Abstract
The objective of the present study is to assess the possible cardioprotective potential effect of resveratrol in myocardial ischemia reperfusion injury induced by ligation of coronary artery in a male rat model. 28 adult male albino rats were randomized into 4 equal groups: (1), Sham group, rats underwent the same anesthetic and surgical procedure as the control group except for LAD ligation; (2), Active control group, rats subjected to regional ischemia for 30 min by ligation of LAD coronary artery and reperfusion for 2 hours; (3), Control vehicle group, rats received dimethyl sulphoxide (DMSO) (vehicle of resveratrol) via IP route and subjected to ischemia for 30 minutes before ligation of LAD coronary artery & reperfusion for 2 hr; (4), Resveratol treated group, rats pretreated with resveratrol 5mg/kg via IP injection 30minutes before ligation of LAD coronary artery & then subjected to reperfusion for 2 hr. In control group, as compared with sham, tissue TNF-α, IL-6, IL-10, caspase-3 and BAX, plasma cTn-T and serum MDA significantly increased (P<0.05), while serum GSH significantly decreased (P<0.05). Histopathologically, control group showed a significant cardiac injury (P<0.05) compared with sham group. Resveratrol significantly counteracted (P<0.05) the increase of TNF-α, IL-6, caspase-3 and BAX and counteracted the increase in plasma cTn-T and serum MDA. Resveratrol produces a significant elevation (P<0.05) in cardiac IL-10 and serum GSH with significant reduction in (P<0.05) cardiac injury. In Conclusions, Resveratrol attenuates myocardial I/R injury in male rats via interfering with inflammatory reactions and apoptosis which were induced by I/R injury.

Key words: Myocardial ischemia, reperfusion, Resveratrol, inflammatory reactions, Apoptosis.
Introduction
Ischemia and reperfusion (I/R) is a pathological condition characterized by an initial restriction of blood supply to an organ followed by the subsequent restoration of perfusion and concomitant reoxygenation [1]. Prolonged organic ischemia is characterized by insufficient oxygen supply resulting in tissue ATP depletion with a transition to activation of anaerobic metabolic pathways which cannot maintain cellular function for prolonged periods lastly leading to cell death [2]. Within myocardial ischemia, tissue pH significantly declines and returns to normal after reperfusion [3]. A difference in metabolic supply and demand within the ischemic organ results in deep tissue hypoxia and microvascular dysfunction [4]. Neutrophils induce inflammatory mediators that amplify recruitment of neutrophil in the ischemic-reperfused myocardium, so expanding myocardial damage [5]. Furthermore, I/R leads to the triggering of cell death programs, involving apoptosis and necrosis [6].

Myocardial ischemia is differentiated with anaerobic metabolism and intracellular acidosis [7]. During reperfusion, the electron transport chain is reactivated, generating ROS. ROS mediate myocardial reperfusion injury by inducing the opening of the MPTP, acting as a neutrophil chemoattractant. This contributes to intracellular Ca\(^2+\) overload and damages the cell membrane by lipid peroxidation, inducing enzyme denaturation and causing direct oxidative damage to DNA. Several hours after the onset of myocardial reperfusion, neutrophils accumulate in the infarcted myocardial tissue in response to the release of the chemoattractants ROS, cytokines, and activated complement [8]. Actually the reperfusion can be more injurious than the pre-reperfusion ischemia\[7\]. Resveratrol is a phytoalexin polyphenol activated cyclic Adenosine Monophosphate (cAMP) signalling pathway [9] leading to amended mitochondrial function and protection against metabolism [10]. Resveratrol provides signal towards anti-oxidative enzyme activating as showed by the up-regulation of activities of Glutathione reductase (GPx) and Superoxide dismutase (SOD)\[11\]. Resveratrol contributes to cell survival, it decreases intracellular calcium level, it inhibits caspase-3 activity and leads to low expression of Bax and high expression of B-cell lymphoma-2 protein (Bcl-2), also protect against apoptosis induced by Hydrogen peroxide (H2O2) through enhancing the antioxidant defenses, SOD activities and GSH contents, and inhibiting the digression of the mitochondrial membrane potential [12].

Method
Materials
Pure Resveratol powder (99%)(Santa crus, USA), normal saline (KSA) ketamine (Hikma, Jordan), Xylazine (RompunTM, 2% vials, Bayer AG, Leverkusen, Germany). Rat TNF-α, IL-6, IL-10, caspase3, BAX and cTnT (ELISA) kits were purchased from Biotangusa, USA. Trichloroacetic acid (TCA)Merck-Germany, Ethylene diaminetetraacetic acid disodium (EDTA)BDH, U.K. Thiobarbituricacid (TBA) Fluka company, Switzerland 5,5-Dithiobis (2-nitrobenzoic acid) DTNB Sigma company Ltd. Reduced glutathione Biochemical, USA and Methanol Fluka company, Switzerland. regarding instruments, High Intensity Ultrasonic Liquid Processor (Sonics & materials Inc., USA), Digital Spectrophotometer EMCLAB/ Germany, Bio-Elisa Reader, BioTek Instruments, USA and ventilator (Harvard. USA).

Animal
After the approval that has been established by the Institutional Animal Care and Use Committee (IACUC) and submission the required applications, 28 male albino rats weighting (180-320 g) were purchased from Animal Resource Center. They were housed in the animal house (for one week) in a temperature-controlled (25°±1°C) room (humidity was kept at (60–65%) with alternating 12-h light/12-h dark cycles and were allowed to access freely regarding water and chow diet until the time of starting the experimental study.

Study design
After the 1st week of accommodation, the 28 rats were randomly divided into 4 groups (7 rats in each) as follow:
1. (Sham group): Rats underwent the same anesthetic and surgical procedures but without ligation for the LAD.
2. Active control (MI/R) group: rats followed surgical operation for LAD ligation and they were subjected to 30 min of ischemia and 120 min of reperfusion.
3. (MI/R) + Vehicle pretreated group: rats were pretreated with DMSO via intraperitoneal injection 30 minutes before ligation of LAD, then underwent surgical LAD ligation, and subjected to 30min of ischemia followed by 120 min of reperfusion.
4. (MI/R) + Resveratrol pretreated group: rats of this group take a single I.P injection of resveratrol in a concentration of 5 mg/kg dissolved in 0.1%
DMSO 30 minutes immediately before ligation of LAD, then subjected to surgical LAD ligation with 30 minutes of ischemia followed by 120 min of reperfusion [13].

**Surgical ligation of the LAD**

Rats were anesthetized with (IP) injection of 100 mg/kg ketamine and 10 mg/kg Xylazine [17]. After intubation of the trachea by a 20 G cannula and the endotracheal tube was connected tightly to the ventilation machine. The ventilation rate was fixed from 120-135 breath/minute with tidal volume 20 ml/kg body weight, with 100% oxygen. Pericardial layer incision was made by administration round end scissors to open the space. The LAD coronary artery was transient ligated 1 to 2 mm below the tip of the left auricle using a tapered needle and a 8-0 polypropylene ligature. Tightening the ligature could then occlude the artery for a 30-minute ischemic period [18]. The chest cavity was closed by bringing together the fourth and fifth ribs with one 2-0 silk suture. Cardiac reperfusion was achieved by releasing the tension applying to the ligature for 120 minutes [19]. The rats were euthanized after reperfusion via injection high dose of anesthesia and the chest was re-opened then the right ventricle was punctured with a syringe needle so that about 3 ml of blood was aspirated for later blood analysis. After that, the heart was isolated and divided into 2 pieces, the apical part used for histological examination and the basal was used for measuring the tissue parameters.

**Blood sampling for measurement of plasma cTn-T, serum MDA and serum reduced GSH**

At the end of experiment, about 2-3 ml of blood sample was placed in a tube containing disodium ethylene diamine tetra acetic acid (EDTA) (22 mg/mL) as anticoagulant and mixed thoroughly and then centrifuged at 3000 rpm for 15 min then the supernatant was used for determination of plasma cTn-T level, whereas the remaining blood was allowed to clot in an ordinary tube at 37 °C then it was centrifuged at 3000 rpm for 15 minutes then the supernatant was taken for MDA and GSH serum levels determination.

**Tissue preparation for TNF-α, IL-6, IL-10, caspase 3 and BAX measurements**

The upper parts of the ventricles were washed with cold normal saline to remove any blood, stored in deep freeze (-20°C), and then homogenized with high intensity liquid processor in 1:10 (w/v) phosphate buffered saline that contain 1% triton X-100 and protease inhibitor cocktail [17]. The homogenate was centrifuged at 14000 rpm 4°C for 20 min. The supernatant was collected for determination of TNF-α, IL-10, IL-6, Bax, and Caspase-3 by ELISA with a commercially available ELISA kit (Literature of kit by life Diagnostic, USA) according to the manufacturer’s instructions.

**Preparation for Histopathology**

The apical parts of the heart were excised immediately, rinsed using ice-cold 0.9% saline and fixed in 10% formalin solution pH 7.4 [18] embedded in paraffin wax. The paraffin-embedded tissues were sectioned (4-μm thick), stained with hematoxylin and eosin (H&E). Damage scores were evaluated according to the following morphological criteria that have been used to evaluate the histopathological damage [19] as follow:

- score 0, no damage; score 1 (mild), interstitial edema and focal necrosis; score 2 (moderate), diffuse myocardial cell swelling and necrosis; score 3 (severe), necrosis with presence of contraction bands and neutrophil infiltrate; score 4 (highly severe), widespread necrosis with presence of contraction bands, neutrophil infiltrate, and hemorrhage.

**Statistical analysis.**

Data were expressed as mean ± SEM. An expert statistical advice was considered for data analysis which were aided by computer. Statistical analysis were done using SPSS version 20.0 computer software (Statistical Package for Social Science). ANOVA (analysis of variance) had been used for measurement (numerical data). Mann-Whitney test had been used for myocardial damage score. P value <0.05 regarded as significant.

**Results:**

**Biochemical results**

**Effect on Pro-inflammatory cytokines (TNF-α and IL-6).**

Results revealed a significant increase (P<0.05) in (TNF-α and IL-6) cardiac tissue levels in the MI/R group as compared with the sham group, while in the MI/R + resveratol pretreated group, resveratol produce a significant decrease (P<0.05) in the (TNF-α and IL-6) cardiac tissue levels as compared with the MI/R group as shown in table 1 and figures 1 and 2.

**Effect on anti-inflammatory cytokine (IL-10).**

Results revealed a significant increase (P<0.05) in (IL-10) cardiac tissue level in the MI/R group as compared with the sham group, while in the MI/R + resveratol pretreated group, resveratol produce a
significant elevation \( (P<0.05) \) in the (IL-10) cardiac tissue level as compared with all other groups (sham group, the MI/R group and MI/R + vehicle group) as shown in table 1 and figure 3.

**Effect on apoptotic markers (caspase-3 and BAX).**
Results revealed a significant increase \( (P<0.05) \) in (caspase-3 and BAX) cardiac tissue levels in the MI/R group as compared with the sham group, while in the MI/R + resveratol pretreated group, resveratol produce a significant reduction \( (P<0.05) \) in (caspase-3 and BAX) cardiac tissue levels as compared with the MI/R group as shown in table 1 and figures 4 and 5.

**Effect on Plasma Level of Troponin T (cTnt).**
Results revealed a significant increase \( (P<0.05) \) in (cTnt) plasma level in the MI/R group as compared with the sham group, while in the MI/R + resveratol pretreated group, resveratol produce a significant reduction \( (P<0.05) \) in the (cTnt) plasma level as compared with the MI/R group as shown in table 1 and figure 6.

**Effect on the serum level of oxidative stress markers (MDA and GSH).**
Results revealed a significant increase \( (P<0.05) \) in the serum level of MDA in the MI/R group as compared with the sham group, while in the MI/R + resveratol pretreated group, resveratol produce a significant reduction \( (P<0.05) \) in MDA serum level as compared with the MI/R group. About GSH, results revealed a significant decrease \( (P<0.05) \) in the serum level of GSH in the MI/R group as compared with the sham group, while in the MI/R + resveratol pretreated group, resveratol produce a significant increase \( (P<0.05) \) in GSH serum level as compared with the MI/R group as shown in table 1 and figures 7 and 8.

**Histopathological Findings**
Histologically, the MI/R group revealed a significant cardiac tissue injury \( (P<0.05) \) compared with the sham group, and this injury was showing sever hemorrhage, presence of interstitial edema, necrosis and neutrophil infiltration in contrast with the cross section of the sham group which showed a 100% normal structure of cardiac tissue with no interstitial edema, no diffuse myocardial cell swelling and necrosis, no neutrophils infiltration, no hemorrhage, no capillary compression and no evidence of apoptosis. Treatment of rats with resveratrol significantly improved \( (P<0.05) \) the injury of cardiac tissue as compared with control group and cross section from this group (MI/R + resveratol) showed mild cardiac injury with absence of necrosis and few interstitial edema and PMN infiltration while there was no significant difference between the MI/R and MI/R + vehicle groups as shown in figures 9 A, B, C, D.

**Discussion**
The common origin of myocardial infarction is occlusion of the coronary artery as a result of the embolization of an unstable coronary plaque [20]. Activation of PMN’s, eicosanoids, cytokines, ROS and complement products have been shown to be involved in the initial ischemic period [21]. The intracellular and extracellular accumulation of these products triggers homeostatic pathways involving necrosis, apoptosis and inflammation that initially occur during acute myocardial infarction. The apoptotic response may then lead to potential permanent tissue or end organ dysfunction. Restoration of blood flow to ischemic myocardium is the current therapy, yet is associated with ischemia/reperfusion injury [22].

**Effects of Resveratol on pro-inflammatory cytokines (TNF-α and IL-6) and on the anti-inflammatory cytokine (IL-10).**
Pretreatment with resveratol before induction of myocardial ischemia produced a significant reduction \( (P<0.05) \) in the myocardial tissue levels of pro-inflammatory cytokines (TNF-α, IL-6 ), with the significant elevation \( (P<0.05) \) in the level of anti-inflammatory cytokine IL-10 compared to control. [24] found that resveratol reduced myocardial TNF-α production compared with control group. Thus, resveratol produces cardioprotective and anti-inflammatory effects [23]. Showed that resveratrol in the rat heart subjected to MI/R significantly reduced serum and myocardial TNF-α production as compared with MI/R [24,25]. To best of our knowledge, there is no study measured the effect of resveratol on IL-6 and IL-10 in myocardial ischemia reperfusion injury.

**Effect of resveratol on Caspase 3 and BAX**
The level of caspase 3 and BAX in cardiac tissue was significantly decreased \( (P<0.05) \) in the resveratol pretreated group compared to the control group. Showed that the expressions of Bax and caspase-3 were significantly increased in the hypoxia group while resveratrol treatment inhibited the hypoxia-induced increase of Bax and caspase in hypoxic myocardial cells [26]. Indicated that resveratrol decreases intracellular calcium level so that
apoptosis triggered by calcium overload is reduced and inhibits caspase-3 activity and leads to the low expression of Bax compared with I/R in neonatal cardiomyocytes injury [12].

Effect of Resveratrol on cTnT level
The cTnT plasma level of resveratrol pretreated group was significantly decreased (P<0.05) compared to the control group. To best of our knowledge, there is no study measured the effect of resveratrol on cTnT in myocardial ischemia reperfusion injury.

Effect of Resveratrol on MDA and reduced GSH level
There was a significant decrease (P<0.05) in serum MDA level with a significant elevation (P<0.05) of GSH serum level in the resveratrol pretreated group compared to the active group. Observed that resveratrol pretreatment exert antioxidant activity in the rat after I/R (30 min occlusion of the left coronary artery followed by a 120 min reperfusion) via decreased MDA content in myocardium compared with the control [27]. Reported that resveratrol exerted beneficial effects on ischemic heart partly by its antioxidant property. Indeed, resveratrol reduced myocardial MDA level and increased superoxide dismutase (SOD) and Peroxidase (POD) activities [28].

There is no data yet available on effect of resveratrol on GSH in myocardial ischemia reperfusion injury. Treatment of rats with resveratrol significantly reduce cardiac injury (P<0.05) as compared with active control. The scores of the control group demonstrates a 28.5% with highly severe myocardial injury and 71.5% with sever myocardial injury, while the score of resveratol treated group were 14.25% of the group had no damage, 57.25% had mild cardiac injury and 28.5% had moderate cardiac injury. Proved that resveratrol reduces infarct size and improves ventricular function after myocardial ischemia in rats [29]. Proved that resveratrol reducing myocardial infarct size and cardiomyocytes apoptosis [30].

Conclusion
It can be concluded that pretreatment with resveratrol modulates myocardial ischemia reperfusion injury via interfering with inflammatory, oxidative pathways and apoptosis.

Abbreviations
LAD (Left Anterior Descending artery), I.P (intraperitoneal), DMSO(dimethyl sulphoxide), MDA (malondialdehyde), GSH (reduced glutathione), TNF-α (Tumor Necrosis Factor alpha), IL-6 (Interleukin 6), IL-10 (Interleukin 10), caspase-3 (cysteine aspartic acid-protease 3), BAX (bcl2 associated X protein), cTn-T (cardiac troponin T), MI/R (myocardial ischemia reperfusion).

References

http://pharmacologyonline.silae.it
ISSN: 1827-8620


Figure 1. The mean of myocardial TNF-α (pg/mg) in the four experimental groups at the end of the experiment. *P<0.05 vs. sham; # P<0.05 vs. Ctrl group.

Figure 2. The mean of myocardial level IL-6 (pg/mg) in the four experimental groups at the end of the experiment. *P<0.05 vs. sham group; #P<0.05 vs. Ctrl group.

Figure 3. The mean of myocardial IL-10 (pg/mg) in the four experimental groups at the end of the experiment. *P<0.05 vs. sham; # P<0.05 vs. Ctrl group.

Figure 4. The myocardial mean of BAX (pg/mg) in the four experimental groups at the end of the experiment. *P<0.05 vs. sham group; # P<0.05 vs. Ctrl group.

Figure 5. The myocardial mean of Caspase-3 (pg/mg) in the four experimental groups at the end of the experiment. *P<0.05 vs. sham group, # P<0.05 vs. Ctrl group.

Figure 6. The mean of plasma cTn-T level (pg/ml) in the four experimental groups at the end of the experiment. P<0.05 vs. sham group, # P<0.05 vs. Ctrl group.
Figure 7. The myocardial mean of MDA (μmol/L) in the four experimental groups at the end of the experiment. *P<0.05 vs. sham group, # P<0.05 vs. Ctrl group.

Figure 8. The myocardial mean of GSH (μmol/L) in the four experimental groups at the end of the experiment. *P<0.05 vs. sham group, # P<0.05 vs. Ctrl group.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham</td>
<td></td>
</tr>
<tr>
<td>2. Control</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>3. Resveratol</td>
<td>&lt;0.05**</td>
</tr>
</tbody>
</table>

Table 1. Comparison according to Mann-Whitney test for scoring regarding histopathological changes.
**Figure 9.** Representative photomicrograph of a section of the heart tissue section stained with Haematoxylin and Eosin (X 40). A) The control group showing hemorrhage, interstitial edema, necrosis and neutrophil infiltration. B) The sham group showing normal architecture. C) The vehicle group showing severe hemorrhage and extravasation of RBC, presence of severe interstitial edema, presence of neutrophil infiltration and necrosis. D) The Resveratol pretreated group showing mild cardiac injury with absence of necrosis and few interstitial edema and PMN infiltration.