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# Ameliorative effects of cerebrolysin against isoproterenol-induced myocardial injury in male rats

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ABSTRACT

*Aims:* Myocardial injury (MI) is the principal cause of death from cardiovascular disease (CVD). The present study was conducted to investigate the ameliorative and antioxidant effects of cerebrolysin (CBL) on isoproterenol-induced MI in rats.

*Methods:* MI was induced in the rats by subcutaneously injecting 100 mg/kg of isoproterenol (ISO) in the first two days. The serum levels of creatine phosphokinase (CK-MB) and cardiac troponin I (cTnI) were measured on the third day to confirm MI. The post-treatment involved intraperitoneally injecting 5 ml/kg of CBL for 7 days. Nitric oxide (NO), malondialdehyde (MDA) in the heart tissue and catalase (CAT) and serum levels of superoxide dismutase (SOD) and glutathione peroxidase (GPX) were measured on the 10th day using the enzyme-linked immunosorbent assay (ELISA). Histopathological examinations of the heart tissue were also performed.

*Findings:* The present results suggested significant increases in CK-MB, cTnI, MDA and NO. A significant decrease was also observed in the ISO-treated rats in certain antioxidant enzymes, including CAT and GPX. CBL administration showed a significant ameliorative increase against the oxidative ISO-induced damage. Moreover, the histopathological findings showed lower levels of the infiltration of inflammatory cells and edema and vascular proliferation in the CBL-treated rats.

*Significance:* The present histopathological and biochemical findings attributed antioxidant properties to CBL in the rat myocardium and suggested protective effects on ISO-induced MI.

## 1. Introduction

Myocardial injury (MI) is a common ischemic heart condition and the principal cause of death from cardiovascular disease (CVD) in industrialized and developing countries [1,2]. The World Health Organization has predicted that CVD will be the most significant cause of mortality in the world [2]. A critical imbalance between the coronary blood supply and myocardial demand causes MI [3]. The accumulation of free radicals has been reported as a mechanism contributing to MI [4].

Research suggests that oxidative stress caused by the generation of reactive oxygen species (ROS) during ischemic damage plays a key role in developing MI [2,5]. ROS can attack intra-membranous polyunsaturated fatty acids (PUFA), and cause chain reactions of lipid peroxidation (LPO) in the membrane [6,7]. Increased tissue levels of malondialdehyde (MDA), as an important LPO by-product, can contribute to the increased generation of free radicals and decreased activities of the antioxidant system [8]. Antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and gluthatione peroxidase (GPX) considered the first-line cellular defense against oxidative stress eliminate ROS in tissues [1].

Nitric oxide (NO) in the heart can regulate many functions, including heart rate and myocardial oxygen consumption [9]. Different endogenous systems produce ROS and reactive nitrogen species (RNS) in normal conditions. Nitrosative/oxidative stress representing an imbalance between the production and elimination of RNS and ROS combined with the reduced production of antioxidants causes oxidative toxic stress [9].

NO is synthesized by three isoforms of nitric oxide synthases (NOS), namely endothelial (eNOS), neuronal (nNOS) and inducible (iNOS), all

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of which can be expressed in the heart [10].

Inducing MI in animals using isoproterenol (ISO) is considered a model established to study the beneficial effects of different cardioprotective agents [11]. ISO, a synthetic β-adrenoceptor agonist, was found to cause intense oxidative stress in the myocardium and an infarcted necrosis of the heart tissue [12]. Besides the positive inotropic and chronotropic effects of ISO via β adrenergic receptors and the induction of partial ischemia due to myocardial hyperactivity and coronary hypotension, the following mechanisms contribute to ISO-induced MI: increased cyclic adenosine monophosphate, intracellular $Ca^{2+}$ overload, diminution of high-energy phosphate supplies, oxidative stress and increased production of cytotoxic free radicals [1,2]. The heart weight significantly increases, and the body weight slightly changes in ISO-induced MI [13,14].

The disadvantages of other experimental animal models of MI, e.g. banding, β-adrenergic agonist microinfusion and ligation, include infection, post-injury complications and a higher mortality [2]. On the other hand, the empirical advantages of ISO-induced MI models include reproducibility, rapidity, simplicity and non-invasiveness with the potential of mimicking acute clinical MI [2] in the subendocardium of the left ventricle with acute extensive myofibrillary degeneration [15].

As a mixture of different free amino acids and neurotrophic factors, cerebrolysin (CBL) is widely used for treating stroke given its ameliorative effects on the nervous system [16]. By promoting CAT and SOD scavenging activities, CBL eliminates free radicals and decreases ROS and free radicals in the mitochondria of nerve cells [16]. The novelties of the present research include exploring the post-treatment antioxidant properties of CBL in MI, which is lacking in literature despite addressing pre-treatments using CBL [17]. The present study was therefore conducted to investigate the ameliorative and antioxidant effects of CBL against ISO-induced MI in rats.

## 2. Materials and methods

### 2.1. Animals

Adult male Wistar rats weighing 150–200 g used in the present study were collected from the Physiology Research Center in Kashan University of Medical Sciences, Kashan, Iran. The animals were kept in standard cages for one week under a 12-hour light–dark cycle at 22–24 °C with 50% humidity before conducting the experiment. The animals had free access to food and water. All the experimental procedures were conducted according to the ethical guidelines of Kashan University of Kashan Medical Sciences, and the study was registered by the Ethics Committee of the university (code: IR.KAUMS.MEDNT.REC.1397.013).

### 2.2. Drugs

ISO was purchased from Merck, Germany, and CBL (vial 5 ml) from EVER Neuro Pharma GmbH, Austria, while every 1 ml contained 215.2 mg of CBL.

### 2.3. MI induction

To empirically induce MI, (100 mg/kg/day) of fresh ISO prepared by being dissolved in physiological saline was subcutaneously injected in the rats every 24 h for two consecutive days [18].

### 2.4. Experimental protocols

After the acclimatization, l8 Wistar rats were divided into three groups of 6. Group 1, i.e. vehicle-treated control rats, were subcutaneously injected with saline for 2 days and intraperitoneally injected with saline for 7 days. Group 2, i.e. ISO-treated (vehicle + ISO) rats were subcutaneously given 100 mg/kg/day of ISO for 2 consecutive days and intraperitoneally injected with saline for 7 days. Group 3, i.e. CBL-treated (CBL + ISO) rats, were subcutaneously given 100 mg/kg/day of ISO for 2 consecutive days and then intraperitoneally injected with 5 ml/kg of CBL for 7 days [17,18]. The drug doses were selected based on previously-conducted studies [18].

### 2.5. Serum biochemical assays

The blood samples were collected from the tail vein on the 3rd day 24 h after the second injection of ISO for the measurement of CK-MB and cTnI markers. Before sacrificing the rats, blood samples were collected via the cardiac puncture on the 10th day 24 h after the final injection of CBL for the measurement of antioxidants and lipid profiles.

### 2.6. Biochemical analysis

#### 2.6.1. Cardiac biomarkers

Commercially available kits (Zellbio, Germany) were used in spectrophotometry to measure the serum levels of CK-MB isoenzyme at 450 nm, and ELISA (Mybiosourc, the US) to measure cTnI as markers for cardiac damage [17].

#### 2.6.2. LPO

MDA levels were measured as per the method proposed by Ohkawa et al. [19] to determine LPO in the heart tissue in terms of nmol/mg protein [17].

#### 2.6.3. Lipid profiles

Serum levels of total cholesterol (TC), triglycerides (TG) and HDL were measured according to routine procedures using commercially available kits (Pars Azmoon, Iran). Serum levels of LDL were measured using the Friedewald formula, i.e. $LDL = TC - [HDL + TG/5]$, and serum levels of VLDL using the Nobert formula, i.e. $VLDL = TG/5$ [20].

#### 2.6.4. Antioxidant markers

The activities of CAT, SOD and GPX were measured using ELISA according to the instructions of the manufacturer (Zellbio, Germany).

#### 2.6.5. NO

NO levels were determined in the heart tissue in terms of nmol/mg protein by measuring its supernatant metabolites using the Griess reagent and according to the method proposed by Moshage et al. [21].

#### 2.6.6. Total protein

Total protein levels were measured in the heart tissue using the Bradford method and the concentrated Coomassie blue reagent and bovine serum albumin as the standard [22].

### 2.7. Histopathological examinations

After terminating the experiments and sacrificing the animals, the heart of each rat was immediately excised and washed with phosphate buffer saline. The cardiac apex of the samples was then excised and fixed in a 10% formaldehyde buffer solution. The apex tissues were embedded in paraffin. The sectioned tissues were then stained with hematoxylin and eosin to evaluate the histological necrosis. The prepared 4-μm thick slides were separately examined by two blinded pathologists under the light microscopy on the histopathological photomicrographs. The histological findings were reported for each specimen in order of the severity of myocardial necrosis. Based on a minimum 10 fields for each specimen, the slides were evaluated for myocardial necrosis, inflammatory cell infiltration and edema and accordingly scored on a scale of severe (+++), moderate (++), mild (+) and nothing (O) [23].

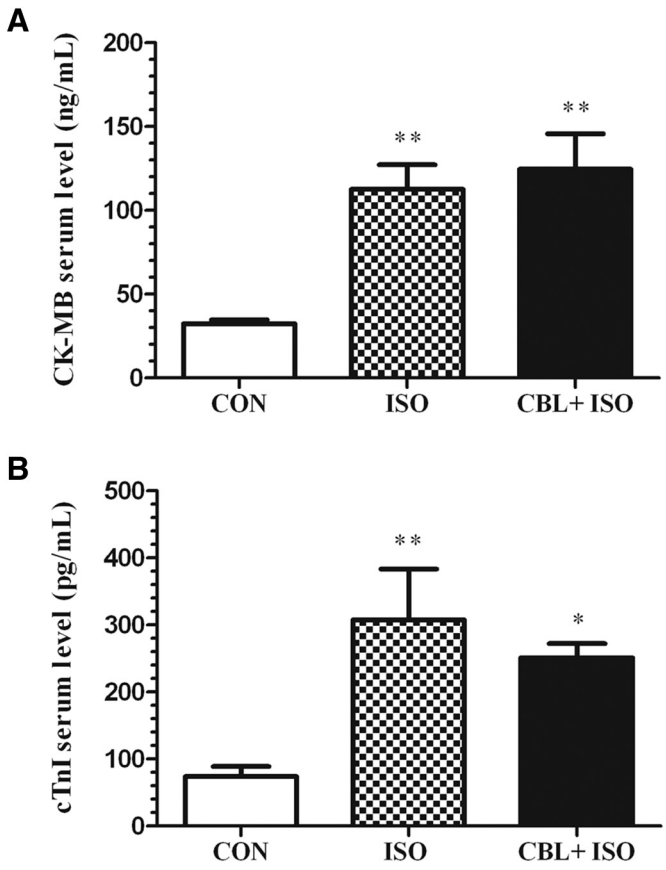


**Fig. 1.** The CK-MB (ng/ml) (A) and the cTnI (pg/ml) (B) levels in serum on the 3rd day, 24 h after the second injection of ISO in three groups (n = 6). CON, control; ISO, isoproterenol; CBL, cerebrolysin; CK-MB, creatine phosphokinase. Data are expressed as mean ± SEM. * indicates significant difference compared to control group ($P < 0.05$).

### 2.8. Statistical analysis

The Kolmogorov-Smirnov test was used to investigate the distribution normality of all the data. The data were presented as mean ± SEM, and analyzed using one-way ANOVA followed by the Tukey's post-hoc test. $P < 0.05$ was set as the level of statistical significance.

## 3. Results

### 3.1. Cardiac biomarkers

Twenty four hours after the second injection of ISO on the third day, the serum levels of cardiac markers (CK-MB and cTnI) significantly increased in the ISO-treated and CBL-treated groups compared to in the controls ($P < 0.05$) (Fig. 1A and B).

### 3.2. Serum antioxidant markers

Fig. 2A–C show the effect of CBL on the activities of serum levels of CAT, GPX and SOD in all the groups. A significant decrease was observed in the antioxidant activities of CAT ($P < 0.05$) and GPX ($P < 0.05$) in the ISO-treated rats compared to in the controls, although the change in SOD was insignificant ($P > 0.05$). A significant increase was also observed in the antioxidant activity of CAT ($P < 0.01$) and GPX ($P < 0.05$) in the CBL-treated group (5 ml/kg/*i.p.*) compared to in the ISO-treated group, although the change in SOD was insignificant ($P > 0.05$).

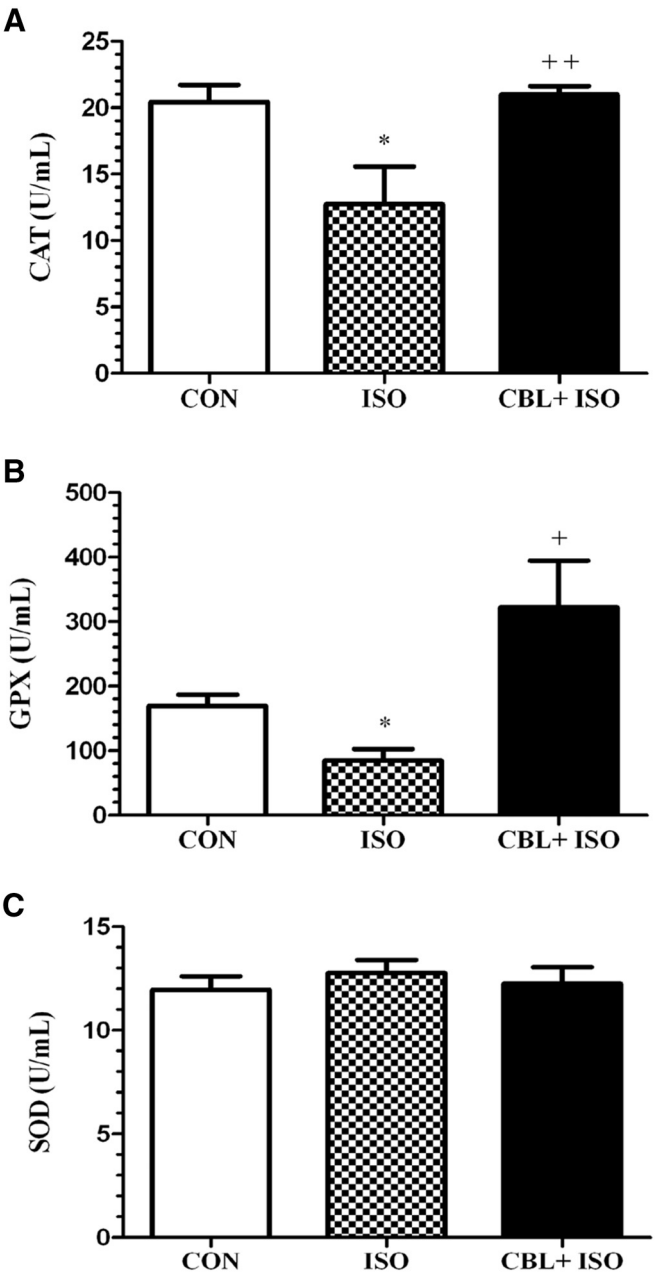


**Fig. 2.** The activity of CAT (U/mL) (A), the activity of GPX (U/mL) (B) and the activity of SOD (U/mL) (C) in serum, one week after ISO treatment in three groups (n = 6). CON, control; ISO, isoproterenol; CAT, catalase; GPX, glutathione peroxidase; SOD, superoxide dismutase; CBL, cerebrolysin. Data are expressed as mean ± SEM. * indicates significant difference compared to control group ($P < 0.05$). + indicates significant difference compared to ISO group ($P < 0.05$).

### 3.3. LPO

Fig. 3A shows the effect of CBL post-treatment on LPO in all the experimental groups. A significant increase in MDA levels was observed in the heart tissue in the ISO-treated group compared to in the controls ($P < 0.05$). The post-treatment with CBL (5 ml/kg/*i.p.*) significantly decreased MDA levels in the heart tissue compared to the ISO-treated group ($P < 0.001$).

### 3.4. NO

A significant increase in NO metabolites measured as nitrite was

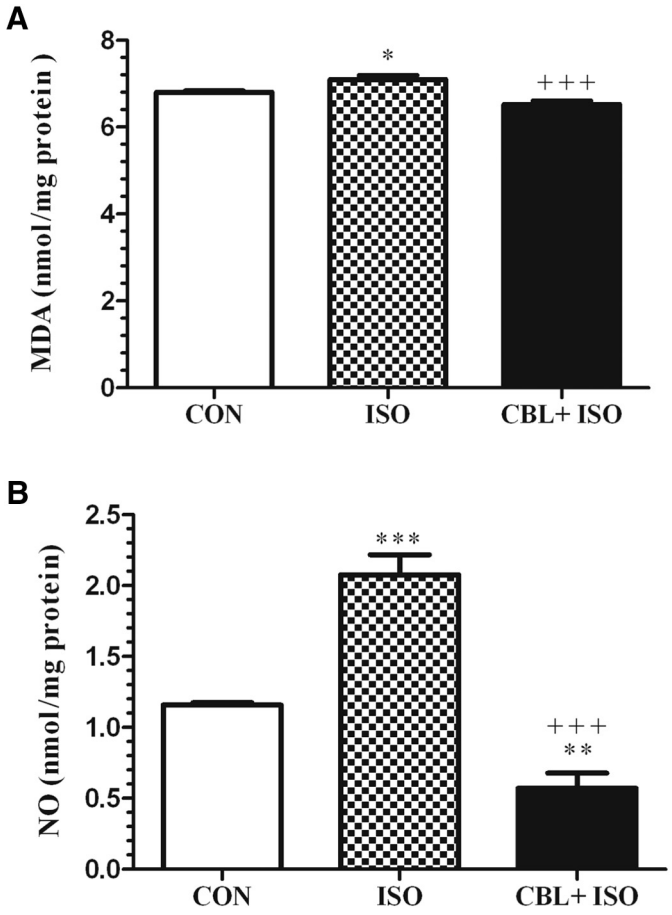


**Fig. 3.** The level of MDA (nmol/mg protein) (A) and the level of NO (nmol/mg protein) (B) in heart tissue one week after ISO treatment in three groups (n = 6). CON, control; ISO, isoproterenol; MDA, malondialdehyde; NO, nitric oxide; CBL, cerebrolysin. Data are expressed as mean ± SEM. * indicates significant difference compared to control group ($P < 0.05$). + indicates significant difference compared to ISO group ($P < 0.05$).

observed in the heart tissue in the ISO-treated group ($P < 0.001$) compared to the controls. A significant reduction in NO metabolite levels was also observed in the heart tissue in the CBL-treated group compared to in the ISO-treated group ($P < 0.001$) (Fig. 3B).

### 3.5. Lipid profiles

Table 1 shows the effect of post-treatment with CBL on lipid profiles in all the groups. No significant differences were observed among the groups in term of lipid profiles ($P > 0.05$).

**Table 1**
Effect of post-treatment with CBL on lipid profile in serum, one week after induction of MI with ISO in three groups (n = 6). CON, control; ISO, isoproterenol; Chol, cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; CBL, cerebrolysin. Data are expressed as mean ± SEM.

| Group | CON | ISO | CBL + ISO |
|---|---|---|---|
| Chol | 68.8 ± 5.4 | 60.5 ± 3.7 | 76 ± 2.1$^{+}$ |
| TG | 100.2 ± 8.4 | 71.5 ± 5.1$^{*}$ | 106.5 ± 7.8$^{++}$ |
| HDL | 31.4 ± 2.2 | 36.7 ± 1.9 | 30.1 ± 1.6 |
| LDL | 11.3 ± 1.06 | 12.3 ± 1.4 | 11.9 ± 2.04 |
| VLDL | 20.04 ± 1.6 | 14.31 ± 1$^{*}$ | 21.3 ± 1.5$^{++}$ |

$^{*}$ Indicates significant difference compared to control group ($P < 0.05$).
$^{+}$ Indicates significant difference compared to ISO group ($P < 0.05$).

**Table 2**
Effect of CBL on histopathological changes in the ISO-induced MI in rats. ISO, isoproterenol: CON, control; CBL, cerebrolysin. CON: received 2 days injections of saline (*sc*) and 7 days injections of saline (*ip*); ISO: received 2 days injections of isoproterenol (100 mg/kg/*sc*) and 7 days injections of saline (*ip*); CBL + ISO: received 2 days injections of isoproterenol (100 mg/kg/*sc*) and 7 days injections of CBL (5 ml/kg/*ip*).

| Group | Myonecrosis | Angiogenesis | Inflammation | Edema |
|---|---|---|---|---|
| CON | O | O | O | O |
| ISO | + + + | O | + + + | + + + |
| CBL + ISO | + + + | + + | + + | + + |

Histopathological changes; (O) no change, (+) mild, (+ +) moderate, (+ + +) severe.

### 3.6. Histopathological examinations

Table 2 and Fig. 4 show the histological changes in myocardial tissues in all the groups. The light microscopy of the myocardium tissue sections showed a normal cell without any myocyte necrosis, edema and inflammatory cells in the control group. The histopathological findings showed an infarcted zone with massive necrosis of myocyte, edema and inflammatory cells in the ISO-treated myocardium. A focal area of infarction with coagulative necrosis and vascular proliferation with mild inflammatory cells and edema were observed in the CBL-treated group.

## 4. Discussion

The present results found CBL administration to cause significant ameliorative effects on the experimental ISO-induced MI in the rats. In addition, a significant increase in serum cTnI and CK-MB in the samples confirmed the development of MI in the rats. These findings are consistent with studies performed on ISO-treated rats [12,24] to confirm MI models. ISO is a synthetic catecholamine that induces MI based on an imbalance between oxidants and antioxidants in the heart [12,25]. Administering ISO causes cardiac hyperactivity and therefore increases the heart demand for oxygen, resulting in a prolonged ischemia and depletion of ATP [26,27]. The damaged cardiac cells with increased muscle contractility increase the cell membrane permeability and allow cardiac enzymes to leak out into the bloodstream [26,27]. cTnI and CK-MB predominantly localized in the heart are considered valuable diagnostic markers for MI given that both are elevated by myocardium-specific damage [25,28]. In line with literature [12,24,27], the present histopathological findings confirmed the ISO-induced myocardial damage.

An increase in oxidative stress during and following MI has been widely reported in literature [2,29]. In this process, the stimulation of β adrenergic receptors by ISO causes the oxidation of lipids and generation of enormous amounts of ROS in the myocardium [7]. Intense LPO caused by ISO can affect the mitochondria and cell membranes, and result in more severe oxidative damage in the heart, and release MDA into the blood circulation [30]. Increased MDA levels contribute to an increased generation of free radicals and decreased activities of the antioxidant defense system [8].

The levels of free radicals in oxidative injuries are controlled by different cellular defense mechanisms, including CAT, GPX and SOD [31]. Administering ISO in the present study caused a significant decrease in endogenous antioxidants, including CAT and GPX and excluding SOD. Administering ISO also markedly increased MDA levels, suggesting the induction of oxidative stress. Furthermore, post-treatment with CBL decreased MDA levels in the heart tissue and increased the activity of serum CAT and GPX. The present findings support the scavenging activity of CBL caused by these antioxidants. CBL was recently shown to reduce oxidative stress and LPO in rats as a result of ISO-induced MI [17]. The antioxidant properties of CBL were reported

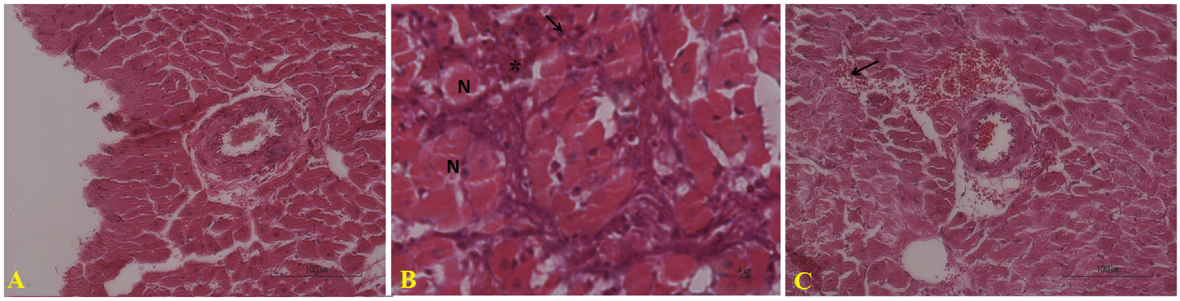


**Fig. 4.** Effect of CBL on the rat heart histopathology in the ISO-induced MI in rats. (A) Control group shows normal histological pattern (H&E; original magnification ×200); (B) ISO group shows cell necrosis (N), infiltration of inflammatory cells (arrow) and fibroblastic proliferation and scar formation (asterisks) (H&E; original magnification ×400); (C) CBL + ISO group shows vascular proliferation (arrow) with less edema (H&E; original magnification ×200); ISO, isoproterenol; CON, control; CBL, cerebrolysin.

to be associated with its ability to promote certain activities of antioxidant enzymes and enhance the ROS scavenger system and reduce free radical reactions to protect the mitochondrial of nerve cells against damage by toxicants [16].

CBL was also found to decrease the serum levels of SOD in rats with brain injuries [32]. In contrast to the present study that did not suggest an increase in SOD activities in post-treatment CBL, pre-treatment CBL has been shown to increase SOD activities in the heart of ISO-induced cardiotoxic rats [17]. Loeper et al. reported that free radicals can inhibit SOD activities [33]. The presence of free radicals by ISO can therefore be assumed to inhibit SOD activities in both ISO and CBL + ISO groups.

Lipids play a key role in CVD by modifying the composition, structure and stability of cell membranes [29]. Cholesterol is also a major component of atherosclerotic plaques associated with MI. In contrast to the finding suggesting that ISO-treated rats develop an elevated TG, LDL and low HDL levels immediately in the first days after the treatment, which causes myocyte membrane damage [29], the present results indicated no significant differences in lipid profiles one week after MI induction. This discrepancy of results can be explained by the fact that the present study evaluated lipid profiles one week after the treatment, which inhibited the detection of changes in lipid profile levels.

The present study showed a significant elevation in the heart tissue levels of NO in the ISO group, whereas post-treatment with CBL significantly attenuated them, which is consistent with previous studies; for instance, Kralova et al. reported that increased NO production in oxidative stress can cause cellular injuries through toxic peroxynitrite formation [10]. Moreover, Dong et al. found CBL to inhibit the abnormal metabolism of NO and reduce apoptosis [16]. Moreover, Zurita et al. found an increase in left ventricular nitrite levels to homogenate in CBL-treated diabetic rats [34]. The mechanisms contributing to the decrease in NO levels with CBL administration could be best explained by the direct inhibitory effects of iNOS by CBL, as CBL inhibits LPO through NO pathways; nevertheless, further studies are recommended to be performed to confirm the present results.

The histopathological findings showed an infarcted zone with myocardial necrosis, interstitial edema, increased macrophage activity and scar formation in the ISO-treated rats. A moderate ameliorative effect on MI was also observed in the CBL-treated group. Administering CBL after inducing an experimental heart injury in the animal models merely showed a moderate reduction in tissue inflammation and edema potentially due to inducing vascular proliferation in the heart tissue [18]. The present results, however, showed that the antioxidant activities of CBL are more potent than its anti-inflammatory effects.

To the best of the authors' knowledge, the antioxidant properties of post-treatment CBL in MI have rarely been reported in literature. The present study was therefore a pioneer of reporting the antioxidant and ameliorative effects of post-treatment CBL in rats with isoproterenol-induced MI.

In a nutshell, the present histopathological and biochemical findings suggest that CBL has antioxidant properties in the myocardium and exerts beneficial effects on ISO-induced MI. The abilities to decrease LPO and NO production and increase the CAT and GPX activity constitute the major ameliorative mechanisms of CBL. The ameliorative and antioxidant effects of CBL on ISO-induced MI are therefore confirmed in rats.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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