Original Article

Aspartic Acid Ameliorates Cholestasis in Bile Duct-Ligated Rats

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ABSTRACT

Background: Amino acids are promising agents in a variety of therapeutic fields including proliferation management. D-aspartic acid (DAA) is a non-essential amino acid, which occurs in many marine and terrestrial animals and has alleviative effects against many liver diseases such as hepatic steatosis, liver fibrosis and lipopolysaccharide-induced liver injury, but its effect has never been tested against cholestasis.

Aim of the Work: Thus, this study tests the effect of D-aspartic acid compared to that of the therapeutic drug for treating cholestatic liver diseases "ursodeoxycholic acid, UDCA".

Patients and Methods: Cholestasis was induced by bile duct ligation (BDL) in adult male albino rats. Three weeks post operation, the animals were allotted into 5 groups: Sham group, bile duct-ligated group, bile duct-ligated rats treated with D-aspartic acid group, bile duct-ligated rats treated with ursodeoxycholic acid group and bile duct-ligated rats treated with D-aspartic acid and ursodeoxycholic acid group, where treatments were orally administered during the fourth week.

Results: Bile duct-ligated rats suffered from lower body weight and higher liver relative weight, in addition to significant increase in the liver biomarkers alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, direct bilirubin, and total bilirubin in comparison with those of sham-operated rats. Histologically, these animals suffered from liver fibrosis, disorganized hepatic lobules, necrosis, and bile duct hyperplasia. After the administration of D-aspartic acid or/ and ursodeoxycholic acid, cholestasis-associated changes were significantly alleviated, especially in the group treated with D-aspartic acid and ursodeoxycholic acid together.

Conclusion: D-aspartic acid has a therapeutic effect in cholestasis of liver in bile duct-ligated rats, especially when used in combination with ursodeoxycholic acid.

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Key Words: Bile duct ligation, cholestasis, d-aspartic acid, rats, ursodeoxycholic acid.

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INTRODUCTION

In order to properly digest fats, absorb lipid-soluble nutrients, protect the small intestine from oxidative stress, eliminate endogenous and xenobiotic chemicals, and maintain the equilibrium of cholesterol and bile acids, normal bile production and secretion are essential[1]. One of the most typical symptoms of liver disorders is cholestasis, or blockage of the bile flow. Hepatic injury results from the accretion of hydrophobic bile acids during cholestasis because of increased oxidative stress and extracellular matrix formation. This is followed by liver fibrosis and liver failure[2,3].

The only medication to treat primary biliary cirrhosis and is approved by FDA is ursodeoxycholic acid (UDCA). UDCA is a naturally occurring bile acid produced from Chinese black bears[4], and is used as a medicinal agent for treating cholestatic liver disorders[5]. UDCA treats cholestasis by improving biliary flow[6], boosting the protective bicarbonate environment on the surface of cholangiocytes[7], protecting the liver from bile acid-induced apoptosis[8], and exerting anti-inflammatory effect[9].

The use of amino acids has quickly risen in a range of therapeutic sectors because they operate as promising agents in the control of proliferative metabolism. In both invertebrate and vertebrate neuroendocrine organs, D-aspartic acid (DAA) is a plentiful endogenous non-essential amino acid[10]. It is crucial to the physiological functioning of the liver[11]. Aspartate consumption has been proven to be able to prevent the growth of liver fibrosis and hepatic steatosis[12]. By reducing the production of pro-inflammatory mediators in liver injury models, aspartate can also lessen liver damage[13]. Additionally, DAA can lessen inflammation and boost all antioxidant capacities[14].

Thus, the aim of the present investigation is to study the therapeutic effect of DAA against cholestasis induced by bile duct ligation, with using of UDCA as a reference drug.

MATERIAL AND METHODS

Drugs and chemicals

Ursodeoxycholic acid (Ursofalk®) was purchased from Minapharm (Cairo, Egypt). D-aspartic acid was obtained from All Max Nutrition (NV, USA). Thiopental sodium was purchased from the Egyptian International Pharmaceutical Industries Company "EIPICO" (Cairo, Egypt). Kits for determining biomarkers of liver injury including serum alanine aminotransferase (ALT; catalog # 264002), aspartate aminotransferase (AST; catalog #

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260002) and alkaline phosphatase (AP; catalog # 217002), were purchased from Spectrum, Egyptian company for biotechnology / S.A.U. (Hannover, Germany), while kits of bilirubin (catalog # BIL099160) were from Bio-Med Diagnostics (Cairo, Egypt).

Animal experiments

Thirty five adult male Wistar rats, initially weighing 220–260g, were obtained from the Animal house of National Research Center (Cairo, Egypt). The animals were relocated at the animal facility at Zoology Department at Faculty of Science, Ain Shams University, and housed under hygienic conditions in plastic cages and acclimatized to laboratory conditions for 1 week before the start of the experiments. Animals were kept under a 12 h light: 12 h darkness schedule. The experimental protocol was approved by Research Committee of Zoology Department, Faculty of Science, Ain Shams University (5/2020).

Bile duct ligation

Bile duct ligation (BDL) is a very reproducible biliary cirrhosis animal model[15]. BDL and sham operation were performed as described previously[16]. In brief, the animals were anaesthetized and maintained throughout surgery using thiopental sodium. Under sterile conditions, a midline abdominal incision was made and the common bile duct was exposed. The bile duct was ligated twice with 4-0 silk, and cut between the ligatures. Sham-operated rats had the same surgery but without bile-duct ligation. Finally, the abdominal incision was closed in layers, and rats were returned to their cages to recover.

Experimental design

One week after the acclimatization period, the animals were allotted to five groups, each of seven rats, as follows: **Group I (Sham-operated group):** rats subjected to sham operation; **group II (BDL group):** rats subjected to bile duct ligation, **group III (DAA group):** BDL, treated with DAA, **group IV (UDCA group):** BDL, treated with UDCA; and **group IV (DAA+UDCA group):** BDL, treated with DAA and UDCA. DAA and UDCA were administrated daily during the fourth week after bile duct ligation. DAA was administered orally, at a dose of 265 mg/kg in saline. This is the rat equivalent dose after converting the human recommended dose (3g/kg b.wt)[17]. UDCA was used as a standard drug for cholestasis, and was administered orally at a dose of 50 mg/ kg b.wt in saline solution[18,19]. Sham and BDL control groups received the vehicle of DAA and UDCA at the same volume and route.

Sample collection

Samples were collected 4 weeks after the surgery. Measurements of the body weight was applied at the time of the surgery, weekly thereafter, then at the time of sacrifice. The rats were fasted overnight, anesthetized and weighed. Blood samples were collected, left to clot in room temperature for 45 min, and then centrifuged at 3000 rpm for 15 min to obtain serum. Serum samples were divided into 200 µl aliquots and frozen at −20◦C until being assayed for bilirubin, and liver enzymes. After necropsy, the liver was dissected out, cleaned of the blood, washed in ice-cold saline, blotted, weighed, examined for gross lesions, and then processed for histology.

Histopathological examination

The left median lobe of liver were fixed in 10% formalin for 24 hr. After that, the samples were washed in running tap water overnight. They were dehydrated in ascending series of alcohol, cleared in terpineol, and then embedded in paraffin wax. Transverse sections, 5 µm thick, were cut on a rotary microtome, stained with hematoxylin and eosin or Masson trichrome[20].

Liver sections were assessed for necrosis, bile duct proliferation and fibrosis. The stage of fibrosis was evaluated according to METAVIR scoring system, where F0: no fibrosis, F1: portal fibrosis without septa, F2: few septa, F3: numerous septa without cirrhosis, and F4: cirrhosis[21].

Liver biomarkers

Serum alanine aminotransferase (ALT), aspartate amino-transferase (AST) and alkaline phosphatase (AP) activities, in addition to total and conjugated bilirubin were assayed according to manufacturer's instructions.

Statistics

Numerical data are reported as mean values and standard deviation. Graph Pad Prism (version 5.0, GraphPad software, San Diego, CA, USA) was used to conduct all statistical analysis. Data were analyzed statistically using One-way ANOVA before applying post hoc multiple comparisons (Tukey's test) for comparative analysis between the groups. $P < 0.05$ was regarded as statistically significant.

RESULTS

Clinical symptoms

Sham-operated rats appeared healthy and active. They steadily increased in body weight in the 28-day period of the study, and gained about 15% of their initial weight. On the other hand, bile duct-ligated control rats appeared weak and hypoactive, and suffered from jaundice. The average weight of BDL rats was significantly lower than that of the sham group, where BDL animals lost about 3.5% of their weight at the end of the experiment. In contrast, treating BDL animals with DAA and/or UDCA resulted in the recovery from the symptoms of bile duct ligation. All animals of the treated groups appeared normal and active. They did not show any sign of jaundice comparable to BDL-operated rats. The animals of all treated groups gained weight after treatment, which reached 6% in DAA-treated group, 3.17% in UDCA-treated group, and ~9% in DAA+UDCA-treated group (Figure 1).

Gross morphology of the liver and reproductive organs

The livers of sham-operated rats had normal appearance, size, and color. On the other hand, livers of BDL rats appeared slightly yellowish and hard, and hypertrophied with significantly increased relative weights in comparison with the livers of the sham group. This was accompanied with light yellowish brown color and finely rough surface typical of liver cirrhosis. On the contrary, the DAA or/ and UDCA-treated rats recovered from hepatomegaly (Table 1; Figure 2).

Biochemical results

The data in (Tables 2,3) illustrates the effect of treatment with DAA or/and UDCA on liver biomarkers in BDL-operated rats. BDL rats showed significant increase in AST, ALT, AP, total and direct bilirubin levels, when compared with sham group. Meanwhile, the same group showed significant decrease in serum concentrations of albumin compared with those of the sham group. Treating ligated rats with DAA or/and UDCA significantly ameliorated the biochemical results compared with BDL control group. Rats treated with DAA or/and UDCA showed reduction in the elevated serum levels liver biomarkers in comparison with untreated BDL group. No statistical difference was noted between the results of all treatment regimes, but the group treated with both DAA and UDCA showed the best results (Figures 4-8).

Histology

The livers of Sham-operated rats had the architecture of a normal liver. The hepatic tissue of this group was arranged into indistinct hepatic lobules (Figure 10a). At the edge of these lobules are the portal spaces, which contain a small hepatic artery branch, a portal vein branch, and a small bile duct. In the center of the hepatic lobules are the central veins that drain out to hepatic veins. Each lobule is composed of hepatocytes that are arranged into branching plates or cords with sinusoids in between. Hepatocytes appeared as polygonal cells with granular eosinophilic cytoplasm and round nuclei (Figure 10b).

In contrast, the hepatic tissue of BDL rats had significantly altered architecture and disorganized hepatic lobules and hepatocytic cords due to the development bridging fibrosis between portal spaces that led to cirrhosis (Metavir score of liver fibrosis= 3.33; Figures 9, 10 c, 11). At the portal spaces, bile ductules showed hypertrophy and hyperplasia, with bile accumulation was shown as yellowish coloration in the periportal area. This was accompanied with fibrosis, inflammatory cell infiltration and hepatocytic necrosis (Figure 10d).

In bile duct-ligated animals treated with DAA, the normal architecture of the hepatic tissue and the normal organization of hepatic lobules were restored. Fibrosis was mild in most animals of this group (Metavir score of liver fibrosis= 1.2; Figures 9, 10 e,11). No signs of inflammation, bile ductular hypertrophy or hyperplasia, or hepatocytic necrosis was observed (Figure 10f).

In bile duct-ligated rats treated with UDCA, the hepatic architecture was slightly altered due to the presence of periportal fibrosis (Metavir score of liver fibrosis= 1.6; Figures 9, 10 g, 11) and moderate bile ductular hypertrophy or hyperplasia (Figure 10h).

In bile duct-ligated animals treated with DAA and UDCA, the hepatic architecture, lobules and hepatocytes were comparable to those of the sham-operated group and showed almost no signs of fibrosis (Metavir score of liver fibrosis= 0.4; Figures 9, 10 i, j, 11).

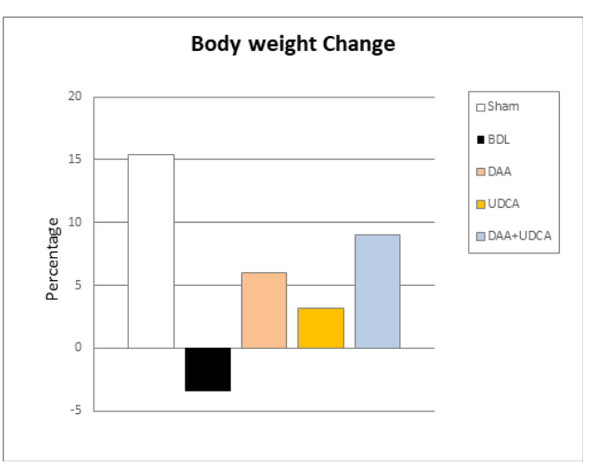

Fig. 1: Effect of DAA or / and UDCA on the body weight change in bile duct-ligated rats

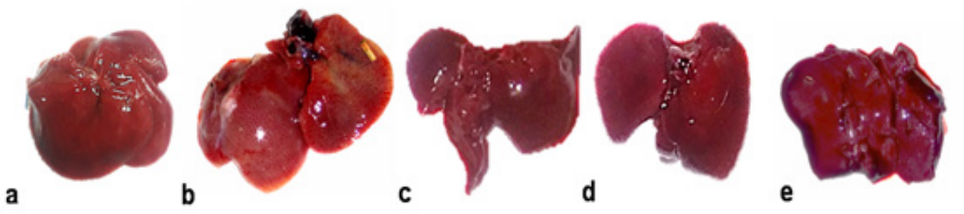

Fig. 2: Effect of DAA or / and UDCA on liver gross morphology in bile duct-ligated rats. (a): Sham-operated, (b): BDL control, (c): DAA-treated, (d): UDCA-treated, and (e): DAA+UDCA-treated group.

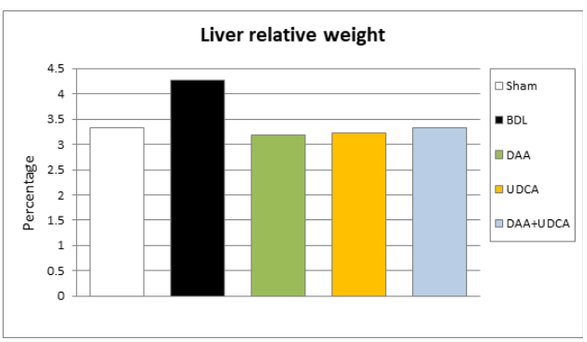

Fig. 3: Effect of DAA and/or UDCA on the liver relative weight in bile duct-ligated rats

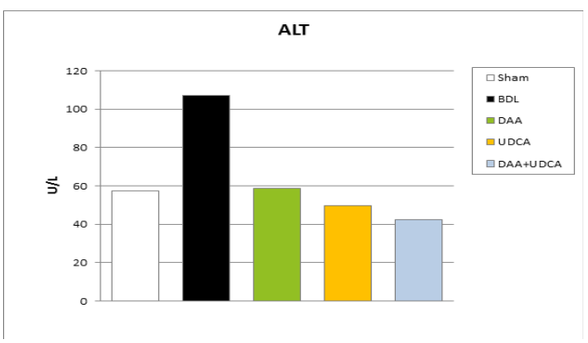

Fig. 4: Effect of DAA and/or UDCA on ALT in bile duct-ligated rats

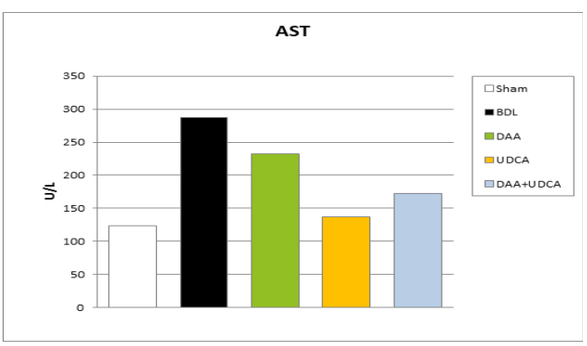

Fig. 5: Effect of DAA and/or UDCA on AST in bile duct-ligated rats

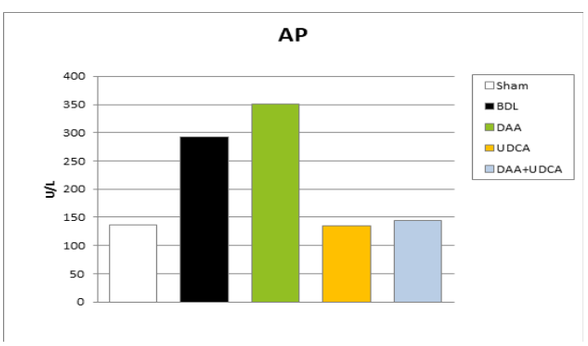

Fig. 6: Effect of DAA and/or UDCA on AP in bile duct-ligated rats

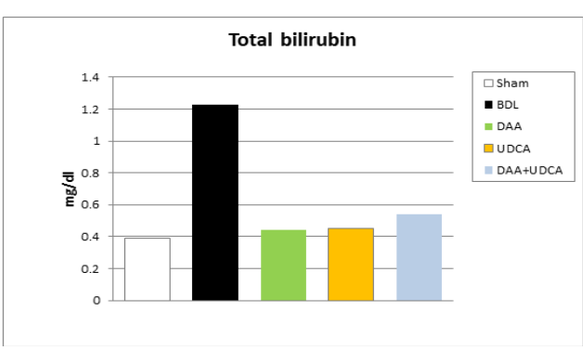

Fig. 7: Effect of DAA and/or UDCA on total bilirubin in bile duct-ligated

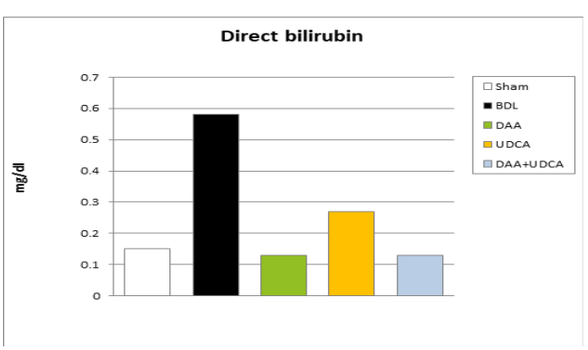

Fig. 8: Effect of DAA and/or UDCA on direct bilirubin in bile duct-ligated rats

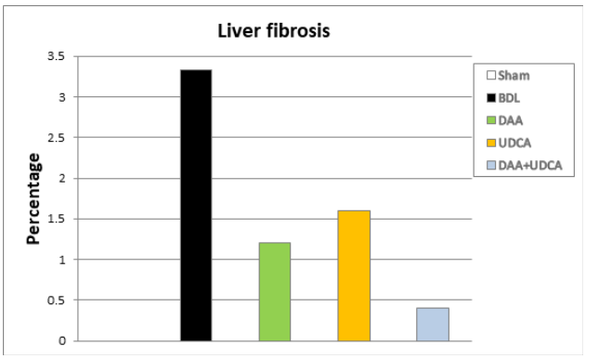

Fig. 9: Effect of DAA or/and UDCA on liver fibrosis in bile duct-ligated rats

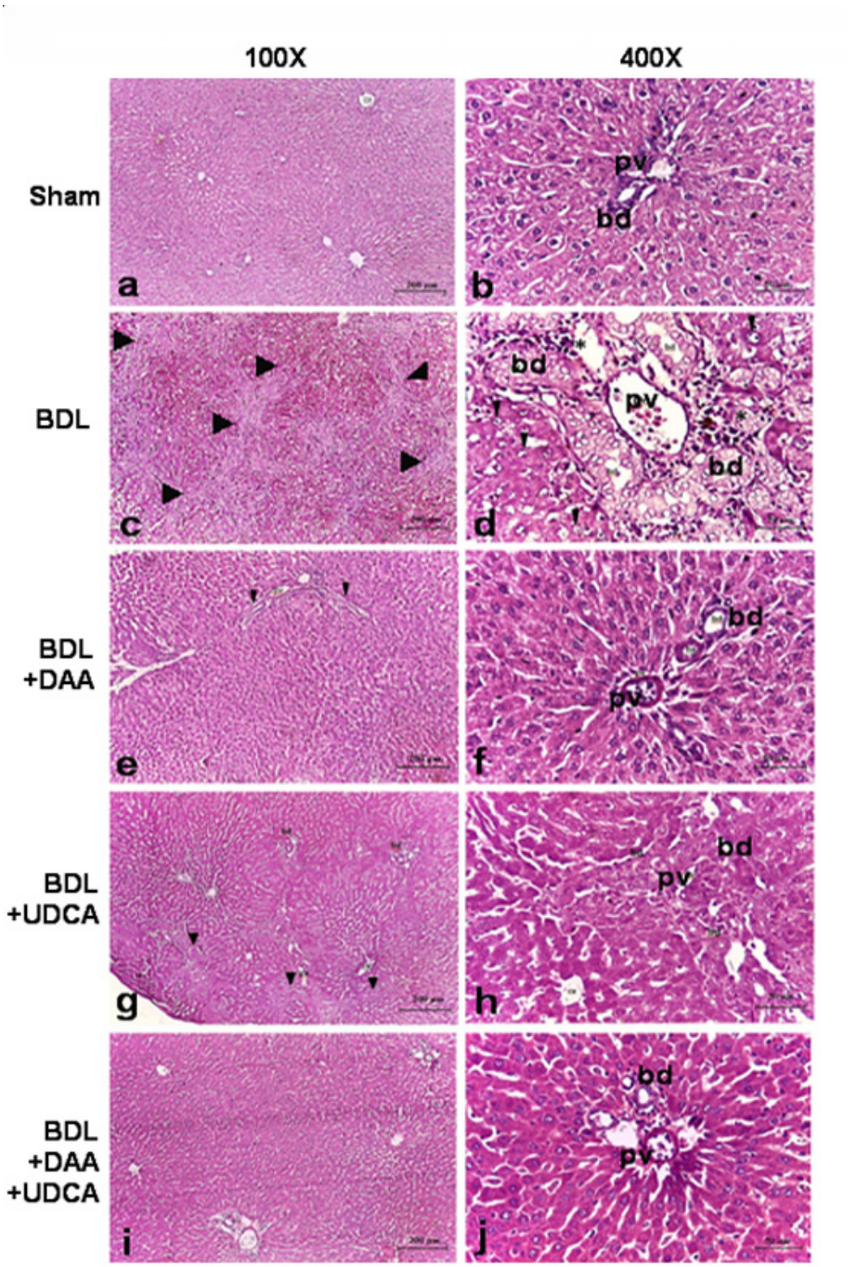

Fig. 10: Effect of DAA or/and UDCA on liver histology of bile duct-ligated rats. (a-b): Sham-operated, (c-d): BDL control, (e-f): DAA-treated, (g-h): UDCA-treated, and (i-j): DAA+UDCA-treated group. Arrowheads: fibrosis; bd: bile ductule; pv: portal vien.

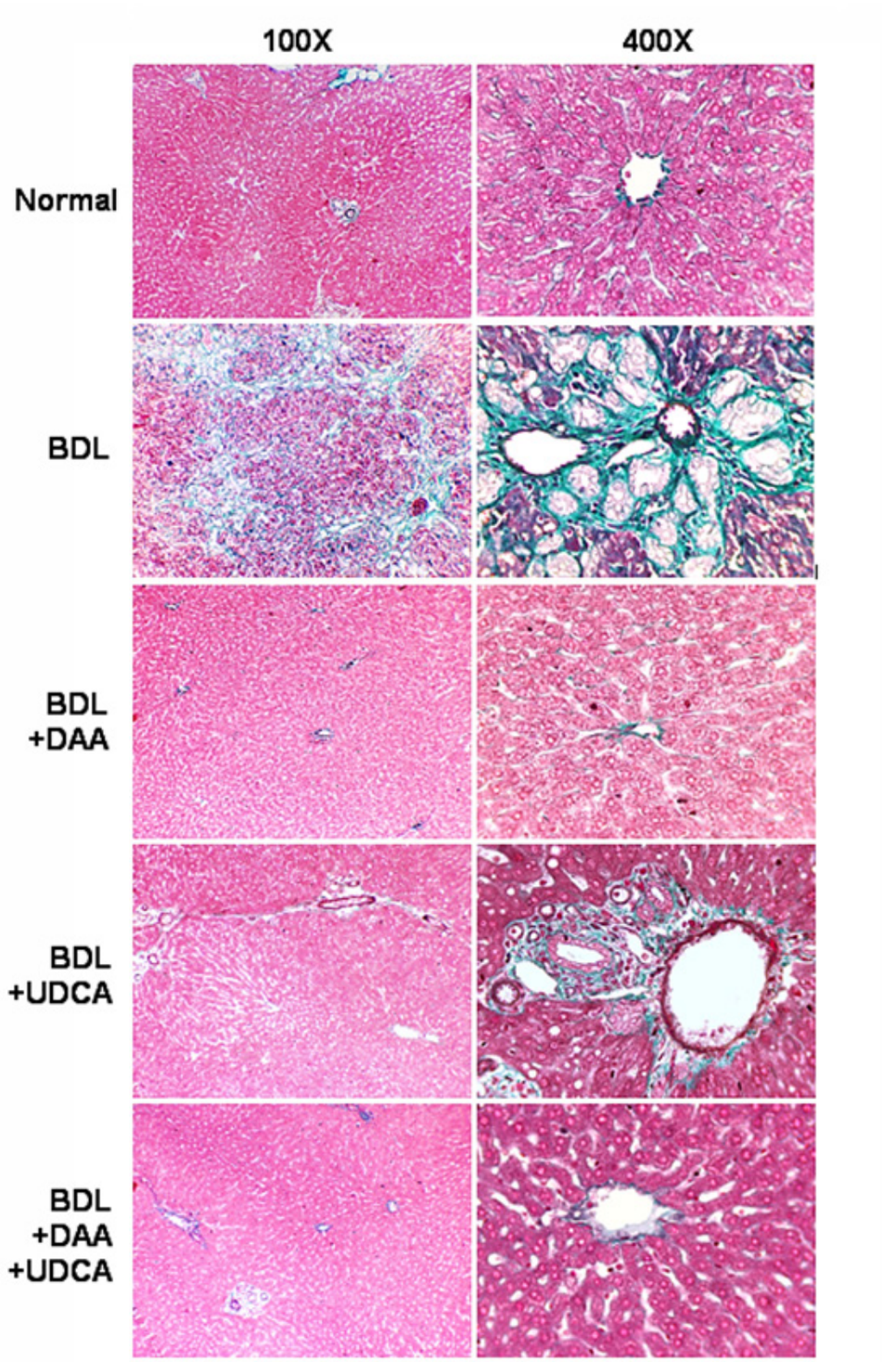

Fig. 11: Effect of DAA or/and UDCA on liver fibrosis in bile duct-ligated rats.

Table 1: Effect of DAA or / and UDCA on liver relative weights in bile duct-ligated rats

Groups	Liver
Sham	3.33 ± 0.08
BDL Control	4.38 ± 0.169††
BDL+DAA	3.19 ± 0.166**
BDL+ UDCA	3.22 ± 0.094**
BDL+UDCA+DAA	3.32 ± 0.156**

Data are expressed as mean ± SEM.
†Symbol represents significance compared with sham group, where †: $P<0.05$ and ††: $P<0.001$.
*Symbol represents significance compared with BDL control group, where *: $P<0.05$ and **: $P<0.001$.

Table 2: Effect of DAA or/and UDCA on hepatic enzymes in bile duct-ligated rats

Groups	ALT (IU/L)	AST (IU/L)	AP (IU/L)
Sham	57.20±5.89	122.8±6.03	136.4±3.96
BDL Control	135. ±3.60††	351.8±13.60††	315.0±21.31††
BDL+DAA	58.60±8.16**	232.2±41.77*	171.2±16.92**
BDL+ UDCA	49.60±3.86**	137.4±3.86**	135.6±7.12**
BDL+UDCA+DAA	42.60±2.23**	172.0±36.47**	144.4±17.33**

Data are expressed as mean ± SEM.
†Symbol represents significance compared with sham group, where †: $P<0.05$ and ††: $P<0.001$.
*Symbol represents significance compared with BDL control group, where *: $P<0.05$ and **: $P<0.001$.

Table 3: Effect of DAA or/and UDCA on non-enzymatic hepatic parameters (total and direct bilirubin) in bile duct-ligated rats

Groups	TBIL (mg/dL)	dBIL (mg/dL)
Sham	0.38 ± 0.007	0.15 ± 0.013
BDL Control	1.52 ± 0.032††	37.71 ± 3.12††
BDL+DAA	0.44 ± 0.022**	0.13 ± 0.013**
BDL+ UDCA	0.45 ± 0.023**	0.27 ± 0.020**
BDL+UDCA+DAA	0.53 ± 0.100**	0.13 ± 0.016*

Data are expressed as mean ± SEM.
†Symbol represents significance compared with sham group, where †: $P<0.05$ and ††: $P<0.001$.
*Symbol represents significance compared with BDL control group, where *: $P<0.05$ and **: $P<0.001$.

DISCUSSION

In order to investigate the curative effects of DAA or/and UDCA on cholestasis, we employed bile duct ligation to cause cholestatic liver impairment in male rats. Cholestasis was well-established 28 days following the surgical biliary blockage. This was demonstrated by the considerable rise of liver enzymes and serum bilirubin, which was seen both here and elsewhere[21-24]. Hepatocytic necrosis, bile ductular hyperplasia, and hepatic cirrhosis were all linked to these biochemical alterations[21,25].

The BDL-operated rats' body weights significantly decreased. According to reports[26,27], liver illness (such as cholestasis), which is typically accompanied by inadequate hepatic function and malnutrition, might be the cause of this weight loss. As previously observed[27,28], hyperbilirubinemia, which accelerates catabolism, may potentially be to blame for this decline.

Hepatomegaly was seen in the current investigation in BDL-operated rats, which is consistent with other findings[21,29]. The following might be used to explain the increase in liver weight: during liver damage, hepatic stellate cells activate and transdifferentiate into cells that resemble myofibroblasts and create extracellular matrix[30].

The levels of AP, ALT, AST, direct and total bilirubin, which are believed to be biochemical indicators of cholestasis, were considerably raised in the current research[31]. The two primary components of bile, bilirubin and bile acids, are raised and maintained in the blood as a result of cholestasis. The toxic bile acids themselves cause hyperbilirubinemia by obstructing the bile excretory route and impairing hepatocyte secretory function[32]. Hepatocytic necrosis and the subsequent leaking of liver enzymes from hepatocytes into the circulation are caused by the detergent-like effects of hydrophobic bile salts on cell membranes. This explains why the blood's level of liver enzymes has increased[32].

Histological analysis revealed bile blockage of interlobular bile ducts, portal enlargement, and bile duct proliferation at the microscopic level. Bile stagnation can be brought on by the retention of bilirubin (bilirubinostasis). Portal edema and centrilobular bilirubinostasis are the early symptoms of acute total blockage of the extrahepatic bile ducts. Cholangitis and parenchymal bilirubinostasis, which spreads into the periportal regions, follow[32,33]. Rats with BDL surgery also exhibited hepatocytic necrosis and liver cirrhosis. According to earlier research[16,34], bile acid buildup and systemic oxidative stress may be responsible for liver injury. The highly abundant protein with the sulfhydryl (-SH) residue is oxidized by reactive oxygen species at the hepatocytes' cell membranes, resulting in necrosis[35].

We employed UDCA as the standard treatment for cholestasis in the current investigation. UDCA reduced body weight, liver relative weight, raised biochemical parameters, and mitigated histological changes brought on by cholestasis. It also decreased the high biochemical parameters. These findings concur with those of other research[21,29,36]. There are a number of causes for UDCA's ameliorative impact. First off, UDCA is the typical treatment for the majority of cholestatic hepatopathies because it lessens liver harm caused by partial or total biliary blockage, which has been shown to reduce inflammation and bile-acid-induced liver damage[5]. Second, UDCA lowers the bile acid pool's hydrophobicity and lowers AP serum activity. UDCA also stimulates intracellular signaling pathways, including protein kinase C and mitogen-activated protein kinase, and reduces liver damage in cholestasis by activating MAPK in response to one of three types of external stimuli: binding of ligand to tyrosine kinase, cytokine receptors, or G-protein coupled receptor, which then transmits extracellular stimuli, in this

case liver damage, to intracellular responses and It boosts biliary HCO3−production and hepatobiliary secretion, protecting hepatocytes and cholangiocytes from the harm caused by hydrophobic bile acid[37]. Moreover, UDCA activates endogenous antioxidants and prevents oxidation that leads to fibrosis[35].

This study is the first to use DAA for treating cholestasis. After treating BDL-operated rats with DAA, all cholestasis-associated alterations including the body weight, liver relative weight, biochemical parameters and histopathological observations were restored to normal. Supplementing with DAA is frequently used to raise testosterone[38]. Due to enhanced protein synthesis, testosterone levels are positively correlated with both muscle growth and weight gain[39]. Aspartate derivatives, such as DAA, function as a substrate for the production of purine and pyrimidine nucleotides, which has a therapeutic effect on liver issues including hepatomegaly by controlling tissue growth. DAA increased the levels of testosterone, which increases transcription rates by binding to the androgen receptor and promoting protein synthesis and cell proliferation[40]. DAA improves liver integrity by down-regulating the expression of pro-inflammatory mediators, such as toll-like receptor-4 (TLR4), nucleotide-binding oligomerization domain protein signaling genes signaling related genes and mRNA expressions of tumor necrosis factor[13]. Additionally, DAA lowers inflammation and regulates interleukin 6 (IL-6), a key mediator of many hepatopathies. The production of antioxidant enzymes is increased by DAA, which reduces oxidative stress, prevents lipid peroxidation of hepatocyte cell membranes, and so preventing the fibrosis process[14,41].

It was obvious that treating cholestatic rats with DAA and UDCA showed better ameliorating results regarding body weight, organ relative weight, liver enzymes and liver histology than using DAA or UDCA alone. This proves a synergistic effect of the chemical and therapeutic properties of the two compounds DAA and UDCA.

CONCLUSION

DAA or/and UDCA could have a therapeutic effect against cholestasis induced by bile duct ligation. However, additional work is needed to recognize the mechanism(s) involved in this effect.

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CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثير العلاجي لحمض الأسبارتك على الركود الصفراوي في الجرذان المربوط قناتها الصفراوية

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المقدمة: تعمل الأحماض الأمينية كعوامل واعدة في مجموعة متنوعة من المجالات العلاجية. حمض الأسبارتيك هو حمض أميني غير أساسي ويعمل علي تخفيف تأثير عدد من أمراض الكبد، ولكن لم يتم اختبار تأثيره ضد الركود الصفراوي وبالتالي تهدف هذه الدراسة إلى اختبار تأثير حمض الأسبارتيك مقارنةً بالدواء العلاجي لعلاج أمراض الكبد الصفراوية حمض الأورثوديوكسيكوليك.

المواد والطرق: تم إحداث الركود الصفراوي عن طريق ربط القناة الصفراوية في ذكور الجرذان البيضاء. بعد ثلاثة أسابيع من العملية، تم تقسيم الحيوانات إلى خمس مجموعات: جرذان لم يتم ربط القناة الصفراوية لها، جرذان تم ربط القناة الصفراوية لها، جرذان تم ربط قناتها الصفراوية ثم علاجها بحمض الأسبارتيك، جرذان تم ربط قناتها الصفراوية ثم علاجها بحمض الأورثوديوكسيكوليك وأخري تم علاجها بكل من حمض الأسبارتيك وحمض الأورثوديوكسيكوليك. تم إعطاء جميع الجرعات العلاجية عن طريق الفم خلال الأسبوع الرابع من إجراء العملية.

النتائج: في نهاية التجربة، تم جمع عينات الدم وأنسجة الكبد وإخضاعها لتحليلات مختلفة. عانت الجرذان التي خضعت لعملية الربط الجراحية من انخفاض وزن الجسم وارتفاع الوزن النسبي للكبد ، بالإضافة إلى زيادة معدل المؤشرات الحيوية للكبد ALT و AST و AP والبيليروبين المباشر والبيليروبين الكلي مقارنة مع جرذان الشام. كما عانت هذه الحيوانات من الناحية النسيجية من تليف الكبد، وعدم انتظام الفصيصات الكبدية وتضخم القناة الصفراوية. وعلى صعيد آخر، تحسنت وظائف وأنسجة الكبد بشكل ملحوظ بعد علاج الفئران بحمض الأسبارتيك وحمض الأورثوديوكسيكوليك بأحدهما أو كليهما.

الاستنتاج: ختاماً، فإن حمض الأسبارتيك له تأثير علاجي على الركود الصفراوي المحدث في الجرذان وخاصة عند استخدامه مع حمض الأورثوديوكسيكوليك.