Effects of PARP Inhibitör 3-Aminobenzamide on Impaired Mesenteric Blood Flow and Organ Injury in CLP-Induced Septic Shock Model

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ABSTRACT

Objective: In various pathophysiological conditions, including septic shock, reactive oxidants cause DNA strand breakage and subsequent activation of the nuclear enzyme poly (ADP ribose) polymerase (PARP). Activation of PARP results in cellular dysfunction. We investigated the 3-Aminobenzamide (3-AB), one of the pure PARP-1 inhibitor, on mesenteric blood flow and organ injury (lung, liver, spleen) in a murine caecal ligation and puncture (CLP) model of septic shock in both gender.

Methods: Female and male Swiss albino mice received 3-AB (10 mg/kg, i.p.) or its solvent saline (0.9% NaCl, w/v) 1 hour after CLP. At 24th hour, the animals were anaesthetized with chloralhydrate (400 mg kg^{-1}, i.p.) and the mesenteric blood flow was monitored for 15 min by using perivascular ultrasonic Doppler-flowmeter. Then the animals were exsanguinated, spleen, liver, and kidneys were isolated accordingly for histopathological examination. Thiobarbituric acid-reacting substances, glutathione and myeloperoxides activities were also determined in tissues.

Results: Although the glutathione levels were decreased and thiobarbituric acid-reacting substances and myeloperoxidase activity were increased by CLP in lung and liver, 3-AB has failed to block these biochemical parameters. In CLP + saline group, the mesenteric arterial blood flow was significantly lower than that of saline group (p<0.0001). 3-AB administration, did not prevent this status. 3-AB also did not attenuate the histopathological injury inflicted by CLP.

Conclusion: Poly (ADP-ribose) polymerase-1 inhibitor 3-AB, controversial to the common results of the recent literature, has no significant effect on splanchnic ischemia or multiple organ injury as histopathogically or biochemically in both gender.

Key words: 3-Aminobenzamide, PARP, septic shock, gender difference, anesthetized mice, sepsis

Introduction

Septic shock is the further state of sepsis with a treatment resistant hypotension and this highly lethal circulatory failure causes to multiorgan failure with a mortality rate of 20–50% (1). In the United States of America it is the second most common cause of death in noncoronary intensive care unit patients and the tenth leading cause of overall death (2) with a huge economic burden as approximately 17$ billion annually. (2,3) Mostly gram (-) bacterial endotoxin in the circulation is found to be responsible for the initiation of the septic cascade that leads to microvascular injury, hypotension, disseminated intravascular coagulation, diminished blood flow to vital organs causing multiple organ failure and eventually death (4). Many factors and mechanisms have been suggested to be responsible in this progression, just as in our previous studies where

we found that many endogenic factors such as nitric oxide and endothelin peptides (5,6), antioxidants (7) or K_{ATP} channel blockers related to pathophysiology.

Although endotoxin is known to be the responsible initiator of sepsis, for the demonstration of an experimental sepsis model, administration of endotoxin to the animals itself, is not sufficient to reflect outcome of the human sepsis precisely, because of the complex immunologic response after the LPS induction with an exaggerated cytokine release (8). Instead, caecal ligation and puncture (CLP) model is accepted to be a superior model of septic shock reflecting systemic response in humans as it mimics the bowel perforation status following the splanchnic ischemia, inducing peritonitis because of its mixed intestinal flora (9).

Poly (ADP-ribose) polymerase (PARP)-1 is the most abundant isoform of PARP enzyme family in the eukaryotic nucleus that participates in the regulation of multiple physiological cellular functions such as DNA repair, gene expression, differentiation, cell cycle progression and apoptosis (10,11). Oxidative and nitrosative stress lead to the DNA single strand breakage and as the DNA nick sensor, PARP protein binds to DNA triggering the poly(ADP-ribosyl)ation reaction (12–14). Activated PARP uses substantial amounts of NAD^+ as a substrate and cleaves it into nicotinamide and ADP-ribose to form long branches of ADP-ribose polymers on glutamate residues of proteins and histones. Binding of negatively charged ADP-ribose polymers to the histones generates an electrostatic repulsion between histones permitting the access of repairing enzymes to the damaged DNA (14–16).

Massive overactivation of PARP depletes the NAD^+ stores thus leading to cellular energetic depletion, mitochondrial dysfunction and eventually necrosis since the rate of the mechanisms using NAD^+ such as glycolysis, electron transport and ATP formation slow down (17,18). In addition, overactivated PARP-1 also facilitates the expression of numerous transcription factors, DNA replication factors and signalling molecules such as nuclear factor-κB (NF-κB), AP-1 and Akt activation (19), leading to increased inflammation and tissue injury consequently (20,21). In multiple studies regarding ischemia-reperfusion injury (22), local inflammation (23,24) or hemorrhagic shock (25), genetic inactivation or pharmacologically inhibition of PARP have shown to improve the results. In our last study, we investigated the effects of the membrane permeable superoxide radical scavenger tempol in septic shock model and as one of its results we found that it had attenuated the PARP immunostaining in tissues.

While PARP activity can modulate other factors, it also can also be modulated by others such as phosphorylation by various kinases such as mitogen activated protein kinases (26) or protein kinase C (27), by various exogeneous factors such as caffeine (28), theophylline (29), tetracycline antibiotics as well as by many endogenous factors including thyroid hormones (30), NAD^+ metabolites, retinoic acid (31) and estrogen (32). Since estrogen has already been found to protect females from the inflammation by inhibiting the PARP activity, PARP inhibition improved the responses to inflammation in males more than females (33). 3-AB is one of the PARP-1 inhibitors that has been used in variable studies. We aimed to investigate the effects of the PARP-1 inhibitor 3-AB on mesenteric blood flow and organ injury (lung, liver, spleen) in a more relevant CLP septic shock model in both female and male mice.

Materials and Methods

Animals

Swiss albino mice (25–40 g) were obtained from the in-bred colony of the Laboratory Animal Husbandry facility of the Department of Pharmacology, Hacettepe University Faculty of Medicine. The mice were housed under environmentally controlled conditions at 21±2°C and 30–70% relative humidity with 12 h dark/12 h light illumination sequence (the lights were on between 7 am and 7 pm) with *ad libitum* access to tap water (drinking bottle) and standard pellet dairy chow (Korkutelim Yem Sanayi, Antalya, Turkey). The Guiding Principles in the Care and Use of Laboratory Animals together with The Recommendations from the Decleration of Helsinki were strictly adhered to during the execution of all the procedures described within this manuscript. This project was approved by the Institutional Experimental Animal Care and Use Ethics Comittee of Hacettepe University before the commencement of any intervention.

Polymicrobial Sepsis Model

Polymicrobial sepsis was induced in mice by CLP that was detailed in our previous publication (34). In brief, animals were fastened overnight, but were allowed *ad libitum* access to drinking water prior to the experiment. The mice were then anaesthetized with chloralhydrate (400 mg kg⁻¹, i.p.) and a 1 cm midline incision was made. The caecum was then exposed, ligated just distal to the ileocaecal valve to avoid any intestinal obstruction, and punctured twice with a 22 Gauge needle. The punctured caecum was gently squeezed to expel a small amount of fecal material and the abdominal incision was then closed in two layers by using atraumatic 4–0 silk sutures. Sham-operated animals underwent the same surgical procedure except that the caecum was neither ligated nor punctured. Animals also received normal saline (1 mL) subcutaneously immediately after the surgery, which is essential for producing the hyperdynamic phase of sepsis during earlier stages in this experimental model.

Experimental Protocol

3-Aminobenzamide (3-AB, 10 mg kg⁻¹, i.p. ; in 1 mL) or nonpyrogenic sterile saline (NaCl 0.9%, w/v, dissolved in pyrogen-free distilled water; i.p, in 1 mL) was given 1 h after CLP. Control mice received 1 mL of the nonpyrogenic sterile saline as the vehicle 1 h after the procedure. All drugs were prepared daily, dissolved in sterile saline and warmed to body temperature (approximately 37°C) before the injection. Drug solutions were kept in dark containers until injection in order to protect them from a possible light-induced decomposition.

Surgical Procedure

Twenty-four h after CLP, the animals were anaesthetized with chloralhydrate (400 mg kg⁻¹, i.p.). the mice were allowed to breathe room air spontaneously and the body temperature of mice were kept at 37.0±0.1°C by a rectal thermistor probe controlled incandescent lamp (100W) placed approximately 30 cm above the animals.

A midline incision was made to the mice and perivascular ultrasonic Doppler-flow probe, connected to a Transonic Small Animal Flowmeter System T106 (Transonic Inc., Ithaca, USA), was placed around the common mesenteric artery. The signals from the flowmeter were also recorded on a computer by using a MP35 Biopac data recording system (Goleta, California, USA) as in millilitres per minute and for standardization, these values were normalized for each individual mice by dividing to the body weight of the animal and the result of the absolute blood flow was

expressed as mL/min/kg body weight. After 10 minutes period of blod flow measurement the mice were sacrificed by cardiac puncture from the apex and blood sample was collected. After the exsanguination liver, lung and spleen samples were extracted from the body and while some parts of the organs was stored at -80°C for biochemical analysis, the rest of them were fixed in 10% neutral buffered formaldehyde solutions at room temperature and later was paraffin embedded by conventional techniques for histopathological examinations.

Histological examination

The lung, liver and spleen samples were fixed in 10% buffered formaldehyde and processed according to routine light microscopic tissue processing technique. 5 μm sections were stained with hematoxylin-eosin and examined and photographed with Olympus BH-2 (Japan) conventional light microscope.

Biochemical analyses

Thiobarbituric acid-reacting substances of lipid peroxidation (TBARS), glutathione (GSH) levels, and myeloperoxidase (MPO) activities in liver, malonyldialdehyde (MDA) levels in plasma, MDA, MPO and GSH levels in lung were determined.

The blood samples were centrifuged (1610 x g for 3 min at room temperature) to separate plasma. All plasma samples were analysed within 24 h by a clinical laboratory using standard laboratory techniques. Tissues were homogenized in 150 mM ice-cold potassium chloride (KCl) to make a 10% homogenate by using a glass Teflon homogenizer. All of the biochemical determinations were carried out on this homogenate. Two ml of homogenate was immediately pipetted into a tube containing 2 ml of cold 8% (v/v) perchloric acid (HClO4). The mixture was shaken vigorously and kept cold until centrifugation.

Determination of lipid peroxides

The levels of lipid peroxides in tissues, as an indicator of oxidative damage (stress), were determined by the method of Uchiama and Mihara (35). Three ml of 1% phosphoric acid (H3PO4) and 1 ml of thiobarbituric acid solution were added to 0.5 ml of 10% tissue homogenate and pipetted into a tube. The mixture was heated in boiling water for 45 min. After cooling, the colored complex was extracted into 4 ml of n-butanol and the absorbance was measured at 532 nm (ε=1.56 x 105/M/cm). The amount of lipid peroxides was calculated as TBARS products of lipid peroxidation and expressed as nanomols/mg of protein.

Quantification of lipid membrane peroxidation in plasma and lung have been made by the measurement of MDA, a highly toxic by-product formed in part by lipid peroxidation derived free radicals. MDA levels were measured in all frozen samples by the TBARS assay.

Determination of glutathione levels

Reduced glutathione, as an indicator of antioxidant capacity, was measured through total sulfhydryl groups by using Ellman Reagent (5.5'-Dithio-bis 2-Nitrobenzoic acid). Tissue homogenate was deproteinized as described above and neutralized by using 0.7 M K3PO4. The resulting precipitate was removed by centrifugation and the supernatant was used for glutathione determination as described earlier (36). Glutathione levels were then calculated as millimoles/mg of protein.

Determination of myeloperoxidases

Standard reaction mixture consisted of 500 μl detergent-containing buffer (160 mM potassium phosphate buffer, pH 5.4, 1% HETAP), 100μl 3.3–5.5 Tetramethylbenzidine (TMB) (16 mM, dissolved in DMF (N, N-Dimethyl formamide), 50μl sample homogenate and 300 μl water. The reaction was initiated by the addition of 50 μl H_2O_2 (diluted to 0.06% for tissue samples and 0.003% for leukocytes) at 37°C. The rate of myeloperoxidases-catalyzed oxidation of TMB was followed by recording the increase of absorbance at 655 nm. Considering the initial and the linear phase of the reaction, we measured the absorbance change per minute and one enzyme unit was defined as the amount of enzyme producing one absorbance change per one minute under assay conditions. Enzyme activity, as an indicator of inflammation, was calculated as units per mg of protein for tissue samples (37).

Drugs

The following drugs and reagents were used: 3-Aminobenzamide (Sigma-Aldrich, USA), chloralhydrate, sodium chloride, hematoxylin, eosin, formaldehyde (Merck, USA), paraffin (Thermo Shandon Histoplast, UK).

Statistical Analysis

Statistical differences between mesenteric blood flow groups were assessed by using two-way analysis of variance for repeated measures. Biochemical values were compared by using one-way analysis of variance (ANOVA) where appropriate. When a significant interaction was found in ANOVA, then a posthoc Tukey-Kramer test for multiple contrasts was performed. All values were expresssed as the arithmetic mean ± Standard error of the mean (S.E.M.) of number (n) of experiments. The differences were considered statistically significant when $P<0.05$.

Results

Biochemical Analyses

In the lung, MDA and MPO activity levels in both sexes increased and GSH levels decreased in CLP groups compared to sham groups significantly ($P<0.0001$) and the effect of neither 3-AB, nor sex is significant in both sham and CLP groups (Figure 1–3)
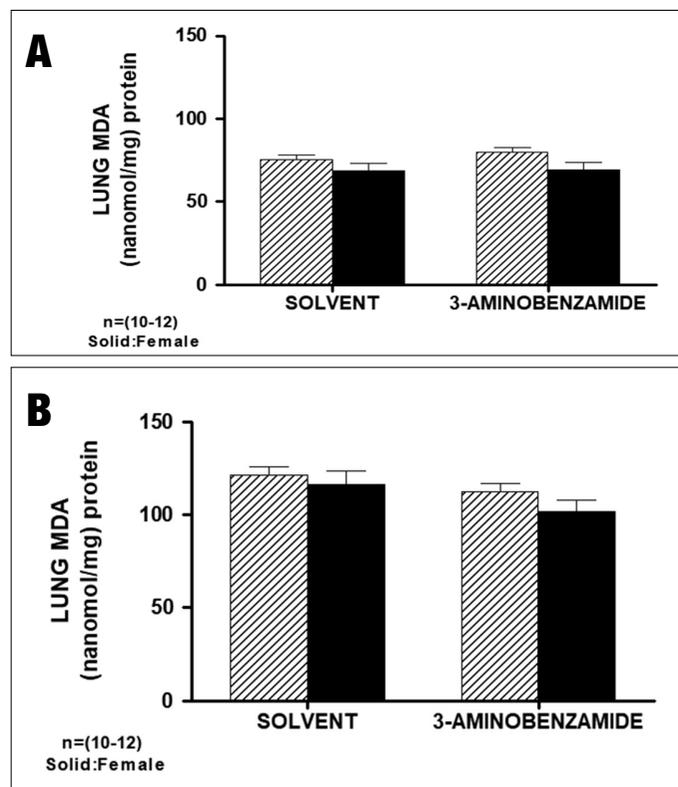
In the liver the results were more complicated especially in females. When two way ANOVA applied to female groups for MPO activity assessment the effect of CLP is not significant ($P=0.1087$) and the effect of 3-Aminobenzamide (3-AB) administration is found statistically significant ($P=0.003$) whereas interaction between CLP and 3-AB is also found significant ($P=0.0477$). Because of this interaction, the significant effect of 3-AB in females was interrogated. In males CLP significantly ($P<0.0001$) increased the levels and
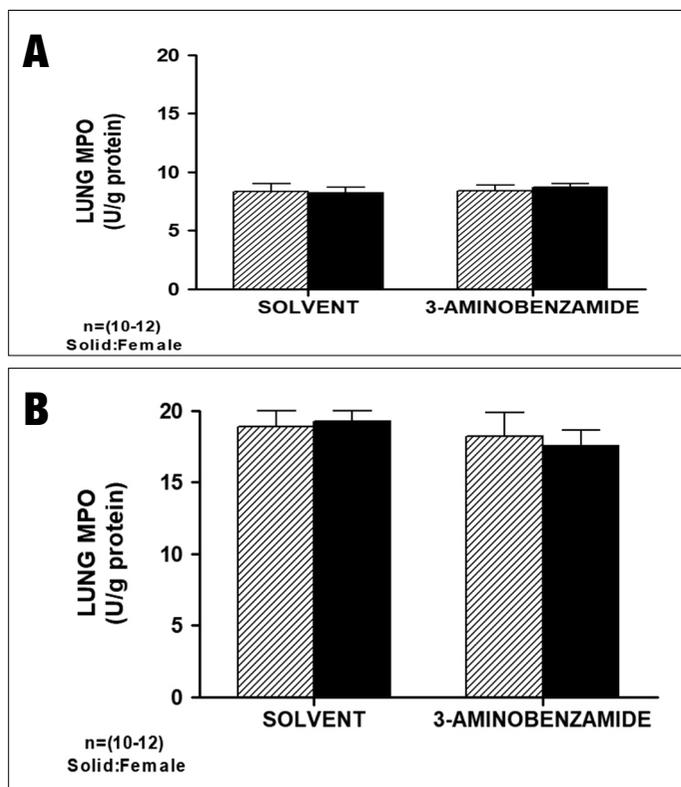
Figure 1 a, b. The effect of 3-Aminobenzamide on lung myeloperoxidase (MPO) activity in male (*n*=10, hatched columns) and female mice (*n*=12, solid black columns) in sham operated **(a)** and caecal ligation and puncture (CLP) sepsis model **(b)** groups in comparison to solvent (saline)-treated ones. Vertical bars indicate the SEM of number *(n)* of experiments. Two-way ANOVA applied to all four bars in sham-operated groups indicated the effect of sex and the effect of 3-Aminobenzamide (3-AB) administration is not significant (*P*=0.8634; 0.5519, respectively). Interaction between sex and 3-AB is not significant (*P*=0.6913) **(a)**. Two-way ANOVA applied to all four bars in the CLP groups indicated the effect of sex and the effect of 3-Aminobenzamide (3-AB) administration is not significant (*P*=0.8634; 0.3094, respectively). Interaction between sex and 3-AB is not significant (*P*=0.6736) **(b)**.

Figure 2 a, b. The effect of 3-Aminobenzamide on lung malonyldialdehyde (MDA) levels in male (*n*=10, hatched columns) and female mice (*n*=12, solid black columns) in sham operated **(a)** and caecal ligation and puncture (CLP) sepsis model **(b)** groups in comparison to solvent (saline)-treated ones. Vertical bars indicate the SEM of number *(n)* of experiments. Two-way ANOVA applied to all four bars in sham-operated groups indicated the effect of sex (*P*=0.0401) and the effect of 3-Aminobenzamide (3-AB) administration is not significant (*P*=0.5358). Interaction between sex and 3-AB is not significant (*P*=0.6198) **(a)**. Two-way ANOVA applied to all four bars in the CLP groups indicated the effect of sex and the effect of 3-Aminobenzamide (3-AB) administration is not significant (*P*=0.1897; 0.0629, respectively). Interaction between sex and 3-AB is not significant (*P*=0.6129) **(b)**.

3-AB could not decrease them to the control ones. When two way ANOVA applied to the sham and CLP groups the effect of sex (P=0.0347; 0.0002, respectively) and the effect of 3-AB (P=0.0326; 0.0003, respectively) found significant whereas the interactions are also found significant (P=0.0162; 0.0045, respectively) making the effects of sex and 3-AB interrogated (Figure 4).

The similar results were also eligible for the assessment of the lipid peroxidation in the liver by the accumulation of TBARS. CLP significantly increased the levels (*P*<0.0001) and 3-AB administration significantly increased them (*P*=0.0083) whereas interaction between CLP and 3-AB is found marginally significant (*P*=0.0517). Because of this interaction, the significant effect of 3-AB in females is interrogated. In males CLP significantly (*P*<0.0001) increased the levels and 3-AB could not decrease them to the control ones. When two way ANOVA applied to the sham groups the effect of sex and 3-AB is found significant (P=0.0032; 0.0114, respectively) whereas the interaction between them is also found significant (*P*<0.0001) and the effects are ignored statistically. When two way ANOVA applied to the CLP groups the effect of sex, 3-AB are found insignificant (Figure 5).

For liver antioxidant capacity the glutathione (GSH) levels were measured. In females CLP significantly reduced the levels (*P*<0.0001) and 3-AB significantly increased (*P*<0.0001) them to control ones whereas the interaction is not significant. In males 3-AB could not get the decreased CLP levels (*P*<0.0001) to the control ones and the interaction is not significant. When two way ANOVA applied to the sham and CLP groups the effect of 3-AB administration is found significant (P=0.0399; <0.0001, respectively) whereas sex is found not significant while the interaction between them is not significant (Figure 6).

CLP did not differ plasma MDA levels significantly in both sexes and 3-AB and sex effect was not significant in both control and CLP groups, either (Figure 7).

The Effect of 3-Aminobenzamide on Absolute Mesenteric Blood Flow

24 h after CLP absolute mesenteric blood flow values (AMBF) were measured as blood flow per body weight for standardization (Figure 8). Two-way analysis of variance for repeated measures
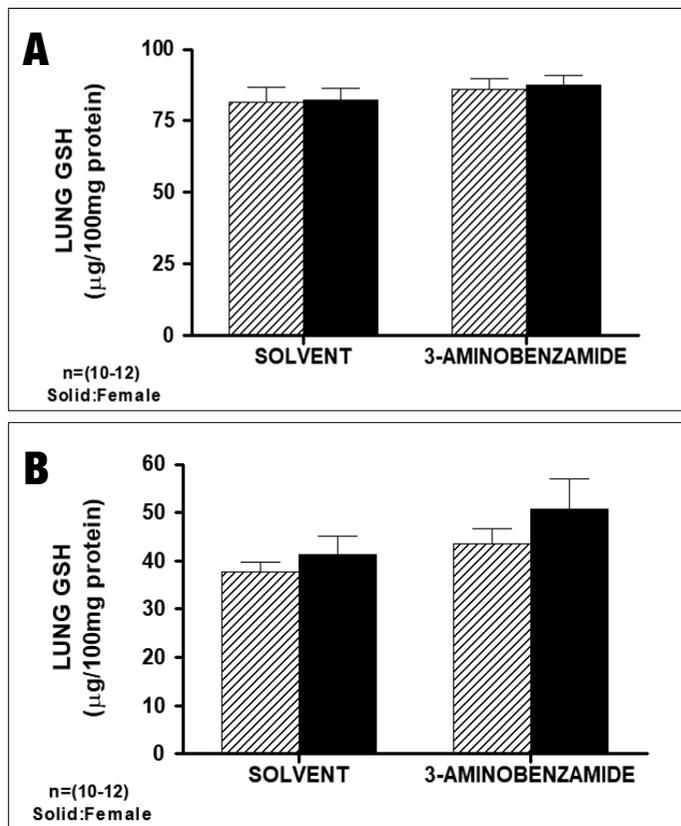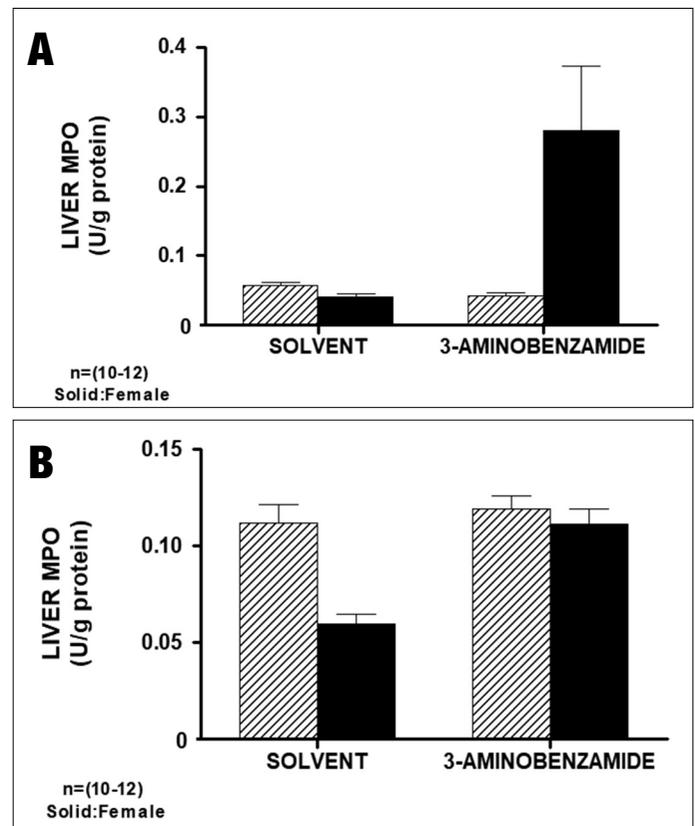
Figure 3 a, b. The effect of 3-Aminobenzamide on lung glutathione (GSH) levels in male (n=10, hatched columns) and female mice (n=12, solid black columns) in sham operated **(a)** and caecal ligation and puncture (CLP) sepsis model **(b)** groups in comparison to solvent (saline)-treated ones. Vertical bars indicate the SEM of number (n) of experiments. Two-way ANOVA applied to all four bars in sham-operated groups indicated the effect of sex and the effect of 3-Aminobenzamide (3-AB) administration is not significant (P=0.8242; 0.2592, respectively). Interaction between sex and 3-AB is not significant (P=0.9208) **(a)**. Two-way ANOVA applied to all four bars in the CLP groups indicated the effect of sex and the effect of 3-Aminobenzamide (3-AB) administration is not significant (P=0.2218; 0.0864, respectively). Interaction between sex and 3-AB is not significant (P=0.6736) **(b)**.

Figure 4 a, b. The effect of 3-Aminobenzamide on liver myeloperoxidase (MPO) activity in male (n=10, hatched columns) and female mice (n=12, solid black columns) in sham operated **(a)** and caecal ligation and puncture (CLP) sepsis model **(b)** groups in comparison to solvent (saline)-treated ones. Vertical bars indicate the SEM of number (n) of experiments. Two-way ANOVA applied to all four bars in sham-operated groups indicated the effect of sex (P=0.0347) and the effect of 3-Aminobenzamide (3-AB) administration (P=0.0326). Interaction between sex and 3-AB is significant (P=0.0162) **(a)**. Two-way ANOVA applied to all four bars in CLP groups indicated the effect of sex (P=0.0002) and the effect of 3-Aminobenzamide (3-AB) administration (P=0.0003). Interaction between sex and 3-AB is significant, P=0.0045 **(b)**.

applied to control *versus* CLP curves indicated significance (P<0.0001; P=0.0171, respectively) in both female and male groups whereas CLP+3-AB vs. CLP (P=0.0967; 0.9214, respectively) or 3-AB vs. control curves did not indicate any significance (P=0.7119; 1, respectively) and the curves of CLP+3-AB vs. control indicated significance as P=0.0009; 0.0525 respectively).

When two-way analysis of variance for repeated measures applied to control and CLP curves of the female and male groups sex effect did not indicate any significance with P=0.471 for sham and P=0.022 for CLP groups with a significant interaction as P=0.0002 (Figure 9)

Histopatological Examination of Lung, Liver and Spleen

In females CLP induced the histopatalogical changes in organs as in sepsis related multiorgan failure. In the lung most of the alveoli were collapsed and emphysematous changes as thickened interalveolar septa and profuse mononuclear cell infiltration in interalveolar septa and bronchioles were seen. Epithelium of the bronchioles was sloughed off (Figure 10a). 3-AB administration to the CLP group could not improve

the organ injury as infiltration and thickening of interalveolar septum with the collapsed alveoli were also seen in those samples (Figure 10d).

In CLP induced female livers, hepatocytes were swollen and degenerated. Some of them had picnotic nuclei whereas some of them were apoptotic with both picnotic nuclei and eosinophilic cytoplasm. The sinusoids between the hepatocytes and central veins were dilated (Figure 10b). In the 3-AB administrated CLP group sinusoids between the swollen hepatocytes were not visible. There were also hepatocytes with picnotic nuclei and eosinophilic cytoplasm, similar to the CLP group (Figure 10e).

Also in the spleens of females, histological changes were similar in both CLP and 3-AB subjected CLP groups. Follicular hyperplasia was prominent and periarterial lymphatic sheath was also enlarged. Between the white pulp and in the red pulp, venous sinusoids were congested (Figure 10c, 10f).

In male organs, CLP induced less severe injury histopatalogically and furthermore, 3-AB administration seem to worsen the conjuncture. Minimal thickening and inflammatory cell
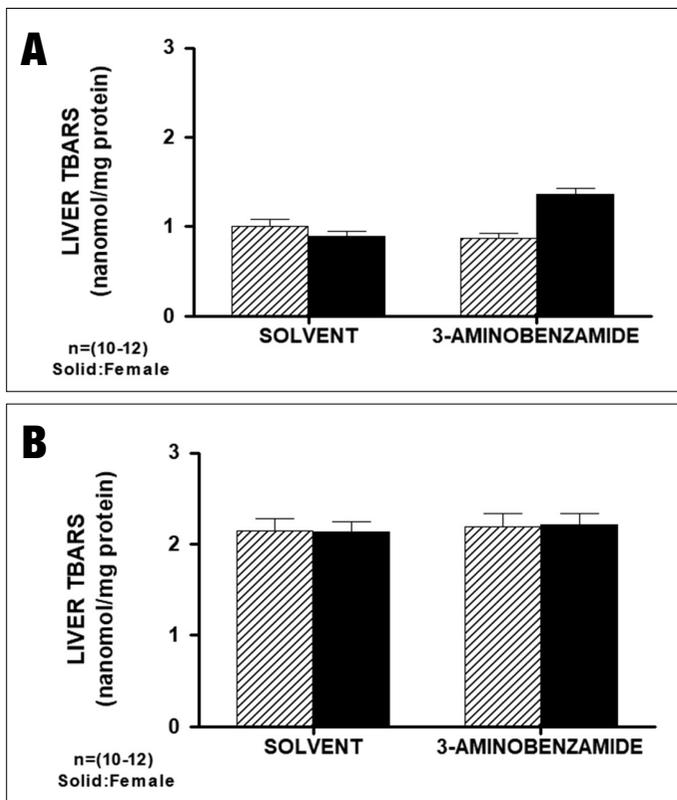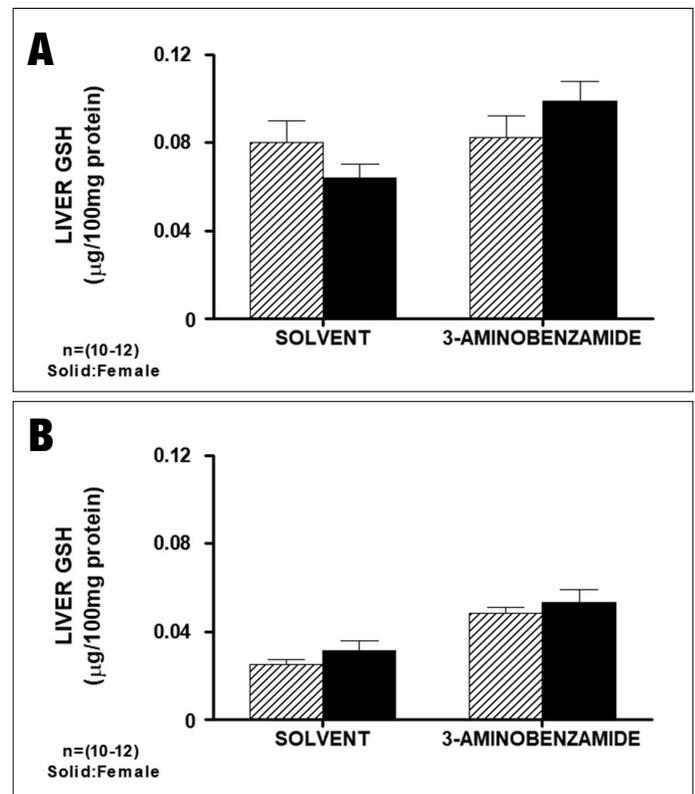
Figure 5 a, b. The effect of 3-Aminobenzamide on liver TBARS of lipid peroxidation in male (n=10, hatched columns) and female mice (n=12, solid black columns) in sham operated **(a)** and caecal ligation and puncture (CLP) sepsis model **(b)** groups in comparison to solvent (saline)-treated ones. Vertical bars indicate the SEM of number (n) of experiments. Two-way ANOVA applied to all four bars in sham-operated groups indicated the effect of sex (P=0.0032) and the effect of 3-Aminobenzamide (3-AB) administration (P=0.0114). Interaction between sex and 3-AB is significant (P<0.0001) **(a)**. Two-way ANOVA applied to all four bars in CLP groups indicated the effect of sex and the effect of 3-Aminobenzamide (3-AB) administration is not significant (P=0.6823; 0.5015, respectively). Interaction between sex and 3-AB is not significant (P=0.7657) **(b)**.

Figure 6 a, b. The effect of 3-Aminobenzamide on liver glutathione (GSH) levels in male (n=10, hatched columns) and female mice (n=12, solid black columns) in sham operated **(a)** and caecal ligation and puncture (CLP) sepsis model **(b)** groups in comparison to solvent (saline)-treated ones. Vertical bars indicate the SEM of number (n) of experiments. Two-way ANOVA applied to all four bars in sham-operated groups indicated the effect of sex is not significant (P=0.9208) and the effect of 3-Aminobenzamide (3-AB) administration (P=0.0399). Interaction between sex and 3-AB is not significant (P=0.0657) **(a)**. Two-way ANOVA applied to all four bars in CLP groups indicated the effect of sex is not significant (P=0.2557) and the effect of 3-Aminobenzamide (3-AB) administration (P<0.0001). Interaction between sex and 3-AB is not significant (P=0.8882) **(b)**.

infiltration in the interalveolar septum were present in the alveoli close to bronchi. Emphysematous areas were not seen in the CLP group (Figure 11a) whereas in the 3-AB treated CLP group interalveolar septa were thickened, some alveoli were collapsed, infiltration and emphysematous changes in some areas were obvious (Figure 11d).

In male liver samples, degenerative changes were observed in hepatocytes as some of them had picnotic whereas some had larger but degenerated nuclei and also the central veins were enlarged (Figure 11b). In the 3-AB induced CLP group, degeneration in the hepatocytes were more severe and in diffuse pattern. There was ballooning degeneration in some hepatocytes (Figure 11e).

In CLP induced male spleens, histological appearance was almost normal. White pulp and red pulp with cords and venous sinuses were easily identified with obvious multinuclear giant cells (Figure 11c). In the 3-AB induced CLP group, white pulp was enlarged and the structure of follicles were destroyed. In the red pulp venous sinusoids, congestion and multinuclear giant cells were also seen as in the saline induced CLP group (Figure 11f).

Discussion

In this study, our results showed that in CLP experimental sepsis model, Poly (ADP-ribose) polymerase-1 inhibitor 3-Aminobenzamide has no significant effect on splanchnic ischemia or multiple organ injury as histopathogically or biochemically. Also we could not show the effect of gender difference on septic results or on PARP-1 activity, either. Nevertheless, these results are controversial to the common results of the recent literature.

It's noteworty that circulatory failure in septic shock is the major cause of multiple organ injury leading to death as blood supply to organs are not effectively and fairly distributed. While the system tries to supply enough blood to the vital organs like brain or heart, the other systems especially the gastrointestinal system gets less blood than it necessitates and the following ischemia concludes with epithelial barrier impairment that allows the passage of the resident microorganisms from the lumen into the circulation leading to much more severe bacteremia and clinical outcomes. That's why the splanchnic blood flow is mightily important for the progression of septic shock (38–41). However, in this study, 3-AB treatment could not improve the vital mesenteric blood flow.
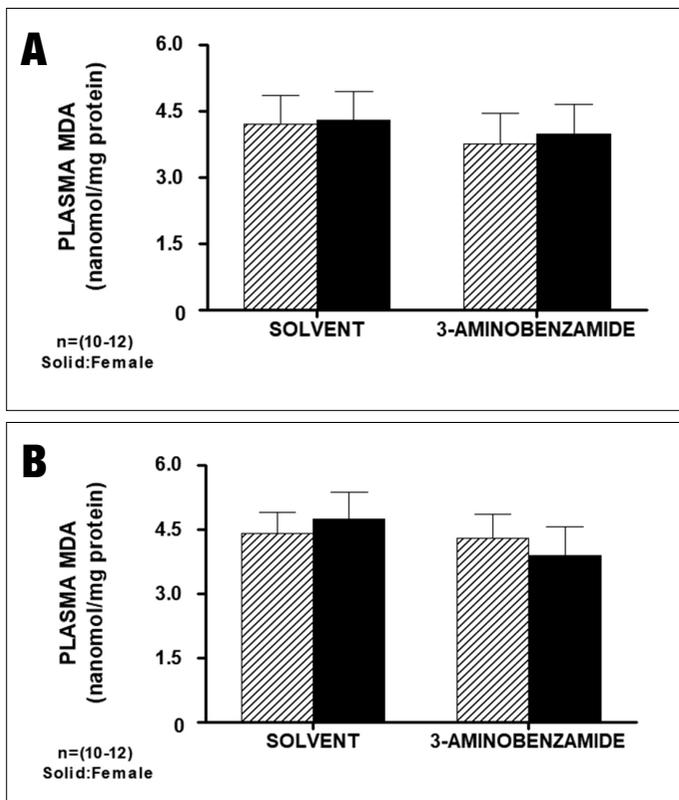
Figure 7 a, b. The effect of 3-Aminobenzamide on plasma malonyldialdehyde (MDA) levels in male (*n*=10, hatched columns) and female mice (*n*=12, solid black columns) in sham operated **(a)** and caecal ligation and puncture (CLP) sepsis model **(b)** groups in comparison to solvent (saline)-treated ones. Vertical bars indicate the SEM of number *(n)* of experiments. Two-way ANOVA applied to all four bars in sham-operated groups indicated the effect of sex and the effect of 3-Aminobenzamide (3-AB) administration is not significant (P=0.8242; 0.5689, respectively). Interaction between sex and 3-AB is not significant (P=0.9208) **(a)**. Two-way ANOVA applied to all four bars in the CLP groups indicated the effect of sex and the effect of 3-Aminobenzamide (3-AB) administration is not significant (P=0.9208; 0.4431, respectively). Interaction between sex and 3-AB is not significant (P=0.5464) **(b)**.

Although there are some controversial results, in many of the other studies dealing with variable pathologies including sepsis, PARP inhibition by pharmacologically or genetically have improved the outcomes (42). Differences in the timing or the type of agents in the treatment, the experimental methods or the stages of the experimental septic shock may be responsible for these controversies. For instance, in the study of Soriano et al., PARP –/– mice were found to be more resistant to inflammatory parameters and survived longer than the wild types (43). In this work, it is found that the absence of PARP function improved the liver injury at the early stages as in 12 h but at the later phases such as 24 h the results were not observed and the survival rates were not different after the 5 days-period. In our study we worked on the later phase of septic shock and got the samples and measurements at 24 h. This might be one of the reasons of the conflicting results.

Goldfarb et al. studied sepsis in pigs by intraperitoneal implantation of *E.Coli* O111: B4-laden fibrin clots and potent PARP inhibitor PJ34 was administered 1 h before clot implantation and was infused continuously for 96 h (44). In this model the animals were not resuscitated and a hypodynamic shock state was exhibited. In our model the animals were
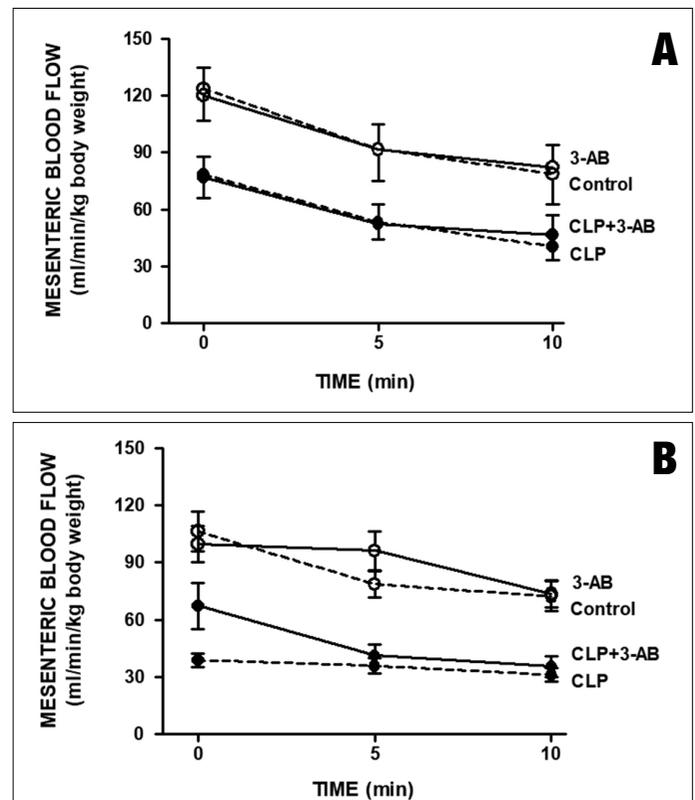
Figure 8 a, b. Mesenteric arterial blood flow (mL/min/kg body weight) values of male (***top,*** *n*=10) and female (***bottom,*** *n*=12) mice observed 24 h after caecal ligation and puncture (CLP), which also received 3-Aminobenzamide (3-AB, 10 mg/kg, at 1 h after CLP) or its solvent (saline, 1 mL/kg, i.p.). Values are mean ± SEM of number (*n*) of experiments. Some error bars have been omitted for the sake of clarity. Two-way analysis of variance for repeated measures applied to "CLP" vs. "CLP + 3-AB" curves did not indicate a significant difference between them in both male **(a)** and female **(b)** groups (P=0.9214; 0.0967, respectively) and the curves of "CLP+3-AB" vs. "Control" indicated (P=0.0525; 0.0009, respectively). There was no significant difference between the curves of "Control" vs. "3-AB" in both male and female groups but contrarily, "3-AB" vs. "CLP + 3-AB" curves differed significantly (P=0.0263; 0.0007, respectively) when pairwise comparisons were performed by using two-way analysis of variance for repeated measures using the "time" and "groups" as the two variables.
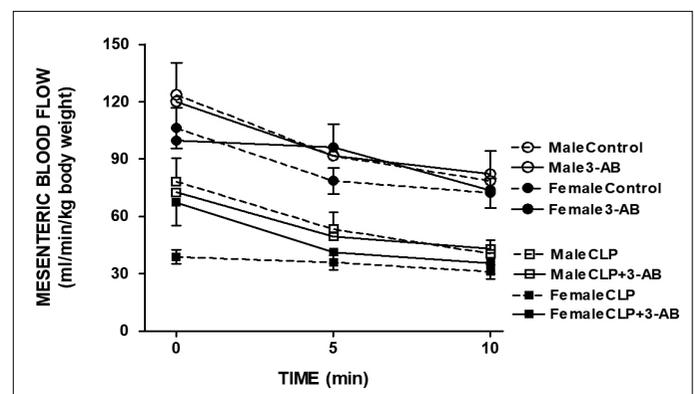

Figure 9. Mesenteric arterial blood flow (mL/min/kg body weight) values of male (*n*=10) and female (*n*=12) mice observed 24 h after caecal ligation and puncture (CLP), which also received 3-Aminobenzamide (3-AB, 10 mg/kg, at 1 h after CLP) or its solvent (saline, 1 mL/kg, i.p.). Values are mean ± SEM of number (*n*) of experiments. Some error bars have been omitted for the sake of clarity. Two-way analysis of variance for repeated measures applied to "Male" vs. "Female" Control curves did not indicate a significant difference between them in both solvent and 3-AB groups (P=0.471; 0.5899, respectively). On the contrary the CLP curves of males and females differed significantly (P=0.02159) and 3-AB administration to CLP groups abolished that significance (P=0.376) when pairwise comparisons were performed by using two-way analysis of variance for repeated measures using the "time" and "groups" as the two variables.
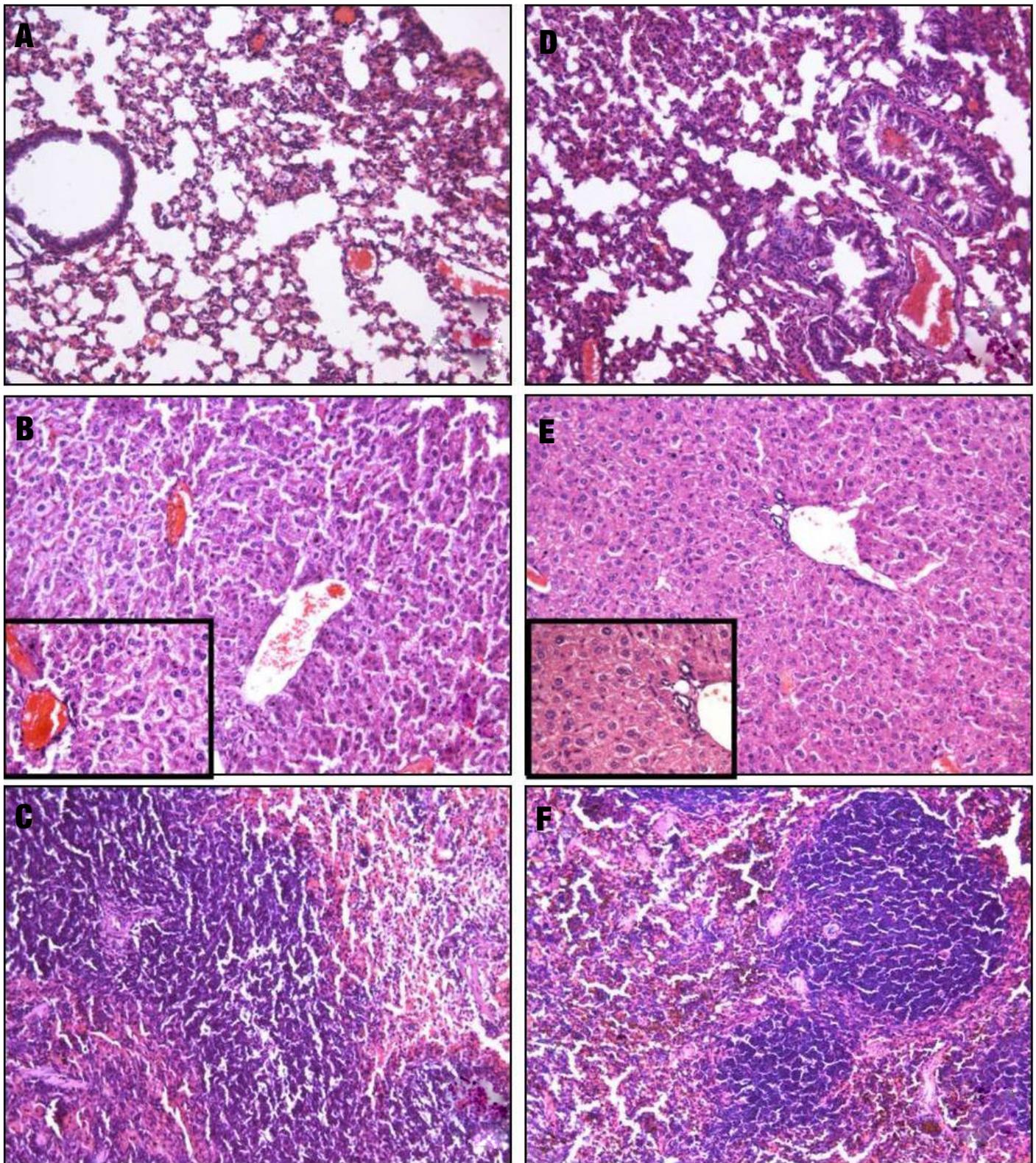
Figure 10 a–f. The photographs on the left column are the histological sections of lung **(a)**, liver **(b)** and spleen **(c)** from CLP group in comparison to those obtained from 3-AB treated female animals on the right column **(d–f)**. Hematoxylin-Eosin ×200, (inset **b** and **e**, ×400).

resuscitated regarding the hyperdynamic state that is much more relevant to the human septic shock with high volume resuscitation. On the other hand while we posttreated the animals at 1 h, the pretreatment in this study at 1 h before the implantation is not relevant to human septic states either, as it can not be predicted.

As a matter of fact, different inhibitors of PARP activity had different effects on the results of the studies. Another PARP inhibitor PJ34 is approximately 10.000 times more potent than 3-AB as their EC_{50} are 20nM and 200µM, respectively (45). For instance, while Wray et al. could not find any effects of the PARP inhibitors, 3-aminobenzamide, nicotinamide,
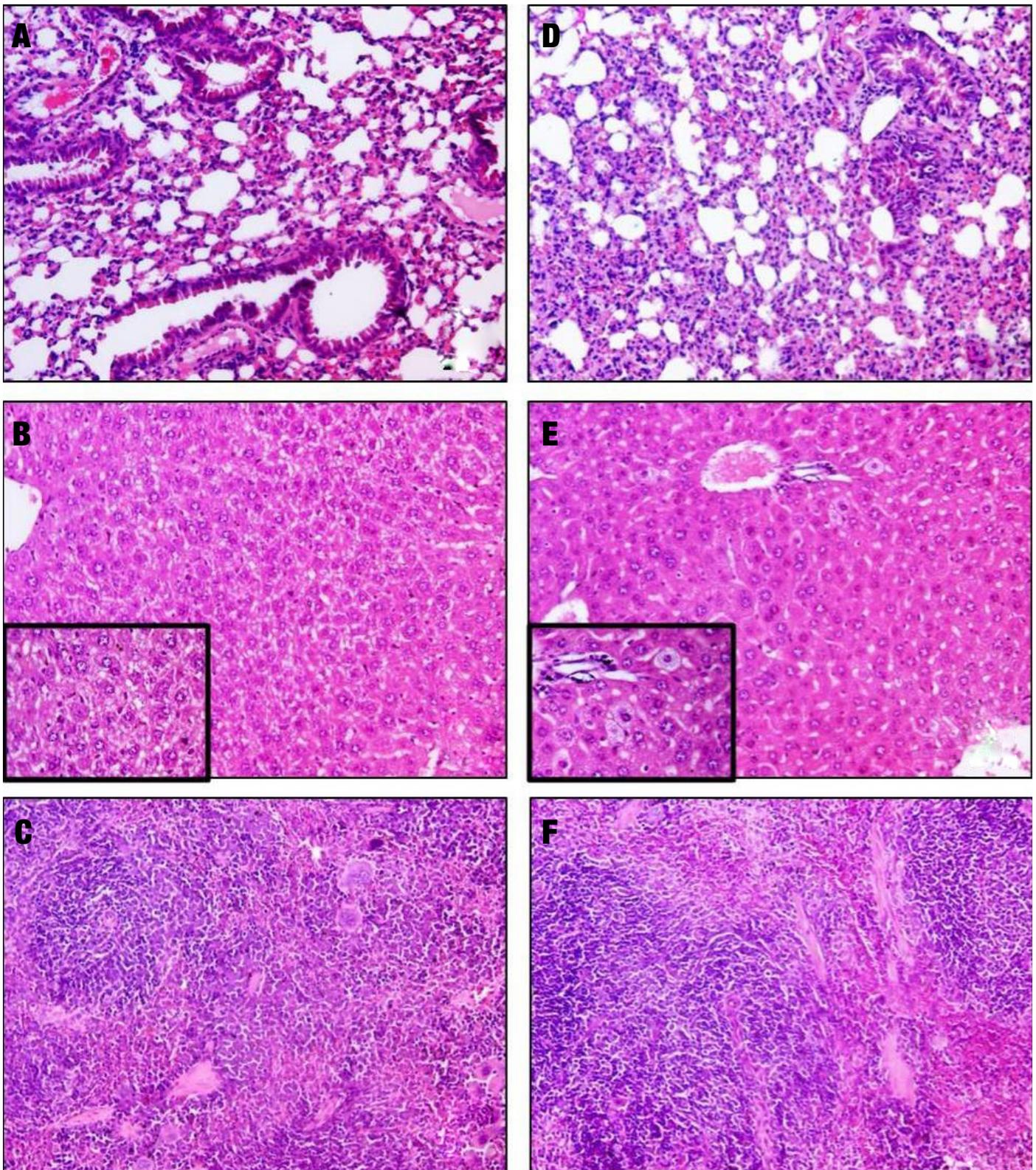
Figure 11 a–f. The photographs on the left column are the histological sections of lung **(a)**, liver **(b)** and spleen **(c)** from CLP group in comparison to those obtained from 3-AB treated male animals on the right column **(d–f)**. Hematoxylin-Eosin ×200, (inset **b** and **e**, ×400).

1.5-dihydroxyisoquinoline on circulatory failure or organ dysfunction in LPS induced rats (46), Jagtap et al., had shown the ameliorated renal and liver dysfunction in LPS induced mice by PJ34 (47). The effects of PJ34 on caecal ligation and puncture sepsis model has not been studied, yet. On the other hand additive pharmacological effects are also seen by these inhibitors such as while PJ34 has immunomodulator effects as one of the phenanthridinones (48), 3-AB has free radical scavenging activities (24). The real PARP activity inhibition effects on inflammation and sepsis are needed to be further studied.

Although gender difference is found to be effective on PARP activity recently (32,33,49), we could not find any difference on the results of PARP inhibition by 3-AB between male and female mice neither in the controls nor in the septic mice.

In conclusion although the increased PARP activity is proven to be increased in septic shock, in caecal ligation and puncture model of sepsis, PARP inhibition by 3-AB did not improve the results. Further clinical studies are needed to illuminate the therapeutic importance of PARP inhibition in human sepsis.

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