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The Cardioprotective Potential of Sildenafil in Myocardial Ischemia Reperfusion Injury.

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ABSTRACT

The aim of this study is to assess the possible ameliorating effect of sildenafil on myocardial ischemia reperfusion injury induced by LAD ligation in male rats through its anti-oxidant, anti-inflammatory and anti-apoptotic activities. 28 male rats were randomized into 4 groups (7 rats per group); Sham , rats underwent the same anesthetic and surgical procedure except for LAD ligation; MI/R , rats underwent LAD ligation for 30 minutes and reperfusion for 2 hours; MI/R+ vehicle , rats treated with I.P 0.9% normal saline, the sildenafil solvent 30 minutes before the ligation; MI/R+sildenafil group, rats pretreated with sildenafil 0.7mg/kg I.P 30 minutes before LAD ligation. Blood samples used for measurement of plasma troponin T additionally to serum MDA and GSH. Heart was divided into two parts, the apex for histopathology and the remaining used for tissue TNF-α, IL-6, IL-10, caspase-3 and BAX measurement. In control group, as compared with sham, tissue TNF-α, IL-6, IL-10, caspase-3 and BAX, plasma cTnT 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. Sildenafil significantly counteracted ($P<0.05$) the increase of TNF-α, IL-6, caspase-3 and BAX and counteracted the increase in plasma cTnT and serum MDA. Sildenafil produces a significant elevation ($P<0.05$) in cardiac IL-10 and serum GSH with significant reduction in ($P<0.05$) cardiac injury. In addition to its activity as a PDE5 enzyme inhibitor, and according to our results, sildenafil have the ability to attenuates myocardial I/R injury in male rats through interfering with inflammatory reactions and apoptosis .

Keywords: Myocardial ischemia, reperfusion, Sildenafil, Cytokines, Apoptosis.

Abbreviations: LAD (Left Anterior Descending artery), I.P (intraperitoneal), 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), MI/R (myocardial ischemia reperfusion).

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INTRODUCTION

Normally, the level of free radicals in cardiac tissue is under control, because of the presence of a balance between the antioxidant defense system and the generation of reactive oxygen species. And any interruption to this balance, by reduction in the endogenous antioxidant defense or increase production of free radicals or both, this will produce a deleterious effect on the antioxidant status and increases oxidative stress in cardiac tissue and cause activation of the inflammatory cascade with its consequence endothelial dysfunction and changes on cardiac function and structure [1-3].

Ischemic heart diseases are the major cause of human death with big cost on individual patients and governmental system regarding health care. Briefly, ischemia is the state at which there is a lack or decrease of blood supply to an organ or tissue, leads to a reduction of oxygen supply. If there was no restoration for blood and oxygen, bad prognosis such as necrosis and death may occur to ischemic tissues. Restoration of blood supply, or reperfusion, is the only treatment for ischemia. As its well-known that ischemia is a critical situation, but more injurious damages to the tissues suffering from ischemia occur on reperfusion state, known as reperfusion injury. Many events leads to ischemia reperfusion injury (e.g. organ transplantation, myocardial infarction, stroke, hemorrhage, trauma and shock). These events will results in a series of related injurious interactions as a consequence for IR which includes oxidative stress, endothelial dysfunction, and stimulation of inflammatory cascade by activation of immune system in it's both branches, innate and adaptive via activation of its toll like receptors and the C5a compartment of complement system [4]. The early reperfusion of the ischemic tissue is the cornerstone to minimize IR injury as there is no specific therapeutic agents available for the treatment of this situation. Numerous interventions may help to ameliorates IR injury, starting with primary percutaneous coronary intervention(PPCI), ischemic preconditioning, postconditioning, and remote preconditioning [5]. Example of drugs that represent the pharmacological branch for ameliorating IR are allopurinol as antioxidants, Cyclosporine A as inhibitors of mitochondrial permeability transition pore (mPTP) opening [6].

PDE-5 enzyme is presents in many tissues including smooth muscles of the corpora cavernosa, platelets, coronary and pulmonary arteries, veins , skeletal muscles, and smooth muscles of bronchi and viscera. Smooth muscle cell relaxation and vasodilatation produced by sildenafil is achieved by the chemical structure of the sildenafil which was derived from cGMP, so it acts as a substrate for PDE5 enzyme instead of cGMP (i.e. act as competitive inhibitor for PDE5 enzyme) [7] .

NO availability is crucial for starting NO/cGMP/PKGI protective pathway ,but the potent negative feedback mechanism is responsible for the termination of its action. This termination arise from the fact that cGMP binds first to the allosteric domain of the PDE5 enzyme which will produce a conformational changes at the catalytic side for the PDE5 thus increasing its activity and affinity towards the hydrolysis of the cGMP, this is accompanied with the concomitant phosphorylation of the PKGI (that was activated by cGMP) to a single serine located in the regulatory domain of the PDE5 enzyme and increasing the affinity of PDE5 enzyme towards cGMP hydrolysis by the catalytic domain (8). As its mentioned at the beginning , that cardiomyocyte death which resulted from ischemia reperfusion injury from acute (MI) have been attributed to a complex events linked with each other, started by the reduction of normal oxygen level required for energy production and reaching to the step at which the cell will die or survive and to which extent it will affect cardiac remodeling after survival. Involvement of ROS, complement, chemokines, pro and anti-inflammatory cytokines, caspases, cells of the immune system particularly neutrophils in the pathogenesis of IRI was documented. Additionally, endothelial dysfunction as a result of ROS and neutrophil infiltration, or due to the presence of comorbidities as DM ,atherosclerosis or other underlying causes are strongly contributed to bad prognosis regarding myocardial IRI (9,10). Until now, there is no research has measured the effect of sildenafil on the levels of pro-inflammatory cytokines (TNF-alpha, IL-6) or the anti-inflammatory cytokine (IL-10) and the pro-apoptotic proteins (BAX, Caspase 3) neither the oxidative stress marker (MDA) and cTnT, the cardiac cell injury marker , nor the level of endogenous anti-oxidant GSH in myocardial ischemia reperfusion injury. So, the aim of the study is to illustrate possible ameliorating effect of sildenafil against reperfusion injury followed myocardial ischemia in male rats through its pleotropic activity as anti-oxidant, anti-apoptotic and anti-inflammatory drug that will cause activation of salvage kinase pathway and produce smooth muscle cell relaxation and vasodilatation.

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METHODS

Material

Pure Sildenafil powder (Sigma, USA),normal saline (KSA) ketamine (Hikma, Jordan), Xylazine (RompunTM, 2% vials, Bayer AG, Leverkusen, Germany). Rat tumor necrosis factor-α (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) in KUFA university and submission the required applications, 28 male albino rats weighting (180-220 g) were purchased from Animal Resource Center, National Center for Drug Control and Research. They were housed in the animal house (for one week) in a temperature-controlled (25°±1C) 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 [11] :

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 normal saline 0.9% 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) + Sildenafil citrate pretreated group: rats of this group take a single I.P injection of sildenafil in a concentration of 0.7 mg/kg dissolved in 0.9% normal saline 30 minutes immediately before ligation of LAD, then subjected to surgical LAD ligation with 30 minutes of ischemia followed by 120 min of reperfusion [12]

Surgical ligation of the LAD artery

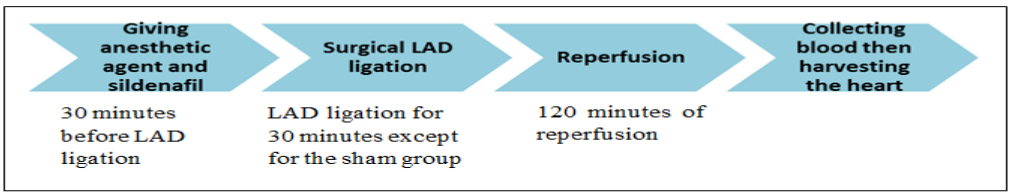

Figure 1 : Schematic diagram for surgical procedure

Anesthesia was done to all rats were through intraperitoneal (IP) injection of ketamine in a dose of (100 mg/kg) and xylazine with a dose of 5 mg/kg (13) . After intubation of the trachea by a 20 fg 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. The intercostal muscle layer was gradually cut with micro fine scissors After that the pericardium was opened the left ventricle was visible , the LAD was ligated with an 8-0 Prolene suture. The chest wall was closed and at the end of reperfusion time, the animal was re-anesthetized by (IP) mixture of 100 mg/kg ketamine and 5 mg/kg xylazine

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, illustration for this procedure summarized in figure 1 .

Collection of Samples

At the end of experiment, about 2-3 ml of blood was collected by disposable syringe from the heart of each rat via cardiac puncture. Heart was excised and divided into two parts, the apex for tissue homogenization, and the other part conserved for histopathology.

Measurement of plasma cTnT, serum MDA and serum reduced GSH

About 1-1.5 ml of the collected blood was placed immediately in EDTA tube containing disodium EDTA (22 mg/ ml) as anticoagulant and after mixing the tube thoroughly, it was centrifuged at 3000 rpm for 15 min then the supernatant was used for determination of plasma cTnT level, while the remaining blood was allowed to clot in an ordinary tube at 37 ^{o}C then it was centrifuged at 3000 rpm for 15 minutes then the supernatant was taken for MDA and GSH serum levels determination .

Preparation for TNF-α, IL-6, IL-10, caspase 3 and BAX measurements

The heart of each rat which was excised at the end of the reperfusion time, any remaining parts of the atria were removed and was washed to exclude clots, then homogenization for the apex part of cardiac tissue was done with a high intensity ultrasonic liquid processor in 1:10 (w/v) phosphate buffered saline that contained 1% Triton X-100 and a protease inhibitor cocktail (14). Then centrifugation at 14000 rpm for 20 min at 4°C was done for tissue homogenate . After this step, the collection of the tissue homogenate supernatant was done in order to detect the levels of TNF-α, IL-6, IL-10, caspase 3 and BAX by the commercially available ELISA kit (literature of kits by Biotangusa, USA.) according to the manufacturer's instructions .

Preparation for Histopathology

The myocardial tissue that reserved for histopathological study was fixed in 10% formalin and embedded in a block of paraffin. The 5µm sections which were cut from each block were stained by hematoxylin and eosin (H&E) after fixation. Damage scores were evaluated according to the following morphological criteria that have been used to evaluate the histopathological damage (15) as follow:

1. score 0, no damage.
2. score 1 (mild), interstitial edema with necrosis.
3. score 2 (moderate), swelling of myocardial cell and necrosis.
4. score 3 (severe), necrosis, neutrophil infiltration and the capillaries were compressed.
5. score 4 (highly severe), necrosis (in a widespread manner), neutrophil infiltration, capillaries compressing 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), LSD for post-hoc test. 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 +

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sildenafil pretreated group, sildenafil produce a significant decrease (P<0.05) in the (TNF-α and IL-6) cardiac tissue levels as compared with the MI/R group and MI/R +vehicle group as shown in table 1 and figures2 and 3.

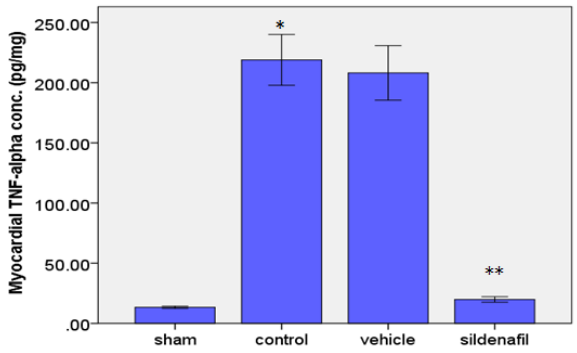


Figure 2: Mean concentration of TNF-α in (pg/mg)

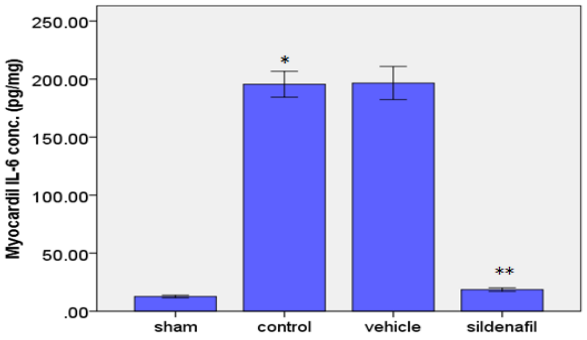


Figure 3: Mean concentration of IL-6 in (pg/mg) .

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 +sildenafil pretreated group, sildenafil 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 4.

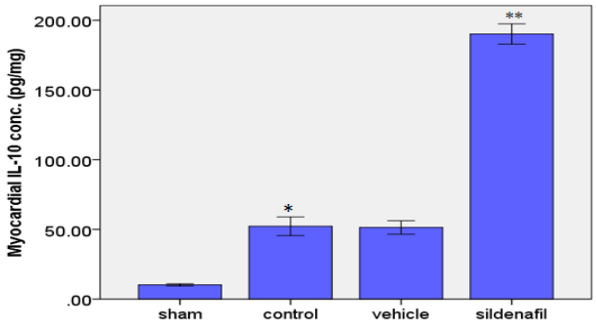
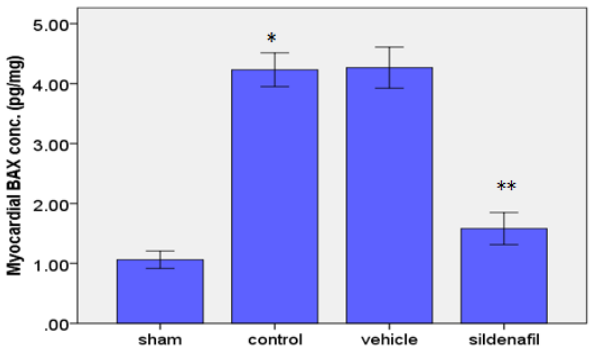


Figure 4: Mean concentration of IL-10 in (pg/mg)

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 +sildenafil pretreated group, sildenafil produce a significant reduction (P<0.05) in the (caspase-3 and BAX) cardiac tissue levels as compared with the MI/R group and MI/R +vehicle group as shown in table 1 and figures 5 and 6.



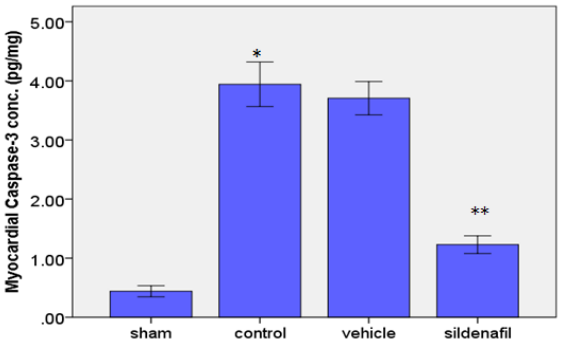


Figure 5: Mean concentration of BAX in (pg/mg) **Figure 6: Mean concentration of Caspase-3 in (pg/mg)**

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 +sildenafil pretreated group,

sildenafil produce a significant reduction (*P*<0.05)in the (cTnT) plasma level as compared with the MI/R group and MI/R +vehicle group as shown in table 1 and figure 7.

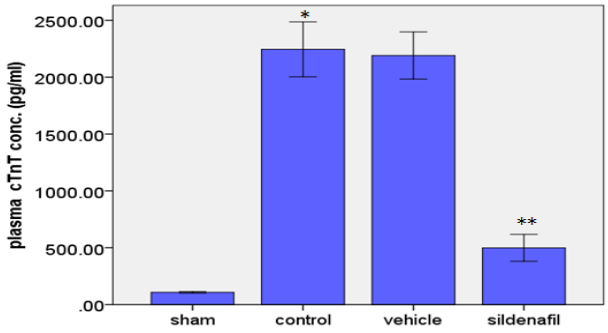


Figure 7 : Mean plasma concentration of cTnT in (pg/ml)

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 +sildenafil pretreated group, sildenafil produce a significant reduction (*P*<0.05) in MDA serum level as compared with the MI/R group and MI/R +vehicle group. Regarding 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 +sildenafil pretreated group, sildenafil produce a significant increase (*P*<0.05) in GSH serum level as compared with the MI/R group and MI/R +vehicle group as shown in table 1 and figures 8 and 9.

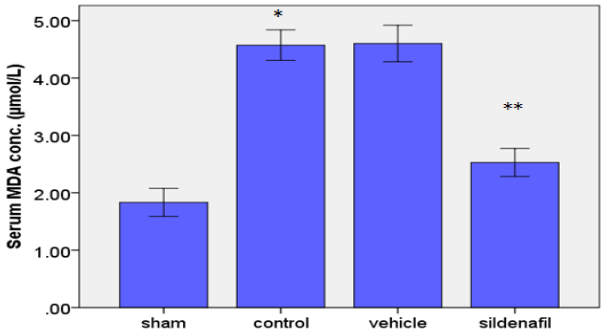


Figure 8: Mean serum concentration of MDA in (µmol/L).

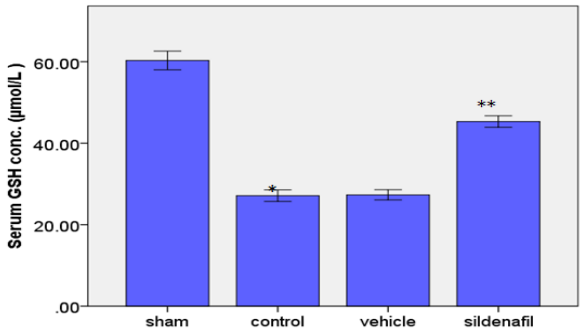


Figure 9: Mean serum concentration of GSH in (µmol/L) .

Table 1: study parameter levels among the four experimental groups

Markers	Sham	MI/R	MI/R +Vehicle	MI/R+Sildenafil
TNF pg/mg	13.3700 ±0.4522	218.9629 ±10.5656*	208.1571 ±11.3442	19.8829 ± 1.1490**
IL-6 pg/mg	12.6557 ±0.5714	195.5343± 5.5652*	196.5857 ± 7.1603	18.6114 ±0.7578**
IL-10 pg/mg	10.2271 ±0.4176	52.2486 ± 3.3470*	51.3914 ± 2.4247	190.1571 ±3.6177**
BAX pg/mg	1.0614± 0.0726	4 .2314± 0.1408*	4 .2657± 0.1715	1.5814± 0.1341**
Caspase 3 pg/mg	0.4400± 0.04695	3.9429± 0.1887*	3.7071± 0.1410	1.2286± 0.0744**
CTnT pg/ml	107.2671 ±3.4133	2245.00 ± 120.6646*	2190.7143±103.6809	499.4286±59.0414**
MDA (µmol/L)	1.8329 ±0.1219	4.5729 ± 0.1332*	4.6014±0.1590	2.5286±0.1219**
GSH (µmol/L)	60.3143 ±1.1509	27.1200 ± 0.7112*	27.3186±0.6281	45.3200±0.7077**

* (P< 0.05) with sham group, ** (P< 0.05) with its respective controls (**MI/R, MI/R +Vehicle**)

Histopathlogical Findings

Examination of a cross section from 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 and extravasation of RBC, presence of sever interstitial edema, presence of neutrophil infiltration and necrosis on the contrast of the cross section of the sham group which showed a 100% normal structure regarding cardiac tissue.

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Treatment of rats with sildenafil significantly decrease (P<0.05) the injury of cardiac tissue and cross section from this group (MI/R+ sildenafil) showed near normal cardiac tissue with absence of edema, absence of neutrophil infiltration , absence of necrosis, and only congested capillary structure while there was no significant difference between the MI/R and MI/R +vehicle groups as shown in figures 10 and 11.

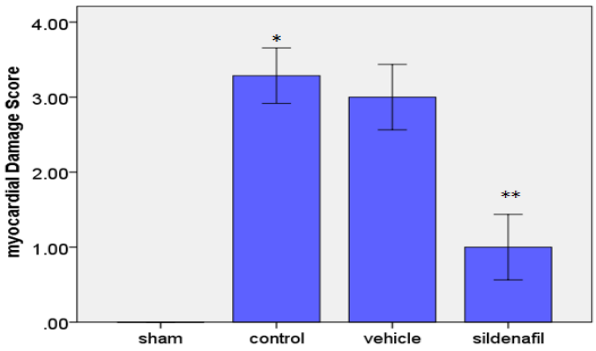

Figure 10: Mean myocardial damage score

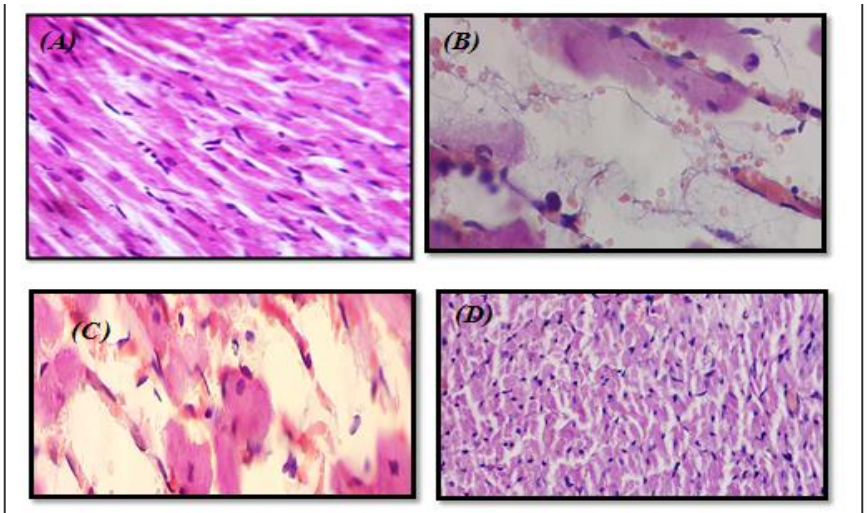

Figure (11) : Histopathlogical findings in the four experimental groups stained with Haematoxylin and Eosin (X40). A) The sham group showing normal architecture . B) The MI/R group showing sever hemorrhage, extravasation of RBC, presence of sever interstitial edema, presence of neutrophil infiltration and necrosis. C) The MI/R + vehicle group showing sever hemorrhage, extravasation of RBC, presence of sever interstitial edema, presence of neutrophil infiltration and necrosis. D) The MI/R + Sildenafil group showing near normal cardiac tissue with absence of edema, absence of neutrophil infiltration, absence of necrosis, and only congested capillary structure.

DISCUSSION

The core of the protective strategies to reduce myocardial RI is to minimize the size of the infarcted area and preserve the integrity of the cardiac tissue and coronary vasculature as possible. This can be achieved through the interference and decreasing the detrimental effect of the mediators that involved in IRI which includes ROS, pronounced inflammatory responses, endothelial dysfunction and activation of apoptosis which are associated with reperfusion to the ischemic myocardium and resulted in a deleterious effects on Cardiomyocyte [16].

Liu *et al* (2014) showed that improvement of heart function and inhibition of ventricular remodeling post myocardial infarction in a rat model was associated with the reduction of TNF-α and IL-6 level [17]. Cai *et al* (2012) proved that up regulation of IL-10 during remote ischemia preconditioning protects

cardiomyocytes against myocardial ischemia reperfusion injury in mice model [18]. Markowski *et al* (2013) confirmed that Pre-conditioning by the TLR-9 agonist CpG-oligonucleotides reduces myocardial IRI through up regulation of IL-10 [19]. Dhingra *et al* (2011) clarified that treatment of isolated rat cardiomyocyte with IL-10 improves its viability and inhibits apoptosis produced by TNF-α [20], our results are in consistence with these studies concerning the effect of sildenafil on cardiac tissue levels of IL-10, TNF-α and IL-6 .

Regarding the level of caspase 3 and BAX in cardiac tissue, Das *et al* (2009) showed that the protective effect of sildenafil against myocardial ischemia reperfusion injury in decreasing apoptosis and necrosis associated with reduction of BAX expression and the significant increase the bcl2/BAX ratio additionally to decreased activation of caspase-3 in cardiac tissue in an *in vivo* mice model [21], also (Das et al., 2005) proved that sildenafil anti-apoptotic effect in a simulating conditions of ischemia re-oxygenation appears through the reduction of BAX level and elevation of the bcl2/BAX ratio that depends on NO pathway in an *in vitro* mice cultured cells [22]. Choi *et al* (2009) clarified that pretreatment with sildenafil significantly reduce the level of Bax and caspase 3 accompanied by significant elevation of the anti-apoptotic bcl2 protein in the reperfused kidneys after induction of renal ischemia in a rat model [23].

The cTnT plasma level of sildenafil pretreated group was significantly decreased ($P<0.05$) compared to the MI\R group and the MI/R +vehicle group. Hassan et al (2005) demonstrated that treatment with sildenafil significantly reduce blood cTnT level in rat model of induced myocardial hypertrophy indicating sildenafil cardio-protective effect [24].

Pretreatment with sildenafil significantly decrease serum MDA level with a significant elevation of GSH serum level. In a study by Koupparis *et al*. (2005) clarified that potent antioxidant effect of sildenafil occur through its inhibition to the production of main free radical, the superoxide anion, by its inhibitory effect on the expression and action of NADPH oxidase enzyme and by increasing the level of cGMP [25]. Beheshtian *et al*. (2008) clarified that the anti-oxidant effect of sildenafil in a rat model of testicular reperfusion injury after ischemic period induced by torsion/de-torsion, is through its ability to reduce the MDA level and increases the activity of anti-oxidant mitochondrial enzymes like SOD, CAT and glutathione reductase (GSH-Px) the enzyme that reduces the oxidized form of glutathione (GSSG) to the active reduced GSH [26]. Sildenafil increase the bioavailability of nitric oxide (NO) and decrease the formation of peroxynitrite ($ONOO^-$) as a result of its ability to suppress the formation of superoxide anion from NADPH oxidase and increase the gene expression of iNOS and eNOS mediated by NO/cGMP/PKGIα pathway [27] Sildenafil also have a cardio-protective effect through activation of ERK1/2 salvage kinases mediated by cGMP dependent PKGI, this ERK1/2 pathway produces its cardio-protective effect through up-regulation of inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) [28].

Treatment of rats with sildenafil significantly reduce cardiac injury. The scores of the control group were 28.5% with highly severe myocardial injury and 71.5% with sever myocardial injury, while the score of sildenafil treated group were14.25% with no damage, 71.5% with mild cardiac tissue injury and 14.25 with a moderate cardiac tissue injury. Milano *et al* (2011) proved that pretreatment with sildenafil significantly reduce the infarct size and apoptosis after the reperfusion of ischemic heart in a rat model [29]. Fisher *et al* (2005) clarified that sildenafil reduces the apoptosis and left ventricular dysfunction in a mice model of chronic cardiotoxicity with doxorubicin [30]. The cardio-protective effect of sildenafil demonstrated through its ability to reduced apoptosis and necrosis in cardiac tissue by a NO/cGMP/PKGI pathway in adult cardiomyocytes in a nitric oxide dependent manner [22]. From the above findings and previous related studies there are a reliable evidence for the effectiveness of sildenafil as anti-oxidant, anti-inflammatory and anti-apoptotic drug that could reduce the injury of myocardial ischemia reperfusion in a rat model.

Study limitations

Western blot analysis and infarct size measurement was not done for further result documentation.

CONCLUSION

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

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