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CARDIOPROTECTIVE EFFECT OF GINGER IN A RAT MODEL OF MYOCARDIAL DAMAGE AND ITS POSSIBLE INTERVENTION IN PERK-ATF4-CHOP-PUMA APOPTOTIC PATHWAY

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For today the exact mechanisms of myocardial infarction and ischemia/reperfusion injury are still not fully understood. ER stress and integrated stress response pathways are thought to play an essential role in myocardial damage. This includes activation of endoplasmic reticulum kinase (PERK), induction of activating transcription factor 4 (ATF4), expression of pro-apoptotic transcription factor (CHOP) and P53 up-regulated modulator of apoptosis (PUMA) involved in apoptosis control. We used a rat model of isoproterenol-induced myocardial damage to elucidate the possible cardioprotective effect of Ginger through the influence on ER stress-induced apoptotic pathway. We also compared its effect with Captopril, inhibitor of angiotensin-converting enzyme. Male albino Wistar rats received 1.0 or 2.0 ml of Zingiber officinale (Ginger) powder suspension (200 mg/ml) daily by intra-gastric intubation for 28 days. Isoproterenol at a dose of 85 mg/kg was IP injected on the 27th and 28th days. Serum aspartate transaminase (AST) level was measured using kinetic kit. Heart tissue was used for RNA extraction, evaluation of gene expression by Q-RT-PCR, immunohistochemical determination of caspase-3 expression and histopathological studies. Our results showed that Isoproterenol administration increased CHOP-mRNA expression 4 folds in cardiac muscle tissue compared to normal control. Ginger pretreatment significantly decreased both CHOP and ATF4, and PUMA mRNA expression compared to Isoproterenol-treated groups. A significant reduction in ATF4 mRNA expression in a group pretreated with Captopril and Ginger compared to normal control group was observed. The results showed that Ginger reduced AST serum levels which correlated with results of histopathological studies of heart tissue. Our findings suggest that the protective effects of Ginger against myocardium damage induced by Isoproterenol may be mediated by reducing the endoplasmic reticulum stress by affecting the ATF4-CHOP-PUMA pathway.

K e y w o r d s: myocardial damage, ischemia-reperfusion, ATF4, CHOP, PUMA, AST, Ginger, Captopril, Isoproterenol.

Coronary heart disease is considered the first leading cause of mortality worldwide. A yearly estimation of deaths is 3.8 and 3.4 million in men and women respectively [1].

Reperfusion after acute myocardial damage is an important rescue mechanism of myocardium against ischemia. Sudden restoration or reperfusion of injured myocardium may result in more injury without restoration of the affected tissue. Modifying the conditions of the myocardium during the phase of reperfusion may reduce the post-infarction injury [2]. This may be through the protective effect of the antihypertensive ACE inhibitor captopril [3] or ginger that has an effect comparable to that of captopril [4].

Receptor-interacting protein kinase-3 (RIPK3) pathway is implicated in cardiac injury and necroptosis of cardiomyocytes in rats exposed to excessive β-adrenergic stimulation [5]. RIPK3, or RIP3, a protein that seems to have a facilitative role in tissue damage and is essential in the apoptosis pathway called necroptosis. This protein was proposed to be a regulator of apoptosis downstream of the tumor necrosis factor (TNF) with caspase activation [6]. Protective agents against isoproterenol-induced chronic

heart failure act through the PI3K Akt, p38 MAPK and NF κB pathways [7].

Hypoxic stress induces the unfolded protein response in the endoplasmic reticulum (ER) via the protein kinase RNA-like endoplasmic reticulum kinase (PERK) with an elevation of the Eukaryotic Initiation Factor 2 alpha (eIF2-α). The eIF2-α results in suppression of global protein translation with the arrest of the cell cycle in the G1 phase and induction of the activating transcription factor 4 (ATF4) that up-regulates gene expression responsible for cell homeostasis restoration. Promotion of the signaling pathway that results in selective translation of ATF4 with a downstream CCAAT-enhancer-binding protein homologous protein (C-EBP homologous protein, CHOP) and thus ends in the induction of pro-apoptotic genes and initiation of an apoptotic cascade [8].

The endoplasmic reticulum is an organelle that plays a role in signal transduction in cell survival and death. Conditions that induce ER stress, like myocardial infarction conditions, induce the accumulation of unfolded proteins within the ER or Ca^{2+} overload. ER stress triggers apoptosis by induction of CHOP, and production of pro-apoptotic factors (caspase-12). Apoptosis caused by caspase-12 and triggered by ER stress, is one of the characteristic features of ischemia/reperfusion injury, so reducing or suppressing ER stress can protect the myocardium from damage [9].

Control of cell fate, whether to survive or proceed through apoptosis, is a consequence of imbalance between the survival and apoptotic signals imposed by ER stress. Cell fate imposed by ER stress is dependent on the duration of stress and is achieved through the unfolded protein response (UPR), the translation of which is driven by eIF2 α phosphorylation. The PERK/eIF2α-dependent induction of pro-apoptotic transcription factor CHOP is an important target driven by ATF4 [10].

ATF4 expression triggered by stress initiates an adaptive cellular response by regulating target gene expression. The target genes driven by ATF4 are responsible for regulating activities involved in cellular differentiation, angiogenesis, tumor metastasis, and amino acid biosynthesis. Numerous genes are induced by hypoxia. ATF4 is one of these genes that has a mediator role in the UPR [11].

CHOP was described to be involved in the induction of cell cycle arrest in response to stress and this was dependent on time and strength of stress. Early stress with CHOP expression participates in cell survival whereas on prolonged stress, CHOP induces apoptosis [12]. This is also proved by the reduced P53 up-regulated modulator of apoptosis (PUMA) expression following CHOP knockdown [13].

ER stress is a pro-apoptotic event that occurs when cells are exposed to stressful hypoxia in tumors with activation of the CHOP apoptotic pathway as a part of the PERK-ATF4-CHOP pathway. ER stress serves a pivotal role as contributing to pro-apoptotic and anti-apoptotic pathways. ATF4 plays a crucial role in cellular adaptation to stress whether short or prolonged as it stimulates CHOP that initiates an apoptotic cascade ending in cell death [14].

PUMA is a component of the cell death pathway induced by the endoplasmic reticulum stress. Deletion of PUMA resulted in improving cardiac function and reduction of cardiomyocyte death in rats exposed to myocardial ischemia and reperfusion. Cardiomyocyte apoptosis was also found to be increased when PUMA was expressed by induction or by ectopic expression [15]. PUMA protein starts to increase 6 h after starting mechanical stretch and remained up to 18 h. They also found that PUMA expression and apoptosis induced by cyclic stretch produced by the AV shunt were inhibited by atorvastatin, a cholesterol lowering drug [16]. ATF4 was also implicated in induction of another transcriptional activator which is PUMA that mediates apoptotic cell death by inducing DNA damage as well as its role in apoptosis induced by ER stress [17].

Captopril, an orally active ester pro-drug, is used as an angiotensin-converting enzyme (ACE) inhibitor for reducing mean arterial blood pressure and improving heart failure symptoms. Captopril has been shown to have potential effects on the cardiovascular system through reduction in preload and afterload, which contribute towards preservation of cardiac contractility and improvement of survival following myocardial infarction, reduces the incidence of congestive heart failure and left ventricular dysfunction. Captopril significantly reduces of blood pressure and improves heart failure symptoms [18]. Although other ACE inhibitors were associated with a lower patient mortality rate compared to Captopril, Captopril seems to protect against myocardial necrosis induced by isoproterenol [19].

High doses of catecholamines administered may deplete the energy reserve of cardiac muscle

cells and thus may result in biochemical and structural changes which are responsible for the development of irreversible damage that occurs under stress conditions.

Isoproterenol was administered to rats to induce stress in the myocardium resulting in myocardial damage [20]. Isoproterenol treatment induced toxic effects that result in a high increase in lipid peroxidation and ROS generation and oxidative stress [21].

Isoproterenol-induced necrosis was shown to occur in the subendocardial region of the left ventricle and in the interventricular septum with observed cardiac hypertrophy caused by pressure overload [22].

ACE inhibitors appeared to be the medications of choice to reduce cardiovascular events and prevent myocardial damage as they show a great reduction in the risk of myocardial damage and stroke [23]. Captopril administered to patients with AMI after interventional therapy effectively improved the cardiac function and reduced the level of inflammation [24]. Captopril pretreatment prevented post-myocardial infarction hypertrophy mostly by preservation of aerobic metabolism in cardiac muscle [25]. Captopril preconditioning also attenuates myocardial ischemia/reperfusion injury by reducing oxidative stress and inflammation [26].

A compensatory effect occurs when Isoproterenol induces cardiac dilatation and heart failure with significant increases of echo-cardiographically measured left ventricular end-diastolic posterior wall thickness and left ventricular end-diastolic pressure was also markedly increased that are significantly attenuated by ramipril treatment [24].

Looking for a cardio-protective agent after ischemia-reperfusion is vital for cardiac function preservation to effectively prevent cardiac disorders. In this study, we evaluated the potential effect of *Zingiber officinale* (Ginger) extract in isoproterenol-induced myocardial damage in male Wistar rats and its underlying mechanism of action. We also compared its effect to the ACE inhibitor Captopril that has been shown to have potential effects on the cardiovascular system through reduction in mean arterial pressure, preload, and afterload which contribute towards the preservation of cardiac contractility.

The aim of this study is to evaluate the role of Ginger extract in reducing the post-ischemic injury following myocardial damage and its possible intervention at the PERK-ATF4-CHOP-PUMA apoptotic pathway compared the effect of an antihypertensive drug Captopril.

Materials and Methods

Chemicals. Isoproterenol, molecular formula ($C_{11}H_{17}NO_3 \cdot HCl$) supplied as a crystalline solid analytical grade was obtained from the Sigma Chemical Company, St. Louis, MO, USA. Ginger powder was obtained from Mepaco Co, Egypt. Captopril (ACE inhibitor); Captopril: (0.3-0.5 mg/kg/dose) obtained from Alexandria Co, Egypt.

Formulation and administration of Ginger. Ginger powder and 0.5% carboxy methyl cellulose (CMC) were suspended in physiological saline and each rat received 1.0 ml or 2.0 ml of ginger suspension at a dose of 200 or 400 mg/ml (for the low and high doses respectively) daily by intra-gastric intubation for 28 days.

Induction of myocardial damage. The myocardial damage was induced by intra-peritoneal (IP) injections of two consecutive doses of Isoproterenol hydrochloride (Iso). Isoproterenol was freshly prepared in distilled water at the time of induction of myocardial damage. Isoproterenol (85 mg/kg) is injected via IP route in rats for two consecutive days on the 27^{th} and 28^{th} days, respectively, with 24 h interval [27].

Experimental protocol. Forty-eight male albino Wistar rats weighing 200-250 g were selected for the study. Groups were housed in steel cages with filter tops under controlled conditions of 12 hourly cycles of light and dark, 50% humidity at 28°C. Diet was supplied as a standard pellet fed throughout the experimental period and water was given ad libitum. The study was conducted after obtaining a clearance from the faculty of medicine Animal Ethical Committee. The experiment was conducted in accordance with the rules set by the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986).

Forty-eight rats were randomly divided into 8 groups with 6 rats in each group.

Group I (Saline group; normal control rats) received oral physiological saline solution with 0.5% carboxymethylcellulose (1 ml/day) for 28 days and on the 27^{th} and 28^{th} days, this group received two consecutive IP injections of 0.1 ml physiological saline given at 24 h interval.

Group II (Saline + Iso group) served as damage induced group; rats received physiological saline so-

lution with 0.5% carboxymethylcellulose (1 ml/day) orally for 28 days and on the 27th and 28th days, they received two consecutive IP injections of isoproterenol (85 mg/kg) at 24 h interval.

Groups III (Ginger 200 + Iso group) received Ginger extract (200 mg/day) orally for 28 days on the 27th and 28th days, they received two consecutive IP injections of isoproterenol (85 mg/kg) at 24 h interval.

Groups IV (Ginger 400 + Iso group) received Ginger extract (400 mg/day) orally for 28 days on the 27th and 28th days, they received two consecutive IP injections of isoproterenol (85 mg/kg) at 24 h interval.

Group V (Captopril + Saline group) received Captopril (1 mg/kg/day) orally for 28 days and on the 27th and 28th days, they received two consecutive IP injections of 0.1 ml physiological saline at 24 h interval.

Group VI (Captopril + Iso group) received Captopril (1 mg/kg/day) orally for 28 days and on the 27th and 28th days, they received two consecutive IP injections of isoproterenol (85 mg/kg) at 24 h interval.

Group VII (Ginger 400 + Captopril + Iso group) received Ginger extract 400 mg/ day, in combination with Captopril (1 mg/kg/day) orally for 28 days and on the 27th and 28th days, they received two consecutive IP injections of isoproterenol (85 mg/kg) at 24 h interval.

Group VIII (Ginger 400 group) served as preservative group; rats received only Ginger extract (400 mg/day) orally for 28 days.

Biochemical parameters. Twenty-four hours after the last dose, blood samples were collected from the tail vein, and serum was separated. Then, all animals are sacrificed after being anaesthetized by IP injection of urethane (1.39 mg/kg) given as 25% freshly prepared solution [28]. Hearts were dissected out for histological studies and serum for the biomarker aspartate aminotransferase (AST) estimation was preserved at -20°C.

Serum AST levels were measured by chemical analyzer (TC 3300-Teco diagnostic, Anaheim, USA) using kinetic kits (Human biochemical and diagnostic kits, Wiesbaden, Germany) according to the standard procedures and instructions manual [29]. Heart tissue was excised after sacrificing animals and divided into 3 parts for histopathology and immunohistochemistry (IHC) studies, RNA extraction and protein estimation.

Tissue samples for IHC were preserved in formalin and sent for histopathological processing, whereas those for protein estimation or RNA extraction were kept at -80°C.

RNA extraction. For RNA extraction, 100 mg of heart tissue were homogenized in 1 ml of ribozol solution by ultrasonic homogenizer (Sonic-Vibracell, Sonics and materials Inc., Newtown, USA) according to manufacturer's instructions for total RNA extraction. RNA was dissolved in Tris EDTA (TE) buffer and the concentration of RNA is estimated by Genova spectrophotometer (Genova Plus, Jenway, Stone, Staffs, UK); by measuring the OD of each sample at wavelength, 260 and 280, the ratio of OD at 260/280 was between 1.8-2.0 and with a conversion factor 1 A260 nm unit = 40 µg/ml for single-stranded RNA [30]. After that the RNA integrity was confirmed by running of RNA samples on 1% standard Agarose gel, where RNA bands were examined (data are not shown). Extracted RNA was stored at -20°C for further use.

Quantitative RT-PCR. One µg of extracted total RNA (equivalent to 3-4 µl of extracted RNA)

Table. Forward and reverse primers of PUMA, ATF4 and CHOP and β-actin primers as the house keeping for β-actin

Primer name	Primer sequences	GenBank accession number
PUMA	5′-CTGTCCCCACGCTGTC (forward) and 5′-GCTTGCTTGCTGGTGTCG (reverse)	Gene ID: 317673
CHOP	5′-GACAAGTTCAGGAAGGACAGC (forward) and 5′-CGGAGGAGGTGAGTGAGTCA (reverse)	Gene ID: 29467
ATF	(F): 5′-ATGGCCGGCTATGGATGAT-3′ and (R): 5′-CGAAGTCAAACTCTTTCAGATCCATT-3′	Gene ID: 79255
β-actin	(F):5′-CGTGGGCCGCCCTAGGCACCA-3′ and (R): 5′-TTGGCTTAGGGTTCAGGGGGG-3′	Gene ID: 81822

was reverse transcribed in triplicate by HERA CYBER GREEN RT-qPCR kit (WF1030300X). (Willowfort, UK) according to manufacturer's instructions thermal cycler (Applied Biosystems Step One TM Real-Time PCR system Thermal Cycling Block 7500 fast, ThermoFisher Scientific, Singapore, LTD, USA). Table represents forward and reverse sequences of primers used in this study.

Gene expression results by Q-PCR were presented and calculated. Genes expression levels were scaled and graphed relative to control samples [31].

Histopathological evaluation. The apical regions of hearts, fixed in a solution of 10% buffered formalin, were embedded in paraffin for 24 h after the onset of fixation. Cuts measuring 4-µm sections were stained with hematoxylin eosin stain (HE) and examined microscopically. The severity and extent of myocardial damage were observed for each case. The pathologist did not know to which group each slide corresponded. The range of histologic myocardial injury findings was classified into the following grades: (0) – No change, (1) – Mild focal myocyte damage or small multifocal degeneration with slight degree of inflammation, (2) – Moderate extensive myofibrillar degeneration and/or diffuse inflammatory process, (3) – Severe necrosis with diffuse inflammatory process [32].

Immunohistochemistry (IHC). Selected heart tissues were excised and fixed in 10% buffered formalin (pH 7.4) prior to being embedded in paraffin. Paraffin-embedded tissue sections of 4-µm thickness on positive charged slides were deparaffinized in xylene and rehydrated through descending grades of ethyl alcohol. To enhance antigen retrieval, the slides were heated in 10 mM sodium citrate buffer, pH 6.0, (Cat. #RB-1197-R7, 7 ml, Ready-To – Use for Immunohistology) using a microwave oven for 30 min at 750 W. Endogenous peroxidase activity was blocked by incubating slides in 0.3% hydrogen peroxide in methanol for 15 min.

Slides were incubated at 4°C overnight with rabbit polyclonal antibody: caspase-3 (CPP32) Ab-4 (NEOMARKERS' Cat. #AP-9003, 1:200 dilution), as primary antibody and goat anti-mouse as a secondary antibody (all from Abcam, Cambridge, UK). Sections were then incubated with streptavidin-biotin immune-peroxidase conjugated secondary antibody at a dilution of 1 mg/ml in 10 mM phosphate buffered saline, pH 7.4, with 0.2% BSA and 0.09% sodium-azide for 1 h. Signal detection was obtained using an immune-enzymatic assay with streptavidin-biotin complex (Thermo Fisher Scientific Anatomical Pathology 46360 Fremont Blvd. Fremont, CA 94538, USA).

IHC-optimized staining was achieved using positive and negative control slides. Negative control for all runs was performed by omitting the primary antibodies. Positive control was cancer colon tissue sections.

Heart sections were examined by light microscopy at 100-200X power. Data were semi-quantitatively analyzed and scoring was performed by determining the intensity of cytoplasmic expression and the percentage of positive cells.

The findings of immunohistochemical expression of caspase were scored as weak (less than 10% of myocytes) immune-positive reaction or focal cytoplasmic expression of caspase-3, mild (30-45% of myocytes), mild to moderate (50-55% of myocytes), strong (60-65% of myocytes) [33].

Statistical analysis. Statistical analysis was carried out using GraphPad Prism 8.0. Data were expressed as mean ± SEM. One Way Anova (ANOVA) is used to compare groups of data and one sample *T*-test and values were considered significant when $P < 0.05$.

Results

In this study we evaluated the role of ginger extract in reducing the post-ischemic injury following myocardial damage induced by IP injections of Isoproterenol, and its possible intervention at the PERK-ATF4-CHOP-PUMA apoptotic pathway. We also compared its effect to Captopril in ischemia/reperfusion injury.

The data shown in Fig. 1 showed that Isoproterenol administration up-regulated the expression of CHOP mRNA compared to normal control (4-fold increase). Low and high dose pretreatment with Ginger produced a significant down-regulation of CHOP mRNA expression in cardiac muscle tissue compared to normal and Isoproterenol-only treated group. Captopril-pretreated group did not show a significant change in CHOP mRNA expression but seemed to reduce damage induced by isoproterenol when they were combined, may be by another mechanism. There was also a significant reduction in expression when the group was pretreated with Captopril and Ginger compared to normal control group (>7 and 8-fold reduction).

The data presented in Fig. 2 showed that isoproterenol administration produced a significant in-

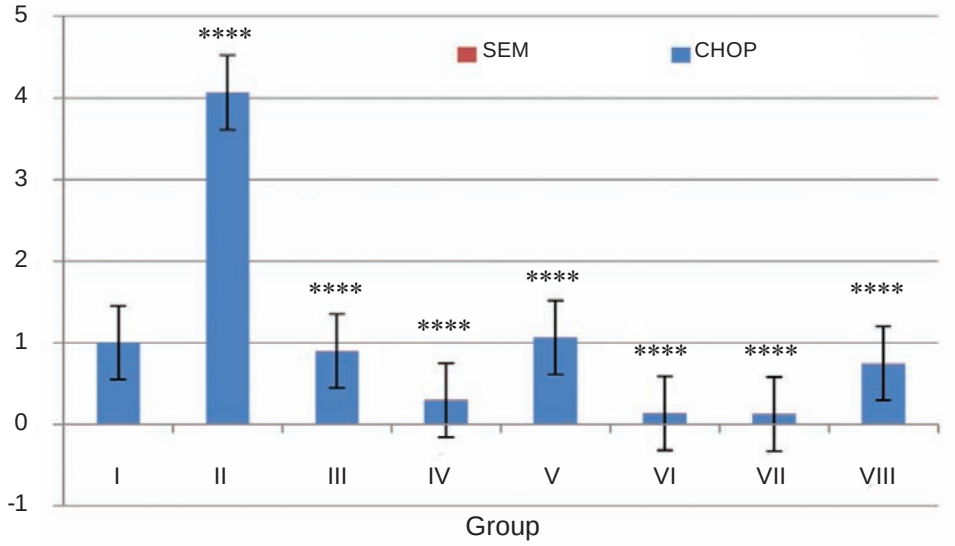

*Fig. 1. Effects of Ginger and/or Captopril on the expression of CHOP mRNA in cardiac muscle tissue: I – Saline group, normal control rats; II – Saline + Iso group; III – Ginger 200 + Iso group; IV – Ginger 400 + Iso group; V – Captopril + Saline group; VI – Captopril + Iso group; VII – Ginger 400 + Captopril+ Iso group; VIII – Ginger 400 group. Data are presented as mean ± SEM, ****significance from control, P < 0.0001, (n = 6)*

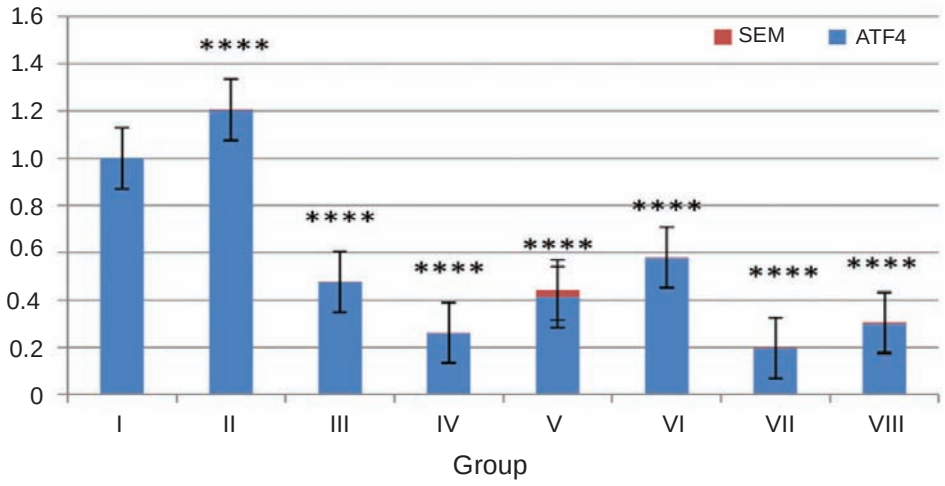

*Fig. 2. Effects of Ginger and/or Captopril on the expression of ATF4 mRNA in cardiac muscle tissue: I – Saline group, normal control rats; II – Saline + Iso group; III – Ginger 200 + Iso group; IV – Ginger 400 + Iso group; V – Captopril + Saline group; VI – Captopril + Iso group; VII – Ginger 400 + Captopril+ Iso group; VIII – Ginger 400 group. Data are presented as mean ± SEM, ****significance from control, P < 0.0001, (n = 6)*

crease in the expression of ATF mRNA compared to normal control (20% increase). Low and high dose pretreatment with Ginger produced a significant down-regulation of ATF mRNA expression in cardiac muscle tissue compared to normal and Isoproterenol only treated group. Captopril pretreated group showed a significant decrease in ATF mRNA expression. There was also a significant reduction in ATF mRNA expression when the group is pretreated with Captopril and Ginger compared to normal control group (4-fold reduction).

The data in Fig. 3 showed that isoproterenol administration increases the mRNA expression level of PUMA as expected when administered

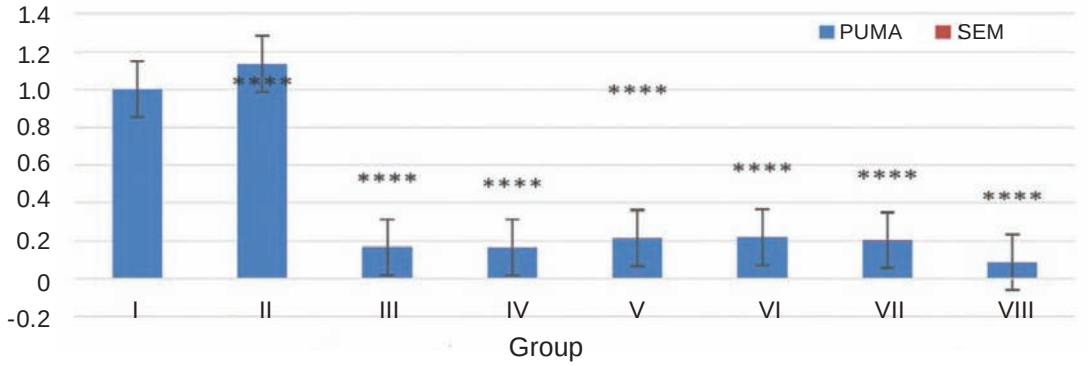

*Fig. 3. Effects of Ginger and/or Captopril on the expression of PUMA mRNA in cardiac muscle tissue: I – Saline group, normal control rats; II – Saline + Iso group; III – Ginger 200 + Iso group; IV – Ginger 400 + Iso group; V – Captopril + Saline group; VI – Captopril + Iso group; VII – Ginger 400 + Captopril+ Iso group; VIII – Ginger 400 group. Data are presented as mean ± SEM, ****significance from control, P < 0.0001, (n = 6)*

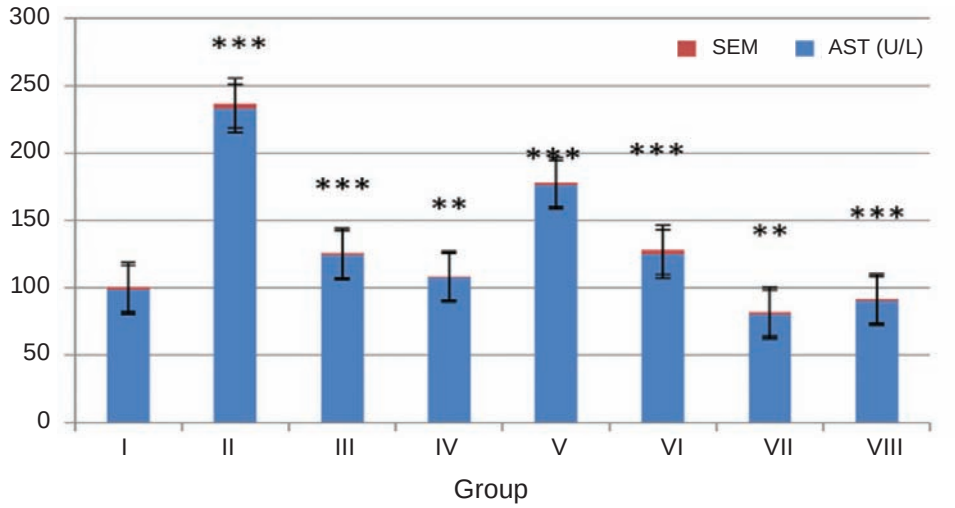

*Fig. 4. Serum levels of the cardiac enzyme AST: I – Saline group; normal control rats; II – Saline + Iso group; III – Ginger 200 + Iso group; IV – Ginger 400 + Iso group; V – Captopril + Saline group; VI – Captopril + Iso group; VII – Ginger 400 + Captopril+ Iso group; VIII – Ginger 400 group. Data are presented as mean ± SEM, **significance from control (P < 0.01), ***significance from control (P < 0.001), (n = 6)*

with saline. Puma expression was significantly reduced ($P < 0.0001$ – paired T test and $P < 0.0001$ ANOVA) when Ginger was combined with Isoproterenol in the low and high doses compared to the Isoproterenol with the saline group. When Ginger was combined with Captopril and Isoproterenol, no observable difference was noted from the Isoproterenol with Ginger or Captopril, although all data were significantly reduced below those of the control group. The Captopril-pretreated group showed a significant reduction of expression of PUMA below control but almost comparable to the Ginger-Captopril-Isoproterenol group suggesting a minimal effect of Captopril on PUMA expression.

Cardiac enzyme estimation: levels of AST have been used to diagnose myocardial infarction or damage although not as specific as cardiac enzymes; troponin and CK-MB; but can serve as an index for assessment of severity of myocardial infarction in the present study. An elevated serum level of this biomarker indicates myocardial damage but is not indicative of the mechanism of damage induced by Isoproterenol.

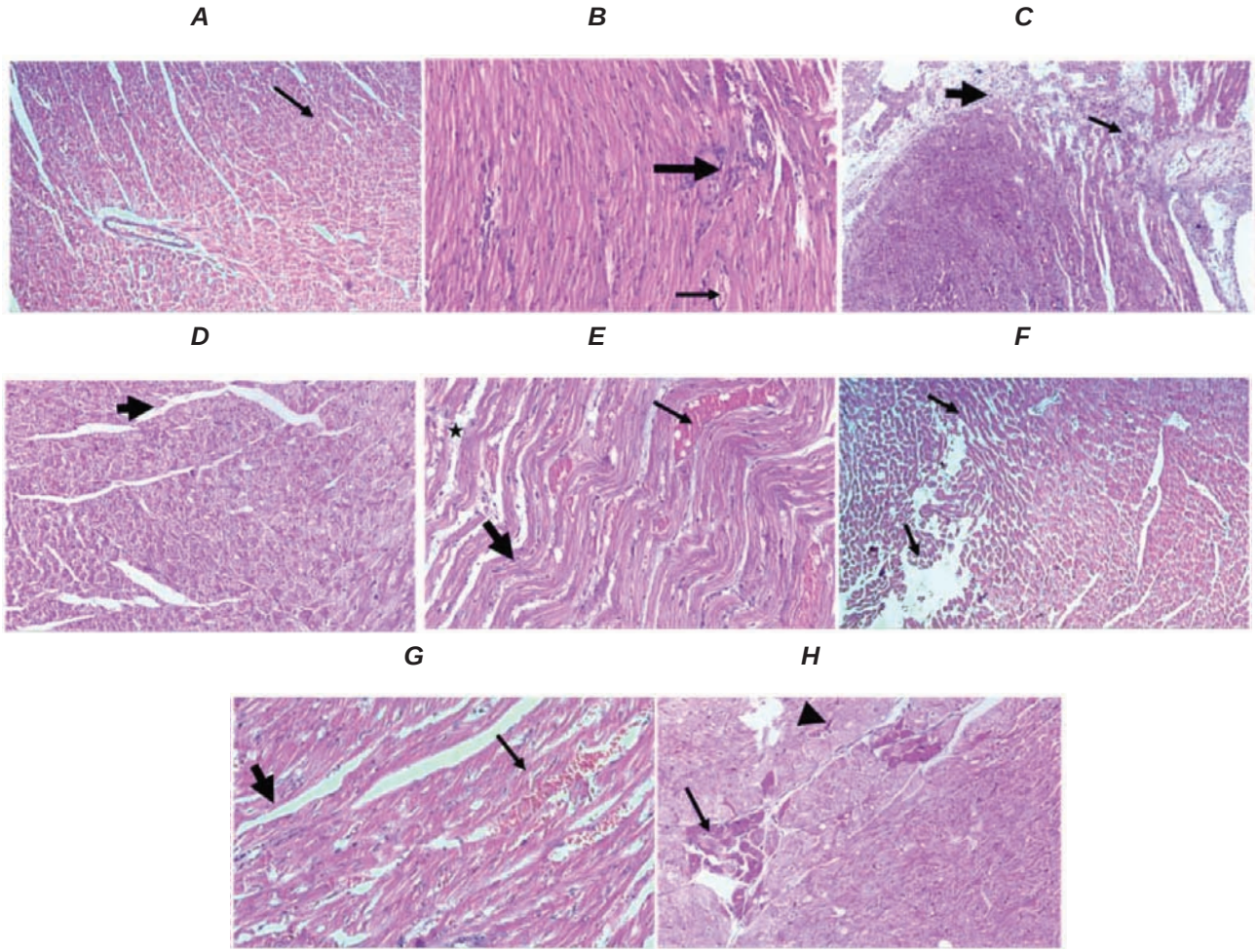

*Fig. 5. Histopathological findings (light photomicrographs; histopathological sections of H&E-stained myocardial tissue. Scale 200×). **A** – (grade 0) normal cardiac muscle architecture; **B** – (grade 2) myocardial tissue with moderate inflammation (thick arrow) and mild hemorrhage (thin arrow); **C** – (grade 1) myocardial tissue with moderate myofibrillar degeneration (thin arrow) and inflammation (thick arrow); **D** – (grade 1) myocardial tissue with mild inflammation (thick arrow), **E** – (grade 2) myocardial tissue with moderate myofibrillar degeneration (star), mild inflammation (thick arrow) and congested vascular spaces (thin arrow); **F** – (grade 2) Moderate – focal myofibrillar degeneration, the moderate inflammatory process in the form of infiltration of neutrophils and plasma cells; **G** – (grade 1) Mild - focal myocyte degeneration with a slight degree of inflammation in the pericardium and mild hemorrhage; **H** – (grade 2) myocardial tissue with moderate myofibrillar degeneration in the form of cloudy swelling (thin arrow), slight inflammatory process (thick arow) with mild hemorrhage between myocytes*

The data presented in Fig. 4 showed that Isoproterenol administration induced a significant increase in the serum enzyme AST level (> 2 folds elevation) compared to normal control. Low and high dose pretreatment with Ginger produced a significant reduction in cardiac enzyme levels compared to Isoproterenol-only treated group and this may be due to the protective effect of ginger. A significant reduction of the AST level in the Captopril pretreated group compared to the Iso-group (25% reduction) and the Captopril-Iso group (50% reduction) although shown to be above the control group. Also, a significant reduction in AST level in the group with a combined pretreatment with Captopril and Ginger (Ginger-Captopril-Iso group) compared to Iso-treated group (nearly 3-fold reduction) and the Captopril pretreated groups although being all elevated above normal.

Our study revealed that Ginger pretreatment at different doses (low and high doses) resulted in reduction of AST levels in a dose-dependent manner and thus helped the preservation of myocardial

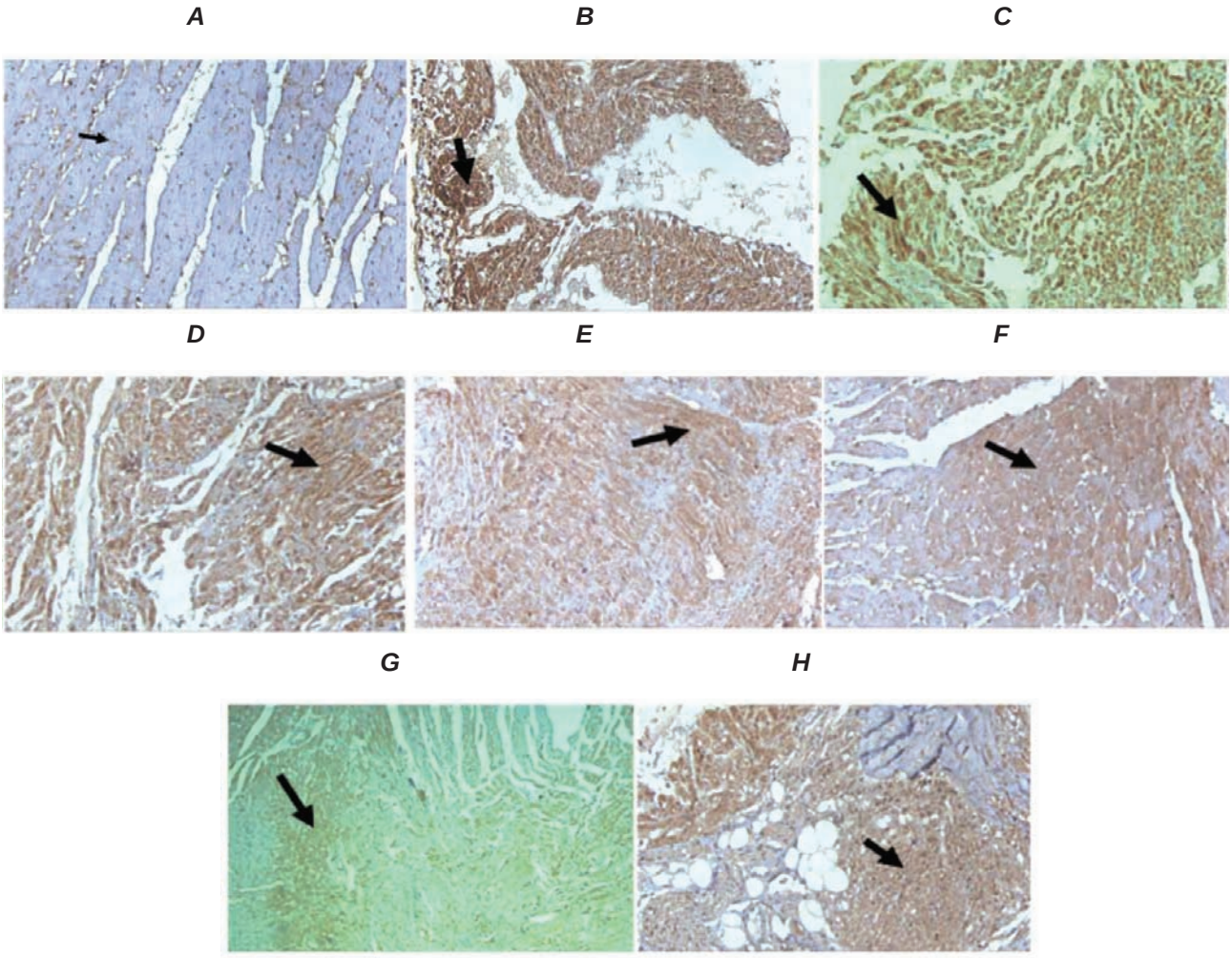

*Fig. 6. Immunohistochemistry findings (light photomicrographs of immunohistochemical caspase 3 sections of stained myocardial tissue. Scale 200×). **A** – Weak focal cytoplasmic expression of caspase 3 in less than 10% of cells; **B** – strong cytoplasmic expression in 60% of cells; **C** – moderate cytoplasmic expression in 65% of cells; **D** – Moderate cytoplasmic expression in 50% of cells; **E** – Mild to moderate cytoplasmic expression in 55% of cells. F – Moderate cytoplasmic expression in 45% of cells; **G** –Moderate cytoplasmic expression of caspase 3 in 30% of cells; **H** – mild to moderate cytoplasmic expression in 38% of cells*

cells. This is supported by studies that showed a correlation between AST enzyme level and the damage induced by Isoproterenol. Also, the effect of pretreatment with Ginger [34-36], and the histopathological findings were consistent with the data presented (Fig. 5, Fig. 6).

Discussion

To evaluate the ER stress state of cardiac muscle tissue *in vivo*, animals were treated with Isoproterenol 85 mg/kg once, sacrificed one day after administration and samples collected for investigation.

Previous studies using some herbal extracts of Ginger, Nigella Sativa and Fenugreek administration have been implicated in reducing ROS generation and oxidative stress damage of tissues. Our study proposed the role of Ginger powder in improving post-infarction reperfusion injury by affecting the ATF4-CHOP pathway involved in apoptosis and result in improving cell survival.

Our RT-PCR studies showed a reduction of CHOP expression and resulted in downstream effects involving reduced caspase expression and concomitant histological changes consistent with reduced myocardial damage. Additional evidence was a reduction of enzyme levels when Ginger is co-administered with Isoproterenol indicating a protective role of Ginger in post-ischemic injury reduction.

Our study also demonstrated a significant reduction of expression of ATF4 and CHOP mRNA, a reduction of the cardiac enzyme levels as well as improved histological pattern and caspase-3 expression in the Ginger-treated groups with isoproterenol-induced myocardial damage compared to ISO treated group. Administration of Ginger as a pretreatment had a role in adaptation of the gene expression of the ATF4-CHOP pathway. The data in the current study also pointed to the effects of Captopril. Also, the pharmacological intervention with the ACE inhibitor Captopril showed a protective effect against myocardial damage.

It was demonstrated that activation of the ATF4-CHOP signaling pathway following endoplasmic reticulum stress by hypoxia or damage is sufficient to induce caspase-3 expression and apoptosis. The findings in our work produced a reasonable platform to rely on in using Ginger to down-regulate the ATF4-CHOP pathway and reduce caspase-3 production.

CHOP, a key regulator of apoptosis, has been reported to have an ATF4 binding site, and both play a role in inducing caspase-3 and regulation of apoptosis. Our results demonstrate that Ginger has a significant reducing effect on CHOP expression and down-regulation of apoptosis. CHOP expression levels are elevated with ER stress and curcumin pretreatment reduced the ischemia/reperfusion injury and ameliorated the effects of ischemia/reperfusion injury [37].

The ER stress-activated kinase (PERK) is responsible for phosphorylating the eukaryotic translation initiation factor 2 alpha subunit (eIF2 α) with subsequent reduction in its activity and activation of ATF4 mRNA. The ATF protein product binds the CHOP promoter and activates it with a resulting sensitization to apoptosis mediated by the ATF-CHOP pathway through activating the pro-apoptotic genes and suppressing anti-apoptotic Bcl-2 protein [17, 38].

ATF4, CHOP and caspase-3 protein levels were elevated with the ischemia/reperfusion injury and reduced when pretreated with Barbaloin – a myocardial protective agent [39].

Ginger-Isoproterenol-treated rats showed a reduction in ATF4 and CHOP levels and this finding is consistent with reports showing that CHOP deletion resulted in protection against apoptosis in cultured fibroblasts [12, 40]. In our study, Ginger pretreatment led to a reduction in the expression of ATF4 and CHOP and markers of myocardial muscle damage and reduced histological changes as well.

In this study, the mRNA expression of ATF4 was increased in the Isoproterenol-induced myocardial damage compared to normal control rats. Ginger pretreatment decreased ATF4 mRNA expression indicating that the protective effect of Ginger on cardiac cell apoptosis was associated with inhibition of ATF4 expression. The expression of CHOP was upregulated in the myocardial damage compared to normal control animals. However, rats treated with Ginger showed significant reduction of CHOP mRNA expression in pretreated animals [21, 41].

Valsartan, an angiotensin receptor blocker used to relax blood vessels was found to block the CHOP/PUMA mediated apoptosis in myocardial cells induced in streptozotocin-induced diabetes in rats and thus ameliorates endoplasmic reticulum stress [42].

It was revealed that mechanical stretch resulted in a transient increase in expression of PUMA which started 6 h after stress due to exposure to stretch and reached its maximum after 18 h and may then decline [43]. However, PUMA expression was found to be significantly increased 48-120 h in neurons after neuronal ischemia, which correlates with neuronal degeneration upon prolonged ER stress [44]. Exposure to methamphetamine was found to increase the expression of CHOP and PUMA resulting in activation of the caspase-3 dependent cascade and initiation of the mitochondrial apoptosis pathway. Also, PUMA expression was reduced following CHOP knockdown in the same study indicating that PUMA is the downstream protein of CHOP [13].

This may explain the delayed expression of PUMA in our study where PUMA expression appeared to be close to normal controls in the Iso group with saline. The groups pretreated with Ginger showed significant reduction below normal control. The exception for the reduced PUMA expression was the Captopril pretreated group which showed PUMA expression levels comparable to the Captopril-Iso and Ginger-Cap-Iso. This coincideg with the findings showing Captopril had a protective effect against apoptosis when used as a blood pressure-lowering drug and to lower the cardiac afterload in heart failure cases [3]. Ginger pretreatment also prevented myocardial hypertrophy through its inhibitory effect on ACE an effect comparable to captopril [4, 25] and attenuates myocardial ischemia/reperfusion injury [26].

This is consistent with our findings in the group pretreated with Captopril where there was a (grade 2) moderate myofibrillar degeneration and

mild inflammatory reaction in the form of neutrophil and plasma cell infiltration and congested myofibrillar vascular spaces.

There was a significant reduction in ALT, AST, CK-MB enzyme activities in the serum of rats pretreated with Ginger extract when compared with ISO-control rats [34], also our histopathological findings are consistent with the data presented.

Limitations of this study. This study hypothesizes a mechanism for the protective effect of Ginger on the damaging effect of Isoproterenol besides the mechanisms provided by other researchers [4]. But this hypothesis may be clinically limited and applied to the experimental model presented but the mechanism shown seems to be promising, however, complementary studies are needed to further explore other possible mechanisms by which ginger protects the myocardium against damage and its beneficial effects on the cardiovascular system.

Conclusion. Collectively, our results confirmed a hypothesis that the administration of Ginger concomitantly with ISO ameliorated the destructive effect of ISO on cardiac muscle cells with preservation of the histologic architecture of cardiac muscle cells. These results demonstrated that Ginger has a protective potential in myocardial damage and may ameliorate the clinical severity of myocardial damage and reperfusion injury following myocardial damage with its effects comparable to those of the ACE inhibitor Captopril. This is mediated through reducing endoplasmic reticulum stress by modulating the ATF-CHOP pathway.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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КАРДІОПРОТЕКТОРНИЙ ЕФЕКТ ІМБИРУ НА МОДЕЛІ ПОШКОДЖЕННЯ МІОКАРДА У ЩУРІВ ТА ЙОГО МОЖЛИВИЙ ВПЛИВ НА АПОПТИЧНИЙ ШЛЯХ PERK-ATF4-CHOP-PUMA

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Точні механізми інфаркту міокарда та ішемічно-реперфузійного пошкодження все ще до кінця нез'ясовані. Вважається, що у пошкодженні міокарда важливу роль відіграють стрес ендоплазматичного ретикулума (ЕПР) та шляхи інтегрованої відповіді на стрес. До них належать активація протеїнкінази ендоплазматичного ретикулума (PERK), індукція фактора транскрипції (ATF4), експресія про-апоптичного фактора транскрипції (CHOP) та P53 регуляторного модулятора апоптозу (PUMA), що бере участь у його контролі. В роботі використовували модель ізопротеренол-індукованого пошкодження міокарда у щурів, щоб оцінити можливий кардіопротекторний ефект імбиру через його вплив на ЕПР стрес-індукований апоптоз. Порівнювали дію імбиру з дією каптоприла, інгібітора ангіотензин-перетворювального ензиму. Щури-альбіноси лінії Wistar щодня отримували 1,0 або 2,0 мл порошкової суспензії *Zingiber officinale* (імбир, 200 мг/мл) шляхом внутрішньошлункової інтубації протягом 28 днів. Ізопротеренол у дозі 85 мг/кг вводили внутрішньочеревною ін'єкцією на 27-й та 28-й дні. Рівень аспартатамінази (АСТ) у сироватці крові вимірювали за допомогою кінетичного набору. Тканини сер-

ця використовували для екстракції РНК, оцінки експресії генів методом кількісної ПЛР, імуногістохімічного визначення експресії каспази 3 та гістопатологічних досліджень. Показано, що введення ізопротеренолу збільшувало експресію CHOP-мРНК у 4 рази в тканині серцевого м'яза порівняно з контролем. Крім того, за введення суспензії імбиру, значно знизились як CHOP, так і ATF4, а також експресія мРНК PUMA порівняно з групами, які отримували ізопротеренол. Відмічено значне зниження експресії мРНК ATF4 у групі, яка отримувала каптоприл та імбир, порівняно з контрольною групою. Показано, що імбир знижував рівень АСТ у сироватці крові, що корелювало з результатами гістопатологічних досліджень тканин серця. Здобуті результати свідчать про те, що протекторна дія імбиру за ізопротеренол-індукованого пошкодження міокарда у щурів може бути зумовлена зниженням стресу ендоплазматичного ретикулума через вплив на шлях ATF4-CHOP-PUMA.

Ключові слова: пошкодження міокарда, ішемія-реперфузія, ATF4, CHOP, PUMA, AST, імбир, каптоприл, ізопротеренол.

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