

Effect of cerebrolysin on oxidative stress-induced apoptosis in an experimental rat model of myocardial ischemia

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Apoptosis plays a role in the process of tissue damage after myocardial infarction (MI). This study was designed to investigate the possible effect of cerebrolysin against apoptosis triggered by oxidative cell stress in myocardial ischemia induced by isoproterenol in rat. Rats were pretreated with cerebrolysin 5 mL/kg intraperitoneally for 7 days and intoxicated with isoproterenol (ISO, 85 mg/kg, sc) on the last 2 days. Hearts were excised and stained to detect the infarction size. Serum levels of cardiotoxicity indices as creatine kinase isoenzyme (CK-MB) and troponin I (cTnI) as well as the cardiac oxidative stress parameters as thiobarbituric acid reactive substances and superoxide dismutase were estimated. The expression of prodeath gene p53 and antideath gene Bcl-2 was also assessed from the excised heart tissues. Leakage of cardiac enzymes, elevated oxidative stress markers, and apoptotic indices confirmed the MI occurring as a consequence of isoproterenol-induced ischemia. Cerebrolysin pretreatment caused significant attenuation of the oxidative stress-induced apoptosis in the ischemic myocardial tissue. These findings provided an evidence that cerebrolysin could protect rat myocardium against ischemic insult that was attributed to its antioxidant as well as its anti-apoptotic properties.

Keywords: cerebrolysin, myocardial ischemia, oxidative stress, p53, Bcl-2

Introduction

The role of apoptosis in tissue damage after myocardial infarction (MI) has many pathological and therapeutic implications (16). Studies in the ventricular myocytes of the hearts of patients with acute MI have shown that apoptosis plays a role in the process of cell death of cardiomyocytes (33). Permanent occlusion of a coronary vessel in rats led to apoptotic changes in the ischemic region, the immediately bordering area, and the remote area from ischemia region suggesting that apoptosis is the major determinant of infarct size (27). Apoptosis is a highly regulated process in which several regulatory proteins play a part, and in which the balance between an arrays of regulatory proteins decides the fate of the cell. The expression of the two regulatory proteins, Bcl-2 and Bax, has been studied in the hearts of patients with MI (22). Bcl-2, an apoptosis inhibitor (13), is expressed in cardiomyocytes surrounding the infarcted areas after the onset of infarction. Bax is a member of the Bcl-2 family that accelerates apoptosis when overexpressed (25). Therefore, the high Bax to Bcl-2 ratio is related to apoptotic cell death in the old infarction (22). One hypothesized that the

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general molecular mediator of hypoxia-induced apoptosis is the tumor suppressor transcription factor p53. It is suggested that p53 may induce apoptosis by stimulating the expression of Bax and/or repression of Bcl-2 expression (23). Increased oxidative stress was also found to coexist with apoptosis in the rat myocardium after MI (18).

Cerebrolysin is a mixture of peptide fragment preparation, which mimics the action of neurotrophic factors and has been proved to be neuroprotective in a number of conditions such as hyperthermia-induced neurotoxicity, Alzheimer's disease, vascular dementia, traumatic brain injury, and stroke (12). Cerebrolysin was shown to exert neuroprotective and neurotrophic effects *in vivo* and *in vitro*. It induces neuron outgrowth and decreases apoptosis triggered by growth factor withdrawal in cultivated neurons (11). Also, it has been shown to reduce the effects of oxidative cell stress in animals following cerebral ischemia (9).

The aim of this study was to investigate the effects of cerebrolysin on the apoptotic and oxidative stress markers in the rat myocardial tissue as a result of isoproterenol-induced myocardial ischemia.

Materials and Methods

Chemicals

Cerebrolysin (EVER Neuro Pharma GmbH, Austria, ampoule 1 mL). Isoproterenol hydrochloride (ISO, Sigma Chemical Co., St. Louis, MO, USA).

Experimental animals

This study was carried out with 48 Sprague-Dawley male rats weighing approximately 150–200 g. They were maintained under standard conditions of temperature 22 ± 2 °C with regular 12 h light/12 h dark cycle and allowed free access to standard laboratory food and water. All experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Mansoura University.

Experimental protocols

Rats were randomly divided into four main groups (12 rats for each group):

- Group I – Control group: Rats received saline 1-mL/kg body weight intraperitoneally (IP) for seven successive days.
- Group II – Cerebrolysin-treated group: Rats treated with cerebrolysin 5-mL/kg body weight IP for seven successive days. This dose of cerebrolysin was chosen because of its antioxidant effect (36).
- Group III – Isoproterenol (ISO)-treated group: Rats received saline and were subjected to myocardial ischemia by ISO treatment in the last two days.
- Group IV – Cerebrolysin- + ISO-treated group: Rats treated with cerebrolysin and were subjected to myocardial ischemia by ISO treatment.

Induction of myocardial ischemia

The last two groups were submitted to myocardial ischemia on the last two days (days 6 and 7) via subcutaneous injection of two equal doses of isoproterenol hydrochloride (ISO, 85-mg/kg body weight), dissolved in physiological saline spaced 24 h apart. The ISO injection started 10 min after the usual dose of saline or cerebrolysin. The dosage used and time sequence were previously determined as offering a combination of high survival rate, induction of myocardial necrosis in virtually all of the treated rats (35).

Twenty four hours after the second dose of ISO injection (day 8), the rats were sacrificed with over dose of anesthesia (thiopental sodium 2.5% 60-mg/kg IP) and blood samples were collected by cardiac puncture. Blood was centrifuged and serum was separated and stored at $-20\text{ }^{\circ}\text{C}$ for measuring the cardiac biomarker. The hearts of each group were excised immediately, washed with chilled isotonic saline, and randomly divided into two groups (6 each). The first section was for the measurement of myocardial infarct size. The other section was dissected, homogenized, and kept at $-80\text{ }^{\circ}\text{C}$ until further biochemical assay.

Measurement of myocardial infarct size

Direct triphenyl tetrazolium chloride (TTC) assay was used to determine the myocardial infarct size according to the method of Lie et al. (19). In brief, the heart was transversely cut across the left ventricle, and sections of 2–3 mm thick were incubated in 1% TTC solution prepared in phosphate buffer pH (7.4) for 30 min at $37\text{ }^{\circ}\text{C}$, following which they were fixed with 10% formalin. The non-ischemic myocardium and viable ischemic myocardium were stained red, while the infarcted myocardium appeared pale gray or white. The slices were photographed on white background using Olympus[®] digital camera with a ring flash at high resolution and converted to TIFF. The % infarction in each image was analyzed on Intel[®] Core I3[®]-based computer using VideoTest Morphology[®] software Russia with a specific built-in routine for area and % area measurement.

Assay of cardiac marker enzymes

Serum creatine phosphokinase (CK-MB) activity and troponin I (cTnI) level were measured as markers for cardiac muscle damage. CK-MB activity was determined spectrophotometrically at 340 nm using commercially available kits (Stanbio Laboratory, Inc., TX, USA). The levels of cardiac troponin I (cTnI) in serum were estimated spectrophotometrically at 450 nm using enzyme-linked immunosorbent assay kits (DRG International, Inc., NJ, USA).

Estimation of lipid peroxidation products

Lipid peroxide level in heart was determined as thiobarbituric acid reactive substances (TBARs) by the methods of Ohkawa et al. (24). The amount of lipid peroxides was measured as the production of MDA, which in combination with TBA forms a pink chromogen compound whose absorbance at 532 nm was measured. The result was expressed as nmol/mg protein.

Estimation of antioxidants' activity

Myocardial superoxide dismutase (SOD) activity was measured through the inhibition of nitroblue tetrazolium reduction by O_2 generated by the xanthine/xanthine oxidase system. One SOD activity unit was defined as the enzyme amount causing 50% inhibition in 1-mL reaction solution per milligram tissue protein and the result was expressed as U/mg protein (46). The total protein content in myocardium was determined according to Lowery's method (20).

Assay of p53 and Bcl-2 expression of rat mRNA in cardiac tissues by total RNA extraction and Reverse transcription (RT-PCR)

Total RNA extraction: Total RNA was extracted using Vivantis nucleic acid extraction kit (GF-1 Total RNA extraction kit, Malaysia) according to the manufacturer's instructions.

Table I. Sequence of all primers used

Gene	Primer sequence	PCR product size (bp)
p53	F: 5'-TCT GTC ATC TTC CGT CCC TTC TC-3 R: 5'-AAC ACG AAC CTC AAA GCT GTC CCG-3	547 (39)
Bcl-2	F: 5'-CCT GCC CCA AACAAA TAT GAA AAG-3' R: 5'-TTG ACC ATT TGCCTGAAT GTG TG-3	174 (15)
GAPDH	F: 5'-GTC TTC ACC ACC ATG GAG-3' R: 5'-CGA TGC CAA AGT TGT CAT G-3'	211 (26)

Reverse transcription reaction: Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using Maxima First Strand complementary DNA (cDNA) Synthesis Kit for cDNA synthesis provided (Thermo Scientific, USA, Cat. No. #K1641) using PTC-200 thermal cycler (MJ Research, Essex, UK). After that, following the protocol instructions the reaction mixture was incubated for 10 min at 25 °C followed by 15 min at 50 °C and terminated by heating at 85 °C for 5 min. cDNA products were amplified by PCR using gene specific primers, their sequences were shown in (Table I), purchased from Biosearch Technologies (South McDowell Blvd, Petaluma, Canada). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping control gene. Amplification was done using Emerald Amp GT PCR Master Mix (2X) (Takara, Japan). The PCR condition was as follows: initial denaturation at 94 °C for 3 min followed by 35 cycles of (a) denaturation at 94 °C for 1 min; (b) annealing at 60 °C for 2 min, 58 °C for 30 s and 55 °C for 30 s for p53, Bcl-2, and GAPDH, respectively; and (c) extension at 72 °C for 1 min with subsequent 10 min at 72 °C. The reaction products were subjected to 2% agarose gel electrophoresis stained with ethidium bromide to visualize the bands.

The results were analyzed with Scion Image[®] release Alpha 4.0.3.2. Software for windows[®] which performs bands detection and conversion to peaks. Area under each peak was calculated in square pixels and used for quantification. p53 and Bcl-2 mRNA expression levels were determined by calculating the ratio between the square pixel values of the target bands in relation to the control bands of GAPDH. The identity of the amplification was confirmed by the determination of the molecular size on agarose gel electrophoresis with a 100-bp DNA molecular marker (GIBCO).

Statistical analysis

Data were presented as mean \pm SEM. Differences among groups within an experiment were analyzed by the one-way analysis of variance (ANOVA) of data followed by post hoc test of Tukey's HSD. A *p* value of <0.05 was considered significant.

Results

None of the rats died in any experimental group over the entire treatment period. Representative illustrations of infarction size stained with TTC are shown in Fig. 1. Rats intoxicated with ISO showed a large infarcted unstained area ($11.7 \pm 1.1\%$) in comparison to the control rats. Pretreatment with cerebrolysin caused significant reduction of the infarcted size to $1.5 \pm 0.1\%$ with the major portion stained positively indicating tissue viability.

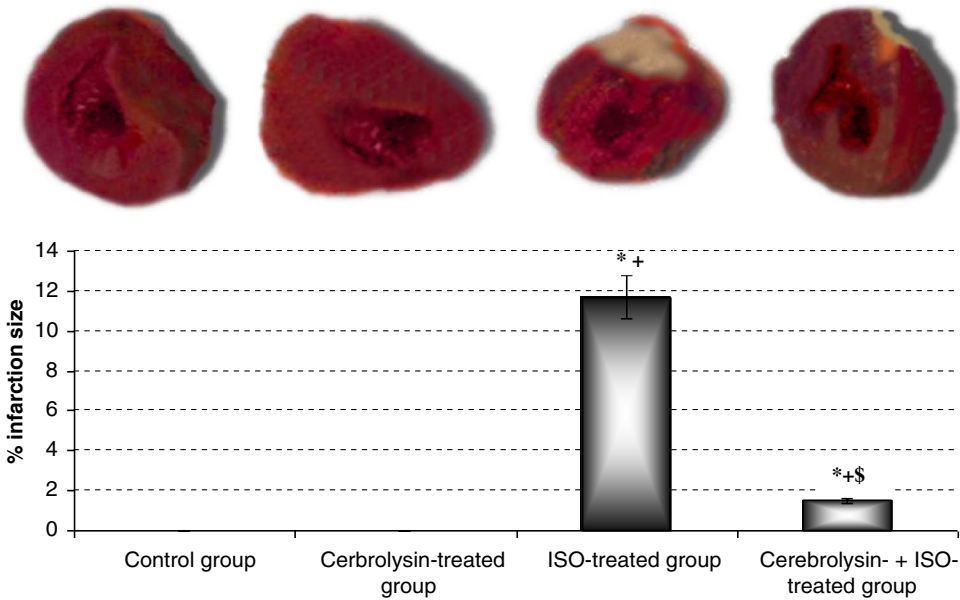


Fig. 1. Effect of pretreatment of cerebrolysin (5 mL/kg) on the cardiac infarction size using TTC stain in ISO-treated rats. Statistical analysis was carried out by one-way ANOVA followed by Tukey's HSD multiple comparison test. All values are presented as means of six rats \pm SEM. *Indicates significant change from control values at $p < 0.05$. +Indicates significant change from cerebrolysin-treated groups at $p < 0.05$. §Indicates significant change from ISO-treated groups at $p < 0.05$

ISO administration showed a significant increase in the oxidative stress parameters in the myocardial tissue represented as significant rise of the mean levels of TBARs and decrease in SOD as compared to the control group. Pretreatment with cerebrolysin along with ISO showed significant decrease in the level of TBARs and increase in SOD as compared to the ISO-treated group (Fig. 2).

ISO-treated group showed significant elevation in the serum creatine phosphokinase (CK-MB) activity and troponin I (cTnI) level as compared to normal control rats. Pretreatment with cerebrolysin showed significant decrease in the levels of the cardiac enzymes when compared to the ISO-treated group (Table II).

RT-PCR analysis of the myocardial tissue showed that administration of ISO caused significant rise in the expression of the apoptotic marker; p53 and significant decrease in the expression of the anti-apoptotic marker; Bcl-2 in comparison to the control group. Pretreatment with cerebrolysin showed significant decrease in the p53 and increase in the Bcl-2 expression as compared to the ISO-treated group (Figs 3 and 4).

Discussion

In this study, pretreatment with cerebrolysin (5 mL/kg/d for 7 days) exerts a cardioprotection in ISO-induced MI in rats.

MI induced by dual injection of ISO (85 mg/kg/d) is a standardized model to study the effect of various drugs (42). In this study, isoproterenol produces a defined myocardial

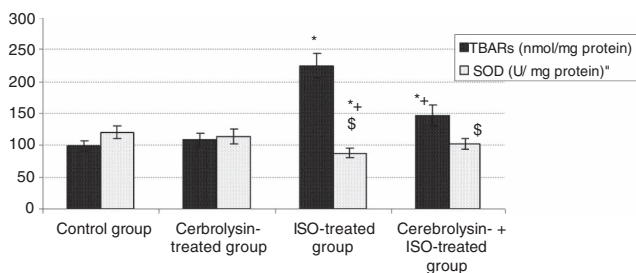


Fig. 2. Effect of pretreatment of cerebrolysin (5 mL/kg) on the mean levels of cardiac oxidative stress parameters in ISO-treated rats. Statistical analysis was carried out by one-way ANOVA followed by Tukey's HSD multiple comparison test. All values are presented as means of six rats \pm SEM. *Indicates significant change from control values at $p < 0.05$. +Indicates significant change from cerebrolysin-treated groups at $p < 0.05$. \$Indicates significant change from ISO-treated groups at $p < 0.05$. TBARs = thiobarbituric acid reactive substances; SOD = superoxide dismutase

Table II. Effect of pretreatment of cerebrolysin (5 mL/kg) on the mean serum levels of cardiotoxicity indices in ISO-treated rats

	Control group	Cerebrolysin-treated group	ISO-treated group	Cerebrolysin + ISO-treated group
CK-MB (U/L)	259.67 \pm 22.34	276.56 \pm 25.78	896.60 \pm 5.32*+	472.11 \pm 39.76*+§
Troponin I (ng/mL)	2.55 \pm 0.12	2.34 \pm 0.10	6.54 \pm 0.56*+	4.44 \pm 0.28*+§

Statistical analysis was carried out by one-way ANOVA followed by Tukey's HSD multiple comparison test. All values are presented as means of six rats \pm SEM

*Indicates significant change from control values at $p < 0.05$

+Indicates significant change from cerebrolysin-treated groups at $p < 0.05$

§Indicates significant change from ISO-treated groups at $p < 0.05$

infarcted area as shown by TTC staining. This ischemic insult was associated with cardiac damage that results in significant leakage of the cardiac enzymes, elevated oxidative stress markers, and apoptotic indices. It has been deduced that the detrimental action of isoproterenol is mainly due to β -adrenergic stimulation with rapid heart rhythm. Electrical depression and failure in the globally ischemic heart may contribute to adverse outcomes as asystole and ventricular fibrillation (7, 41). The observed increased level of serum CK and troponin I in ISO-treated rats in this study was in consonance with an earlier report (1, 34). Myocardial ischemia induced by isoproterenol results in deficient oxygen supply that leads to myocardial cell damage and rupture with the leakage of their enzyme content decreasing their activities in the heart of rats (21). It was proved that the plasma levels of troponin found in patients with unstable angina pectoris provide information about the severity of myocardial ischemia that caused degradation of cellular troponin and release of troponin degradation products in the circulation (43). The use of cerebrolysin seemed to preserve the functional and structural integrity and the permeability of the cardiac membrane, and thus restricting the leakage of these enzymes from the myocardium, as evident from the markedly reduced levels of these enzymes in cerebrolysin-treated ischemic group when compared to the non-treated ischemic group, thereby establishing the cardioprotective effect of cerebrolysin.

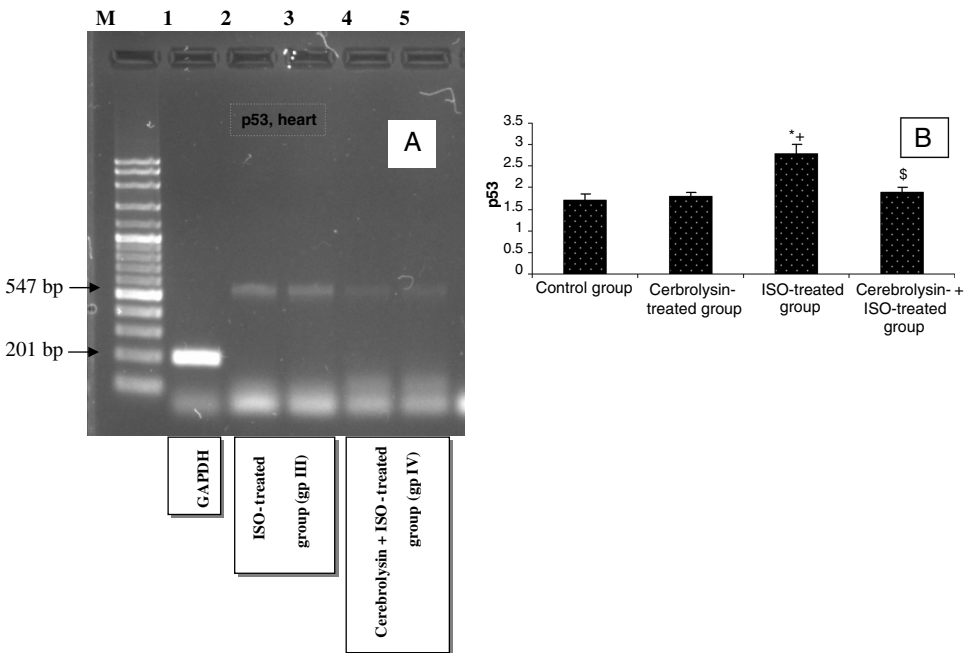


Fig. 3. Effect of pretreatment of cerebrollysin (5 mL/kg) on the mean levels of cardiac p53. (A) Expression analysis of p53 mRNA from cardiac tissues by RT-PCR. Lanes 2 and 3: ischemic cardiac tissues (gp III; ISO-treated group). Lanes 4 and 5: cardiac tissues after treatment with cerebrollysin (gp IV; cerebrollysin + ISO-treated group). M = 100-bp DNA ladder. The upper 547-bp bands represent the amplification of p53 in groups III and IV; and the lower 201-bp band represents the internal control GAPDH (Lane 1). (B) Quantitative analysis of p53 mRNA. Statistical analysis was carried out by one-way ANOVA followed by Tukey's HSD multiple comparison test. All values are presented as means of six rats \pm SEM. *Indicates significant change from control values at $p < 0.05$. +Indicates significant change from cerebrollysin-treated groups at $p < 0.05$. \$Indicates significant change from ISO-treated groups at $p < 0.05$

The deterioration of the oxidative stress markers in ISO-treated rats observed in this study (increase in the lipid peroxidation and decrease in the antioxidant activity) were in keeping with the previous findings (32). TBARs' content is considered an indirect marker of cellular damage degree as it reflects the extent of systemic lipid peroxidation. The antioxidant SOD activity reflects the cellular capacity to scavenge oxygen free radicals (31). ISO may induce myocardial ischemia due to cytosolic Calcium ion (Ca^{2+}) overload (38). The oxidative stress may be induced by quinone metabolites of ISO, which reacts with oxygen to produce reactive oxygen species (ROS) (30) and interfere with glutathione reductase and superoxide dismutase (31). However, after the first dose of ISO, the heart showed decreased lipid peroxidation. This is because the initial hypertrophy of the heart can protect itself to a limited extent against oxidative stress. The second dose of ISO, administered 24 h after the first treatment, showed toxic effects resulting in a higher increase in lipid peroxidation (30).

Pretreatment with cerebrollysin decreased the levels of lipid peroxidation in terms of TBARs while it increased the level of SOD in the heart of ISO-induced cardiotoxic rats when compared to non-treated ischemic group. The previous study had shown that cerebrollysin already decreased the TBARs' levels in the brain (14, 40) and heart tissue (29) with their resulting consequences. Cerebrollysin induces a decrease in the serum SOD levels in rats with

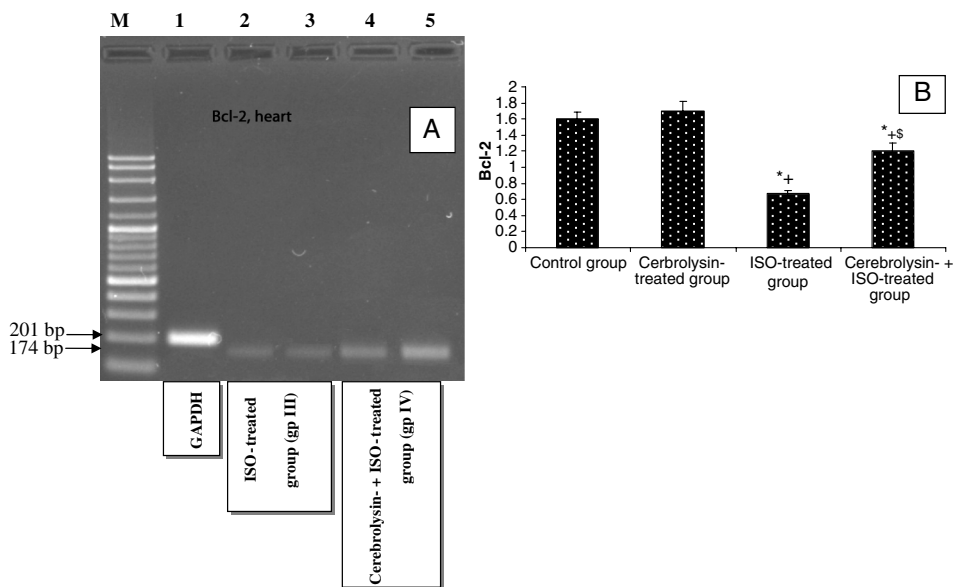


Fig. 4. Effect of pretreatment of cerebrolysin (5 mL/kg) on the mean levels of cardiac Bcl-2. (A) Expression analysis of Bcl-2 mRNA from cardiac tissues by RT-PCR. Lanes 2 and 3: ischemic cardiac myocytes (gp III; ISO-treated group). Lanes 4 and 5: cardiac tissues after treatment with cerebrolysin (gp IV; cerebrolysin- + ISO-treated group). M = 100-bp DNA ladder. The upper 201-bp band represents the internal control GAPDH (Lane 1) and the lower 174-bp bands represent the amplification of Bcl-2 in groups III and IV. (B) Quantitative analysis of Bcl-2 mRNA. Statistical analysis was carried out by one-way ANOVA followed by Tukey's HSD multiple comparison test. All values are presented as means of six rats \pm SEM. *Indicates significant change from control values at $p < 0.05$. +Indicates significant change from cerebrolysin-treated groups at $p < 0.05$. SIndicates significant change from ISO-treated groups at $p < 0.05$

brain injury (9). Deviatkina et al. (4) also reported the antioxidant effect of cerebrolysin in the brain, liver, thymus, spleen, and serum of rats under acute stress conditions. This positive antioxidant effect of cerebrolysin in the heart suggests its possible use in the experimental treatment of heart disturbances (29).

In addition to necrosis, apoptosis also plays a role in the process of tissue damage after MI. It is suggested that hypoxia-induced apoptosis has general molecular mediator, which is p53 that may induce apoptosis by stimulating the expression of Bax and/or repression of Bcl-2 expression (3, 16, 23). Being apoptotic markers, the increase in p53 expression and decrease in Bcl-2 expression in the ISO-induced ischemic infarction group compared to the control one shown in this study could be explained by hypoxia induced by ischemia. During acute MI, the formation of ROS in infarction areas may exceed the anti-oxidative capacity to initiate mitochondrial apoptotic signaling and activate Bax, a proapoptotic member of Bcl-2 family proteins (45). In healthy cells, the majority of Bax is localized in the cytosol, but with initiation of apoptotic signaling, activated Bax rapidly translocates to the mitochondria and undergoes a conformation shift to form protein-permeable pores on mitochondrial membranes. This results in the release of proapoptotic factors and cytochrome c from the mitochondria to the cytosol. Cytochrome c in turn forms apoptosomes, and activates Caspase-3, leading to cell death. On the contrary, the overexpression of anti-apoptotic Bcl-2 family protein was shown to intercept the release of cytochrome c in response to a variety of

apoptotic signals and therefore it inhibits apoptosis (10). It has been already reported that oxidative stress due to ISO administration provokes cardiac DNA fragmentation and apoptosis (6). In addition, ROS-induced apoptosis in neonatal rat cardiomyocytes was associated with an increase in p53 protein content, whereas protein concentrations of Bcl-2 and Bax were unaltered (44). Furthermore, a natural antioxidant, Chinonin, significantly attenuated hypoxia-/reoxygenation-induced apoptosis, downregulated p53 expression, and induced Bcl-2 upregulation (37). Inflammation has also been recognized as a major driving force in the ischemic process with increasing incidence of inflammatory markers (8). TNF- α can also induce cardiomyocyte apoptosis and participate in ventricular remodeling (49).

In contrast, it was found that ischemia-induced apoptosis occurred as readily in the p53 knockout mice as in wild-type mice indicating the existence of other, p53 independent, mechanisms of post-ischemic apoptosis (2). This may involve the stress-activated protein kinase (SAPK) signaling pathway as an important mediator of apoptosis where its activity is dramatically increased in rabbit hearts after ischemia/reperfusion (48).

Cerebrolysin restored the p53/Bcl-2 balance, thus providing an anti-apoptosis effect against MI damage in this study. These results are in line with Formichi et al., (5) who recorded a protective effect of cerebrolysin on oxidative stress-induced apoptosis in peripheral blood lymphocytes. Also, cerebrolysin markedly caused downregulation of Bcl-2 and upregulation of Bax in the ipsilateral thalamus after cerebral infarction in rats (47).

This study demonstrated that ISO-induced rats showed increase in myocardial infarct size with less TTC absorbing capacity. The induction of MI by ISO was previously reported by Li et al. (17). TTC dye forms a red formazan precipitate in the presence of intact lactate dehydrogenase enzyme activity, while the infarcted myocardium in lack of this dehydrogenase system fails to be stained with it. Unstained area of infarction had leakage of dehydrogenases and loss of membrane integrity (28). Pretreatment with cerebrolysin significantly decreased the infarction size which might be due to its potent antioxidant and anti-apoptotic activity that prevents cell death and leakage of lactate dehydrogenase enzymes.

Conclusion

Cerebrolysin could protect rat myocardium against ischemic insult through attenuation of oxidative stress-induced apoptosis, and thereby cerebrolysin could have a future recommendation in the prevention and/or treatment of ischemic heart diseases.

Conflict of interest

The authors declare no conflict of interest.

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