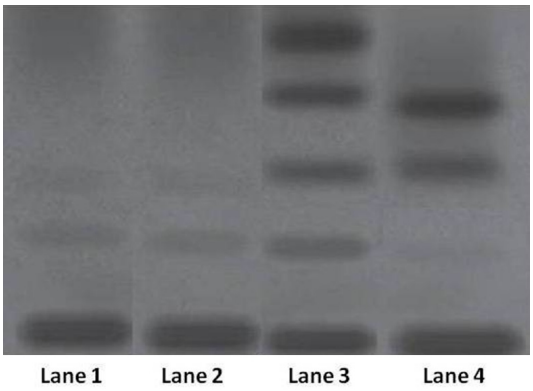

Figure 3. Effect of indirubin on Lactate Dehydrogenase (LDH)-isoenzymes in serum of normal and ISO-induced myocardial infracted rats. Lane 1: normal control, Lane 2: normal control + indirubin (10 mg/kg), Lane 3: ISO control (100 mg/kg), Lane 4: ISO + indirubin (10 mg/kg).

as compared to the normal group rats. The ISO control rats treated with indirubin (10 mg/kg) causes significant ($P<0.05$) improvement in the non-enzymic antioxidants levels and significantly ($P<0.05$) reduction in the uric acid level as compared to the ISO control rats (**Table 4**).

The **Figure 4A-D** displayed the heart histopathology of normal, indirubin and ISO induced MI rats. The normal control rats explained the cardiac fibers without infarction (**Figure 4A**). Whereas, the normal control rats received the indirubin (10 mg/kg) displayed tnormal cardiac muscle with no infarction bundles without any injury (**Figure 4B**). The ISO induced MI rats confirmed the area of infarction through disturbance in the cardiac muscle fibers and development of inflammatory cells (**Figure 4C**). Whereas, the rats treated with Indirubin (10 mg/kg) showed mild hyalinization in the cardiac muscle fibres (**Figure 4D**).

From the above results, it could be corroborated that, the ISO induced rats treated with the Indirubin (10 mg/kg) confirmed the significant ($P<0.05$) modulation of the entire tested biochemical parameters. It was also indicated that, ISO control rats treated with the Indirubin confirmed the defensive effect on the histopathology of heart. The normal rats and the normal control rats received indirubin (10 mg/kg) did not revealed any considerable change in any of the biochemical and histopathological parameters over the treatment of 10 days.

Discussion

Some of the serum parameters viz., CK-MB, CK, LDH, ALT and AST are considered as important biochemical markers of the MI. During the myocardial infarction, the cells are break or injured due to the incomplete oxygen or glucose supply, which render cardiac membrane porous or may burst causing secretion of these enzymes. After release from the tissue, their level has been found elevated in the blood as well as in serum. As shown in the present study, the serum level of all enzymes showed reduction in the ISO control rats received indirubin rats possibly due to the defensive effect of indirubin on myocardium. It results in the improvement of the myocardial injury, persuaded by ISO, suggested by limiting the secretion of enzymes form myocardium [10].

The increased level of troponin usually forecast the risk of both infarction and cardiac fatality. In the current investigation, we have found enhanced cTnT level in the ISO induced MI rats. The LDH (cytosolic enzymes), which is commonly found in most of the tissues concerned in the glycolysis and exists in the five various isoforms such as LDH-1 to 5. In cardiac tissues, LDH (1 and 2) preponderate. Therefore, the estimation of the increased level of this enzymes secretion into the blood from the injured tissue has turn into ultimate analysis and predictive standard for various diseases and disorder. It can be distinguished from the additional kind of the tis-

Table 2. Effect of Indirubin on the level of antioxidant marker in the normal control and ISOinduced myocardial infracted rats

S.NO	Groups	Plasma LOOH (values $\times 10^{-5}$ mmol/dl)	Heart LOOH (mmol/100 g wet tissue)	Serum TBARS (nmol/ml)	Heart TBARS (mmol/100 g wet tissue)
1	Normal Control	20.32 ± 1.26	27.65 ± 2.93	7.43 ± 0.98	1.65 ± 0.54
2	Normal Control + Indirubin (10 mg/kg)	20.04 ± 1.41	27.47 ± 3.11	7.12 ± 0.88	1.52 ± 0.83
3	ISO (100 mg/kg)	44.34 ± 2.93*	66.54 ± 4.82*	22.54 ± 2.32*	8.32 ± 1.73*
4	ISO + Indirubin (10 mg/kg)	24.32 ± 1.29#	36.54 ± 1.82#	11.82 ± 2.72#	3.43 ± 1.53#

The data presented as the mean ± standard error of the mean; *P<0.05, vs. control group; #P<0.05, vs. ISO group.

Table 3. Effect of indirubin on the activities of enzymic antioxidants in the heart of normal andisoproterenol (ISO)-induced myocardial infarcted rats

S.NO	Groups	Catalase (_mol of H_2O_2 consumed/ min/mg protein)	SOD (units/ mg protein)	GPx (_g of GSH oxidized/min/ mg protein)	GRx (nmol of NADPH oxidized/min/100 mg protein)	GST (nmol of CDNB conjugated/ min/mg protein)
1	Normal Control	12.54 ± 1.43	16.54 ± 1.65	5.65 ± 0.62	8.54 ± 0.65	795 ± 80.43
2	Normal Control + Indirubin (10 mg/kg)	12.11 ± 1.73	16.34 ± 1.95	5.65 ± 0.43	8.47 ± 0.82	789.6 ± 86.43
3	ISO (100 mg/kg)	5.11 ± 1.03	6.89 ± 1.34	2.15 ± 0.51	3.67 ± 0.45	280.32 ± 65.75
4	ISO + Indirubin (10 mg/kg)	9.32 ± 1.54#	14.02 ± 1.43#	4.32 ± 0.25#	6.54 ± 0.83#	654.11 ± 87.86#

The data presented as the mean ± standard error of the mean; *P<0.05, vs. control group; #P<0.05, vs. ISO group.

sue injury, while the level of the LDH starts to be enhance in first 12-24 h subsequently after MI and reach in the higher level within 2-3 days and slowly dissolved in 5-14 days. In the current investigation, we have observed the enhanced level of LDH (1 and 2) bands intensity in ISO control rats. ISO control rats showed the enhanced strength of the LDH (1 and 2) in the serum due to the heart necrosis. ISO received rats treated with the indirubin causes significant (P<0.05) decline in the level of the serum cTnT and strength of the LDH-1 and 2 bands. This might be due to the diminution of the degree of injury in the myocardium via indirubin thereby thwarting their secretion.

In the current times, the attention towards the role of free radicals in disease progression and generation has made critical awareness to find novel ways of antioxidant therapy, e.g. neutraceuticals. Consequently, more herbal medicine or their products need to be screened for their free radical protection potential. LPO, a type of the oxidative worsening of PUFAs has been associated with the modulated enzyme inactivation and membrane structure. As shown, the ISO control rats confirmed the enhanced level of the lipid peroxidation (LOOH and TBARS) products in the heart and plasma. The enhanced level of LPO appears due to the preliminary stage of the tissue creation which makes it additional prone to the oxidative or free radical damage. The ISO induced rats treated with Indirubin showed decline in the levels of LPO in a dose dependent manner [13, 14].

The antioxidant property of the indirubin may delay the ROS which are generated through Isoproterenol. The antioxidant constitutes the chief protection method that restricts the toxicity connected with free radicals and oxidative stress. The balance between free radicals and antioxidant is an imperative feature for the effectual elimination of the intracellular organelles in oxidative stress. Still, in MI pathological condition, the production of the ROS can considerably disturb this equilibrium with increase supply of the antioxidant protection method. Free radical scavenging system viz., CAT, SOD, GST and GPx are used as the initial stage of cellular protection against the oxidative damage. These first lines antioxidant enzymes are minimized due to increase in the level of the lipid peroxidation. During the injury, the site of the MI initiates the generation of the superoxide radicals and altered the activity of the CAT and SOD, resulting deposition of superoxide anion, which also showed the effect on the injury of the myocardium. The ISO induced rats pretreated with the indirubin demonstrated the enhanced level of antioxidant enzymes which confirm that the indirubin may have the capabil-

Table 4. Effect of indirubin on the activities of non-enzymic antioxidants in plasma and the heart of normal and ISO-induced myocardial infarcted rats

S.NO	Groups	Plasma vitamin C (mg/dl)	Heart vitamin C (mmol/mg protein)	Plasma vitamin E (mg/dl)	Heart vitamin E (mmol/mg protein)	Plasma GSH (mg/dl)	Heart GSH (mmol/g wet tissue)	Plasma uric acid (mg/dl)
1	Normal Control	5.32 ± 0.45	1.88 ± 0.23	4.42 ± 0.43	1.88 ± 0.15	24.54 ± 2.76	12.43 ± 1.03	8.43 ± 1.02
2	Normal Control + Indirubin (10 mg/kg)	5.43 ± 0.65	1.94 ± 0.13	4.56 ± 0.93	1.93 ± 0.19	25.11 ± 3.54	12.14 ± 1.76	8.54 ± 1.94
3	ISO (100 mg/kg)	2.43 ± 0.74*	0.96 ± 0.12*	2.32 ± 0.53*	0.89 ± 0.11*	13.43 ± 2.94*	5.21 ± 0.54*	15.67 ± 2.12*
4	ISO + Indirubin (10 mg/kg)	4.43 ± 0.43#	1.54 ± 0.11#	4.01±0.32#	1.54 ± 0.31#	21.98 ± 3.11#	10.1± 0.98#	11.65 ± 1.94#

The data presented as the mean ± standard error of the mean; *P<0.05, vs. control group; #P<0.05, vs. ISO group.

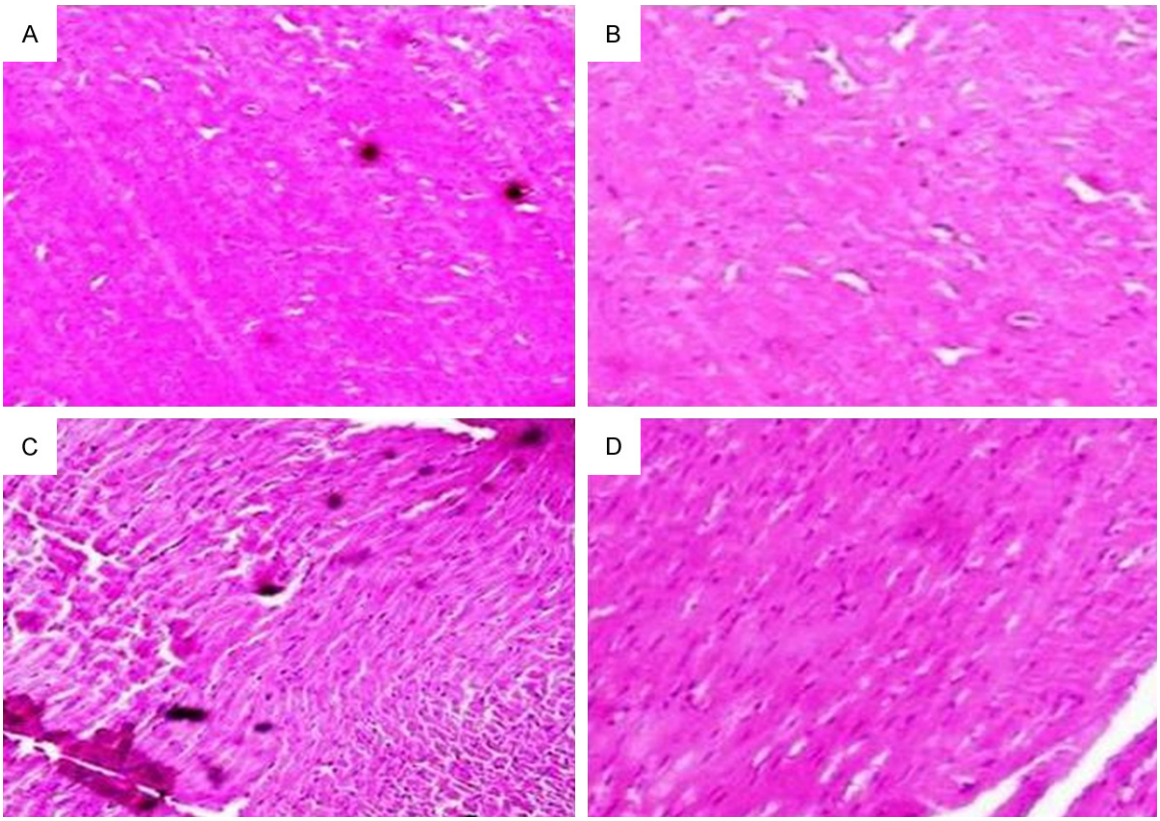

Figure 4. Effect of indirubin on the normal and ISO induced myocardial infarcted rats. A: Group I-normal control heart showing normal cardiac muscle fibres. B: Group II-normal + indirubin (10 mg/kg) treated heart showing normal muscle fibres without any pathological changes. C: Group III-ISO (100 mg/kg) control heart showing cardiac muscle fibres with muscle separation and inflammatory cells. D: Group IV-indirubin (10 mg/kg) + ISO-treated heart showing hyperplastic muscle fibres with focal hyalinized muscle bundles and absence of inflammatory cells.

ity to avoid the toxic effects induced via free radicals. The level of GST and GPx was found to be decreased in the ISO control group rats, which could be due to the decline in the accessibility of GSH. The decreased accessibility of the GPx in the heart leads to the deposition of the oxidized glutathione (GSSG) in the heart. The inactivation of the GSSG may be due to the holding the SH-group and reduction in the protein synthesis [15-17]. The ISO induced rats treated with the indirubin enhances the activity of the GRx, GPx and GST and confirmed the antioxidant protection against the free radicals. Another antioxidant enzymes such as GSH, is an imperative enzyme protecting the myocardium against the free radical intervened damage and thus reduction in the cellular GSH level could damage the cardiac tissues. The ISO induced control group rats showed the reduced level of the GSH in the heart and plasma. The reduction in the level of GSH might be due to enhanced consumption in the defensive "SH" containing lipids peroxidase and proteins. The main role of the vitamin C is directly involved in the scavenging of the superoxide, singlet superoxide and hydroxyl radicals. The vitamin C also inhibits the risk of the cardio vascular disease (CVD) via reduction in the blood cholesterol, blood pressure and the generation of the oxidized LDL cholesterol in the blood. Another vitamin such as, vitamin E is the potential lipid soluble antioxidant found in the biological system. The vitamin E is also implicated in the minimization of the LPO and redevelops the declined GSH and vitamin C. The protection the myocardial membrane and reducing the oxidation of lipoproteins, it causes reduction of the membrane peroxidation injury and atherogenesis. In the current investigation, it was found that, ISO induced control group rats showed reduction in the plasma and heart level of the vitamin C and E, which may be due to these non enzymatic antioxidants and increased level of LPO. Another factor for the estimation of the expression of the MI is the uric acid level. In the current investigation, we have found a consider-

able increase in the level of uric acid in the plasma of the ISO control group rats, which might be due to the enhanced level of free radical generated by the ISO. In the hypoxic tissue, the ATP reduction happens which starts with the deposition of hypoxanthine. During the tissue multiplication, the xanthine dehydrogensae is changed to the xanthine oxidase via the 'SH' group oxidation. The xanthine oxidase is responsible for the catalyzing the alteration of hypoxanthine to superoxide, uric acid and xanthine. This is considered to be the main reason for the increase in the uric acid level in ISO control group rats. ISO control rats treated with indirubin confirmed the significant ($P<0.05$) enhanced level of GSH and vitamin (C & E) in the heart and plasma together with considerable reduction in the level of uric acid in the plasma. The enhanced level of the GSH and vitamin (C and E) in the heart and plasma together with declined level of the uric acid in the plasma in ISO rats pre-treated with indirubin, confirmed the antioxidant potential of indirubin against the damage induced via free radicals [18, 19].

The histopathological observation of the ISO rats treated with indirubin showed the average cardiac muscle morphology along with no sign of necrosis as compared to the ISO control group rats. The previous studies showed that the indirubin has potential effect on the ALT, AST, GSH and LPO in carbon tetrachloride (CCl4) induced damage in Wistar rats. Earlier reported confirmed that the indirubin protecting the heart from the LPO because it scavenges the toxic radical such as hydroxyl and superoxide. The possible mechanism of action of indirubin may be attributed to the ability of directly scavenging the free radicals/oxidative stress and escort to the inactivation which then could repress the intracellular level of free radicals. The histopathology study of the indirubin pretreated rats explained the effectual effect of indirubin in ISO induced MI rats [20, 21].

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Disclosure of conflict of interest

None.

Authors' contribution

S. Zheng and C. Sun designed and performed the experimental study. J. Chen analyzed the data and edits the manuscript. All authors read and approved the final manuscript.

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