

The effects of aspirin, flurbiprofen, and NO-donating acetylsalicylic acid (NCX 4016) on mice models of endotoxic and septic shock

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Background/aim: Nitric oxide-donating nonsteroidal antiinflammatory drugs (NO-NSAIDs) are a promising new class of antiinflammatory agents, which are obtained by adding NO-donating moieties to the existing conventional NSAID molecules. The aim of this study was to investigate the effects of aspirin, flurbiprofen, and NO-donating acetylsalicylic acid (NCX 4016) on cecal ligation and puncture (CLP) and endotoxin-induced septic shock (LPS) models in mice.

Materials and methods: Overall survival and spleen and liver weights were monitored in LPS and CLP models. Histopathological examinations of liver and spleen were performed at the end of the experimental protocols.

Results: NCX 4016 was able to reverse the increased spleen weight in CLP-operated animals, whereas aspirin or flurbiprofen did not. Similar to the results of the CLP model, none of the drugs modified the survival rates in the LPS model. Flurbiprofen in particular produced significant histopathological damage in spleens and livers, which was less significant with aspirin. NCX 4016 did not cause any liver damage.

Conclusion: NCX 4016 has the potential to be used in septic states, while special attention has to be paid to the effects of aspirin and flurbiprofen on the liver and spleen.

Key words: Aspirin, flurbiprofen, NCX 4016, septic shock, cecal ligation and puncture, hepatotoxicity

1. Introduction

Sepsis is a severe systemic inflammatory response to an infection. The causative agents are microorganisms or their toxins, which spread from a local infection site. In the majority of cases, the clinical outcome is worsened by the development of multiple organ dysfunction, which leads to an unrelenting state of acute circulatory failure, namely septic shock (1). Septic shock remains one of the major causes of death in intensive care units (2). Several pathways, such as the arachidonic acid-cyclooxygenase pathway, have been investigated to explain the pathophysiology of septic shock (3). Among these, the maldistribution of blood flow due to potent vasoactive mediators, such as nitric oxide (NO) (4), has always retained its popularity.

NO-donating nonsteroidal antiinflammatory drugs (NO-NSAIDs) constitute a promising class of new antiinflammatory agents, which are obtained by adding an NO-donating moiety to the existing conventional NSAID molecules (5). They are reported to be active in several experimental disease models, including sepsis (6–

9). The underlying mechanism of their beneficial effects is suggested to be the reduction of gut injury due to the release of NO (10). Since the inhibition of arachidonic acid metabolites is known to be beneficial in endotoxemia (11) and they have a pivotal role in the development of acute respiratory distress syndrome in sepsis (12), NO-NSAIDs, i.e. cyclooxygenase inhibitors that donate NO, appear to be promising for the reduction of septic shock symptoms.

The role of NO in the pathophysiology of sepsis and septic shock is controversial and currently under scrutiny (13). The pro- and antiinflammatory effects of NO, as well as its oxidant and antioxidant properties (14), have been the source of interest and debate in sepsis research. It is becoming clearer that NO may act both as friend and foe in septic states (15). Since many deleterious effects of endotoxins, such as hypotension (16) and vascular hyporesponsiveness (17) to vasoconstrictors (18) and vasodilators (19), could be explained by excess NO production (20), the inhibition of NO to combat septic shock was regarded as essential until recently. The

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usefulness of an NO-inhibiting drug for the treatment of human toxic shock syndrome in an isolated case report (21) has enhanced this belief. We showed the potential beneficial effect of NO-induced local vasodilation, particularly in the mesenteric circulation (22). Since splanchnic ischemia increases mortality in septic shock (23), the restoration of tissue perfusion by local vasodilation via NO may reduce the bacterial translocation (24) and improve survival (25).

Therefore, we aimed to investigate the effects of NCX 4016, an NO-donating derivative of aspirin, in experimental models of septic and endotoxic shock, namely cecal ligation and puncture (CLP)-septic and endotoxin-induced endotoxic shock models, by comparing it with aspirin and flurbiprofen. Although our main focus was on overall survival, spleen weight, which reflects the severity of septic state (22), and the histopathological alterations in spleen and liver were also investigated.

2. Materials and methods

2.1. Animals

Swiss albino mice (35–45 g) were obtained from the Laboratory of Animal Husbandry of the Department of Pharmacology, Hacettepe University, and were housed under environmentally controlled conditions at 21 ± 2 °C and 30%–70% relative humidity with a 12-h dark/light illumination sequence (the lights were on between 0700 and 1900 hours), and ad libitum access to tap water (drinking bottle) and standard pellet rodent chow (Korkutelim Yem Sanayi, Antalya, Turkey). All animal experiments were performed with strict adherence to European Community guidelines and the Declarations of Helsinki and Tokyo, and they were approved by the Institutional Experimental Animal Care and Use Ethics Committee of Hacettepe University before the commencement of any interventions (Approval Number: 2004/55-4).

2.2. Mice model of septic shock induced by cecal ligation and puncture

Polymicrobial sepsis was induced by adhering to the same protocol of our previous study (26), which was based on the detailed guidance of other publications (27,28). In brief, mice were fasted overnight, but were allowed ad libitum access to drinking water prior to the experiment. The animals were then anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed on a heat-insulated cork sheet-covered operating table. The animals were allowed to breathe room air spontaneously. After the obtundation of all responses to painful stimuli (pinching of the plantar skin with tweezers), a 1-cm midline abdominal incision was made. The cecum was then exposed, ligated by using atraumatic 3-0 silk sutures to distal the ileocecal valve in order to avoid any intestinal obstruction, and punctured twice with a 22-gauge needle. The punctured cecum 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 cecum was neither ligated nor punctured. Immediately after the surgery all animals received a subcutaneous injection of 1 mL of sterile saline (NaCl 0.9 %, w/v, dissolved in pyrogen-free distilled water).

After recovery from the surgery, the animals were randomly allocated to different treatment groups ($n = 14$ – 24) to receive twice-daily intraperitoneal injections of one of the following: (i) conventional aspirin (acetylsalicylic acid; 30 mg/kg per day), (ii) its nitric oxide-donating derivative (NCX 4016; 50 mg/kg per day), or (iii) flurbiprofen (6 mg/kg per day) until tissue harvesting (maximum of 7 days, depending on the survival time of an individual animal). These particular doses of the test drugs were chosen on the basis of their activity in animals obtained from published data. Likewise, the animals in matching control groups received equal volumes of either sterile saline or the vehicle mixture that was used to dissolve the compounds (i.e. 50% (v/v) polyethylene glycol 400, 45% (v/v) nonpyrogenic sterile saline, and 5% (v/v) dimethyl sulfoxide). NCX 4016, aspirin, and flurbiprofen were dissolved daily in this vehicle, kept in dark containers until the injections in order to protect them from light-induced decomposition, and warmed to body temperature (approximately 37 °C) before injections.

Survival rates were recorded at 12-h intervals for 168 h (7 days) after surgery, and all living animals were killed humanely at the end of our observational period. Since our previous study revealed a direct correlation between the enlargement of the spleen and the presence and severity of septic challenge, the spleens of all animals were isolated and weighed as an indication of the success of the surgical intervention.

In addition to spleens, livers were also isolated in some randomly selected animals. The tissues were fixed in 4% paraformaldehyde solution immediately after removal and were then embedded in paraffin. The histopathological sections were prepared and stained with hematoxylin-eosin staining. When the preparations exhibited fibrosis, the corresponding adjacent sections were stained with trichrome to confirm the presence of fibrotic changes. The slides were examined under a conventional light microscope (Zeiss Axioskop, Germany) by an appropriately blinded pathologist.

2.3. Mice model of endotoxic shock induced by lipopolysaccharide

Endotoxin derived from *Escherichia coli* (lipopolysaccharide (LPS) O55:B5, 60 mg/kg, i.p.) or its vehicle saline (NaCl 0.9 %, w/v, dissolved in pyrogen-free distilled water) was given by intraperitoneal injection to animals at a volume of 0.2 mL. This particular dose of endotoxin was chosen on

the basis of our previous experience, which had revealed a remarkable mortality rate within the first 24 h in mice (22). After the injection of LPS, the animals were placed in separate cages to recover from the intervention and were randomly allocated to groups of 12–14 to receive either aspirin or NCX 4016 treatments twice daily for an observational period of 72 h (3 days). The control animals received the same volume of the vehicles of LPS (i.e. saline), aspirin, or NCX 4016 at the same time points. Survival rates were then recorded for the following 72 h at intervals of 1 h during the first 6 h, and at 12-h intervals until the end of the observational period.

2.4. Drugs

Aspirin, flurbiprofen, and NCX 4016 were kindly provided by NicOx S.A. (Sophia Antipolis, France). Chloral hydrate, sodium chloride, dimethyl sulfoxide, hematoxylin (Merck, USA), lipopolysaccharide O55:B5, polyethylene glycol 400, eosin, trichrome (Sigma, USA), paraffin (Shandon, UK), and formaldehyde (Carlo Erba, Italy) were purchased.

2.5. Statistical analysis

Fisher's exact test for 2 × 2 tables was used to compare the percent survival at selected time points (i.e. at the end of 72-h or 168-h observation periods), while Cox regression analysis was used to compare the trends of the survival curves. The data related to the weight of the spleen were analyzed with two-way analysis of variance (ANOVA), while the rest of the pair-wise comparisons were performed either with the Mann–Whitney U test or Student's t-test, where appropriate, depending on the similarity of the standard deviations of the populations. P < 0.05 was considered to be statistically significant, and the actual values of P are indicated throughout the manuscript.

3. Results

3.1. Saline/vehicle controls in cecal ligation and puncture model

The survival in saline-treated sham-operated animals was 92.9% (13 of 14) and it was 35.7% in the CLP group (5 of 14, P < 0.01) on day 7 (168 h) after the surgery. As shown by the survival curves in Figure 1, the deaths occurred consequently on a linear basis beginning at 24 h with no apparent aggregation at any time point. There was no significant effect of the vehicle, which was used to dissolve test drugs in any of the parameters related to the survival. The survival rate in the vehicle-treated CLP group was 45.8% (11 of 24) at 168 h, and this was not significantly different than the corresponding value obtained from saline-treated CLP animals (Figure 1).

In saline-treated animals, CLP significantly increased spleen weight (g/kg body weight; sham-operated: 5.5 ± 0.6, n = 14 vs. CLP: 8.13 ± 0.8, n = 14; P = 0.0157). These findings were further analyzed by using vehicle and CLP as the variables in a two-way ANOVA test applied to all 4 groups, which revealed a significant interaction between the aforementioned variables (P = 0.008) (Figure 2).

3.2. The effects of aspirin, flurbiprofen, and NCX 4016 on the CLP model

As mentioned earlier, CLP resulted in a 45.8% (11 of 24) survival rate at 168 h in vehicle-treated animals. When the animals were treated with aspirin, the overall survival rate was the same at 168 h. As such, the curves were almost superimposable (Figure 3). Although NCX 4016 treatment resulted in an apparently better overall survival at 168 h (70.8%, 17 of 24), the difference from its corresponding value failed to reach statistical significance (P = 0.1425).

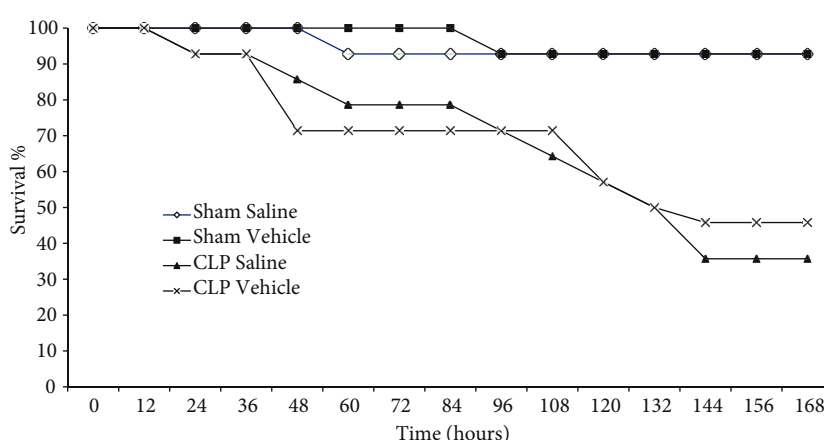


Figure 1. Survival rates obtained from mice that underwent sham or cecal ligation and puncture (CLP) surgery and were then treated either with saline or vehicle, which was used to dissolve drugs. Asterisk indicates significant difference (P < 0.05, two-tailed value, Fisher's exact test) from the corresponding value obtained from sham-operated animals at 168 h. n = 14 in sham saline, sham vehicle, and CLP saline groups, n = 24 in CLP vehicle group.

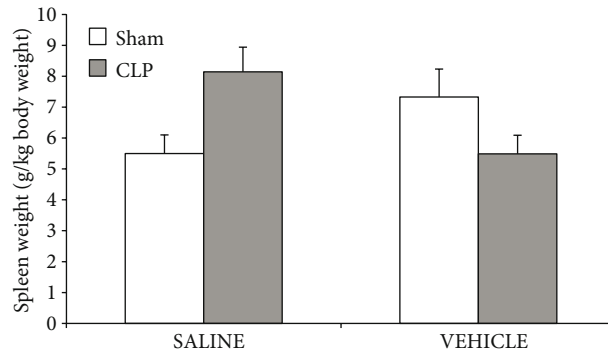


Figure 2. The spleen weights (g/kg body weight, mean ± SEM) obtained from mice that underwent sham or cecal ligation and puncture (CLP) surgery and were treated either with saline or vehicle, which was used to dissolve drugs. Asterisk indicates significant difference ($P = 0.0157$, two-tailed value, Mann–Whitney U test) from the corresponding value indicated in the adjacent column. Two-way ANOVA, applied to the data obtained from all 4 groups by using CLP and vehicle as the variables, indicated a significant ($P = 0.008$) interaction between them. $n = 14$ in sham saline, sham vehicle, and CLP saline groups, $n = 24$ in CLP vehicle group.

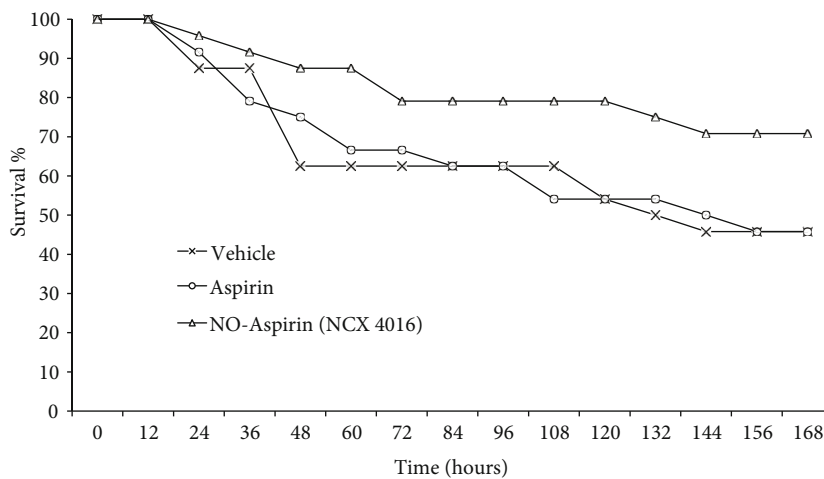


Figure 3. Survival rates obtained from mice that underwent cecal ligation and puncture (CLP) surgery and were treated either with vehicle or conventional aspirin or its NO-donating derivative, NCX 4016. $n = 24$ in all groups.

Similarly, Cox regression analysis of the survival curves did not indicate any significant difference (Figure 3).

The weights of spleen obtained from aspirin-treated animals (8.23 ± 1.06 g/kg body weight, $n = 24$) were significantly ($P = 0.0282$) greater than those of vehicle-treated CLP animals (5.49 ± 0.57 g/kg body weight, $n = 24$), while such an increase in spleen weight was not observed in NCX 4016-treated animals (5.64 ± 0.71 g/kg body weight, $n = 24$) (Figure 4).

In contrast to the results obtained by NCX 4016, flurbiprofen failed to improve the survival rate (Figure 5). In addition, spleen weight was significantly ($P = 0.0475$) higher (8.23 ± 1.14 g/kg body weight, $n = 14$)

in flurbiprofen-treated animals when compared to the vehicle-treated CLP animals (5.49 ± 0.57 g/kg body weight, $n = 24$) (Figure 6).

3.3. The effects of aspirin and NCX 4016 on LPS-induced model of septic shock

The survival rate was 100% in groups treated with vehicle ($n = 14$), aspirin ($n = 12$), or NCX 4016 ($n = 12$), and it was 28.6 % in the LPS group (10 of 14, $P = 0.0002$ vs. controls). Deaths were observed within 3 h of the LPS injection (Figure 7). When the animals were treated either with aspirin or NCX 4016 ($n = 12$ for each group) in addition to LPS, mortality was 100% by hour 12. (Figure 7).

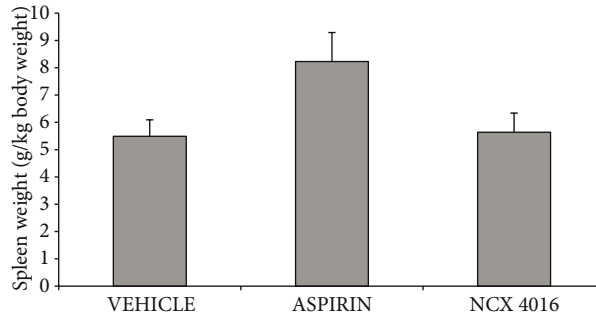


Figure 4. The spleen weight (g/kg body weight, mean \pm SEM) obtained from mice that underwent cecal ligation and puncture (CLP) surgery and were also treated either with vehicle, plain aspirin, or its NO-donating derivative, NCX 4016. Asterisk indicates significant difference ($P = 0.0282$, two-tailed value, Student's unpaired t-test) from the corresponding value obtained from vehicle-treated animals. $n = 24$ in all groups.

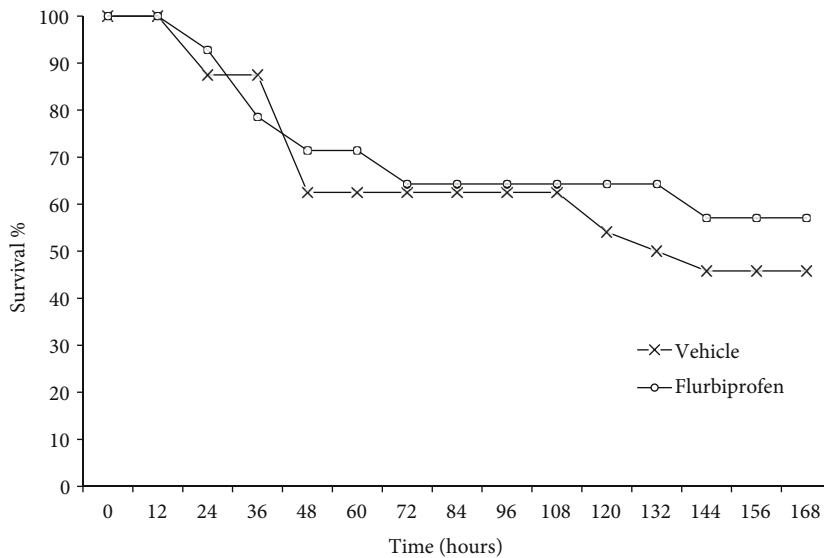


Figure 5. Survival rates obtained from mice that underwent cecal ligation and puncture (CLP) surgery and were treated either with vehicle or flurbiprofen. $n = 24$ in vehicle and flurbiprofen groups.

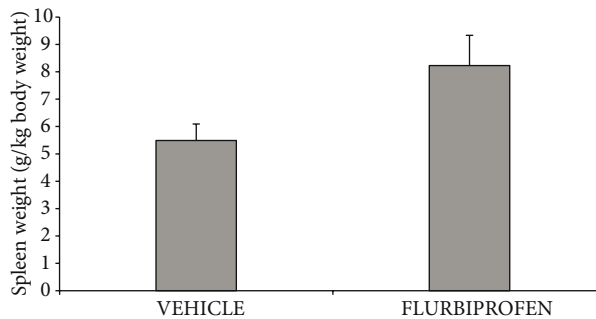


Figure 6. Spleen weight (g/kg body weight, mean \pm SEM) obtained from mice that underwent cecal ligation and puncture (CLP) surgery and were treated either with vehicle or flurbiprofen. Asterisk indicates significant difference ($P < 0.05$, two-tailed value, Mann-Whitney U test) from the corresponding value obtained from vehicle treated animals. $n = 24$ in vehicle and flurbiprofen groups.

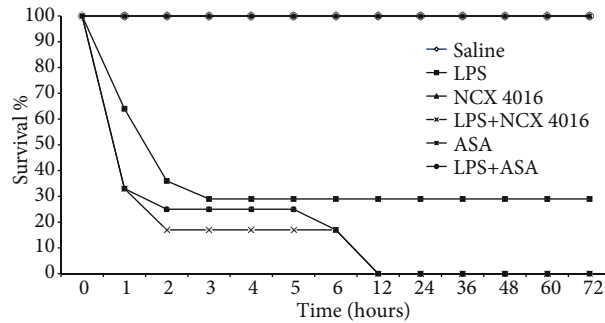


Figure 7. Survival rates obtained from mice that were given lipopolysaccharide (LPS) or its vehicle, i.e. saline, which were also treated either with plain aspirin, its NO-donating derivative, NCX 4016, or the vehicle. Single asterisk indicates significant difference ($P = 0.0002$, two-tailed value, Mann-Whitney U-test) from the corresponding value obtained from saline-treated animals. Double asterisk indicates significant difference ($P < 0.0001$, two-tailed value, Mann-Whitney U test) from the corresponding value obtained from vehicle-treated animals. $n = 12-14$ in each group.

3.4. The effects of drugs on spleen and liver histopathology in CLP

CLP resulted in severe congestion (6 of 6 animals), minimal fibrosis (2 of 6 animals), and capsulitis (1 of 6 animals) in the spleens of randomly selected animals ($n = 6$). In the aspirin-treated animals, both fibrosis and congestion in the spleen were moderately less. However, NCX 4016 treatment caused milder histopathological findings than in aspirin-treated animals. In contrast, flurbiprofen caused remarkable congestion and severe fibrosis of the spleen in all of the randomly selected specimens ($n = 3$) (Figure 8).

CLP resulted in mild focal “spotty” necrosis (5 of 6 randomly selected animals) and minimum degree of congestion and capsulitis (2 of 6 randomly selected animals) with no sign of fibrosis or any other degeneration in liver. However, all of the test drugs caused severe focal necrosis, moderate congestion, and capsulitis in the liver. Severe fibrosis was detected in the livers of all randomly selected flurbiprofen-treated animals, which was almost

completely absent in aspirin- or NCX 4016-treated animals (Figures 9a and 9b).

4. Discussion

Despite the advances in research on the pathophysiological mechanisms of sepsis and the refinements in the management of septic patients, the high mortality and morbidity rates in surgical intensive care units due to sepsis, septic shock, and multiple organ failure have not been significantly reduced in the past two decades (1). Although an extensive amount of studies that demonstrated significant beneficial effects of various procedures in experimental animals exists, there is controversy about their validity in terms of their relevance to human sepsis.

Most of the experimental models of endotoxic and septic shock, such as infusion of bacterial endotoxin or cecal ligation and puncture procedure, are rather acute models of sepsis, and these models are used to improve or to investigate short-term mortality and organ failure. The

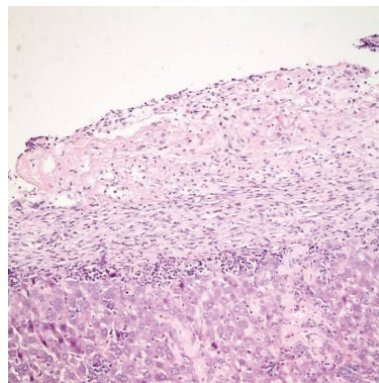


Figure 8. The presence of capsulitis characterized by the presence of fibrin, polymorphonuclear leukocytes, and lymphocytes on the peritoneal side of the spleen of an animal treated with flurbiprofen, which also underwent cecal ligation and puncture (hematoxylin and eosin, 200 \times).

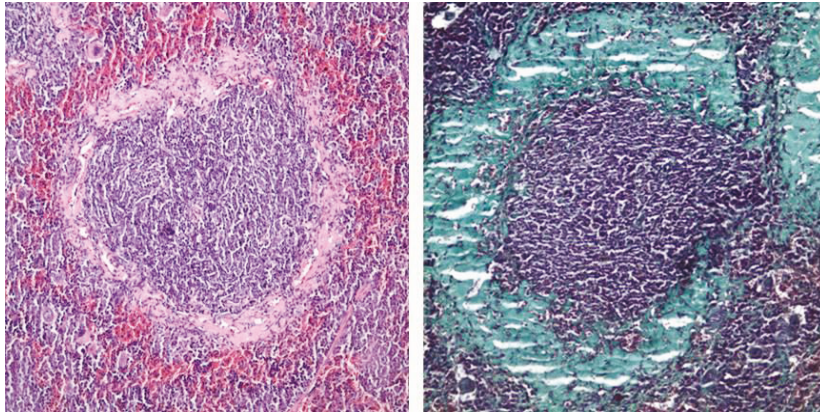


Figure 9. Hematoxylin and eosin (200 \times , on the left) or trichrome (200 \times , on the right) staining showing the presence of pericentral fibrosis in the liver of an animal treated with flurbiprofen, which also underwent cecal ligation and puncture.

two different models of shock in the present study were efficient, consistent, and in line with our previous work and other publications (25–27). LPS-induced respiratory failure model is an effective model for testing COX inhibitors (12), and CLP is the best model of septic shock that represents the clinical situation. The validity of CLP has also been supported by previous publications where it was shown that the blockade of tumor necrosis factor alpha failed to prevent death in this model (29). This was also shown in human clinical trials.

None of the test drugs in this study produced a beneficial effect on the overall survival rates, nor did they alleviate the spleen or liver injury in these septic models, although some degree of improvement/protection was obtained in the NCX 4016-treated group. The data are consistent within themselves since aspirin and NCX 4016 survival curves displayed the same trend in the LPS model. The mortality rates in both models were at such a level that we were able to detect a worsening trend with aspirin and NCX 4016 in the LPS model and some benefit with NCX 4016 in CLP. Spleen weight was not measured in the LPS model, since the majority of the animals were lost within a few hours, a time period that was not enough for spleen enlargement.

The drug doses and their administration route can be questioned. Twice-daily injections of aspirin (2×15 mg/kg) and flurbiprofen (2×3 mg/kg) were sufficient to saturate the tissues and, unquestionably, to systemically inhibit the COX. The particular doses used in this study were supported by the literature (30). Even when strong survival endpoints were not statistically reached, test drugs were absorbed, since NCX 4016 displayed some limited benefit and flurbiprofen was able to produce fibrosis, necrosis, and spleen enlargement. Since one of the solutions in the mixture of the vehicle, polyethylene glycol 400, is incompatible with salicylic acid, the vehicle should be questioned. We found that the vehicle selected interacts

with the CLP procedure (Figure 2). Therefore, this could have an influence on the results.

We think that our study is a valuable addition to the common knowledge in the field of septic and endotoxic shock. Although we reported mostly negative results with aspirin and flurbiprofen on overall mortality, other groups have previously reported positive results on endotoxic and septic shock models with some of the test drugs (8,9). The differences in the findings could be explained by the different techniques, animal strains, and endpoints that were used by us and by other investigators. In our model, the endpoint measurements were obtained on day 7 in the CLP model, whereas shorter protocols were used by the other investigators (31).

In conclusion, this study indicates that the choice of vehicle for test drugs could have an influence on the measurements of overall activity. NCX 4016 showed some potential beneficial effect on overall mortality in septic shock, which needs to be further confirmed with another vehicle. In the present study, aspirin and flurbiprofen did not improve overall mortality at 1 week after the induction of CLP. However, the effects of arachidonic acid-cyclooxygenase pathway blockade in endotoxic and septic shock have to be extensively investigated before discussing the relevance of the molecules in the treatment of human septic shock.

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