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Suppression of cirrhosis-related renal injury by N-acetyl cysteine

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ABSTRACT

Cirrhosis-induced renal injury or cholemic nephropathy (CN) is a serious clinical complication with poor prognosis. CN could finally lead to renal failure and the need for organ transplantation. Unfortunately, there is no specific pharmacological intervention against CN to date. On the other hand, various studies mentioned the role of oxidative stress and mitochondrial impairment in the pathogenesis of CN. The current study aimed to evaluate the potential protective effects of NAC as a thiol-reducing agent and antioxidant in CN. Bile duct ligation (BDL) was used as a reliable animal model of cholestasis. BDL animals received NAC (0.25% and 1% w: v) in drinking water for 28 consecutive days. Finally, urine, blood, and kidney samples were collected and analyzed. Significant elevation in serum biomarkers of renal injury, along with urine markers of kidney damage, was evident in the BDL group. Moreover, markers of oxidative stress, including reactive oxygen species (ROS) formation, lipid peroxidation, protein carbonylation, and increased oxidized glutathione (GSSG) were evident detected in the kidney of cholestatic rats. Renal tissue antioxidant capacity and reduced glutathione (GSH) were also significantly depleted in the BDL group. Significant mitochondrial depolarization, depleted ATP content, and mitochondrial permeabilization was also detected in mitochondria isolated from the kidney of cholestatic animals. Renal histopathological alterations consisted of significant tissue fibrosis, interstitial inflammation, and tubular atrophy. It was found that NAC (0.25 and 1% in drinking water for 28 consecutive days) blunted histopathological changes, decreased markers of oxidative stress, and improved mitochondrial indices in the kidney of cirrhotic rats. Moreover, serum and urine biomarkers of renal injury were also mitigated in upon NAC treatment. These data indicate a potential renoprotective role for NAC in cholestasis. The effects of NAC on cellular redox state and mitochondrial function seem to play a fundamental role in its renoprotective effects during CN.

1. Introduction

Cholestasis is a severe complication that affects not only the liver function but also could damage other organs. The kidney is the main extrahepatic organ affected by cholestasis (Fickert et al., 2013; Fickert & Rosenkranz, 2020; Krones et al., 2015, 2018). Cholestasis-induced renal injury is recognized as cholemic nephropathy (CN). The accumulation of cytotoxic compositions of the bile (*e.g.*, hydrophobic bile acids and bilirubin) in the body could severely affect renal function. Bile cast nephropathy, severe inflammation, tissue necrosis, and fibrotic changes are induced in CN (Fickert et al., 2013; Fickert & Rosenkranz, 2020; Krones et al., 2015, 2018). Although CN has a high prevalence among cirrhotic patients, there is no specific pharmacological intervention against this complication. CN could lead to fulminant renal failure and the need for organ transplantation.

Mechanistically, various studies mentioned the role of mitochondrial impairment and oxidative stress in the pathogenesis of renal injury during cholestasis (Copple et al., 2010; Orellana et al., 2000; Panozzo et al., 1995; Siavashpour et al., 2020; Sokol et al., 2006). It has been found that the activity of several antioxidant enzymes has been severely influenced by cholestasis (Ara et al., 2005; Copple et al., 2010; Orellana et al., 2000; Siavashpour et al., 2020; Sokol et al., 2006). On the other hand, mitochondrial depolarization, depleted mitochondrial ATP content, decreased dehydrogenases activity, and mitochondrial permeabilization occurred in

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CN models (Gores et al., 1998; Heidari et al., 2019; Heidari & Niknahad, 2019; Rolo et al., 2000). As the kidney is a very high energy demand organ, the energy crisis in this organ could lead to severe consequences such as electrolytes waste (Heidari, 2019; Yamaguchi et al., 2015). Actually, several cases of Fanconi syndrome (serum electrolyte disturbances) have been documented in cholestatic patients (Yamaguchi et al., 2015; Alalawi et al., 2015; Lino et al., 2005; R. A & O, 2018). Impaired cellular antioxidants function, excessive production of reactive species, and damage of different cellular components (*e.g.*, lipids and proteins) have also occurred in the kidney during cholestasis (Copple et al., 2010; Heidari et al., 2019; Heidari & Niknahad, 2019; Orellana et al., 2000; Siavashpour et al., 2020; Sokol et al., 2006). Based on these data, agents with antioxidant and mitochondria protecting properties could be potential candidates for the alleviation of CN symptoms.

N-acetyl cysteine (NAC) is an old drug known as a thiol-reducing agent (Niknahad et al., 2017; Ommati et al., 2017). NAC is clinically used as the acetaminophen antidote as well as a mucolytic agent (Mokhtari et al., 2017). On the other hand, many other pharmacological properties have been attributed to NAC (Mokhtari et al., 2017a, 2017b). This compound has been tested for neurodegenerative disorders, hepatic diseases, cardiovascular abnormalities, kidney injury, or even cosmetics (Bavarsad Shahripour et al., 2014; Dludla et al., 2018; Fishbane et al., 2004; Heidari et al., 2014; Morley et al., 2018; Tardiolo et al., 2018). The effects of NAC on cellular enzymatic and non-enzymatic antioxidants, such as its ability in scavenging reactive radicals, are involved in its antioxidant properties in biological systems (Aldini et al., 2018; Aruoma et al., 1989; Bémeur et al., 2010; Ortiz et al., 2016; Pastor et al., 1997). We recently administered this drug in cholestatic/cirrhotic animals for managing hepatic encephalopathy and locomotor activities (Mohammadi et al., 2020; Ommati et al., 2017). It was found that NAC significantly blunted oxidative stress in the brain of hyperammonemic rats (Mohammadi et al., 2020; Ommati et al., 2017). Moreover, NAC improved brain energy metabolism and locomotor activity during hepatic encephalopathy (Mohammadi et al., 2020). NAC is also able to blunt mitochondria-mediated cell death and apoptosis (Mokhtari et al., 2017a, 2017b). Beside all these effects, the antifibrotic properties of NAC also have been reported in different experimental models (Galicia-Moreno et al., 2012; Marian et al., 2006). All these data mention the potential protective effects of NAC in cirrhosis.

The current study was designed to evaluate the effects of NAC administration on jaundice-related renal injury (cholemic nephropathy; CN). Cholestasis was persuaded by bile duct ligation (BDL), and animals received NAC in their drinking water (0.25 and 1%) for 28 consecutive days. Finally, urine, blood, and kidney samples were collected and assessed. Moreover, kidney mitochondria were isolated from different experimental groups, and several mitochondrial indices were evaluated.

2. Materials and methods

2.1. Chemicals

N-chloro tosylamide (Chloramine-T), citric acid, n-propanol, p-dimethyl amino benzaldehyde, dithiothreitol, sucrose, 2,4,6-Tri(2-pyridyl)-s-triazine, thiobarbituric acid, sodium citrate, ethylenediamine tetra-acetic acid, trichloroacetic acid, 3-(N-morpholino)propanesulfonic acid (MOPS), D-mannitol, sodium acetate, potassium chloride, meta-phosphoric acid, 2-amino-2-hydroxymethyl-propane-1,3-diol-hydrochloride (Tris–HCl), were obtained from Merck (Darmstadt, Germany). N-acetylcysteine, 2′, 7′-dichlorodihydrofluorescein diacetate (DFC-DA), and reduced (GSH) and oxidized (GSSG) glutathione were purchased from Sigma–Aldrich (St. Louis, MO, USA). Kits for evaluating biomarkers of organ injury were obtained from Pars Azmun® (Tehran, Iran).

2.2. Animals

Male Sprague–Dawley rats (n = 32; 250–300 g) were obtained from Shiraz University of Medical Sciences, Shiraz, Iran. Rats were housed in a standard environment (temperature 23±1 °C, a 12 h light: 12 h dark photoschedule along with 40% of relative humidity). Animals had free access to a commercial rodent's diet (RoyanFeed®, Esfahan, Iran) and tap water. All experiments were performed in conformity with the guidelines for care and use of laboratory animals and approved by the ethics committee of Shiraz University of Medical Sciences, Shiraz, Iran (# 97-01-36-19355).

2.3. Cholestasis induction and experimental setup

Animals were anesthetized (10 mg/kg of xylazine and 70 mg/kg of ketamine, i.p), a midline incision was made, and the common bile duct was identified, doubly ligated, and cut between the ligatures (Heidari & Niknahad, 2019; Ommati et al., 2020). The sham operation consisted of laparotomy and bile duct identification without ligation (Heidari & Niknahad, 2019). Animals were equally allotted into four groups (n = 8/group). Rats were treated as follows: **1**) Sham-operated (Vehicle--treated); **2**) BDL; **3**) BDL + NAC (0.25% in drinking water); **4**) BDL + NAC (1% in drinking water) (Ommati et al., 2020; Ommati et al., 2020). Animals were assessed 28 days after BDL surgery (Cirrhosis model) (Heidari et al., 2018; Heidari & Niknahad, 2019).

2.4. Urinalysis and serum biochemistry

Urine samples were collected (200 μL) were collected during animal handling, diluted with ice-cooled normal saline (200 μL, 4 °C), and centrifuged (1000 g, 10 min, 4 °C) (Ommati et al., 2020; Ommati et al., 2020). The clear supernatant was used for urinalysis. Then, animals were deeply anesthetized (80 mg/kg, i.p), and blood samples were collected from the inferior *vena cava*. Samples were centrifuged (3000 g, 20 min, 4 °C), and the serum was collected. A Mindray® auto analyzer and commercial kits (Pars Azmun®, Tehran, Iran) were used for urinalysis and serum biochemical measurements.

2.5. Kidney histopathology and organ weight index

Samples of kidney tissue were fixed in a buffered formalin solution (10% v: v of formaldehyde in phosphate buffer, pH = 7.4). Paraffin-embedded sections of prepared tissues (5 μm) were prepared and stained with hematoxylin and eosin (H&E). Kidney fibrosis was determined by Masson's trichrome staining (Brunt, 2000; Goodman, 2007). The organs (liver, spleen, and kidney) weight indices were measured as organ weight index = [Wet organ weight (g)/Body weight (g)] × 100.

2.6. Renal reactive oxygen species (ROS) formation

Reactive oxygen species (ROS) formation in the kidney of cholestatic rats was estimated using 2′, 7′-dichlorofluorescein diacetate (DCF-DA) as a fluorescent probe (Ahmadian et al., 2018; Heidari et al., 2016; Heidari & Niknahad, 2019; Jamshidzadeh et al., 2017). Briefly, 200 mg of the kidney tissue was homogenized in 5 mL of ice-cooled Tris–HCl buffer (250 mM, 4 °C, pH = 7.4). Then, 100 μL of the tissue homogenate was added to Tris–HCl buffer (1 mL) and DCF-DA (Final concentration 10 μM) (Heidari et al., 2016). The mixture was incubated at 37 °C (15 min, in the dark). Finally, the fluorescence intensity of the samples was measured FLUOstar Omega® fluorimeter ($\lambda_{excitation}$ = 485 nm and $\lambda_{emission}$ = 525 nm) (Heidari et al., 2019; Heidari & Niknahad, 2019; Ommati et al., 2018).

2.7. Lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) were assessed as an index of lipid peroxidation in the kidney of cholestatic rats (Heidari et al., 2016; Heidari & Niknahad, 2019; Jamshidzadeh et al., 2016). Briefly, 500 μL of tissue homogenate (10% w: v in KCl, 1.15% w: v) was mixed with 1 mL of thiobarbituric acid (0.375%, w: v), and 3 mL of meta-phosphoric acid (1% w: v, pH = 2) (Heidari et al., 2016; Ommati

et al., 2019). Samples were mixed well and heated (100 °C water bath, 45 min). After cooling to room temperature, 2 mL of n-butanol was added. Then, samples were mixed well and centrifuged (10,000 *g* for 10 min) (Heidari et al., 2017; Heidari & Niknahad, 2019). Finally, the absorbance of the upper phase was measured (λ = 532 nm, EPOCH® plate reader, BioTek®, USA) (Heidari et al., 2018; Heidari & Niknahad, 2019).

2.8. Ferric reducing antioxidant power (FRAP) of the kidney

The FRAP assay was used to estimate the total antioxidant capacity of the kidney in cholestatic animals (Heidari et al., 2018; Heidari & Niknahad, 2019; Katalinic et al., 2005). Briefly, the FRAP reagent was freshly prepared by mixing 25 mL of acetate buffer (300 mM, pH = 3.6), with 2.5 mL of TPTZ (10 mM in HCl), and 2.5 mL of ferric chloride ($FeCl_3.6H_2O$, 20 mM). The kidney was homogenized in an ice-cooled 250 mM Tris–HCl buffer. Then, 100 μL of tissue homogenate (10% w: v) was added to 900 μL of the FRAP reagent (Heidari & Niknahad, 2019). The mixture was incubated at 37 °C for 5 min (in the dark). Finally, samples were centrifuged (12,000 g, 2 min, 4 °C), and the absorbance was measured at λ = 595 nm (EPOCH® plate reader, BioTek®, USA) (Alía et al., 2003; Heidari & Niknahad, 2019; Jamshidzadeh et al., 2017; Ommati et al., 2020).

2.9. Renal glutathione levels

The reduced (GSH) and oxidized (GSSG) glutathione content in the kidney of cholestatic animals was measured by the HPLC analysis of samples after derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene (DNFB). An NH_2 column (25 cm Bischoff® chromatography, Leonberg, Germany) was used as the stationary phase (Meeks & Harrison, 1991). The mobile phase consisted of buffer A (Acetate buffer: Water; 1:4 v: v) and buffer B (Water: Methanol: 1:4 v: v), and a gradient method was used with a steady increase of buffer B to 95% in 30 min. The flow rate was 1 mL/min, and the UV detector was set at λ = 254 nm (Heidari et al., 2018; Meeks & Harrison, 1991; Ommati et al., 2019). For sample preparation, kidney tissue (200 mg) was homogenized in Tris–HCl buffer (250 mM; pH = 7.4; 4 °C). Then, 500 μL of TCA (50% w: v, 4 °C) was added to 1 mL of the tissue homogenate. Samples were mixed well and incubated on ice (20 min, 4 °C). Afterward, samples were centrifuged (17,000 g, 30 min, 4 °C), and the supernatant was collected in 5 mL tubes. NaOH: $NaHCO_3$ (300 μL of the 2 M: 2 M solution) was added until the gas production was stopped. Then, 100 μL of iodoacetic acid (1.5% w: v in double-distilled water) was added, and samples were incubated in the dark (1 h, 4 °C). After the incubation period, DNFB (500 μL; 1.5% v: v in ethanol) was added and mixed. Samples were incubated in the dark (25 °C, 24 h). Finally, samples were centrifuged (17,000 g, 30 min, 4 °C) and injected (20 μL) into the described HPLC apparatus (Meeks & Harrison, 1991; Truong et al., 2006).

2.10. Kidney mitochondria isolation

The kidney was washed with normal saline (NaCl 0.9% w: v, 4 °C), and minced in the ice-cold mitochondria isolation buffer containing 70 mM mannitol, 0.5 mM EGTA, 220 mM sucrose, 2 mM HEPES, and 0.1% BSA (pH = 7.4). Minced tissue was transported into fresh isolation buffer (5 mL buffer: 1 g tissue) and homogenized. Isolated mitochondria were prepared based on the differential centrifugation method (Fernández--Vizarra et al., 2010; Niknahad et al., 2020). For this purpose, the tissue homogenate was centrifuged (1000 *g*, 20 min, 4 °C) to pellet unbroken cells and nuclei. Then, the supernatant was collected and centrifuged at 10,000 *g* (20 min, 4 °C). The second centrifugation round (10,000 *g*, 20 min, 4 °C) was repeated four times using a fresh isolation buffer medium. Final mitochondrial pellets were resuspended in a buffer (5 mL buffer/g tissue) containing 70 mM mannitol, 220 mM sucrose, and 2 mM HEPES (pH = 7.4). The mitochondria fractions used to measure mitochondrial swelling and mitochondrial depolarization were suspended in mitochondria swelling assay buffer (65 mM KCl, 125 mM sucrose, 10 mM HEPES, pH = 7.2), and depolarization assay buffer (220 mM sucrose, 5 mM KH_2PO_4, 10 mM KCl, 2 mM $MgCl_2$, 68 mM D-mannitol, 50 μM EGTA, and 10 mM HEPES, pH = 7.2) respectively (Fernández-Vizarra et al., 2010). Samples protein concentrations were determined using the Bradford method.

2.11. Mitochondrial ATP content

The kidney mitochondrial ATP level in cholestatic rats was assessed by an HPLC method (Chen et al., 2007; Heidari et al., 2018). Briefly, isolated mitochondria (1 mg protein/mL) were treated with 100 μL of ice-cooled meta-phosphoric acid (50% w: v, 4 °C), incubated on ice (10 min), and centrifuged (30 min, 17,000 *g*, 4 °C). Afterward, the supernatant was treated with 15 μL of ice-cooled KOH solution (1 M). Samples (25 μL) were injected into an HPLC system consisted of an LC-18 column (μ-Bondapak®, 15 cm). The mobile phase was composed of mono-basic potassium hydrogen phosphate (100 mM KH_2PO_4, pH = 7), 1 mM tetrabutylammonium hydroxide, and acetonitrile (2.5% v: v). An isocratic method with the flow rate was 1 mL/min was used. The UV detector was set at λ = 254 nm (Chen et al., 2007; Heidari et al., 2018).

2.12. Mitochondrial permeabilization and swelling

Mitochondrial permeabilization was assessed by analyzing the changes in the absorbance at λ = 540 nm during 30 min of incubation (30 °C) (Emadi et al., 2019; Jamshidzadeh et al., 2017; Jamshidzadeh et al., 2017). Briefly, isolated mitochondria (0.5 mg protein/mL) were suspended in swelling buffer (125 mM Sucrose, 10 mM HEPES, and 65 mM KCl; pH = 7.2), and the absorbance was monitored (30 min, 30 °C), using an EPOCH® plate reader (BioTek®, USA). A decrease in absorbance is connected with an increase in mitochondrial swelling. The results are reported as maximal mitochondrial permeabilization amplitude (ΔOD at λ = 540 nm) (Ahmadi et al., 2018; Caro et al., 2012; Heidari et al., 2018).

2.13. Mitochondrial depolarization

Mitochondrial uptake of rhodamine 123 was used for the estimation of mitochondrial depolarization (Ahmadian et al., 2017; Eftekhari et al., 2018; Heidari et al., 2018; Niknahad et al., 2017). Briefly, isolated kidney mitochondria (0.5 mg protein/mL; in the depolarization assay buffer) was incubated with 10 μL of rhodamine 123 (10 μM final concentration, 10 min, 37 °C, in the dark). Then, samples were centrifuged (17,000 g, 2 min, 4 °C) to precipitate the mitochondria fraction. Finally, the fluorescence intensity of the supernatant was measured with a fluorimeter (FLUOstar Omega®, Germany; λ_{excit} = 485 nm and λ_{em} = 525 nm) (Ahmadian et al., 2017; Ahmadian et al., 2017; Eftekhari et al., 2018; Jamshidzadeh et al., 2017).

2.14. Statistical methods

Data are given as mean ± SD. The comparison of data sets was performed by the one-way analysis of variance (ANOVA) with Tukey's multiple comparisons as the *post hoc* test. The scores of histopathological changes are given as median and quartiles (n = 8 random pictures/group). The statistical analysis of histopathological scores was performed by the Kruskal–Wallis and the Mann Whitney *U* test. Values of $P < 0.05$ were considered as a statistically significant difference.

3. Results

As NAC was supplemented in animals drinking water in the current model, the daily water consumption was assessed (Fig. 1). No significant differences in daily water consumption between different experimental groups were detected in the current model (Fig. 1). On the other hand,

the assessment of animals' weight indices revealed significant hepatomegaly and splenomegaly in cirrhotic rats (Fig. 1). On the other hand, the kidney weight index was significantly decreased in the BDL group (Fig. 1). It was found that NAC administration normalized organs weight indices in cirrhotic animals (Fig. 1). The effect of organ weight indices was not dose-dependent in the current study (Fig. 1).

Serum biochemical measurements revealed a significant elevation in serum ALT, AST, LDH, ALP, γ-GT, total bilirubin, and bile acids (Table 1). It was found that NAC (0.25 and 1% w: v in drinking water) significantly mitigated the biomarkers of organ injury in cholestatic animals (Table 1). On the other hand, it is noteworthy to mention that some markers such as bilirubin, bile acids, γ-GT, and ALP were permanently increased in the current experimental model. The effect of NAC on serum markers of organ injury was not dose-dependent (Table 1).

Serum biomarkers of renal injury, including BUN and Cr, were significantly increased in cholestatic animals 28 days after the BDL surgery (Fig. 2). Moreover, urine levels of protein, ALP, γ-GT, and glucose were significantly increased in cirrhotic rats (Fig. 2). NAC (0.25 and 1% w: v in drinking water, 28 consecutive days) significantly alleviated the biomarkers of renal injury in the serum and urine of BDL animals (Fig. 2). It is noteworthy to mention that the urine level of bilirubin and bile acids were persistently higher than the average levels in the current animal model of cirrhosis (Fig. 2). Moreover, the effect of NAC on urine markers was not dose-dependent in the current model (Fig. 2).

Markers of oxidative stress were significantly increased in the kidney of cholestatic rats (Fig. 3). Significant ROS formation, lipid peroxidation, depleted glutathione reservoirs, protein carbonylation, and decreased antioxidant capacity were detected in the kidney of BDL rats (Fig. 3). NAC treatment (0.25 and 1% w: v in drinking water) significantly decreased oxidative stress in the kidney of cirrhotic rats (Fig. 3).

Significant depletion of mitochondrial ATP content, mitochondrial depolarization, and mitochondrial permeabilization and swelling was evident in the kidney of BDL rats (Fig. 4). It was found that NAC (0.25 and 1% w: v in drinking water) significantly improved mitochondrial indices in the kidney of BDL rats (Fig. 4). The effect of NAC on renal mitochondrial indices was not dose-dependent in the current study (Fig. 4).

Tubular atrophy and interstitial nephritis were the predominant histopathological alterations in the kidney of BDL rats (Fig. 5). On the other hand, significant collagen deposition and increased hydroxyproline content were also detected in the cirrhotic animals (Fig. 5). It was found that NAC (0.25 and 1% w: v) significantly blunted kidney histopathological changes as assessed 28 days after BDL surgery (Fig. 5). The effects of NAC on CN-induced histopathological alterations was not dose-dependent in the current study (Table 2).

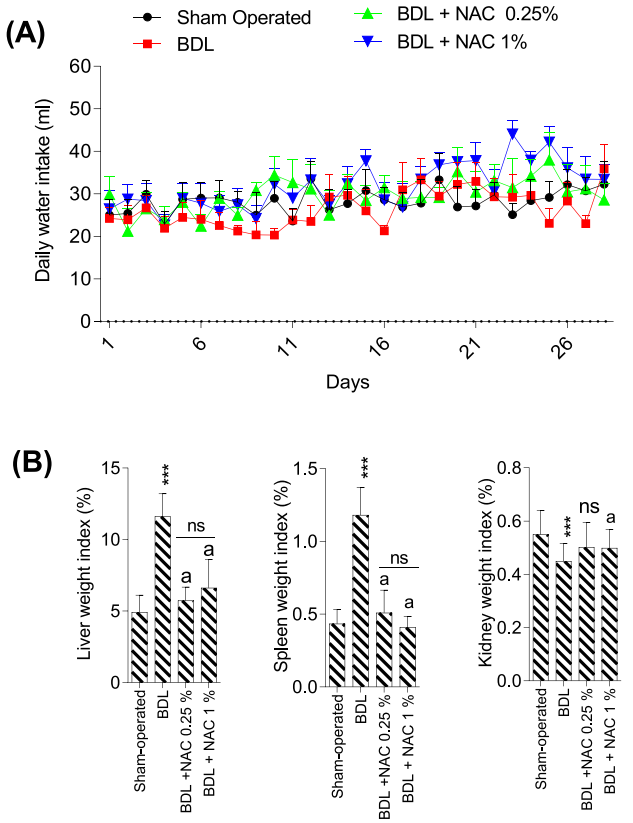

Fig. 1. Daily water intake in different experimental groups. Data are given as mean ± SD (n = 8). There was no significant difference in daily water intake between experimental groups in the current investigation (A). *** Indicate significantly different as compared with the sham-operated group (P < 0.001) (B). [a]Indicates significantly different as compared with control (P < 0.001) (B). ns: not significant.

4. Discussion

Cholestasis-induced renal injury or cholemic nephropathy (CN) is a serious clinical complication that could lead to acute kidney injury and renal failure (Panozzo et al., 1995; R. A & O, 2018; Heidari et al., 2018; Holt et al., 1999). Unfortunately, there is no pharmacological intervention to stop or blunt CN to date. In the current investigation, it was found that NAC (0.25 and 1% w: v in drinking water for 28 consecutive days) significantly mitigated CN. The effect of NAC on oxidative stress parameters, as well as the positive impact of this compound on mitochondrial function, seems to play a pivotal role in its renoprotective properties.

Although the precise mechanism(s) of renal injury during CN is far from clear, several lines of evidence indicate the fundamental role of oxidative stress in this complication (Heidari et al., 2019; Holt et al., 1999). Interestingly, the occurrence of oxidative stress has also been reported in human cases of CN (R. A & O, 2018; Lino et al., 2005; Yamaguchi et al., 2015; Martínez-Cecilia et al., 2016). Basically, it has been mentioned that oxidative stress is a general phenomenon in the cholestasis (Ljubuncic et al., 2000; Sheen et al., 2010). In the kidney tissue, the increased ROS formation depleted antioxidant capacity, and disruption of various cellular targets such as lipids and proteins has been documented (Copple et al., 2010; Orellana et al., 2000; Siavashpour et al., 2020). Cellular thiol content and thiol-related antioxidants have also impaired in the kidney tissue during cholestasis (Heidari et al., 2018; Heidari & Niknahad, 2019). Hence, administration of thiol-reducing agents such as NAC could serve as a therapeutic strategy against this complication. In the current study, we found that biomarkers of oxidative

Table 1
Serum biochemical measurements in cirrhotic rats.

Serum biomarker	Sham	BDL	BDL + NAC 0.25%	BDL + NAC 1%
ALT (U/l)	46 ± 11	295 ± 54	134 ± 19[a]	111 ± 8[a]
AST (U/l)	108 ± 12	199 ± 39*	106 ± 14[a]	121 ± 15[a]
LDH (U/l)	387 ± 148	2089 ± 707*	1121 ± 157	928 ± 116[a]
ALP (U/l)	1325 ± 443	2926 ± 539*	2443 ± 353	1947 ± 481
γ-GT (U/l)	28 ± 9	262 ± 79*	190 ± 81	265 ± 84
Total bilirubin (mg/dl)	0.1 ± 0.04	12 ± 2.3*	9.02 ± 2.04	9.6 ± 3.2
Bile acids (nmol/l)	4 ± 2	78 ± 19*	85 ± 18	67 ± 18

Data are given as mean ± SD (n = 8). The effect of NAC on serum biochemical measurements was not dose-dependent in the current study.
*Indicates significantly different as compared with the sham group (P < 0.001).
[a]Indicates significantly different as compared with the BDL group (P < 0.05).

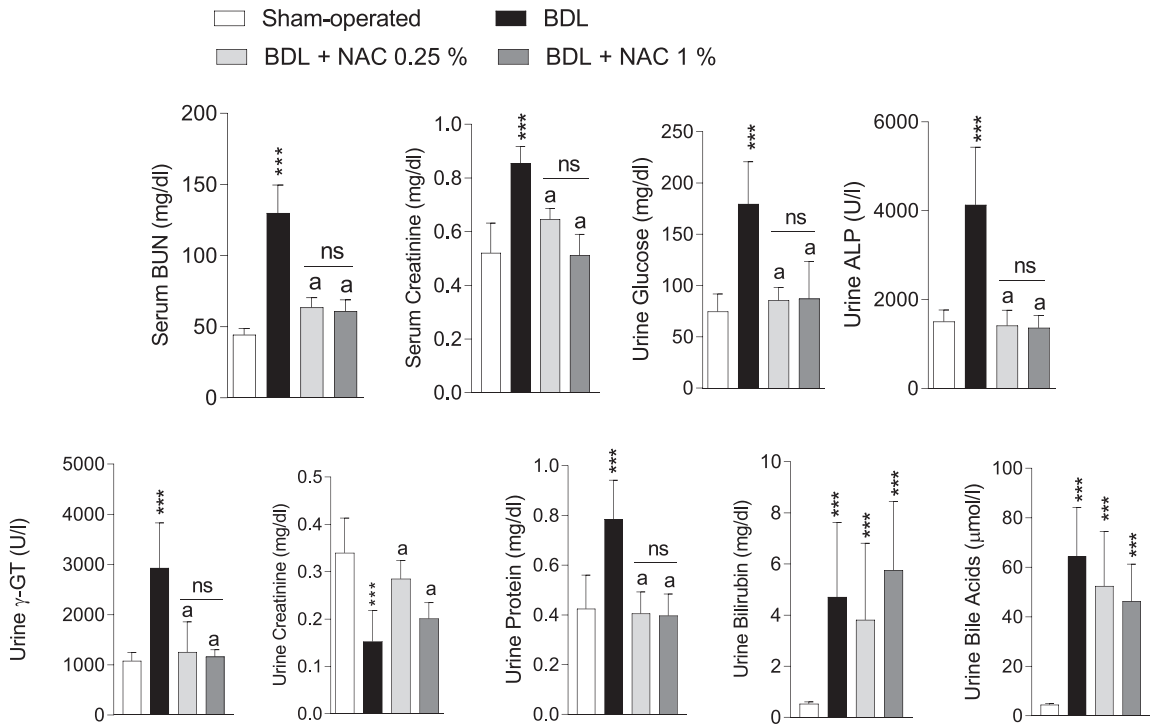

Fig. 2. Urinalysis and serum biomarkers of renal injury in cirrhotic rats. BDL: Bile duct ligation. Data are given as mean ± SD (n = 8). *** indicate significantly different as compared with sham-operated group (***P < 0.001). [a]Indicates significantly different as compared with control (P < 0.001). ns: not significant.

stress were significantly increased in the kidney of cirrhotic rats. These data are in line with previous studies in this field. On the other hand, NAC significantly decreased oxidative stress in the kidney during cholestasis. Hence, the effect of NAC on oxidative stress parameters play a fundamental role in its renoprotective properties.

Hydrophobic bile acids are the most suspected toxic molecules responsible for the oxidative stress and organ injury in CN. These molecules could directly disrupt biomembranes lipids because of their surfactant activity (Erlinger, 2014; Krones et al., 2015; Li & Chiang, 2017, pp. 143–172). On the other hand, the deactivation of the critical cellular antioxidant mechanisms also has been reported upon bile acid exposure (Orellana et al., 2000). Toxic bile acids are also able to affect protein structure and function. These events could finally lead to cell death and organ injury.

The antioxidant effect of NAC is repeatedly documented in different experimental models (Aldini et al., 2018; Aruoma et al., 1989; Bémeur et al., 2010; Ortiz et al., 2016; Pastor et al., 1997). NAC could induce its antioxidant activity through various routes (Aldini et al., 2018). First, it has been found that NAC could act as a direct radical scavenger (Aldini et al., 2018; Aruoma et al., 1989). It has been found that NAC could significantly scavenge species such as hydroxyl and hydrogen peroxide radicals (Aruoma et al., 1989). NAC is also a potent scavenger of the hypochlorous acid (HOOCl) produced by the interaction of enzymes and ROS by inflammatory cells (Aruoma et al., 1989). Second, NAC could act as a precursor for cellular glutathione (GSH) synthesis (Aldini et al., 2018). It has been found that GSH reservoirs are significantly replenished during NAC treatment (Aldini et al., 2018). Finally, NAC is a thiol-reducing agent that could reduce oxidized thiol groups (S–S cross-links) in damaged proteins (Aldini et al., 2018). This action could preserve the functionality of vital cellular proteins (Aldini et al., 2018). In the current study, we found that NAC effectively decreased markers of oxidative stress in the kidney of cholestatic animals (Fig. 3). Moreover, cellular antioxidant capacity and GSH content were preserved at a higher level in NAC-treated cholestatic rats (Fig. 3). These findings are in line with previous studies indicating the antioxidant activity of NAC in other experimental models.

Several investigations also mentioned the importance of inflammatory response in the pathogenesis of cholestasis-induced organ injury (Kosters & Karpen, 2010). In the current study, significant inflammatory cells infiltration was detected in the renal tissue of cholestatic rats (Fig. 5 and Table 2). The NFkB signaling is the major pathway involved in the inflammatory response and the expression of pro-inflammatory cytokines (Ahmadian et al., 2018; Pires et al., 2018; Zhang & Sun, 2015). Interestingly, it has been found that NAC could significantly mitigate NFkB-related mechanisms of cell death and organ injury (Zhang et al., 2018; Zmora et al., 2020). On the other hand, many other mechanisms such as lysosomal disintegrity, nuclear or mitochondrial DNA injury, and endoplasmic reticulum (ER) stress are also firmly interconnected with oxidative stress and mitochondrial impairment (Brookes et al., 2004; Tang et al., 2019). Therefore, further studies on the role of these parameters in the pathogenesis of CN could provide a better insight into the potential therapeutic options against this complication.

The positive effects of NAC on renal mitochondrial function during cholestasis was another novel finding of the current study (Fig. 4). Previously, we found that mitochondrial impairment could play an essential role in the pathogenesis of CN (Heidari et al., 2019). Cellular mitochondria are the primary sources of ROS (Brookes et al., 2004). Hence, mitochondrial impairment is firmly interconnected with oxidative stress (Brookes et al., 2004). A fundamental cause of oxidative stress in the kidney of cholestatic animals could be associated with mitochondrial impairment. On the other hand, the kidney requires a high level of ATP for proper reabsorption of chemicals. It has been found that the waste of chemical (the Fanconi syndrome) occur in cholestatic patients (Yamaguchi et al., 2015; Alalawi et al., 2015; Lino et al., 2005; R. A & O, 2018). This adverse effect could be attributed to mitochondrial impairment and the energy crisis in the kidney. The positive impact of NAC on cellular mitochondria guarantees enough level of ATP for proper renal function during cholestasis.

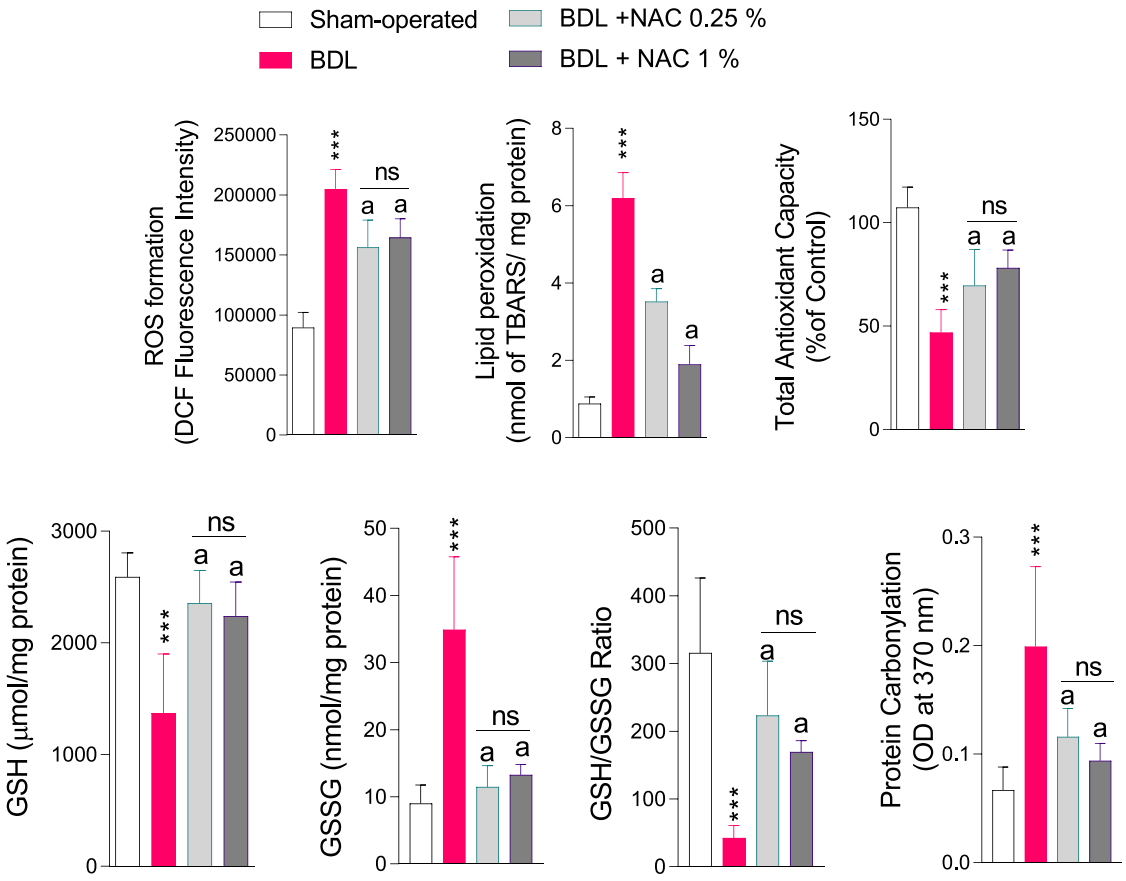

Fig. 3. Kidney oxidative stress markers in bile duct ligated (BDL) rats. NAC: N-acetyl cysteine; ROS: Reactive oxygen species; DCF: Dichlorodihydrofluorescein; GSH: Glutathione; TBARS: Thiobarbituric acid reactive substances. Data are given as mean ± SD (n = 8). ***Indicates significantly different as compared with the sham group ($P < 0.001$). [a] Indicates significantly different as compared with the BDL group ($P < 0.01$). ns: not significant.

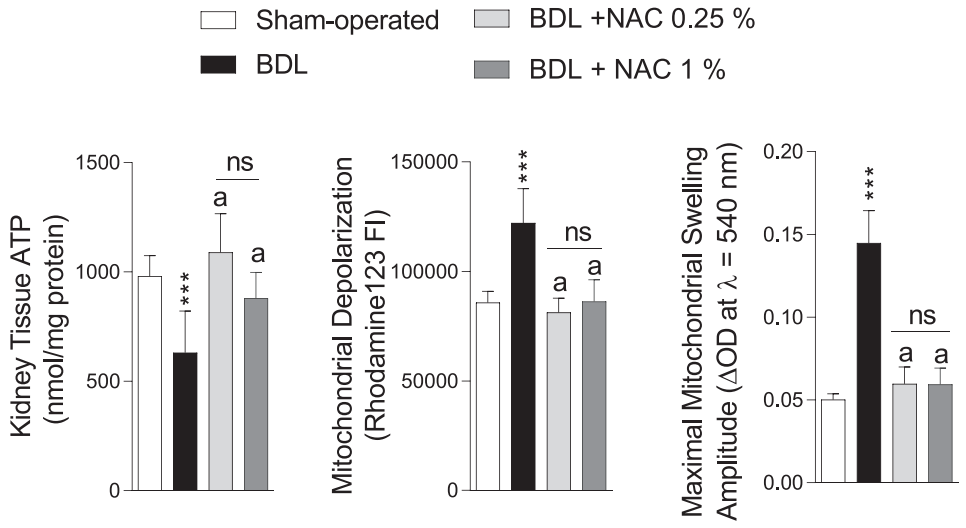

Fig. 4. Mitochondrial indices in the renal tissue of cirrhotic animals. Data are represented as mean ± SD (n = 8). ***Indicates significantly different as compared with the sham group ($P < 0.001$). [a] Indicates significantly different as compared with control ($P < 0.001$). ns: not significant.

The data obtained from this study revealed the nephroprotective effects of NAC against cholestasis-induced renal injury. The protective effects of NAC seem to be mediated by alleviating oxidative stress and modulating mitochondrial function. More investigations are warranted to reveal other cytoprotective mechanisms of NAC and, finally, its application in the clinical settings.

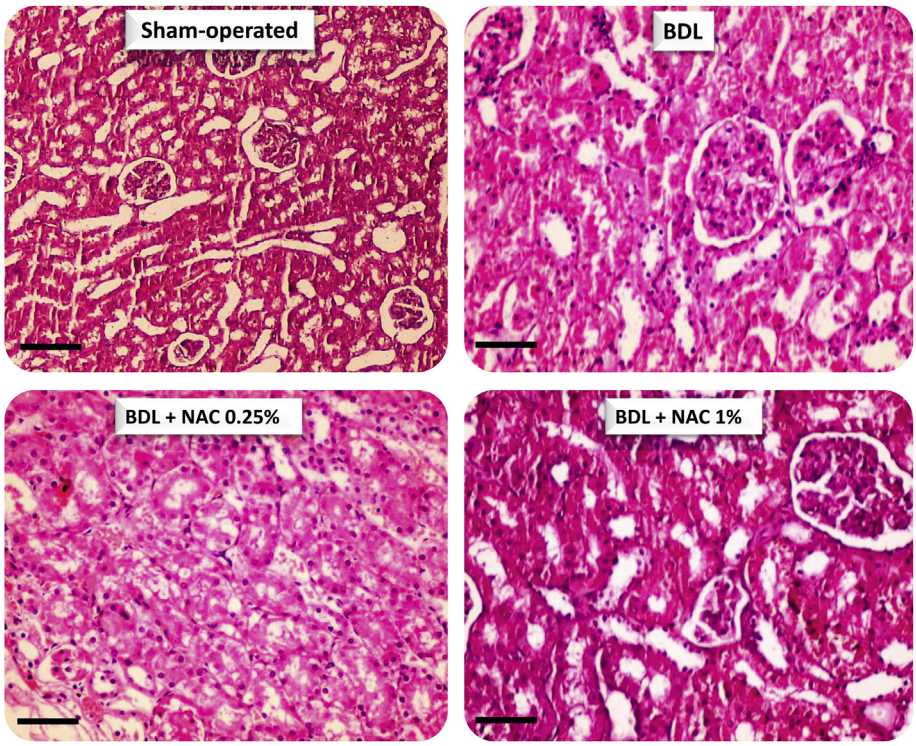

Fig. 5. Renal histopathological alterations in cirrhotic rats (H&E stain; scale bar 100 μm). Significant tubular atrophy and interstitial inflammation were revealed in the kidney of cholestatic animals. Scores of renal tissue histopathological alterations and its statistical analysis are given in Table 2.

Table 2
Grade of renal histopathological alterations in N-acetylcysteine (NAC)-treated bile duct ligated (BDL) rats.

Treatments	Interstitial inflammation	Tubular degeneration	Fibrosis
Control	0 (0, 0)#	0 (0, 0)#	0 (0, 0)#
BDL	2 (2, 2)	2 (2, 3)	3 (3, 3)
BDL + NAC 0.25%	1 (0, 1)#	1 (0, 1)#	1(0, 1)#
BDL + NAC 1%	0 (0, 0)#	0 (0, 0)#	1 (0,1)#

0 = absent; 1 = mild; 2 = moderate; 3 = severe histopathological changes.
Data are represented as median and quartiles for eight random pictures per group.
#Indicates significantly different as compared with the BDL group ($P < 0.05$).

Author contributions

Mohammad Mehdi Ommati, Khadijeh Mousavi, and Issa Sadeghian were involved in data collection and experimental setup. Narges Abdoli and Reza Heidari were involved in manuscript draft preparation and revision. Reza Heidari, Mohammad Mehdi Ommati, and Narges Abdoli performed data analysis and statistical analysis. Professor Negar Azarpira carried out histopathological evaluations. All authors read, checked, and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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