Cardioprotective Potential of Zinc and Vitamin E Against Isoprenaline-Induced Myocardial Infarction in Albino Rats by Targeting Autophagy: A Histological and Biochemical Study

Original Article

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ABSTRACT

Background: Myocardial infarction (MI) is a leading cause of morbidity and mortality. It is associated with oxidative stress, apoptosis and inflammation. Zinc (Zn) and vitamin E (VE) are known to exert antioxidant and anti-inflammatory effects.

Aim of the Work: Evaluate the cardioprotective potential of Zn, VE and their combination against isoprenaline (ISO)-induced myocardial infarction in adult male albino rats.

Materials and Methods: Forty rats divided into five groups; I (control), II (ISO group): rats were injected subcutaneously (SC) with ISO (100 mg/kg) on the 20th and 21st days at interval of 24 h. Groups III (Zn group), IV (VE group) and V (ZE group): rats received respectively daily Zn (30 mg/kg), VE (100 mg/kg) or combination of both orally for 21 days and injected with ISO as group II. On the 22nd day, electrocardiography, biochemical and histological studies were done. Myocardial sections were subjected to H&E, caspase-3 and beclin 1 immunohistochemical stains. This was followed by morphometric and statistical analysis.

Results: Group II exhibited significant electrocardiographic and biochemical changes compared to the control; deterioration of cardiac function with elevated cardiac enzymes, MDA, TNF-α and mTOR, in addition to reduced SOD, IL-10 and AMPK. Myocardial sections showed disturbed architecture with marked inflammatory infiltration and significantly increased caspase-3 and decreased beclin 1 immunoexpression. Groups III and IV revealed decreased cardiac enzymes, MDA, TNF-α and mTOR, in addition to elevated SOD, IL-10 and AMPK. Myocardial sections showed nearly normal histology with significantly decreased caspase-3 and increased beclin 1 immunoexpression. Group V presented the most protection with results significant to both groups III and IV.

Conclusion: Combined Zn and VE pretreatment proved to have protective effect against ISO-induced MI more than using either of them alone regarding the electrocardiography, biochemical and histological parameters and this was through targeting autophagy and modulating its AMPK-mTOR pathway.

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Key Words: Apoptosis; autophagy; myocardial infarction; vitamin E; zinc.

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INTRODUCTION

Myocardial infarction (MI) is a major worldwide cause of death and puts surviving patients at risk to develop other vascular diseases as cardiac hypertrophy and myocardial fibrosis[1]. Though definite drugs are used to treat MI including calcium channel blockers, angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists, they have limited application because of their serious side effects as proarrhythmia or cardiac depression plus their inability to reduce infarct size and fibrosis. Therefore, developing novel therapeutic approaches is an urgent obligation to minimize the damage caused by MI, especially natural products with cardioprotective potential[2]. Research for new therapeutic agents has been facilitated by the use of animal models; administration of isoprenaline (ISO), a β-adrenergic agonist, has been widely used to mimic the characteristics of MI in rats; being simple non-surgical and non-invasive method with low mortality rate. The physiologic and morphologic alterations of this noninvasive model are comparable with those of MI in humans; as electrocardiographic, biochemical and histological alterations[3,4].

Myocardial infarction (MI) results from imbalance between myocardial demand and coronary blood supply, leading to ischemic cardiac muscle necrosis. The specific mechanism involved in MI was linked to oxidative stress (OS), inflammation and apoptosis; as it is accompanied

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with formation of reactive oxygen species (ROS) and lipid peroxides (LPO), defect in antioxidant defense system, release of inflammatory mediators and destruction of cell membranes[1,2]. Zinc (Zn) is a trace element essential to maintain normal cell structure and function; it has a vital role in keeping human health, mainly in terms of anti-oxidative and anti-inflammatory stress[5,6]. Vitamin E (VE) is a fat-soluble substance including eight different compounds; the most copious and biologically active is α-tocopherol which has anti-oxidant, anti- inflammatory and anti-cancer activities[1].

Autophagy is a physiological catabolic process aims at recycling damaged organelles and cellular components via lysosome-mediated self-digestion. It principally serves an adaptive role protecting organisms during periods of enhanced cellular stress to maintain cell homeostasis. Nevertheless, starvation, excessive ROS production, inflammatory cytokines have emerged as negative regulators of autophagy in some cell lines[7]. Multiple studies have demonstrated the presence of autophagy in cardiovascular tissues, where it remains at a low basal level in normal conditions and plays a pivotal role in maintaining the homeostasis of cardiovascular system, but its level rises or falls under different conditions. It could exert both protective and harmful effects, yet in most cases autophagy reflects a programmed cell survival mechanism[8].

Autophagy involves the sequestration of the cellular cytoplasmic components in double-membrane vesicles termed autophagosomes, which degrade their contents by lysosomal proteolysis to generate raw material for the synthesis of vital macromolecules and generation of cellular energy. A preliminary step in the assembly of autophagosomes is the recruitment and activation of pro-autophagic class III phosphatidylinositol 3-kinase complex (PI3K) that consists of beclin 1, vacuolar protein sorting protein (VPS) 34, VPS15 and autophagy related gene/protein (ATG) 14[9]. Hence, beclin 1 is a key regulator of autophagy; playing a fundamental role in the formation of autophagosomes that engulf damaged cellular components[10]. Mammalian target of rapamycin (mTOR), a conserved serine/threonine kinase, is classified as a member of the phosphatidylinositol kinase-related kinase family and has been shown to regulate cell growth and to have an inhibitory role in autophagy[11]. Adenosine monophosphate-activated protein kinase (AMPK) is a main sensor to the increase in AMP/ATP and ADP/ATP ratios then restores the energy balance by inhibiting anabolic processes consuming ATP and promoting catabolic processes generating ATP[12]. Besides, it regulates cellular metabolism, proliferation and apoptosis and promotes autophagy via regulating numerous autophagic proteins and inhibiting mTOR[13]. Moreover, AMPK initiates autophagy by direct beclin 1 phosphorylation necessary for activating the pro-autophagic complex[9].

This study aims at investigating the cardioprotective potential of zinc and vitamin E and their combination in a rat model of ISO-induced MI and their mechanisms of action, in addition to exploring their modulatory effect on AMPK-mTOR pathway and beclin 1 expression.

MATERIALS AND METHODS

Drugs

Isoprenaline (ISO) was supplied as isoproterenol hydrochloride powder and dissolved in normal saline. Zinc (Zn) was supplied as zinc sulfate monohydrate powder that was dissolved in distilled water. Vitamin E (VE) was supplied as α-tocopherol liquid and dissolved in corn oil. All drugs were purchased from Sigma-Aldrich Co., St. Louis, MO, USA.

Animals

A total of 40 adult male albino rats, with average weights ranging from 180-200 grams, were used in this study and were kept in the animal house of Kasr Al-Aini, Faculty of Medicine. All rats were housed under the same environmental conditions at 24 ± 1°C with normal light/dark cycle. They were provided with ordinary rat chow with free access to water and food.

Experimental Design

The experiment was accomplished in strict compliance with the guidelines of Cairo University Committee for Animal Care and Use. Rats were randomly assigned into five equal groups as follows:

- Group I (control group); 8 rats subdivided equally into:
    - Subgroup IA: left without any intervention
    - Subgroup IB: injected subcutaneously (SC) with normal saline on the 20th and 21st days at an interval of 24 h.
    - Subgroup IC: received distilled water by oral gavage for 21 days and were injected SC with normal saline as in subgroup 1B.
    - Subgroup ID: received corn oil by oral gavage for 21 days and were injected SC with normal saline as in subgroup 1B.
- Group II (ISO group): 8 rats injected SC with isoprenaline (ISO, 100 mg/kg) on the 20th and 21st days from the beginning of experiment at an interval of 24 h between the two doses[2].
- Group III (Zn group): 8 rats received Zn (30 mg/kg/day) by oral gavage for 21 days[14] and injected with ISO (100 mg/kg) as in group II.
- Group IV (VE group): 8 rats received VE (100 mg/kg/day) by oral gavage for 21 days[15] and were SC injected with ISO (100 mg/kg) as in group II.
- Group V (ZE group): 8 rats received a combination of Zn (30 mg/kg/day) and VE (100 mg/kg/day) for 21 days then injected with ISO (100 mg/kg) as in group II.

Echocardiography

It was performed at the end of the experiment, on the 22nd day, 24 h after the last ISO dose, using an echocardiography system equipped with 8-10MHz liner transducer (Samsung

Madison, SONOACE-R3-Korea). The transducer was placed over the left parasternal area and moved over the heart from apex to base to obtain two-dimensional short axis B-mode of the heart, and the ejection fraction (EF%) and fractional shortening (FS%) were recorded.

Serological investigations

Blood samples were collected from the tail veins for measuring serum levels of cardiac enzymes; cardiac troponin I (cTnI), lactate dehydrogenase (LDH) and creatine kinase myocardial band (CKMB) according to the manufacturer's instructions of the routine laboratory kits (Pars Azmoon, Tehran). Measuring the serum levels of inflammatory markers; tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) were done according to manufacturer's instructions using ELISA kit supplied by R&D system USA.

Heart weight index measurement

Body weights of the rats were recorded then they were euthanized using overdose of intraperitoneal ketamine (150 mg/kg) and xylazine (15 mg/kg)[14]. Hearts were dissected out, rinsed and weighed after separating the aorta and adipose tissue. The heart weight index (HWI) was calculated by dividing heart weight by body weight[16]. Heart specimens were processed for tissue homogenates and histological studies.

Detection of oxidative markers

After tissue homogenization and centrifugation, the supernatant was removed for assessment of oxidative markers; malondialdehyde (MDA) and superoxide dismutase (SOD)[17].

Detection of AMPK and mTOR

Total and phosphorylated AMPK and mTOR were detected by western blot analysis[18]. Cardiac samples were lysed using a specific buffer containing protease and phosphatase inhibitors (Pierce). Bio-Rad Mini-Protein II systems were used for fractionation of proteins by SDS-PAGE (10% acrylamide gel). After being transferred on membranes of polyvinylidene fluoride, tissue proteins were washed with phosphate buffer saline (PBS) then were blocked at room temperature for 1 h with 5% (w/v) skimmed milk powder in PBS. The phosphorylated AMPK and phosphorylated mTOR were measured in approximately 20 mg of cardiac tissue. The blots were obtained by using primary anti-AMPKα antibody (#2532), anti-phospho-AMPKα (Thr172) antibody (#2535), anti-mTOR antibody (#2972), anti-phospho-mTOR (Ser2448) antibody (#2971) and beta actin antibodies (Cell signaling technology), all were overnight incubated at PH 7.6 and 4°C. Peroxidase-labeled secondary antibodies were used for 3 times wash, each continued 10 minutes then the membranes were incubated for 2 h with secondary antibody at room temperature. Analysis of the band intensity was done by ChemiDocTM imaging system with Image LabTM software version 5.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA). Finally the results were expressed as arbitrary units after normalization for the house keeping gene β-actin protein.

Histological studies

Specimens from the lower third of the left ventricles were fixed in 10% formol saline, paraffin-embedded, then were cut at 5-7 µm thickness and subjected to:

1) Hematoxylin and eosin stain[19].

2) Immunohistochemical staining[19]; sections were boiled in 10 mM citrate buffer (AP9003) at pH 6 for 10 minutes to retrieve antigen, then incubated for 1h with the primary antibodies; caspase-3 (rabbit polyclonal antibody, ab13847) and beclin 1 (rabbit polyclonal antibody, ab62557). Both were purchased from Abcam, MA, USA and both showed cytoplasmic localization. This was completed using Ultravision detection system (TP-015-HD). Sections were counter stained with Mayer's hematoxylin stain (TA-060-MH) and negative control was prepared by omitting the primary antibody. Citrate buffer, Ultravision detection system and Mayer's hematoxylin were purchased from Labvision Thermo Scientific, Fremont, California, USA.

Morphometric study

This was done using Leica Qwin 500 image analyzer (Leica image system Ltd, Cambridge, England). Diameters of the left ventricular wall and cardiac fibers were measured using an objective lens of x10 magnification. Area percent of caspase-3 and beclin-1 immunoreaction was measured using an objective lens of x40 magnification and within 10 non overlapping fields for each section.

Statistical Analysis

This was done for the echocardiography, biochemical and morphometric results using the Statistical Package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). Data was summarized using mean and standard deviation. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test. Correlations between quantitative variables were done using Pearson correlation coefficient. *P-values* less than 0.05 were considered statistically significant[20].

RESULTS

No mortalities were detected throughout the experimental duration and the control subgroups displayed similar results so they were titled collectively control group.

Echocardiography results

Isoprenaline administration resulted in deterioration of cardiac function, whereas, pretreatment with Zn and VE showed significant cardioprotection (Table 1, Figure 1).

Results of cardiac, inflammatory and oxidative markers

Isoprenaline resulted in elevated cardiac, inflammatory and oxidative markers, while Zn and VE presented significant cardioprotection manifested by reduced cardiac, inflammatory and oxidative markers (Table 2).

Results of AMPK and mTOR

AMPK showed significant reduction in group II compared to the control with significant elevation after Zn and VE treatment, yet, in group V it was significantly elevated compared to both groups III and IV. Regarding mTOR, there was significant rise in group II compared to the control and significant decrease in both groups III and IV compared to group II. However, group IV showed significant reduction compared to group III and group V displayed significant decrease compared to both groups III and IV (Figure 2).

Histological results

H&E results

Group I (control group) showed normal myocardial architecture without inflammatory infiltration or congestion. The myofibers were intact, branching and cylindrical with acidophilic cytoplasm and exhibited vesicular nuclei, transverse striations and obvious intercalated discs. They were separated by scanty connective tissue containing fibroblasts that were identified by their flat nuclei (Figure 3). Group II (ISO group) displayed multiple focal areas of disturbed myocardial architecture with marked inflammatory infiltration, congestion and extravasation. This was observed in the apex of left ventricle mainly in the inner subendocardial half of the myocardium. Many myofibers were swollen and interrupted with vacuolations and wide separations, while others had intensely eosinophilic and hyaline appearance with lost striations and intercalated discs. Most nuclei were fading and dissolved (Figure 4). Both groups III and IV (Zn and VE groups) showed nearly normal myocardial architecture, apart from mild inflammatory infiltration and congestion. Most myofibers were intact while others had fading dissolved nuclei. Few myofibers had intensely acidophilic cytoplasm (Figures 5,6). Group V (ZE group) presented almost normal myocardium with minimal inflammatory infiltration but without congestion. Most myofibers were intact while few had fading dissolved nuclei (Figure 7).

Caspase-3 immunohistochemical results

Both groups I and V (the control and ZE groups) exhibited negative caspase-3 immunoreaction. Whereas in group II (ISO group), most myofibers were immunoreactive. While in both groups III and IV (Zn and VE), few myofibers displayed positive immunoreaction (Figure 8).

Beclin 1 immunohistochemical results

Both groups I and V displayed positive immunoreaction to beclin 1 in many myofibers. Concurrently, the immunoreactivity was seen in few myofibers from the ISO group. Whilst both groups; III and IV showed immunoreactivity in some myofibers (Figure 9).

Heart weight index and morphometric results

These are illustrated in (Table 3).

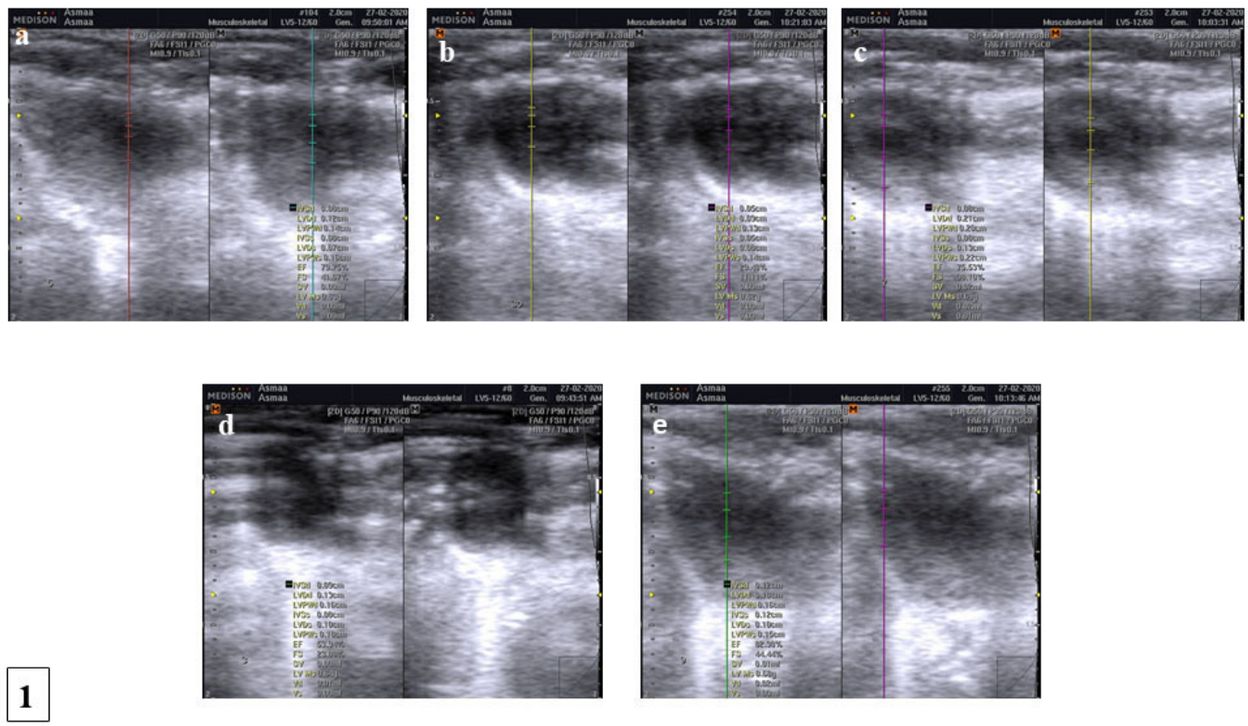

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Fig. 1: Estimated EF% and FS% at the end of the study in all groups: (a) control, (b) ISO group, (c) Zn group, (d) VE group and (e) ZE group

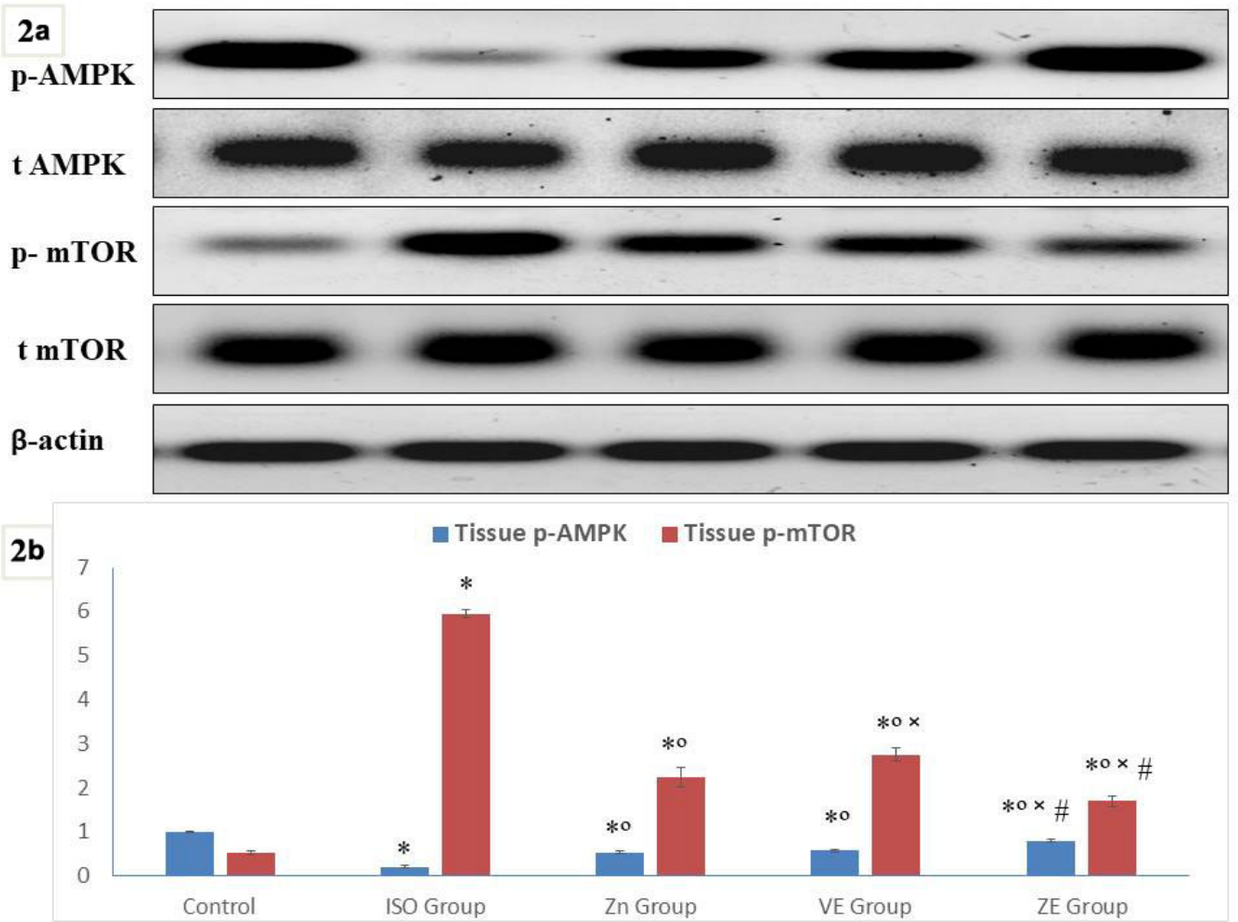

Fig. 2: a) Protein bands of western blot analysis for AMPK (phosphorylated and total), mTOR (phosphorylated and total) and β-actin in the studied groups. b) Mean ± SD of western blot protein load analysis of phosphorylated AMPK and phosphorylated mTOR in the studied groups.
*Significant compared to the control group
ºSignificant compared to ISO group
×Significant compared to Zn group
#Significant compared to VE group.

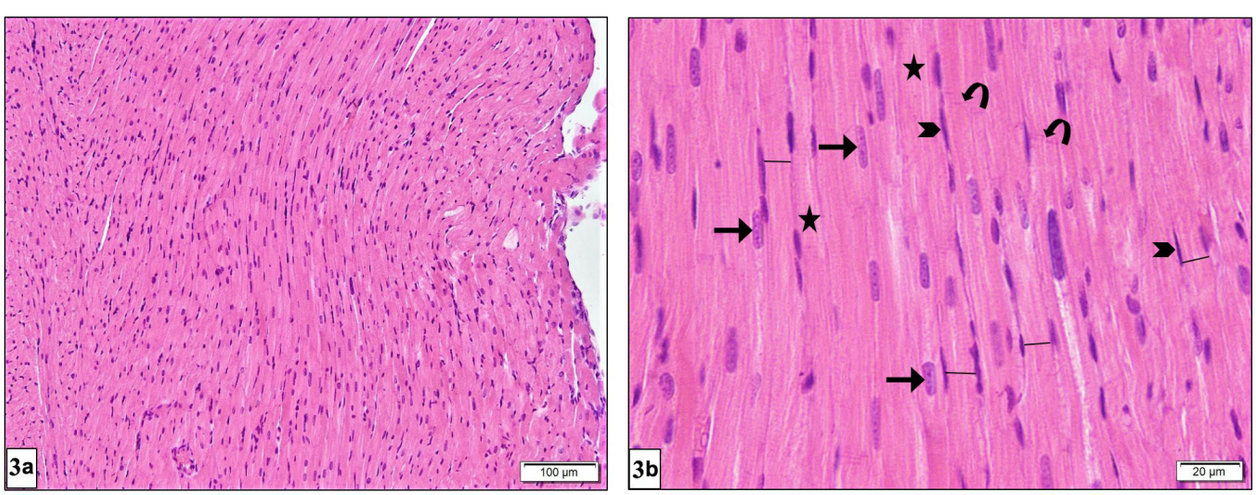

Fig. 3 (a and b): Photomicrograph of heart tissue from the control group (group I) showing normal myocardial architecture without inflammatory infiltrate or congestion. Myofibers (lines) are intact branching cylindrical with acidophilic cytoplasm. Myofibers are separated by scanty connective tissue containing fibroblasts (arrow heads). Most myofibers have vesicular nuclei (arrows) and show transverse striations (stars) and intercalated discs (curved arrows).
(H&E; ax100, bx400)

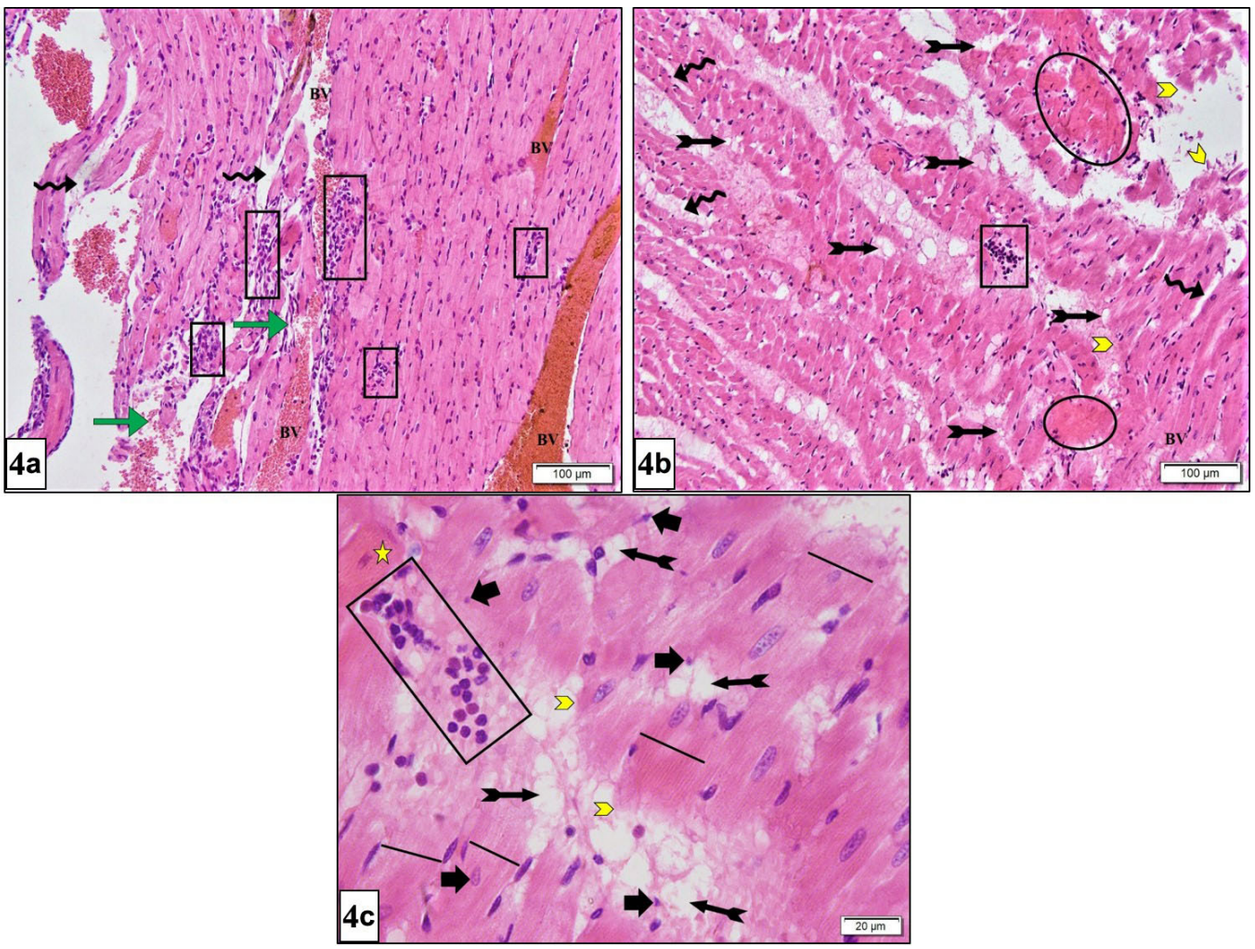

Fig. 4 (a, b and c): Photomicrograph of heart tissue from ISO group (group II) showing disturbed myocardial architecture with marked inflammatory infiltrate (rectangles). Blood vessels are congested (BV) and ruptured with extravasation (green arrows). Myofibers are interrupted with wide separations (wavy arrows). Many vacuolations (bifid arrows) are interrupting myofibers. Myofibers have intensely eosinophilic and hyaline appearance (ovals). Myofibers are swollen (lines) and ruptured (yellow arrowheads). Myofibers have lost their striations and intercalated discs. One myofiber is seen with intensely acidophilic hyaline cytoplasm (yellow star). Most nuclei are fading and dissolved (thick arrows). (H&E; a&bx100, cx400)

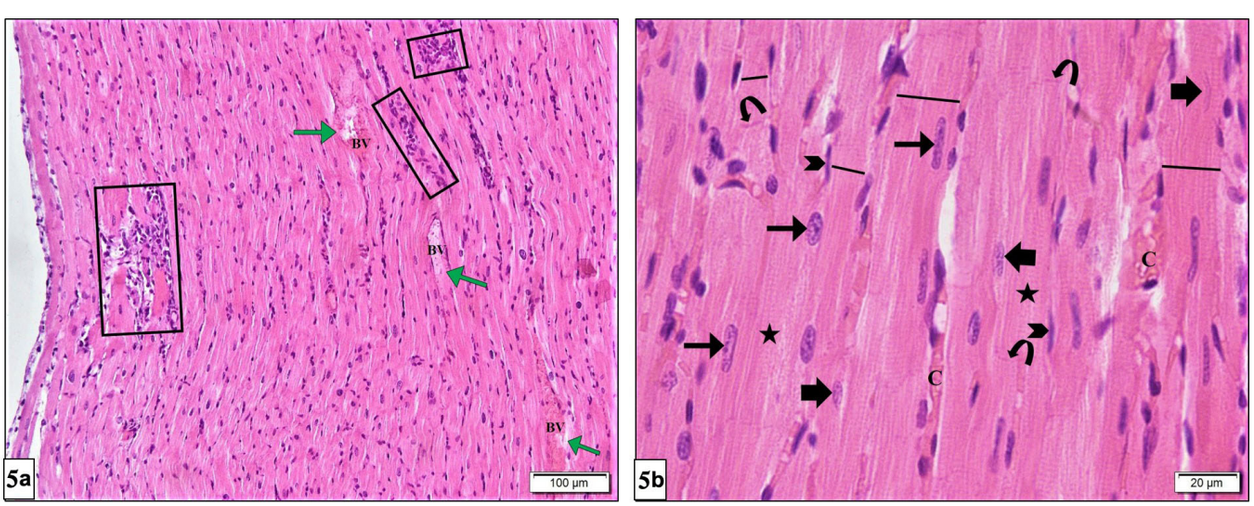

Fig. 5 (a and b): Photomicrograph of heart tissue from Zn group (group III) showing apparently nearly normal myocardial architecture with mild inflammatory infiltrate (rectangles). Blood vessels are congested (BV) and ruptured (green arrows). Most myofibers (lines) are intact branching cylindrical with acidophilic cytoplasm and vesicular nuclei (arrows) while others have fading dissolved nuclei (thick arrows). Some myofibers show transverse striations (stars) and intercalated discs (curved arrows). Myofibers are separated by scanty connective tissue which contains congested capillaries (c) and fibroblasts (arrow heads) identified by its flat nuclei. (H&E; ax100, bx400)

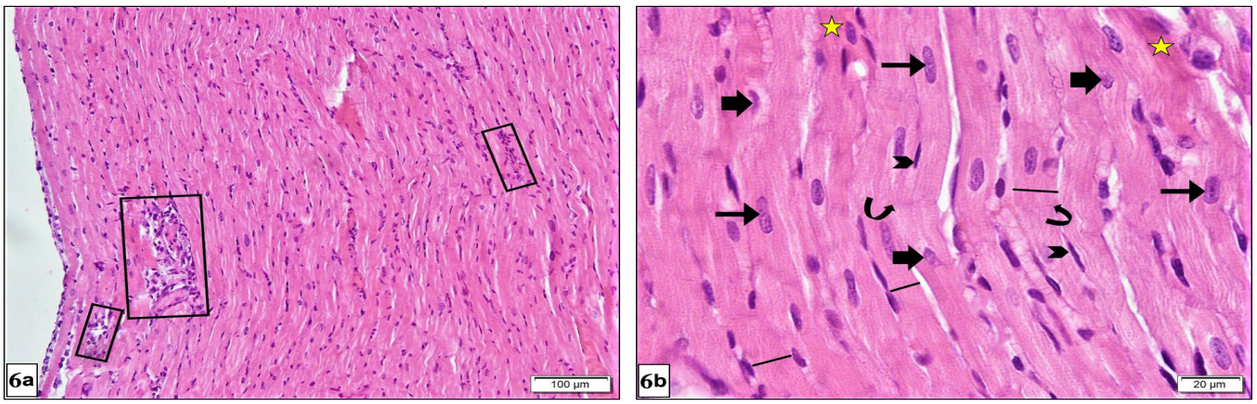

Fig. 6 (a and b): Photomicrograph of heart tissue from VE group (group IV) showing apparently nearly normal myocardial architecture with mild inflammatory infiltrate (rectangles). Most myofibers (lines) are intact branching cylindrical with acidophilic cytoplasm and separated by minimal connective tissue which contains fibroblasts (arrow heads). Most myofibers have vesicular nuclei (arrows) while others have fading dissolved nuclei (thick arrows). Some myofibers show intercalated discs (curved arrows) while transverse striations are not clear. Few myofibers have intensely acidophilic cytoplasm (yellow stars). (H&E; ax100, bx400)

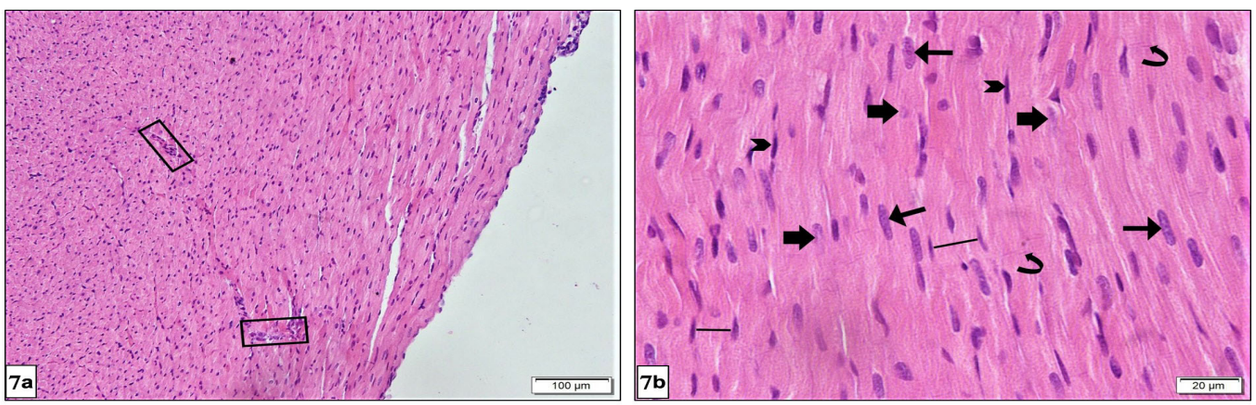

Fig. 7 (a and b): Photomicrograph of heart tissue from ZE group (group V) showing apparently normal myocardial architecture with minimal inflammatory infiltrate (rectangles). Myofibers (lines) are intact branching cylindrical with acidophilic cytoplasm and separated by scanty connective tissue which contains fibroblasts (arrow heads) identified by its flat nuclei. Most myofibers have vesicular nuclei (arrows) while others have fading dissolved nuclei (thick arrows). Some myofibers show intercalated discs (curved arrows), while transverse striations are not clear. (H&E; ax100, bx400)

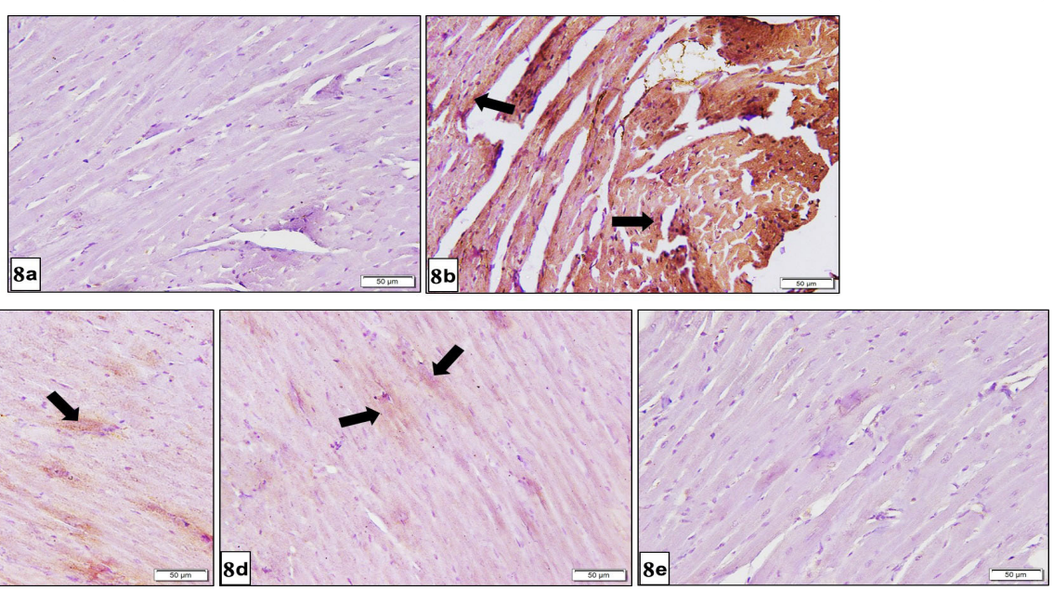

Fig. 8: Photomicrograph of caspase-3 immunostained heart sections. Negative immunoreactivity is seen in the control (a) and ZE groups (e). The immunoreactivity (arrows) is cytoplasmic, widespread and seen in most myofibers of ISO group (b). Both Zn group (c) and VE group (d) show immunoreactivity within few myofibers. (Caspase-3 immunostaining; x200)

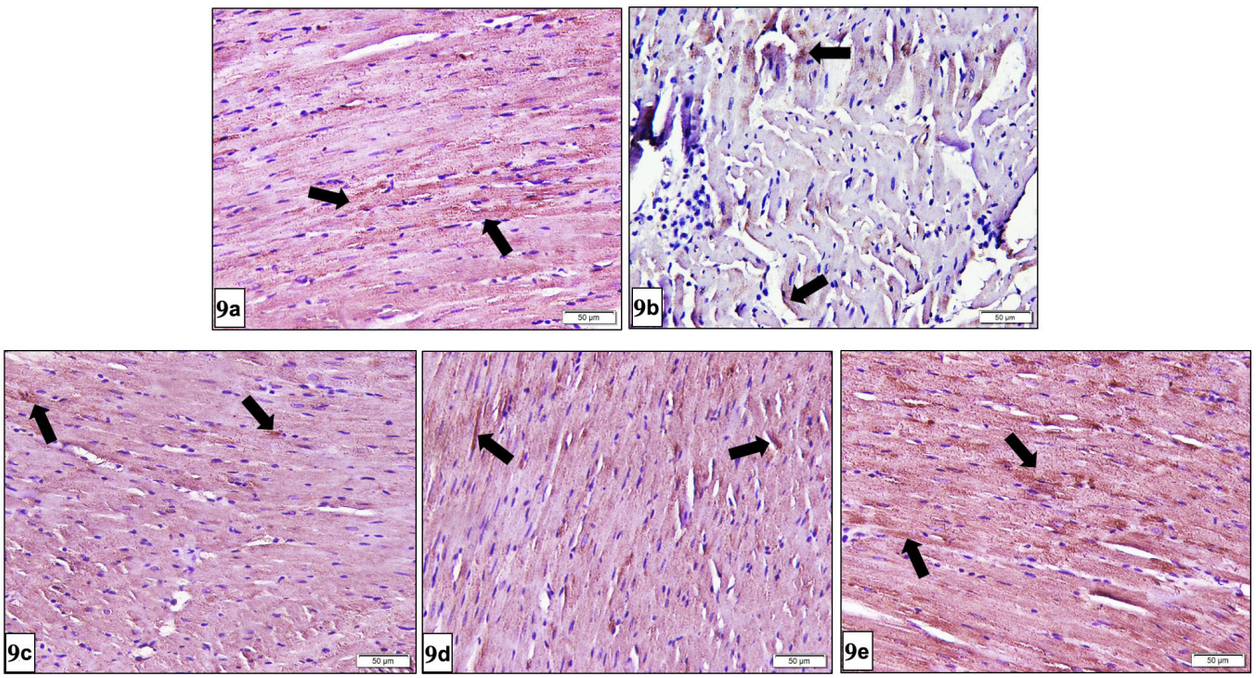

Fig. 9: Photomicrograph of beclin 1 immunostained heart sections. Positive cytoplasmic immunoreactivity (arrows) is detected in many myofibers of the control (a) and ZE (e) groups. The immunoreactivity is seen in few myofibers of ISO group (b). Both Zn group (c) and VE group (d) show immunoreactivity in some myofibers. (Beclin 1 immunostaining x200)

Table 1: Mean ejection fraction (EF%) and fractional shortening (FS%) ±SD in the experimental groups

	Group I (control)	Group II (ISO)	Group III (Zn)	Group IV (VE)	Group V (ZE)
EF%	77.72±1.28	32.17±3.31*	63.01±5.42*#	68.95±3.61*#	75.14±2.19#$@
FS%	39.2±2.49	12.17±1.17*	31.51±7.08*#	35.68±4.4#	38.68±1.53#@

* Significant compared to control group
# Significant compared to ISO group
@ Significant compared to Zn group
$ Significant compared to VE group

Table 2: Mean Levels of cardiac, inflammatory and oxidative markers (± SD) in the studied groups

	Group I (control)	Group II (ISO)	Group III (Zn)	Group IV (VE)	Group V (ZE)
cTnI (ng/mL)	0.04±0.01	1.03±0.06*	0.36±0.03 *º	0.49±0.03 *º ×	0.17±0.01*º#×
Serum LDH (U/mL)	116.67±2.15	222.83±2.57*	172.08±4.39*º	160.18±2.52*º×	132.97±1.85*º#×
Serum CKMB (U/mL)	163.18±1.02	318.2±3.23*	195.95±1.29*º	197.95±3.15*º	155.55±0.93*º#×
Serum TNF-α (pg/mL)	15.4±0.91	60.37±3.05*	27.92±1.59*º	29.67±1.45*º	19.38±1.47*º#×
Serum IL-10 (pg/ml)	123.93±1.25	56.55±0.88*	90.13±3.02*º	92.35±5.61*º	111.32±1.62*º#×
MDA (nmol/mg Protein)	44.03±5.68	145.57±5.37*	80.9±1.73*º	88.05±8.72*º	53.35±1.27*#×
SOD (U/mg protein)	17.81±1.1	6.58±0.78*	10.93±0.71*º	11.7±0.84*º	15.68±0.3*º#×

* Significant compared to the control group
º Significant compared to ISO group
× Significant compared to Zn group
Significant compared to VE group

Table 3: Heart weight index and morphometric results in the studied groups (mean ±SD)

	Control group	ISO group	Zn group	VE group	ZE group
HWI (mg/gm)	2.7±0.04	4.65 ±0.06*	3.72±0.05*º	3.4±0.05*º×	3.01±0.04*º×#
Diameter of left ventricular wall (µm)	707.66±27.06	1224.31±31.98*	1068.79±22.59*º	977.93±23.74*º×	848.04±23.98*º×#
Diameter of myofibers (µm)	23.67±3.20	58.96±3.19*	44.35±2.69*º	46.07±2.37*º	32.40±2.25*º×#
Area % of caspase-3	0.083 ±0.034	36.20±1.25*	15.06±1.17*º	15.11±1.06*º	4.40±0.78*º×#
Area % of beclin I	49.99±1.44	15.10±1.02*	35.14±1.16*º	36.15±1.38*º	42.71±1.68*º×#

* Significant compared to the control group
º Significant compared to ISO group
× Significant compared to Zn group
Significant compared to VE group

DISCUSSION

In the current work, isoprenaline (ISO) is used to induce MI as it has been proved to result in myocardial changes similar to the characteristics of MI[4]. Functionally, ISO results in reduced EF% and FS% in group II and this goes with previous studies[21,22] that clarified it by systolic and diastolic dysfunctions. Cardiac enzymes are diagnostic markers for cardiotoxicity, cTnI is shown to be a specific highly sensitive marker for myocardial cell injury; it is undetectable normally in serum and released only following myocardial necrosis, predicting the risk for cardiac cell death and infarction[23]. LDH and CKMB are present largely in the myocardium and released into the blood stream when cell membranes rupture. They serve as diagnostic tool for MI and their blood concentrations reflect the degree of cell damage, plus being indicative of inflammatory changes[1,22,24]. ISO group showed significantly elevated cTnI, LDH and CKMB compared to the control which matches numerous former studies[1,22-26]. This marked increase in serum level of cardiac enzymes denotes necrotic damage and leakiness of myocardial cell membrane[23,27] and could be explained by the enhanced production of free radicals with subsequent lysosomal degradation and alteration of myocardial structure as well as function[28].

Researchers have documented oxidative stress as a chief mechanism in ISO-induced cardiotoxicity; once entering the cell, ISO generates highly cytotoxic free radicals and produce excessive reactive oxygen species (ROS), resulting in lost integrity and function of myocardial membranes[23,25]. In addition, ISO affects the enzymes of antioxidant system leading to heart tissues damage, noticed from the correlation between the degree of oxidative stress and the severity of damage[1]. Produced free radicals are removed by intracellular antioxidant enzymes like SOD and catalase, whereas their intracellular accumulation promotes lipid peroxidation causing cell damage. The main product of lipid peroxidation is MDA, so its tissue content reflects the balance between free radicals and antioxidant defense system[29]. In this context, current work showed significant increased MDA and decreased SOD in ISO group compared to the control, and this is in agreement with previous studies[1,22,24-26,30].

In addition, growing evidence proves that inflammation is a key process engaged in myocardial tissue damage after ISO administration; neutrophils infiltrate the infarcted area and release proteolytic enzymes, countless inflammatory cytokines and chemokines, along with ROS production[1]. In the present study, ISO group showed significant increase in the pro-inflammatory cytokine TNF-α and significant decrease in the anti-inflammatory one IL-10, which is consistent with former studies[1,24,25,30]. In case of MI, ischemia and anoxia activate cardiomyocytes and macrophages to produce big amounts of TNF-α in the infarcted zone and its border and this is stimulated by increased IL-1α accompanying hypoxia[30]. The anti-inflammatory cytokine IL-10 could modulate the inflammatory pathways[25]; it suppresses the inflammatory response by inhibiting the production of IL-1β, IL-6 and TNFα[31].

One of the major regulators of autophagy is AMPK; initiates it by direct beclin 1 phosphorylation plus suppressing its key inhibitory regulator, mTOR[9,13]. In the current work, AMPK expression level was significantly decreased and that of mTOR was significantly increased in the ISO group compared to control group, which is consistent with former studies[21,22] and indicative of suppressed autophagy in ISO-induced MI that was further confirmed by the significant reduction in beclin 1 expression. An earlier study proved that mTOR activation and failure of autophagy induction resulted in cardiac pathogenesis, decline in cardiac function, increased inflammation and occurrence of fibrotic injury[32]. Suppressed autophagy could be correlated to increased ROS production and TNF-α that induce nuclear factor kappa which has emerged as a negative regulator of autophagy[7]. Moreover, AMPK reduction endorses oxidative stress injury as well as apoptosis in ISO-treated cardiac tissue[22] which was manifested in this work.

Regarding the heart weight index, it was significantly increased in ISO group compared to the control, which goes in line with earlier studies[2,16,22] and is explained by cardiac hypertrophy. That might be due to increased water content and edema and because of increased plasma membrane permeability[27]. Also might be due to massive necrosis in cardiac muscle fibers with subsequent invasion of the damaged tissues by inflammatory cells[2,22].

Histologically, the ISO group in the present work showed marked myocardial injury with disturbed architecture; many myofibers were swollen, interrupted, with vacuolations in addition to wide separations. This vacuolar degeneration was reported previously and explained by myocytolysis detected in the myofibers especially at the margin of the infarct[33]. Other myofibers appeared with intensely eosinophilic cytoplasm, hyaline appearance, lost striations and intercalated discs and most nuclei were fading and dissolved. This picture points to coagulative necrosis as stated by former investigators[34] and are in accordance with Harsh Mohan[33] who further described oedematous swollen myofibers that matches the findings in this study, which was established by the significant increase in myofibers diameters in ISO group compared to the control. Moreover, cardiac sections from ISO group displayed marked inflammatory infiltration, vascular congestion and extravasation. This goes in line with previous studies[2,4] which documented swollen cardiomyocytes, fragmented myofibrils, loss of transverse striations, appearance of vacuoles and necrosis after ISO administration. They also reported intense infiltration of inflammatory cells, vascular damage and interstitial edema. The edema was manifested in this work by the wide separations between myofibers and may explain the significant increase in the ventricular diameter and HWI detected in ISO group. Furthermore, it was proved that ISO administration in rats resulted in dilated congested or ruptured capillaries with hemorrhagic foci between the fibers followed by perivascular inflammatory cells[28].

The myocardial damage seen after ISO administration could be attributed to its mode of action; a positive

chronotropic and inotropic effect (ß1 effect) leading to cardiac hyperfunction. On the other hand, it causes hypotension in the coronary bed secondary to its depressor effect on circulation (ß2 effect). Thus, the imbalance between coronary blood flow and cardiac demand leads to ischemia[3]. Besides the release of highly cytotoxic ROS that lead to intense oxidative stress and infarct like necrosis[27].

It is interesting to note that the myocardial findings in the current work were focal and multiple and were observed in the apex of left ventricle of the heart mainly occupying the inner subendocardial half of the myocardium. This is in accordance with earlier studies[34,35] which recorded that massive myocardial necrosis characterizes human myocardial infarction whereas catecholamine model of myocardial necrosis is presented in the form of focal coagulative necrosis[34]. Additionally, the changes in the subendocardial layer and the apex left ventricle could be explained by the least coronary perfusion of subendocardial myocardium and the thick wall and more metabolic requirements of left ventricle thus are more vulnerable to any reduction in blood supply[33].

Sections from ISO group showed significantly increased caspase-3 immunoreactivity compared to the control group which is in harmony with former studies[1,24,27]. Caspase-3 is the most important apoptosis-regulating protein that is activated by diverse apoptotic stimuli[24]. A major stimulus is oxidative stress that causes mitochondrial and lysosomal destabilization, release of pro-apoptotic factors into the cytosol, endoplasmic reticulum stress and activation of pro-caspases[1]. In addition, high levels of pro-inflammatory cytokines like TNF-α and IL-1β facilitate myocardial cell necrosis and apoptosis, consequently inducing heart failure[24].

Recent findings have highlighted a potential cross talk between apoptosis, necrosis and autophagy; where autophagy precedes apoptosis. Numerous stimuli that induce autophagy follow that by inducing cell death pathways once autophagy fails to cope with stress or the autophagic process is dysregulated[36]. Beclin 1 is localized within cytoplasmic structures; mitochondria, endoplasmic reticulum, Golgi apparatus and perinuclear membrane and its mislocalization or mutations resulted in impaired autophagic activity[37]. It has been stated that caspases -3, -8 and 7 can cleave beclin 1, generating N and C-terminal fragments that change their localizations, become unable to induce autophagy and are transferred to mitochondria as apoptotic signals[38]. The aberrant expression or post-translational modification of beclin 1 has been reported in diverse heart diseases including MI, ischemia-reperfusion, cardiac hypertrophy plus heart failure, possibly through altered myocardial autophagy and apoptosis[37]. In addition autophagosomes engulf the apoptotic proteins and damaged ROS-producing mitochondria to promote cell survival under stress conditions, thus inhibition of autophagy increases ROS production and apoptosis[39]. This could explain the findings in ISO group where increased apoptosis was accompanied by significant decrease in beclin 1 immunoexpression and this points to suppressed autophagy in MI as recorded previously[40].

Up till now, the gross mortality among patients with acute MI is still high and more efficient therapeutic strategies are yet demanded[41]. Given that MI has been proved to be associated with oxidative stress[25], inflammation[1] and apoptosis[24], so their inhibition could be an important therapeutic target for protection against MI. Therefore, this study investigated the potential cardioprotective role of zinc (Zn) and Vitamin E (VE), either singly or in combination; as antioxidants are uniquely dissimilar and when used together in proper combination can work most effectively[42]. In the present work, supplementation with either Zn (group III) or VE (group IV) or both together (group V) resulted in cardioprotection; manifested functionally by the echocardiograph results and by the biochemical and histopathological findings, with ZE group revealing significant cardioprotection compared to either Zinc or VE monotherapy. The three groups showed significantly augmented EF% and FS% with significant reduction in cardiac enzymes, MDA and TNF-α, as well as significantly increased SOD and IL-10 as compared to ISO group. However, ZE group presented significant values compared to both Zinc and VE groups. This is in harmony with former studies; where Zn had exerted a cardioprotective effect in experimental diabetic rats[14] and in MI combined with selenium and chromium[43]. Also, VE had protected against cardiotoxicity induced by aflatoxin[15] or by cisplatin[17], in MI together with green tea[44] or L-carnitine[28] and in myocardial ischemia/reperfusion injury[45].

This could be ascribed to the participation of Zn in different cell functions; being a crucial component of the main antioxidant enzymes including SOD and catalase, when deficient, it disrupts their synthesis promoting oxidative stress. In addition, it is employed in the synthesis of metallothionein antioxidant that adds up to free radical scavenging[14]. Also it competes with iron and copper for negative charges in the lipid bilayer of cell membranes protecting them from lipid peroxidation[5]. Regarding the anti-inflammatory and anti-apoptotic actions, Zn is known to decrease inflammatory cytokines including IL-6, IL-1β and TNF-α, and to inhibit NF-κB activation. Also it inhibits the activation of procaspases-3, 6 and 9[46]. Vitamin E has been recognized to be the most efficient lipid soluble anti-oxidant scavenging ROS, preventing lipid peroxidation and consequently oxidative tissue damage[17], also it might have a structural role in stabilizing membranes[42]. Besides, it is known to be one of most efficient anti-inflammatory agents; as it affects the migration of neutrophils and monocytes into inflamed areas, thus preventing the release of pro-inflammatory cytokines[45].

Autophagy has a beneficial role in heart as it prevents accumulation of damaged organelles or abnormal proteins that disrupt the survival and growth of cardiomyocytes[22] and degradation of these proteins produces big number of free fatty acids and amino acids to keep the mitochondrial energy supply[47]. Additionally, autophagy appears to be inflammatory suppressor by limiting the availability of activators and/or components of inflammasomes as ROS and mitochondrial DNA, plus its inhibitory effect on

inflammatory mediators as nuclear factor of kappa[32,41] and this adds up to the reduced inflammation found in this work in Zn and VE treated groups. Consequently, enhancement of autophagy could reverse or even attenuate cardiac injury that leads to heart failure thus may be a promising therapeutic approach in acute MI[22]. Results of the current work provided evidence that Zn and VE could promote autophagy; where AMPK expression in cardiac tissue was significantly elevated with reduction of mTOR protein level compared to the ISO group. However synergistic effect of combined Zn and VE (ZE group) was manifested; as it revealed significant results compared to either Zn or VE only, and this was further confirmed by the significantly elevated beclin 1 expression found in these groups.

Earlier literatures have reported that Zn and VE could up-regulate autophagy, still few researchers have investigated this role in MI. In neuronal tissues, activation of AMPK was observed after exposure to Zn[48]. In a study conducted on hepatic lipid deposition, Zn supplementation stimulated lipolysis by autophagy-mediated mechanism called lipophagy and this was partially mediated by AMPK activation[49]. Also, VE supplementation was proved to induce autophagy via regulating AMPK-mTOR pathway[50] and has exhibited neuroprotective effect in chronic unpredictable mild stress in mice by the same mechanism[51].

AMPK signaling comes out to have wide implications in cardiovascular diseases like endothelial dysfunction and atherosclerosis, myocardial ischemia reperfusion injury, hypertension and cardiac remodeling, in addition to protection against ISO-induced oxidative stress injury and apoptosis[22]. Moreover, previous studies stated that it protects cardiomyocytes from hypoxia induced injury both in-vivo and in-vitro; as it regulates cell energy, balances the cell metabolism and promotes autophagy by inhibiting mTOR[52]. Further, inhibition of mTOR alleviates endoplasmic reticulum stress by accelerating the degradation of unfolded protein via autophagy that in turn protects cardiomyocytes from apoptosis and improve cardiac function[53]. This was established by the significant reduction in caspase-3 and significant improvement of EF% and FS% found in Zn and VE treated groups compared to ISO group.

In the present work, sections from groups III, IV and V showed almost normal myocardial architecture with mild inflammatory infiltration and congestion. The cardioprotective role of Zn and VE was proved previously[14,17,28]. This indicated that Zn and VE pretreatments could prevent most of the pathological changes induced by ISO administration and might be attributed to their anti-oxidant, anti-inflammatory and anti-apoptotic effects proved in this study. These groups also displayed significantly reduced caspase-3 immunoexpression compared to ISO group; with ZE group being significant compared to the other two. Zinc and VE have been previously recorded to prevent apoptosis and reduce caspase-3 expression[14,45] and this contributes to the obvious improvement in cardiac function seen in the echocardiography. This down-regulation in apoptosis might be attributed to the decline in oxidative stress and inflammation, also might be attributed to the up-regulation of autophagy manifested by the modulation of APMK-mTOR signaling pathway and this was documented by diverse studies[54,55,56,57].

Herein, the up-regulation of autophagy was also manifested by the significant elevation in beclin 1 immunoexpression observed in Zn and VE treated groups. Consistently, studies have shown that Zn is a critical positive regulator for both basal and induced autophagy; on the other hand, its depletion resulted in its suppression[5,58,59]. One of the mechanisms by which Zn modulates autophagy is the phosphorylation of extracellular-signal-regulated kinases that activate autophagy either through activation of beclin 1/PI3K complex or by promoting disassembly of mTOR complex[5]. In addition, Zn can regulate autophagy through microRNA expression regulation and modulation of genes involved in autophagy. Furthermore, Zn is needed for proper lysosomal function and plays an essential role in cargo degradation[60]. Regarding VE, it has been proved to act as an enhancer of autophagy via AMPK-mTOR signaling pathway; as it up-regulates AMPK phosphorylation and inhibits mTOR activity, with increased beclin 1 expression[49,50,61].

CONCLUSION

Zinc and vitamin E could protect against isoprenaline-induced myocardial infarction, especially when used together. Their actions might be related to their anti-oxidant and anti-inflammatory effects and also to their ability to induce autophagy via the modulation of AMPK-mTOR signaling pathway.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

إمكانية الحماية القلبية للزنك وفيتامين هـ ضد احتشاء عضلة القلب الناجم عن الأيزوبرينالين في الجرذان البيضاء من خلال استهداف البلعمة الذاتية : دراسة هستولوجية وبيوكيميائية

Q2

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الخلفية: احتشاء عضلة القلب (MI) سبب رئيسي للمراضة والوفيات. ويترافق مع الإجهاد التأكسدي والاستماتة والالتهاب. من المعروف أن الزنك (Zn) وفيتامين هـ ((VEيمارسان تأثيرات مضادة للأكسدة ومضادة للالتهابات.
الهدف: تقييم إمكانية حماية القلب بالزنك وفيتامين هـ ومزيجهما ضد احتشاء عضلة القلب الناجم عن الأيزوبرينالين(ISO) في ذكور الجرذان البيضاء البالغة.
الأدوات و الطرق: تم تقسيم ٤٠ من الجرذان إلى خمس مجموعات. المجموعة I (المجموعة الضابطة). المجموعة الثانية (مجموعة (ISO: تم حقن الجرذان تحت الجلد بالأيزوبرنالين ١٠٠ (ملج / كج) في اليوم ٢٠ و ٢١ بفارق فترة ٢٤ ساعة. المجموعات III (مجموعة Zn) ، و IV (مجموعة VE) و V (مجموعة ZE): تلقت الجرذان ٣٠) Zn مجم / كجم) ، ١٠٠) VEمجم / كجم) أو مزيج من كليهما على التوالي عن طريق الفم يوميا لمدة ٢١ يومًا ، وتم حقنهم باستخدام ISO كما فى المجموعة II. في اليوم الثاني والعشرين ، تم إجراء تخطيط كهربية القلب والدراسات البيوكيميائية والنسيجية. تعرضت عينات عضلة القلب لصبغات الهيماتوكسيلين والإيوسين ، والصبغات الهستوكيميائية المناعية ضد كاسباس-٣ وبكلين١. تبع ذلك التحليل القياسى والإحصائي.
النتائج: أظهرت المجموعة II تغييرات كبيرة في تخطيط القلب والدراسات البيوكيميائية مقارنة بالمجموعة الضابطة ؛ تدهور وظيفة القلب مع ارتفاع إنزيمات القلب ، TNF-α ، MDA و mTOR ، بالإضافة إلى انخفاض SOD و IL-١٠ و AMPK. أظهرت عينات عضلة القلب بنية مضطربة مع تسلل التهابي ملحوظ ، وزيادة ذات دلالة إحصائية من التعبير المناعي لكاسباس-٣ وانخفاض بيكلن ١. كشفت المجموعتان III و IV انخفاض إنزيمات القلب ، ، MDA TNF-α و mTOR ، بالإضافة إلى ارتفاع SOD و IL-١٠ و AMPK. أظهرت عينات عضلة القلب تقريبا أنسجة طبيعية مع انخفاض التعبير المناعي لكاسباس-٣ انخفاض ذو دلالة إحصائية وزيادة بيكلين ١. قدمت المجموعة V أكبر قدر من الحماية مع نتائج بارزة مقارنة بالمجموعتين III وIV.
الخلاصة: ثبت أن المعالجة المسبقة بالزنك وفيتامين هـ معا لها تأثير وقائي ضد احتشاء عضلة القلب الناجم عن الأيزوبرينالين أكثر من استخدام أي منهما بمفرده ؛ فيما يتعلق بمعايير تخطيط القلب الكهربائي والبيوكيميائية والنسيجية ، وذلك من خلال استهداف البلعمة الذاتية وتعديل مسار AMPK-mTOR