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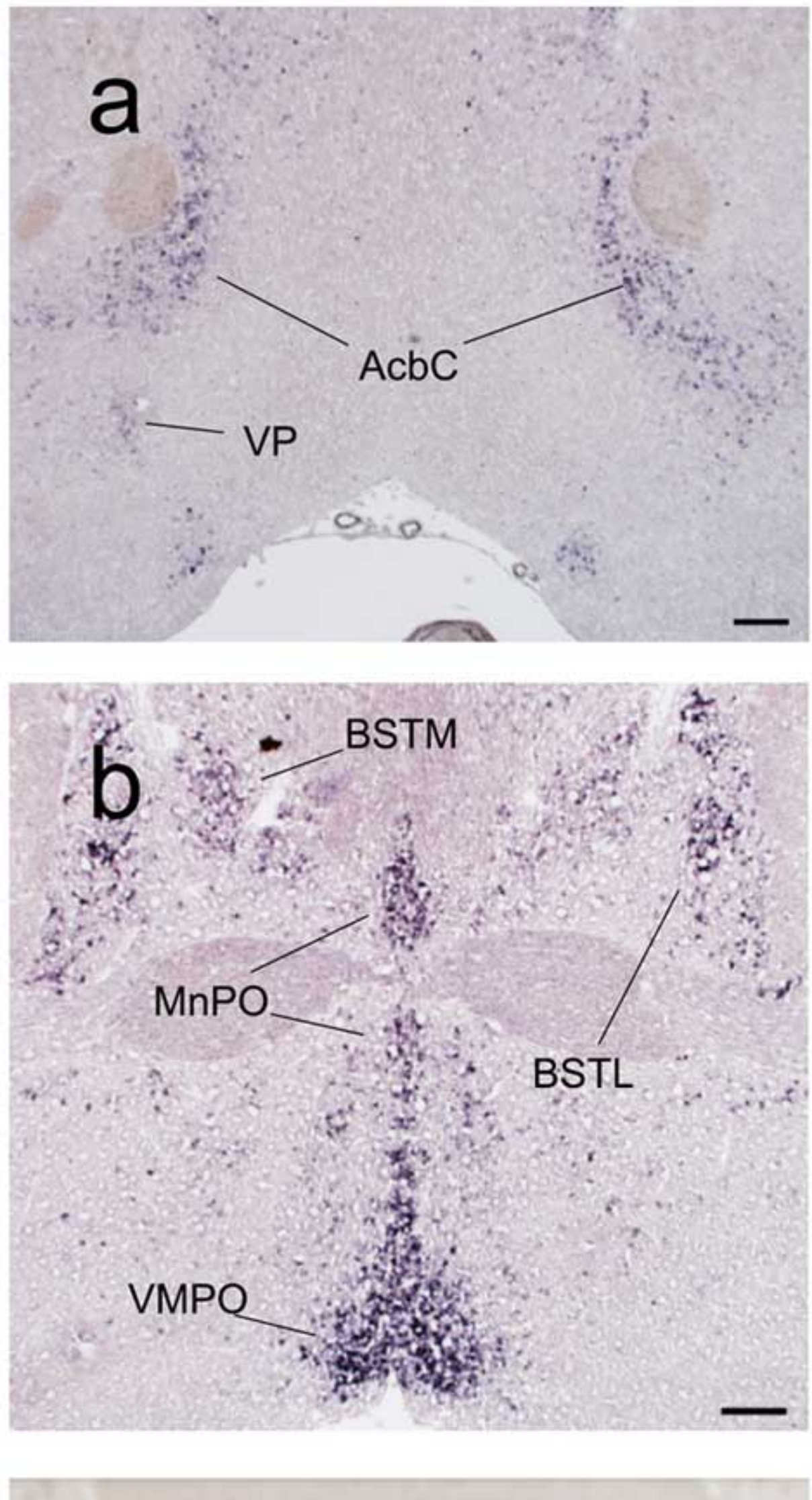

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ORIGINAL ARTICLE

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Liver ischemia/reperfusion injury, a setting in which the functional mass is reduced and the role of PDE5 inhibitor

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SUMARY

Liver ischemia reperfusion is induced during surgical procedures like liver transplantation and resection. Multiple mechanisms have been postulated to liver damage following liver ischemia reperfusion injury, such as oxidative stress and inflammatory reactions. The present study declares the possible mechanism of tadalafil, toward modulating the inflammatory response. Forty-eight rats were divided into 4 groups as follows; Sham group subjected to midline laparotomy only. Tadalafil group administered Tadalafil 10 mg/kg intraperitoneal 45 min before sham operation. I/R (Ischemia-reperfusion) group, rats undergo 60 min of hepatic ischemia followed by 60 min of reperfusion. Tadalafil + I/R group rats undergo a similar pattern of I/R after the treatment with Tadalafil 10 mg/kg, 45 min before ischemia. At the end of the reperfusion, the blood samples were collected for estimation of biochemical markers including liver enzymes using colorimetric assay method and serum: TNF-α (tumor necrosis factor-α), IL-6 (interleukin 6) levels, ICAM- 1 (Intercellular Adhesion Molecule-1) were measured. Tissues were evaluated by semi-quantitative and morphometrical approaches. Tadalafil succeeded in restoring normal levels of liver enzymes and ameliorating the oxidative stress as evidenced by decreasing MDA and restoring reduced glutathione levels in liver tissue homogenate. Also, Tadalafil exhibits anti-inflammatory effects, as it significantly decreased the levels of TNF-α, IL6 and ICAM-1. The findings are supported by BCL-2, TNF-α immunomarkers. It is concluded that modulation of the inflammatory response might be one of the mechanisms of Tadalafil-mediated hepatoprotection, so it is recommended as an adjuvant therapy in liver surgery.

Key words: Ischemia/reperfusion injury – Oxidative stress – Apoptosis – TNF-α – BCL-2

INTRODUCTION

Hepatic I/R (ischemia/reperfusion) injury is a common complication following surgical procedures such as liver resection and transplantation that involves a variable period of ischemia, and may result in complicated medical conditions such as shock, trauma, or low cardiac output (Ye et al., 2015). In addition, it may occur during liver surgery when Pringle's maneuver (ligation of the hepatoduodenal ligament) to reduce blood loss is done

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(Freitas et al., 2017).

Ischemia initiates a series of events that cause necrosis and cellular dysfunction; the reperfusion of blood flow can paradoxically generate more tissue injury. Excessive inflammatory reaction is considered as a key mechanism. A complex cascade of inflammatory mediators is activated by liver ischemia and reperfusion (Zhai et al., 2011). I/R injury is mediated by the activation of proinflammatory cytokines such as TNF-α (tumor necrosis factor alpha), release of free radicals and the accumulation of inflammatory cells, accumulation of intracellular sodium and calcium, and induction of hepatocyte apoptosis (Rao et al., 2013).

During hepatic I/R injury, many microvascular and endothelial alternations occur as initiation of coagulation cascade, variations in the molecular vasoactive products such as endothelin (ET) and NO (nitric oxide), and upregulation of endothelial adhesion molecules such as ICAM-1 (intracellular adhesion molecule-1). These alternations are suggested targets for therapeutic strategies (Camara-Lemarroy et al., 2014). Safe clearance of damaged cells is the apoptosis (programmed cell death). BCL-2 family are proteins that are considered as a non-death signal (antiapoptosis) (Hegazy et al., 2018).

Phosphodiesterase (PDE) inhibitors are the compounds that inhibit the biosynthesis or actions of PDEs. PDE inhibitors are currently widely used in treatment of erectile dysfunction, as well as in pulmonary arterial hypertension (Sawamura et al., 2009). PDE inhibitors have protective effects on myocardial muscles and vascular structures (Korkmaz-Icoz et al., 2018). The effects of PDE inhibitors have been widely studied in I/R in different organs. Moreover, PDE is the family of enzymes that adjust the cellular levels of second messengers, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (Reffelmann and Kloner, 2009).

Tadalafil (TDF) is a selective and effective inhibitor of PDE5 (phosphodiesterase type-5) that has been broadly used in the treatment of erectile dysfunction due to its capability to prevent the break of cGMP, which is the second messenger of NO (nitric oxide) (Kucuk et al., 2012). The purpose of this study was to declare the beneficial effect of tadalafil on hepatic I/R-injury.

MATERIALS AND METHODS

Drugs

Tadalafil was purchased from Lilly Co. and given via intraperitoneal injection with dose of 10 mg/kg (Bektas et al., 2016).

Animals

Mature male Wistar albino rats, their weighs ranging from 200 to 250g. They were obtained from the Animal House. They were preserved in cages with optimum temperature, 60% humidity under 12h dark and light cycles. Rats were provided standard pellet diet and water for one week before the experiment for adaptation.

Ethics Statement

This study was approved by the biomedical Ethics Research Committee [Reference No 229-19].

Experimental Design

Forty-eight rats were separated into four groups (12 each) in this way; Sham group underwent midline laparotomy only. Tadalafil group administered Tadalafil 10 mg/kg intraperitoneal 45 min before sham operation (Bektas et al., 2016). Ischemia-reperfusion (I/R) group, rats exposed to 60 min of hepatic ischemia, then they were exposed to 60 min of reperfusion (Liu et al., 2016). Tadalafil+ I/R group rats were subjected to a similar pattern of I/R after the administration of Tadalafil 10 mg/kg, 45 minutes before ischemia (Bektas et al., 2016).

Surgical Procedure

All surgical procedures were performed under complete aseptic conditions, and the anesthesia, with combination of ketamine 100 mg/kg and xylazine 10 mg/kg, administered through intraperitoneal route (Savvanis et al., 2014). Rats were exposed to midline abdominal incision, liver lobes and the portal triad identified, and rats subjected to 60 minutes of hepatic ischemia by clamping the portal triad with a micro-vascular clamp after the bifurcate of the right lobe, interrupting the portal triad flow to the left and median lobes, inducing ischemia for 60 min, and after that the clamp removed to allow 60 min of reperfusion (Hueper et al., 2018). At the end of the reperfusion period, the blood samples were collected from the abdominal aorta and biopsies were taken from the ischemic hepatic lobes.

Collection for blood samples

Blood was centrifuged (700×g, 4°C, 20 min) for assessment of liver enzymes.

Liver tissue Extracts

Liver was homogenized in phosphate buffer saline (PBS) [10%]. The first part was centrifuged at 15000 g for 10 min, and the supernatant was gathered and kept at -80C° to measure oxidative stress markers and inflammatory cytokines in liver tissue homogenates. The second part was subjected to repeated freeze-thaw cycle twice in order to break the cell membranes, then centrifuged at 5000×g for 5 min and kept at -80C° for the measurement of the other parameters.

Hepatic biochemical parameters in serum

Serum ALT and AST were determined by using colorimetric assay kits provided by Elabscience, Houston, Texas, USA, (Catalogues E-BC-K235, E-

Table 1: Effect of Tadalafil on serum liver functions.

Groups	Sham N=12	Tadalafil N=12	I/R N=12	Tadalafil +I/R N=12
ALT (U/L)	293.00±71.39	304.33±57.90 [1]P= 0.686 [2]P= 0.000	491.75±85.35 [1]P= 0.000	299.75±.53.26 [1]P= 0.809 [2]P= 0.000
AST (U/L)	260.50±58.77	268.83±50.59 [1]P= 0.863 [2]P= 0.000	791.25±126.37 [1]P= 0.000	378.25±181.79 [1]P= 0.018 [2]P= 0.000

Values are Mean± SD: standard deviation. Comparison between groups done by ANOVA followed by LSD post hoc test. [1]P: compared to Sham group. [2]P: compared to I/R (group subjected to ischemia reperfusion injury).
ALT (Alanine transaminase), AST (Aspartate transaminase). U/L unit/ liter.

BC-K236 respectively) in accordance with the manufacturer's instructions.

Oxidative stress markers activities in liver tissue homogenates

Estimation of MDA (malondialdehyde) and GSH (reduced glutathione) levels utilizing colorimetric assay kits (Catalogues No.MD 25 29, GR 25 11 respectively) according to the manufacturer's instructions (Bio Diagnostic, Cairo, Egypt).

Assessment of hepatic cytokines in liver tissue homogenates

TNF-α (Tumor necrosis factor-α) and IL-6 (interleukin 6) levels were measured using ELISA (enzyme-linked immunosorbent assay) kit; (TNF-α: catalogue number RAB0479 Sigma), RayBio Rat IL-6 ELISA Kit: (IL6: catalogue number ELR-IL6-001) in accordance with the manufacturer's instructions. Measurement of ICAM-1 (Intercellular Adhesion Molecule-1): quantitative determination of ICAM-1 was determined by ELISA using Kit provided by RayBio Rat ICAM-1 (ICAM-1: catalogue number ELR-ICAM1) according to the manufacturer's instructions.

Histopathological analysis

Paraffin sections of 5μm thickness prepared, for each specimen, at least 3-5 slides stained with Hematoxylin and Eosin (H&E) to examine hepatic histoarchitecture, periodic acid-Schiff (PAS) to demonstrate the glycogen deposition in hepatocytes, and Masson's trichrome (MT) for distinguishing collagen. The examination using Olympus BX53 microscope equipped with an Olympus DP73 camera (Olympus, Tokyo, Japan) (Mustafa, 2016).

Immunohistochemical study

Using the streptavidin-biotin-peroxidase technique, the endogenous peroxidase activity was eliminated using 10% H_2O_2 for 15min. Sections were incubated for 1h with primary antibody against BCL-2 associated X (BAX) protein, a monoclonal antibody (Dako, Carpinteria, CA, USA; dilution: 1:200; cytoplasmic), as a marker for apoptotic death. They were similarly incubated with the primary antibody against tumor necrosis factor alpha (TNF-α), a mouse monoclonal antibody (Dako; 5-10 μg/ml; cytoplasmic), as a marker for inflammatory cytokines. Sections were incubated for 20min in DAB (3, 30-diaminobenzidine) chromogen and counterstained with Mayer's hematoxylin. Negative control sections were prepared by omitting the primary antibody. Absence of staining was recognized as a negative result (-), while the presence of brown staining was recognized as a positive result (+) (Hegazy et al., 2018).

Semi-quantitative assessment of the severity of the liver damage using the following parameters: congestion, hepatocyte vacuolization, sinusoidal dilatation and congestion, central vein dilatation, and loss of the glycogen deposition in hepatocytes. Microscopic damage was scored as no change (-), minimal (+), moderate (++), and severe (+++), for each parameter, and was assessed in a blinded manner (Sahin et al., 2013; Tas Hekimoglu et al., 2013).

Morphometric analysis

About 30 sections were analyzed at magnifications ×200 and ×400 with the use of Image-Pro Plus v6.0 (Media Cybernetics, Maryland, USA) for area percentage of collagen and BCL-2 and the optical density (OD) of TNF-α immunopositive cells (Hegazy et al., 2018).

Statistical analysis

Quantitative data were expressed as mean and standard deviation of different parameters between treated groups. Data analyzed using One Way Analysis of Variance (ANOVA) followed by Tukey's posthoc test. All statistical analysis was implemented using SPSS version 24. The values considered significant when $P<0.05$ (Mustafa et al., 2017).

RESULTS

Effect of Tadalafil on survival rate and body weight of different groups

Neither deaths nor significant changes in body weight had been documented in each group.

Effect of Tadalafil on serum liver functions

Tadalafil succeeded in significant decrease in serum levels of ALT in Tadalafil +I/R) when compared with IR group. ALT is considered a vital diagnostic marker for liver function and Tadalafil reduced its level by near the normal level of Sham group (Table 1). Moreover, Tadalafil induced significant decrease in serum level of AST in (Tadalafil +I/R) when compared with IR group (Table 1).

Table 2: Effect of Tadalafil on some inflammatory cytokines in liver tissue homogenate.

Groups	Sham N=12	Tadalafil N=12	I/R N=12	Tadalafil+I/R N=12
TNF-α Pg/gm liver tissue	1330.25±103.33	1341.08±80.20 [1]P= 0. 838 [2]P= 0.000	1943.25±2.38 [1]P= 0.000	1608.00±.86.76 [1]P= 0.000 [2]P= 0.000
IL–6 Pg/gm liver tissue	7.25±0.87	7.50±.0.15 [1]P= 0.645 [2]P= 0.000	28.75±2.38 [1]P= 0.000	11.50±0.52 [1]P= 0.000 [2]P= 0.000
ICAM-1 Pg/gm liver tissue	9.75±0.45	9.65±0.44 [1]P= 0.985 [2]P= 0.000	39.75±0.2.01 [1]P= 0.000	16.25±2.80 [1]P= 0.000 [2]P= 0.000

Values are Mean± SD: standard deviation. Comparison between groups done by ANOVA followed by LSD post hoc test. [1]P: compared to Sham group. [2]P: compared to I/R (group subjected to ischemia reperfusion injury). TNF-α (Tumor Necrosis Factor alpha), IL-6 (Interleuin-6), ICAM-1 (Intracellular adhesion molecule-1). Pg/gm liver tissue (Picogram/gram liver Tissue).

Table 3: Effect of Tadalafil on some oxidative stress markers in liver tissue homogenate.

Groups	Sham N=12	Tadalafil N=12	I/R N=12	Tadalafil +I/R N=12
Malondialdhyde (MDA) (Nano mole /mg liver tissue)	159.25±44.88	164.25±38.93 [1]P= 0.744 [2]P= 0.000	494.25±85.72 [1]P= 0.000	215.25±40.35 [1]P= 0.018 [2]P= 0.000
Reduced Glutathione (GSH) (Micromole/gm liver tissue)	19.50±1.3.80	19.92±3.18 [1]P= 0. 828 [2]P= 0.000	9.00±1.28 [1]P= 0.000	15.50 ±3.50 [1]P= 0.003 [2]P= 0.000

Values are Mean± SD: standard deviation. Comparison between groups done by ANOVA followed by LSD post hoc test. [1]P: compared to Sham group. 2P: compared to I/R (group subjected to ischemia reperfusion injury). TNF-α (Tumor Necrosis Factor alpha), IL-6 (Interleuin-6), ICAM-1 (Intracellular [a]dhesion molecule-1). Pg/gm liver tissue (Picogram/gram liver Tissue).

Table 4: Histopathological findings in the different study groups.

	Sham N=12	Tadalafil N=12	I/R N=12	Tadalafil + I/R N=12
Congestion	--	--	+	--
Hepatocyte vacuolization and necrosis	--	--	++	+
Sinusoidal dilatation and congestion	--	--	+++	++
Central vein dilatation	--	--	+++	++
loss of the glycogen deposition in hepatocytes	+++	+++	+	++

Effect of Tadalafil on TNF alpha, IL-6 and ICAM -1 levels in liver tissue homogenate TNF alpha, IL-6 and ICAM-1

Tadalafil achieved significant decrease in the level of TNF alpha, IL-6 and ICAM-1 in (Icariin +I/R) when compared with IR group (Table 2).

Effect of Tadalafil on some oxidative stress markers in liver tissue homogenate

Tadalafil ameliorated oxidative stress by significant reduction of lipid peroxidation, as indicated by decrease of MDA levels, and restoring reduced glutathione levels but still significantly different from sham group (Table 3).

Histological Assessment

Tadalafil + I/R group showed majority of the hepatocytes and blood sinusoids appear preserved (Fig. 1, Table 4). Also, it showed noticeable increment in glycogen content in hepatocytes (Fig. 2, Table 4). In addition, it showed decreased collagen fibers around the portal areas as well as in the perisinusoidal region (Fig. 3, Table 5).

Immunohistochemical Assessment

I/R showed negative BCL-2 expression, while Tadalafil + I/R showed positive BCL-2 expression and area percentage of BCL-2 reactions was significantly increased in Tadalafil + I/R group (Fig. 4, Table 5). I/R group showed strong positive immunoreaction for TNF-α in the hepatocytes cytoplasm and in the sinusoids wall. Tadalafil + I/R group showed weak immunoreaction for TNF- α in in some hepatocytes cytoplasm and in the sinusoids wall. Optical density (OD) of TNF-α supports the descriptive findings (Fig. 5, Table 5).

DISCUSSION

I/R is one of the clear components of liver injury

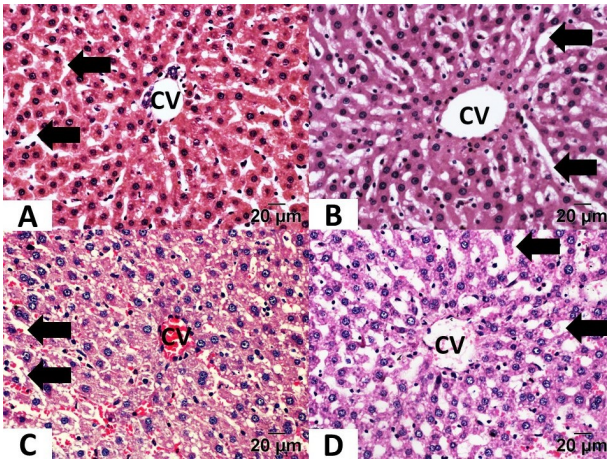

Fig 1. A: Sham group showed polyhedral hepatocytes radiating from the central vein (CV), with rounded vesicular nuclei and acidophilic cytoplasm separated by blood sinusoids (arrow). **B:** Tadalafil group showed architecture being nearly similar to sham group. **C:** I/R group showed majority of hepatocytes around the central vein (CV) appear necrotic while the remaining appeared vacuolated or with acidophilic cytoplasm and dark nuclei. Also, disorganized hepatic architecture around the central vein (CV). The central veins and the blood sinusoids (arrow) are dilated and congested. **D:** Tadalafil + I/R group showed most of the hepatocytes and blood sinusoids (arrow) appear preserved. Some hepatocytes appeared vacuolated or with acidophilic cytoplasm and dark nuclei. The central vein (CV) and some blood sinusoids are still dilated and congested (H&E, Scale bar: 20 µm).

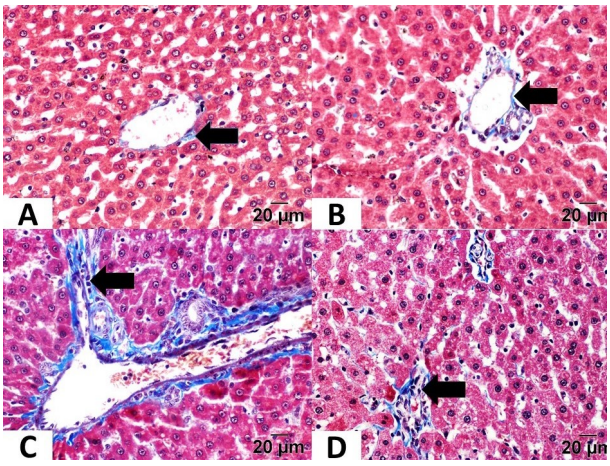

Fig 3. A: Sham group showed minimal collagen around the central vein (arrow). **B:** Tadalafil group showed minimal collagen fibers in the portal tract area (arrow). **C:** I/R group showed increased deposition of collagen fibers in the portal tract area (arrow) and in the perisinusoidal spaces. **D:** Tadalafil + I/R group showed decreased collagen deposition in the portal tract area (arrow) and in the perisinusoidal spaces (Masson trichrome, Scale bar: 20 µm).

that is evidenced in liver transplantation and liver resection. Other conditions in which I/R occur is sepsis, hepatic artery ligation, trauma and hemorrhagic shock (Liu et al., 2016). Liver I/R injury is elicited by more than one mechanism, mainly the oxidative stress that results in damage in various organs. Injury in liver I/R consists of 2 phases. The

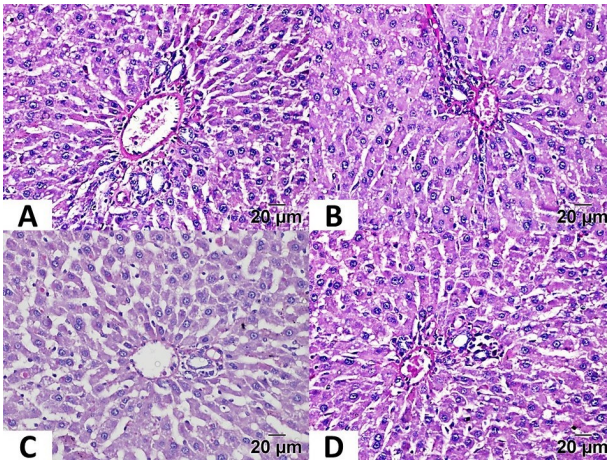

Fig 2. A: Sham group showed positive PAS reaction of magenta staining in which glycogen is present within hepatocytes. **B:** Tadalafil group showed nearly similar to sham group. **C:** I/R group showed decreased glycogen storage in hepatocytes. **D:** Tadalafil + I/R group showed marked increment in glycogen content in hepatocytes (PAS, Scale bar: 20 µm).

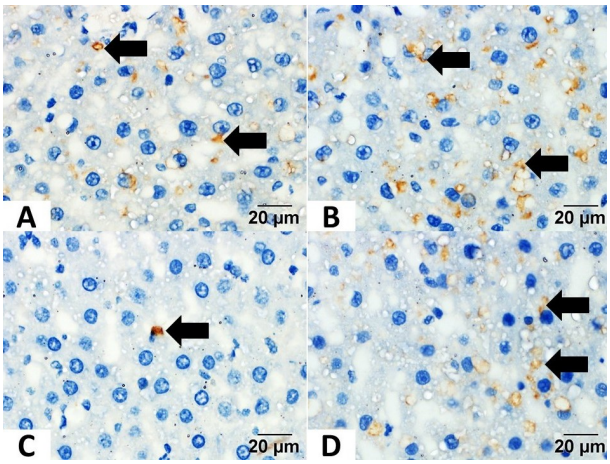

Fig 4. A: Sham group showed positive BCL-2 expression (arrow). **B:** Tadalafil group showed positive BCL-2 expression (arrow). **C:** I/R group showed minimal BCL-2 expression (arrow). **D:** Tadalafil + I/R group showed decreased collagen deposition in the portal tract area (arrow) (BCL-2, Scale bar: 20 µm).

early phase is due to ischemia caused by lack of oxygen, the late phase due to reperfusion and it is characterized by the activation of Kupffer cells and the release of various mediators (Peralta et al., 2013).

The current study highlights the mechanism of tadalafil toward the attenuation of liver ischemia. Serum ALT and AST levels are widely used as markers of liver cell damage. In this, tadalafil at a dose of 10 mg/kg ameliorated hepatic I/R injury, as demonstrated by reduction in AST and ALT levels, ameliorated oxidative stress status and cytokines' profile, in addition to decreased histopathological alterations. These results are in conformity with other studies that show the beneficial effects of other phosphodiesterase inhibitors in depleting elevated ALT and AST in I/R induced liver injury

Table 5: Means±SD of the area percent of collagen and BCL-2 in the studied groups.

	Sham N=12	Tadalafil N=12	I/R N=12	Tadalafil + I/R N=12
Area percent of collagen	0.28 ± 0.04	0.31± 0.02	3.81± 0.98 [1]P<0.001 [2]P<0.001	1.21 ± 0.6 [1]P<0.01 [2]P<0.01 [3]P<0.001
Area percentage of BCL -2	4.76 ± 0.02	4.72 ± 0.05	2.32 ± 0.08 [1]P<0.001 [2]P<0.001	3.64 ± 0.04 [1]P<0.001 [2]P<0.001 [3]P<0.001
Optical density (OD) of TNF-α	0.84±0.03	0.95±0.01	6.22±0.41 [1]P<0.001 [2]P<0.001	2.51±0.12 [1]P<0.001 [2]P<0.001 [3]P<0.001

[1]P comparison with sham, [2]P comparison with Tadalafil, [3]P comparison with I/R group.

(Genoves et al., 2014).

Cyclic nucleotides (cAMP and cGMP) are playing a pivotal role in signal transduction in many physiological processes, as they are working as second messengers. Their Intracellular levels are controlled by adenylyl and guanylyl cyclases, which are used for their synthesis while they are degraded by PDEs (Gulati and Singh, 2014).

Reactive oxygen species (ROS) are considered one of the main constituents involved in I/R. The Cellular antioxidant enzymatic and non-enzymatic defense system plays a main role in the mitigation of tissue injury elicited by free radicals. Hepatic cellular injury occurs because of the ROS's direct effect on biological components (Sehitoglu et al., 2015). Eradication of ROS in healthy cells is maintained by a protective scavenging system that eliminate the excessively released ROS as (CAT), superoxide dismutase (SOD), and GSH. Oxidative stress happens due to the excessive release of ROS and the decreases antioxidant defense system (Sheweita et al., 2015).

GSH is oxidized with the enzymatic effect of glutathione peroxidase to remove the ROS, and hence release oxidized glutathione (GSSG) in the hepatic cells, which explains the important role of GSH in protection against oxidative stress (Peralta et al., 2013).

During I/R, GSH decreases, as the release of ROS consumes it, leading to further oxidation and degradation of vital structures in the cell as lipids, proteins and DNA (Hegazy et al., 2018). In this study, tadalafil significantly elevated the depleted GSH level attenuating the oxidative stress elicited by I/R in rats. These results are in harmony with previous studies that stated that PDE inhibitors restoring GSH level (Luo et al., 2015). Lipid peroxidation is another main mediator in the oxidative stress produced in different organ injuries. Oxidation of the lipids in cellular membranes lead to cellular damage and the end-product in MDA (Mustafa et al., 2015). In the present study, I/R injury resulted in an excessive amount of MDA levels in the liver. The liver MDA level was more

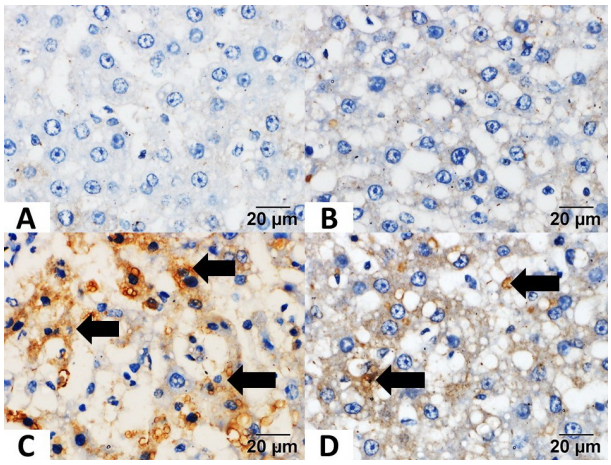

Fig 5. A: Sham group showed negative immunoreaction for TNF-α protein expression in the cytoplasm of hepatocytes (arrow). **B:** Tadalafil group showed minimal immunoreaction for TNF-α (arrow). **C:** I/R group showed strong positive immunoreaction for TNF-α in the wall of blood sinusoids and in the cytoplasm of hepatocytes (arrow). **D:** Tadalafil + I/R group showed weak immunoreaction for TNF- α in the wall of blood sinusoids and in the cytoplasm of some hepatocytes (arrow) (TNF-α, Scale bar: 20 µm).

than that of the normal liver, which is in harmony with other previous studies that showed the significant elevation of MDA in ischemic liver (Cakir et al., 2016).

Tadalafil significantly depleted the elevated MDA levels in the liver of I/R rats, and attenuated the lipid peroxidation effectively. From the main sources of ROS in I/R hepatic injury is the kupffer cells and the polymorphonuclear neutrophils (Datta et al., 2013), as their number and activity determines the severity of oxidative activity. Moreover, the activated Kupffer cells produce cytokines, especially TNF-α and IL-1β (Peralta et al., 2013).

In the current study, tadalafil is associated with some sinusoidal dilatation. This can be attributed to the fact that this drug is metabolized in the liver via the cytochrome P450 system (CYP 3A4 and 2C9), and a toxic or immunogenic intermediate may account for the rare instances of hepatic inju-

ry (Graziano et al., 2017; Osayame and Ewek, 2011). In support of this, previous studies found that the tadalafil may be associated with some cytoarchitectural distortion, occasional central vein and portal vessels dilatation (Osayame and Ewek, 2011; Nna et al., 2015; Jarrar and Almansour, 2015).

Local and systemic inflammatory response elicited by I/R is controlled mainly by released cytokines such as TNF-α, and affect significantly organ injury (Rao et al., 2013). During hepatic I/R, leukocyte recruitment is visible in liver injury, and elevated expression of ICAM-1 in endothelial cells promotes leukocyte adhesion and induces clot formation that changes sinusoidal perfusion (Camara-Lemarroy et al., 2014). Beside the increased local expression of ICAM-1 after hepatic I/R, increased expression of ICAM-1 in other distant organs has been shown, and that explains the multiorgan failure detected after I/R (Camara-Lemarroy et al., 2014; Rao et al., 2013).

Conclusion

Tadalafil effects suggest that modulation of the inflammatory response might be one of the mechanisms of tadalafil-mediated hepatoprotection.

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