Early Ascorbic Acid Administration Prevents Vascular Endothelial Cell Damage In Septic Mice

Yutaro Madokoro
Kagoshima University Graduate School of Medical and Dental Sciences
Chinatsu Kamikokuryo
Kagoshima University Graduate School of Medical and Dental Sciences
Shuhei Niiyama
Kagoshima University Graduate School of Medical and Dental Sciences
Takashi Ito
Kumamoto University
Satoshi Hara
Tokyo Institute of Technology
Hiroshi Ichinose
Tokyo Institute of Technology
Yasuyuki Kakihana (kakihana@m3.kufm.kagoshima-u.ac.jp)
Kagoshima University Graduate School of Medical and Dental Sciences

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Abstract

Ascorbic acid (AsA) therapy for sepsis is thought to have a protective effect on vascular endothelial cells, but the effect of AsA therapy on endothelial cell dysfunction over time and the appropriate timing for AsA administration to demonstrate efficacy is unclear. Septic mice, induced by cecal ligation and puncture (CLP), were examined for the effect of AsA administration (200 mg/kg) on vascular endothelial cell dysfunction at two administration timings: early group (AsA was administered immediately after CLP) and late group (AsA was administered 12 h after CLP). Survival rates were compared between the early and late administration groups, and vascular endothelial cell damage, indicated by the dihydrobiopterin/tetrahydrobiopterin ratio, serum syndecan-1, and endothelial nitric oxide synthase, as well as liver damage, were examined. The early group showed significantly improved survival compared to the non-treatment group ($p < 0.05$), while the late group showed no improved survival compared to the non-treatment group. Early AsA administration suppressed damage to the vascular endothelial system and liver compared to the non-treatment group. In septic mice, early AsA administration immediately after CLP may have protective effects on vascular endothelial cells, resulting in reduced organ dysfunction and improved survival.

Background

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. The World Health Organization reports that although sepsis mortality rates have declined in recent decades, it still causes 11 million deaths annually [2]. Currently, there is no definitive treatment for sepsis, and the recommended treatment includes early detection, early antibiotic administration, appropriate infusion therapy, and optimal timing of vasopressor administration; however, the mortality rate still remains high [3].

Sepsis progresses to septic shock, which is caused by increased vascular permeability due to endothelial dysfunction and vasodilatory hypotension caused by elevated levels of nitric oxide (NO), resulting in multiple organ failure and significantly reduced survival [4, 5]. One of the causes of vascular endothelial dysfunction is reactive oxygen species produced by the uncoupling of endothelial nitric oxide synthase (eNOS) [6]. eNOS is an important molecule that regulates vascular homeostasis by generating NO, with tetrahydrobiopterin (BH_4) as a cofactor. BH_4 is produced from guanosine triphosphate and acts as an essential cofactor for various enzymes. BH_4 is easily oxidized to dihydrobiopterin (BH_2), and the binding affinities of BH_4 and BH_2 to eNOS are equal. NO is produced when BH_4 binds to eNOS, whereas superoxide is produced when BH_2 binds to eNOS [7]. The reaction that produces superoxide is referred to as “uncoupling” of nitric oxide synthase. When availability of BH_4 is limited, the BH_2/BH_4 ratio, rather than the absolute amounts of BH_4 and BH_2, is associated with vascular endothelial function [8-10]. Endothelial dysfunction decreases the permeability barrier function, increases glycocalyx shedding and leukocyte adhesion, and initiates a pro-coagulant and anti-fibrinolytic state [5]. In particular, glycocalyx shedding plays a major role in exacerbating sepsis. One of the core proteins that constitute the

glycocalyx is syndecan-1, which is released from the vascular endothelium into the bloodstream when vascular endothelial cells are damaged; therefore, serum syndecan-1 levels are used as a marker of glycocalyx damage [11].

Ascorbic acid (AsA), also known as vitamin C, is an important antioxidant that prevents the oxidation of various substances, including BH_4. Vitamin C levels are decreased in critically ill patients, such as those with sepsis [12]. In sepsis, AsA administration has been reported to improve survival and protect microvascular functions [13]. However, the optimal timing for AsA administration to protect endothelial cells remains unclear. In this study, we administered AsA at the early and late phases and evaluated the effect of AsA on endothelial cells in a mouse model of sepsis.

Methods

Animals

Adult C57BL/6 mice (9–11-week-old males) weighing 25 g were obtained from Kyudo (Fukuoka, Japan), housed under standard environmental conditions, and maintained at 23 ± 1°C with a 12-h light/dark cycle. All animal experiments were conducted under the rules approved by the Institutional Animal Care and Use Committee of Kagoshima University (approval number MD18126). As this was an animal study, consents for participation and publication were not applicable. We carried out the study in compliance with the ARRIVE guidelines (https://arriveguidelines.org), and the Guideline for the Proper Conduct of Animal Experiments established by the Science Council of Japan.

Cecal Ligation and Puncture (CLP)

The cecal ligation and puncture (CLP) model is the gold standard for experimental models of sepsis. Septic shock was induced by CLP, as previously described, with slight modifications [14]. Briefly, mice were anesthetized with isoflurane, and the mouse cecum was ligated with a 3-0 silk suture and punctured in one place with a 21-gauge needle. The cecum was retracted into the abdominal cavity, and the incision was sutured with 3-0 nylon. For sham-operated mice, only open and closed abdominal procedures were performed without CLP. At different times during each experiment, blood, liver, and heart tissues were collected and analyzed under inhalation anesthesia. At 6, 12, and 24 h after CLP and sham operation, the blood of mice was collected by inferior vena cava puncture after which the animals were sacrificed. At the same time point, myocardium and liver were collected. Blood samples were centrifuged at 2000 g for 10 min to collect plasma and stored at -80°C until analysis. After the operation, buprenorphine (0.05 mg/kg) was repeatedly administered every 12 h by subcutaneous injection.

Experimental design

Survival experiment: CLP vs CLP + AsA (early)

Mice were randomized into the following groups: 1) sham (n = 10), 2) sham + AsA (early) (n = 10), 3) CLP (n = 10), and 4) CLP + AsA (early) groups (n = 9). These mice received 40 ml/kg of normal saline or AsA

(200 mg/kg) by subcutaneous injection immediately after the operation and were monitored for 72 h (The data for experimental design is shown in Supplementary figure 1a and table 1). In the study, n refers to number of animals. The numbers for each group were taken from similar experiment reported in the literature.

Survival experiment: CLP vs CLP + AsA (late)

Mice were randomized into the following groups: 1) sham (n = 10), 2) CLP (n = 10), and 3) CLP + AsA (late) (n = 9) Mice received 40 ml/kg of normal saline by subcutaneous injection immediately after the operation, and 10 ml/kg of normal saline or AsA (200 mg/kg) by subcutaneous injection at 12 h after the operation. The mice were monitored for 72 h. (The data for experimental design is shown in Supplementary figure 1b and table 1)

Measurement of BH_4 and BH_2 and calculation of BH_2/BH_4

BH_4 is a substance that oxidizes easily, and oxidation was prevented by adding 0.2% dithioerythritol (a final concentration). BH_4 and BH_2 were measured separately by the post-column oxidation method using high-performance liquid chromatography with a fluorescence detector [15]. The plasma samples (100 μL) were deproteinized by adding 25 μL of 1 M perchloric acid containing 0.5 mM EDTA, followed by centrifugation. The supernatants were filtered through a 0.2 μm filter. The BH_2/BH_4 ratio was calculated by dividing BH_2 by BH_4. The number of mice in each group after 6hr, 12hr and 24hr of operation in Sham + NS group was 7, 4, 4 respectively, in CLP+ NS group was 8, 6, 7 respectively, in CLP+ AsA group was 7, 7, 7 respectively. The number of mice in control group was 5. (The data for experimental design is shown in Supplementary figure 1a and table 2.)

Measurement of Syndecan-1

Plasma syndecan-1 levels were measured using a Murine CD138 ELISA Kit (Diaclone, France). The number of mice in each group after 6hr, 12hr and 24hr of operation in Sham + NS group was 7, 6, 7 respectively, in CLP+ NS group was 7, 12, 11 respectively, in CLP+ AsA group was 7, 7, 7 respectively. (The data for experimental design is shown in Supplementary figure 1a and table 2.)

Western blotting analysis

The heart was homogenized in a buffer solution (T-PER Tissue Protein Extraction Reagent; Thermo scientific, Rockford, USA). The extracted proteins were quantified (TaKaRa BCA Protein Assay Kit, Takara Holdings Inc, Japan) and the amount of protein to be applied to the gel was adjusted. The protein samples (1 μg of protein) were electrophoresed on 10% SDS-PAGE and transferred to the PVDF membrane. The membrane was blocked for 1 h (BLOCK ACE®, MEGMILK SNOW BRAND, Japan) and incubated with primary antibodies (eNOS, 1:1,000, Purified Mouse Anti-eNOS/NOS Type III, BDbioscience, USA; GAPDH, 1:20,000, Anti-GAPDH Loading Control ab8245, Abcam, UK) at 4℃ overnight. After washing with Phosphate Buffered Saline with Tween (PBST) buffer, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000, Goat Anti-Mouse IgG H&L HRP

ab205719, Abcam, UK) for 1 h at room temperature. Blots were washed with PBST, and immunoreactive bands were detected using an enhanced chemiluminescence system (ImmunoStar®, FUJIFILM Wako Chemical Corporation, Japan). (The data for experimental design is shown in Supplementary figure 1a.)

Histologic Examination

Liver tissue specimens were fixed in 10% formalin and embedded in paraffin. They were stained with hematoxylin and eosin to evaluate the degree of injury. (The data for experimental design is shown in Supplementary figure 1a.)

Statistics analysis

Survival rates were analyzed using the Kaplan-Meier method. Survival times were compared using the log-rank test. Data are expressed as mean ± standard error. The Kruskal-Wallis test was used to detect differences between the groups. Significant differences were considered if P value was <0.05.

Results

Early administration of AsA improves survival rate of mice with CLP-induced sepsis compared to late administration of AsA.

We compared the survival rates after operation between Sham, Sham + AsA (early), CLP, and CLP + AsA (early) (Figure 1A). None of the CLP mice used in this study survived to 45 h after the operation. All Sham + NS and Sham+AsA (early) mice survived for 72 h. In the CLP + AsA (early) mice group, 3 of 9 mice survived after operation (33%).

The CLP + AsA (early) mice group showed significantly higher survival rates than the CLP mice group.

Second, we compared the survival rates after operation among sham, CLP, and CLP + AsA (late) groups (Figure 1B). All sham mice survived for 72 h. In the CLP and CLP + AsA (late) mice groups, 1 of 9 mice survived after operation (11%). The CLP + AsA (late) mice group showed no difference in survival rates compared to the CLP mice group.

BH_2/BH_4 ratio increased 6 hours after the operation and continued to increase over time. Early administration of AsA prevented an increase of BH_2/BH_4 ratio.

To elucidate the dynamics of BH_4 and BH_2 in CLP-induced sepsis and how the dynamics of BH_2/BH_4 ratio change with the administration of AsA (early), we measured BH_4 and BH_2 and then calculated BH_2/BH_4. Serum BH_4 and BH_2 levels were determined in CLP and sham mice at 6, 12, and 24 h after the operation.

Both BH_4 and BH_2 showed a significant increase 24 h after the operation (Figure 2). The ratio of BH_2 to BH_4 was significantly elevated in the CLP group compared to the early AsA group starting at 12 h.

Syndecan-1 levels increased after 12 h, but early administration of AsA suppressed this increase. The expression of eNOS in myocardial tissues was also maintained by the early administration of AsA.

Syndecan-1 level and eNOS expression in myocardial tissues were measured to evaluate whether early administration of AsA protects vascular endothelial cells.

Syndecan-1 at 12 h was significantly higher in the CLP group than in the early AsA group (Figure 3). The expression of eNOS was measured to evaluate vascular endothelial cells and was assessed in myocardial tissue 12 h after operation. Two samples from each of the normal, CLP, and CLP + AsA (early) groups were collected and evaluated by western blotting. eNOS expression was lower in the CLP group than in the normal group. Compared to the CLP group, the early AsA group maintained eNOS expression.

Liver organ damage was reduced by early AsA administration

Finally, liver tissue was stained with hematoxylin and eosin and observed under a microscope to evaluate organ damage due to sepsis. Each sample was collected 12 h after the operation.

No histological differences were observed between the control and sham mice. In CLP mice, the arrangement of hepatocytes was markedly disorganized (Figure 4). In contrast, hepatocyte disarrangement was reduced in CLP + AsA (early) mice, although not as orderly as in the control and sham mice.

Discussion

In this study, we showed that early AsA administration compared to late AsA administration may improve the survival rate of a mouse model of sepsis by reducing vascular endothelial cell damage. Despite reports that administration of AsA to septic mice improves prognosis, there are no reports of differences in prognosis depending on the timing of administration [13, 16]. Here, we discuss the mechanism underlying the effectiveness of early AsA administration in reducing vascular endothelial cell damage and the appropriate timing of AsA administration.

The severity of illness and death in septic patients is related to organ damage caused by microcirculatory disturbances and increased vascular permeability due to vascular endothelial dysfunction [4]. The BH_2/BH_4 ratio correlates with vascular endothelial function [8, 17]. Although AsA has antioxidant properties and inhibits BH_4 oxidation, it is ineffective in reducing the already oxidized BH_2. In animal experiments using guinea pigs, increased BH_2/BH_4 ratio has been shown in AsA-deficient models. AsA levels in the body are decreased in critically ill patients, who often experience difficulties in taking AsA orally, and, therefore, require supplemental therapy [13]. In our septic mice, we also confirmed a decrease in AsA plasma concentration in preliminary experiments (data not shown). In this septic mouse, the BH_2/BH_4 ratio increased, suggesting that the lack of AsA could not inhibit BH_4 oxidation. In case of

absolute or relative lack of BH_4, eNOS undergoes an uncoupling reaction, which produces superoxide, instead of NO, that reacts to form peroxynitrite ($ONOO^-$), a powerful oxidant [7, 18, 19]. Our study showed that the BH_2/BH_4 ratio increased at 6 h after CLP. Early AsA administration immediately after operation significantly suppressed BH_2/BH_4 ratio increase. AsA administration has been reported to inhibit BH_4 oxidation [20]. Although the optimal AsA dose is unknown, the antioxidant capacity of vitamin C is dose-dependent, and a plasma vitamin C concentration of 175 mg/l (1000 μmol/l) or higher is required to demonstrate radical scavenging capacity. Vitamin C (10 g) is required to achieve a plasma concentration of more than 1000 μmol/l [21]. In humans, the AsA dosage for sepsis is 200 mg/kg/day in most cases, but a very high volume of AsA (66 mg/kg/hour) was shown to be effective in the very early stage of burn injury [22]. In addition, another study reported that vitamin C therapy at doses as high as 10 g within 2 days of admission reduced mortality in patients with severe burns [23], and no major side effects were observed, even with such a very high dose. Therefore, the optimal dosage of AsA should be investigated. Thus, although the effective dosage of vitamin C for critically ill patients varies according to the disease state, 200 mg/kg/day of AsA was considered the optimal dosage for this experiment as its effectiveness has been demonstrated previously in septic mice [14, 18, 24, 25]. As a result, early AsA administration may improve $ONOO^-$ generation, suggesting a protective effect on endothelial cells. In the AsA-non-treated group, increased serum syndecan-1 levels, an indicator of endothelial cell damage, and decreased eNOS expression, an indicator of endothelial cell protection, were observed 12 h after the operation. By contrast, in the early AsA administration group, both serum syndecan-1 levels and eNOS expression showed protective effect on the vascular endothelium. Increased serum syndecan-1 levels in sepsis are associated with vascular permeability and organ damage [26]. Serum syndecan-1 levels are likely to be useful in diagnosing sepsis and may be related to its severity [27]. In our single-center study, syndecan-1 was a predictor of fatal outcome in septic patients [28]. Moreover, we examined liver tissues for organ damage. Indeed, organ damage occurred after 12 h in the group with elevated syndecan-1, but it was reduced in the group with suppressed syndecan-1 following early AsA administration. The difference in survival may be due to the protection of vascular endothelial cells by early AsA administration, thus suppressing organ damage.

In recent years, the effects of AsA administration in patients with septic shock have received much attention [29, 30]. The administration of AsA alone, as well as the simultaneous administration of vitamin B1 and hydrocortisone, have been studied widely. In particular, the simultaneous administration of vitamin B1 and hydrocortisone is known as HAT therapy [31-34]. Given that several recent studies have shown no positive effect of AsA administration in septic shock patients, it remains controversial whether AsA should be administered to these patients [35-37]. Some studies have cited delayed administration as a limiting factor to obtaining a good effect of AsA in septic shock [36]. In our septic mouse, an increase in the BH_2/BH_4 ratio, which causes $ONOO^-$, had already occurred after 6 h. In addition, organ damage and vascular endothelial cell damage also occurred; thus, administration of AsA at this time would not have been effective. Therefore, in studies reporting no effect of AsA therapy, it is possible that AsA was administered after the BH_2/BH_4 ratio had already increased, as we have shown in this study. Early high-dose AsA administration is effective in patients with sepsis [38], including those with hypoalbuminemia

or severe organ failure [39]. In this study, we only mentioned the protective effect of AsA on vascular endothelial cells by suppressing the increase in BH_2/BH_4 ratio, but AsA has additional effects, such as catecholamine production [40], adrenocorticotropic hormone production [41], and direct scavenging of free radicals [42], which may improve the prognosis of sepsis through various pathways [43].

This study has several limitations. First, it is unclear whether the results of this mouse sepsis model would be similar to those of human sepsis. Changes in BH_4 and BH_2 over time may differ between humans and mice. Second, because mice can synthesize AsA in their bodies, the dynamics of AsA concentration in their bodies may be different from those of humans. The appropriate dosage needs to be discussed in both human and animal studies. However, our present findings indicate that the timing of AsA administration affects prognosis and that the BH_2/BH_4 ratio is related to the mechanism of septic shock.

In the CLP mouse model, an increase in the BH_2/BH_4 ratio, which causes vascular endothelial cell damage, occurred 6 h after the disease onset. In the present study, administration of AsA at an earlier time before the increase in the BH_2/BH_4 ratio improved the prognosis of the CLP mouse model by protecting the vascular endothelium. In the future, it will be necessary to evaluate the time course of the BH_2/BH_4 ratio and the post-onset dynamics of syndecan-1 in humans, as well as study the appropriate timing for AsA administration.

Declarations

We carried out the study in compliance with the ARRIVE guidelines (https://arriveguidelines.org)

Availability of materials and data

Not applicable.

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Authors' contributions

YM, CK, SH, and HI contributed to the data acquisition, analysis, and interpretation. All authors contributed to data interpretation, critically revised the manuscript, and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Data availability

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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Figures

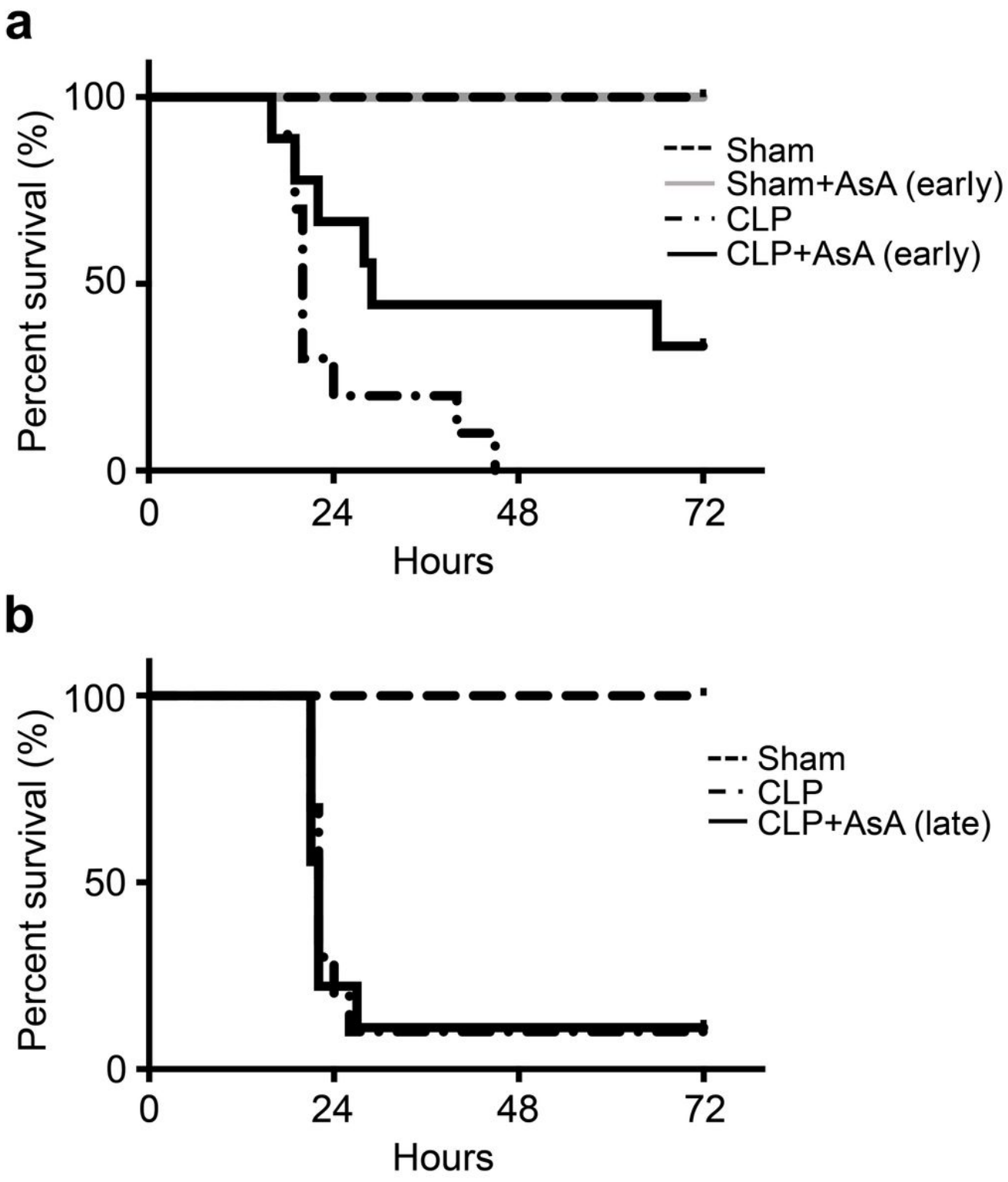

Figure 1

(A) Mice subjected to CLP, as described in the Methods section, for 72 h survival study. In the AsA administration group, AsA was injected subcutaneously immediately after the operation. The CLP + AsA (early) mice group had a significantly prolonged survival rate compared to the CLP group. *P <0.05 versus CLP. (B) Mice subjected to CLP, as described in the Methods section, for 72 h survival study. AsA was injected subcutaneously 12 h after the operation. CLP + AsA (late) mice showed no difference in the

survival rate from the CLP group. *P <0.05 versus CLP. Abbreviations: AsA, ascorbic acid; CLP, cecal ligation and puncture

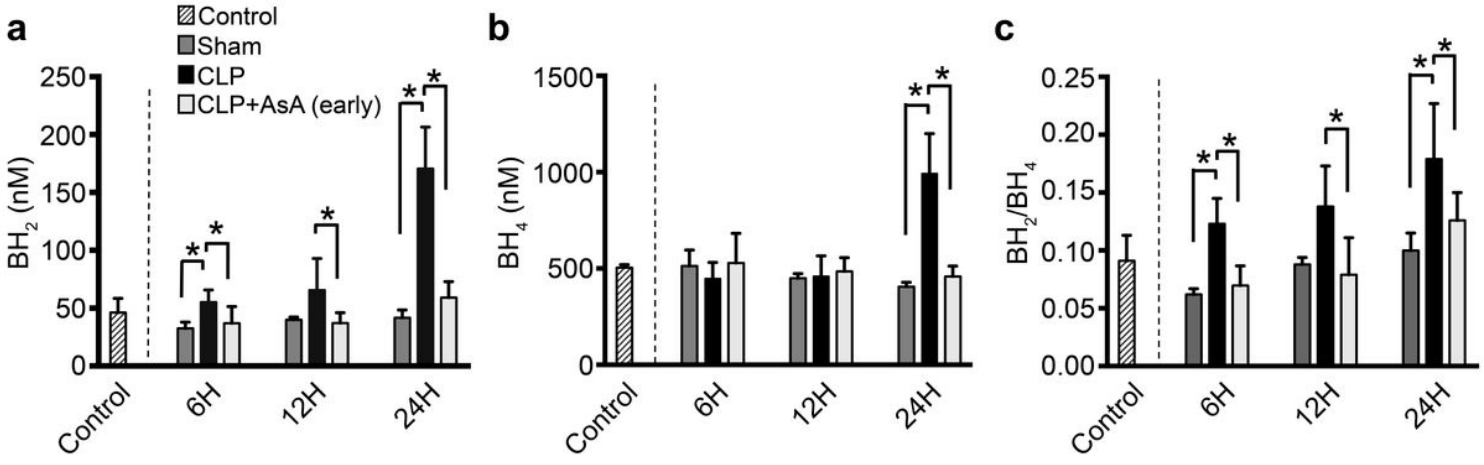

Figure 2

(A) BH2 concentration in the plasma was measured by HPLC. At 6 and 24 h after the operation, CLP group had significantly elevated BH2 concentration compared to CLP+ AsA (early) and Sham groups. At 12 h after the operation, CLP group had significantly elevated BH2 concentration compared to CLP+ AsA (early) group. Error bars represent SE. *P <0.05. (B) BH4 concentration in the plasma was measured by HPLC. At 24 h after the operation, CLP group had significantly elevated BH4 concentration compared to CLP+ AsA (early) and Sham groups. At 6 and 12 h after the operation, there was no significant differences between the groups. Error bars represent SE. *P <0.05. (C) BH2/BH4 ratio was calculated by dividing BH2 by BH4. At 6 and 24 h after the operation, CLP group had significantly elevated BH2/BH4 ratio compared to CLP+ AsA (early) and Sham groups. At 12 h after the operation, CLP group had significantly elevated BH2/BH4 ratio compared to CLP+ AsA (early) group. Error bars represent SE. *P <0.05. Abbreviations: AsA, ascorbic acid; CLP, cecal ligation and puncture; BH4, tetrahydrobiopterin; BH2, dihydrobiopterin; SE, standard error; HPLC, high-performance liquid chromatography

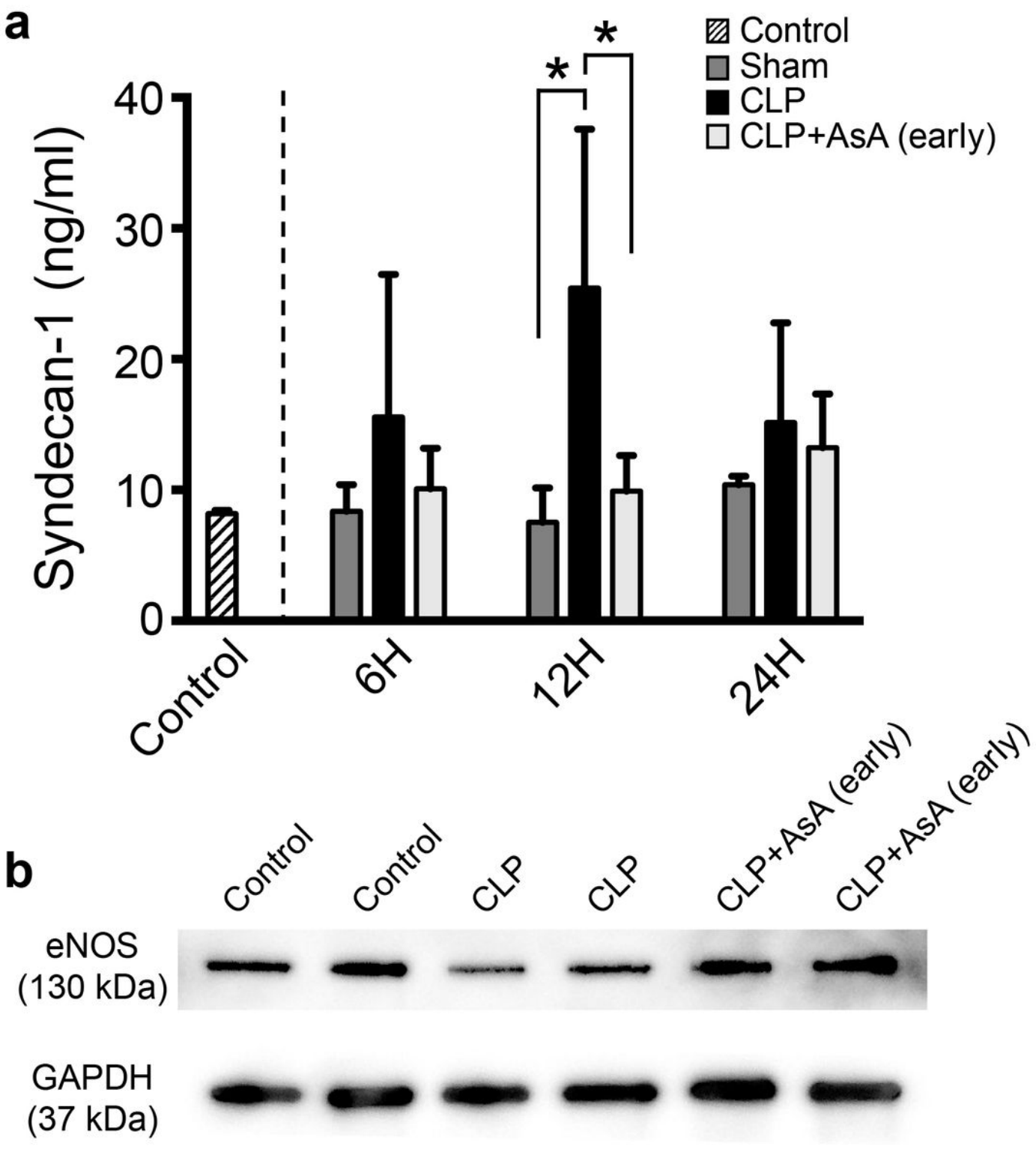

Figure 3

(A) Plasma syndecan-1 was measured by ELISA kit. At 12 h after the operation, CLP group had significantly elevated syndecan-1 level compared to the CLP+ AsA (early) and Sham groups. Error bars represent SE. *P <0.05. (B) All samples were taken 12 h postoperatively. The expression of eNOS (130 kDa) in the heart was measured by western blotting; it was decreased in the CLP group but maintained in the early AsA group. The data for the original strip are shown in Supplementary Figure S2a and S2b.

Abbreviations: AsA, ascorbic acid; CLP, cecal ligation and puncture; eNOS, endothelial NO synthase; SE, standard error

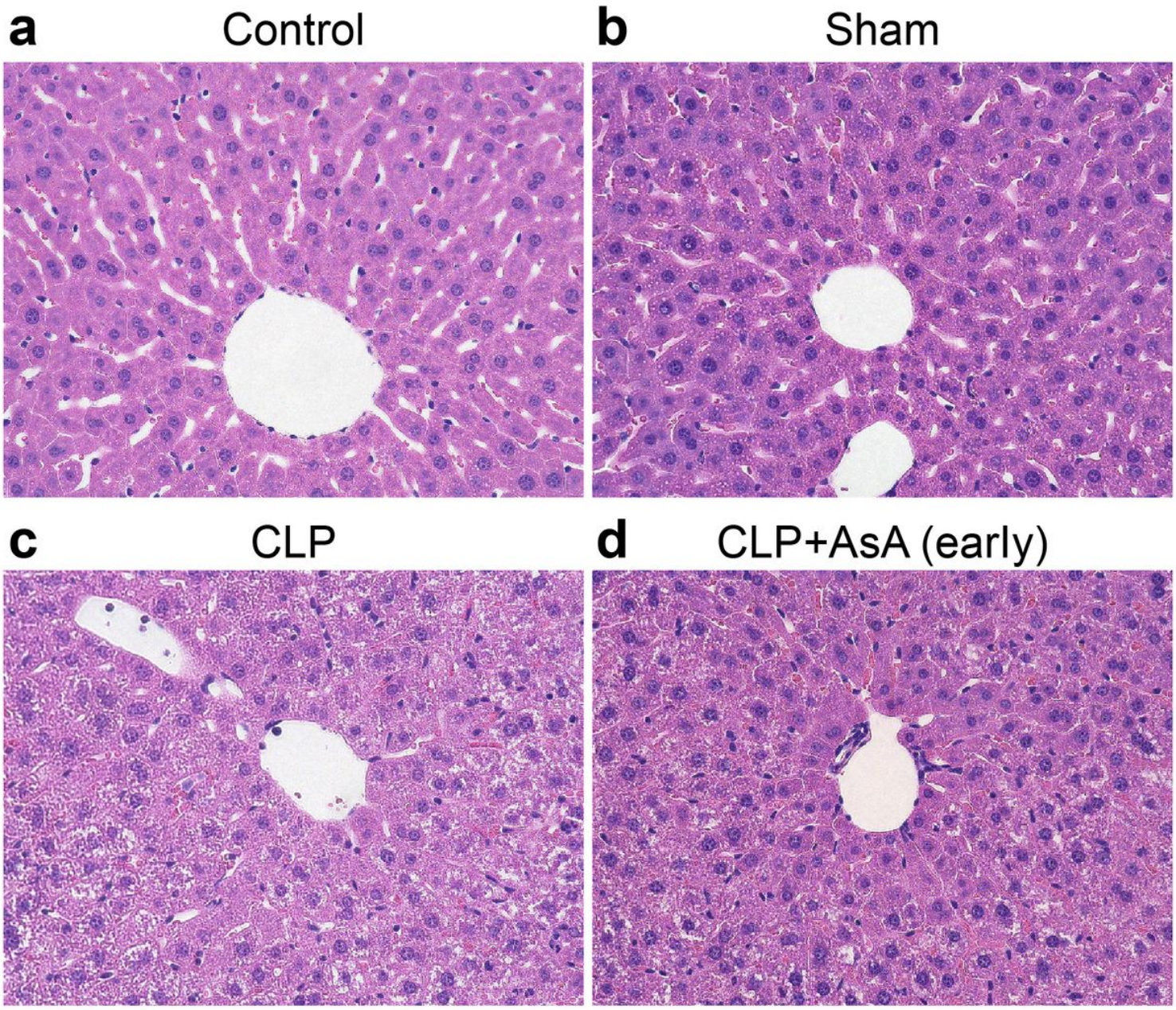

Figure 4

Histopathological examination of the liver of CLP-treated and untreated mice. A) B) Normal histology of liver tissues obtained from sham and control mice. C) Representative CLP induced liver damage. D) Representative liver of CLP mice treated with early AsA administration. Sham and CLP mice were killed 12 h after the operation. Original magnification, ×40 Abbreviations: AsA, ascorbic acid; CLP, cecal ligation and puncture

Supplementary Files

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- Supplementalfigure.docx