VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 82, No. 3, pp. 668–676
DOI: 10.5603/FM.a2022.0049

ISSN 0015–5659
eISSN 1644–3284
journals.viamedica.pl

Protective effect of liriodendrin against liver ischaemia/reperfusion injury in mice via modulating oxidative stress, inflammation and nuclear factor kappa B/toll-like receptor 4 pathway

Z.Y. Yu[1], G. Cheng[2, 3, 4]

[1]*Department of General Surgery, Panjin Central Hospital, Panjin, Liaoning Province, China*
[2]*Department of Intensive Care Unit, The People's Hospital of Shimen County, Changde, Hunan Province, China*
[3]*Department of Clinical Medicine, Yiyang Medical College, Yiyang, Hunan Province, China*
[4]*Changsha Medical University, Changsha, Hunan Province, China*

[Received: 9 February 2022; Accepted: 7 April 2022; Early publication date: 28 April 2022]

Background: *The aim of the present study was to investigate the protective effect and mechanism of liriodendrin (LDN) is a lignan diglucoside in hepatic ischaemia/reperfusion (I/R) injury.*

Materials and methods: *The liver I/R was established in male C57BL/6 mice. The effect of LDN is initially investigated on hepatic I/R injury via estimating histopathology of liver. The level of metabolic enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) was studied along with apoptosis of mouse hepatocytes via TUNEL and flow cytometry analysis. The effect of LDN was investigated on oxidative stress biomarkers (glutathione [GSH] content, malondialdehyde [MDA] and superoxide dismutase [SOD] activities) and pro-inflammatory cytokines (tumour necrosis factor alpha [TNF-α], interleukin [IL]-1β and IL-6). Western blot study was also conducted to elucidate the effect of LDN on toll-like receptor 4/nuclear factor kappa B (TLR4/NF-κB).*

Results: *Liriodendrin alleviates liver I/R injury, as manifested by decreased plasma ALT, AST and ALP with improvement in liver necrotic area. LDN also reduces apoptosis of mouse hepatocytes with reduction of oxidative stress and generation of pro-inflammatory cytokines. It significantly reduces the expression of TLR4 and NF-κB.*

Conclusions: *The study demonstrated that LDN reduces liver injury and prevented apoptosis of hepatocytes following I/R injury. In addition, LDN also reduces oxidative stress, inflammation, and TLR4/NF-κB in I/R injured mice.* (Folia Morphol 2023; 82, 3: 668–676)

Key words: liriodendrin, liver ischaemia/reperfusion injury, apoptosis, inflammation

Address for correspondence: Dr. G. Cheng, Department of Intensive Care Unit, The People's Hospital of Shimen County, Changde, Hunan Province, China, e-mail: kongk1976@sina.com

INTRODUCTION

Liver ischaemia and reperfusion injury has been the topic of intense study during the last decades since it is implicated in many clinical scenarios, including haemorrhagic shock and resuscitation, trauma, liver resections [8]. It can seriously impair liver function, even producing irreversible damage, which causes a cascade of multiple organ dysfunction [1, 12]. Among the causes that affect liver, ischaemic/reperfusion (IR) injury is major cause resulting from the alteration in the blood flow with instant oxygen deficiency of cells. Although the nature of I/R injury has been studied extensively, the mechanisms by which organ damage occurs are unclear [27]. Various studies have shown oxidative stress is a main causative factor for the hepatic I/R injury. It is originated due to excessive production of reactive oxygen species (ROS) which hinder cellular redox status and innervate to hepatocyte necrosis. Furthermore, in response to stress, Kupffer–Browicz cells also mediate hepatocyte injury via the production of pro-inflammatory cytokines which further aggravate the injury [36, 39]. However, the current treatment options to manage this condition are merely supportive in nature. Thus, it is more imperative to identify novel agent which provides therapeutic support to hepatic I/R injury.

Plant-based drugs have been providing therapeutic benefit to the mankind since the ancient days. Modern science has also shown the significant benefit of plant-based drugs in many ailments, such as, cancer, diabetes, cardiovascular diseases, and so on [4]. These plant-based drugs have strong free radical scavenging and anti-inflammatory properties. Considering the importance of oxidative stress and inflammation in the hepatic I/R injury, the plant-based drugs have shown great benefit than conventional therapy and provide the impetus for the search of novel agents from the plant origin [33].

Liriodendrin (LDN) is a lignan diglucoside obtained from the inner bark of Yellow Poplar (*Liriodendron tulipifera L.*). Various studies have enumerated the pharmacological activity LDN in various *in-vitro* and *in-vivo* models [9]. It possesses anti-inflammatory, anti-nociceptive [13], anti-arrhythmic [9], hypoglycaemic [37], and inhibitory activity on HepG-2 cells [26], resisting glutamate-induced PC12 cell damage [10], increasing of heat shock factor 1 expression [22]. It also reduces sciatic endometriosis-associated pain [11].

However, despite its strong anti-oxidant and anti-inflammatory properties, none of the study has reported the effect of LDN on hepatic I/R injury. Hence, the present investigation is an attempt to study the pharmacological effect of LDN in hepatic I/R injury.

MATERIALS AND METHODS

Chemical

The LDN study was obtained from Sigma Aldrich, USA. The reagents and solvents used were procured from the Sigma Aldrich, USA and used without further purification unless otherwise stated.

Animals

Male C57BL/6 mice weighing 18–22 g (6–8 weeks) were after obtaining from the institutional animal house was housed under a controlled temperature and humidity, in strict hygienic condition. The mice were allowed to acclimatise with laboratory conditions and were supplied with food and water *ad-libitum* in alternate day and night cycle of 12 h. The study has been approved by Institutional Animal Ethical Committee for Biomedical Experiments of Changsha Medical University, China.

Establishment of the liver I/R model

A 70% liver ischaemia model was established following a previously described method [16, 17]. Briefly, ketamine (70 mg/kg body weight) and xylazine (5 mg/kg body weight) were used to anaesthetize the animals intraperitoneally, and abdominal cavity was exposed by creating the median incision in the upper abdomen. The middle lobe and the right lobe of the liver were separated, followed by the separation of the left lobe and the caudate lobe. The hepatic left artery, portal vein, and bile duct running together for about 0.5 cm, and then splitting into their respective lobe was observed. By slightly separating the posterior hepatic vein from anterior wall, hepatic artery, portal vein, and bile duct was clamped using non-invasive small blood vessel clamp in the common running section without separating them individually. After blocking the running for 60 min, the establishment of nearly 70% of the liver with ischaemia was achieved. Evidence of ischaemia was based on visualizing the pale blanching of the ischaemic lobes. The right lobe and caudate lobe of liver blood flow were not blocked to avoid gastrointestinal congestion. After 1 h of ischaemia, the clamp was removed, and reperfusion was performed. The sham group underwent the same procedure, but without clamping blood vessels. Blood and liver

tissue samples were collected at the end of the reperfusion for further analysis.

Drug treatment protocol

The mice were randomly divided into five groups (10 mice in each group): group 1: sham, group 2: I/R, group 3: LDN (5 mg/kg) + IR, group 4: LDN (10 mg/kg) + IR, group 5: LDN (20 mg/kg) + IR.

The LDN was dissolved in dimethyl sulfoxide and then diluted with normal saline. The final concentration of dimethyl sulfoxide (DMSO) was 0.4%. The LDN treated groups were gavaged with LDN for 1 week before the experiment. The sham and I/R groups received an equivalent volume of saline containing 0.4% DMSO.

Biochemical assessments

The alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels in mouse plasma were measured using commercial kits (JianCheng Bioengineering Institute, Nanjing, China) according to manufacturer's instructions.

Haematoxylin and eosin staining

Liver tissues were fixed in 10% formaldehyde solution for 24 h and embedded in paraffin. The tissue was cut into 5-μm sections and stained with haematoxylin and eosin (Servicebio, Wuhan, China). The images were photographed using a light microscope (Olympus, Tokyo, Japan).

TUNEL staining

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining was performed using an in-situ apoptosis detection kit (Servicebio, Wuhan, China), according to the manufacturer's instructions. The paraffin-embedded tissue was cut into 5-μm sections and stained with TUNEL reagents at room temperature for 1 h in the dark. DAPI (Servicebio, Wuhan, China) was used to stain the nucleus at room temperature for 10 min, followed by the addition of an anti-fluorescence quenching solution (Servicebio, Wuhan, China). Images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

Annexin V/PI assay

Primary hepatocyte isolation and culture were performed as previously described [38]. The apoptosis in hepatocytes was recorded by using Annexin V-FITC and PI (BioVision, Mountain View, CA, USA) staining as per the manufacturer instruction and analysed with a flow cytometer (BD FACS Canto™; BD Biosciences).

Estimation of oxidative stress biomarkers

The level of antioxidant biomarkers glutathione (GSH) content, malondialdehyde (MDA) and superoxide dismutase (SOD) activities were estimated using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as per the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

The determination of tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β and IL-6 were performed using commercially available ELISA kits (Thermo Fisher Scientific) as per the manufacturer's instructions.

Western blot analysis

The isolated proteins were subjected to sodium dodecyl sulphate polyacrylamide electrophoresis and transferred onto nitrocellulose membranes (Merck Millipore, Billerica, MA, USA). The membranes were blocked and incubated overnight at 4°C with primary antibodies, followed by incubation with appropriate secondary antibodies. Antibody binding was detected with ECL chemiluminescence reagents (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis

The data were articulated as mean ± standard error of mean (SEM). Statistical analysis was executed using ANOVA pursued by Bonferroni *post hoc* multiple comparison test using GraphPad Prism 5.0 (California, USA). The p value < 0.05 was measured as statistically significant.

RESULTS

LDN reduces hepatic I/R injury

The level of ALT, AST and ALP were studied in the different treated-experimental subjects and results were presented in Figure 1B–D, respectively. The histopathology of liver was also studied to determine the degree of injury in the liver and effect of LDN (Fig. 1A). As shown in Figure 1, the plasma levels of ALT, AST and ALP were found to be increased in I/R injury model group as compared to sham, which is further found in agreement with the liver injury of I/R group. The liver injury was found to be reduced significantly with considerable improvement in plas-

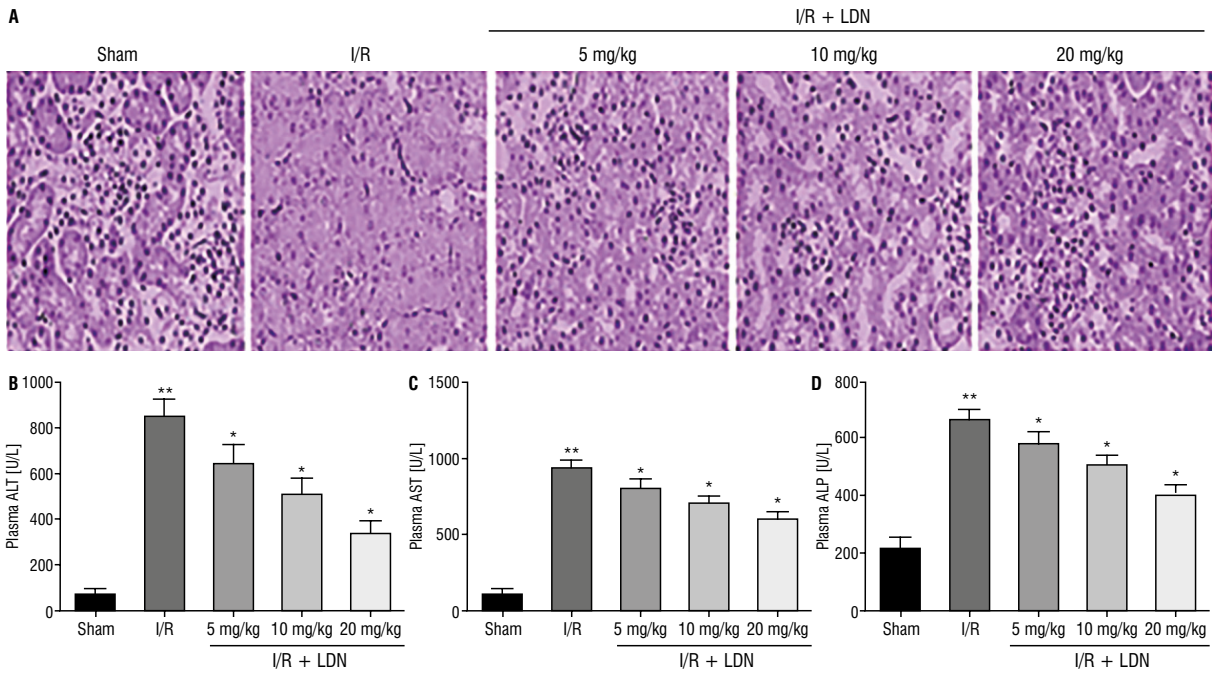

Figure 1. Liriodendrin (LDN) reduces hepatic ischaemia/reperfusion (I/R) injury histopathology of liver sections (**A**), plasma level of alanine aminotransferase (ALT) (**B**), aspartate aminotransferase (AST) (**C**), and alkaline phosphatase (ALP) (**D**). Values represent the mean ± standard error of mean; **$p < 0.05$ vs. sham; *$p < 0.05$ vs. I/R, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

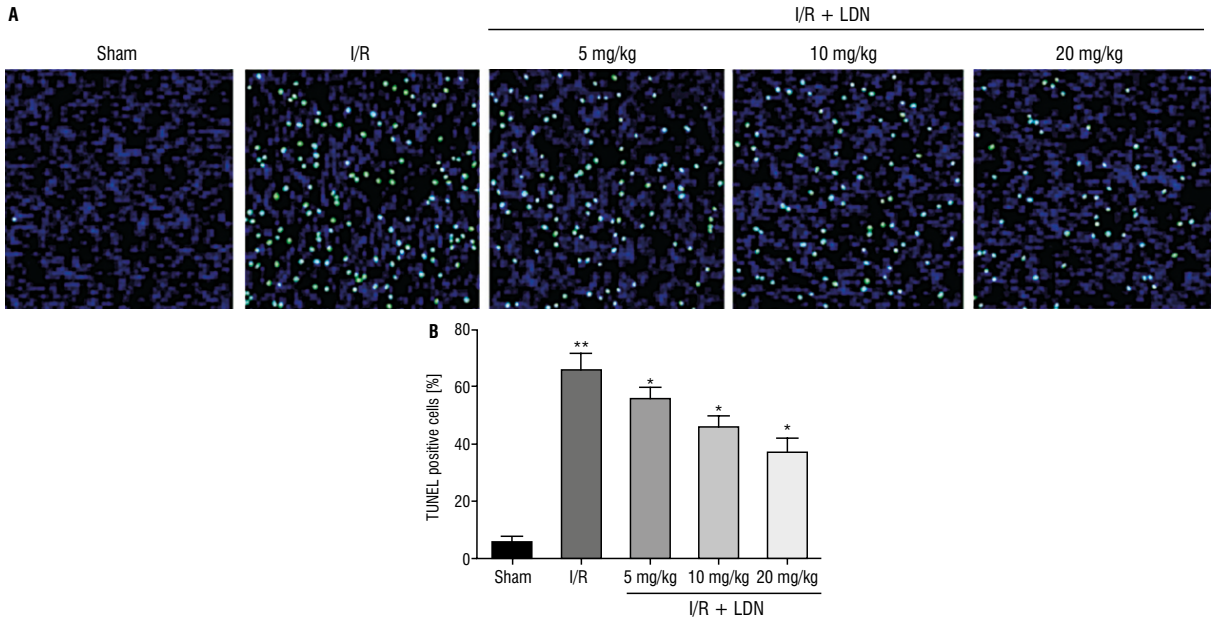

Figure 2. Liriodendrin (LDN) reduces apoptosis of hepatocytes after hepatic ischaemia/reperfusion (I/R) injury. Representative TUNEL staining of hepatocyte apoptosis (**A**) and quantification of TUNEL(+) cells (**B**). Values represent the mean ± standard error of mean; **$p < 0.05$ vs. sham; *$p < 0.05$ vs. I/R, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

ma levels of ALT, AST and ALP in LDN treated group in comparison to I/R injury. These results propose that LDN can effectively mitigate the liver injury and provide protective effect against I/R injury.

LDN reduces hepatocyte apoptosis

As shown in Figure 2, TUNEL positive cells were found significantly increased in I/R injury group as compared to sham. However, the LDN-treated group

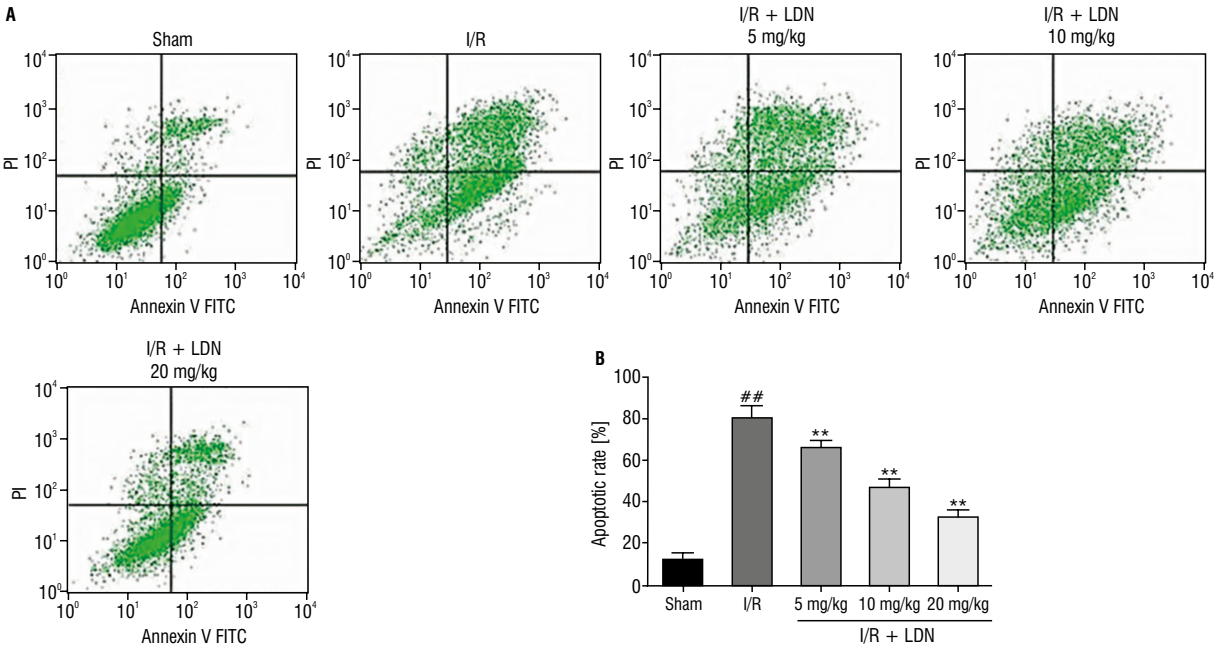

Figure 3. Liriodendrin (LDN) reduces apoptosis of hepatocytes following the hepatic ischaemia/reperfusion (I/R) injury by flow cytometry (**A**), representative bar-graph of apoptotic rate (**B**). Values represent the mean ± standard error of mean; ##p < 0.05 vs. sham; **p < 0.05 vs. I/R, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test; PI — propidium iodide; Annexin V FITC — annexin V conjugated with fluorescein isothiocyante.

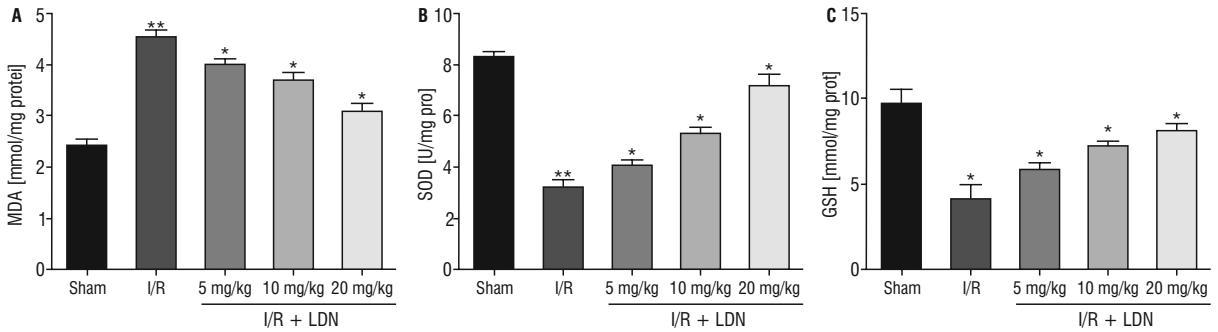

Figure 4. Liriodendrin (LDN) inhibits oxidative stress after hepatic ischaemia/reperfusion (I/R) injury. Expression of malondialdehyde (MDA) (**A**), superoxide dismutase (SOD) (**B**), and glutathione (GSH) (**C**). Values represent the mean ± standard error of mean; **p < 0.05 vs. sham; *p < 0.05 vs. I/R, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

showed dose-dependent reduction of TUNEL positive cells as compared to I//R injury group. In flow cytometry analysis (Fig. 3), similar results were obtained where LDN treated group significantly reduces apoptosis (Fig. 3). These results suggest that LDN provides significant protective effect against I/R injury in mice possibly due to inhibition of hepatocyte apoptosis.

LDN reduces oxidative stress

As shown in Figure 4, following I/R injury, the level of MDA was found elevated with reduction in SOD and GSH as compared to sham. However, in LDN treated experimental subject the level of these studied biomarker were restored near to normal in a significant way in dose-dependent manner. These results suggest that LDN significantly reduces oxidative stress following the I/R injury.

LDN reduces hepatic inflammation

The level of tested cytokines TNF-α, IL-1β, and IL-6 were found significantly elevated in I/R injury group as compared to sham. However, in LDN treated group, the levels of these cytokines were found significantly reduced as compared to I/R group (Fig. 5).

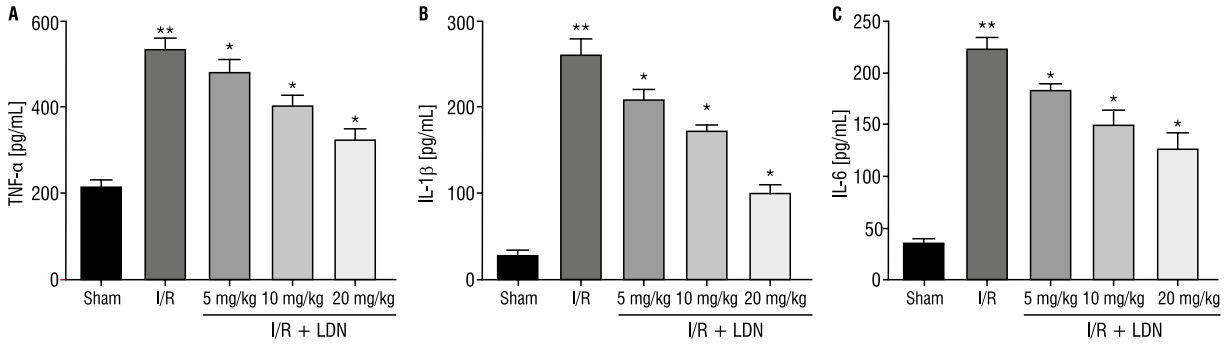

Figure 5. Effect of liriodendrin (LDN) on various cytokines tumour necrosis factor alpha (TNF-α) (**A**), interleukin (IL)-1β (**B**), and IL-6 (**C**). Values represent the mean ± standard error of mean; **p < 0.05 vs. sham; *p < 0.05 vs. ischaemia/reperfusion (I/R), one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

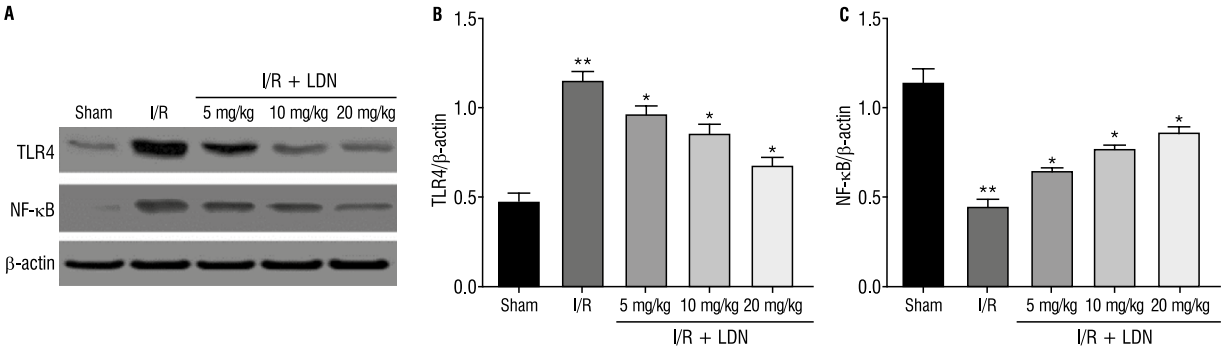

Figure 6. Effect of liriodendrin (LDN) on the toll-like receptor 4/nuclear factor kappa B (TLR4/NF-κB) by Western blot analysis (**A**), representative quantitative bar-graph for TLR4 (**B**), and NF-κB (**C**). Values represent the mean ± standard error of mean. **p < 0.05 vs. sham; *p < 0.05 vs. ischaemia/reperfusion (I/R), one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

LDN inhibited TLR4/NF-κB

As shown in Figure 6, the level of both toll-like receptor 4/nuclear factor kappa B (TLR4/NF-κB) was found significantly elevated after I/R injury in experimental subjects as compared to sham. However, LDN causes dose-dependent reduction of these tested biomarkers in treated group as compared to I/R group.

DISCUSSION

Ischaemia causes metabolic changes in hepatocytes, especially in mitochondria. After reintroduction of oxygen to ischaemic tissues, toxic ROS are released from mitochondria, mainly as the main causes of I/R injury [8]. Studies have shown that hepatic I/R injury is a multifactorial process [7]. This has put selective pressure for the discovery and development of novel agent which acts via multiple pathways to provide protective effect against I/R injury. Towards this, various natural products have shown tremendous success against hepatic I/R injury, such as hesperidin [16], methyl eugenol [32], galangin [17], curcumin [5], etc. Impressed by the above studies and excellent anti-oxidant and anti-inflammatory effect of LDN, for the first time, we have evaluated the effect of LDN in live I/R injury. Results of the present study showed that I/R induced acute kidney injury led to liver injury, which manifested itself as elevated levels of AST, ALT, and ALP as well as increased oxidative stress (MDA rise), diminished total antioxidant capacity (SOD, and GSH decline), increased expression of TLR4/NF-κB. The animals were healthy throughout the study and did not observe any mortality in them in the whole experimental protocol.

In the current study, we have found that I/R injury provoke acute hepatic injury which results in elevation of various hepatic enzymes such as, AST, ALT, and ALP. Both aminotransferases (ALT, and AST) are highly concentrated in the liver, and any an increase in serum levels is, therefore, more specific for liver damage [1]. ALP is an enzyme that transports metabolites across cell membranes. Studies have shown that these biomarkers are considered as key parameter depicting

the state of liver and their elevated levels greatly correlate with liver damage [3].

Reperfusion injury can cause liver dysfunction after cold storage and warm ischaemia [18]. Recently it has been suggested that more than 50% of hepatocytes and sinusoidal endothelial cells are undergoing apoptosis during the first 24 h of reperfusion [35]. Thus, apoptosis is the main determinant in both acute and chronic graft rejection [34]. Therefore we have studied the effect of LDN via TUNEL and flow-cytometry analysis on the apoptosis of hepatocytes in I/R injury mice. The results of the study suggested that LDN significantly reduces the apoptosis of hepatocytes. Studies have shown that compounds inhibiting apoptosis in I/R injury have significant benefit against the disease and have less changes of late allograft rejection (Acubine, and others).

A considerable number of experimental studies have indicated that I/R-induced liver injury occurs in a biphasic manner [15]. Data obtained by several different research groups suggest that in both early and late phases of reperfusion injury, oxidative stress is one of the main pathogenic mechanisms [20, 24, 30, 31]. SOD and GSH-Px are important antioxidants, and MDA is a product of lipid peroxidation. Thus, these compounds are important indicators of oxidative stress. After liver I/R injury, SOD, GSH-Px activities and cell activity decreased significantly, and MDA content was found to be increased significantly [6, 29]. In current study, LDN treatment provides a significant reduction of oxidative stress damage in the I/R injury model.

Data from murine models have indicated that liver I/R injury has hypoxic cellular stress and inflammation-mediated injury components [14, 19, 20, 31, 40]. Local circulatory damage first induces endogenous reactive oxygen species production causing hepatocyte death. This cellular damage initiates the second phase by recruiting and activating innate immune cells at the site of injury [25]. Thus, activated Kupffer cells secrete TNF-α, IL-1β, IL-6 cytokines, which is an indicator of both oxidative stress and inflammatory reaction [15]. In the present study, I/R group showed significantly higher level of these cytokines than sham group. According to the results, LDN significantly reduces the elevated levels of these cytokines in dose-dependent manner showing LDN have good anti-inflammatory effect. In order to understand the mechanism behind the protective effect of LDN, lastly, we have examined the effect of LDN on the expression of TLR4/NF-κB [21, 28]. The TLR4/NF-κB signalling pathway is a classic pathway that mediates inflammation and plays an important role in ischaemic injury of the heart, brain, liver, lung, and other important organs [2]. During liver I/R injury, ROS promote expression of the early response transcription factor IRF-1, enhance activity of histone acetyl transferase, and promotes acetylation of HMGB1. Acetylated HMGB1 is transferred from intracellular to extracellular environments, binds to TLR4 and RAGE, activates the nuclear transcription factor NF-κB P65, inhibits IκB-α kinase activity, and mediates the release of inflammatory factors TNF-α and IL-1β [23]. Results of the study suggested that was able to reduce the inflammatory response caused by liver ischaemia and reperfusion injury by inhibiting the expression of both TLR4 and NF-κB. Moreover, the current study has some limitations, first, the sample size is very low; second, other mice/rat species and different animal models may also be used to completely characterise the benefit of LDN; third, pharmacokinetic and metabolic assessment were need to be performed; fourth, the study does not provide any idea about the effect of LDN on the entry of Ca^{2+} ion, which is responsible for the progression of I/R injury; fifth, the specific mechanism through which LDN regulates mitochondria injury mediated apoptosis remains unclear and further research is needed.

CONCLUSIONS

Present study demonstrated the LDN significantly ameliorate the liver I/R injury in *in-vivo* experiment. Results suggested that LDN reduces liver injury and prevented apoptosis of hepatocytes following I/R injury. In addition, LDN also reduces oxidative stress, inflammation, and TLR4/NF-κB in I/R injured mice.

Funding

Authors are thankful to Hunan Science and Technology Project (2014SK3247) for funding this study.

Conflict of interest: None declared

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