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The effect of alpha-lipoic acid on oxidative parameters, *SCUBE-1* and *SCUBE-2* in hepatic ischemia-reperfusion injury in cholestatic rats

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Abstract. – OBJECTIVE: The aim of the study was to evaluate the protective effects of alpha-lipoic acid (ALA) on the liver, oxidative parameters, and signal peptide-CUB-epidermal growth factor-like domain-containing proteins 1 and 2 (*SCUBE-1* and *-2*) in an experimental cholestatic hepatic ischemia-reperfusion (IR) model.

MATERIALS AND METHODS: Twenty-four female rats were included in the study and divided into four groups of six rats each. Group 1 was the control group, in which only laparotomy was performed; Group 2 underwent laparotomy and received alpha-lipoic acid (ALA) on a daily basis; bile duct ligation was performed in Group 3; bile duct ligation was performed, and ALA was administered to Group 4. All rats underwent relaparotomy on the seventh day, followed by 30 minutes of hepatic ischemia and 60 minutes of reperfusion in Groups 3 and 4. Liver tissue and blood samples were taken for histopathological and biochemical examinations. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), direct bilirubin (DBIL), albumin, ischemia modified albumin (IMA), *SCUBE-1*, *SCUBE-2*, total antioxidant status (TAS) and total oxidant status (TOS) levels were also examined.

RESULTS: The SCUBE-1 and SCUBE-2 values in Group 4 were lower than in Group 3, but no significant difference was observed between all the groups. The AST, TBIL, and DBIL levels were significantly higher in Groups 3 and 4 than in Groups 1 and 2 ($p<0.0001$). Although TOS was the highest in Group 3, the measurements were similar across the groups ($p=0.464$). IMA and TAS were similar between Groups 3 and 4 but significantly higher in these groups than in Groups 1 and 2 ($p=0.001$). The hepatic injury observed in Groups 3 and 4 was significantly higher than that observed in Groups 1 and 2 ($p<0.0001$). In the histopathological examination, neutrophilic infiltration and bile duct proliferation were less commonly detected in the portal areas in Group 4 than in Group 3, and necrotic foci were not observed due to the administration of ALA.

CONCLUSIONS: The promising effects of ALA, known for its powerful antioxidant properties, on the IR injury of the liver can allow it to enter clinical practice in the future.

Key Words:
Alpha lipoic acid, Liver, Cholestasis, Ischemia-reperfusion.

Introduction

The liver is the largest gland in the human body. It is involved in a multitude of vital functions, with its most remarkable feature being its regenerative capacity, while other functions include production, storage, metabolism, and secretion. The liver plays a role in carbohydrate and lipid metabolisms, the production of bile and fatty acids, the synthesis of various plasma proteins in circulation, and the detoxification of many substances[1]. The Pringle maneuver or total vascular clamping can be used for the temporary control of bleeding during hepatic resection

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procedures performed due to trauma, tumors, or bile duct malignancies. The interruption of blood flow to the liver has significant consequences, particularly in cases in which the flow of bile to the bowel is arrested with a consequent increase in blood bilirubin levels, a condition known as cholestasis. Impaired perfusion as a result of a significant reduction or complete interruption of blood flow to an organ or tissue is termed ischemia. Oxygen deprivation in ischemic tissue over a certain period of time can result in tissue damage and necrosis. In addition, blood flow is interrupted temporarily during the cold ischemia period between resection and re-anastomosis that occurs in transplant surgery for the treatment of hepatic insufficiency, and the reperfusion that occurs after the restoration of the blood flow can cause more damage to the ischemic tissue than the ischemia itself, which is known as ischemia-reperfusion (IR) injury[2]. Hepatic ischemia-reperfusion (IR) injury that occurs as a result of clinical conditions, such as hemorrhagic, cardiogenic, and septic shock, and liver surgery and transplantation, in particular, is associated with high mortality and morbidity. In IR injury, also referred to as the oxygen paradox, the degree of injury depends on the duration of ischemia and the structural and biochemical characteristics of the affected tissues. Oxygen concentration, temperature, and pH also affect the extent of tissue damage[3]. The Kupffer cells that become activated in the liver during the ischemia period release reactive oxygen species (ROS) and cytokines during reperfusion that contribute to endothelial and hepatocyte damage. The delicate balance between antioxidants and pro-oxidants must be maintained for the maintenance of vital functions, as any impairment in the balance in favor of pro-oxidants may lead to oxidative stress and damage. An increase in oxidative stress results in oxidative damage to biomolecules such as lipids, DNA, and proteins. The status of all oxidants can be determined from a measurement of total oxidant status (TOS)[4,5], and total antioxidant status (TAS) reflects the total oxidant defense of an organism against attack by free radicals in plasma. The oxidative stress index (OSI) is the ratio of plasma TOS to TAS and is an indicator of oxidative stress. Plasma TAS, TOS, and OSI all reflect the redox balance between oxidation and antioxidation. The measurement of TAS and TOS can be considered a useful approach to the estimation of oxidative status[6]. Signal peptide-CUB-epidermal growth factor-like domain-containing protein 1 (*SCUBE-1*) is a cell surface glycoprotein released during early embryogenesis. It is found in platelets and endothelial cells and is deposited in the alpha granules. *SCUBE* genes are present in humans, mice, and zebrafish. Studies[7,8] have revealed the biological function of these genes in cases of acute coronary syndrome and acute ischemic stroke. Dai et al[7] reported that the *SCUBE-1* protein becomes detectable within six hours of the onset of ischemic symptoms and remains detectable for three to four days; therefore, it can be considered a good but non-sensitive marker of epidermal growth factor-like (EGF)-like repeats acute thrombotic diseases. One gene family, *SCUBE,* encodes secreted proteins, harboring nine copies of, a spacer region, three cysteine-rich domains, and one CUB domain at the C terminus. There are three members of the *SCUBE* family, namely *SCUBE-1, SCUBE-2*, and *SCUBE-3*, all of which play a role in pathological angiogenesis processes. The biological function of *SCUBE-1* in atherosclerosis and thrombus formation has yet to be fully elucidated. Similar to *SCUBE-1, SCUBE-2* has been defined as a vascular endothelium cell surface protein and is known to be a novel tumor suppressor and a useful prognostic marker in breast cancer. *SCUBE-2* is expressed in the endothelium, as identified during embryogenesis, and mainly in the neuroepithelium, the third ventricle of the central nervous system, the nasal septum, the tongue mesoderm, and the genioglossus muscle[8]. An increased expression of *SCUBE-3* has been detected in osteoblasts[9]. *SCUBE-1* has been investigated as a potential marker for the early diagnosis of various ischemic pathologies. Acute mesenteric ischemia, acute coronary syndrome, and acute ischemic stroke are among the diseases for which *SCUBE* research has been carried out to facilitate early diagnosis[10]. Alpha lipoic acid (ALA) is synthesized from octanoic acid and cysteine in the mitochondria of plants and animals and acts as a cofactor of pyruvate dehydrogenase and alpha-ketoglutarate in the body. It has been reported that both the reduced and oxidized forms of alpha lipoic acid have antioxidant properties. The therapeutic use of ALA has been reported in diabetes mellitus, IR injury, heavy-metal poisoning, radiation damage, neurodegeneration, cataract formation, and HIV infection. In addition, ALA can act as a redox regulator of proteins such as lipoate, myoglobin, prolactin, and

thioredoxin[11]. This study aimed to evaluate the effect of alpha lipoic acid on liver tissue damage, hepatic TOS, *SCUBE-1,* and *SCUBE-2* and explore its use as an early marker in the diagnosis of ischemia in a cholestatic rat model of hepatic IR injury.

Materials and Methods

Animals

In this study, 24 female Wistar Albino rats weighing between 250-300 g were used. During both preoperative and postoperative periods, the rats were housed in cages under constant environmental conditions (temperature: 23°C and humidity: 55.5%) and fed with standard laboratory feed and tap water. Access to food and water was stopped 12 and two hours before anesthesia, respectively.

Surgery and Experimental Protocol

This research aimed to investigate the effect of ALA on oxidative parameters and *SCUBE* in the hepatic IR injury of the cholestatic liver. The rats were randomized into four groups as follows:

Group 1 (control)

The rats in this group only underwent laparotomy. No other therapies were administered. The rats were sacrificed at the end of the study, and blood and tissue samples were collected.

Group 2 (control + ALA)

The rats in this group underwent only laparotomy as a surgical procedure and were then administered 100 mg/kg of ALA (Alpha lipoic acid; Jiangsu Tohope Pharmaceutical Co., Ltd., Changshu City, China) intraperitoneally for seven postoperative days. The rats were sacrificed at the end of the study for the collection of blood and tissue samples.

Group 3 (IR)

After laparotomy, the common bile duct was dissected from the adjacent tissues and ligated with 3/0 silk sutures. The abdominal incision was then closed with 3/0 polypropylene sutures. The rats were subsequently allowed to feed. A re-laparotomy was performed on day 7 after surgery. A vascular clamp was placed on the hepatic artery and the portal vein to induce hepatic ischemia for 30 minutes, and the clamp was opened to allow hepatic reperfusion for 60 minutes. No other intervention was made, and no medication was administered. Liver tissue samples and blood samples from the aorta were taken for histopathological and biochemical analyses, and the rats were sacrificed.

Group 4 (IR + ALA)

Similar to Group 3, the common bile duct was ligated following laparotomy to induce cholestasis. The rats were administered 100 mg/kg of ALA intraperitoneally for seven days. The vascular pedicle was clamped for 30 minutes after re-laparotomy on postoperative day 7 to induce hepatic ischemia, and then the clamp was opened to allow reperfusion for 60 minutes. Liver tissue samples and blood samples were collected for histopathological and biochemical analyses, and the rats were sacrificed.

Biochemical Analysis

Routine parameters

The serum albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), and direct bilirubin (DBIL) levels of the rats were determined using commercially available kits (Beckman Coulter Diagnostics, Brea, CA, USA) and an autoanalyzer (Beckman AU5800; Beckman Coulter Diagnostics, Brea, CA, USA).

Measurements of SCUBE-1 and SCUBE-2

The serum *SCUBE-1* and *SCUBE-2* levels of the rats were quantified using a commercially available ELISA kit (Bioassay Technology Laboratory, Shanghai, CHINA) according to the manufacturer's instructions. The kit's sensitivity was 0.35 ng/mL for *SCUBE-1* and 0.07 ng/mL for *SCUBE-2*. The inter- and intra-assay coefficients of variation for *SCUBE-1* and *SCUBE-2* were <10%. The assay results were expressed as ng/mL.

Measurement of Ischemia-Modified Albumin (IMA)

The decrease in cobalt-albumin-binding capacity (IMA level) was detected using a colorimetric method[12]. 200 µL of rat serum was pipetted into a glass tube, to which 50 µL of 0.1 % Cobalt (II) - chloride hexahydrate ($CoCl_2$-$6H_2O$, Sigma Aldrich, St. Louis, MO, USA) was added. After shaking, the mixture was held for 10 minutes to ensure proper cobalt-albumin binding. Then, 50 µl of 1.5 mg/ml dithiothreitol (DTT) (Cleland's reagent) (Sigma Aldrich, St. Louis, MO, USA)

was pipetted as the dye. After two minutes, 1 ml of 0.9% NaCl was pipetted to stop the binding between cobalt and albumin. A blank was designed for each sample using 50 microliters of distilled water instead of 50 µl of 1.5 mg/ml DTT during the DTT addition step. Absorbance was recorded at 470 nm using a spectrophotometer (UV1201, Shimadzu, Kyoto, Japan). The color development in the DTT samples was compared to that in the dummy tubes, and the results were expressed in absorbance units. To maintain the IMA ratio (IMAR), the formula IMA levels/individual serum albumin concentration was used to avoid the effects of albumin concentration differences between groups.

Measurement of Serum Total Oxidant Status (TOS)

Serum TOS levels were detected utilizing a colorimetric strategy, in which oxidants within the test oxidize the ferrous ion-chelated complex to a ferric particle, which occurs as a colored mixture with a chromogen at an acidic pH. The color escalated, which can be detected by spectrophotometry, is related to the overall sum of oxidant atoms-molecules displayed within the test. The result is expressed in terms of micromolar hydrogen peroxide identical per liter (µmol H_2O_2 equiv./l)[13].

Measurement of Serum Total Antioxidants Status (TAS)

Serum TAS values were detected employing a colorimetric measurement method[14]. In this strategy, antioxidants of the test decrease the dull blue-green colored ABTS [22-.′-azino-bis (3-ethylbenzthiazoline-6-sulfonic corrosive)] radicals to a colorless diminished ABTS complex. The alteration of absorbance at 660 nm is involved in the whole antioxidant level of the test. This strategy determined the anti-oxidative impact of the test against the strong free radical responses initiated by the delivered hydroxyl radical. The result is obtained as micromolar Trolox proportionate per liter (µmol Trolox equiv./l).

Oxidative Stress Index (OSI)

The ratio of the TOS level to the TAS level provides the OSI. For this calculation, the micromolar unit of TAS was changed, and the OSI value was calculated according to the following equation: OSI (arbitrary unit) = TOS (µmol Hmillimoles per liter$_2$ O_2 equiv./l)/TAS (µmol Trolox equiv./l).

Histopathological Evaluation

Liver samples excised from the rats were fixed in a 10% neutral formalin solution. After 48 hours of fixation, tissues were dehydrated through a series of graded alcohols embedded in paraffin and cut into 4-µm sections using a microtome (Leica RM 2125, Leica Microsystems Nussloch GmbH, Germany) and stained with hematoxylin and eosin. Additionally, Masson's trichrome stain was used to evaluate fibrosis. These preparations were blindly evaluated by a pathologist under a light microscope. Hepatic injury was evaluated for severity using an ordinal scale as follows: Grade 0 = minimal or no evidence of injury; Grade 1 = mild injury with cytoplasmic vacuolation and focal nuclear pyknosis; Grade 2 = moderate-to-severe injury with nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and Grade 3 = severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration. The presence of cholestasis and fibrosis in the liver was also noted[15].

Statistical Analysis

Descriptive statistics included frequency, percentage, mean, standard deviation, median, minimum, and maximum values. Fisher's exact test was used to analyze categorical data since the percentage of cells with an expected value of less than 5 was higher than 20%. The column ratios were compared using the z-test with the Bonferroni correction. The normality assumption was tested using the Shapiro-Wilk test. When the data were normally distributed, the one-way analysis of variance test was used for the analysis of the difference between the numeric data of the groups. In the presence of a significant difference, the Tukey test was used to make pairwise comparisons. The Kruskal-Wallis H test was used when the data were not normally distributed. In the presence of a significant difference, the Dunn-Bonferroni procedure was used, and Spearman's correlation coefficient was determined to test the relationship between numeric variables as they did not fit a normal distribution. The Statistical Package for the Social Sciences (SPSS) version 23.0 (IBM Corp., Armonk, NY, USA) software package was used for the analysis of the study data. A *p*-value lower than 0.05 was considered statistically significant.

Results

The results of the analysis of the biochemical parameters of the groups are presented in Table I.

Table I. Comparison of laboratory findings of study groups.

	Group 1 (control) mean ± SD (min.-max.) median (Q1-Q3)	Group 2 (control+ALA) mean ± SD (min.-max.) median (Q1-Q3)	Group 3 (IR) mean ± SD (min.-max.) median (Q1-Q3)	GROUP 4 (IR+ALA) mean ± SD (min.-max.) median (Q1-Q3)	*p*
SCUBE-1	26.92 ± 5.22 (18.07-32.26)	24.00 ± 9.05 (12.57-37.66)	33.20 ± 9.09 (18.23-43)	25.72 ± 8.74 (17.39-40)	0.2611
(ng/mL)	27.74 (25.04-30.67)	23.25 (17.39-29.89)	33.36 (29.26-42)	23.41 (18.12-32)	
SCUBE-2	1.80 ± 0.54 (1.29-2.80)	2.08 ± 0.12 (1.92-2.30)	2.17 ± 0.64 (1.68-3)	1.79 ± 0.36 (1.26-2.21)	0.2442
(ng/mL)	1.6 (1.46-1.96)	2.08 (2.00-2.13)	1.80 (1.73-3)	1.87 (1.49-2.02)	
AST	193 ± 150.69 (102-498)[b]	115.5 ± 20.65 (94-151)[b]	1218.33 ± 310.68 (956-1819)[a]	840.17 ± 388.42 (529-1538)[a]	< 0.00012
(U/L)	136 (124-162)	111 (100-126)	1140 (1025-1230)	725 (534-990)	
ALT	117 ± 161.27 (41-446)[a,b]	55.17 ± 18.06 (36-87)[b]	258.33 ± 128.55 (110-497)[a]	150.33 ± 38.96 (101-189)[a,b]	0.0032
(U/L)	54.5(50-56)	49.5 (45-64)	235 (213-260)	154.5 (119-184)	
ALP	93.33 ± 44.03(34-147)[c]	122.33 ± 39.78 (82-178)[b,c]	335.67 ± 160.94 (182-641)[a]	229.83 ± 71.53 (116-310)[a,b]	0.0011
(U/L)	87.5 (68-136)	107.5 (95-164)	301.5 (244-344)	224 (202-303)	
DBIL	0.02 ± 0.01 (0.01-0.03)[b]	0.02 ± 0.01(0.01-0.02)[b]	10.1 ± 5.58 (4.16-17)[a]	9.79 ± 2.77 (5.87-13.39)[a]	< 0.00012
(mg/dL)	0.02 (0.02-0.03)	0.02 (0.01-0.02)	9.22 (4.99-16)	9.34 (8.34-12.46)	
TBIL	0.17 ± 0.03 (0.12-0.21)[b]	0.15 ± 0.01 (0.13-0.17)[b]	15.64 ± 8.24 (6.73-25)[a]	15.61 ± 5.33 (8.77-23.23)[a]	< 0.00011
(mg/dL)	0.18(0.15-0.2)	0.16 (0.14-0.16)	14.99(8.16-24)	14.18 (12.75-20.55)	
ALBUMIN	31.95 ± 1.69 (29.3-34.1)[a]	31.28 ± 4.64 (25.1-36.2)[a]	24.82 ± 1.49 (23.6-27.6)[b]	25.93 ± 1.85 (23.3-27.6)[b]	< 0.00011
(g/dL)	32.1 (31-33.1)	33.05 (25.9-34.4)	24.45 (23.8-25)	26.45 (24.3-27.5)	
IMA	0.12 ± 0.03 (0.09-0.17)[b]	0.12 ± 0.03(0.07-0.18)[b]	1.005 ± 0.782 (0.277-2)[a]	1.15 ± 0.38 (0.72-1.69)[a]	0.0012
(absorbance unit)	0.11 (0.10-0.15)	0.11 (0.09-0.15)	0.63 (0.48-2)	1.10 (0.82-1.46)	
TOS	19.90 ± 3.80 (13.78-25.24)	20.38 ± 7.10 (11.55-28.49)	24.34 ± 3.86 (18.55-30.36)	21.94 ± 5.34 (14.51-28.14)	0.4641
(μmol H_2O_2 equiv./L)	20.28 (18.17-21.63)	20.93 (14.49-25.87)	24.5 (22.64-25.52)	23.29 (16.52-25.9)	
TAS	0.62 ± 0.03 (0.55-0.65)[b]	0.62 ± 0.09 (0.47-0.72)[b]	1.01 ± 0.04 (0.97-1.11)[a]	1.02 ± 0.06 (0.93-1.08)[a]	0.0012
(μmol Trolox equiv./L)	0.64 (0.63-0.64)	0.65 (0.55-0.67)	1.003 (1-1.025)	1.05 (0.95-1.07)	
OSI	32.04 ± 7.95 (21.74-45.81)[a]	32.20 ± 7.87 (22.29-39.41)[a]	27.01 ± 6.93 (16.70-36.37)[a,b]	21.42 ± 5.15 (15.24-27.67)[b]	0.0491
(TOS/TAS)	31.60 (28-33.48)	33.97 (24.23-39.34)	28.23 (22.09-30.45)	21.94 (15.69-26.06)	
IMAR	0.004 ± 0.001 (0.002-0.005)[b]	0.004 ± 0.001 (0.002-0.005)[b]	0.04 ± 0.03 (0.01-0.08)[a]	0.04 ± 0.01 (0.03-0.06)[a]	< 0.00011
(IMA/Albumin)	0.003 (0.002-0.004)	0.004 (0.003-0.004)	0.02 (0.02-0.08)	0.04 (0.03-0.05)	

Q1: 25th Percentile; Q3: 75th Percentile; p^1: One Way ANOVA; p^2: Kruskal Wallis H test has been applied. Different lowercase letters indicate that the column ratios are statistically different from each other (a,b,c). Lowercase a,b, not statistically different from both a and b. ($p < 0.05$). *SCUBE-1*: Signal peptide-CUB-epidermal growth factor-like domain-containing protein 1; *SCUBE-2*: Signal peptide-CUB-epidermal growth factor-like domain-containing protein 2; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; DBIL: Direct bilirubin; TBIL: Total bilirubin; IMA: Ischemia modified albumin; TOS: Total oxidant status; TAS: Total antioxidant status; OSI: Oxidative stress index.

The serum *SCUBE-1* and *SCUBE-2* levels were the highest in Group 3. They were also lower in Group 4 than in Group 3, but they did not differ significantly between these two groups (p = 0.261 and p = 0.244, respectively). There was no difference in the AST values of Groups 3 and 4, although these values were significantly higher when compared to those of Groups 1 and 2 (p < 0.0001). The ALT levels significantly differed only between Group 2 and Group 3 (p = 0.003). The ALP levels were the highest in Group 3 and significantly higher in Group 3 than in Groups 1 and 2 (p = 0.001). In the comparison of the TBIL and DBIL measurements, these values were higher in Groups 3 and 4 than in Groups 1 and 2 (p < 0.0001), while there was no significant difference between Group 3 and Group 4. The albumin levels were similar between Group 3 and Group 4, but significantly lower in Groups 3 and 4 than in Groups 1 and 2 (p < 0.0001).

The IMA levels were similar between Group 3 and Group 4 and significantly higher in Groups 3 and 4 than in Groups 1 and 2 (p = 0.001). TOS was the highest in Group 3, but its levels were similar across all groups (p = 0.464). TAS was similar between Group 4 and Group 3, but its levels were significantly higher in Groups 3 and 4 than in Groups 1 and 2 (p = 0.001). OSI was significantly lower in Group 4 than in Groups 1 and 2 (p = 0.049). IMAR measurements did not significantly differ between Groups 3 and 4 but were significantly higher in Groups 3 and 4 than in Groups 1 and 2 (p < 0.0001). The comparison of the SCUBE-1, SCUBE-2, TOS, and TAS levels between the groups is shown in Figure 1. A weak positive correlation was found between *SCUBE-1* and ALP (r = 0.438, p = 0.032) and TOS (r = 0.427, p = 0.038). There was no correlation between *SCUBE-2* and any of the remaining variables. TAS was determined to have a moderate positive correlation with AST, ALT, ALP, TBIL, DBIL, and IMA and a strong positive correlation with IMA (r = 0.701, p < 0.0001).

The relationship between the groups in terms of hepatic injury, cholestasis, and fibrosis scores was investigated with a histopathological examination (Table II). According to the degree of hepatic injury, the subjects were categorized as grade 0, grade 1, grade 2, and grade 3 (Figure 2). There was a statistical relationship between the hepatic injury scores and the experimental groups. A significant difference was found in the hepatic injury score 3 of Group 3 and the remaining groups, as well as the hepatic injury score 2 of Group 4 and the remaining groups (p < 0.0001). There was no significant difference between the cholestasis scores of the groups (p = 0.573), but a significant difference was observed between the groups in terms of the fibrosis score (p = 0.003). For fibrosis score 1, there was a difference between Group 3 and Groups 1 and 2, but not Group 4. No cholestasis or fibrosis was

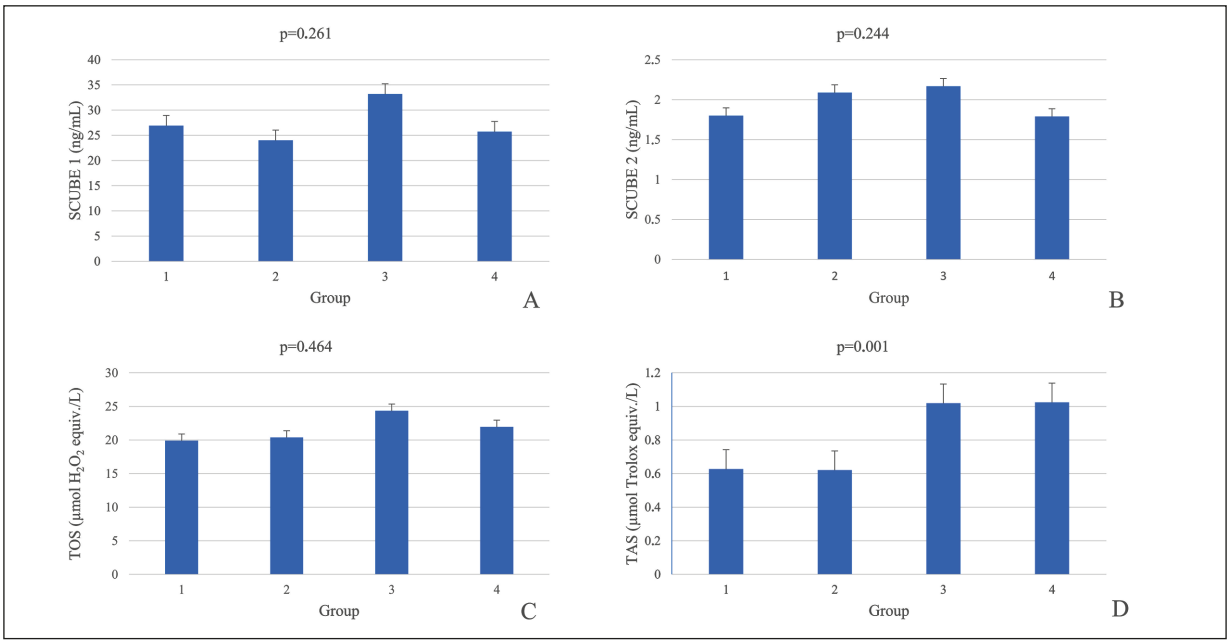

Figure 1. Inter-group comparisons of the investigated variables: **A**, *SCUBE-1*, **B**, *SCUBE-2*, **C**, TOS, and **D**, TAS.

Table II. Relationship of hepatic damage, cholestasis and fibrosis scores with groups.

	Group 1 N (%)	Group 2 N (%)	Group 3 N (%)	Group 4 N (%)	N (%)	*p*
Hepatic damage						
0	2 (33.3)^{a,b}	6 (100)^{b}	0 (0)^{a}	0 (0)^{a}	8 (33.3)	< 0.0001
1	4 (66.7)^{a}	0(0)^{a}	0 (0)^{a}	0 (0)^{a}	4 (16.7)	
2	0 (0)^{a}	0 (0)^{a}	1 (16.7)^{a}	6 (100)^{b}	7 (29.2)	
3	0 (0)^{a}	0 (0)^{a}	5 (83.3)^{b}	0 (0)^{a}	5 (20.8)	
Cholestasis						
0	6 (100)	6 (100)	4 (66.7)	5 (83.3)	21 (87.5)	0.573
1	0 (0)	0 (0)	2 (33.3)	1 (16.7)	3 (12.5)	
Fibrosis						
0	6 (100)^{a}	6 (100)^{a}	1 (16.7)^{b}	2 (33.3)^{a,b}	15 (62.5)	0.003
1	0 (0)^{a}	0 (0)^{a}	5 (0.8)^{b}	4 (66.7)^{a,b}	9 (37.5)	

Fisher's Exact test was used. Different superscript letters (^{a,b}) indicate that the column ratios are statistically different from each other ($p < 0.05$).

observed in a histopathological examination of the liver samples in Groups 1 and 2. Hepatic injury was minimal or absent in the control subjects, and no parenchymal injury was observed in Group 2. A histopathological examination of the subjects in Group 3 revealed bile duct proliferation, mild-to-moderate neutrophilic infiltration, and dilation related to fibrosis. Foci of necrosis,

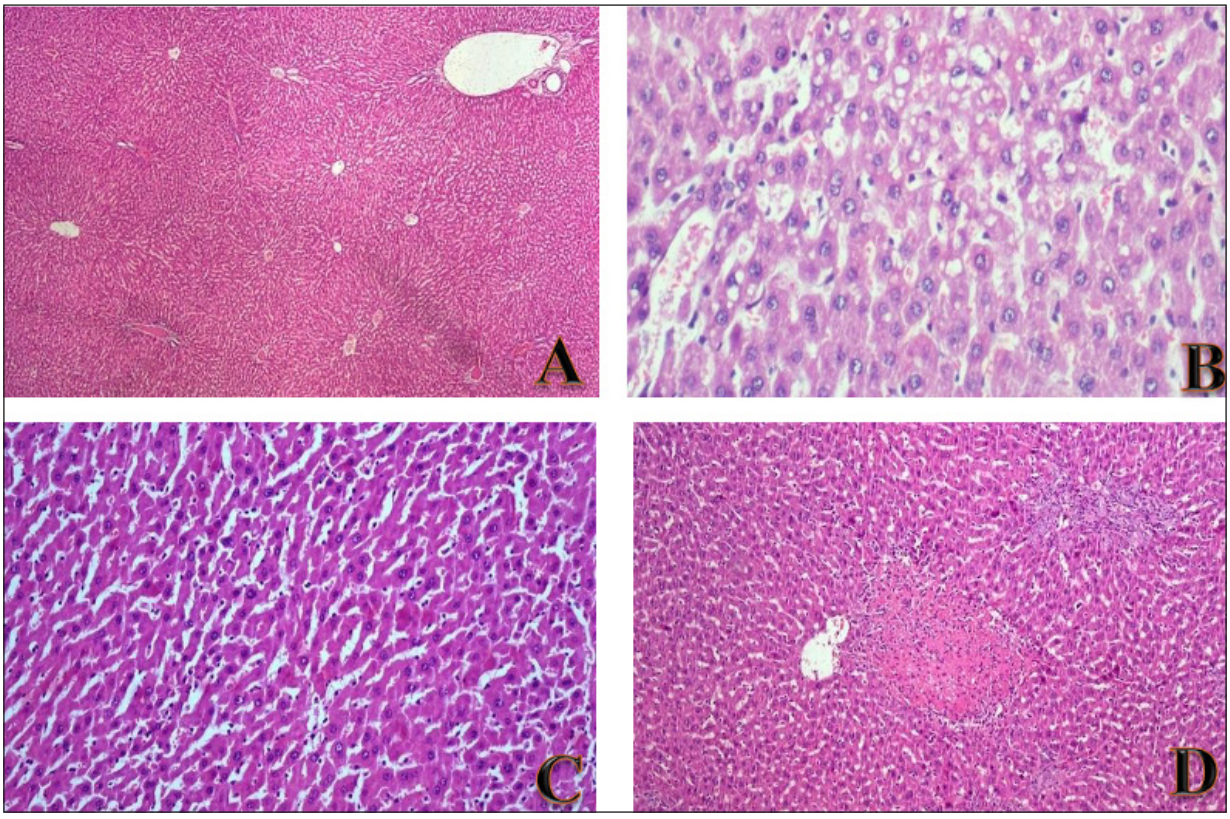

Figure 2. Histopathological examination of the samples obtained from the rats with different hepatic injury grades. **A**, Grade 0, no evidence of injury is observed in the liver parenchyma (×40, H&E). **B**, Grade 1, balloon degeneration, single cell necrosis, and intercellular dissociation are observed in damaged hepatocytes (×200, H&E). **C**, Grade 2, nuclear pyknosis, cytoplasmic hypereosinophilia, mitosis, and focal loss of intercellular borders are seen in hepatocytes with the progression of liver damage (×200, H&E). **D**, Grade 3, necrosis and neutrophil infiltration are observed in the damaged areas (×100, H&E).

hemorrhage, and debris were observed in lobular areas (Figure 3). Intracytoplasmic bile pigment deposition was observed in two subjects in Group 3. The histopathological examination revealed less extensive neutrophilic infiltration in the portal areas and bile duct proliferation in Group 4 than in Group 3, and no foci of necrosis were observed in the former.

Discussion

IR injury to the liver is among the most significant problems faced during major operations, such as transplant surgery, particularly those performed due to tumors and trauma. The production of ROS that is primarily responsible for this injury can be explained by the xanthine oxidase-mediated, mitochondrial electron transport chain, nitric oxide synthase, and phagocytic cell-mediated interrelated biochemical mechanisms[16]. Aside from the duration of ischemia, the severity of tissue damage is closely related to the specific structural and biochemical characteristics of the affected tissues and organs. During ischemia, ATP production stops, and intracellular ATP is catabolized. It has been demonstrated that in cases of IR injury, neutrophils are increased at the tissue level, as well as systemically, in many organs[17].

It is well known that endothelial cells play an active role in the production of ROS by releasing adhesion molecules, such as selectin and integrin, which are known to trigger the recruitment of leukocytes in hepatocytes[18]. Reactive radical nitric oxide (NO) results in the production of potentially toxic nitrogen trioxide (N_2O_3) together with superoxide radicals, whose levels are increased in ischemia-reperfusion injury.

IR injury has a complex pathophysiology involving many factors[19]. The production of inflammatory mediators and the recruitment of neutrophils, macrophages, and lymphocytes to the area expand the injury and are responsible for the systemic effects of IR injury. All of these mechanisms vary in different tissues, depending on the duration and severity of ischemia. IR injury may not be confined to the affected site and may cause damage to distant organs and systems. Distant organ injury depends primarily on the affected organ or the inflammatory mediators in the circulation. Particularly in hepatic IR injury, studies[20] have identified increased levels of xanthine oxidase-mediated reactive oxygen species in the lungs. To date, the focus of research has been on therapies intended to prevent the life-threatening oxidative stress response associated with ischemia in many organs other than the liver. To the best of our knowledge, the current study is the first in the literature to investigate the hepatoprotective effects of ALA and its effects on the recently described glycoproteins *SCUBE-1* and *SCUBE-2* in cholestatic rats with obstructive jaundice and IR injury.

SCUBE-1 is deposited in the alpha granules of platelets and becomes activated on the cell surface upon stimulation of platelets. The literature reported increased *SCUBE-1* levels six hours after the activation of platelets, and plasma levels are measurable for three to four days. Increased *SCUBE-1* levels have been identified in cases of pulmonary thromboembolism and experimental-

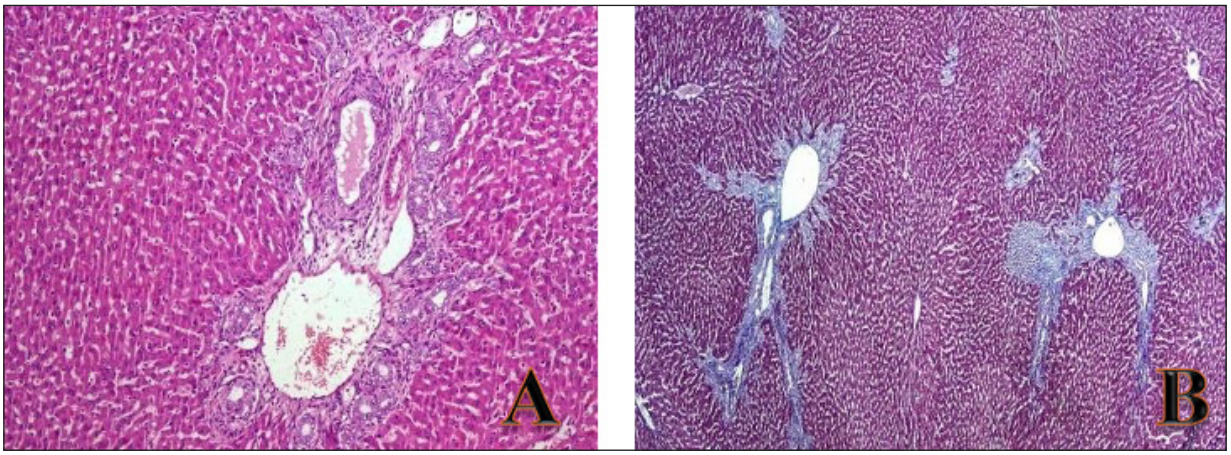

Figure 3. Histopathological appearance of a case in group 3. **A**, Bile duct proliferation (×200, H&E). **B**, Fibrosis in portal areas (×40, Masson's trichrome).

ly-induced ischemic stroke. Similarly, increased *SCUBE-1* levels have been reported within two hours of acute mesenteric ischemia and in hemodialysis patients as a result of platelet dysfunction[21-24]. In the current study, the *SCUBE-1* and *SCUBE-2* levels were found to be elevated in Groups 3 and 4, in which cholestasis was induced. These levels were lower in Group 4, which received ALA than in Group 3, although the differences between the two groups were not statistically significant ($p = 0.261$).

It has been demonstrated that oxidative stress parameters are increased in cases of gastric cancer and that *SCUBE-1* could be a marker of recurrence after treatment[25,26]. Many tumors cause an elevation in the levels of procoagulant substances and trigger coagulation through an inflammatory response. Tu et al[27] demonstrated that *SCUBE-1* is expressed not only by vascular endothelial cells but also by platelets.

SCUBE-2 is a vascular endothelium cell surface protein that is closely related to *SCUBE-1* and has been demonstrated to be involved in the development and progression of coronary artery atherosclerosis[28]. Song et al[29] reported a lower recurrence rate and better survival in *SCUBE-2*-positive patients with colorectal cancer. The relationship between *SCUBE-1, -2*, and *-3* levels and clinical findings has been investigated in patients with Hashimoto thyroiditis, psoriasis, and systemic sclerosis caused by angiogenesis and inflammatory reactions[30]. It has been shown that the *SCUBE-1* level is higher in patients with hypothyroidism due to Hashimoto's thyroiditis compared to healthy individuals[31].

In conditions such as diabetes mellitus, hyperglycemia, and dyslipidemia, which are associated with an increase in vascular complications, *SCUBE-2* has been suggested[32] to play a role in endothelial dysfunction and the accumulation of ROS.

The effects of various antioxidants on many disorders that cause an increase in oxidative stress are a popular research subject. The present study investigated the hepatoprotective effects of ALA, a strong antioxidant against oxidative stress, in a cholestatic model of IR injury. The accumulation of ROS in conditions that cause an increase in oxidative stress further worsens existing pathologies in conditions such as neurodegenerative disorders, stroke, and cancer. Various antioxidants are currently available in clinical practice, including Edaravone for acute ischemic stroke, N-Acetylcysteine for dry eye syndrome and acetaminophen overdose, and ALA for diabetic neuropathies, as strong antioxidants[33]. In a meta-analysis conducted by Ziegler et al[34], ALA was shown to provide significant symptom relief in patients with diabetic polyneuropathy[35]. It has also been shown that ALA supports the improvement of the metabolic profile, providing glycemic control and weight loss without affecting hormones such as testosterone and estradiol in female patients with polycystic ovary syndrome[36].

The benefits of ALA for the treatment of distal sensory-motor neuropathy were reported in patients with type 2 diabetes mellitus and good glycemic control[37]. In another study[38], the neuroprotective effect of pretreatment with ALA was demonstrated in an experimental animal sciatic nerve injury model, in which an increase in superoxide dismutase and catalase levels suggested a protective effect in response to increased ROS levels. A review of the literature[39] examining more than 100 articles related to the consumption of ALA and pathologies associated with oxidative stress reported that ALA acted as a prooxidant in cancer and central sensitization diseases and controlled cell apoptosis. The therapeutic effects of oral ALA administered to mice have been identified in Ehrlich ascites carcinoma cells and hepatic antioxidant status. It has also been reported that ALA regulates liver enzymes and acts as a cancer inhibitor[40].

The hepatoprotective effects of ALA have been reported in conditions such as alcohol, mushroom, and heavy metal poisoning. In a study[41] conducted on rats, lipoic acid was shown to reduce methotrexate-induced oxidative damage in hepatocytes used in the treatment of inflammatory diseases, such as rheumatoid arthritis and psoriasis, and as a chemotherapeutic cytotoxic agent in primary malignancies, such as leukemia. In an experimental animal study, the protective effects of ALA on oxidative damage caused by methotrexate in the rat kidney were shown[42].

It has been demonstrated that ALA restores impairments in the balance between ROS and antioxidants by reducing virus-induced oxidative stress and inflammation. Aside from its immunomodulatory effects in various chronic inflammatory disorders, such as diabetic neuropathy and metabolic syndrome, the efficacy of ALA has been demonstrated in the treatment of various viral infections. Recent studies[43] have also recommended its use in the treatment algorithm

for coronavirus disease-19. The positive effects of ALA on anemia, inflammation, and glycemic control have been demonstrated in diabetic patients receiving hemodialysis treatment[44].

Despite the improvements in surgical techniques and medical therapies, IR injury continues to be a significant cause of morbidity and mortality during major operations, such as hepatobiliary system tumor surgery, biliary obstruction surgery, liver transplantation, and trauma surgery. In experimental jaundice rat models, ALA has been reported to reduce hepatic and intestinal injury and to induce the biosynthesis of antioxidant mediators[45-47]. ALA is a very important intramolecular redox system. As a coenzyme, it plays an active role in the citric acid cycle and has enzymatic and cytoprotective effects[35]. ALA is present in mitochondria as a cofactor of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase and functions as a free oxygen radical scavenger. It has been shown that ALA inhibits lipid peroxidation and neutrophil infiltration in rat liver tissues and protects cell membranes and intracellular proteins from oxidative tissue damage. It has also been reported that ALA decreases increased myeloperoxidase activity, plasma tumor necrosis factor-alpha, and Interleukin-1-beta levels, which are indicators of the systemic inflammatory response[41,48]. We consider that these remarkable biological activities of ALA can protect tissues from oxidative stress damage in IR injury. This study demonstrated the positive effects of ALA on hepatic IR injury in a cholestatic rat model. We observed positive effects, especially at the cellular pathological level. In the biochemical examination, the *SCUBE-1, SCUBE-2*, TOS, liver enzyme, and bilirubin levels were found to be lower in the treatment group receiving ALA than in the cholestatic group. In the histopathological examination of the liver, neutrophilic infiltration, necrosis, and bile duct proliferation were less common in the group treated with ALA than in the cholestatic group. The fact that cellular pathological damage was significantly less and more limited in the ALA group compared to the cholestatic group was much more remarkable and prominent than the improvement in biochemical parameters.

Limitations

Limitations of this study include the use of fixed-dose ALA and the ischemia-reperfusion time, as well as the relatively small sample size.

Conclusions

There have been many clinical and experimental studies reporting the strong antioxidant properties of ALA. However, to the best of our knowledge, this study is the first to evaluate the effect of ALA on IR injury in an experimental model of cholestasis. The histopathological findings of the study showed that ALA reduced hepatic damage, inflammation, and necrosis, while the biochemical examination revealed a reduction in TOS and positive effects on antioxidant capacity. We histopathologically observed the hepatoprotective effects of ALA on IR damage, especially at the cellular level. Although it is exciting to consider the possibility that ALA may enter clinical practice in the future, further studies are needed to better understand the mechanisms underlying its protective effects.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Ethics Approval

Approval (No. 48) was obtained on May 23, 2022, from the Animal Experiments Local Ethics Committee of Akdeniz University for this experimental animal study. All procedures were carried out at the Experimental Medicine and Animal Laboratory of the university between June and August 2022, in light of national animal experimentation principles.

Data Availability

The datasets are available from the corresponding author upon request.

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Authors' Contribution

Ugur Dogan: Conceptualization, Data curation, Methodology, Project administration, Supervision, Writing-review and editing. Abdullah Hilmi Yilmaz: Methodology, Project administration, Visualization. Senay Yildirim: Data curation, Validation, Visualization. Hamit Yasar Ellidag: Da-

ta curation, Validation, Visualization. Arif Aslaner: Formal analysis, Supervision. Remzi Can Cakir: Formal analysis, Supervision. Belkis Koctekin: Formal analysis, Validation. Baris Rafet Karakas: Validation, Visualization. Tugrul Cakir: Conceptualization, Supervision.

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