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The reno-protective effect of Celastrol mediated by Nrf2/HO-1 activation and NF-κB/NLRP3 suppression in hepatic ischemia/reperfusion model

Shaimaa G. Ibrahim[1], Eman A. Mohamed [2*], Mohamed F. Abd Ellah [3] and Azza A. Ali [2]

[1]Department of Pharmacology and Toxicology, Faculty of Pharmacy, October 6 University, Cairo, Egypt
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt
[3]Department of Pharmacology and Toxicology, Faculty of Pharmacy (Boys), Al-Azhar University, Cairo, Egypt

***** Correspondence: emanabdelwahed@yahoo.com, emanabdelwahed@azhar.edu.eg ; Tel.: +202 01222345536

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Abstract: Background: Hepatic ischemia reperfusion injury (IRI) takes place in clinical cases like liver transplantation and resection. Hepatic IRI is mediated by induction of oxidative stress and inflammation. Hepatic IRI results in remote organ injury. Acute kidney injury (AKI) is common and increases mortality and morbidity. Extensive experimentations showed that celastrol (CEL) has therapeutic characteristics in curing the inflammatory disorders and kidney disorders. **Objective**: The current study pointed to estimate the potential protective effect of CEL against hepatic IRI-induced AKI, and identify the uderlying mechanisms. **Materials and methods**: CEL (4mg/kg, IP, once) was given to rats. After 1 hour rats liver was exposed to 90 min ischemia then 4 hrs of reperfusion (90/4 I/R). At the end of surgery kidney tissue and blood were collected to evaluate the AKI. **Results**: Results revealed that compared to I/R group, CEL protected group provided reduction in renal injury. This was confirmed by a remarkable cutdown in BUN, Cr levels in sera and amended histopathological results. Renal protection obtained by CEL was driven by increasing Nrf2, HO-1, TAC content and decreasing MDA content. Furthermore, CEL protection significantly reduced the inflammatory markers (NF-κB, NLRP3, CASP1, IL-1β, HSP90 and HSF-1) and neutrophilic infiltration assessed as MPO. **Conclusion**: Reno-protection by CEL might be resulted from its anti-inflammatory and antioxidant properties mediated by (Nrf2/HO-1), (NF-κB/NLRP3) inflammasome, and (HSP90/HSF-1) pathways. Thereby, CEL therapy could be a possible strategy to improve the clinical outcomes of liver surgery.

Keywords: Hepatic ischemia reperfusion injury; Celastrol; Nrf2/HO-1; NF-κB/NLRP3 inflammasome; Renal injury

1. INTRODUCTION

Ischemia/reperfusion injury (IRI) is a tissue damage produced by blood flow prevention then reperfusion, during which many mediators are released leading to cellular then organ dysfunction [(1)]. Liver known as one of the most affected organs [(2)]. This insult is a serious reason for liver damage which happens in many surgeries like liver resection or transplantation. It is considered as the basic reason for graft dysfunction after transplantation. Hepatic IRI is mediated mainly by the formation of reactive oxygen species (ROS) in addition to inflammatory cytokines [(1)].

This insult is frequently causes acute liver failure (ALF) which is a popular complication following hepatic resection and transplantation [(3)]. Acute kidney injury (AKI) takes place subsequently in patients have ALF, it poses a major problem post-operatively which increases the morbidity and mortality [(4)].

The mechanism of renal dysfunction after liver IRI begins with portal hypertension due to portal vein occlusion. This produces splanchnic vasodilation [(5)], hypotension and activates the renin-angiotensin system (RAAS). Activation of RAAS results in profound reduction in glomerular filtration rate, renal ischemia, tubular necrosis and renal dysfunction [(6)]. Moreover, the pro-inflammatory cytokines and the transcription factor increased and released from liver which could drive inflammatory alterations in kidneys following liver I/R [(7)].

Besides, oxidative stress considered as serious cause of liver I/R induced AKI as the activate

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neutrophils accumulates in sub-endothelium area and exonerate ROS, and cytokines, resulting in direct kidney injury [8, 9]. Thus, many pathological processes are included in the development of renal injury, including generation of ROS and the activation of the immune response [10].

The NOD like receptor protein-3 (NLRP3) is a cytosolic receptor which mainly expressed on kupffer cells (KCs) and activated by various stimuli, for instance by the sterile inflammation that mediated by endogenous patterns [11]. The activation of NLRP3 inflammasome has a deep role in regulating inflammation [12]. Its activation establishes a complex which activates caspase-1, which break-down the inactive pro- interleukin (IL)-1β to the active secreted form. [13].

Heat shock proteins (Hsps) are chaperones that aid folding the newly formed proteins, repairing mis-folded ones, and preventing their harmful aggregation [14]. When NLRP3 is translated next to the prompting signal, it will be removed from the cells except if protected by a complex holding Hsp90 [15]. Piippo et al. [16] showed that the absence of Hsp90 causes the destruction of NLRP3 in proteasome. The presence of HSP 90 is also activates NF-κB, following the depletion of HSP90 the activation of NF-κB is prevented [17]. Heat shock transcription factor-1 (HSF-1) was shown to support cell survival and proliferation against the serious stress insults [18]. It is induced by diverse stressors, including the OS. It is also one of the HSP90 clients. The HSP90 inhibition activates HSF-1 [19]

Among the important factors that implicated in liver IRI the antioxidant nuclear factor erythroid-2-related factor 2 (Nrf2). It is a positive regulator that leads the expression of antioxidant enzymes among which heme oxygenase-1 (HO-1) that breaks down heme and yield biliverdin, CO and free iron. De facto, HO-1 overexpression exerts strong cytoprotective functions in IRI [20].

Celastrol is extracted from Tripterygium Wilfordii (TW) [21] that belongs to the Celastraceae family. It has been shown to provide anti-oxidant and anti-inflammatory activities [22].

2. METHODS

1.1. Animals:

Adult albino male rats of (200-300g) obtained from the animal house of National Research Center (Cairo, Egypt). Animals were kept in proper laboratory conditions, in well-ventilated polypropylene cages, with 12 hrs light/dark cycles at temperature of 25±2°C. Rats were fed the standard pellets and allowed tap water ad libitum throughout the period of the experiment. The experiments were executed in accordance with the guidelines of the "Research Ethical Committee" of the Faculty of pharmacy, Al-Azhar University (Girls), Cairo, Egypt. (Permit Number: 225).

1.2. Drugs and chemicals

Celastrol and the chemicals were bought from Sigma Aldrich chemical Co. (St. Louis, MO, USA) except those mentioned elsewhere. The other used chemicals were of the highest c0mmercially available quality.

1.3. Inducement of hepatic ischemia/reperfusion

Rats were fasted (16-18 hrs) before the induction of I/R, but were allowed free access to water. After anesthetizing rats using urethane (1.25g/kg, IP) [23], they were immobilized by taping the rat's legs and arms. The abdomen was then shaved and cleaned by 70% ethanol solution [24]. An upper abdominal midline incision 3cm and laparotomy were made using small scissors to expose the abdominal contents. Liver hilus was then exposed to find the triad of the lobes of the left lateral and median. Ischemia was made by clamping the portal triad with a vascular microclip; ischemia is confirmed by the change in the operated lobes from the normal reddish brown color to the pale color.

After 90 min ischemia, reperfusion was allowed by removing the clamp and confirmed by the gradual color restoration within 1 min. Laparotomy was then closed by suturing the layer of muscle then skin layer and leaves the animals to recover for the reperfusion period (4hrs). By finishing the reperfusion time, blood was gathered from abdominal aorta before killing rats.

1.4. Experimental Design

Rats posited at random in 5 groups, (n=6 rats in each group), and were treated as following scheme:

1. **Control group:** Rats were neither exposed to laparotomy nor received the vehicle.
2. **Sham group:** Animals administered the vehicle (1% DMSO in normal saline, 20U, IP, once) 1 hour before laparotomy. After 1 hour, animals were laparotomized for 90 min and neither exposed to hepatectomy nor vascular clamping.
3. **CEL group:** Rats received with CEL (4mg/kg, IP, once) [25] 1hour before killing. Rats were not exposed to I/R.
4. **I/R group:** Animals received the vehicle (1% DMSO in normal saline, IP, once). After 1 hour animals were exposed to 90 min ischemia then 4 hrs of reperfusion (90/4 I/R).
5. **CEL + I/R group:** rats received with CEL (4mg/kg, IP, once) 1hour before subjected to 90/4 I/R.

1.5. Samples collection and preparation

After the experiment termination, blood was taken from the abdominal aorta in non-heparinized tubes and left to clot for 30 minutes at room temperature. Sera was isolated via centrifugation operated at 4000 round per minute (rpm) for 20 min. sera were removed in order to be used in measuring

both blood urea nitrogen (BUN) and creatinine (Cr) sera levels. Kidney tissues were isolated then washed in ice cold saline and dried. For histopathological examination, a sample of each tissue was fixed using 10% formalin. Then for the investigation of various biochemical markers, other specimens were homogenized in ice cold phosphate buffer saline (PBS, pH 7.4).

1.6. Histopathological analysis

Fixed kidney tissues were dehydrated and embedded in paraffin, then sectioned at a of 5 µm thickness. The sections were stained with hematoxylin and eosin (H&E) and examined with a light electric microscope. To avoid bias, all histopathological steps and evaluations were performed by an experienced observer.

1.7. Biochemical analysis

1.7.1. Assessment of renal functions

The sera were used for measuring BUN level besides Cr. These were measured to estimate renal injury with a commercially supplied kit (BioAssay systems CO., Hayward, California, U.S.A.).

1.7.2. Enzyme linked immunosorbent assay (ELISA)

To obtain 10% homogenate, kidney tissues were homogenized in ice cold PBS, supplemented with protease inhibitor at 4 C°, using tissue homogenizer (Omni Tissue Master 125, TM 125-220, England). The obtained supernatant was used to measure caspase-1 (CASP1) (Catalog # E1357Ra,Bioassay Technology Laboratory, Shanghai Korain Biotech Co., China), heme oxygenase 1(HO-1) (Catalog # CSB-E08267r,Cusabio Co., Houston, U.S.A.), interleukin 1β (IL-1β) (Catalog # CSB-E08055r,Cusabio Co., Houston, U.S.A.), malondialdehyde (MDA) (Catalog # MBS738685,MyBiosource Co., San Diego, California, U.S.A.), myeloperoxidase (MPO) (Catal0g # LS-F26022, Lifespan biosciences Co., Washington, U.S.A.), nuclear factor-κB P65 (NF-κB-P65) (Catalog # MBS015549 ,MyBiosource Co., San Diego, California, U.S.A.), and total antioxidant capacity (TAC) (Catalog # MBS1600693 ,MyBiosource Co., San Diego, California, U.S.A.). Kits were used in agreement with the instructions of manufacturer.

1.7.3. Protein expressions of HSP 90, HSF-1, NLRP3, and Nrf2 using Western Blotting

Briefly, the Bradford method was used to determine protein concentrations. SDS-PAGE gel electrophoresis was used to separate proteins, which were then transferred to nitr0cellulose membranes. The primary antib0dies used are for HSP 90 (Catalog # PA3-013, Thermo Fisher Scientific, USA), HSF-1 (Catal0g # PA3-017, Therm0 Fisher Scientific, USA), NLRP3 (Catalog # PA5-88709, Thermo Fisher Scientific, USA), and Nrf2 (Catalog # PA5-27882, Thermo Fisher Scientific, USA). A secondary h0rseradish per0xidase c0njugated antibody (Thermo Fisher Scientific, USA; Catalog # G-21234) was used to visualize the protein-antibody complex. The chemiluminescent substrate (ClarityTM Western ECL substrate – BIO-RAD, USA cat#170-5060) was applied to the blot according to the manufacturer's recommendation. The chemiluminescent signals were captured by CCD camera based imager. Image analysis software was used to read the band intensity of the measured proteins against control after normalization by beta actin (β-actin) on the Chemi Doc MP imager. For HSP 90, HSF-1, NLRP3, and Nrf2 the results were expressed as arbitrary units after normalizating with β-actin protein.

1.8. Statistical analysis

Data were expressed as mean ± SEM. The difference between groups was statistically analyzed by GraphPad Prism 5 (La Jolla, CA, USA), using one-way ANOVA followed by Tukey's Multiple Comparison Test. P value < 0.05 was considered as significant.

3. RESULTS

3.1. Effect of CEL on renal functions following hepatic ischemia/reperfusion

Renal function was estimated by measuring BUN and Cr sera levels. The I/R deteriorated kidney function evidenced by significant rising of BUN (Fig. 1A) and Cr (Fig. 1B) serum level as compared to sham group. While, rats pre-treated with CEL had significant lower levels of kidney functions when compared to I/R-group ($p < 0.05$).

3.2. Effect of CEL on renal oxidative stress markers following hepatic ischemia/reperfusion

The antioxidant effect of CEL against ROS production induced by I/R was evaluated by estimating the contents of MDA, TAC, Nrf2, and HO-1. Induction of I/R provided significant lowering in renal TAC, Nrf2, and HO-1 by about 57.7%, 55.4% and 60.7% respectively, as well as significant rising in lipid peroxidation evaluated by MDA level 4.6 times compared with sham group ($p < 0.05$). Protection with CEL prevented the reduction in TAC, Nrf2, and HO-1 contents by about 83.2%, 77.8% and 106.4% respectively. Moreover, MDA content was reduced in CEL protected group by 66.4% when compared to I/R group at ($p < 0.05$) as shown in Fig.2.

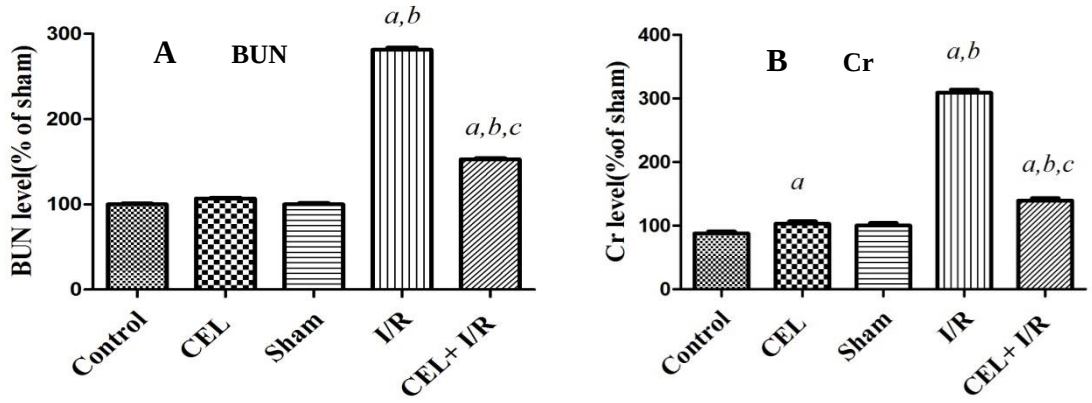

Figure 1. Effect of CEL on serum blood urea nitrogen (A) and creatinine (B). Data were expressed as mean ± S.E.M (n=6). Celastrol (CEL) was given at a dose (4mg/kg, IP, once) 1 hour before I/R. Ischemia/reperfusion (90/4 I/R) was induced at the same day, ischemia operated for 90 minutes followed by reperfusion for 4 hours. Statistical analysis was performed using ANOVA followed by Turkey's multiple comparison test. a: significantly different from the control group, b: significantly different from the sham group, c: significantly different from the I/R operated group at $p < 0.05$.

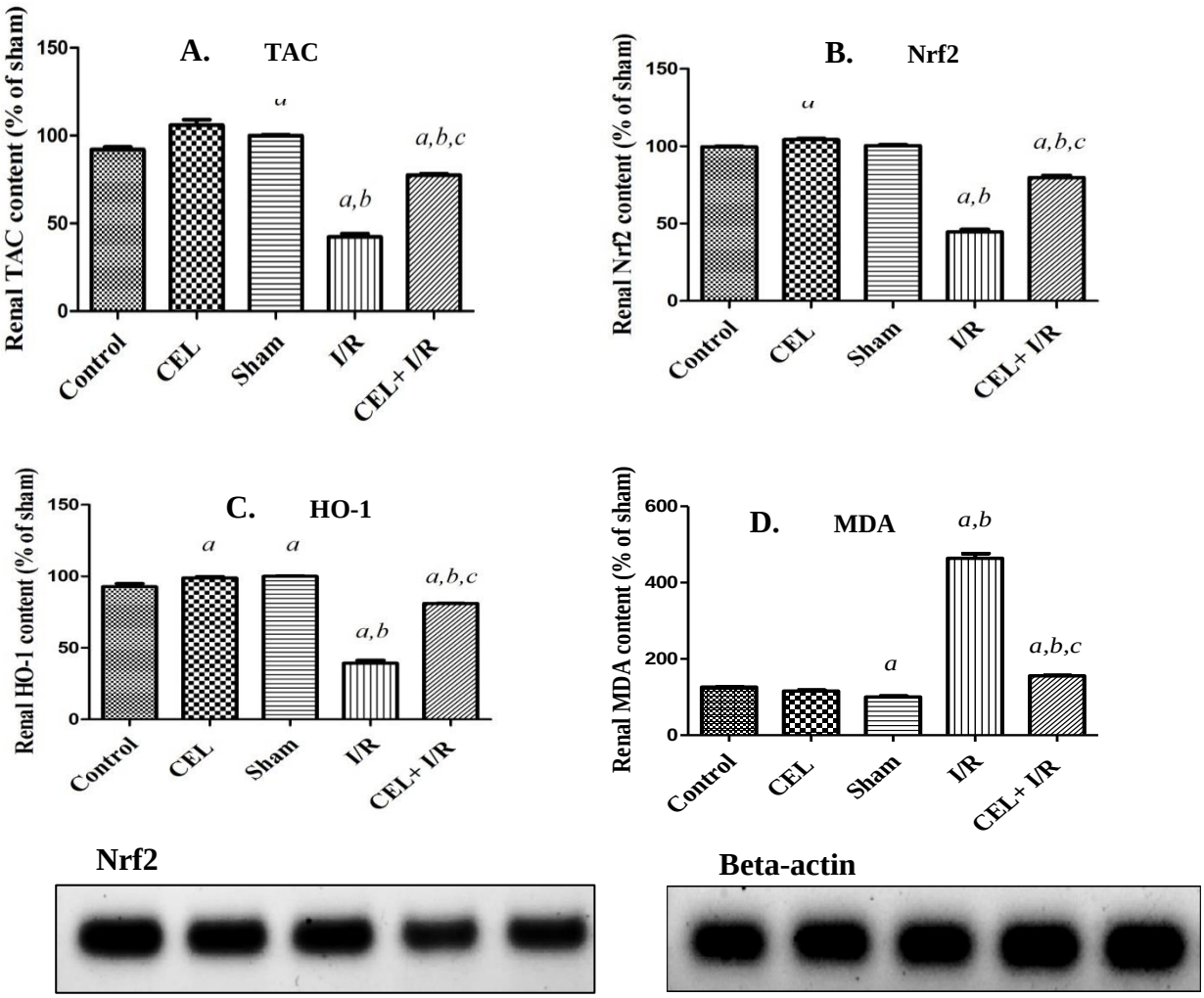

Figure 2. Effect of CEL on serum TAC (A), Nrf2 (B), HO-1 (C) and MDA (D). Data were expressed as mean ± S.E.M (n=6). Celastrol (CEL) was given at a dose (4mg/kg, IP, once) 1 hour before I/R. Ischemia/reperfusion (90/4 I/R) was induced at the same day, ischemia operated for 90 minutes followed by reperfusion for 4 hours. Statistical analysis was performed using ANOVA followed by Turkey's multiple comparison test. a: significantly different from the control group, b: significantly different from the sham group, c: significantly different from the I/R operated group at $p < 0.05$.

3.3. Effect of CEL on renal inflammatory markers (NF-κB/ NLRP3) and (HSP 90/HSF-1) signaling pathways following hepatic ischemia/reperfusion

3.3.1. Effect of CEL on NF-κB/ NLRP3 inflammasome pathway markers

Ischemia/reperfusion significantly increased the renal contents of NF-κB, NLRP3, CASP1, and IL-1β as well as MPO by about 2.5, 4.8, 2.3 and 3.7 folds respectively. However, pretreatment with CEL significantly reduced these protein contents by about 45.4%, 61.4%, 43.6%, 58% and 45.9% respectively as compared to the I/R group ($p < 0.05$) as offered in Fig. 3.1.

3.3.2. Effect of CEL on HSP 90/HSF-1 pathway markers

I/R significantly increased the renal contents of HSP 90 and HSF-1 by about 6.5 and 7.3 folds respectively, while pretreatment with CEL significantly reduced these protein contents by about 75.9% and 66.4% respectively as compared to I/R group ($p < 0.05$) as presented in Figure (3.2).

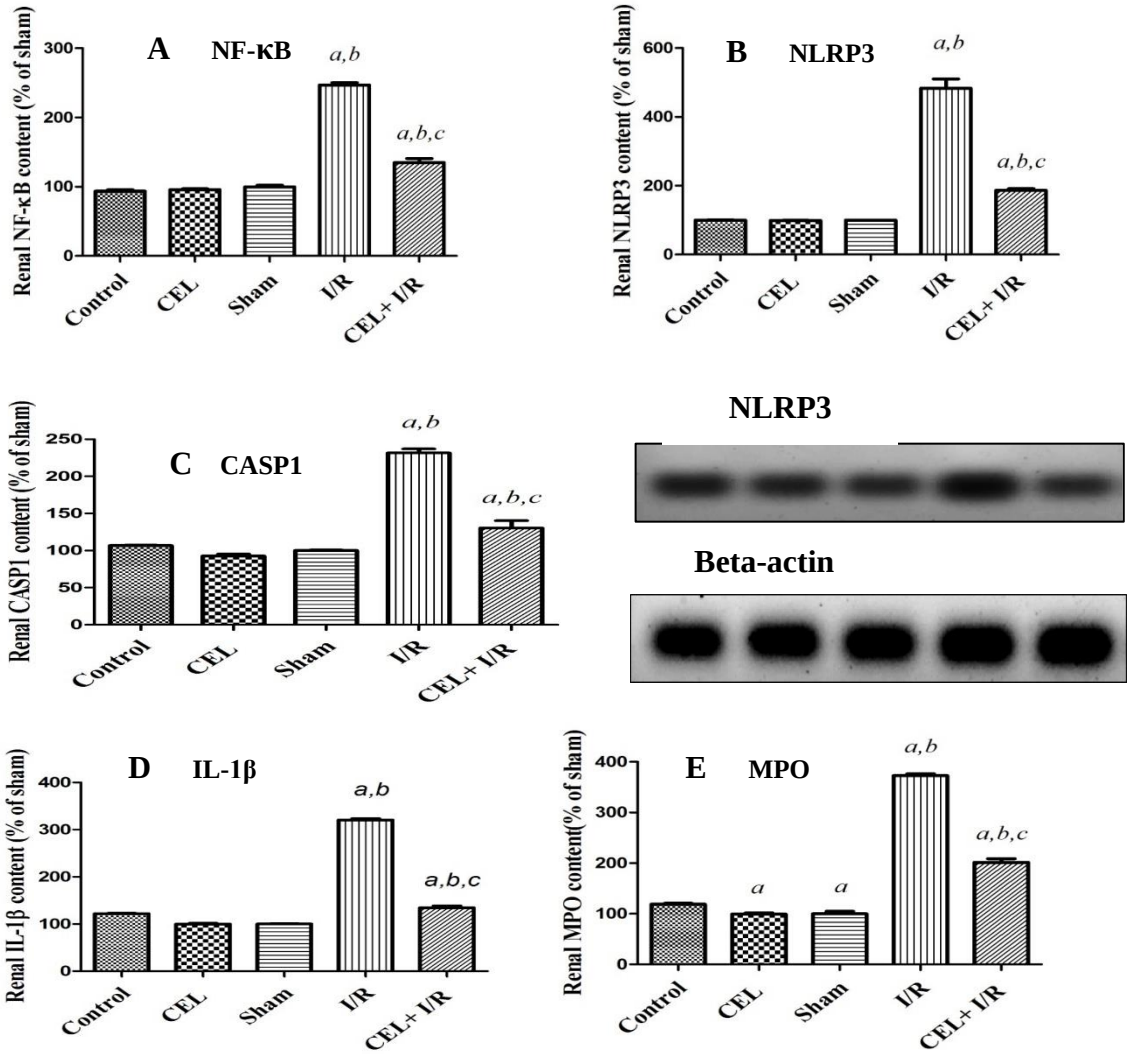

Figure 3.1. Effect of CEL on NF-κB (A), NLRP3 (B), CASP1 (C), IL-1β (D) and MPO (E). Data were expressed as mean ± S.E.M (n=6). Celastrol (CEL) was given at a dose (4mg/kg, IP, once) 1 hour before I/R. Ischemia/reperfusion (90/4 I/R) was induced at the same day, ischemia operated for 90 minutes followed by reperfusion for 4 hours. Statistical analysis was performed using ANOVA followed by Turkey's multiple comparison test. a: significantly different from the control group, b: significantly different from the sham group, c: significantly different from the I/R operated group at $p < 0.05$.

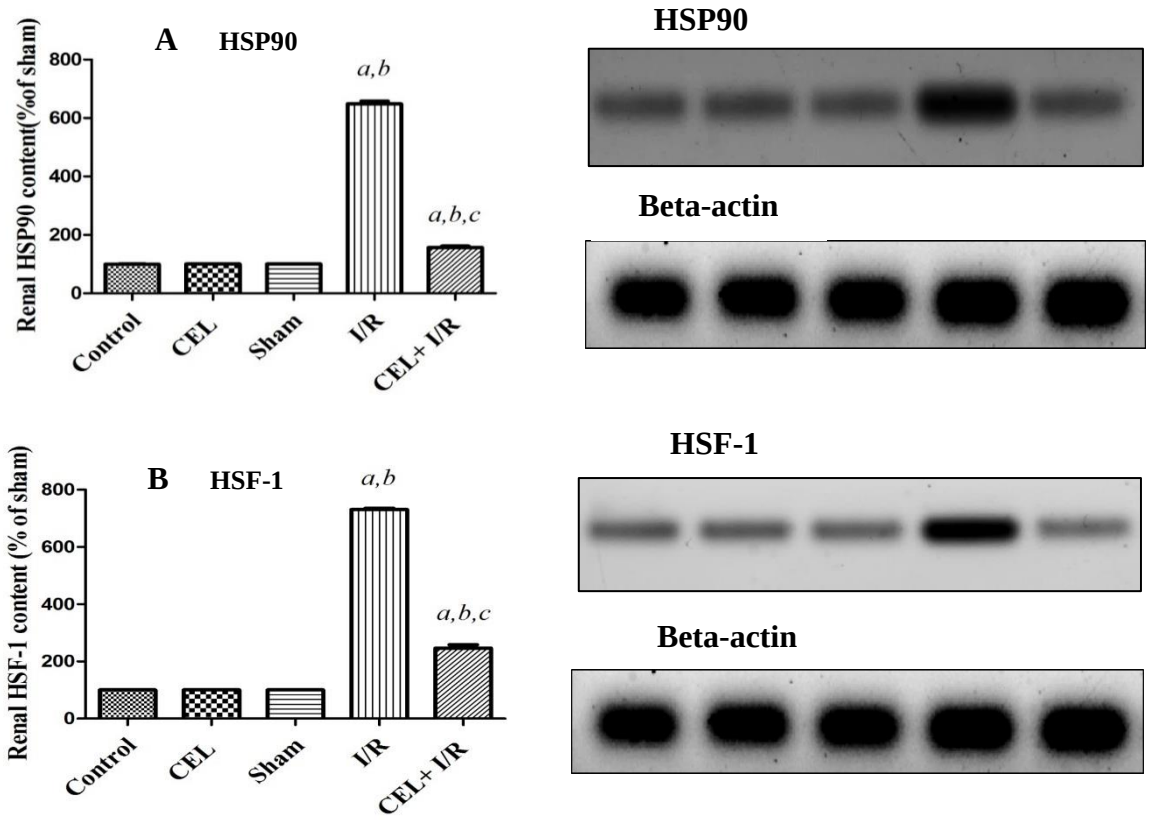

Figure 3.2. Effect of CEL on HSP90 (A) and HSF-1 (B). Data were expressed as mean ± S.E.M (n=6). Celastrol (CEL) was given at a dose (4mg/kg, IP, once) 1 hour before I/R. Ischemia/reperfusion (90/4 I/R) was induced at the same day, ischemia operated for 90 minutes followed by reperfusion for 4 hours. Statistical analysis was performed using ANOVA followed by Turkey's multiple comparison test. a: significantly different from the control group, b: significantly different from the sham group, c: significantly different from the I/R operated group at $p < 0.05$.

3.4. Evaluation of histological changes

As presented in Figure (4), kidney sections of the control, CEL and sham groups showed no histopathological changes and normal kidney structure. However, I/R group exhibited vacuolization and swelling in lining endothelium of glomerular tufts associated with focal inflammatory cells infiltration in between the tubules at the cortex, coagulative necrosis in some tubules at crticomedullary junction with cystic dilatation in others. On the other hand, protection of rats with CEL repressed the histopathological alterations induced by I/R as a marked reduction in both the vacuolization in the lining endothelium of renal glomerular tufts, and in the cystic dilatation in the tubules at the corticomedullary junction were detected, with the absence of both the inflammatory cells infiltration in between the tubules and in the coagulative necrosis were detected.

4. DISCUSSION

Hepatic IRI occurs by many clinical settings including liver resection, liver transplantation, as well as in trauma, and hemorrhagic shock [26]. Hepatic I/R results in remote organ injury, in particular, acute kidney injury (AKI) is common and greatly rises mortality and morbidity in patients [27]. During I/R liver generates ROS and pro-inflammatory cytokines that are remotely released factors strongly implicated in the establishment of AKI [27]. Celastrol (CEL) is a bioactive compound obtained from a Chinese medicinal plant named Tripterygium wilfordii [21]. CEL has been shown to afford anti-oxidant and anti-inflammatory activities [22].

In the presented work, applying 90/4 I/R in liver was resulted in remote kidney injury, this injury was confirmed by a reduction in renal functions that evidenced by the significant elevation in BUN and Cr levels in sera of the I/R group, as compared to the sham group. These findings are going with various studies in which liver I/R formed changes in renal functions [7, 27]. The elevation in BUN and Cr may be attributed to the reduction in GFR due to up-regulation of RAAS that leads to a subsequent intra-renal ischemia that eventually result in renal tubular necrosis and renal dysfunction [8]. Additionally, it may be attributed to the circulating pro-inflammatory cytokines which permit up-regulation of the adhesion molecules in kidneys resulting in leukocyte infiltration that release ROS and cytokines creating

direct renal injury [9]. In the current study pretreatment with CEL (4mg/kg, IP, once) 1 hr before liver I/R alleviated the increase in the renal functions (BUN and Cr) compared to the I/R group, which indicate improvement in renal functions. These findings are in concordance with Chu et al. [28] who demonstrated that CEL could ameliorate the acute kidney injury that was induced by renal I/R in rats and suppress the elevated renal function markers by inhibiting NF-κB activation and inflammation, which goes in harmony with the findings of Yu et al. [29] in cisplatin-induced kidney injury model.

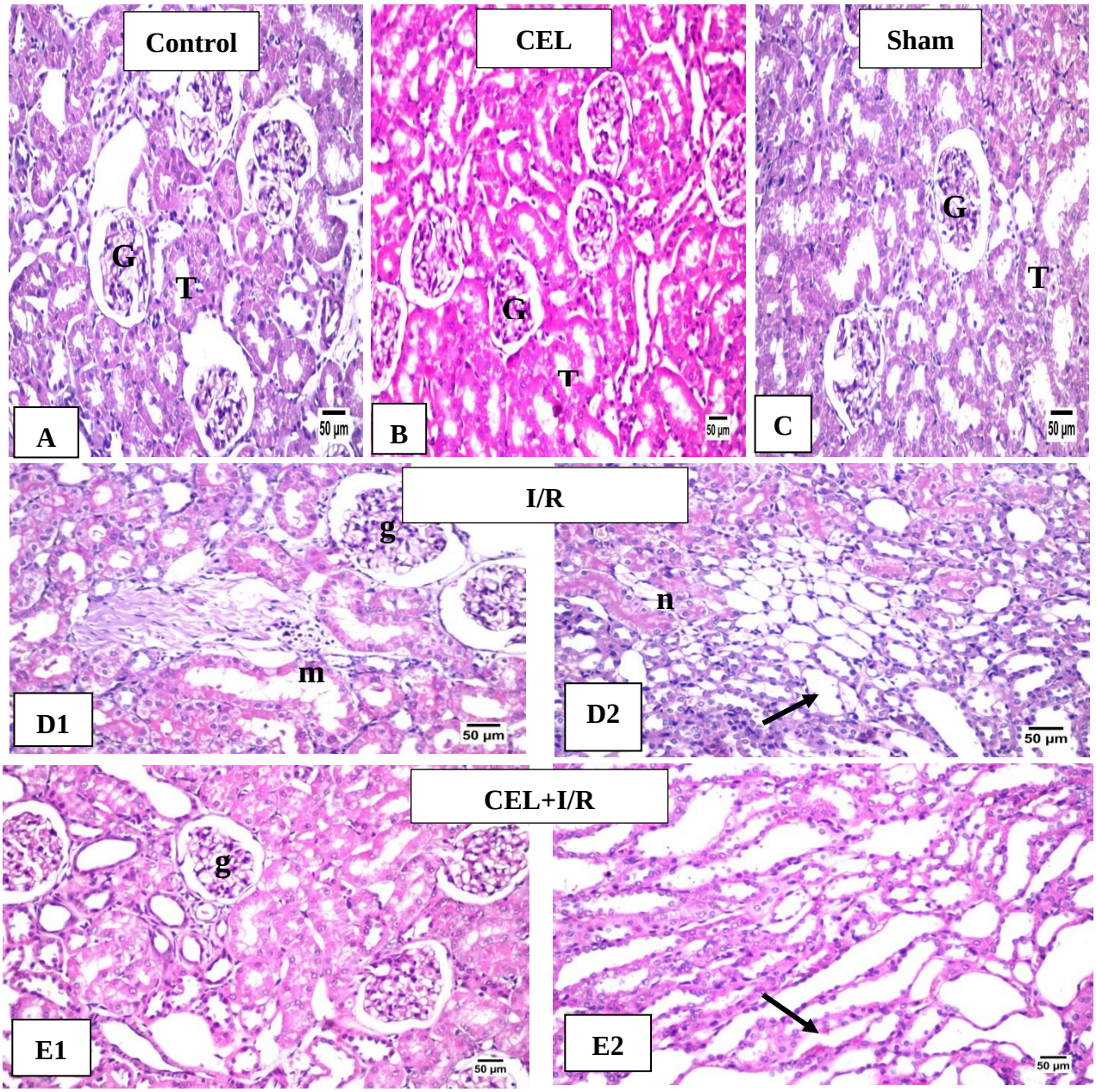

Figure 4. Photomicrograph for sections of kidney stained with H&E, ×40. Control (A): No histopathological changes in the glomeruli (G) and tubules (T) and normal kidney structure. **CEL (B):** No histopathological changes in the glomeruli (G) and tubules (T) and normal kidney structure. **Sham (C):** No histopathological changes in the glomeruli (G) and tubules (T) and normal kidney structure. **I/R (D):** Vacuolization and swelling (g) in lining endothelium of glomerular tufts associated with focal inflammatory cells infiltration (m) in between the tubules at the cortex (D1), coagulative necrosis (n) in some tubules at crticomedullary junction with cystic dilatation in others (arrow) (D2). **CEL+I/R (E):** A marked reduction in both the vacuolization (g) in the lining endothelium of renal glomerular tufts, and in the cystic dilatation (arrow) in the tubules at the corticomedullary junction, with the absence of both the inflammatory cells infiltration in between the tubules and in the coagulative necrosis.

Oxidative stress and ROS perform an important role in both hepatic damage [30] and kidney damage [31] during liver I/R. Among the important transcription factors implicated in IRI there is Nrf2 which is critical in the control of many antioxidants that keep the oxidation-reduction balance and protect from inflammation [32]. After activation by ROS, Nrf2 translocates to inside nucleus, and engines the expression of multiple protective antioxidant enzymes like HO-1 [33] and subsequently could increase TAC of the cell [34, 35]. In the present work, 90/4 I/R insult showed lessened hepatic and renal concentrations of Nrf2, HO-1, and TAC as well as elevated MDA content, which goes in harmony with the findings of Ibrahim et al. [34] who induced liver injury by liver (45/24 I/R) insult and Ragab et al. [35] who induced kidney injury by renal (45/24 I/R). Kudoh et al. [36] study showed that Nrf2 is a significant factor that is able to prevent the hepatocyte death during hepatic I/R in mice. Their study demonstrated that Nrf2 shortage aggravated OS, inflammation, and hepatocyte damage, while the activation of Nrf2 before ischemia significantly repressed OS, MDA level, and the extent of hepatocytes damage resulted from I/R. Furthermore, it reduced the activation of IκB and subsequently inhibited the nuclear translocation of NF-κB [37] which can provide an explanation for the NF-κB findings in the current study that will be illustrated later. Additionally, Younis et al. [38] showed that the stimulation of Nrf2/HO-1 signaling pathway could protect kidney from cisplatin induced nephrotoxicity in rats by exerting antioxidant and anti-inflammatory properties and increasing TAC. Another study performed by Ragab et al. [35] in renal I/R model revealed that the induced expression of Nrf2 by Nrf2 activator could enhance renal TAC, inhibit lipid peroxidation, besides inhibiting pro-activator caspase-1 via the suppression of NF-κB. Thus, establishment of effective and specific Nrf2 activator may confer a distinct strategy for renal- protection contra hepatic IRI. Despite proven renal protective role of CEL in different kidney injury models induced by renal IRI [28, 39], the present study is the first to document its impact against the remote kidney injury induced by liver I/R insult. CEL is known to stimulate the activity of the cytoprotective Nrf2/HO-1 pathway and up-regulate the expression of antioxidant enzymes [40]. Hence, pretreatment with CEL afford its anti-oxidant role, where it was able to antagonize the effect of I/R in liver and kidney by inducing Nrf2 and HO-1, enhancing TAC, and dropping lipid peroxidation. Our results concur with the study of Yang et al. [40] in which CEL has alleviated the OS and vascular calcificationin CKD rats via increaseing the mRNA and protein levels of HO-1. Another study performed by Francis et al. [41] was reported the protective role of CEL against aminoglycoside-induced ototoxicity as a result of inducing the expression of both Nrf2 and HO-1. The same study also reported that HSF-1 was not required for inducing the expression of H0-1 as it was still expressed in HSF-$1^{-/-}$ mice, suggesting that CEL induction of HO-1 in the absence of HSF-1 might be occure through activation of Nrf2. MDA is an important indicator for lipid peroxidation and cell damage under OS, and the elevation of MDA associated with the remote kidney injury suggesting excessive generation of ROS and cellular damage [7, 42]. This was shown in the current study which goes in harmony with the study of Kadkhodaee et al. [7] who performed liver I/R model for 90 min ischemia followed by reperfusion for 4 hrs and reported a significant elevation in renal MDA content. The current work showed that CEL protected groups showed a significant lowering in the increased renal MDA contents, suggesting that CEL possess a protective activity against the damage induced by I/R via hindering MDA elevation through its ability to reduce OS by increasing the intracellular antioxidants which can be confirmed by the results of TAC and HO-1 which illustrated previously. Moreover, the reduced MDA content observed in CEL protected group coincides with previous observations of Chu et al. [28] which showed reduced renal MDA content in renal (45/6 I/R) model induced in rats.

The activation of NLRP3 inflammasome has a pivotal role in inflammation [12]. Active NLRP3 forms a complex called the inflammasome which mediates caspase-1 activation. Caspase-1 rives the pro-inflammatory cytokine pro-IL-1β to active mature form, which is secreted. The released IL-1β may then activate the IL-1 receptor which amplify the inflammas0me signaling [43]. The current study demonstrated that hepatic I/R produced a salient increase in renal protein content of NLRP3, caspase-1, and IL-1β compared to the sham group. These results in accordance with Zhou et al. [44] that was reported a significant elevation of IL-1β content in renal tissue of the rats exposed to liver ischemia for 30 min followed by 24 hr of reperfusion which was thought to be mediated by the activation of NF-κB that resulted in stimulation of the glomerular cells to produce TNF-α which could impair filtration or tubular function. In the present study the increase in renal protein content of NLRP3, caspase-1, and IL-1β may be attributed to the induced expression of NF-κB observed in our results. It was reported that the activation of NLRP3 inflammasome requires 2 steps: firstly, needs an initiation signals such as interleukin-1 receptor (IL-1R) activation that activates NF-κB. This induces the expression of inflammasome components (NLRP3, pro-caspase-1

and pro- IL-1) [11]. The present study showed that rats protected by CEL showed a significant detraction in renal protein content of NF-κB p65, NLRP3, CASP1, and IL-1β. These outcomes are inconsistent with Chu et al. [28] who induced renal I/R via occlusion of the bilateral renal pedicles for 45min followed by reperfusion for 6hr in rats. In their study rats were injected with CEL 30 min before renal ischemia, which could prevent the acute kidney injury by preventing I/R-induced expression of pro-inflammatory mediat0rs via suppression of NF-κB subunit p65, decreasing the level of IL-1β, and suppressing the elevated renal function markers BUN and Cr. The accumulation of neutrophils can be confirmed by measuring the myeloperoxidase (MPO) which is considered as an indicator not only for neutrophil infiltration, which induces inflammation, but also as an index for oxidative damage [45]. In the present study hepatic IRI resulted in a significant elevation in MPO content. These results go in harmony with many of previous studies either directly [46] or remotely [47] which could be attributed to the induced expression of NF-κB and/or IL-1β that accomplished in the present work. The pretreatment with CEL could provide a significant cut down in the renal MPO content. These outcomes match with the findings of Chu et al. [28] who showed that rats protected by CEL 30 min before the insult of renal I/R for 45/6 showed a significant reduction in the MPO activity.

Heat shock protein 90 (Hsp90) is a chaperone which regulates the stability and the activation of several proteins (clients) [49]. Several regulators of signaling pathways that have critical roles in driving inflammatory responses are clients of HSP90 among which NF-κB [48] and NLRP3 [16]. Mayor et al. [49] and Piippo et al. [16] showed that in the absence of HSP90, NLRP3 becomes degraded by the proteasome. Therefore the inhibition and preventing the expression of HSP90 could lead to the degradation of both NF-κB and NLRP3 which could result in inhibiting NF-κB/NLRP3/CASP1 axis and prevent inflammation. In the present study hepatic IRI resulted in a noticeable elevation in HSP90 content, these findings go in harmony with the findings of various I/R models including myocardial I/R [50] which was attributed to the increased HSF-1, such explanation is in accordance with the HSF-1 outcomes of the present study. The same findings reported in cerebral I/R model [51] and other I/R models [52, 53]. Our findings showed that pretreatment with CEL could provide a significant cut down in renal HSP90 content. These outcomes are inconsistent with the outcomes of Aceros et al. [53] who performed combining *in vitro* cell culture using rat myocardial cell line exposed to ischemic and I/R stresse, and ex vivo Langendorff rat heart perfusion I/R model, they reported that CEL through HSP90 activity modulation could provide cardio-protection through the activation of cytoprotective HSPs such as HO-1. Such inhibitory effect of HSP90 was reported in various studies. Such inhibition may be resulted from inducing the degradation of HSP90 and its client proteins [54]. Another explanation could be obtained from the HSF-1 findings of the present study in which CEL could reduce the expression of HSF-1 which responsible for the expression of the HSPs including HSP90 [55]. Insights taken from the published data suggest that HSP90 inhibition activates HSF-1, HSP90 was proposed to sequester HSF-1 in unstressed cells [19]. Heat shock factor 1 induces the expression of other cytoprotective stress proteins such as HO-1[56]. Under unstressed conditions HSF-1 present in its inactive form in which it is united with HSP90 [57].

Stress can dissociate HSF-1 from chaperones, allowing HSF-1 activation and up-regulation of HSP90, thus enhancing the chaperone capacity and help protein correctly folding. In the present study, the 90/4 I/R resulted in increased renal contents of HSF-1. These results go in harmony with Kuboki et al. [58] who performed ischemia till 90 min and 1hr of reperfusion in mice liver and Nair and Sharma [50] in myocardial I/R model in which the ischemic stress in the heart induced an increase in HSP90 level which was directed by a simultaneous increase in HSF-1and ROS accumulation, all provide a sufficient explanation for our findings. Hence, the induction of OS by I/R which has been proven by our findings in OS markers could result in inducing the expression of HSF-1 which activated directly by OS that trigger HSF-1 homo-multimerization and activation [55] and induce the expression of HSP90.

Nevertheless, the induced expression of HSF-1 could not induce the expression of HSP32 in I/R groups. These findings may indicate that HSF-1 is responsible for the expression of HSP90 but not, at least in part, required for HSP32 expression which could be mediated mainly by Nrf2-ARE pathway that is confirmed by our Nrf2 findings. Therefore, greatly interesting is that HSF-1 and Nrf2 could compensate for one another. For instance, methionine privation was shown to enhance the HSP70 expressi0n in an Nrf2-dependending mechanism and independent 0f HSF-1 [59]. Our findings showed that pretreatment with CEL could provide a significant cut down in renal HSF-1 content. These findings may be attributed to its anti-oxidant activity which proved in the present study by the reduction of the OS markers and increasing the TAC together with induced expression of Nrf2 and its product HO-1 which possess both anti-oxidant and anti-inflammatory properties.

Finally, the present study demonstrated remote kidney injury after hepatic I/R insult that were confirmed by the histopathological examination of

kidney tissues of the I/R group. Kidney sections showed vacuolization and swelling in lining endothelium of glomerular tufts associated with focal inflammatory cells infiltration in between the tubules at the cortex, coagulative necrosis in some tubules at crticomedullary junction with cystic dilatation in others. These observations are consistent with several reported findings in which hepatic I/R caused alterations and changes in kidney histology [27, 60]. Pretreatment with CEL attenuated kidney damage induced by hepatic I/R as CEL significantly improved our histopathological alterations as compared to the control groups.

5. CONCLUSIONS

The current study highlighted many positive effects achieved by CEL on the protection of kidney in a partial hepatic I/R model. The mechanistic cassette in includes inducing the expression of the antioxidant enzyme HO-1, increasing TAC by inducing Nrf2 leading to inhibition of OS, and attenuation of the inflammatory response. Hence, we conclude that CEL could be effective prophylaxis for AKI. More investigations are required to assess the potential therapeutic efficacy of CEL using different doses and different pathways.

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Ethical Statement: The study was performed in line with the ethical rules and policies set out by the Ethics Committee of Faculty of Pharmacy, (Girls), Al-Azhar University (No.225) that is compatible with the NIH guidelines for laboratory animals.

Author Contribution: SI performed the experiment, collected the data, performed the graphical and statistical analysis, and wrote the manuscript. EM supervised the data analysis, writing, and revised the manuscript. MA shared in designing the research idea and the execution of the experiment and revised the manuscript. AA revised the manuscript.

List of Abbreviations: AKI: Acute kidney injury; BUN: Blood urea nitrogen; CASP1: Caspase-1; CEL: Celastrol; Cr: Creatinine; HO-1: Heme oxygenase-1; HSF1: Heat shock proteins; Hsp90: Heat shock protein 90; Hsps: Heat shock proteins; I/R: Ischemia/reperfusion; IL-1β: Interleukin-1 beta; IRI: Ischemia/reperfusion injury; MDA: Malondialdehyde; MPO: Myeloperoxidase; NF-κB: Nuclear factor-kappa B; NLRP3: NOD-like receptor protein-3; OS: Oxidative stress; ROS: Reactive oxygen species; TAC: Total antioxidant capacity; TNF-α: Tumor necrosis factor-alpha; TW: Tripterygium Wilfordii

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