Clinical and Experimental HEPATOLOGY

Clin Exp HEPATOL 2021; 7, 1: 30–40
DOI: https://doi.org/10.5114/ceh.2021.104675
Received: 7.09.2020, **Accepted:** 30.10.2020, **Published:** 25.03.2021

Original paper

Taurine mitigates bile duct obstruction-associated cholemic nephropathy: effect on oxidative stress and mitochondrial parameters

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Abstract

Aim of the study: Cholestasis is a serious complication affecting other organs such as the liver and kidney. Oxidative stress and mitochondrial impairment are proposed as the primary mechanisms for cholestasis-induced organ injury. Taurine (TAU) is the most abundant free amino acid in the human body, which is not incorporated in the structure of proteins. Several pharmacological effects have been attributed to TAU. It has been reported that TAU effectively mitigated oxidative stress and modulated mitochondrial function. The current study aimed to evaluate the impact of TAU on oxidative stress biomarkers and mitochondrial parameters in the kidney of cholestatic animals.

Material and methods: Bile duct ligated (BDL) rats were used as an antioxidant model of cholestasis. Animals were treated with TAU (500 and 1000 mg/kg, oral) for seven consecutive days. Animals were anesthetized (thiopental 80 mg/kg, i.p.), and kidney and blood specimens were collected.

Results: Severe elevation in serum and urine biomarkers of renal injury was evident in the BDL group. Significant lipid peroxidation, reactive oxygen species (ROS) formation, and protein carbonylation were detected in the kidney of BDL animals. Furthermore, depleted glutathione reservoirs and a significant decrease in the antioxidant capacity of renal tissue were detected in cholestatic rats. Renal tubular atrophy and interstitial inflammation were evident in BDL animals. Cholestasis also caused significant mitochondrial dysfunction in the kidney. TAU significantly prevented cholestasis-induced renal injury by inhibiting oxidative stress and mitochondrial impairment.

Conclusions: These data indicate TAU as a potential therapeutic agent in the management of cholestasis-induced renal injury.

Key words: amino acids, bile acids, cirrhosis, cholestasis, nephropathy.

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Introduction

Taurine (TAU) is the most abundant free amino acid in the human body that does not become incorporated in protein structure. However, several physiological roles, such as osmoregulatory effects, have been attributed to TAU. On the other hand, it has been found that TAU significantly provided a positive impact on different diseases [1-7]. The impact of TAU on cardiovascular diseases, central nervous system (CNS) disorders, and liver damage has been widely investigated [6, 8-16]. It has also been found that TAU also significantly alleviated renal disorders [17].

The effects of TAU on reactive oxygen species (ROS) formation and oxidative stress have been mentioned as a primary mechanism for its cytoprotective properties [12-14, 18-26]. It has been found that TAU significantly mitigated oxidative stress in different experimental

models [12-14, 18-26]. On the other hand, the effects of TAU on mitochondrial function and mitochondria-associated cell injury mechanisms are among the most exciting mechanisms of cytoprotection provided by this amino acid [12, 26-41]. It has been found that TAU is essential for the proper synthesis of mitochondrial electron chain transport components, preserving mitochondrial membrane matrix pH, preventing mitochondrial depolarization, and decreasing mitochondria-mediated ROS formation [12, 26-41].

Cholemic nephropathy (CN) is a clinical complication associated with cholestasis/cirrhosis. CN could lead to renal failure or the need for organ transplantation. Although the only promising option is identifying the etiology of CN and its eradication, preserving renal function and protecting this organ during cholestasis is a critical issue. It has been evident that oxidative stress and mitochondrial impairment play a key role in the pathogenesis of renal injury in CN [32, 42-45]. Therefore, the administration of antioxidants and mitochondria protecting agents could be useful.

In the current study, TAU (500 and 1000 mg/kg, oral) was administered to cholestatic animals. Then, markers of oxidative stress and mitochondrial indices were evaluated. As TAU is a safe amino acid and could be readily administered to patients, the results of this study could help in the development of therapeutic strategies against cholestasis-induced renal injury.

Material and methods

Reagents

N-chloro tosylamide (chloramine-T), trichloroacetic acid, sodium acetate, citric acid, n-propanol, meta-phosphoric acid, p-dimethyl amino benzaldehyde, 2,4,6-Tri(2-pyridyl)-s-triazine, thiobarbituric acid, sodium citrate, ethylenediamine tetra-acetic acid (EDTA), and 2amino2-hydroxymethyl-propane-1,3-diol-hydrochloride (Tris-HCl) were obtained from Merck (Darmstadt, Germany). Taurine, 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 purchased from Pars Azmun (Tehran, Iran). All salts used for making buffer solutions were of analytical grade and purchased from Merck (Darmstadt, Germany).

Animals

Male Sprague-Dawley rats ($n = 60$, 200-250 g weight) were obtained from Shiraz University of Medical Sciences, Shiraz, Iran. Rats were housed in a standard environment (temperature of 23 ±1°C, a 12 light : 12 dark photoschedule, and 40% relative humidity). Animals had free access to a regular rat's diet (RoyanFeed, Esfahan, Iran) and tap water. All experiments were performed in conformity with the guidelines for care and use of experimental animals and approved by the ethics committee of Shiraz University of Medical Sciences, Shiraz, Iran (#97-01-36-19359).

Bile duct ligation surgery and experimental setup

Animals were anesthetized (10 mg/kg of xylazine and 70 mg/kg of ketamine, i.p.). A midline incision was made (~2 cm), and the common bile duct was localized, doubly ligated, and cut between the ligatures [46, 47]. The sham operation consisted of laparotomy and bile duct identification and manipulation without ligation. Animals were equally allotted to four groups containing 12 rats in each. Rats were treated as follows: 1) sham-operated (vehicle-treated); 2) bile duct ligated (BDL); 3) BDL + taurine (500 mg/kg, oral); 4) BDL + taurine (1000 mg/kg, oral) [48]. TAU was administered for seven consecutive days, and its effect on the cholestasis-induced renal injury was assessed [46, 49].

Organ weight index

Animals were weighed, and the organs' (liver, spleen, and kidney) weight indices were measured as organ weight index = [wet organ weight (g)/body weight (g)] × 100.

Urinalysis and serum biochemistry

Urine samples were collected during animal handling (200 µl) and diluted with 200 µl of ice-cooled normal saline (0.9% NaCl, 4°C). Samples were centrifuged (1000 g, 5 min 4°C), and the clear supernatant was used for urinalysis [50]. Then, animals were anesthetized (thiopental 80 mg/kg), and blood samples were collected from the abdominal aorta. Samples were centrifuged (3000 g, 15 min 4°C), and the separated serum was used. A Mindray auto analyzer and commercial kits (Pars-Azmun, Tehran, Iran) were used to assess biomarkers of organ injury in urine and serum of cholestatic animals [51].

Renal histopathological alterations

Samples of kidney tissue were fixed in a buffered formalin solution (10% formaldehyde in phosphate buffer, pH = 7.4). Paraffin-embedded kidney tissue

(5 μm sections) were prepared and stained with hematoxylin and eosin (H&E). Kidney and liver fibrotic changes were determined by Masson's trichrome staining in BDL rats [52, 53].

Reactive oxygen species formation

Reactive oxygen species formation in the kidney was estimated based on a previously described procedure [54-56]. Briefly, 200 mg of the kidney tissue was homogenized in 5 ml of ice-cooled Tris-HCl buffer (40 mM, pH = 7.4). Samples of the resultant tissue homogenate (100 μl) were mixed with 1 ml of Tris-HCl buffer and 2',7'-dichlorofluorescein diacetate; DCF-DA (final concentration 10 μM). The mixture was incubated at 37°C (15 min, in the dark). Finally, the fluorescence intensity of samples was assessed using a FLUOstar Omega multifunctional fluorimeter ($\lambda_{excitation}$ = 485 nm and $\lambda_{emission}$ = 525 nm) [54, 57, 58].

Lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) were measured as an index of lipid peroxidation in cholestatic rats' kidney tissue [59-61]. The reaction mixture consisted of 500 μl of tissue homogenate (10% w/v in KCl, 1.15% w/v), 1 ml of thiobarbituric acid (0.375%, w/v), and 3 ml of phosphoric acid (1% w/v, pH = 2). Samples were mixed well and heated (100°C water bath, 45 min). Then, the mixture was cooled to room temperature, and 2 ml of n-butanol was added. Samples were mixed well and centrifuged (10,000 g for 10 min) [55, 62]. Finally, the absorbance of developed color in the n-butanol phase was measured at 532 nm (EPOCH plate reader, BioTek, USA) [59, 63, 64].

Renal glutathione content

The reduced (GSH) and oxidized (GSSG) glutathione levels in the kidney of cholestatic animals were measured using a gradient HPLC method [65]. Briefly, the mobile phases consisted of buffer A (acetate buffer : water; 1 : 4 v : v) and buffer B (water : methanol; 1 : 4 v : v). There was a steady increase of buffer B to 95% in 30 min, and the flow rate was 1 ml/min was applied [65]. For tissue preparation, the kidney sample (200 mg) was homogenized in Tris-HCl buffer (250 mM, pH = 7.4, 4°C), and 500 μl of TCA (50% w/v) was added to 1 ml of the tissue homogenate. Samples were mixed well and incubated on ice (10 min). Samples were centrifuged (17,000 g, 30 min, 4°C), and the supernatant was collected in 5 ml tubes. Then, 300 μl of the NaOH : $NaHCO_3$ (2 M : 2 M) was added until the gas production was stopped. Afterward, 100 μl of iodoacetic acid (1.5% w/v in deionized 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, mixed well, and incubated in the dark (24 h, 25°C). Finally, samples were centrifuged (17,000 g, 30 min, 4°C) and injected (50 μl) into the described HPLC apparatus [65, 66]. An NH_2 column was used as the stationary phase (25 cm, Bischoff chromatography, Leonberg, Germany) and the UV detector was set at λ = 254 nm.

Ferric reducing antioxidant power of kidney tissue

Ferric reducing antioxidant power (FRAP) assay measures the formation of a blue-colored Fe^{2+}-tripyridyltriazine compound from the colorless oxidized Fe^{3+} form by the action of electron-donating antioxidants [67, 68]. In the current study, the working FRAP reagent was freshly prepared by mixing acetate buffer (10 volume of 300 mmol/l, pH = 3.6) with TPTZ (1 volume of 10 mmol/l in 40 mmol/l HCl) and ferric chloride (1 volume of 20 mmol/l $FeCl_3.6H_2O$). Kidney tissue (200 mg) was homogenized in an ice-cooled Tris-HCl buffer (250 mM Tris-HCl, pH = 7.4, 4°C). Afterward, 100 μl of tissue homogenate and 150 μl of deionized water were added to 1.5 ml of the FRAP reagent [69, 70]. The reaction mixture was incubated in the dark (37°C, 5 min). Finally, the absorbance of developed color was measured at 595 nm (EPOCH plate reader, BioTek, USA) [55, 71].

Mitochondria isolation from the rat kidney

The kidney was washed in normal saline (NaCl 0.9% w/v, 4°C) and minced in an ice-cold isolation buffer containing 70 mM D-mannitol, 220 mM sucrose, 2 mM HEPES, 0.5 mM EGTA and 0.1% BSA (pH = 7.4). Minced tissue was transported into mitochondria isolation buffer (5 ml buffer : 1 g tissue) and homogenized. The differential centrifugation method was used to isolate kidney mitochondria [26, 72, 73]. For this purpose, the kidney homogenate was centrifuged (1000 g, 20 min, 4°C) to pellet unbroken cells and nuclei. The supernatant was then further centrifuged (10,000 g, 20 min, 4°C) to pellet the mitochondria fraction. The second centrifugation step was repeated four times using a fresh mitochondria isolation buffer medium. Finally, isolated kidney mitochondria were re-suspended in a buffer (5 ml buffer/1 g tissue) containing 70 mM D-mannitol, 2 mM HEPES, and

220 mM sucrose (pH = 7.4). The mitochondria fractions used to assess mitochondrial permeabilization and mitochondrial depolarization were suspended in swelling buffer (125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH = 7.2), and mitochondria membrane potential assay buffer (220 mM sucrose, 10 mM KCl, 68 mM D-mannitol, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 50 μM EGTA, and 10 mM HEPES, pH = 7.2) [72, 74]. The protein content of the samples was determined based on the Bradford method.

Mitochondrial ATP levels

A method based on the luciferase-luciferin reaction (ENLITEN kit from Promega) was used to assess mitochondrial ATP content [26, 75]. Samples and buffer solutions were made based on the kit instructions, and the luminescence intensity of samples was measured (λ = 560 nm using a FLUOstar Omega fluorimeter) [76].

Mitochondrial depolarization assay

Mitochondrial uptake of rhodamine 123 was used to assess mitochondrial depolarization [77-79]. Briefly, kidney isolated mitochondria (0.5 mg protein/ml; in the depolarization assay buffer) were incubated with rhodamine 123 (30 min, 37°C, in the dark). Afterward, samples were centrifuged (17,000 g, 5 min, 4°C), and the fluorescence intensity of the supernatant was monitored with a fluorimeter (FLUOstar Omega, Germany; $\lambda_{excitation}$ = 485 nm and $\lambda_{emission}$ = 525 nm) [77, 80].

Lipid peroxidation in kidney mitochondria

Thiobarbituric acid-reactive substances (TBARS) were assessed in kidney mitochondria isolated from cholestatic animals. Previous studies mentioned that sucrose interrupts the lipid peroxidation test in isolated mitochondria [81]. Therefore, sucrose was removed by washing mitochondria preparation in ice-cooled MOPS-KCl buffer (50 mM MOPS, 10 μM Trolox, and 100 mM KCl, 4°C, pH = 7.4). For this purpose, 1 ml of isolated kidney mitochondria (1 mg protein/ml) was suspended in 5 ml of MOPS-KCl buffer and centrifuged (15,000 g, 20 min). The pellet was re-suspended in 1 mM of MOPS-KCl buffer and used for TBARS assay [82, 83]. For this purpose, the mitochondrial suspension (1 mg protein/ml) was mixed with 1 ml of TBARS assay reagent containing trichloroacetic acid (15% w/v), HCl (240 mM), thiobarbituric acid (0.375% w/v), and 10 μl of Trolox (500 μM). Samples were heated for 15 min at 100°C [81]. Afterward, samples were centrifuged (17,000 g, 20 min, 4°C), and the absorbance was measured at λ = 532 nm (EPOCH plate reader, BioTek Instruments, USA) [81].

Statistical methods

Data are given as mean ± SD. A comparison of data sets was performed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons as the *post hoc* test. Values of $p < 0.05$ were considered statistically significant.

Results

A significant increase in serum biomarkers of organ injury [alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH), alkaline phosphatase (ALP), γ-glutamyltransferase (γ-GT), bile acids, and bilirubin) was detected in the BDL model of cholestasis. On the other hand, serum

Table 1. Serum biochemical measurements in cirrhotic rats

Parameters assessed	Sham-operated	BDL	BDL + TAU 500 mg/kg	BDL + TAU 1000 mg/kg
ALT (U/l)	53 ±16	232 ±90	118 ±37[a]	108 ±25[a]
AST (U/l)	107 ±17	207 ±38*	128 ±27	118 ±13[a]
LDH (U/l)	493 ±169	1580 ±432*	1105 ±292	950 ±76[a]
ALP (U/l)	1265 ±332	2676 ±652*	2781 ±569	1893 ±133[a]
γ-GT (U/l)	27 ±6	242 ±82*	178 ±84	204 ±77
Total bilirubin (mg/dl)	0.1 ±0.07	11.9 ±1.30*	9.89 ±1.88	11 ±1.72
Albumin (mg/dl)	3.96 ±0.14	3.22 ±0.36*	3.5 ±0.20	3.94 ±0.54[a]
BUN (mg/dl)	30 ±12	41 ±9	42 ±11	36 ±11
Creatinine (mg/dl)	0.22 ±0.04	0.69 ±0.17*	0.3 ±0.08[a]	0.28 ±0.11[a]

Data are given as mean ± SD (n = 8). The effect of taurine (TAU) on serum biomarkers of organ injury 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 ($p < 0.05$).

creatinine as a renal injury marker was significantly higher in BDL rats. No significant BUN changes were detected seven days after the BDL operation in the current study. It was found that TAU (500 and 1000 mg/kg, seven consecutive days) mitigated serum markers of hepatic and renal injury (Table 1). Hepatomegaly and splenomegaly were also evident in BDL rats, which confirm the occurrence of cholestasis. No significant kidney weight index changes were detected seven days after the BDL surgery. TAU (500 and 1000 mg/kg, oral) significantly decreased hepatomegaly and splenomegaly in BDL rats (Fig. 1). The effect of TAU on serum biomarkers of organ injury (Table 1), as well as liver and spleen weight indices (Fig. 1), was not dose-dependent in the current study.

Urinalysis revealed a significant increase in glucose, ALP, γ-GT, bile acids, and bilirubin in cholestatic rats. It was found that TAU treatment significantly alleviated urine markers of renal injury in BDL rats. The effects of TAU on urine biomarkers were not dose-dependent in the current model (Fig. 2).

Decreased mitochondrial dehydrogenase activity, mitochondrial depolarization, decreased ATP stores, lipid peroxidation, and mitochondrial permeabilization were evident in the kidney mitochondria isolated from cholestatic animals. TAU (500 and 1000 mg/kg, oral) significantly improved mitochondrial indices in BDL rats. The effect of TAU on renal mitochondrial indices was not dose-dependent in cholestatic rats (Fig. 3).

Tubular atrophy and interstitial inflammation were the most prominent renal histopathological alterations even days after the BDL surgery (Fig. 4 and Table 3). On the other hand, it was found that TAU treatment significantly ameliorated cholestasis-induced renal injury in BDL animals (Fig. 4 and Table 3). The effects of TAU on renal histopathological alterations were not dose-dependent (Fig. 4 and Table 2).

Discussion

Cholestasis-induced renal injury (also known as cholemic nephropathy, CN) is a severe clinical complication that could lead to renal failure or the need for organ transplantation. Although the only promising option is identifying the etiology of CN and its eradication (e.g., gall stones), preserving renal function and protecting this organ during cholestasis is a critical issue. In the current study, we found that administration of TAU (500 and 1000 mg/kg, oral, seven consecutive days) to cholestatic animals could significantly preserve renal function and prevent cholestasis-induced renal injury. The effects of TAU on oxidative stress markers and mitochondrial indices seem to be the fundamental mechanisms for this amino acid's nephroprotective effects in the current model.

Oxidative stress and its associated events such as lipid peroxidation, protein carbonylation, and defect in enzymatic and non-enzymatic antioxidant systems have been mentioned as key mechanisms involved in the pathogenesis of CN [42-45]. Several studies have mentioned the positive effects of antioxidants against cholestasis [84-86]. N-acetyl cysteine, proline, α-lipoic acid, betaine, selenium, glycine, boldine, agmatine, and several other agents have been used to ameliorate cholestasis-induced organ injury [42, 62, 73, 87-92].

It has repeatedly been mentioned that TAU could alleviate oxidative stress status in various experimental models [12-14, 18-26]. The effects of TAU on enzymatic and non-enzymatic antioxidant systems have been noted as a cytoprotective mechanism for this amino acid [12-14, 18-26]. On the other hand, it has been

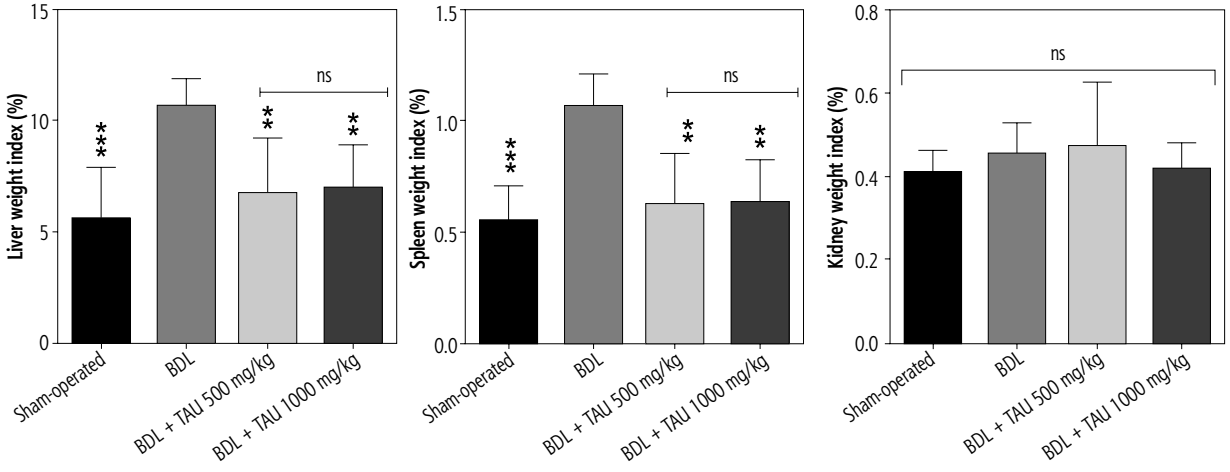

Fig. 1. Organ weight index in cirrhotic rats. Data are given as mean ± SD (n = 8). **Indicates significantly different as compared with the BDL group (p < 0.01)

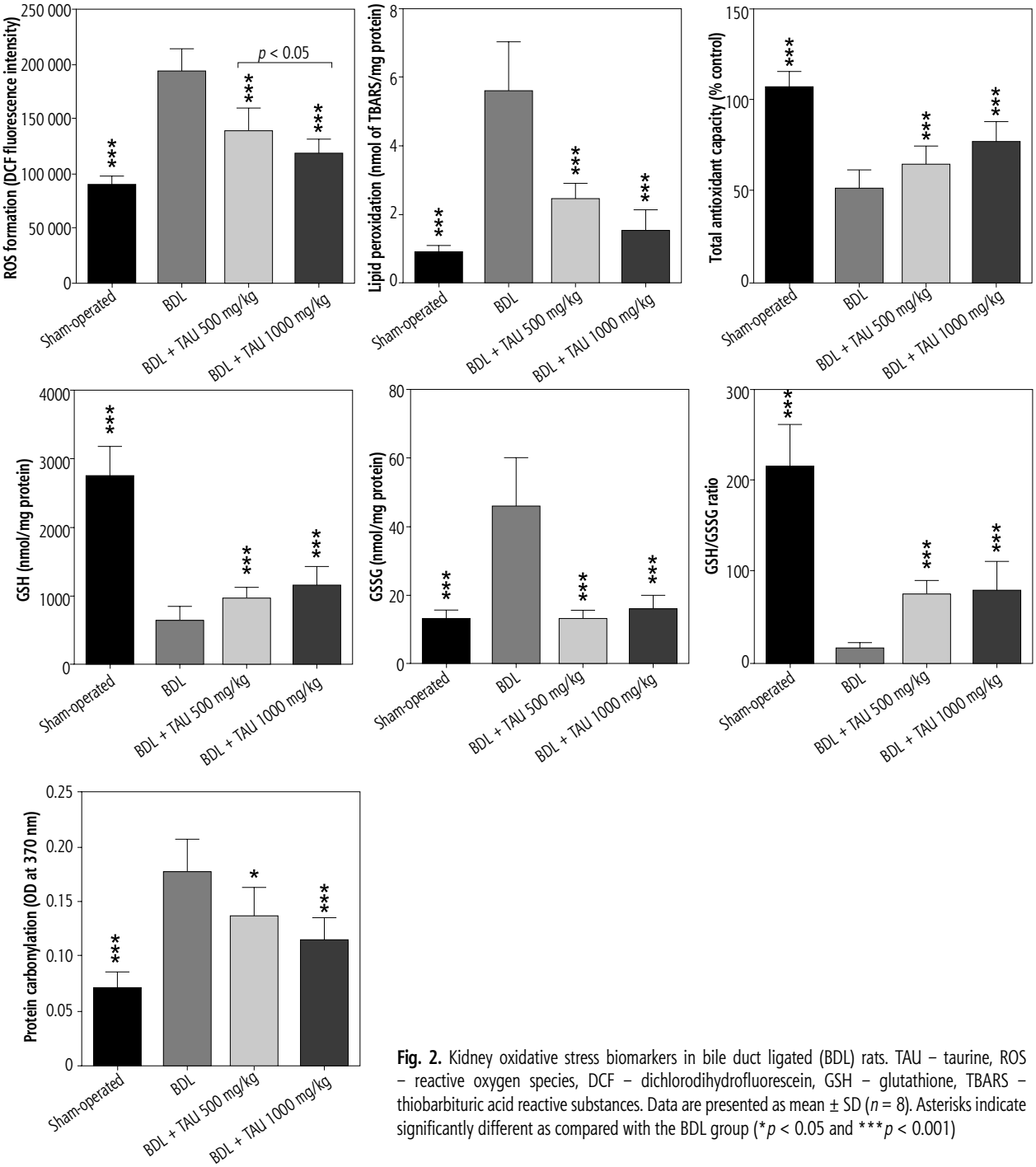

Fig. 2. Kidney oxidative stress biomarkers in bile duct ligated (BDL) rats. TAU – taurine, ROS – reactive oxygen species, DCF – dichlorodihydrofluorescein, GSH – glutathione, TBARS – thiobarbituric acid reactive substances. Data are presented as mean ± SD (n = 8). Asterisks indicate significantly different as compared with the BDL group (*p < 0.05 and ***p < 0.001)

found that TAU is not an excellent radical scavenger. Hence several studies mention other mechanisms for the cytoprotection provided by this amino acid.

The effect of TAU on cellular mitochondria is a new and exciting mechanism of action provided by this amino acid [12, 26-41]. Recent studies mentioned that the most crucial antioxidant mechanism of TAU is mediated through its effects on cellular mitochondria [12, 26-41, 93]. It has been found that TAU effectively mitigated mitochondria-mediated ROS formation [26, 29, 35, 94-96]. TAU also regulates the synthesis of mitochondria electron transport chain components and enhances mitochondrial ATP [26, 29, 35, 94-97]. Our data are in agreement with investigations indicating the occurrence of oxidative stress in the kidney of cholestatic animals. On the other hand, we found that mitochondrial impairment in renal tissue of cholestatic rats could act as a significant source of ROS and oxidative stress in this disease. We found that TAU mitigated oxidative stress in the renal tissue of cholestatic

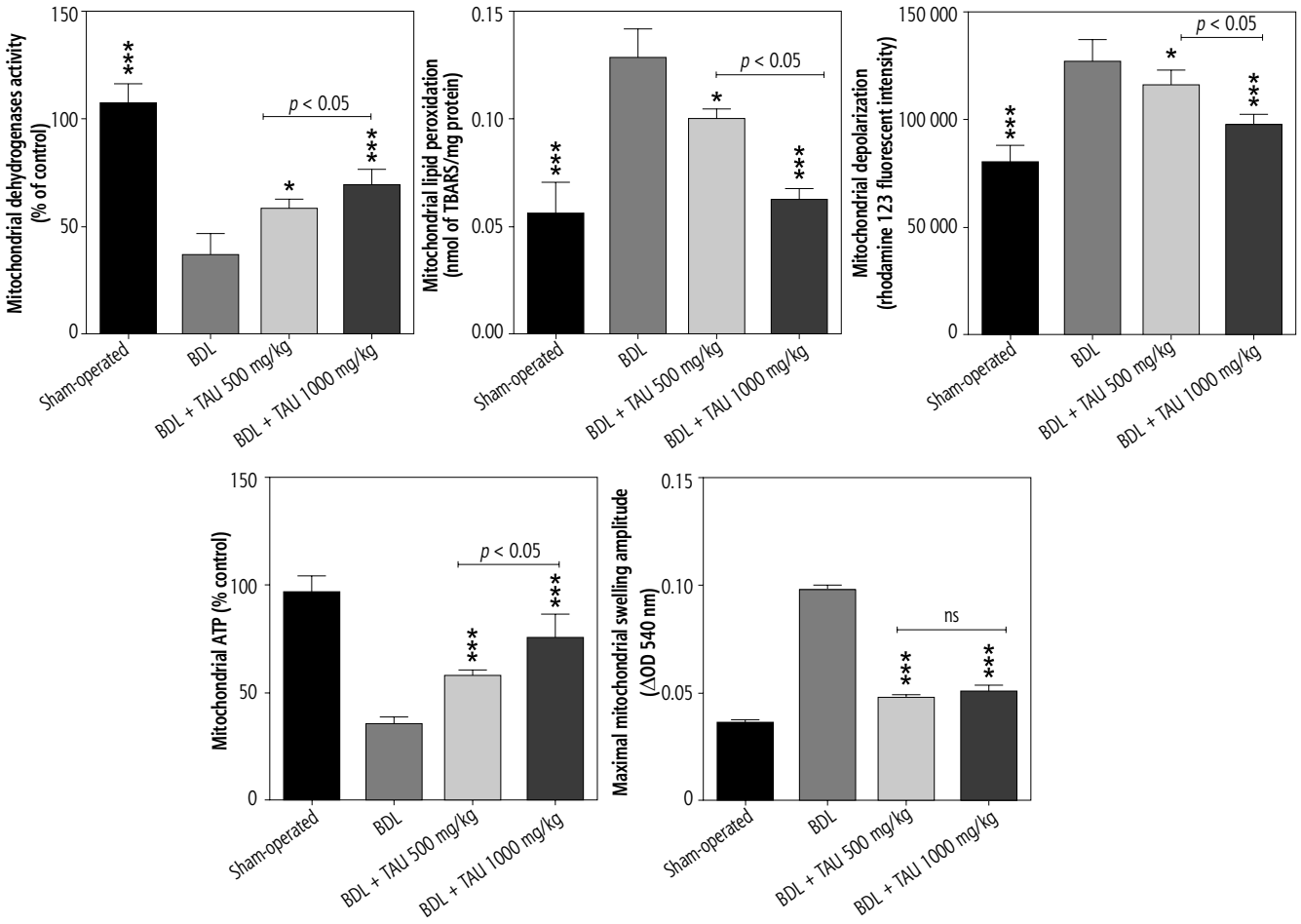

Fig. 3. Mitochondrial indices in the kidney of cholestatic animals. TAU – taurine, TBARS – thiobarbituric acid reactive substances. Data are given as mean ± SD (n = 8). Asterisks indicate significantly different as compared with the BDL group (*p < 0.05 and ***p < 0.001)

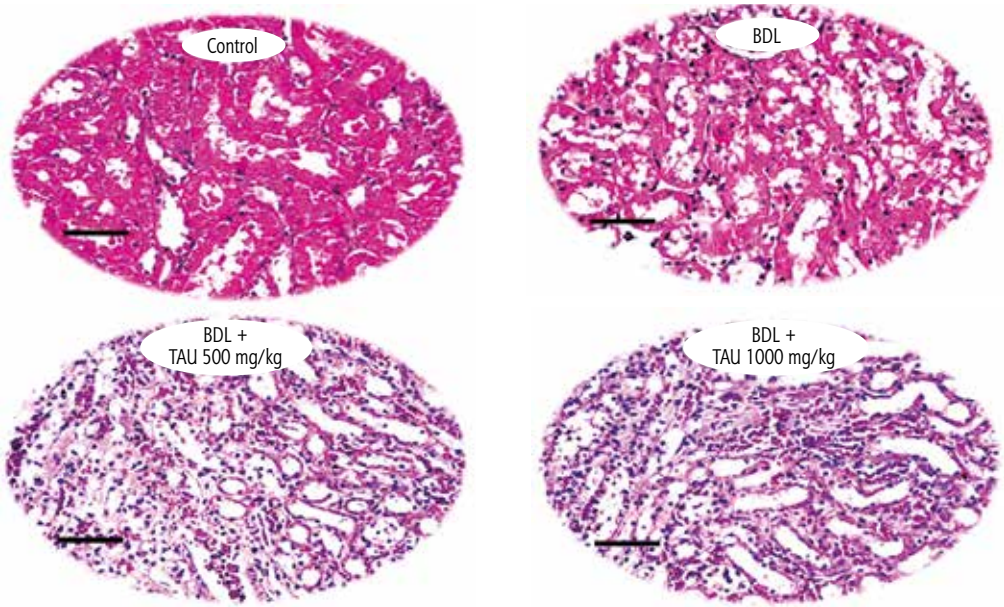

Fig. 4. Taurine (TAU) treatment mitigates renal histopathological changes in cholestatic rats. BDL – bile duct ligated. H&E staining (scale bar 100 µm). Tubular atrophy and interstitial inflammation were the prominent histopathological alterations in the BDL animals (7 days after BDL surgery) (Table 3). It was found that taurine (500 and 1000 mg/kg, oral) mitigated renal histopathological changes in BDL rats (Table 3)

Table 2. Urinalysis of bile duct ligated (BDL) rats treated with taurine (TAU)

Parameters assessed	Sham-operated	BDL	BDL + TAU 500 mg/kg	BDL + TAU 1000 mg/kg
Glucose (mg/dl)	67 ±9	141 ±14*	112 ±6[a]	109 ±11[a]
ALP (U/l)	1450 ±248	4333 ±564*	30100 ±559[a]	2792 ±343[a]
γ-GT (U/l)	2142 ±392	4361 ±810*	2822 ±305[a]	2541 ±300[a]
Total bilirubin (mg/dl)	0.49 ±0.13	4.8 ±1*	4.77 ±1	3.4 ±1.5
Bile acids (mg/dl)	3.9 ±1.1	55.3 ±11*	64 ±15	72 ±11

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 < 0.05).

Table 3. Renal tissue histopathological alterations in bile duct ligated (BDL) rats seven days after BDL surgery

Group	Tubular atrophy	Interstitial inflammation
Sham-operated	–	–
BDL	+++	++
BDL + TAU 500 mg/kg	+	+
BDL + TAU 1000 mg/kg	+	–

+++, ++, and + indicate severe, moderate, and mild histopathological alterations.
TAU – taurine

animals. The antioxidative mechanism of TAU in this study might be mediated through its effects on renal mitochondrial function.

TAU is a safe compound [98]. On the other hand, this amino acid is under clinical trials for the management of several diseases [99]. Previous studies mentioned the positive effects of TAU on cholestasis/cirrhosis [14, 78, 84, 86, 99-105]. Therefore, TAU might be readily administered in cholestatic patients to prevent renal injury. Finally, our results suggest the potential protective effects of taurine on cirrhosis-associated renal injury. Nevertheless, the precise impact of TAU on the renal function in cholestasis and the clinical relevance of these data require further studies for clarification.

Acknowledgments

This investigation was financially supported by the Vice-Chancellor of Research Affairs of Shiraz University of Medical Sciences (Grant number: 19359/14883). The authors thank the Pharmaceutical Sciences Research Center of Shiraz University of Medical Sciences for providing technical facilities to carry out this study. The Shanxi Government Scholarship also supported this study for International Research Assistant (National Natural Science Foundation of China (CN); Grant No. 2018YJ33; provided by Dr. M. Mehdi Ommati), and outstanding doctors volunteering to work in Shanxi Province (No. K271999031; by Dr. M. Mehdi Ommati), Shanxi province, China.

Disclosure

The authors declare no conflict of interest.

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