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Effects of Sulfasalazine on Lipid Peroxidation and Histologic Liver Damage in a Rat Model of Obstructive Jaundice and Obstructive Jaundice With Lipopolysaccharide-Induced Sepsis

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ABSTRACT

BACKGROUND: Sulfasalazine, an inhibitor of cyclooxygenase, 5-lipoxygenase, and nuclear factor κB (NF-κB), has been found to alleviate oxidative damage, pro-inflammatory cytokine production, bile-duct proliferation, neutrophil infiltration, and fibrosis. Therefore, it may have a potential effect in attenuating lipid peroxidation and histologic liver damage in patients with biliary obstruction and biliary obstruction with sepsis.

OBJECTIVE: The aim of this study was to investigate the effect of sulfasalazine on lipid peroxidation and histologic liver damage due to obstructive jaundice (OJ) and to OJ with lipopolysaccharide (LPS)-induced sepsis in an experimental model.

METHODS: Male Wistar rats, weighing 150 to 220 g, were randomized into 6 groups: OJ; OJ + LPS; OJ + sulfasalazine; OJ + sulfasalazine + LPS (sulfasalazine administered before sepsis); OJ + LPS + sulfasalazine (sulfasalazine administered after sepsis); and sham. Liver malondialdehyde (MDA) and myeloperoxidase (MPO) activities were assessed to monitor lipid peroxidation and neutrophil infiltration in liver tissue. Histologic liver damage was evaluated with hematoxylin-eosin stained slides. Liver tissue NF-κB and caspase-3 expression were studied immunohistopathologically to evaluate lipid peroxidation, liver damage, and hepatocyte apoptosis.

RESULTS: Forty-eight rats were evenly randomized into 6 groups of 8. MDA ($P = 0.001$), MPO ($P = 0.001$), NF-κB ($P = 0.003$), caspase-3 expression ($P = 0.002$), and liver injury scores ($P = 0.002$) increased significantly in the OJ group compared with the sham group. Compared with the OJ group, MDA ($P = 0.030$) and MPO levels ($P = 0.001$), and liver injury scores ($P = 0.033$) were decreased significantly in the OJ + sulfasalazine group. In the OJ + sulfasalazine + LPS and OJ + LPS + sulfasalazine groups, MDA ($P = 0.008$ and $P = 0.023$, respectively) and MPO (both, $P = 0.001$) were significantly decreased; however, liver NF-κB, caspase-3 expression,

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and liver injury scores were not significantly different compared with the OJ + LPS group. There was no significant difference between the OJ + LPS + sulfasalazine and OJ + sulfasalazine + LPS groups in regard to all end points when comparing the effects of sulfasalazine administered before or after sepsis.

CONCLUSIONS: Sulfasalazine was associated with decreased neutrophil accumulation and lipid peroxidation in these rats with OJ. Administration of sulfasalazine before or after LPS-induced sepsis was associated with a reduction in lipid peroxidation and neutrophil accumulation; however, it did not attenuate histologic liver damage. There was no difference between the findings when sulfasalazine was administered before or after sepsis in OJ. (*Curr Ther Res Clin Exp.* 2009;70:299–315)

KEY WORDS: sulfasalazine, lipid peroxidation, nuclear factor κB, caspase-3, liver injury.

INTRODUCTION

Obstructive jaundice (OJ) is a condition caused by occlusion of the common bile duct or its tributaries.[1,2] OJ leads to complications such as biliary infection, septic shock, hepatic parenchymal injury, and multiple organ dysfunctions that carry a high risk for mortality. OJ is associated with many clinical conditions such as gallstones, benign stricture or tumor of bile duct, and complication of biliary surgery or pancreatitis, and may lead to serious complications such as wound breakdown, sepsis, coagulopathy, gastrointestinal hemorrhage, cardiovascular problems, immune depression, and hepatic and renal failure.[1]

The mechanisms and mediators responsible for the pathogenesis of liver damage from acute biliary obstruction remain largely unknown. Intrahepatic accumulation of toxic bile salts is thought to be one of the primary causes.[3,4] Increased production of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β and IL-6, has been implicated.[5] Data also suggest that oxidative stress is an important factor.[6] Lipid peroxidation is an important problem in OJ, in which free radical production is increased and antioxidative activity is reduced.[7] Nitric oxide formation and increased expression of inducible nitric oxide synthase (iNOS) take place.[8] Nitric oxide reacts with free oxygen radicals, and this leads to the formation of the harmful peroxynitrite anion ($ONOO^-$), which can lead to lipid peroxidation, cellular damage, and apoptosis.

Another important problem associated with OJ is the increased incidence of endotoxemia.[1,9–12] The absence of bile in the gastrointestinal tract stimulates bacterial overgrowth and bacterial translocation. Increased concentrations of bacteria and endotoxin in the portal blood lead to systemic endotoxemia.[13,14] Systemic endotoxemia developed in OJ is the result of the depression in the clearance of lipopolysaccharide (LPS) and other endotoxins in the portal circulation by Kupffer cells in the liver. In OJ, hepatic Kupffer cells' endocytosis is impaired and their endotoxin clearance ability is decreased. Portal endotoxemia, which is the result of an impaired intestinal barrier, and systemic endotoxemia, which is developed as a result of impaired function of Kupffer cells, lead to sepsis.[1,12,15,16]

In sepsis, neutrophilic inflammation appears to be the result of the local production of cytokines, chemokines, endothelial-leukocyte adhesion molecules, and enzymes, such as iNOS and cyclooxygenase-2 (COX-2). The expression of iNOS can be induced by injection of LPS.[17] The production of iNOS and COX-2 are regulated by the ubiquitous transcription factor, complex nuclear factor κB (NF-κB). NF-κB is a DNA-binding protein necessary for directing high-level transcription of many proinflammatory genes.[18] $ONOO^-$, free oxygen radicals, and environment factors lead to DNA strand breakage, which triggers the activation of poly (adenosine diphosphate-ribose) synthase (PARS).[19,20] PARS is an energy-requiring enzyme that plays a role in the repair of strand breaks in DNA. Its activation results in a substantial depletion of nicotinamide adenine dinucleotide, thus leading to cell dysfunction.[21]

Hepatocyte injury during cholestasis depends in part on the release of proinflammatory mediators that cause neutrophils to accumulate in the liver and become activated, damaging hepatocytes.[22] On the other hand, cellular and molecular events during the course of liver injury in OJ have centered on the role of resident hepatic macrophages, or Kupffer cells. Activation of macrophages leads to invasion polymorphonuclear leukocytes, secretion and upregulation of proinflammatory mediators, such as TNF-α, IL-1, and IL-6, and recruitment of systemic macrophages to the site of injury.[22,23] In addition to sinusoids, neutrophils adhere in portal and postsinusoidal venules and extravasate from these locations.[24,25]

Sulfasalazine consists of 5-aminosalicylic acid (5-ASA) and sulfapyridine, linked by an azo band. Sulfapyridine and 5-ASA inhibit cyclooxygenase and 5-lipoxygenase. Research has suggested that sulfasalazine is a potent and specific inhibitor of NF-κB.[26] In addition, sulfasalazine has antioxidant properties; it is a strong scavenger of free radicals.[27] Treatment with sulfasalazine may alleviate liver oxidative damage, proinflammatory cytokine production, ductular proliferation, neutrophil infiltration, and fibrosis in bile-duct ligated rats.[28]

The beneficial effects of sulfasalazine suggest that it might be useful in attenuating liver damage in patients with biliary obstruction and sepsis. The aim of this study was to investigate the effect of sulfasalazine on lipid peroxidation and histologic liver damage due to OJ and to OJ with LPS-induced sepsis in a rat model.

MATERIALS AND METHODS

The experiments described in this article were performed in adherence to the Turkish National Institutes of Health guidelines on the use of experimental animals.[29] Approval was obtained from the ethics committee of the Mersin University Medical School, Mersin, Turkey. Male Wistar rats, weighing 150 to 220 g, were housed at constant temperature with a light/dark exposure period of 14/10 hours. Animals were allowed access to standard rat chow and water ad libitum with an acclimation period of ≥5 days prior to use in these experiments. The experiments were performed in The Animal Experimental Laboratory of the Mersin University Medical School.

We randomized the rats into 6 groups of equal size. The randomization was performed with Minitab version 13 (Minitab Inc., State College, Pennsylvania).

In the OJ group, OJ was constituted by common bile-duct ligation (BDL) for 5 days. In the OJ + LPS group, after constituting OJ with BDL, LPS 10 mg/kg (*Escherichia coli* LPS serotype 0127:B8, 100 mg, Sigma Chemical Co., St. Louis, Missouri) was injected intraperitoneally on the sixth day. LPS was used to establish sepsis. In the OJ + sulfasalazine group, starting on the sixth day of BDL, sulfasalazine 100 mg/kg (S0883-10G, Sigma Chemical Co.) was injected intraperitoneally for 5 days. In the OJ + sulfasalazine + LPS group, starting on the sixth day of BDL, sulfasalazine 100 mg/kg was injected intraperitoneally for 5 days and LPS 10 mg/kg was injected intraperitoneally 6 hours before the rats were euthanized on the tenth day. In the OJ + LPS + sulfasalazine group, LPS 10 mg/kg was injected intraperitoneally on the sixth day of BDL and sulfasalazine 100 mg/kg was injected intraperitoneally for 5 days. Sham operation was performed in the sham group.

SURGICAL PROCEDURES

Rats were anesthetized with IM ketamine 50 mg/kg and xylazine 7 mg/kg. The chest and abdomen were shaved and each animal was fixed in a supine position on the operating table. The abdomen was cleaned with 1% polyvinyl iodine and, when dry, the operating field was covered with a sterile drape and median laparotomy was performed.

The sham operation consisted of only mobilization of the common bile duct, without ligation.

The experimental jaundice was created by ligation of the common bile duct according to the technique described by Lee.[30] The common bile duct was exposed and ligated twice by silk suture, and the portion between the ligatures was resected. The abdominal wall was closed with interrupted silk sutures and the edges of the skin were approximated with a subcuticular stitch. OJ was constituted by BDL for 5 days.

After completion of each arm of the study, rats in all arms were euthanized at the end of the tenth day. Before being euthanized, rats were anesthetized with IM ketamine 50 mg/kg, and hepatectomies were performed through repeat laparotomy. Liver malondialdehyde (MDA) and myeloperoxidase (MPO) activities were determined to assess lipid peroxidation and neutrophil infiltration in liver tissue. Histologic liver damage was evaluated by light microscopic examination of hematoxylin-eosin stained slides. Liver tissue NF-κB level and caspase-3 expression were studied immunohistopathologically to evaluate lipid peroxidation, liver damage, and hepatocyte apoptosis. The study investigators were blinded to group assignments.

The sham and OJ groups were compared to evaluate the effects of jaundice. Comparison of the OJ and OJ + LPS groups was made to evaluate the effects of sepsis in OJ. The OJ and OJ + sulfasalazine groups were compared to evaluate the potential therapeutic effect of sulfasalazine in OJ. Comparison of the OJ + sulfasalazine + LPS and OJ + LPS + sulfasalazine groups with the OJ + LPS group was made to evaluate the effects of sulfasalazine in OJ + LPS. The OJ + sulfasalazine + LPS and OJ + LPS + sulfasalazine groups were designed to evaluate the protective (before establishment of sepsis) or therapeutic (after establishment of sepsis) effect of sulfasalazine in OJ + LPS, respectively. The OJ + sulfasalazine + LPS and OJ + LPS + sulfasalazine groups were compared to determine when sulfasalazine was more effective, before or after establishment of sepsis.

LIPID PEROXIDE ASSAYS

Liver Malondialdehyde Determination

The MDA levels, as an index of lipid peroxidation, were determined by thiobarbituric acid (TBA) reaction according to Yagi.[31] The principle of the method depends on measurement of the pink color spectrophotometrically produced by interaction of TBA with MDA elaborated as a result of LPS. The colored reaction was determined at 553 nm using 1,1,3,3-tetraethoxypropane as the primary standard.[31] The results were expressed as nmol of MDA per gram of tissue.

Liver Myeloperoxidase Determination

The determination of tissue MPO activity, as an index of neutrophil infiltration, depends on the fact that it reduces *o*-dianisidine. Reduced *o*-dianisidine was measured at 410 nm by spectrophotometry.[32] The MPO activity was expressed as U/g of tissue.

HISTOLOGIC EVALUATION

Evaluation of Liver Injury With Light Microscopy

The extracted liver tissues were fixed in 10% neutral formalin and subsequently embedded in paraffin. Sections (5 μm) were deparaffinized in xylene and dehydrated through graded concentrations of ethanol and stained with hematoxylin-eosin.

Liver sections were graded with histopathology as described previously[33]: grade 0 = no hepatocellular damage; grade 1 = minimal hepatocellular damage; grade 2 = minimal centrolobular damage; grade 3 = severe centrolobular damage; grade 4 = centrolobular and midzonal damage; grade 5 = severe centrolobular and midzonal damage; and grade 6 = total hepatocellular destruction.

Immunohistochemical Staining Method for Nuclear Factor-κB and Caspase-3 Expression

After blocking endogenous peroxidase activity with hydrogen peroxide, the sections were heated in 0.01 mol/L citrate buffer in a microwave cooker for 20 minutes. Sections were incubated using caspase-3 polyclonal antibody (dilution 1/100, RB-1197-B0 LabVision/Neomarkers, Fremont, California) and anti–NF-κB/p65 (Rel A) Ab–1 antibody (dilution 1/100RB–1638, LabVision/Neomarkers) for 1 hour at room temperature in a humidified chamber, and were then stained using the avidin-biotin complex (ABC) immunoperoxidase technique with a commercially available reagent (ABC kit, LabVision). The sections were counterstained with Mayer's hematoxylin and mounted with mounting media (LabVision).

Evaluation of Immunohistochemical Reactivity

Stained slides were categorized using a semiquantitative score according to the staining intensity (SI) and the percentage of immunostained cells (SP) for each marker (caspase-3 and NF-kB/p65) as described previously.[34] SI was graded from 0 to 3 as follows: grade 0 = no staining; grade 1 = weak staining; grade 2 = moderate staining; and grade 3 = intense staining. SP was graded as follows: grade 0 = no staining; grade 1 = <25% staining of liver tissue; grade 2 = 25% to 50% staining of liver tissue;

grade 3 = >50% to 75% staining of liver tissue; and grade 4 = >75% staining of liver tissue. Total staining score (TSS) was obtained as the sum of the SP and SI scores. TSS was recorded using a scale of 0 to 7 (0 = no staining; 1–2 = mild staining; 3–4 = moderate staining; 5–7 = highest staining).

Statistical Analysis

Biochemical values were described as mean (SEM). Statistical differences for liver MDA and MPO activity values were evaluated using 1-way ANOVA followed by the Tukey post hoc test in SPSS version 11.5 (SPSS Inc., Chicago, Illinois). Histopathologic examinations were presented as medians (interquartile range [IQR]). Comparisons of liver injury scores, staining scores for NF-κB, and caspase-3 expression were analyzed using the Kruskal-Wallis test followed by the Dunn test (Statistica 6.1, Statsoft Inc., Tulsa, Oklahoma). $P < 0.05$ was considered statistically significant. Box-plot graphics were used to show the medians of liver injury, NF-κB, and caspase-3 expression scores.

RESULTS

Forty-eight rats were evenly randomized to 6 groups of 8.

Biochemical Examination

Liver Malondialdehyde

Mean (SEM) levels of liver MDA were 34.26 (7.14) in the OJ group (n = 8), 45.71 (6.45) in the OJ + LPS group (n = 8), 18.88 (0.83) in the OJ + sulfasalazine group (n = 8), 26.34 (0.80) in the OJ + sulfasalazine + LPS group (n = 8), 28.50 (1.13) in the OJ + LPS + sulfasalazine group (n = 8), and 12.42 (2.04) in the sham group (n = 8) (**Figure 1**).

Liver MDA increased significantly in the OJ group compared with the sham group ($P = 0.001$). Liver MDA was not significantly different between the OJ + LPS and OJ groups. Liver MDA in the OJ + sulfasalazine group was significantly lower than in the OJ group ($P = 0.03$). In the OJ + sulfasalazine + LPS and OJ + LPS + sulfasalazine groups, liver MDA was significantly lower than in the OJ + LPS group ($P = 0.008$ and $P = 0.023$, respectively). There was no statistically significant difference between the OJ + sulfasalazine + LPS and OJ + LPS + sulfasalazine groups.

Liver Myeloperoxidase

Mean (SEM) levels of liver MPO were 3.01 (0.07) in the OJ group, 4.64 (0.32) in the OJ + LPS group, 0.94 (0.07) in the OJ + sulfasalazine group, 1.89 (0.10) in the OJ + sulfasalazine + LPS group, 2.23 (0.32) in the OJ + LPS + sulfasalazine group, and 0.44 (0.04) in the sham group (**Figure 2**).

Liver MPO was increased significantly in the OJ group compared with the sham group ($P = 0.001$). Liver MPO in the OJ + LPS group was significantly higher than that in the OJ group ($P = 0.001$). Liver MPO was significantly lower in the OJ + sulfasalazine group than in the OJ group ($P = 0.001$). In both the OJ + sulfasalazine + LPS and OJ + LPS + sulfasalazine groups, liver MPO levels were significantly lower

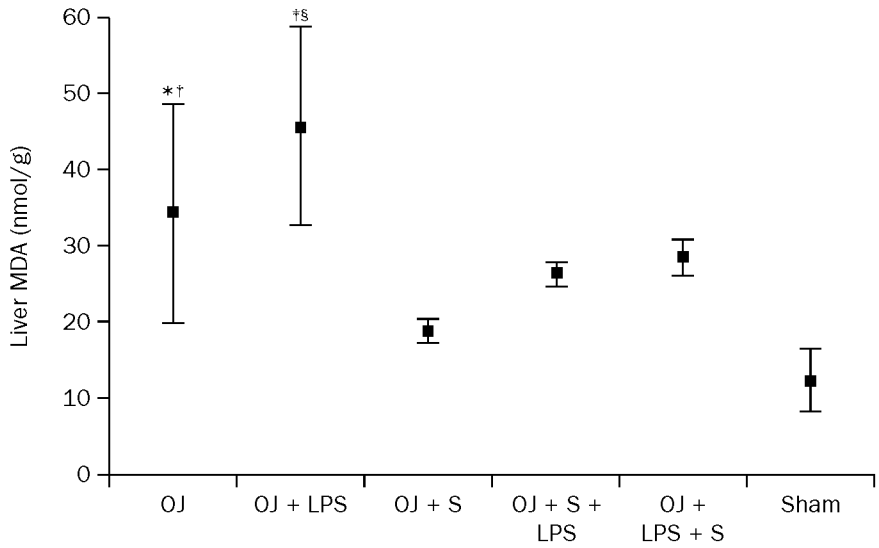

Figure 1. Mean (SEM) liver malondialdehyde (MDA) levels in male Wistar rats with obstructive jaundice (OJ) and lipopolysaccharide (LPS)-induced sepsis administered sulfasalazine (S) before (OJ + S + LPS) and after (OJ + LPS + S) sepsis (N = 48). *P = 0.001 versus the sham group; †P = 0.03 versus the OJ + S group; †P = 0.008 versus the OJ + S + LPS group; §P = 0.023 versus the OJ + LPS + S group.

than that in the OJ + LPS group (both, P = 0.001). There was no statistically significant difference between the OJ + sulfasalazine + LPS and OJ + LPS + sulfasalazine groups.

HISTOPATHOLOGIC EXAMINATION

Light Microscopic Evaluation of Liver Injury

In the OJ + sulfasalazine group, minimal congestion in sinusoids was observed, with degeneration in a few hepatocytes and spare centrolobular focal necrosis (grade 1). In the OJ and OJ + LPS groups, severe centrolobular and midzonal necroses (grade 3) were noted. Focal centrolobular necrosis (grade 2) was noted in the OJ + sulfasalazine + LPS and OJ + LPS + sulfasalazine groups. Liver sections of the sham group showed near-normal morphology (grade 0) (**Figure 3**).

Light Microscopic Evaluation of Immunohistochemical Staining of Nuclear Factor-κB and Caspase-3 Expression in Liver Tissue

In the OJ and OJ + LPS groups, intense cytoplasmic staining (grade 3) was detected in large areas with both NF-κB and caspase-3. Caspase-3 and NF-κB had moderate cytoplasmic staining in perivenular hepatocytes (grade 2) in the OJ + sulfasalazine, OJ + sulfasalazine + LPS, and OJ + LPS + sulfasalazine groups. Both NF-κB and caspase-3 showed no cytoplasmic staining (grade 0) in hepatocytes in the sham group (**Figures 4** and **5**).

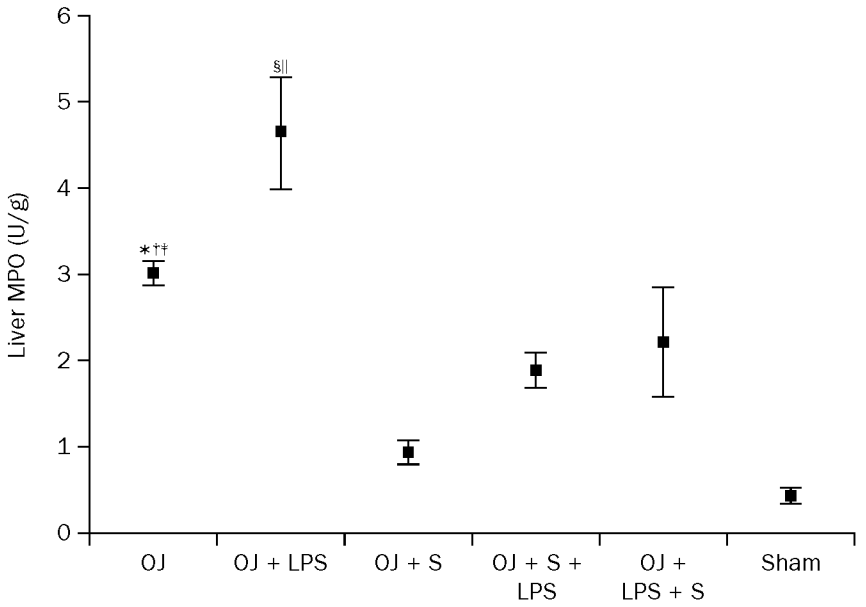

Figure 2. Mean (SEM) liver myeloperoxidase (MPO) levels in male Wistar rats with obstructive jaundice (OJ) and lipopolysaccharide (LPS)-induced sepsis administered sulfasalazine (S) before (OJ + S + LPS) and after (OJ + LPS + S) sepsis (N = 48). *P = 0.001 versus the sham group; †P = 0.001 versus the OJ + LPS group; ‡P = 0.001 versus the OJ + S group; §P = 0.001 versus the OJ + S + LPS group; ||P = 0.001 versus the OJ + LPS + S group.

Statistical Evaluation of Liver Injury Scores

Median (IQR) liver injury scores in the OJ, OJ + LPS, OJ + sulfasalazine, OJ + sulfasalazine + LPS, OJ + LPS + sulfasalazine, and sham groups were 3 (2–3), 3 (2–4), 1 (0–1), 2 (1–3), 2 (2–2), and 0 (0–0), respectively (Figure 6).

Liver injury scores increased significantly in the OJ group compared with the sham group (P = 0.002). There was no statistically significant between-group difference in the OJ and OJ + LPS groups. The mean liver injury score of the OJ + sulfasalazine group was significantly lower than that of the OJ group (P = 0.033). There were no other statistically significant between-group differences in regard to liver injury scores.

Statistical Evaluation of Nuclear Factor κB Expression

Median (IQR) NF-κB TSSs in the OJ, OJ + LPS, OJ + sulfasalazine, OJ + sulfasalazine + LPS, OJ + LPS + sulfasalazine, and sham groups were 4 (3–5), 4 (3.5–4), 3 (2–4), 3 (3–4), 3 (2–4), and 1 (0–2), respectively (Figure 7).

NF-κB staining scores increased significantly in the OJ group compared with those in the sham group (P = 0.003). There was no statistically significant difference between staining scores in the OJ and OJ + LPS groups. There were no other statistically significant between-group differences in regard to NF-κB staining scores.

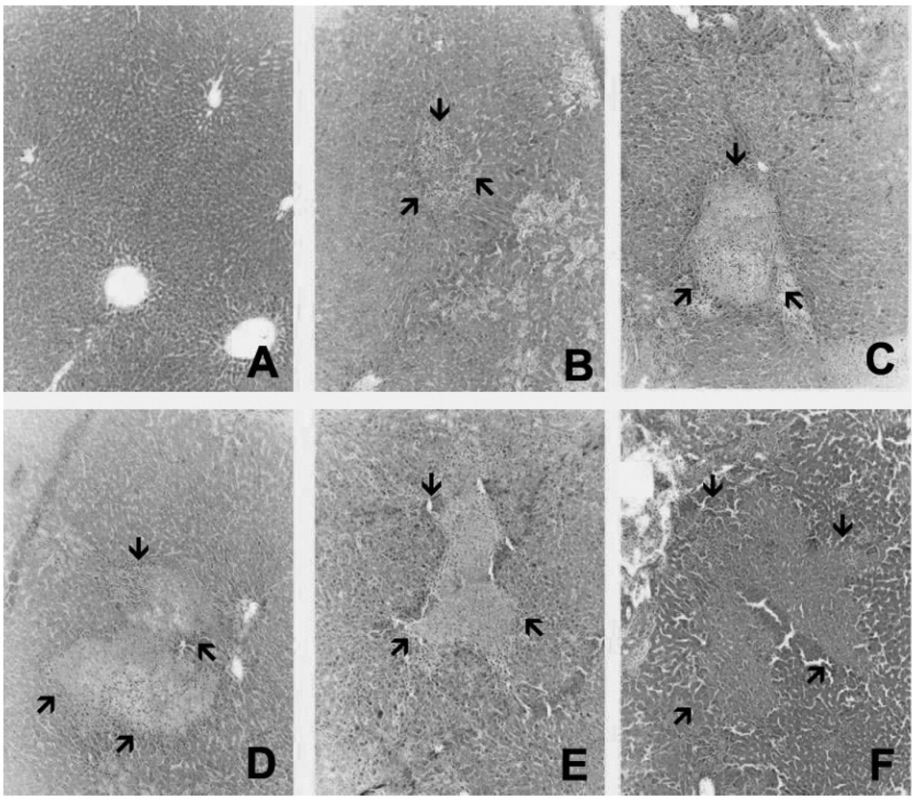

Figure 3. Representative light microscopic liver injury changes in the tissue of male Wistar rats with obstructive jaundice (OJ) and lipopolysaccharide (LPS)-induced sepsis administered sulfasalazine (S) before (OJ + S + LPS) and after (OJ + LPS + S) sepsis (magnification X100). (A) Normal liver parenchyma (grade 0) in the sham group; (B) spare focal necrosis in the pericentral zone (grade 1) in the OJ + S group; focal centrolobular necrosis (grade 2) was noted in the (D) OJ + S + LPS and (E) OJ + LPS + S groups; large necrotic plaque extending from precentral to periportal areas (grade 3) in the (C) OJ and (F) OJ + LPS groups. Grade 0 = no hepatocellular damage; grade 1 = minimal hepatocellular damage; grade 2 = minimal centrolobular damage; grade 3 = severe centrolobular damage; grade 4 = centrolobular and midzonal damage; grade 5 = severe centrolobular and midzonal damage; grade 6 = total hepatocellular destruction.[33]

Statistical Evaluation of Caspase-3 Staining

Median (IQR) of caspase-3 TSSs in the OJ, OJ + LPS, OJ + sulfasalazine, OJ + sulfasalazine + LPS, OJ + LPS + sulfasalazine, and sham groups were 4 (3–5), 5 (4–5), 3 (2–4), 4 (3–5), 4 (3–5), and 0 (0–2), respectively (Figure 8).

Caspase-3 expression scores increased significantly in the OJ group compared with the sham group ($P = 0.002$). There were no other statistically significant between-group differences observed in regard to caspase-3 expression scores.

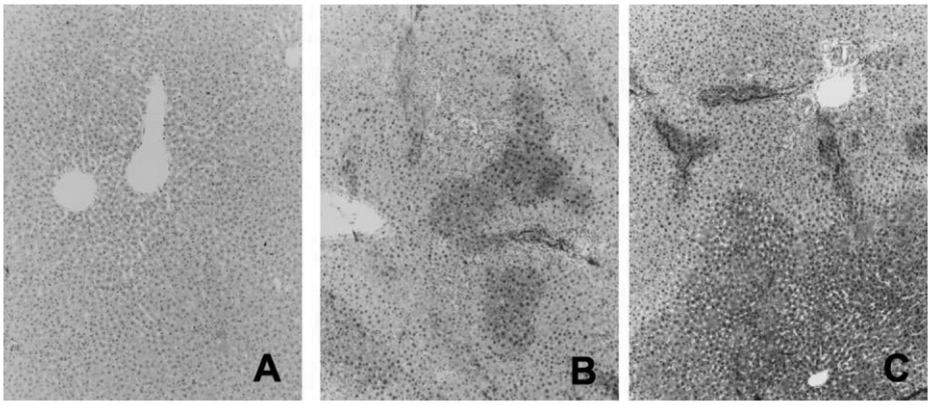

Figure 4. Representative nuclear factor κB expression staining in the liver tissue of male Wistar rats with obstructive jaundice (OJ) and lipopolysaccharide (LPS)-induced sepsis administered sulfasalazine (S) before (OJ + S + LPS) and after (OJ + LPS + S) sepsis (magnification X100). (A) No staining was detected (grade 0) in hepatocytes in the sham group; (B) moderate and focal cytoplasmic staining (grade 2) in hepatocytes in the OJ + S, OJ + S + LPS, and OJ + LPS + S groups; and (C) intense and diffuse cytoplasmic staining (grade 3) in hepatocytes in the OJ and OJ + LPS groups. Staining intensity was graded from 0 to 3 as follows: grade 0 = no staining; grade 1 = weak staining; grade 2 = moderate staining; grade 3 = intense staining.

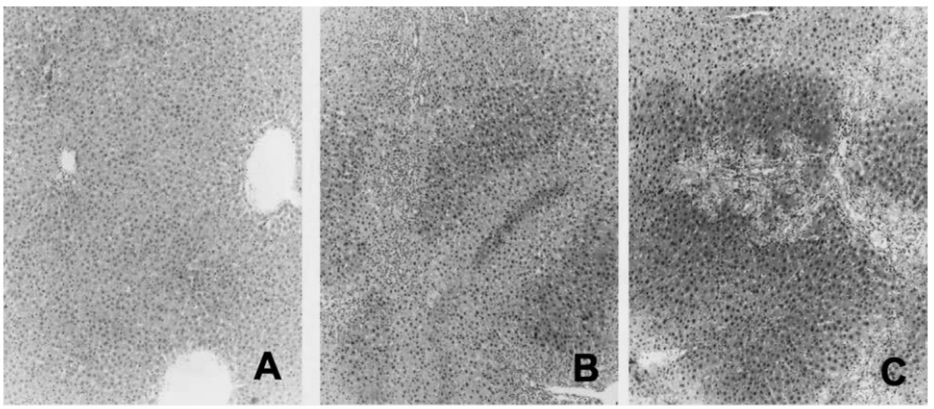

Figure 5. Representative caspase-3 expression staining in the liver tissue of male Wistar rats with obstructive jaundice (OJ) and lipopolysaccharide (LPS)-induced sepsis administered sulfasalazine (S) before (OJ + S + LPS) and after (OJ + LPS + S) sepsis (magnification X100). (A) No staining was detected (grade 0) in hepatocytes in the sham group; (B) moderate and focal caspase-3 staining (grade 2) in hepatocytes in the OJ + S, OJ + S + LPS, and OJ + LPS + S groups; and (C) intense and diffuse caspase-3 staining (grade 3) in hepatocytes in the OJ and OJ + LPS groups. Staining intensity was graded from 0 to 3 as follows: grade 0 = no staining; grade 1 = weak staining; grade 2 = moderate staining; grade 3 = intense staining.

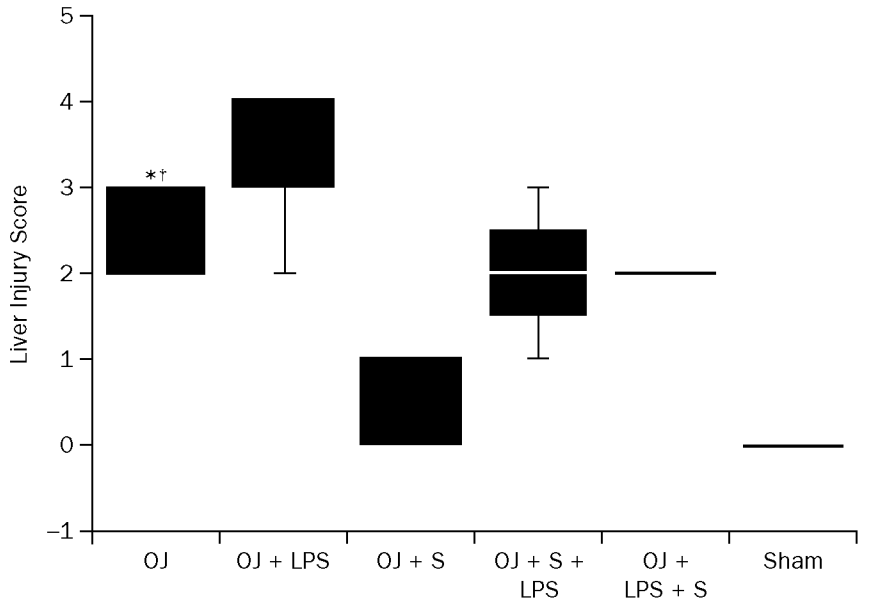

Figure 6. Box plot of median (interquartile range) liver injury scores in male Wistar rats with obstructive jaundice (OJ) and lipopolysaccharide (LPS)-induced sepsis administered sulfasalazine (S) before (OJ + S + LPS) and after (OJ + LPS + S) sepsis (N = 48). *P = 0.002 versus the sham group; †P = 0.033 versus the OJ + S group.

DISCUSSION

The hepatic content of MPO reflects the number of neutrophils in the liver. In the present study, liver MPO, as an index of neutrophil infiltration, increased in association with OJ, as mentioned in the literature.[35] The inflammatory process in both macrophages and neutrophils has been found to be associated with activation of the transcription factor NF-κB.[36] In unstimulated cells, NF-κB is bound to IκB-α, preventing its nuclear localization. Upon activation, IκB-α is degraded and NF-κB translocates to the nucleus, where it binds the promoter elements of genes for inflammatory cytokines and adhesion proteins.[18] In the present study, there was overproduction of NF-κB in the OJ group, as mentioned in the literature. Activation of NF-κB leads to overproduction of cytokines and other proinflammatory molecules such as iNOS and COX-2. These molecules and cytokines lead to high lipid peroxidation and nitric oxide formation.

It has been suggested that oxidative stress associated with lipid peroxidation is involved in the development of liver injury in OJ. Biliary obstruction is associated with an intense state of oxidative stress. Extrahepatic BDL in rats has been found to produce an important reduction of antioxidant defenses and exacerbation of lipid peroxidation in the liver.[37–42] Nitric oxide reacts with free oxygen radicals, leading to the formation of $ONOO^-$, and this anion leads to lipid peroxidation, cellular damage, and apoptosis. In our study, we observed that liver MDA, as an index of lipid peroxidation, increased in the OJ group.

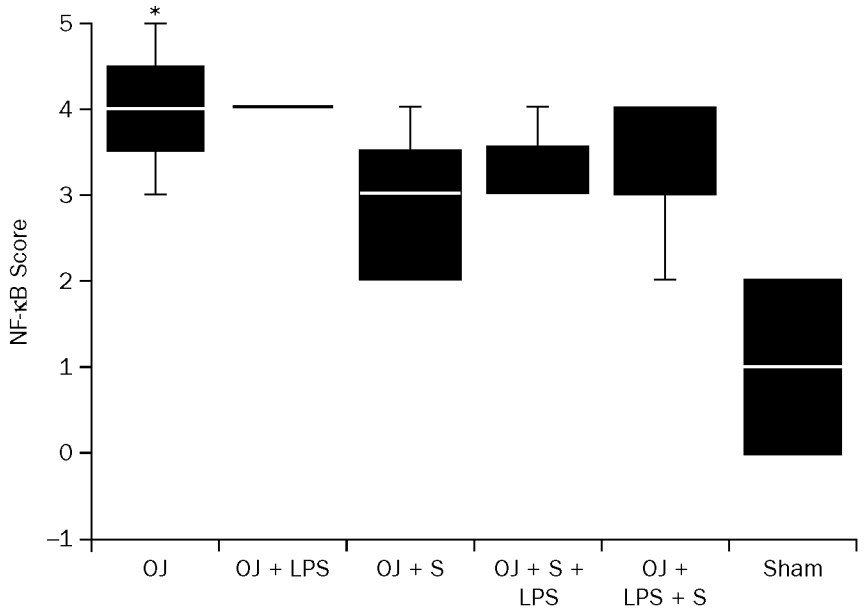

Figure 7. Box plot of median (interquartile range) liver tissue nuclear factor κB (NF-κB) expression scores in male Wistar rats with obstructive jaundice (OJ) and lipopolysaccharide (LPS)-induced sepsis administered sulfasalazine (S) before (OJ + S + LPS) and after (OJ + LPS + S) sepsis (N = 48). *P = 0.003 versus the sham group.

Increased production of proinflammatory cytokines, lipid peroxidation, and endotoxins leads to liver damage and hepatocyte apoptosis; therefore, intrahepatic accumulation of toxic bile acids also induces hepatocyte apoptosis.[37,43] The glycine conjugate of chenodeoxycholate induces hepatocyte apoptosis in vitro, whereas the taurine conjugate does not.[39,44] Studies have reported that bile acids activate cytoplasmic protein kinase cascades and function as ligands for the nuclear receptor farnesoid X receptor.[40,45] The nontoxic bile acid taurocholate has been found to activate phosphatidylinositol 3-kinase,[1,14,41] a potent activator of survival signals.[42,46] Bile salt cytotoxicity both in vivo and in vitro is mediated by the death receptor Fas.[3,47] Toxic bile acids induce Fas oligomerization and activate caspases, most likely caspase-8, resulting in apoptosis.[37,47] NF-κB may suppress bile acid–mediated Fas/caspase-8 activation by upregulating *cIAP-1* expression. This concept suggests that the net effect of bile acids in mediating liver injury reflects a balance between pro- and antiapoptotic processes. For this reason, we observed a high rate of caspase-3 expression in the OJ group in our study. As a result of these mechanisms and mediators responsible for the pathogenesis of liver damage in OJ, we observed a high rate of liver injury scores in the OJ group in the present study.

In the OJ + LPS group, we observed significantly greater neutrophil accumulation, supported by a high level of liver MPO. This can be explained by the fact that in OJ, there is depression in the clearance of LPS and other endotoxins in the portal circula-

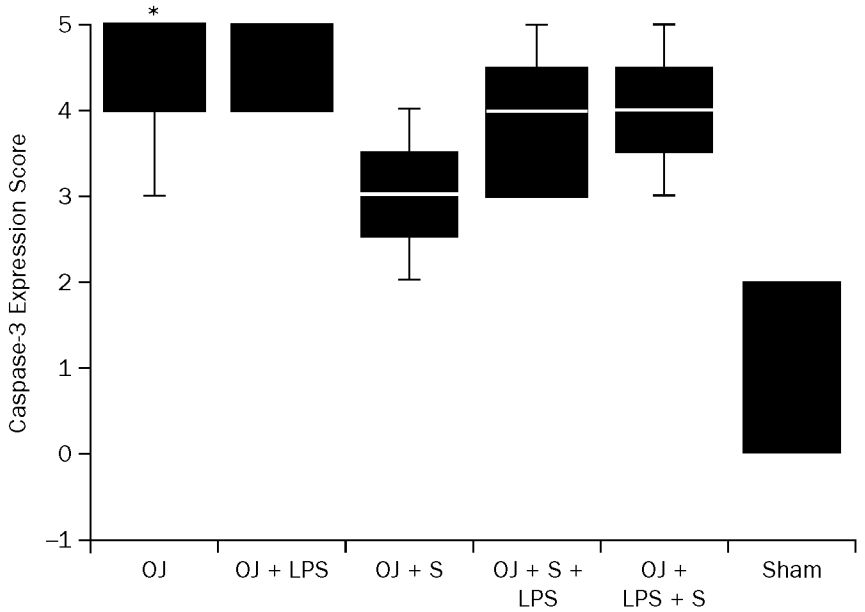

Figure 8. Box plot of median (interquartile range) liver tissue caspase-3 expression scores in male Wistar rats with obstructive jaundice (OJ) and lipopolysaccharide (LPS)-induced sepsis administered sulfasalazine (S) before (OJ + S + LPS) and after (OJ + LPS + S) sepsis (N = 48). *P = 0.002 versus the sham group.

tion by Kuppfer cells in the liver.[48] Increased sensitivity to LPS endotoxin leads to exaggerated lipid peroxidation and accumulation of neutrophils, which then lead to organ dysfunction.[12,15,16] However, NF-κB expression in the OJ + LPS group was as high as NF-κB expression in the OJ group. Although NF-κB expression was high, it did not increase as high as expected. This might be explained by the fact that NF-κB activated by LPS induces IκB and contributes to the downregulation of this intracellular signaling cascade.[18] In our study, we observed that caspase-3 expression in the OJ + LPS group was as high as that in the OJ group. This suggests that NF-κB is also a potent antiapoptotic agent and that massive apoptosis does not occur as one might expect.[49] However, exaggerated lipid peroxidation and accumulation of toxic hydrophobic bile salts within hepatocytes cause hepatocyte toxicity that leads to liver damage. For this reason, there were increased liver injury scores in the OJ + LPS group.

Previous studies have found that sulfasalazine is a potent NF-κB inhibitor.[26–28] Because NF-κB plays a role in lipid peroxidation, neutrophil accumulation, and hepatocyte apoptosis, we administered sulfasalazine to jaundiced rats to see whether it exerted any beneficial effect on these events. In the OJ + sulfasalazine group, we observed that there was a reduction, though not statistically significant, in NF-κB and caspase-3 expression. Blocking a single mediator, especially after the initial attack to the host, might be insufficient to inhibit NF-κB significantly because of the redun-

dancy of proximal mediators with the potential to activate NF-κB.[50,51] However, liver MDA and MPO levels were significantly lower compared with the OJ group. Treatment with sulfasalazine might alleviate liver oxidative damage, proinflammatory cytokine production, ductular proliferation, and neutrophil infiltration.[28] In addition, sulfasalazine has antioxidative properties; it is a strong scavenger of free radicals.[27] Lipid peroxidation was attenuated with the inhibitory effect of sulfasalazine on neutrophil infiltration rather than its inhibitory effect on NF-κB production and caspase-3 expression. Because one of the potential sources of liver injury in OJ is lipid peroxidation, sulfasalazine likely decreases liver injury scores via attenuating lipid peroxidation.

There are several factors reported in the literature that are thought to affect hepatocyte apoptosis besides lipid peroxidation. High NF-κB production suppresses hepatocyte apoptosis.[49] Reduction, though not statistically significant, in NF-κB production with sulfasalazine, as reported in the present study, and retention and accumulation of toxic hydrophobic bile salts within hepatocytes may aggravate hepatocyte apoptosis. Retention and accumulation of nontoxic bile acid taurocholate within hepatocytes may activate phosphatidylinositol 3-kinase, a potent activator of survival signals, and may reduce hepatocyte apoptosis.[42,46] Apoptotic and antiapoptotic processes balance the rate of hepatocyte apoptosis. In the present study, we observed a numerical, though not statistically significant, reduction in caspase-3 expression in the OJ + sulfasalazine group. Therefore, sulfasalazine might attenuate histologic liver damage via attenuating lipid peroxidation rather than inhibiting NF-κB production and caspase-3 expression.

Administration of sulfasalazine in jaundiced rats before or after LPS-induced sepsis (OJ + sulfasalazine + LPS and OJ + LPS + sulfasalazine groups) led to a reduction, though not statistically significant, in NF-κB production, caspase-3 expression, and liver injury scores. Since cholestasis is the initial attack to the host, sepsis established after cholestasis is the second attack. For this reason, sepsis in OJ creates a chaotic and uncontrollable condition in which there is a pool of proinflammatory cytokines, molecules, mediators, and inflammatory cells. Therefore, blocking a single mediator in this chaotic condition might inhibit NF-κB expression insignificantly. Sulfasalazine induces apoptosis in T-lymphocytes and neutrophils.[52] This leads to attenuation in lipid peroxidation and neutrophil accumulation in OJ with sepsis. However, blocking only 1 of the potential sources of liver injury in OJ with sepsis might attenuate liver injury scores and caspase-3 expression insignificantly. For this reason, we observed no statistically significant changes in caspase-3 expression and liver injury scores in these groups. As a result of these findings, we can conclude that sulfasalazine failed to attenuate histologic liver damage in OJ with sepsis. When the OJ + sulfasalazine + LPS and OJ + LPS + sulfasalazine groups were compared, there were no significant differences observed in any of the study end points. Therefore, administration of sulfasalazine in jaundiced rats before or after the induction of sepsis was equivalent, and it failed to attenuate histologic liver damage when there was sepsis.

STUDY LIMITATIONS

The small number of rats in each arm of the study made the interpretation of histopathologic results difficult. Also, the dose of sulfasalazine used in the treatment or

pretreatment of sepsis in OJ might have been too low to inhibit NF-κB, caspase-3 expression, and histologic liver damage. Further investigations with larger numbers of subjects and higher doses of sulfasalazine are needed to clarify the findings of this study.

CONCLUSIONS

Because sulfasalazine did not decrease NF-κB production and caspase-3 expression in these male Wistar rats, histologic liver damage in the OJ + sulfasalazine group was thought to be attenuated via decreased neutrophil accumulation and lipid peroxidation. Administration of sulfasalazine to jaundiced rats prior to or after LPS-induced sepsis was associated with a reduction in lipid peroxidation and neutrophil accumulation, but not in NF-κB production or caspase-3 expression. Sulfasalazine failed to attenuate histologic liver damage in OJ aggravated with sepsis (LPS). There was no difference between the findings when sulfasalazine was administered before (OJ + sulfasalazine + LPS) or after sepsis (OJ + LPS + sulfasalazine).

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