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Colchicine Attenuates Renal Ischemia-Reperfusion-Induced Liver Damage: Implication of TLR4/NF- κ B, TGF- β , BAX and Bcl-2 gene expression

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Abstract

Background: Ischemia/reperfusion injury (IRI) is typically associated with a vigorous inflammatory and oxidative stress response to hypoxia and reperfusion that disturbs the function of the organ. The remote effects of renal IRI on the liver, however, require further study. Renal damage associated with liver disease is a common clinical problem. Colchicine, a polymerization inhibitor of microtubules has been used as an anti-inflammatory and anti-fibrotic drug for liver diseases. **Aims:** The goal of the current study was to investigate the possible protective mechanisms of colchicine on liver injury following renal IRI. **Methods:** Forty rats were divided randomly into four groups; group of sham operation, group of colchicine treated, group of IRI, group of colchicine treated-IRI. **Key findings:** Treatment with colchicine significantly reduced hepatic toll-like receptor 4 {TLR4}, nuclear factor kappa B transcription factor {NF- κ B}, myeloid differentiation factor 88 {MyD88}, and tumor necrosis factor- α {TNF- α } contents, down-regulated BCL2 Associated X Apoptosis Regulator {BAX} gene expression, transforming growth factor- β {TGF- β } content and upregulated hepatic B-cell lymphoma 2 {Bcl-2} gene expression as compared to the IRI group. Finally, hepatic histopathological examinations have confirmed the biochemical results. **Significance:** Renal IRI-induced liver damage in rats was alleviated by colchicine through its anti-inflammatory, anti-apoptotic, and anti-fibrotic actions.

Keywords: Renal ischemia-reperfusion; Colchicine; Liver; Apoptosis; TLR4.

1. Introduction

One of the major causes of acute kidney injury (AKI) which is related to a high mortality rate is ischemia-reperfusion injury (IRI) (**Malek and Nematbakhsh 2015**). The high mortality rate in AKI is primarily due to remote organ dysfunction suggesting that cellular mediators and circulating humoral originating from the ischemic tissues are the main cause of remote organ injuries (**Farag et al. 2016**). Particularly, the remote impact of AKI on the liver in particular, is an important point of concern since there is compelling evidence that AKI patients with liver damage have a poor prognosis and increased mortality (**Lane et al. 2013**).

Inflammation has been identified in renal IRI as one of the most important pathophysiological responses (**Bai et al. 2018**). Toll-like receptor 4 (TLR4) is a key regulator of nuclear factor kappa B (NF- κ B) pro-inflammatory transcription factor by activating NF- κ B nuclear translocation by mediating the myeloid differentiation primary response protein MyD88 (Myd88)-dependent early response pathway, thus inducing a sequence of inflammatory responses (**Wu et al. 2007**). Consequently, suppressing abnormal immune responses via the TLR4/NF- κ B pathway may be a successful measure for attenuating liver damage following renal IRI injury.

Renal IRI is a complex pathological mechanism with cell apoptosis included (**Zheng et al. 2018**). It is suspected that reactive oxygen species (ROS) and oxidative stress directly lead to hepatic apoptosis during renal IRI (**Golab et al. 2009**). Apoptosis is a programmed cell death process that occurs through genes regulation, extracellular molecular activation, and external factors (**Lin et al. 2014**). Many proteins and a number of genes regulate apoptosis, Bcl-2 family play essential roles in cell apoptosis, and regulation of BCL2 Associated X Apoptosis Regulator (BAX) and B-cell lymphoma 2 (Bcl-2) gene expressions can effectively reduce the apoptosis (**Hashemi 2014; Decuypere et al. 2015**).

Therefore, agents that inhibit signaling pathways of apoptosis may attenuate liver injury induced by renal IRI.

Colchicine, a microtubule-disrupting agent, is being used as an anti-inflammatory agent to treat acute gout and other inflammatory disorders such as pericarditis (**Martínez et al. 2015**). Although the mechanism of the anti-inflammatory response of colchicine is not recognized, numerous anti-inflammatory pathways of colchicine have been previously documented including the inhibition of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome protein assembly in macrophages by disrupting the formation of microtubule (**Misawa et al. 2013**), diminishes neutrophil chemotaxis, leukocyte function, and cytokine production (**Slobodnick et al. 2015**), downregulation of tumor necrosis factor-alpha receptor (TNFR) and reduce the levels of inflammatory mediators in several settings (**Wang et al. 2016**). In previous research, colchicine has been shown to have antioxidant properties in addition to anti-inflammatory properties (**He et al. 2006**). It was reported that colchicine improved both acute and chronic liver damage in carbon tetrachloride-treated rats (**Das et al. 2000**). However, it remains unclear if colchicine can ameliorate or avoid renal IRI-induced liver damage. The objective of the present study was to investigate whether colchicine could protect against liver tissue damage after renal IRI and study the possible protective mechanisms of colchicine.

2. Material and methods

2.1 Animals

Forty Sprague Dawley male rats, weighing 200-250g were used. Rats were purchased from The Nile Co. for Pharmaceuticals and Chemical Industries, Cairo, Egypt. They were placed at a constant temperature of $25 \pm 1^{\circ}\text{C}$. Rats were housed under the same adequate conditions and provided standard diet pellets with their daily dietary requirements and ad-libitum was provided with water. The study was performed in agreement with ethical guidelines and policies approved by the Ethics Committee of Faculty of

Pharmacy (Girls), Al-Azhar University (No. 141/2017), which is in harmony with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press).

2.2 Drugs and Chemicals

Colchicine which was used in this study was obtained from Sigma Chemical Co. (St. Louis, MO, USA). It was dissolved in saline. All other chemicals and solvents were of the highest quality.

2.3 Experimental Design

Rats were divided into four groups randomly (10 rats/each): group I (Sham): rats undergone sham surgery without induction of ischemia; group II (colchicine-treated group): rats received colchicine (60µg/kg/day, I.P), for seven consecutive days (Kim et al. 2018); group III (IRI): rats subjected to renal IRI for 45min followed by 24 h of reperfusion; group IV (Colchicine + IRI): rats received colchicine (60µg/kg/day, I.P), for seven consecutive days, one hour after the last dose of colchicine rats subjected to renal IRI for 45min followed by 24 h of reperfusion.

Animals were sacrificed by decapitation at the end of the reperfusion period, and the liver of each rat was separated, one- half of hepatic tissues were fixed in 10% phosphate-buffered formalin for histological analysis, and the remainder of liver tissues were homogenized in saline; the homogenates have been used to assess the inflammatory mediators (TLR4/NF-κB) signaling pathway, MyD88 content, TNF-α; apoptotic markers (Bcl-2 and BAX gene expressions), and fibrotic marker (TGF-β) content.

2.4 Induction of renal ischemia-reperfusion

Rats fasted with free access to water overnight, they were anesthetized with chloral hydrate (400 mg/kg, I.P). To hold the rectal temperature steady (37°C) during the surgery, the anesthetized rat was placed on a heating pad. A midline laparotomy was done and the renal arteries were carefully isolated from the tissues around them. Renal arteries were occluded by non-traumatic microvascular clips for 45min in the

renal IRI groups, then the clamps were removed, and the kidneys were checked to undergo reperfusion for 24 h according to the method described by **Chok et al. (2010)**.

2.5 Biochemical parameters:

2.5.1 Inflammatory markers assessment

The liver homogenates contents of TLR4, MYD88, NF- κ B, and TNF- α were determined using the TLR4 ELISA Kit (MyBioSource, Inc., SanDiego, USA, Cat. No. MBS705488), MYD88 ELISA Kit (MyBioSource, Inc., SanDiego, USA, Cat. No. MBS1600660), NF- κ B ELISA Kit (MyBioSource, Inc., SanDiego, USA, Cat. No. MBS722386), and TNF- α ELISA Kit (Cusabio, Houston, Texas, Cat. No. CSB-E11987r) respectively, according to the manufacturer's guidelines.

2.5.2 Apoptotic markers assessment

The level of Bcl-2 and BAX mRNA expressions were determined in the hepatic tissue using beta-actin as a housekeeping gene using quantitative real-time PCR (qRT-PCR) via SYBR Green Kit (**Pfaffl 2001**). The relative gene expressions were determined using the $2^{-\Delta\Delta CT}$ method and the gene-specific primer pairs were shown in table (1).

Table 1: The primer sequence of the studied gene:

Primer sequence	
Forward primer: 5'-TGTGGATGACTGACTACCTGAACC-3'	Bcl-2
Reverse primer: 5'-CAGCCAGGAGAAATCAAACAGAGG-3'	
Forward primer: 5'-CGGCGAATTGGAGATGAACTGG-3'	BAX
Reverse primer: 5'-CTAGCAAAGTAGAAGAGGGCAACC-3'	
Forward primer: 5'-AAGATCCTGACCGAGCGTGG-3'	β-actin
Reverse primer: 5'-CAGCACTGTGTTGGCATAGAGG-3'	

2.5.3 Fibrotic markers assessment

TGF- β content in liver homogenate was determined by Boster Picokine™ Rat TGF beta 1 ELISA Kit (MyBioSource, Inc., SanDiego, USA, Cat. No. MBS824788) in accordance with the manufacturer's guidelines.

2.6 Histopathological examination of the liver:

Liver tissues were fixed for 24 h in 10% buffered formalin. The samples were washed, dehydrated by alcohol, cleared in xylene, and embedded in paraffin for 24 h at 56°C in a hot air oven. For histopathological analysis, 4 μ m thick sections were cut and stained with hematoxylin and eosin (Bancroft, 2008). A professional blinded observer was used to perform all histopathologic processing and assessment of specimens to avoid bias.

Statistical analysis

The data were interpreted as mean $\pm$ SEM and several comparisons were made using one-way ANOVA followed by Tukey Kramer as a post hoc test at $p < 0.05$. The software GraphPad Prism (ISI®, USA) (version 5) was used to conduct all statistical analyses and graphs.

3. Results

3.1 Effect of colchicine on hepatic {TLR4/NF- κ B} inflammatory signaling pathway in renal I/R-induced liver injury in rats: As shown in fig. 1 (a, b, c, d), renal IRI for 45 min accompanied by 24 h of reperfusion produced a significant elevation in the contents of hepatic protein of TLR4, MyD88, NF- κ B, and TNF- α by 253.22%, 156.11%, 81.92%, and 478.52% respectively, compared to the sham group. On the other hand, colchicine pretreatment (60 μ g/kg, I.P) for seven days significantly reduced the hepatic contents of TLR4, MyD88, NF- κ B, and TNF- α by 47.60%, 38.44%, 25.88%, and 68.52% respectively, compared to the IRI group.

3.2 Effect of colchicine on hepatic apoptotic markers {Bcl-2, BAX} gene expressions in renal I/R-induced liver injury in rats: As shown in fig. 2 (a, b), renal IRI for 45 min accompanied by 24 h of reperfusion produced a significant reduction in hepatic Bcl-2 by 78.36% and a significant elevation in BAX gene expressions by 462.06% compared to the sham group. On the other hand, colchicine pretreatment (60µg/kg, I.P) for seven days significantly elevated hepatic Bcl-2 by 187.72% and significantly reduced hepatic BAX gene expressions by 67.34% compared to the IRI group.

3.3 Effect of colchicine on hepatic fibrotic markers ((TGF-β) content in renal I/R-induced liver injury in rats: As shown in fig. 3 renal IRI for 45min accompanied by 24 h of reperfusion produced a significant elevation in hepatic TGF-β content by 155.66% compared to the sham group. On the other hand, colchicine pretreatment (60µg/kg, I.P) for seven days significantly reduced hepatic TGF-β content by 44.50% compared to the IRI group.

3.4 Histopathological alterations in the liver: As shown in fig. 4 (a, b, c, d), hepatic tissue of sham and colchicine-treated groups (60µg/kg, I.P), for seven days showed no histopathological alteration in the central vein and surrounding hepatocytes. However, renal IRI induced extensive changes in hepatic tissues with the fatty change in the hepatocytes surrounding the dilated central veins and congestion in the portal vein. Finally, colchicine pretreatment (60µg/kg, I.P), for seven days before induction of renal IRI improved hepatic histopathological damages induced by renal IRI as compared to the IRI group.

These data are summarized in table 2 as follow:

Groups		Sham	Colchicine	IRI	Colchicine + IRI
Histopathological lesions					

Fatty changes in hepatocytes	-	-	++	+
Congestion	-	-	++	-

++: Moderate +: Mild -: Nil

Table 2: The protective effect of colchicine on histopathological examination of hepatic tissues in renal I/R-induced liver injury in rats.

4. Discussion

Remote organ damage is one of the complications which are produced following ischemia-reperfusion initiated AKI, dramatically increasing the mortality rate (Mohammadi et al. 2019). The liver is particularly susceptible to an inflammatory attack from the distant renal IRI because it is extensively perfused with systematic circulation. It is accepted that remote liver damage is initiated and supported by proinflammatory cytokines that are released after renal IRI. If the liver repair and regenerative mechanisms are not quickly triggered, acute functional impairment of the liver could occur (Zhao et al. 2018). Renal IRI processes tend to be multifactorial and interdependent including hypoxia, development of ROS with oxidative stress, overproduction of cytokines, and inflammatory responses to eventual cell death (Bonventre and Yang 2011; Malek and Nematbakhsh 2015).

Colchicine, an inhibitor of microtubule polymerization, has an anti-inflammatory property and is widely used to relieve the IRI in the intestine (Yurttutan et al. 2013), myocardium (Bakhta et al. 2018), cerebral tissue (Pratt et al. 1994), and ovarian tissue (Kurt et al. 2015). Although colchicine's mechanism of anti-inflammatory action is not exactly known, it is recognized that it induces TNFR downregulation, inhibits the activation of phospholipase, and decreases neutrophil chemotaxis, leukocyte function, and cytokine formation (Kurt et al. 2015). Colchicine has been used as an anti-inflammatory and anti-fibrotic drug for liver diseases (Lee et al. 2004). Therefore, the potential protective effect of colchicine on liver injury following renal IRI was studied.

Our previous study showed that after ischemia and reperfusion of the kidneys, remote liver damage is observed, as shown by a decline in liver function as indicated by a significant elevation in serum ALT and AST activities and colchicine pretreatment with (60µg/kg, I.P) for seven days significantly enhanced liver function as compared to the sham group (**Awad et al. 2020**).

Renal IRI increases oxidative stress and lipid peroxidation in hepatic tissue which may play a significant role in the activation and expression of the TLR4 signaling pathway, leading to extreme infiltration of inflammatory cells (**Yildirim et al. 2003; Bergler et al. 2013**). Once stimulated, several intracellular adaptor molecules, including MyD88, are associated with TLRs to activate a signaling cascade that activates pro-inflammatory transcription factors, such as NF-κB and TNF-α (**Arumugam et al. 2009; Bai et al. 2018**).

TLR4/NF-κB is a classical inflammatory signal, and liver injury is associated with high expression of TLR4/NF-κB. It has been established that therapeutic intervention of the expression of TLR4 and NF-κB may enhance liver function to a limited degree (**Xu et al. 2018**).

The current study demonstrated that renal IRI resulted in a significant elevation in hepatic protein contents of TLR4, MyD88, NF-κB, and TNF-α compared to the sham group. These results are inconsistent with **Zhao et al. (2018)**, who suggested that infiltrating monocytes or Kupffer cell activation and enhanced inflammation in remote hepatic injury is correlated with IRI in the renal graft which manifested by increased TLR4 and NF-κB expressions. However, colchicine pretreatment (60µg/kg, I.P) for seven days significantly reduced hepatic TLR4, MyD88, NF-κB, and TNF-α contents as compared to the IRI group (**figure 5**).

It is thought that oxidative stress and ROS directly contribute to hepatic apoptosis during renal IRI (**Golab et al. 2009**). Production of ROS during reperfusion is the main cause of uncontrolled oxidative

stress, which contributes to changes in Bcl-2 and BAX expressions, which are markers of mitochondrial dysfunction. In cell apoptosis, Bcl-2 family members play fundamental roles, and regulation of protein expressions of BAX and Bcl-2 can successfully decrease the apoptosis. There are currently two categories of the Bcl-2 family: inhibiting apoptosis such as Bcl-2 or promoting apoptosis such as BAX (**Zhang et al. 2014**). Moreover, the inflammatory response to some extent mediates the damage caused by reperfusion injury. White blood cells transported to the area by the newly returning blood, release inflammatory factors like interleukins and TNF- α (**Hu et al. 2004; Devarajan 2005**). TNF- α activates the assembly of Bcl-2 family member protein from the cytoplasm to the outer mitochondrial membrane. This induces mitochondrial swelling and induces apoptosis (**Hashemi, 2014**).

In this study, renal IRI showed a significant reduction in hepatic Bcl-2 and a significant elevation in hepatic BAX gene expressions in proapoptotic direction, while colchicine pretreatment (60 μ g/kg, I.P) for seven days significantly upregulated Bcl-2 and down-regulated BAX, elucidating the role of apoptosis in mediating the ameliorative action of colchicine in liver injury after IRI. The anti-apoptotic action of colchicine may be due to inhibiting inflammatory cytokine production TNF- α ; therefore, it decreases the inflammatory response and also can inhibit cell injury indirectly (**figure 5**). These outcomes are inconsistent with **Koenig et al. (2006)**, who demonstrated that an important agent to induce cell death after ischemia-reperfusion is cytokines TNF- α that by connecting to its specific receptor TNFR, induce cell death from the extracellular pathway. These results strongly indicate that a hepatoprotective effect of colchicine induced by renal IRI is co-related with a decrease in apoptotic cells. These findings are in accordance with **Li et al. (2002)**, who exhibited that colchicine pretreatment (60 μ g/kg, I.P) for seven days preserved the expression of Bcl-2 protein and subsequently increased the relation of Bcl-2 to BAX protein in chronic cyclosporine nephrotoxicity. Hepatic inflammation is considered a feature of early-stage fibrosis, which can lead to extensive fibrosis and cirrhosis (**Ma et al.**

2015). TGF- β 1 a key member in the TGF- β superfamily plays a vital role in hepatic fibrosis development (**Xu et al. 2016**).

TGF- β 1 has numerous biological effects such as induction of cell apoptosis, differentiation, proliferation, and epithelial-mesenchymal transformation (**Iveta et al. 2012**). Our investigation showed that hepatic TGF- β significantly increased following renal IRI compared to the sham group. Colchicine pretreatment (60 μ g/kg, I.P) for seven days significantly reduced the high level of hepatic TGF- β compared to the IRI group. The inhibitory effect of colchicine on TGF- β may be due to suppressing TLR4. These data are inconsistent with **Pulskens et al. (2010)**, who demonstrated that TLR4 exerts important functions during chronic obstructive nephropathy by exerting profibrogenic properties after unilateral ureteral obstruction (UUO) via increased TGF- β susceptibility. It was shown in a model of hepatic fibrogenesis, that TLR4 can enhance TGF- β signaling, indicating that TLR4 can act as a molecular link in liver tissue between proinflammatory and profibrogenic signals. Similarly, **Seki et al. (2007)**, showed that during chronic obstructive nephropathy, TLR4 exerts important functions by exerting profibrogenic properties after UUO through increased TGF- β susceptibility. In this way, TLR4 inhibition may be a new therapeutic strategy to inhibit chronic renal disease progression.

The anti-fibrotic activity of colchicine is evidenced by **Kim et al. (2018)**, who illustrated that colchicine pretreatment led to the down-regulation of TGF- β expression in UUO kidneys. Additionally, **Disel et al. (2004)**, indicated that colchicine suppresses TGF- β expression and decreases cyclosporine related nephrotoxicity.

Finally, the present study demonstrated that remote liver injury after renal IRI was confirmed by histopathologic alterations, such as leukocyte infiltration, fatty change in the hepatocytes surrounding the dilated central veins, and congestion in the portal vein. These results are in accordance with many

reports in which renal IRI caused changes in hepatic histology (**Golab et al. 2009; Wang et al. 2010; Farag et al. 2016**). Colchicine pretreatment (60µg/kg, I.P) for seven days may help improve renal IR-induced liver damage as colchicine extensively improved histopathologic alterations compared to the IRI group.

In conclusion, our study showed that colchicine pretreatment can protect the liver against the damages induced by renal IRI through its anti-inflammatory, anti-apoptotic, and anti-fibrotic actions. To determine the utilization of colchicine in the treatment of liver injury following renal IRI, additional studies are needed.

Declarations

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Figure captions:

Figure 1 (a, b, c, d): The protective effect of colchicine on hepatic (TLR4/NF- κ B) inflammatory signaling pathway in renal I/R-induced liver injury in rats.

Data expressed as Mean $\pm$ SEM. (n = 10).

a: Significantly different from the sham group at $p < 0.05$.

b: Significantly different from the colchicine group at $p < 0.05$.

c: Significantly different from the IRI group at $p < 0.05$.

Figure 2 (a, b): The protective effect of colchicine on hepatic apoptotic markers (Bcl-2, BAX) gene expressions in renal I/R-induced liver injury in rats.

Data expressed as Mean $\pm$ SEM. (n = 10).

a: Significantly different from the sham group at $p < 0.05$.

b: Significantly different from the colchicine group at $p < 0.05$.

c: Significantly different from the IRI group at $p < 0.05$.

Figure 3: The protective effect of colchicine on hepatic fibrotic markers (TGF- β) content in renal I/R-induced liver injury in rats.

Data expressed as Mean $\pm$ SEM. (n = 10).

a: Significantly different from the sham group at $p < 0.05$.

b: Significantly different from the colchicine group at $p < 0.05$.

c: Significantly different from the IRI group at $p < 0.05$.

Figure 4 (a, b, c, d): Photomicrographs showing the effect of colchicine on hepatic tissue following renal I/R-induced liver injury in rats stained by hematoxylin-eosin (H&E) stain (magnification $\times$

40): (a) Sham group: normal histological structure of the hepatic tissue. **(b)** Colchicine-treated groups: no histopathological alteration in the central vein and surrounding hepatocytes. **(c)** Renal IRI group: extensive changes in hepatic tissues with the fatty change in the hepatocytes surrounding the dilated central veins and congestion in the portal vein. **(d)** Colchicine treated-IRI group: improved hepatic histopathological damages induced by renal IRI.

Figure 5: Schematic diagram showing the effect of acute kidney injury induced by bilateral renal ischemia-reperfusion injury on liver dysfunction and the possible mechanisms underlying the potential effect of colchicine.

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Figure 4 (a, b, c, d): Photomicrographs showing the effect of colchicine on hepatic tissue following renal I/R-induced liver injury in rats stained by hematoxylin-eosin (H&E) stain (magnification $\times 40$): (a) Sham group; (b) Colchicine-treated groups; (c) Renal IRI group; (d) Colchicine treated-IRI group.

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