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METFORMIN AND CARVEDILOL AMELIORATE ISOPRENALINE-INDUCED MYOCARDIAL INFARCTION IN NON-DIABETIC RATS THROUGH AMPK ACTIVATION AND SUPPRESSION OF APOPTOSIS

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The objective of this study was to clarify the ameliorative effect of metformin (MET), carvedilol (CAR), and their combination and the underlying mechanisms in a non-diabetic rat model of isoprenaline (ISO)-induced MI. The adult male Wistar rats were allocated into six groups (n=8). Control group A received saline while control group B received DMSO 0.5% (i.p) for 10 days. ISO-treated group: received ISO (85 mg/kg.i.p.) on the first and second day of the experiment with the injection of normal saline for 10 days from the first day. ISO+MET and ISO+CAR-treated groups: received ISO as previously described and MET (200 mg/kg.i.p.) and CAR (10 mg/kg.i.p.) respectively for 10 days from the first day of the experiment. ISO+MET+CAR-treated group: received ISO, MET, and CAR as previously described. In the ISO group, the rise in serum cardiac biomarkers cTn-I and LDH provided evidence of MI. In addition, cardiac MDA, IL6, caspase-3, and Bax gene levels were significantly elevated, while cardiac SOD, GSH, pAMPK, eNOS, Bcl-2 gene, and Bcl-2/Bax ratio levels were significantly reduced with histopathological changes in cardiac tissue. Whereas posttreatment with MET, CAR, and their combination significantly reversed these overwhelming ISO-induced damaging effects on the heart. In conclusion, MET, CAR, and their combination could improve myocardial injury in ISO-induced MI through AMPK signaling pathway activation, and anti-apoptotic, anti-inflammatory, and antioxidant mechanisms. Subsequently, promote the recovery of cardiomyocyte function.

Keywords: *Metformin, Carvedilol, Myocardial infarction, AMPK, Apoptosis, Gene expression*

INTRODUCTION

Myocardial infarction (MI) is one of the most common cardiovascular emergencies with high mortality. Every year, nearly 10% of patients complaining of chest pain who are admitted to emergency rooms are diagnosed with a heart attack. The most widely used techniques for treating acute MI are thrombolysis, percutaneous coronary intervention (PCI), and coronary artery bypass grafting[1&2]. During infarction, myocardial cells are deprived of oxygen, which results in irretrievable damage. Cardiac damage is caused by a variety of cellular and molecular mechanisms that affect heart function, including altered myocardial redox balance, increased inflammation, and mitochondrial dysfunction[3]. Moreover, after acute MI, cardiomyocytes undergo apoptosis and necrosis which may be an imperative pathway in cardiomyocyte death during acute ischemia[4].

The synthetic catecholamine, isoprenaline (ISO), causes 'infarct-like' myocardial necrosis that resembles human MI. When ISO is administered, ischemia develops as a result of an imbalance between increased cardiac stimulation and reduced coronary blood flow brought on by the depressant effects of the drug on circulation. In addition, ISO produces extremely cytotoxic free radicals that promote the peroxidation of membrane phospholipids and cause significant harm to the cardiac

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membrane[3]. Inflammation and apoptosis caused by ISO also resulted in severe cardiac damage[5].

Metformin (MET), a biguanide drug given orally, is one of the most often prescribed antihyperglycemic medications for type 2 diabetes treatment[6]. In patients with type 2 diabetes, MET can considerably lower the risk of MI and overall mortality[7]. Moreover, over the past decade, researchers have discovered that MET has a cardiovascular protective impact, that can substantially lower the patient's cardiovascular events[8]. Additionally, in animal models of MI, MET significantly reduces infarct size in animals without diabetes and potently prevents ischemia-reperfusion injury[9]. The molecular mechanisms for MET's cardioprotective effect are not entirely understood. According to most of the researches, MET displayed cardioprotective influences by triggering adenosine monophosphate-activated protein kinase (AMPK)[10], however, Xu et al.,[11] found that MET protected against systolic overload-induced heart failure independently of AMPK. Additionally, MET can attain anti-inflammatory and antioxidant effects by activating AMPK and various signals downstream, preventing the nuclear factor-κB signaling pathway, and decreasing reactive oxygen species (ROS) production[12]. Moreover, through the AMPK-dependent phosphorylation, MET activates endothelial nitric oxide synthase (eNOS) thus increasing the local nitric oxide (NO) production which enhances coronary blood flow, afterload, and left ventricular function as it prevents oxidative stress and apoptosis and causes vasodilation[13].

Carvedilol (CAR) is a non-selective β blocker that also blocks α_1-adrenergic receptors providing cardioprotective action with vasodilation. It is a cardiovascular medication with multiple approved uses, including the treatment of hypertension, angina pectoris, MI, cardiac arrhythmias, left ventricular dysfunction, congestive heart failure, and as an antioxidant and antiproliferative agent[14]. CAR has been demonstrated to exhibit potent antioxidant, anti-inflammatory, and anti-apoptotic effects. Additionally, CAR inhibits the expression of numerous genes implicated in myocardial injury and cardiac remodeling[15,16]. Moreover, CAR may shield the heart from ischemia and reperfusion injury by enhancing myocardial salvage, decreasing infarct size, and enhancing left ventricular contractility and remodeling through the activation of the AMPK signaling pathway[17].

AMP-activated protein kinase, which was recognized to play a significant role in regulating both glucose and fatty acid balance and maintaining the overall body's energy metabolism, is one of the main cellular targets for the treatment of cardiovascular disorders. In addition to playing a critical role in regulating intracellular energy metabolism, it has been demonstrated that AMPK phosphorylation enhances ischemic preconditioning or ischemic postconditioning by reducing oxidative and endoplasmic reticulum stress, preventing apoptosis, and triggering anti-inflammatory mechanisms. It also improves myocardial ischemia by activating eNOS. Therefore, activation of AMPK has potential clinical importance in the prevention and treatment of ischemic heart disease[17&18].

The objective of this study was to clarify the ameliorative effect of MET, CAR, and their combination against ISO-induced MI in non-diabetic rats through the activation of AMPK and antiapoptotic signaling pathways and the lessening of inflammatory, oxidative stress, and apoptotic responses.

MATERIALS AND METHODS

Drugs and Chemicals

Metformin and Carvedilol were procured from AK Scientific, Inc. (USA). Pharma Biotech (China) provided isoprenaline HCL 95%. Normal saline (0.9% NaCl) was purchased from the Bio-diagnostic Company, Egypt. GFS chemicals Co (India) provided dimethyl sulfoxide (DMSO).

Interleukin 6 (IL-6) measurement kits were purchased from Elabscience Biotechnology Inc. in the USA. From Novus Biologicals, LLC (USA), kits for the determination of eNOS were acquired. Wuhan Fine Biotech Co., Ltd. (China) provided the kits for measuring phosphorylated AMPK (pAMPK). Superoxide dismutase (SOD), reduced glutathione (GSH), and malondialdehyde (MDA) detection kits were procured from the Bio-diagnostic Company in Egypt. Cardiac Troponin-I (cTn-I) and Lactate dehydrogenase (LDH) were detected using kits

from Kamiya Biomedical Company (USA) and Spinreact Company (Spain) respectively. Analytical-grade chemicals were used.

Animals

We used 48 adult male Wistar rats from the animal house at the Faculty of Medicine, Sohag University, Egypt, weighing 200–220g. Rats were housed under standardized conditions (12-hrs light/dark cycle, temperature 23±2°C), with unlimited access to food and water. The experimental protocol was certified by the Institutional Animal Care and Use Committee of Sohag University, Faculty of Medicine, Egypt (Approval No. Sohag-5-5-2021-03).

Experimental design

The animals were split into six groups at random, each consisting of eight animals, after a week of acclimatization.

Control A group: rats were administered normal saline intraperitoneally (i.p.) daily for 10 days.

Control B group: rats received DMSO 0.5% (i.p.) daily for 10 days.

ISO-treated group: rats were given ISO (85 mg/kg.i.p.)[19] on the first and second day of the experiment with the injection of normal saline daily for 10 days.

ISO+MET-treated group: rats were administered ISO (85 mg/kg.i.p.) on the first and second day of the experiment, and MET (200 mg/kg.i.p.)[20] for 10 days from the first day of the experiment and was given 1 hr after ISO injection in the first 2 days.

ISO+CAR-treated group: rats received ISO (85 mg/kg.i.p.) on the first and second day of the experiment, and CAR (10 mg/kg.i.p.)[21] for 10 days from the first day of the experiment and was given 1 hr after ISO injection in the first 2 days.

ISO+MET+CAR-treated group: rats were given ISO (85 mg/kg.i.p.) on the first and second day of the experiment, and MET (200 mg/kg.i.p.) and CAR (10 mg/kg.i.p.) for 10 days from the first day of the experiment and were given 1 hr after ISO injection in the first 2 days.

Sample collection

All groups of tested rats had light ether anesthesia 24 hrs after the end of the experiment. Serum was extracted from the heart blood samples by centrifugation for the estimation of cTn-I and LDH. Animals were then slaughtered via cervical dislocation to collect tissue samples. Each animal's heart was swiftly extracted. Heart samples were split into three portions; one portion was instantly fixed in 10 %formalin for immunohistochemistry (IHC), and histological assessment. The second portion was swiftly immersed in liquid nitrogen and held at -80 °C for western blot (WB) and real-time quantitative polymerase chain reaction (RT q-PCR) analysis. The remaining cardiac tissue was weighed and homogenized in phosphate-buffered saline (pH 7.4) after being rinsed in ice-cold saline. After centrifuging the tissue homogenate at 3000 rpm for 15 minutes, the supernatant was thrown and kept at -80°C till SOD, GSH, MDA, IL6, eNOS, and pAMPK measurements were made.

Biochemical analysis

Measurements of serum cTn-I and LDH

Cardiac Troponin-I was determined using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations, and its level was indicated in ng/ml. LDH was detected spectrophotometrically (Photometer 5010, Germany) and was recorded as U/L.

Measurements of cardiac SOD, GSH, and MDA

Superoxide dismutase was determined according to Nishikimi et al.,[22] procedure. The level of SOD was indicated in U/g tissue. GSH was assayed according to Beutler et al.,[23] approach, and its level was expressed in mg/g tissue. MDA level was estimated by Ohkawa et al.,[24] method to detect the degree of lipid peroxidation. MDA level was indicated in nmol/g tissue. All parameters were assayed Using a colorimetric technique.

Measurements of cardiac IL-6, eNOS and pAMPK

Using ELISA, the levels of IL-6, eNOS, and pAMPK were assayed according to the instructions from the manufacturer. IL-6 and eNOS were recorded as pg/g tissue, whereas pAMPK was reported as ng/g tissue.

Western Blot Analysis

Western blot analysis was conducted as previously mentioned[25]. The RIPA (RadioImmunoprecipitation Assay) lysis buffer was added to the cardiac tissues, which were subsequently homogenized and centrifuged at a speed of 12000 x g for 20 min at 4°C. Using the BCA protein assay Kit (Thermo Fisher, USA), the protein concentration of each sample was determined. On 10% sodium dodecyl sulfate-polyacrylamide gel, identical amounts of proteins were split and then transferred to a nitrocellulose membrane. Nonspecific reactivity was blocked with 5% non-fat milk for two hrs at room temperature and the nitrocellulose membrane was incubated with the primary antibody at 4°C overnight. Following washing, the membrane was subjected to a second, one-hr incubation with a secondary antibody at room temperature to detect proteins using electrochemiluminescence (ECL) detection. The band intensity was measured using the Image J program. The protein contents were normalized to β-actin.

Real-time q-PCR Analysis

Total RNA is first isolated from cardiac tissue by using a TRI REAGENT from (Bioshop Co, Canada) and then evaluated for quantity and integrity by measuring the optical density at 260 nm (OD260) with a Nanodrop-1000 spectrophotometer. We applied a one-step reaction to reverse transcribe mRNA into cDNA. The real-time PCR assay uses RNA as a template, and reverse transcription takes place while the assay is running. To amplify a segment of the target cDNA, modified gene-specific PCR primers from (Metabione Co, Germany) are employed following the reaction in real time. The steps were carried out using a GoTaqR 1-Step RT-qPCR System from (Promega Company, USA) on a real-time PCR machine (applied biosystems step one plus). The following parameters were used in quantitative PCR for several cycles: denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The relative quantification of gene expression for all samples was quantitated using the $^{2-\Delta\Delta}$CT comparative approach, normalized to GAPDH[26]. **Table (1)** shows the primers used in this study.

Histopathological studies

The cardiac tissue samples were fixed in 10% formaldehyde for 24 hrs, then embedded in paraffin wax and cut into sections with a thickness of 5 micrometers. The sections were then stained with hematoxylin and eosin and examined under a light microscope. Subsequently, photomicrographs were taken for assessment of histopathological changes.

Immunohistochemistry

The immunohistochemical staining methods were performed according to Saber et al.,[27]. Sections were dewaxed and dipped into a 0.05 M citrate buffer solution with a pH of 6.8 for antigen retrieval. After that, these sections were treated with 0.3% H_2O_2 and protein block. Thereafter, sections were incubated with polyclonal anti-caspase-3 antibodies (Invitrogen, USA, dilution 1/100). After being cleaned with phosphate-buffered saline, the samples were incubated for 30 minutes at room temperature with a goat anti-rabbit secondary antibody (EnVision+TM System Horseradish Peroxidase Labelled Polymer; Dako). A DAB kit was used to visualize the slides before Mayer's hematoxylin was used as a counterstain. The immunolabeling index of caspase-3 was estimated by calculating the positive cells per 1000 cardiomyocytes.

Table 1: Sequence of primers used in the study.

Gene	Forward Primer	Reverse primer
Bcl2	5′- GCA GAG ATG TCC AGT CAG C-3′	5′- CCC ACC GAA CTC AAA GAA GG-3′
Bax	5′- GCG AAT TGG AGA TGA ACT GG-3′	5′- GTG AGC GAG GCG GTG AGG AC-3′
GAPDH	5′- GGC ACA GTC AAG GCT GAG AAT G-3′	5′- ATG GTG GTG AAG ACG CCA GTA-3′

Statistical analysis of data

Data were stated as mean ± SE. The one-way analysis of variance (ANOVA) was used to analyze all data using the SPSS program (Statistical Package for the Social Sciences, version 25.0, SPSS Inc, Chicago, IL, USA). Tukey's post hoc test was employed to compare the groups' means. In all sorts of statistical tests, $P< 0.05$ was deemed significant.

RESULTS AND DISCUSSION

Results

Effect of MET, CAR, and their combination on serum levels of cardiac biomarkers in ISO-induced MI in rats

Table (2) proves that in comparison to the control groups, cTn-I and LDH serum levels were significantly increased ($P<0.05$) in the ISO-treated group. While posttreatment of rats with MET, CAR, and their combination revealed a significant decrease ($P<0.05$) in serum cTn-I and LDH when compared to the ISO-treated group. In addition, there was no statistically significant difference ($P> 0.05$) in the cTn-I and LDH levels between ISO+MET, ISO+CAR, and ISO+MET+CAR-treated groups and between them and the control groups.

Effect of MET, CAR, and their combination on cardiac oxidative stress parameters in ISO-induced MI in rats:

As appeared in **Table (3),** when ISO was administered, there was a significant drop ($P< 0.05$) in cardiac SOD and GSH and a significantly higher level ($P< 0.05$) of MDA compared to the control groups. However, there was a substantial increase ($P< 0.05$) in myocardial SOD and GSH activity and a reduction in MDA level after 10 days of posttreatment with MET, CAR, and their combination in comparison to the ISO-treated group. A non-significant difference ($P> 0.05$) also existed between the ISO+MET, ISO+CAR, and ISO+MET+CAR-treated groups and between them and the control groups.

Table 2: Effect of MET (200 mg/kg/day i.p), CAR (10 mg/kg/day i.p), and their combination on serum levels of cardiac biomarkers in ISO (85 mg/kg, twice at 24h interval i.p.)-induced MI.

Groups	cTn-I (ng/ml)	LDH (U/L)
Control A	0.42 ± 0.03	267.75 ± 10.83
Control B	0.41 ± 0.02	263.75 ± 9.37
ISO	2.2 ± 0.14 (a b)	676.38 ± 32.25 (a b)
ISO+MET	0.53 ± 0.04*	289 ± 14.37*
ISO+CAR	0.49 ± 0.03*	301.25 ± 18.31*
ISO+MET+CAR	0.45 ± 0.03*	272.5 ± 14.71*

Data were reported as mean ± SE (n=8). ISO=Isoprenaline, MET=Metformin, CAR=Carvedilol, cTn-I=Cardiac troponin-I, LDH=Lactate dehydrogenase. (a b) $P<0.05$ versus control group A and B respectively, * $P<0.05$ versus ISO-treated group.

Table 3: Effect of MET (200 mg/kg/day i.p), CAR (10 mg/kg/day i.p), and their combination on cardiac oxidative stress parameters in ISO (85 mg/kg, twice at 24h interval i.p.)-induced MI.

Groups	**SOD U/g**	**GSH mg/g**	**MDA nmol/g**
Control A	376±17.21	319.38±16.37	264±14.03
Control B	380.25±20.06	321.13±15.48	261.75±15.22
ISO	148.5±7.98 (a b)	154.75±7.8 (a b)	463.25±21.2 (a b)
ISO+MET	350.63±19.23*	301.63±12.95*	277.13±11.59*
ISO+CAR	344.13±22.91*	292.75±18.09*	287.5±18.24*
ISO+MET+CAR	370.63±23.38*	312.63±15.42*	267±17.61*

Data were reported as mean ± SE (n=8). ISO=Isoprenaline, MET=Metformin, CAR=Carvedilol, SOD=Superoxide dismutase, GSH=Reduced glutathione, MDA=Malondialdehyde. (a b) $P<0.05$ versus control group A and B respectively, * $P<0.05$ versus ISO-treated group.

Effect of MET, CAR, and their combination on cardiac IL-6 in ISO-induced MI in rats:

Fig. (1) demonstrates a substantial rise ($P< 0.05$) in cardiac IL-6 in the ISO-treated group compared to the control groups. In contrast, when compared to the ISO-treated group, posttreatment with MET, CAR, and their combination resulted in a discernible decline ($P< 0.05$) in cardiac IL-6. Additionally, there was no statistically significant difference ($P> 0.05$) between the ISO+MET, ISO+CAR, and ISO+MET+CAR-treated groups and between them and the control groups.

Effect of MET, CAR, and their combination on cardiac eNOS in ISO-induced MI in rats

According to **Fig. (2)**, there was a significant decrease ($P< 0.05$) in cardiac eNOS after giving ISO compared to the control groups. However, as compared to the ISO-treated group, posttreatment with MET, CAR, and their combination was associated with elevated ($P<0.05$) cardiac eNOS levels. The difference between the ISO+MET, ISO+CAR, and ISO+MET+CAR-treated groups and between them and the control groups was also not statistically significant ($P>0.05$).

Effect of MET, CAR, and their combination on cardiac p-AMPK in ISO-induced MI in rats

Fig. (3) reveals that after administering ISO, there was a significant drop ($P< 0.05$) in cardiac p-AMPK compared to the control groups. In contrast, there was an elevation ($P< 0.05$) in cardiac p-AMPK after 10 days of posttreatment with MET, CAR, and their combination when compared to the ISO-treated group. Furthermore, there was no statistically significant change ($P> 0.05$) between the ISO+MET, ISO+CAR, and ISO+MET+CAR-treated groups and between them and the control groups.

Western Blot

It can be inferred from **Fig. (4)**, that in the ISO-treated group, pAMPK and AMPK protein expression was downregulated ($P< 0.05$). Simultaneously, the ratio of pAMPK/AMPK protein expression significantly declined ($P< 0.05$) in comparison to the control group. However, groups of MET, CAR, and their combination posttreatment can remarkably increase ($P< 0.05$) the phosphorylation of AMPK which is characterized by an increase ($P< 0.05$) in the ratio of pAMPK/AMPK protein expression compared to the ISO-group. Furthermore, there was no statistically significant change ($P> 0.05$) between the ISO+MET, ISO+CAR, and ISO+MET+CAR-treated groups and between them and the control groups.

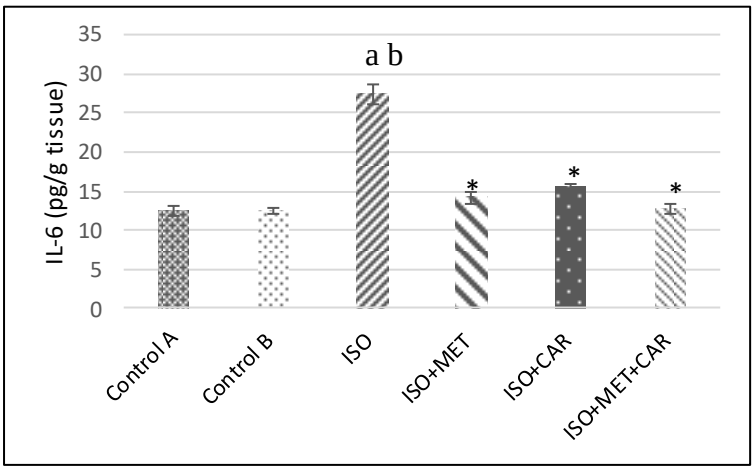

Fig. 1: Effect of MET (200 mg/kg/day i.p), CAR (10 mg/kg/day i.p), and their combination on cardiac IL-6 in ISO (85 mg/kg, twice at 24h interval i.p.)-induced MI. Data were reported as mean ± SE (n=8). ISO=Isoprenaline, MET=Metformin, CAR=Carvedilol, IL6=Interleukin 6. $^{(a\ b)}$ $P<0.05$ versus control group A and B respectively, * $P<0.05$ versus ISO-treated group.

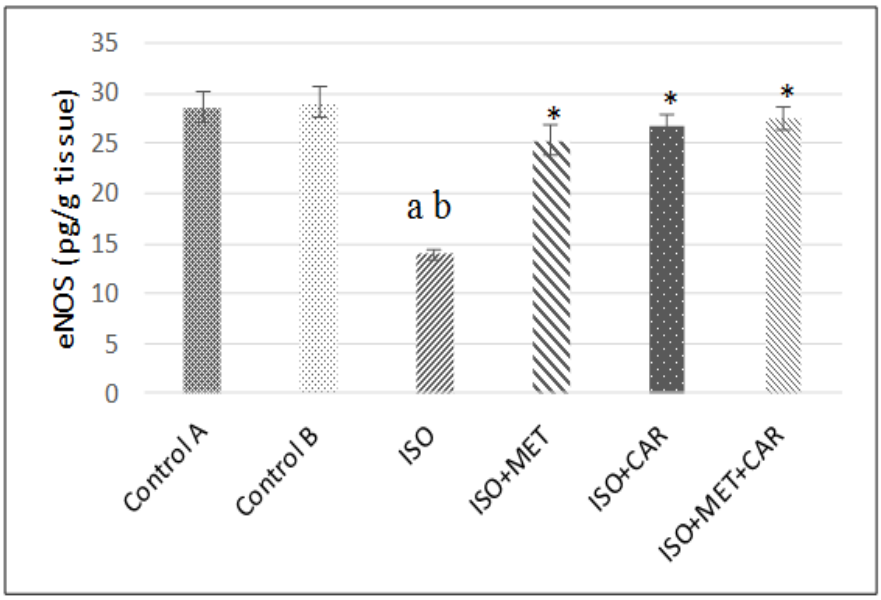

Fig. 2: Effect of MET (200 mg/kg/day i.p), CAR (10 mg/kg/day i.p), and their combination on cardiac eNOS in ISO (85 mg/kg, twice at 24h interval i.p.)-induced MI. Data were reported as mean ± SE (n=8). ISO=Isoprenaline, MET=Metformin, CAR=Carvedilol, eNOS=Endothelial nitric oxide synthase. [a b] $P<0.05$ versus control group A and B respectively, * $P<0.05$ versus ISO-treated group.

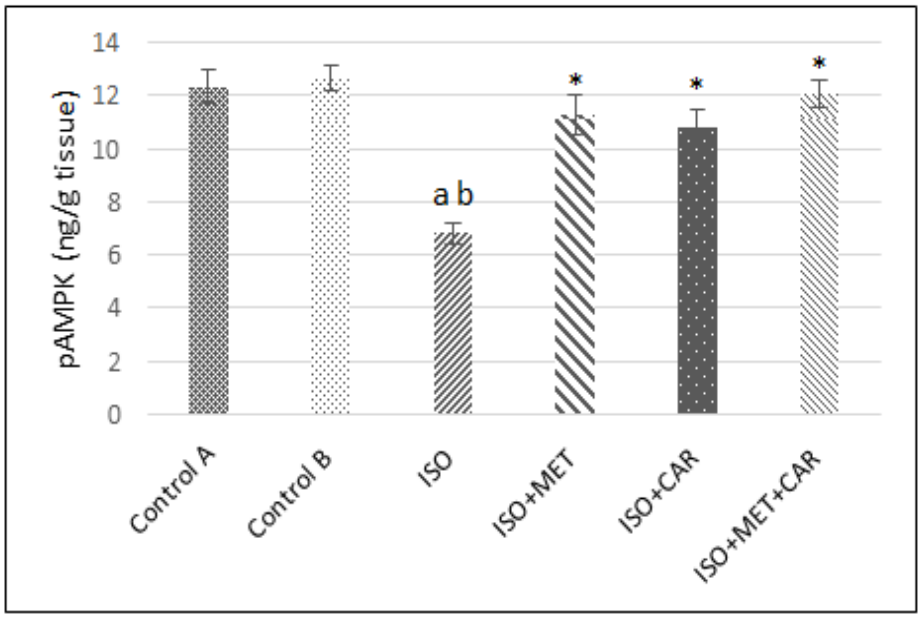

Fig. 3: Effect of MET (200 mg/kg/day i.p), CAR (10 mg/kg/day i.p), and their combination on cardiac p-AMPK in ISO (85 mg/kg, twice at 24h interval i.p.)-induced MI. Data were reported as mean ± SE (n=8). ISO=Isoprenaline, MET=Metformin, CAR=Carvedilol, pAMPK= Phosphorylated adenosine monophosphate activated protein kinase. [a b] $P<0.05$ versus control group A and B respectively, * $P<0.05$ versus ISO-treated group.

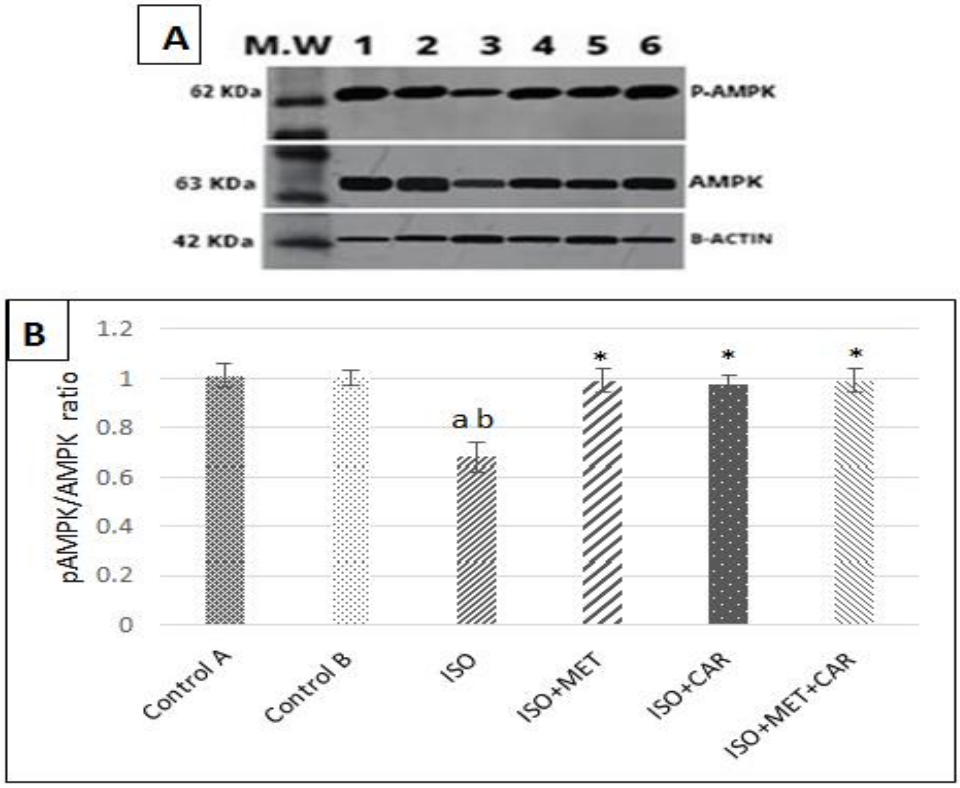

Fig. 4A, B: Effect of MET (200 mg/kg/day i.p), CAR (10 mg/kg/day i.p), and their combination on AMPK signal pathway in ISO (85 mg/kg, twice at 24h interval i.p.)-induced MI. (A) Representative protein expression of p-AMPK, AMPK as determined by Western blotting (B) Quantitative analysis of the ratio of pAMPK/AMPK protein expression. Protein levels were normalized to β-Actin. Data were reported as mean ± SE (n=8). ISO=Isoprenaline, MET=Metformin, CAR=Carvedilol, pAMPK= Phosphorylated adenosine monophosphate activated protein kinase, AMPK= Adenosine monophosphate activated protein kinase. [a b] $P<0.05$ versus control group A and B respectively, * $P<0.05$ versus ISO-treated group.

Effect of MET, CAR, and their combination on the expression of cardiac genes Bcl-2, Bax, and Bcl-2/Bax ratio in ISO-induced MI in rats

Fig. (5A)shows that after administering ISO, the level of mRNA expression of the B-cell lymphoma 2 (Bcl-2) gene was downregulated ($P< 0.05$), while the level of mRNA expression of the Bcl-2 Associated X-protein (Bax) gene was upregulated ($P< 0.05$) compared to the control groups. In contrast, there was an upregulation ($P< 0.05$) in the level of mRNA expression of the Bcl-2 gene and a downregulation ($P< 0.05$) in the level of mRNA expression of the Bax gene after 10 days of posttreatment with MET, CAR, and their combination when compared to the ISO-treated group. Also, the difference between the ISO+MET, ISO+CAR, and ISO+MET+CAR-treated groups and between them and the control groups was not statistically significant ($P> 0.05$).

According to **Fig. (5 B)**, there was a significant decrease ($P< 0.05$) in Bcl-2/Bax ratio in the ISO-treated group compared to the control groups. Alternatively, posttreatment with MET, CAR, and their combination resulted in a significant elevation ($P< 0.05$) in Bcl-2/Bax ratio compared to the ISO-treated group. Furthermore, there was a significant decrease ($P< 0.05$) in Bcl-2/Bax ratio in ISO+MET and ISO+CAR-treated groups compared to the control groups. In addition, there was no statistically significant change ($P>0.05$) between the ISO+MET, ISO+CAR, or ISO + MET + CAR-treated groups and between the ISO+MET+CAR-treated group and the control groups.

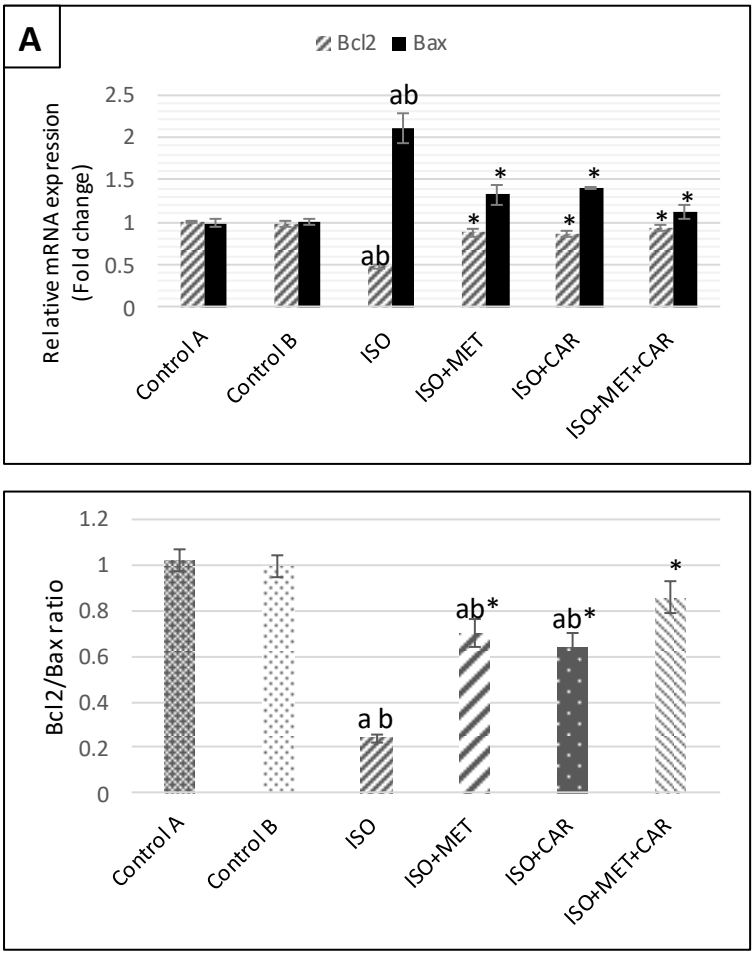

Fig. 5 (A, B): Effect of MET (200 mg/kg/day i.p), CAR (10 mg/kg/day i.p), and their combination on the expression of cardiac genes Bcl2, Bax, and Bcl2/Bax ratio in ISO (85 mg/kg, twice at 24h interval i.p.)-induced MI. Data were reported as mean ± SE (n=8). ISO=Isoprenaline, MET=Metformin, CAR=Carvedilol, Bcl2=B-cell lymphoma 2 , Bax=Bcl-2 Associated X-protein. $^{(a\ b)}$ $P<0.05$ versus control group A and B respectively, * $P<0.05$ versus ISO-treated group.

Histopathological results

Histological alterations of cardiac tissue were evaluated for rats in all groups **(Fig. 6)**. In control rats, cardiac muscle fibers have normal architecture, and cardiac striations were preserved. Alternatively, the treatment of rats with ISO resulted in degenerative changes in cardiomyocytes. The cells showed cloudy swelling, granular cytoplasm, and loss of cardiac muscle striations. The stroma showed interstitial tissue edema and vascular congestion with focal infiltration by neutrophils and lymphocytes. Multiple foci of interstitial tissue hemorrhage were observed. Isolated administration of either MET or CAR showed improvement in the deleterious effect of ISO. Generally, there are focal mild changes including focal cloudy swelling with granular eosinophilic cytoplasm and mild vascular congestion compared to rats treated with ISO. Furthermore, the inflammatory reaction and interstitial tissue hemorrhage were minimal, necrosis and apoptosis were not identified, and cardiac muscle striations were preserved. The augmenting effect of MET and CAR was evaluated. When administered together, MET and CAR induced obvious improvement in the effects of ISO. The cardiac muscle fibers have a nearly normal histological appearance with preserved striations. No encountered hemorrhage, apoptosis, or necrosis of cardiac muscle.

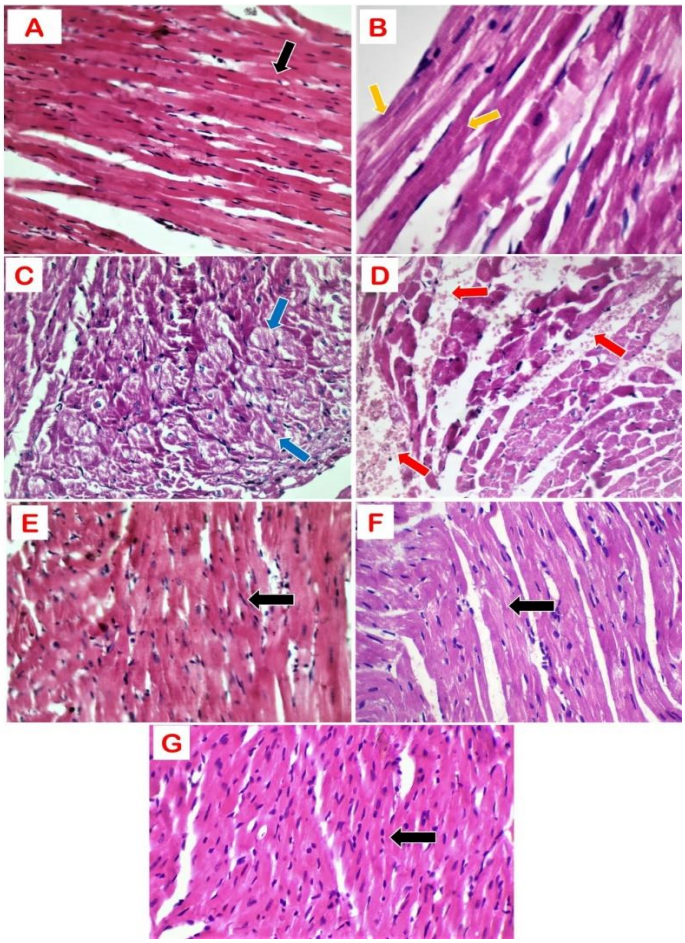

Fig. 6: Photomicrographs showing histological changes of cardiac muscle:Sections of negative control rats showed preserved muscle bundling (black arrow) (**A**), with identified cardiac striations (yellow arrows) (**B).** Sections of rats treated with ISO showed cloudy swelling of myocytes (blue arrows) (**C)** and frequent interstitial tissue hemorrhage (red arrows) (**D).** Sections of MET-treated rats (**E**), CAR-treated rats (**F**), and MET/CAR-treated rats (**G**) showed preserved muscle bundles (black arrows). Magnification is x600 for B and x400 for others.

Immunohistochemical results

As illustrated in **Fig. (7A)**, cardiac sections of control rats displayed a slight and mild immunoreaction for caspase-3 in the cytoplasm of cardiomyocytes (A, B respectively). A marked increase of both cytoplasmic and nuclear expression of caspase-3 in the myocardial fibers was noticed in the ISO-treated group (C). Cardiac sections of ISO+MET, ISO+CAR, and ISO+MET+CAR-treated rats displayed a marked decrease in the expression of caspase-3 antibody within the myocardial fibers (D, E, F).

Caspase-3 labeling apoptotic index

In **Fig. (7, B)**, we observed that the number of cells labeled with the antibody to caspase- 3 was significantly higher ($P< 0.05$) in the ISO-treated group compared to the control groups. In contrast, the number of cells labeled with the antibody to caspase-3 was significantly reduced ($P< 0.05$) in the ISO+MET, ISO+CAR, and ISO+MET+CAR treated groups compared to the ISO-treated group. Moreover, there was a significant increase ($P< 0.05$) in the number of cells labeled with caspase-3 antibody in the ISO+MET, ISO+CAR, and ISO+MET+CAR-treated groups compared to the control groups. On the other hand, there was a significant rise ($P< 0.05$) in the number of cells labeled with caspase-3 antibody in the ISO+MET and ISO+CAR-treated groups compared to the ISO+MET+CAR-treated group.

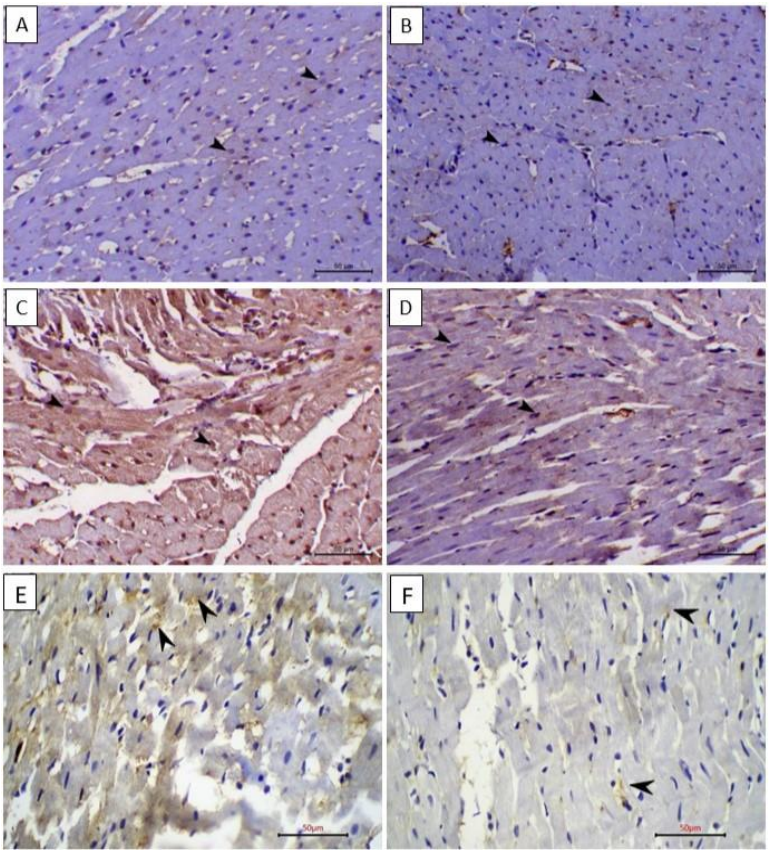

Fig. 7A: Photomicrographs of caspase-3 immunostaining in the studied different animal groups: The heart of the control A group showed mild cytoplasmic immunostaining of caspase-3 antibodies within the myocardial fibers (arrowheads) **(A),** the heart of the control B group showed slight cytoplasmic immunostaining of caspase-3 antibodies within the myocardial fibers (arrowheads) **(B)**, the heart of the ISO-treated group showed a marked increase of both cytoplasmic and nuclear expression of caspase-3 antibodies within the myocardial fibers (arrowheads) **(C)**, the heart of the ISO+MET, ISO+CAR, and ISO+MET+CAR-treated groups showed a marked decrease of the expression of caspase-3 antibodies within the myocardial fibers (arrowheads) **(d, E, F)**.

*All photomicrographs showed caspase-3 IHC, magnification is x400 for E and F and x200 for others, bar= 50 µm

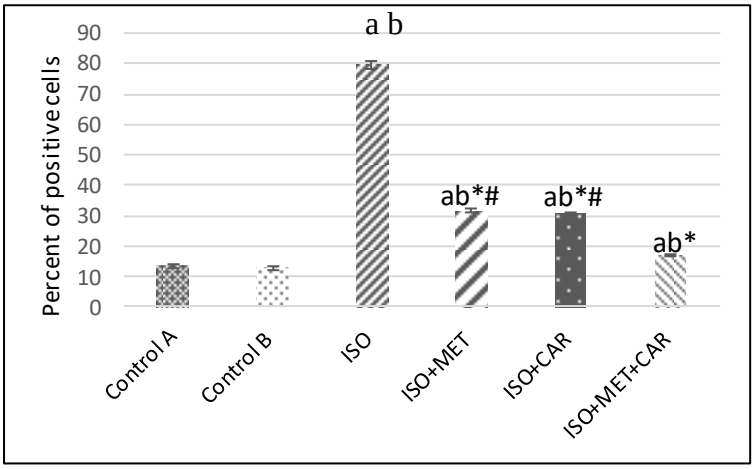

Fig. 7B: Graphical presentation of the percentage of caspase-3 positive cells. Data were reported as mean ± SE (n=8). ISO=Isoprenaline, MET=Metformin, CAR=Carvedilol. [(a b)] $P<0.05$ versus control group A and B respectively, [*] $P<0.05$ versus ISO-treated group, [#] $P<0.05$ versus ISO+MET+CAR-treated group.

Discussion

We designed the current investigation to explain the mechanisms behind MET promising cardioprotective impacts that attracted much attention, by comparison to CAR. The findings of this study demonstrate that MET, CAR, and their combination could improve ISO-induced myocardial damage in a post-MI non-diabetic rat model.

Cardiac troponins, CK-MB as well as LDH are standard diagnostic indicators that are highly sensitive and specific for the diagnosis of myocardial damage in various conditions[28]. In the present study, the elevated levels of serum cardiac injury biomarkers, cTn-I and LDH after ISO administration indicated a significant impairment of myocardial function. Our results are in harmony with the prior reports[29-31]. Cardiomyocyte damage in ISO-induced MI is caused by an excess of ROS and cytotoxic free radicals, which disrupted the integrity and function of myocardial membranes and released intracellular cardiac enzymes like the cTn-I[32]. Our results also revealed that posttreatment with MET or CAR or their combination significantly attenuated the rise of cTn-I and LDH. The results of MET are in accordance with the previous studies[31&33-36]. Additionally, our results coincide with the previous reports[14&37&38] which showed lower levels of cTn-I and LDH after CAR treatment which indicated that MET and CAR possess cardioprotective effects in myocardial injury.

Our data showed that ISO administration was associated with a significant decrease in cardiac SOD and GSH levels, which are vital endogenous antioxidant defenses against oxidative cell damage, and a significant increase in the cardiac oxidative stress marker MDA level. This is consistent with Huwait and Al-Ghamdi, and Hasan et al.,[29&39] findings. ISO-induced cardiac damage is mediated through the production of oxygen free radicals due to the oxidation of ISO, which results in the formation of superoxide anions that eventually lead to the formation of hydrogen peroxide that damages cellular proteins, lipids, and DNA[14]. Our study showed that MET, CAR, and their combination are effective in reducing cardiac MDA and preserving cardiac levels of SOD, and GSH. Our results of MET are in harmony with articles published previously[33-36]. It is remarkable that MET was reported to have antioxidant capabilities reducing the accumulation of free radicals[40]. This suggests that MET can improve cells' capacity to counteract oxidative damage by controlling the actions of SOD, LDH, and other enzymes, as well as greatly accelerate the restoration of myocardial function. Furthermore, our CAR results agreed with the previous studies[15,37,38,41]. CAR possesses antioxidant capabilities that inhibit the generation of ROS in the myocardium and reduce the triggering of transcription factors and apoptosis by free radicals[42].

Our results demonstrated a significant increase in the cardiac IL-6 levels in the ISO-treated group which is agreed with Huwait and Al-Ghamdi,[29] results. This is suggested that the onset of MI was associated with a severe myocardial inflammatory response. Our results also revealed that posttreatment with MET and CAR or their combination led to a significant decrease in cardiac IL-6 levels. This is consistent with Karam and Radwan,[43] who demonstrated that heart IL-6 is markedly decreased in MET-treated rats. Also, Amirshahrokhi and Abzirakan,[15] results coincide with our CAR findings. By preventing excessive nuclear factor-κB expression brought on by MI, MET, and CAR could prevent proinflammatory cytokines release, which may reduce inflammation and cardiomyocyte apoptosis and alleviate early cardiac dysfunction[34,44].

Endothelial NOS is thought to safeguard against cardiac oxidative damage through regulated NO generation[45]. Our results showed a significant decrease in cardiac eNOS levels in the ISO-treated group. This finding is in harmony with Refaie et al.,[46] findings. The inhibitory effect of ISO on eNOS is a main contributing factor in the mediation of MI with endothelial damage and vascular dysfunction[47]. Our results also revealed that posttreatment with MET, CAR, and their combination led to a significant increase in cardiac eNOS levels. In a rat model of ventricular hypertrophy, Zhang and colleagues.,[48] have demonstrated that the improvement in heart structure and function following MET treatment was connected to increased eNOS production. Also, Yin et al.,[9] revealed that MET partially prevented the sharp decrease in eNOS mRNA

levels in MI animals in a rat model of post-MI heart failure. Furthermore, our findings agreed with Refaie et al.,[49] and Hassan et al.,[50] who indicated that CAR treatment resulted in a significant increase in eNOS expression. The activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway may be the cause of the CAR effect on eNOS expression. AKT is regarded as the traditional upstream regulator for the expression of eNOS[50]. MET and CAR stimulate eNOS with the release of NO by endothelial cells which helps to preserve vascular integrity, improve endothelium-dependent relaxation factor, and reduce oxidative stress[49,51].

We found that ISO reduced AMPK phosphorylation and AMPK activation which was significantly reversed by MET and CAR treatment and their combination. This is consistent with the previously published articles[39&52&53] which reported a significant reduction in pAMPK and AMPK in the ISO-treated group which indicated that ISO treatment reduces the activation of the AMPK signaling pathway. Our study is also consistent with the prior reports[9&10&35] findings indicating that MET treatment activated the AMPK signaling pathway. Additionally, MET promotes myocardial protection by activating AMPK to limit intracellular oxidation[54]. Moreover, our results are in harmony with Hu et al.,[17] who revealed that CAR activates the AMPK signaling pathway in cardiomyocytes.

B cell lymphoma-2 family proteins serve as essential regulators of mammalian apoptosis which include proteins that either promote or prevent apoptosis[55]. Our results showed a significant decrease in cardiac Bcl-2 mRNA expression level, a significant increase in cardiac Bax gene level, and a significant decrease in the Bcl-2/Bax ratio in the ISO-treated group. This is agreed with Sun et al.,[53] study. Our results also revealed that posttreatment with MET, CAR, and their combination protected cardiomyocytes against ISO-induced apoptosis, by increasing the cardiac Bcl-2 gene, decreasing the cardiac Bax gene level, and increasing the Bcl-2/Bax ratio. The results of MET conicide with Li et al.,[34] study. Our results are also in accordance with Hu et al.,[56] who revealed that treatment with CAR led to the upregulation of Bcl-2 and downregulation of Bax protein expression, and Yang et al.,[57] who showed that treatment with CAR increases the expression ratio of Bcl-2/Bax. These data together suggest that MET and CAR can reduce cardiomyocyte apoptosis and improve cardiac function.

We examined the levels of caspase-3, a key pro-apoptotic protease involved in the production of apoptotic bodies and the activation of cell death[41]. Our results showed a significant increase in caspase-3 level in the ISO-treated group. This is in accordance with Ibrahim et al.,[31] and Hasan et al.,[39] results indicating enhanced apoptosis of cardiomyocytes. Increased ROS, followed by lipid peroxidation in heart tissue and immediate DNA damage, could account for this. Additionally, oxidative stress can affect calcium ion channels and change the potential of the mitochondrial membrane, releasing cytochrome C that promotes the caspase cascade and DNA damage[49]. Our results also revealed that posttreatment with MET, CAR, and their combination showed a significant decrease in the expression of caspase-3 compared to the ISO-treated group. This is agreed with the prior reports[31&33&34] suggesting that MET possesses antiapoptotic properties. Additionally, our findings agreed with El-Shitany and El-desoky,[37] and Zheng et al.,[41] who reported that CAR treatment downregulated the expression of caspase-3 thus attenuating cardiomyocyte apoptosis. CAR decreased cardiomyocytic apoptosis by downregulating the expression of inflammation-associated genes and apoptosis-associated proteins[58].

A histological examination was carried out to determine the degree of myocardial injury, along with immunohistochemistry, biochemical estimations, and protein expression. The findings of the ISO-treated group agreed with the previous studies[29&52&53] which showed disorganization of cardiac muscle structure with necrosis and congestion of blood capillaries, edema, and infiltration of immune cells in the ISO-treated group. In addition, treatment with MET, CAR, or their combination showed obvious improvement in the deleterious effects of ISO. This is consistent with Huang et al.,[6] and An and Kang,[36] who indicated that MET prevented myocardial damage and also Asdaq et al.,[14] and

El-Shitany and El-desoky,[37] who revealed markedly preserved cardiomyocyte morphology in CAR treatment.

Conclusion

The present findings reveal that MET, CAR, or their combination exert a potent ameliorative effect against the ISO-induced MI in non-diabetic rats through activating the AMPK signaling pathway, enhancing eNOS, preventing myocardium apoptosis, minimizing oxidative stress and inflammation, and improving histopathological changes in cardiac tissues. Moreover, our data indicated that the combination of MET and CAR showed remarkably favorable outcomes over their individual use. So, we recommend adding MET to patients with AMI treated with CAR.

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الميتفورمين و الكارفيديلول يحسنان احتشاء عضلة القلب الناجم عن الأيزوبرينالين في الجرذان غير المصابة بالسكري من خلال تنشيط AMPK وقمع موت الخلايا المبرمج

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أجريت الدراسة الحالية على عدد من فئران التجارب المصابة باحتشاء عضلة القلب الناجم عن الأيزوبرينالين والغير مصابة بداء السكرى لإيضاح تأثير وآليات العمل للميتفورمين و الكارفيديلول كل على حده أومدمجين معا. تم تقسيم ذكور فئران ويستار البالغة إلى ست مجموعات (٨ لكل مجموعة) وتلقت المجموعة الضابطة (أ) محلولاً ملحيًا بينما تلقت المجموعة الضابطة(ب) دايميثايل سلفوكسايد داخل الصفاق لمدة ١٠ أيام. مجموعة الأيزوبرينالين: حصلت على الأيزوبرينالين (٨٥ ملجم / كجم) داخل الصفاق في اليوم الأول والثاني من التجربة مع حقن محلول ملحي لمدة ١٠ أيام من اليوم الأول. المجموعات المعالجة بـ الأيزوبرينالين والميتفورمين و الأيزوبرينالين و الكارفيديلول: تلقت الأيزوبرينالين كما هو موصوف سابقًا و الميتفورمين (٢٠٠ ملجم / كجم) داخل الصفاق و الكارفيديلول (١٠ ملجم / كجم) على التوالي لمدة ١٠ أيام من اليوم الأول للتجربة. المجموعة المعالجة بـ الأيزوبرينالين والميتفورمين و الكارفيديلول: حصلت على الثلاثة أدوية بالجرعات و الطريقة المذكورة سابقا.

و قد أظهرت مجموعة الأيزوبرينالين احتشاء عضلة القلب كما يتضح من الارتفاع في المؤشرات الحيوية القلبية في الدم cTn-I و LDH. بالإضافة إلى ذلك ، كانت مستويات MDA و IL6 و caspase-3 و Bax مرتفعة بشكل ملحوظ ، بينما انخفضت مستويات SOD و GSH و pAMPK و eNOS و Bcl2 و Bcl2 / Bax بشكل ملحوظ مع التغيرات النسيجية في أنسجة القلب. في حين أن العلاج اللاحق باستخدام الميتفورمين و الكارفيديلول ومزيجهما عكس بشكل كبير هذه الآثار الضارة لعضلة القلب التي يسببها الأيزوبرينالين.

وخلصت الدراسة إلي أنه يمكن للميتفورمين و الكارفيديلول ومزيجهم علاج عضلة القلب المحتشية من الأيزوبرينالين من خلال تنشيط مسار إشارات AMPK والآليات المضادة للاستماتة والمضادة للالتهابات و الأكسدة ، وبالتالي تعزيز استرداد وظيفة عضلة القلب.