The effects of ursodeoxycholic acid in the management of sepsis-induced cholestasis in rodents

Randa H. Ainosah

East Jeddah Hospital, Ministry of Health, Saudi Arabia

Magda M. Hagra

Suez Canal University Faculty of Medicine

Sameer Alharthi

King Abdulaziz University, Faculty of Medicine, Department of Pharmacology

Omar I. Saadah (✉ osaadah@kau.edu.sa)

<https://orcid.org/0000-0002-3744-9907>

Research article

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Abstract

Background: Cholestasis is a condition in which there is impairment of bile flow from the liver to the small bowel. It is a common complication of bacterial infection and sepsis. Treatment is usually directed towards the eradication of bacterial infection and consequences of sepsis. Ursodeoxycholic acid (UDCA) has been under investigation as a possible therapeutic option for the treatments of sepsis-associated cholestasis.

Methods: Sixty male albino rats (weighing 100–150g) were subjected to daily doses of UDCA (100 mg/kg, p.o.) for 10 days before or after lipopolysaccharides (LPS) induction of cholestasis. Then, the following liver enzyme activity was assessed: plasma aspartate transferase (AST), plasma alkaline transferase (ALT), plasma alkaline phosphatase (ALP), total bilirubin (TBIL). Hepatocyte apoptosis and immunomodulatory activity were assessed by flow cytometric analysis. Plasma pro-inflammatory cytokines (TNF- α , IL-1 β and IL-4) were measured by ELISA. Liver histology changes were assessed by hematoxylin and eosin (H&E) staining.

Results: Our results showed that LPS-induced cholestasis resulted in a significant rise in the TBIL and liver enzymes including GGT, ALP, AST, and hepatocytes death. UDCA improves serum liver chemistries and halts bile acid cytotoxicity when it was used either as a treatment or prevention, compared to the LPS group. Moreover, UDCA has immunomodulatory properties: the effect of UDCA on the percentage of natural killer (NK) cells did not change in either the treatment or prevention group when compared to LPS induced cholestasis. However, significant decrease in the CD3 has been found in the treatment group as compared to the LPS group, and an unexpected increase in the prevention group compared to the LPS treated group. UDCA failed to ameliorate the increase in plasma TNF- α concentration in the treatment group. On the other hand, UDCA caused reduction in plasma TNF- α in the prevention group. We also found significant reduction in the liver tissue apoptosis in the UDCA treated groups.

Conclusion: Prophylactic treatment and treatment with UDCA appear to exert a beneficial effect against the damaging effect of hydrophobic bile acids by LPS-induced secretory failure. This involved multiple mechanisms of action.

Background

Cholestasis is considered a clinical syndrome that occurs because of disruption of bile formation due to impaired secretion by hepatocytes or blockage in the flow of bile through the intrahepatic or extrahepatic bile ducts (1). Infections may induce cholestasis via bacterial endotoxins such as lipopolysaccharides (LPS), which are released by gram-negative bacteria from the sites of bacterial infection into the circulation; such endotoxins provoke hepatic Kupffer cells to release proinflammatory cytokines, such as tumor necrosis factor- α , (TNF- α), and interleukin-6 (IL-6), which leads to failure of bile secretion (2, 3). Sepsis-induced cholestasis may be an early complication in 20% of hospitalized patients with sepsis (4). Severe sepsis may result in life-threatening organ failure due to dysregulated host immune response

against infection (5). Sepsis-associated hepatic damage may result from cholestasis induced by the impairment of hepatocellular and bile duct formation due to circulating pro-inflammatory cytokines (2). Apart from the control of sepsis with appropriate antibiotics and supportive care, there is no specific treatment for cholestasis (6).

Ursodeoxycholic acid (UDCA) is the first effective modifying therapy for patients with primary biliary cholangitis (PBC)—previously known as primary biliary cirrhosis—which is a chronic immune-mediated liver disease characterized by progressive cholestasis, biliary fibrosis, and eventually cirrhosis (7). UDCA is very effective in about two thirds of treated patients with an expected survival similar to the general population (8). Furthermore, UDCA is the preferred choice for empiric therapy of most cholestatic liver disease, including cholelithiasis (9). UDCA (3, 7-dihydroxy-5-cholanic acid) is a hydrophilic bile acid that is more commonly becoming used for the treatment of various cholestatic disorders (10, 11). It is normally present in human bile acids in low concentration. UDCA is a major component of bile acid in black bear's bile, which has historically been used in Chinese traditional medicine for the treatment of liver disease; however, the Japanese were the first to formally report its effectiveness on patients with liver disease (12). The mechanisms behind the beneficial effects of UDCA in cholestasis are still unclear. Evidence from experimental studies suggests three major mechanisms of action: cytoprotection of cholangiocytes from the detergent effect of bile acids, enhancement of hepatobiliary secretion, and hepatocytes protection from apoptosis-induced by bile acids. One or all of these mechanisms may be relevant to some cholestatic disorders (13). The aims of this study are to investigate the effect of UDCA in the treatment and prevention of LPS-induced cholestasis and to examine the underlying mechanisms, including liver tissue apoptosis, inflammatory mediators, and any possible immunomodulatory mechanisms.

Methods

This study was conducted according to the guidelines of the Research Animal Care Committee-approved protocol at the King Fahd Medical Research Center. All studies conducted on animals were approved by the Bioethical Research Unit of Faculty of Medicine, King Abdulaziz University (Ref: 597–17)

Chemicals

Lipopolysaccharide (LPS) was purchased from (Sigma Chemical Co., St. Louis, MO). It was supplied as a lyophilized powder for reconstitution, as 5 ml sterile saline NaCl 0.9%. Ursodeoxycholic acid (UDCA) was provided as suspension form Ursofalk (Dr. Falk Pharma GmbH, in Freiburg, Germany). Alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TBIL) kits were obtained from "Wako Pure Chemical Industries", in Osaka, Japan. Annexin V-FITC apoptosis detection kits were purchased from Aldrich Chem. Corp., in the USA. TNF-alpha kits and Interleukin Kits (IL1, IL4) were purchased from "ASSAYPRO" Co. (Universal Biologicals (Cambridge) Ltd.,

UK). Enzyme-Linked Immunosorbent Assay (ELISA) Kits and T/B/NK Kits were purchased from the “BD Biosciences” Co., in Franklin Lakes, New Jersey.

Animals

Sixty male albino rats (weighing 100–150g) were obtained from King Fahd Medical Research Center, King Abdulaziz University, in Jeddah, Saudi Arabia. Animals were housed in plastic cages with free access to water and standard chow. They were placed in an adjusted temperature of 20–25°C and controlled humidity of $50 \pm 5\%$ on a 12 h dark/light cycle. They were kept for acclimation one week before the start of experimental work.

Experimental Design

Rats were randomly classified into five groups (12 rats each). The control (untreated) group received saline (0.5 ml/kg, i.p.) and water (0.5 ml/kg, p.o), both used as a vehicle for delivering LPS and UDCA respectively for 10 days. The UDCA-treated group received a daily dose of UDCA (100 mg/kg, p.o) for 10 days (14). In the LPS-induced cholestasis group, rats were injected with a single sublethal dose of LPS (5 mg/kg, i.p.) (3, 15). In the treatment group, rats were injected with LPS; 24 hours later, these rats received UDCA in a dose of 100 mg/kg, p.o. for 10 days (16). In the preventive group, rats received a daily dose of UDCA for 10 days before LPS injection and then 24 hours later the rats were euthanized. At the end of the experiment, rats were exsanguinated by severing the subclavian artery and vein under lethal anesthesia using pentobarbital sodium, in a dose of 50 mg/kg, i.p. Blood samples were collected from the retro-orbital venous plexus with heparinized sterile capillary tubes for further analysis.

Liver enzymes analysis

Sera were collected individually in stoppered polystyrene tubes and stored in a deep freezer at -20°C until the time of analysis for liver enzymes: AST, ALT, ALP, and TBIL activities were analyzed using the Transaminase CII-test Wako. The serum AST:ALT ratio was computed from the results of the serum AST and ALT activities.

Flow Cytometric Analysis of Apoptosis

Livers and spleens were perfused with phosphate buffer saline (PBS), removed, cut into small pieces, and stored in a deep freezer at -20°C until the time of evaluating the anti-apoptotic effect using Annexin V-FITC Apoptosis Detection Kit (eBioscience, San Diego, CA, U.S.) according to the manufacturer's instructions. Flow cytometric analysis of apoptosis was performed on a BD FACSCalibur using Cell Questpro software (BD Biosciences).

Immunological assays

Spleens were used to evaluate the effect T/B/NK cells. The Rat T/B/NK were measured by use of a flow cytometric analysis ("BD Biosciences"), according to the manufacturer's instructions.

Cytokine Quantification

Cytokines (TNF-, IL-1 and IL-4) in the sera or the culture supernatants were measured by ELISA according to the manufacturer's instructions.

Histopathology

Liver tissue was obtained from UDCA-fed or control rats 24 hours after or before LPS injection. Sections were prepared from formalin-fixed (5- μ m-thick slides) paraffin-embedded tissue samples stained with hematoxylin and eosin (H&E) by routine procedure. Stained sections were examined using a microscope (SPI Supplies®) connected to a digital camera (camera fits eyepiece sizes 23mm to 30mm).

Statistical analysis

SPSS version 20 (SPSS, Chicago, IL, USA) was used for data processing. Data are expressed as means $\pm$ S. D. A one-way ANOVA and Tukey's post hoc test were used for multiple comparisons. The number of animals per group was (n = 12): A *P*-value <0.05 was considered statistically significant.

Results

The following variables indicated the effect of UDCA on prevention and treatment groups of LPS-induced cholestasis.

Effect of UDCA on biochemical parameters of liver function

To determine the efficacy of the UDCA, the liver function parameters in the serum were measured as indicators of liver-injury status, and the results for each group are listed in Table 1. The rats on the LPS group had significantly higher values for GGT, ALP, TBIL, and AST compared with that of the control group or UDCA group ($p < 0.05$). The treatment group with UDCA had only significantly lower values for GGT, ALP, and AST ($P < 0.05$). However, in UDCA pretreatment, the values for GGT, ALP, TBIL, and AST were significantly lower than the corresponding values in the LPS group ($p < 0.05$). Moreover, the ALP and TBIL values were significantly lower than those in the treatment group ($p < 0.05$).

Effect of UDCA on hepatocyte apoptosis

Apoptosis is one of the mechanisms underlying UDCA growth inhibition *in vitro*. To determine whether UDCA elicits growth inhibition *in vivo* through apoptosis, liver tissue was isolated and examined using flow cytometric analysis, as demonstrated in *Figures 1&2*. The results indicated that the LPS injected group had significantly increased apoptotic activity as compared to the control group ($p = 0.003$). Both treatment and prevention groups demonstrated reduced apoptosis when compared to the LPS group ($p = 0.013$ and $p = 0.002$, respectively). There was no significant difference found between the control group and the UDCA ($p = 0.63$), treatment ($p = 0.44$), and prevention groups ($p = 0.9$) (*Table 2*). To further investigate the immunomodulatory properties of UDCA, liver tissues were isolated in five rat groups.

Immunoregulatory properties of UDCA

Table 3 shows a decrease in the activity of NK cells in the LPS group compared to the control group, but reduction was not statistically significant ($P = 0.14$). Similarly, no statistical significance was detected in NK cells activity between LPS and either the UDCA treatment group or the pretreatment group ($p = 0.12$, $p = 0.74$, respectively). The effect of UDCA on T-lymphocytes (CD3) activity in the different study groups was examined. The percentage of CD3 when UDCA was used as treatment increased compared to the LPS group ($p = 0.000$), however, the pretreatment group showed that the percentage of CD3 cells had decreased significantly as compared to either LPS group or the control group ($p < 0.05$). No significant difference was found between the treatment and pretreatment groups ($p = 0.17$).

Effect of UDCA on proinflammatory cytokines

The effect of UDCA on the inflammatory mediators in treatment and prevention groups were also investigated, as shown in *Table 4*. The concentration of TNF- in the LPS injected group significantly increased compared to the control. However, treatment with UDCA decreased the concentration of TNF-, with no significant difference compared to the LPS group, while the use of UDCA as pretreatment decreased the concentration of TNF- significantly ($p < 0.05$). There was no significant difference between the treatment and pretreatment groups ($p = 0.5$). The concentration of the inflammatory cytokine IL-1 α in the LPS group significantly increased compared to the control ($p < 0.05$), with no significant difference detected between LPS and either UDCA treatment or pretreatment groups ($p = 0.7$). In addition, the concentration of the IL-4 in the LPS group significantly increased compared to the control group ($p < 0.05$), while no difference was found between LPS group and either treatment or pretreatment group. The IL-4 concentration in the pretreatment group was significantly less than the IL-4 concentration in the treatment group ($p = 0.032$).

The effect of UDCA on liver histology changes

The histopathological features are depicted in *Figure 3*. The hepatocytes in the control rat liver are arranged around central veins and extend in branched plates towards the portal area. They are polyhedral in shape with acidophilic stained cytoplasm. Their nuclei are central and rounded with dispersed

chromatin. Portal tracts showed branches of portal vein, hepatic artery, and bile duct. They are surrounded by fine connective tissue fibers with few cells. The UDCA given to control rats did not alter normal structure of liver parenchymatous tissue. Liver parenchyma of the LPS group showed congested blood sinusoids, hepatocytes with unstained cytoplasmic degenerated regions, and small degenerated or lost nuclei; in the region of necrotic inflammatory infiltrate, apoptotic, degenerated, and inflammatory cells around bile ducts were observed. The liver tissue examination of the prevention group showed normal histopathological changes/features. Hepatocytes looked normal with no degeneration changes in their cytoplasm or nuclei; sinusoids between cells are not congested and are similar to those of the control. Regions near the portal area showed normal hepatocytes but still few degenerated inflammatory cell aggregates. In this group, administration of UDCA as a treatment provided marked histological changes induced by LPS. Hepatocytes, sinusoids, and portal areas looked normal without any degenerative, necrotic, or apoptotic changes. No inflammatory cells were observed in the portal tracts. The effect of UDCA on the nuclear area length, measured as μm in the liver tissue, was also investigated. In both treatment and pretreatment groups, the administration of UDCA provided a significant reduction in the nuclear area length as compared to the LPS group, as demonstrated in *Figure 4*. Taken together, these results indicate the hepatoprotective effects of UDCA administration throughout multi-functionality.

Discussion

Sepsis-induced cholestasis is one of the types of inflammatory cholestasis in which pro-inflammatory cytokines, produced by the effect of LPS endotoxin on the hepatic Kuepfer cells, induces impairment of bile production (3). These pro-inflammatory cytokines cause transcriptional and post-transcriptional impairment of hepatocellular expression of bile acids carriers and glutathione (12, 17).

In this study, the intra-peritoneal injection of LPS induces cholestasis in rats that resulted in significant rise in the TBIL and liver enzymes, comprising GGT, ALP, and AST (Table 1), in agreement with a number of other investigators (15, 18). Liver enzyme ALP is a cellular membrane-bound enzyme that is induced and released into the circulation in response to cellular membrane damage by the endogenous bile acids, and causes hepatocytes damage and lysis (19). The UDCA had anti-cholestatic effects on the LPS-treated rats in our experiment that was manifested by reduction in the mean level of liver enzymes: GGT, ALP, and AST. UDCA had also a preventive role when administered to experimental rats before LPS induction of cholestasis, as evidenced by the comparable liver enzymes and TBIL to the control, and significantly lowered the level of liver enzymes GGT, ALP, and AST compared to LPS-induced rats. This response may be explained partly by its membrane protective effects (20).

The accumulation of endogenous bile acids in the hepatocytes results in cellular damage and hepatocytes death. We found that LPS induced hepatocytes death was evident by higher percentage of apoptosis as compared to the control group; UDCA resulted in a lower percentage of an apoptosis comparable to the control group, which favors the positive effect of UDCA in minimizing or preventing hepatocellular damage and death. This finding was in agreement with one previous study (21).

We investigated the immunomodulatory effect of UDCA in LPS-induced cholestasis. The percentage of natural killer (NK) cells did not change in either the treatment or prevention group when compared to LPS-induced and control rats. However, estimating the percentage of CD3 T-cell co-receptor protein in all groups has shown a significant decrease in the treatment group as compared to the LPS group, and an unexpected increase percentage in the prevention group as compared to the LPS treated group. Our result for the treatment group was consistent with that reported by Manousou *et al.*, who found reduction in the chemokine receptor (CXCR3) expression on T lymphocytes in the group treated by UDCA in comparison with the un-treated group (22).

LPS induced a significant rise in the concentration of TNF- α , IL-1 α , and IL-6 inflammatory cytokines compared to control group, similar to the results reported in Tanasescu *et al.* (23). UDCA attenuated the TNF- α concentration in the treatment and prevention groups but it was statistically significant only in the prevention group. There was no effect for UDCA on either IL-1 α or IL-6. This is consistent with the report of Razori *et al.* (24) who found that attenuation in TNF- α and IL-6 cytokines in LPS treated rats was not statistically significant. Also, Diamond *et al.* (25) showed the lack of effect of UDCA in reducing the expression of cytokines. This may be related to the instrumental role of cytokines in the mechanisms of hepatocytes regeneration (26).

The improvement in the liver enzymes in the groups who received UDCA, either as treatment or prevention, in comparison with the LPS group, coincides with significant improvement in the liver tissue when examining the hepatic histopathology. These findings were consistent with some other reports (16). In addition to the histopathological improvement, we also found significant reduction in the liver tissue apoptosis in the UDCA treated groups. Rodrigues *et al.* (27) showed that co-administration of UDCA with the apoptosis-inducing agents was associated with a 50–100% inhibition of apoptotic changes in all cell types. A recent study (24) has suggested that UDCA can reverse the cytokine-induced impairment in gene expression, and improves localization of the canalicular membrane transporters of bile acids, the bile salt export pump (Bsep) and multidrug resistance-associated protein 2 (Mrp2), by counteracting LPS-induced endocytic internalization. Future efforts will be focused on optimized dosage regimens, as well as on further elucidation of the mechanisms of action of UDCA at the molecular level.

Conclusion

In conclusion, our study showed that UDCA is beneficial for the treatment and prevention of LPS-induced cholestasis in animals; an effect that can be manifested by improvement in liver enzymes and histopathological abnormalities.

Abbreviations

UDCA: Ursodeoxycholic acid, ALT: plasma alkaline transferase, ALP: plasma alkaline phosphatase, TBIL: total bilirubin, GGT: Gamma-glutamyl transferase, NK: natural killer, LPS: lipopolysaccharides.

Declarations

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Authors' contributions

All authors contributed substantially to the work presented in this paper, read and approved the final manuscript. RA designed all the work under the supervision of OS and MH, collected, analyzed and interpreted data, drafted the manuscript. OS and MH and SA designed the research, contributed substantially with data analysis, results interpretations and manuscript editing and approval.

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Availability of data and materials

The information on the chemicals used in this manuscript could be requested from the corresponding author.

Ethics approval

This study was conducted according to the guidelines of the Research Animal Care Committee-approved protocol at the King Fahd Medical Research Center. All studies conducted on animals were approved by the Bioethical Research Unit of Faculty of Medicine, King Abdulaziz University (Ref: 597–17).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ East Jeddah Hospital, Ministry of Health, Kingdom of Saudi Arabia. ²Department of Clinical Pharmacology, Faculty of Medicine, Suez Canal University, Egypt. ³Department of Pharmacology, Faculty of Medicine, King Abdulaziz University, Kingdom of Saudi Arabia. ⁴Gastroenterology Unit, Department of Pediatrics, Faculty of Medicine, King Abdulaziz University, Kingdom of Saudi Arabia.

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Tables

Table 1. Effect of UDCA on liver enzymes activities in the prevention and treatment groups of LPS-induced cholestasis in rats

Parameters	GGT(IU/L)	ALP(IU/L)	TBIL(umol/L)	AST(IU/L)	ALT(IU/L)	AST/ALT Ratio
Group						
Control group (n=12) %change	1.75±0.50 (-46.1)	89.67±23.30 ^b (-14.6)	1.75±0.5 ^{b,c} (-30)	58.5±8.18 ^b (-22.13)	23.61±0.65 (-14.1)	2.47±0.32 (-9.5)
UDCA group (n=12) %change	1.67±0.52 ^b (-48.6)	83.62±21.29 ^b (-20.3)	1.67±0.52 ^{b,c} (-33.2)	62.05±6.72 ^b (-17.4)	24.18±0.53 (-12)	2.50±0.43 (-8.4)
LPS group (n=12)	3.25±0.50 ^a	105.64±15.48 ^a	2.5±0.58 ^a	75.13±24.89 ^a	27.50±4.25	2.73±1.01
Treatment group (n=12) %change	2.2±0.10 ^b (-32.3)	99.26±26.27 ^b (-5.4)	2.5±0.53 (0)	60.60±11.32 ^b (-16.6)	24.18±4.76 (-12.3)	2.50±0.52 (-8.4)
Prevention group (n=12) %change	2.1±0.20 ^b (-35.3)	50.90±23.08 ^{b,c} (-22.9)	1.14±38 ^{b,c} (-54.4)	62.11±6.27 ^b (-17.3)	24.84±7.07 (-9.67)	2.50±0.3 (-8.4)
^a Significant change compared to control at P<0.05 ^b Significant change compared to LPS group at P<0.05 ^c Significant change compared to treatment group at P<0.05.						

Table 2. Effect of UDCA on percentage of apoptosis of liver tissue in the prevention and treatment groups of LPS-induced cholestasis in rats.

Group	Percentage of Apoptosis
	Mean $\pm$ SD
Cotrol group (n=12)	4.33 $\pm$ 2.7 ^b
UDCA group (n=12)	5.37 $\pm$ 1.1 ^b
LPS group (n=12)	10.75 $\pm$ 4.93 ^{a,c}
Treatment group (n=12)	5.89 $\pm$ 2.19 ^b
Prevention group (n=12)	4.58 $\pm$ 3.9 ^b
Data are presented as mean $\pm$ S.D. ^a Significant change compared to control at P<0.05 ^b Significant change compared to LPS group at P<0.05. ^c Significant change compared to treatment group at P<0.05 .	

Table 3. Effect of UDCA on the percentage of natural killer (NK) cells & clusters of differentiation (CD3) in preventive and treated groups of LPS- induced cholestasis in rats.

Groups	Percentage of NK cells Mean±SD	Percentage CD3 (T cells co-receptor protein) Mean±SD
Control group (n=12)	8.63±2.35	62.14±3.20 ^c
UDCA group (n=12)	8.63±2.35	62.14±3.20
LPS group (n=12)	5.26±2.9	57.86±9.83 ^c
Treatment group (n=12)	8.58±4.12	37.29±4.30 ^{a,b}
Prevention group (n=12)	5.9±4.5	68.03±5.42 ^b
Data are presented as mean± S.D. ^a Significant change compared to control at P<0.05 ^b Significant change compared to LPS group at P<0.05. ^c Significant change compared to treatment group at P<0.05 .		

Table 4. Effect of UDCA on the concentration of inflammatory mediators in liver tissue in the prevention and treatment of cholestasis induced by LPS in rats.

Groups	TNF-a Mean±SD	IL-1a Mean±SD	IL-4 Mean±SD
Control group (n=12)	37.40±2.47 ^{b,c}	714.60±73.32 ^b	625.60±124.25
UDCA group (n=12)	39.60±7.42 ^b	700.90±54.25 ^b	600.80±100.57
LPS group (n=12)	75.63±3.72 ^a	898.50±63.56	804.25±61.01
Treatment group (n=12)	62.83±9.20 ^a	851.33±130.38	851.67±51.00
Prevention group (n=12)	55.38±13.15 ^b	832.63±80.01	749.63±89.55 ^c
Data are presented as mean± S.D. ^a Significant change compared to control at P<0.05 ^b Significant change compared to LPS group at P<0.05. ^c Significant change compared to treatment group at P<0.05 .			

Figures

Figure 1

Showing the effect of UDCA on the percentage of apoptotic cells in rat liver tissue in control, LPS, treatment, and prevention groups.

Figure 2

Flow cytometry dot plot showing the effect of UDCA on the LPS-induced apoptosis in all groups compared to the control group. Apoptotic cells were identified and quantified using the Annexin V-FITC apoptosis detection kit. Dot-plot shows early apoptotic cells in the lower right quadrant, late apoptotic cells in the upper right quadrant, dead cells in the upper left quadrant, and life cells in the lower left quadrant.

Figure 3

Histological feature of rat liver in all groups: Control & UDCA: shows a normal radial arrangement of hepatocytes around the central vein. They have acidophilic cytoplasm and large vesicular active nuclei (thin black arrows). Notice the intervening blood sinusoids lined by flat endothelial cells (stars). LPS: hepatocytes with ill-defined borders, regions of unstained degenerated cytoplasm, small or lost nuclei (black arrows), and large size nuclei (karyomegaly) (small black arrows). Treatment: sections from rat

liver treatment group shows marked improvement of changes induced by LPS. Hepatocytes (black arrow) & blood sinusoid (black stars) looked normal with no necrotic or apoptotic changes. Prevention: marked protection. Hepatocytes looked normal with no degenerative changes (white arrows). No congestion in blood sinusoids (stars).

Figure 4

Effect of UDCA on the nuclear area length in liver tissue in the prevention and treatment of cholestasis induced by LPS in rats.

Supplementary Files

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- [NC3RsARRIVEGuidelinesChecklist201410092019.docx](#)