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CILOSTAZOL HEPATOPROTECTIVE EFFECT AGAINST ISCHEMIA/REPERFUSION: INVOLVEMENT OF GSK-3β, CYCLIN D1 and WNT/β-CATENIN PATHWAY

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Abstract- Cilostazol is an antiplatelet that acts by inhibiting phosphodiesterase-3 and that was proven to be effective in models of ischemia/reperfusion (I/R) injury; however, its possible role in hepatic I/R remains indistinct, which is the aim of the current work. To fulfill this goal, rats were randomized into sham, I/R and cilostazol (60mg/kg, p.o) groups. The hepatic artery and portal vein to the left and median liver lobes were occluded for 30 min and then declamped for reperfusion to establish a model of segmental (70%) warm hepatic ischemia. Pretreatment of animals with cilostazol for two weeks prior to I/R insult significantly decreased serum alanine aminotransferase, and inhibited I/R-induced hepatocytes apoptotic death signified by inhibition of caspase-3. Moreover, cilostazol increased ATP content and lowered the level of lipid peroxidation assessed as malondialdehyde. The drug also elevated the nitric oxide content and decreased that of tumor necrosis factor-α, as well as the myeloperoxidase activity, a marker of neutrophil infiltration. Mechanistic studies revealed that cilostazol protected the liver and enhanced its proliferation ability, where it markedly increased the level of β-catenin and cyclin D1, but blocked the phosphorylation of GSK-3β at Ser9. In conclusion, cilostazol pre-administration protected hepatocytes against I/R insult by virtue of its antioxidant, anti-inflammatory, and antiapoptotic effects; besides, the drug increased hepatocytes proliferation by increasing the level of cyclin D1. It increased also the Wnt/ β-catenin pathway, which aid in its hepatoprotective action along with blocking the GSK-3β phosphorylation at the ser9, which had injurious role in this work.

Keywords- Cilostazol, hepatic ischemia/reperfusion, GSK-3β, β-catenin, ATP, cyclin D1

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Introduction

The hepatic ischemia-reperfusion (I/R) injury occurs in two main settings, the first arose following systemic hypoxia or low flow states associated with sepsis or shock The second setting takes place after the liver resection or transplantation due to anoxia or ischemia of the liver itself [1,2], where it is considered as the main hurdle, especially in the two latter operations. During the I/R insult, viz., the warm I/R, the insufficient blood flow increases oxidative stress and impairs the hepatic mitochondrial energy metabolism, depresses oxidative phosphorylation, and decreases ATP concentrations [3]. This ensues the activation of anaerobic metabolic pathways [4] resulting in liver and/or multi-system organs failure. Albeit reinstatement of the blood flow is necessary to restore cellular function, yet the re-oxygenation itself triggers a cascade of tissue injury following a period of ischemia [5]. Therefore, the I/R insult-induced liver injury can be subdivided into two distinct phases. The first is the acute phase of injury that emerges within the initial few hours after reperfusion and is allied with the production of reactive oxygen species (ROS) from Kupffer cells. In this phase, mild liver injury occurs, where the hepatocytes and sinusoidal endothelial cells undergo apoptosis [6,7].

The second phase then follows, which is the hepatic inflammation phase of injury, prompted by the release of inflammatory mediators, including tumor necrosis factor alpha (TNF-α) and interleukins (IL)-1, 12, 23, released by the hepatocytes and the activated Kupffer cells. These inflammatory mediators provoke the expression of chemokines and adhesion molecules, which recruit activated neutrophils from the liver microcirculation into the parenchyma [8]. The neutrophils infiltration process encompasses a cascade of concerted events leading to the capture, adhesion and extravasation of the leukocytes [5]. The recruited neutrophils, 6 h after reperfusion, mediate cellular injury and alter the nitric oxide (NO) level, which has various protective effects on cells during I/R [4,9]. In addition, the neutrophils oxidative burst is considered the main source of ROS production in the later stages [10]. In a previous study, Diao, et al [10] confirmed also that intercellular adhesion molecule-1 (ICAM-1), along with TNF-α are involved together in the pathogenesis during reperfusion injury in liver transplantation. Hence, suppression of TNF-α is highly effective in attenuating acute postischemia-mediated inflammation injury [11] and apoptotic cell death [12].

One of the molecules that play an important role in the liver physiology and pathology is glycogen synthase kinase 3 (GSK-3), which is a constitutively active serine/threonine protein kinase. GSK-3 exists in two isoforms, GSK-3α and GSK-3β [13] and, apart from phosphorylating and inactivating glycogen synthase [14], it regulates various basic cellular events, including apoptosis, proliferation, differentiation, and oxidative stress [15]. Hence, modulating GSK-3β was reported to ameliorate hepatic I/R injury through maintaining mitochondrial function and hepatic energy-balance [16], and/or regulating immune responses [17]. The liver cell proliferation is a vital process for liver regeneration and cell survival after an injurious insult [18] and was found to be controlled by a cyclin, known as cyclin D1[19], which makes it a surrogate marker for cell cycle progression in hepatocytes. Once the hepatocytes express cyclin D1, they have passed the G1 restriction point and are committed to DNA replication [20].

Cilostazol, a selective type-3 phosphodiesterase inhibitor (PDEI), is known to inhibit platelet aggregation by increasing the intracellular c -AMP concentration, by blocking its conversion into adenosine monophosphate [21]. Phosphodiesterase inhibitors have been used in a wide spectrum of experimental and clinical studies on cardiac failure, pulmonary hypertension, vascular diseases, platelet functions, reduction of I/R injury, and preservation of graft viability [22, 23]. In addition, cilostazol itself, besides its antiplatelet effect, has various pharmacological effects including anti-inflammatory, antioxidant and anti-apoptotic effects via the c-AMP -dependent and -independent pathways [24]. In the liver, cilostazol has shown a beneficial effect on liver steatosis [25], ethanol [24] and carbon tetrachloride- induced liver fibrosis [26]. However, its potential hepatoprotective effect against a hepatic I/R insult in rats has not been evaluated, which is the aim of the present study. In addition, the possible mechanistic cassette has been tackled.

Materials and Methods

Animals

Adult male *Wistar* rats (200-250g) were purchased from "El-Salam Breeding Center", Cairo, Egypt. Rats were housed at the animal facility of Faculty of Pharmacy, October 6 University, for one week prior to the experimentation and were allowed free access to standard pellet diet and tap water. Animals were housed under suitable laboratory conditions, with 12h light/dark cycles at an ambient temperature of 22° C ±2°C and a humidity of 65-70%. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study protocol was approved by the guidelines of the Research Ethical Committee of the Faculty of Pharmacy, Cairo University, Cairo, Egypt (PT 240). All surgery was performed under thiopental anesthesia, and all efforts were made to minimize suffering.

Experimental Design

Animals were randomly allocated into three groups (n= 7-9). The first group was the sham control group and the second was the I/R group, in which rats were subjected to I/R. Rats in both groups received the vehicle (1% Tween 80, p.o) daily for 14 days. The last group was administered Cilostazol (60mg/kg p.o; Otsuka, Al Sharqia, Egypt), for 2 weeks, after which animals underwent I/R as in the second group.

Surgical Technique

Under aseptic conditions, animals underwent a midline laparotomy under thiopental anesthesia followed by a delicate dissection of the round ligament of the liver and identification and exposure of the hepatic pedicle. To induce hepatic ischemia, the hepatic pedicle that supplies the left and median liver lobes (70% of liver mass) was occluded with a microvascular clamp for 30 min. Ischemia was confirmed by observing the pale appearance of the clamped liver lobes. After the pedicular clamping period the clamp was carefully removed to allow for reperfusion for 24 hrs; reperfusion was evaluated based on the immediate color recovery. Sham animals underwent anesthesia, laparotomy, and exposure of the hepatic pedicle without clamping.

Determination of Biomarkers

Twenty four hours after reperfusion and under thiopental anesthesia, blood samples were collected from tail vein and were centrifuged to separate the serum, which was used for the determination of alanine aminotransferase (ALT) according to the method described by Reitman and Frankel [27] using a test reagent kit (Biodiagnostic Co., Cairo, Egypt). After blood collection, the animals were then euthanized and livers were excised, weighed, homogenized in saline and kept in aliquots at -80°C for the estimation of the following biomarkers.

Assessment of Glutathione (GSH) and lipid Peroxidation

One aliquot of the homogenized liver was used for the assessment of GSH by adopting the method described by Ahmed, et al [28], in which GSH reacts with Ellman's reagent after precipitation of protein SH-groups using sulfosalicylic acid. The reaction with Ellman's reagent formed a stable yellow color of 5 mercapto-2-nitrobenzoic acid, which was measured colorimetrically at 412 nm (mg/g tissue). Another aliquot was used for the estimation of lipid peroxidation (nmol/g tissue) according to the method of Mihara and Uchiyama [29], where the thiobarbituric acid (TBA) reactive substances, measured as malondialdehyde (MDA), was used as an index of lipid peroxides. The MDA-TBA adduct develops pink color, which was extracted by n-butanol and measured at two wave lengths, viz., 520 and 535 nm.

Assessment of Total Nitric Oxide (NOx)

Hepatic NOx level was estimated indirectly according to the method of Miranda, et al [30]. NO content (μM/g tissue) was quantified as total nitrite by the use of vanadium trichloride to reduce nitrate into nitrite. The pink azo-dye produced by the reaction of nitrite with sulfanilic acid followed by the subsequent coupling with N-(1-naphthyl)-ethylene diamine was measured colorimetrically at 540 nm.

Assessment of Myeloperoxidase (MPO) Activity

The method of Bradley, et al [31] was adopted for the assessment of MPO activity. The method is based on the measurement of the hydrogen peroxide-dependent oxidation of o-dianisidine, catalyzed by MPO, which results in the formation of a compound exhibiting an increased absorbance at 460 nm.

Assessment of other Parameters

The levels of the following parameters were determined using the corresponding commercial ELISA kits: GSK-3β(pS9) and β-catenin (Invitrogen Corporation;Camarillo, CA); rat cyclin D1 (Wuhan Eiaab

Science Co., Wuhan, China); rat caspase-3 (Uscn Life Science Inc., Wuhan, China); rat ATP (KAMIYA Biomedical Co., Seattle, USA) and rat TNF-α (Ray Biotech, Inc., Norcross GA, USA). For analysis of these parameters, the liver homogenate aliquots were processed according to the manufacturer's instructions.

Histopathological Examination

Parts of the isolated hepatic tissue were fixed in 10% formalin solution and then dehydrated in ascending grades of alcohol and embedded in paraffin. Four micron-thickness sections were taken, stained with hematoxylin and eosin solutions and examined under light microscope. The specimens were examined blindly by a single pathologist without knowledge of the groups.

Statistical Analysis

Values were expressed as mean ± SEM (n=7-9). Statistical difference between groups were computed by one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. The level of significance was accepted at $p < 0.05$.

Results

[Table-1] depicts the 2.9 folds elevation in the serum ALT level in the I/R group as compared to the normal level in the sham-operated one; this elevation was hindered by the cilostazol pretreatment. Similarly, the I/R insult boosted the hepatic MDA level (9.6 folds), an effect that was impeded by cilostazol (67.42%), as compared with the I/R effect. However, the hepatic NOx content was sharply decreased (83.45%) by the insult, while the cilostazol pretreatment opposed this inhibition. As presented in [Table-2], the I/R model caused a significant elevation in the activity of MPO and caspase-3, as well as the content of TNF-α mounted to 2.4, 8.1, and 1.65 folds, respectively. However, cilostazol pretreatment hindered these increments by 25.48, 61.4 and17.6 %, respectively. On the other hand, the table illustrated the sharp decline in the ATP content caused by the I/R insult (6.94±0.60 pg/g), as compared to the sham control value (26.90±1.06 pg/g), an effect that was increased significantly even above the normal value by the PDEI, cilostazol (35.7±2.23 pg/g).

Table 1- Effect of Cilostazol (60 mg/kg; $Cilo_{60}$) on serum alanine aminotransferase (ALT) and the hepatic content of reduced glutathione (GSH), lipid peroxide (MDA) and total nitrate/nitrite content (NOx) in hepatic ischemia/ reperfused rats.

Groups	ALT (U/L)	GSH (mg/g tissue)	MDA (nmol/g tissue)	NOx (μM/g tissue)
Sham	31.5± 3.02	0.50± 0.04	12.9± 1.12	69.5± 3.24
I/R	90.8± 3.98*	0.57± 0.03	124± 4.17*	11.5± 0.49*
I/R+$Cilo_{60}$	64.5± 4.80*#	0.49 ± 0.03	40.4±3.39*#	24.4±0.98*#

Cilostazol was administered orally for 2 weeks and ischemia followed by reperfusion was induced on the 15th day. Values are expressed as mean ± S.E (n=7-9). Statistical analysis was carried out using one way ANOVA followed by Tukey-Kramer multiple comparison test. As compared with sham-operated (*) and I/R-operated (#) groups, P < 0.05.

[Fig-1] illustrated the 4 folds elevation in the GSK-3β (pS9) caused by the I/R insult in comparison to the sham-operated control group, indicating an increase in its phosphorylation and the corresponding decrease in its activity. On the contrary, the pre-administration of cilostazol hampered the phosphorylation of GSK-3β and increased its activity. The I/R succeeded also to inhibit the β-catenin level (3.82±0.15 ng/g), to reach less than half its content as compared to the sham control group (9.25±0.21ng/g). Pretreatment with cilostazol significantly opposed the inhibitory effect of I/R, where β-catenin level reached 6.08±0.20 ng/g [Fig-2]. Additionally, hepatic cyclin D1 content was markedly decreased in the I/R group by 86.47% compared to the sham control group; this effect was significantly impeded by the pre-administration of cilostazol [Fig-3]. The histopathological photomicrographs presented in [Fig-4], revealed the presence of normal hepatic architecture (h) and cell organelles with no pathological appearances in the sham-operated group; however, the I/R injury was manifested by the presence of focal areas of coagulative necrosis (Fn) in the infarcted parenchyma, congestion of the sinusoids (arrow), and cellular derangements with inflammatory cells infiltration in the portal area. On the other hand, pre-ischemic treatment with cilostazol markedly attenuated the I/R-induced histopathological changes in rat liver evidenced by the marked reduction in the number of the infiltered neutrophils, with only mild sinusoidal congestion, and the absence of the coagulative necrosis with virtually normal structure.

Table 2- Effect of Cilostazol (60 mg/kg; $Cilo_{60}$) on the hepatic activity/content of myeloperoxidase (MPO), caspase-3, tumor necrosis factor-α (TNF-α) and adenosine triphosphate (ATP) in hepatic ischemia/ reperfused rats.

Groups	MPO (U/g tissue)	Caspase-3 (ng/g tissue)	TNF- α (pg/mg tissue)	ATP (pg/g tissue)
Sham	0.085±0.001	12.4± 0.53	5.71± 0.04	26.9±1.06
I/R	0.208±0.011*	101± 3.66*	9.45± 0.06*	6.94±0.60*
I/R+$Cilo_{60}$	0.155±0.008*#	39± 2.35*#	7.78±0.06*#	35.7±2.23*#

Cilostazol was administered orally for 2 weeks and ischemia followed by reperfusion was induced on the 15th day. Values are expressed as mean ± S.E (n=7-9). Statistical analysis was carried out using one way ANOVA followed by Tukey-Kramer multiple comparison test. As compared with sham-operated (*) and I/R-operated (#) groups, P < 0.05.

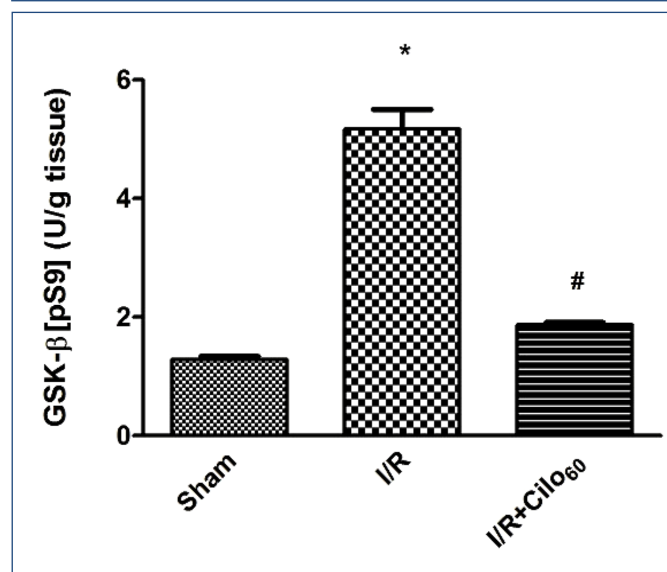

Fig. 1- Effect of Cilostazol (60mg/kg; $Cilo_{60}$) on the liver phosphorylated glycogen synthase kinase-3β (GSK-3β(pS9)) activity in hepatic ischemia/ reperfused rats. Cilostazol was administered orally for 2 weeks and ischemia followed by reperfusion was induced on the 15th day. Values are expressed as mean ± S.E (n=7-9). Statistical analysis was carried out using one way ANOVA followed by Tukey-Kramer multiple comparison test. As compared with sham-operated (*) and I/R-operated (#) groups, P < 0.05.

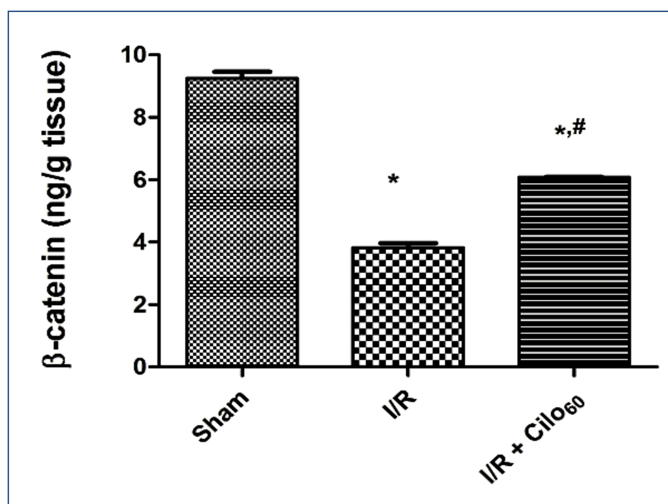

Fig. 2- Effect of Cilostazol (60 mg/kg; $Cilo_{60}$) on the liver β-catenin content in hepatic ischemia/ reperfused rats. Cilostazol was administered orally for 2 weeks and ischemia followed by reperfusion was induced on the 15th day. Values are expressed as mean ± S.E (n=7 -9). Statistical analysis was carried out using one way ANOVA followed by Tukey-Kramer multiple comparison test. As compared with sham-operated (*) and I/R-operated (#) groups, $P < 0.05$.

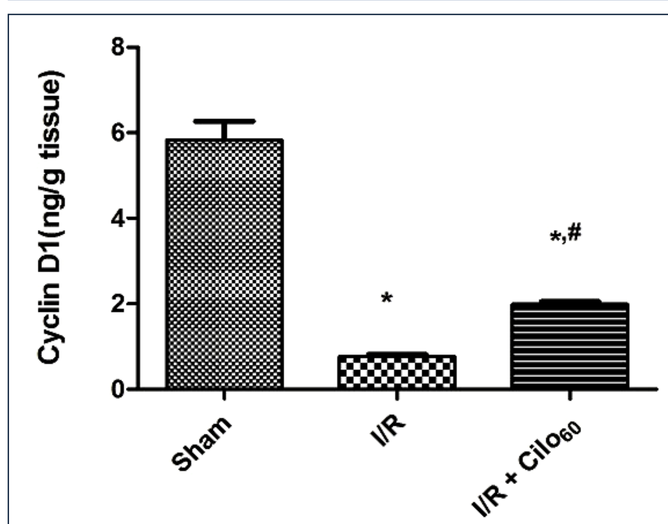

Fig. 3- Effect of Cilostazol (60mg/kg; $Cilo_{60}$) on the liver cyclin-D1 content in hepatic ischemia/ reperfused rats. Cilostazol was administered orally for 2 weeks and ischemia followed by reperfusion was induced on the 15th day. Values are expressed as mean ± S.E (n=7 -9). Statistical analysis was carried out using one way ANOVA followed by Tukey-Kramer multiple comparison test. As compared with sham-operated (*) and I/R-operated (#) groups, $P < 0.05$.

Discussion

Experimentally-induced hepatic I/R results in a sharp decrease in the hepatic ATP level as documented in the present study and others [32], via increasing the release of reactive oxygen and nitrogen species (ROS, RNS). These radicals promote intracellular damage and ensue disruption of the oxidative phosphorylation process and the cytosolic ions/ pumps resulting in mitochondrial dysfunction and ATP failure [33]. Cilostazol interfered with the I/R devastating effect on ATP, an effect that was proven in other I/R models [34,35]. The insult provokes the release of ROS that injure cell membranes through lipid peroxidation, exemplified by the increased level of MDA herein and in previous studies [36,37]. Besides, ROS induce alteration in hepatocyte transport function and membrane permeability leading to the leakage of liver enzymes into the circulation [38] as reflected here by the increased serum level of ALT. Cilostazol improved cellular integrity and lowered ALT and MDA levels, pointing, thus, to its free radical scavenging and/or antioxidant properties; effects that were verified previously [39] via inhibiting NADPH oxidase-derived ROS production [40] or by its ability to trap the released ROS [21].

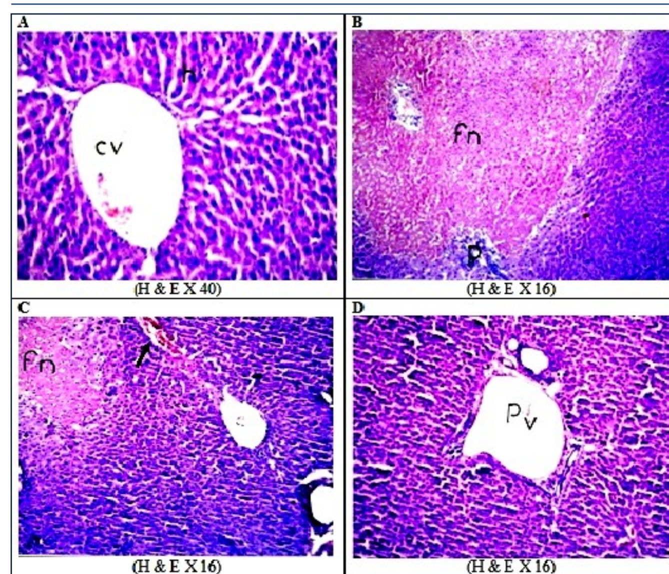

Fig. 4- Representative photomicrographs of hepatic tissue 24 h after sham-operation or ischemia/ reperfusion with or without pretreatment with Cilostazol (60 mg/kg, p.o). **(A)** Sham-operated rat section showing normal hepatic histological architecture of the central vein (cv) and hepatocytes (h) in the surrounding parenchyma. **(B & C)** Ischemia/ reperfusion sections show the presence of focal areas of coagulative necrosis in the infarcted area of the hepatic parenchyma (Fn), congested sinusoids (arrow), cellular derangements with inflammatory cells infiltration (P) in the portal area. **(D)** Cilostazol pretreatment section shows a virtually normal structure with a marked reduction in the number of the infiltered neutrophils, a mild sinusoidal congestion and the absence of the coagulative necrosis. Original magnification×40 and 16 (H & E staining).

The increased release of ROS can overwhelm the endogenous antioxidants, including GSH, leading to its depletion. Nevertheless, this is not always the case, where although several studies have pointed to the I/R-mediated GSH depletion or inhibition, our study and others [41,42] showed no alteration in the GSH level.

In the current investigation, the I/R-induced NO inhibition, supported by previous studies [43,44], is associated with the increased liver injury, a correlation that is appreciated recently to two reasons. The first is the I/R-induced low availability of tissue arginine and low concentrations of endothelial NOS (e-NOS) resulting in a reduction in NO production [10] and the second is the decreased NO bioavailability, wasted in the reaction with the abundant ROS, such as superoxide anion [45]. However, pre-administration of cilostazol elevated the level of NO, possibly by increasing its synthesis, at least partially through a c-AMP-dependent pathway [46] or via the upregulation of e-NOS [47]. The elevation of NO can be one factor for

the cilostazol positive effect as NO mediates various protective effects on cells during I/R, via inhibiting oxidative stress, cytokine release, leukocyte endothelial adhesion, apoptosis and TNF-α [9].

The I/R injury increased the infiltration of neutrophils as manifested by the increased MPO activity; a result that goes in line with previous findings [48,49]. These cells contribute to microcirculatory disturbances and the re-release of ROS, proteases and various cytokines [50]. Moreover, they may be an additive cause for lipid peroxidation, where MPO can convert hydroperoxides into free radicals, triggering thus, lipid peroxidation [51]. The pre-administration of cilostazol attenuated the infiltration of neutrophils in the liver, offering thus, another possible mechanism for preventing hepatic malfunction, besides increasing NO, ATP and scavenging the free radicals. The cilostazol-mediated MPO suppression can be accredited to the increased level of NO [45]. Moreover, a direct relation between recruitment of neutrophils and TNF-α production has been previously reported [52]. This fact has been adopted by Lee, et al [53], who attributed the cilostazol effect on MPO to its ability to suppress TNF-α release, an explanation that concurs with our findings.

During the early phase of hepatic reperfusion injury (at zero time-1day of reperfusion), the activated Kupffer cells produce high concentrations of TNF-α, a fact that supports the I/R-induced TNF-α release reported in the present work and others [45,54], {2005 #4687}. Cilostazol-mediated inhibition of TNF-α production can be ascribed to the increase in c-AMP [55] and the scavenging of free radicals [21].

TNF-α is also the major mediator of hepatic apoptosis [56], a fact that agrees with our results and others [57,58]. Cilostazol hindered the I/R effect on caspase-3 activity herein and previously [59,60], an effect that can be linked, in part, to the decrease in TNF-α [56], the elevation of NO [45] and the decrease in ROS [61], all of which are documented in the present work. Moreover, other pathways involve the increase of c-AMP-responsive element binding protein phosphorylation, the up-regulation of Bcl-2 and the down-regulation of Bax protein [21].

GSK-3β is involved in the I/R-induced liver injury [16,17,62,63], however the exact role and mechanism are not clear. Although several studies have pointed to the protective action mediated by inhibiting GSK-3β activity, yet in the current study the phosphorylated inactive GSK-3β has been elevated by 4 folds after 24 h reperfusion. This finding coincides with that of Ren, et al [17], who stated that the phosphorylated GSK-3β (at serine 9) level was slightly reduced by ischemia itself (time 0) and then rapidly increased at reperfusion and remained phosphorylated throughout the reperfusion phase, via a phosphoinositide-3 kinase (PI3K)-GSK-3β signaling. Additionally, the study by Xia and his team [62] revealed that the phospho-GSK-3β was just detectable in the sham group, while the total GSK-3β expression was strong, and that the expression of phospho-GSK-3β increased with I/R. Whether increasing phospho-GSK-3β can mediate a protective role or not, is again a point of debate. Ren, et al [17] implied that PI3K-GSK-3β signaling was a self-regulatory mechanism preventing the excessive I/R-hepatocellular damage, while others had an opposite point of view. In one study, the acute and chronic treatment with lithium chloride (an inhibitor of GSK-3β activity) caused serious damage in the hepatic I/R injury, including increased apoptosis and oxidative stress [63]. Furthermore, Wang, et al [64] have attributed the harmful effect of GSK-3β inhibition in liver I/R to the increase of macrophage IFN-β production, which is a key pro-inflammatory mediator in the liver I/R along with its downstream gene product CXCL10 [65]. Additionally, mice with GSK-3β genetic deletion showed severe liver degeneration and massive hepatocyte apoptosis during their development [66], and the inhibition of GSK-3β increased hepatocyte apoptosis mainly via the activation of TNF-α [67]. The latter findings strengthen our results, where the I/R group represents the highest level in caspase-3 and TNF- α along with the phosphorylated GSK-3β. Thus, GSK-3β inhibition may exert multifaceted functions in the liver during I/R and it remains questionable as to whether GSK-3β inhibition can ameliorate the liver I/R cascade. The definite relation between cilostazol and GSK-3β is also uncertain, where in one study [53] cilostazol suppressed Aβ (1-40)-induced neurotoxicity via upregulating the GSK-3β phosphorylation at Ser9/β-catenin phosphorylation at Ser675. Nevertheless, in another study [68] cilostazol inhibited the proliferation of vascular smooth muscle cells, without affecting the phosphorylated GSK-3 β. To our knowledge, this is the first study that verifies the cilostazol effect on GSK-3β in a hepatic I/R model. In this work cilostazol pretreatment for two weeks maintained GSK-3β in its active form and blocked the I/R-mediated GSK-3β phosphorylation at Ser9, possibly via its antiapoptotic effect and/or the inhibition of TNF-α [69].

The Wnt/β-catenin signaling regulates multifaceted hepatocelluler processes, as development, proliferation and regeneration, and recently in regulating hepatic energy metabolism and mitochondrial function [70,71]. GSK-3β regulates the Wnt/ β-catenin target gene expression by controlling the level of cytoplasmic β-catenin and its nuclear shuttle [69]. However, not only the GSK-3β directs the Wnt/ β-catenin pathway, but also the redox imbalance has emerged as a key player in the development of several hepatic diseases and in the regulation of different cellular signaling pathways including the Wnt/ β-catenin pathway [71]. β-Catenin has been proven to be hepatoprotective through its interaction with hypoxia inducible factor (HIF-1)-α signaling in the liver [70] and/or the inhibition of the fork-head transcription factor FoxO3 [71]. Furthermore, Kuncewitch, et al [72] documented that hepatic expression of β-catenin and its downstream target gene Axin2 were decreased after I/R and that the administration of a Wnt agonist has dampened the I/R related MPO, iNOS, and IL-6. To this extent, these studies and others support our findings, where the I/R group, after 24 hrs of reperfusion, has experienced a 60% decrease in the β-catenin level, along with a disturbed histological structure; whereas, pretreatment with cilostazol elevated the β-catenin content and improved the hepatocytes structure emphasizing the impact of catenin on the hepatocytes maturation, expansion and survival [73]. This effect can further pin down the hepatoprotective effect of cilostazol and offered additional mechanism for the antiplatelet drug. Again, to our knowledge, this is the first study that tested the effect of cilostazol on β-catenin in liver I/R model. A previous study pointed to the positive effect of cilostazol against etoposide-induced apoptosis in articular chondrocytes via stimulating the accumulation of β-catenin and reducing the expression of type II collagen in primary rat articular chondrocytes [74]. Moreover, cilostazol was reported to retain β-catenin in a study of brain acute ischemia, in which cilostazol inhibited the endothelial permeability [75].

Cyclin D1 is a member of cell cycle proteins that modulate the progression of cells through the cell cycle [19,20]. In a recent study by Sakai, et al [8], the activation of cyclin D1, besides nuclear factor-kB and p38 mitogen-activated protein kinase (MAPK), reduced the I/R-induced hepatocellular injury. In the current study, I/R insult de-

creased the hepatic cyclin D1 levels, while pre-administration of cilostazol enhanced liver cyclin D1 content compared to the I/R group, in a manner similar to that seen in the β-catenin. The hepatic cell proliferation occurs in a Wnt/β-catenin dependent manner, where this pathway activates normal proliferation of adult hepatic oval cells [76] and that induced by the thyroid hormone [77]. Another pathway was offered by Ke, et al [78], who demonstrated that HIF-1α-mediated overexpression of HO-1/Cyclin D1 facilitates cytoprotection by limiting hepatic inflammatory responses, and hepatocellular necrosis/ apoptosis in a PI3K-dependent manner. In this context, the cilostazol hepatoprotective effect involves the enhancement of cellular proliferation.

Conclusion

The present study highlighted the hepatoprotective effect of the antiplatelet drug cilostazol in an I/R model. The mechanistic cassette of cilostazol involves preventing the phosphorylation of GSK-3 β and keeping it in its normal active form, and increasing both β-catenin and cyclin D1, hence, protecting the liver and promoting its proliferation. Additionally, cilostazol boosted hepatocellular ATP content, elevated NO, and inhibited hepatocyte oxidative stress, apoptosis, neutrophils infiltration and TNF-α production.

Conflict of Interest: No conflict of interest.

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