Original Article

Erythropoietin ameliorates severe hemorrhagic shock-induced serum proinflammatory cytokines and biochemical changes in spontaneously hypertensive rats

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ABSTRACT

Objective: Hypertensive patients have higher mortality rates from hemorrhagic shock (HS) than normotensive patients. HS can produce several proinflammatory mediators such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), leading to multiple organ dysfunction and death. Erythropoietin (EPO) has pleiotropic cytoprotective actions. We investigated the effects of EPO (300 U/kg) treatment on HS-induced serum proinflammatory cytokines and biochemical changes in spontaneously hypertensive rats (SHRs).
Materials and Methods: Severe HS was induced by withdrawing 60% of the rat's total blood volume via a femoral arterial catheter (6 mL/100 g body weight) over 30 minutes. The mean arterial pressure (MAP) and heart rate (HR) were monitored continuously for 2 hours after the start of blood withdrawal. Levels of biochemical and cytokine parameters, including glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), blood urea nitrogen (BUN), creatinine (Cre), creatine phosphokinase (CPK), lactate, TNF-α, and IL-6 were measured 1 hour after HS had been induced.
Results: HS significantly increased HR and blood GOT, GPT, BUN, Cre, CPK, lactate, TNF-α, and IL-6 levels, and decreased hemoglobin level and MAP, in SHRs. Pretreatment with EPO improved the survival rate at 2 hours, preserved the MAP, decreased tachycardia and markers of organ injury (GOT, GPT, BUN, Cre, CPK, lactate), and suppressed the release of TNF-α and IL-6 after HS in SHRs.
Conclusion: Pretreatment with EPO suppresses the release of serum TNF-α and IL-6, and decreases the levels of markers of organ injury associated with HS in SHRs.

1. Introduction

Hemorrhagic shock (HS) leads to hemodynamic instability, decreases tissue perfusion, and causes cellular hypoxia, organ damage, and death [1,2]. After HS, nuclear factor kappa κB (NF-κB) is involved and further increases the inflammatory cascade [3,4]. Overwhelming production of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) leads to multiple organ dysfunction and death after HS [5].

Erythropoietin (EPO) has been found to interact with its receptor in a large variety of non-hematopoietic tissues to induce cytoprotective responses [6–8]. EPO promotes the degradation to inhibitory κB (IκB), further decreases NF-κB, and decreases proinflammatory cytokine production after HS [9]. Hypertensive patients have a higher mortality rate from hemorrhage after trauma than normotensive patients [10,11]. In the present study, we examined the effects of pretreatment with EPO on cytokines induced by severe HS (TNF-α and IL-6) and organ damage in conscious spontaneously hypertensive rats (SHRs).

2. Materials and methods

2.1. Preparation of animals

Twenty-four male SHRs weighing 250–300 g were purchased from the National Animal Center (Taipei, Taiwan). They were

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housed in the university Animal Center in a controlled environment at a temperature of $22 \pm 1°C$ with a 12-hour light/dark cycle. Food and water were provided *ad libitum*. The Animal Care and Use Committee of Tzu Chi University approved the experimental protocol.

The animals were anesthetized with ether inhalation for about 15 minutes. During the period of anesthesia, a femoral artery was cannulated and connected to a pressure transducer (Gould Instruments Systems Inc, Cleveland, OH, USA) to record arterial pressure and heart rate (HR) on a polygraph recorder (PowerLab; ADInstruments, Mountain View, CA, USA). A femoral vein was catheterized for intravenous administration of drugs or fluid. The operation was completed within 15 minutes, leaving a small section wound (less than $0.5 \, cm^2$). After the operation, the animals were placed in a metabolic cage (Shingshieying Instruments, Hualien, Taiwan). The rats soon awakened after the operation [12–14].

2.2. Hemorrhagic shock

HS was induced 24 hours later after the operation. HS was induced by drawing blood from the femoral arterial catheter into a 10 mL syringe. An infusion pump controlled the withdrawal rate to mimic a typical bleeding event. The blood volume withdrawn was 60% of the total blood volume (6 mL/100 g body weight) [15,16]. The duration of blood withdrawal was 30 minutes. Normal saline 0.5 mL was infused as fluid resuscitation at 30 minutes before induction of HS, and at 0, 1, and 2 hours after the start or end of blood withdrawal. After blood withdrawal, the animals were continuously observed for 2 hours [12–14].

2.3. Experimental design

The animals were randomly divided into three groups. Rats in the control group ($n = 8$), were given 300 U/kg EPO (Roche, Mannheim, Germany) in 0.5 mL normal saline intravenously over 10 minutes and were not subjected to HS [13,17]. Rats in the HS group ($n = 8$), were given 0.5 mL of an intravenous drip of normal saline over 10 minutes, and then HS was induced. In the EPO + HS group ($n = 8$), rats received 300 U/kg EPO in 0.5 mL normal saline intravenously over 10 minutes, after which HS was induced.

2.4. Blood sample analysis

Blood samples (0.5 mL) were collected for measurement of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), blood urea nitrogen (BUN), creatinine (Cre), creatine phosphokinase (CPK), and lactate at 1 hour after the start and end of blood withdrawal.

Blood samples of about 0.1 mL for hemoglobin tests (Sysmex K-1000; Sysmex America Inc, Mundelein, IL, USA) and 0.4 mL for the other tests were immediately centrifuged at 3000 g for 10 minutes. The serum was decanted and separated into two parts; one part was stored at 4°C within 1 hour after collection for biochemical analysis. Serum levels of GOT, GPT, BUN, Cre, CPK, and lactate were measured with an autoanalyzer (COBAS Integra C111; Roche Diagnostics, Basel, Switzerland) to obtain various biochemical data. Another portion of the serum collected at 1 hour after HS was stored at −80°C for later measurement of TNF-α and IL-6 concentrations [12–14].

2.5. TNF-α and IL-6 measurements by ELISA

TNF-α and IL-6 concentrations in blood samples were measured separately by antibody enzyme-linked immunosorbent assay (ELISA) with a commercial antibody pair, recombinant standard and biotin−streptavidin−peroxidase detection system (Endogen, Rockford, IL, USA) as described previously [12–14]. Blood samples were collected in serum-separated tubes. All reagents, samples, and working standards were brought to room temperature and prepared according to the manufacturer's directions. Reactions were quantified by optical density using an automated ELISA reader (Sunrise; Tecan Co., Grödingen, Austria) at a wavelength of 450/540 nm.

2.6. Data analysis

Data were expressed as mean ± standard error of the mean. Statistical comparisons between different groups at corresponding time points were made by repeated measures one- or two-way analysis of variance followed by a *post hoc* test (Bonferroni's method). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Survival rate and hemoglobin

All rats had survived at 1 hour after HS. However, the survival rate at 2 hours after induction of HS was 12.5% in the HS group, 100% in the control group, and 75% in the EPO + HS group (Fig. 1A). The mortality rate in the EPO + HS group was significantly lower than in the HS group ($^\#p < 0.05$; Fig. 1A). The hemoglobin level was decreased after induction of HS (Fig. 1B). Pretreatment with EPO did
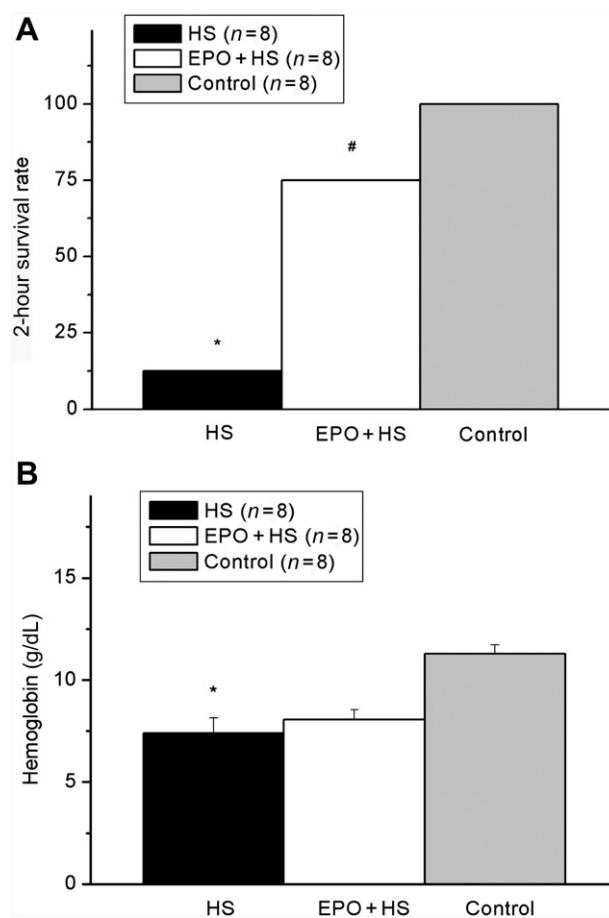
Fig. 1. (A) Two-hour survival rates for spontaneously hypertensive rats during severe hemorrhagic shock (HS). (B) Change in hemoglobin level after HS in spontaneously hypertensive rats. $^*p < 0.05$ for the HS group compared with the control group. $^\#p < 0.05$ for the erythropoietin (EPO) + HS group compared with the HS group.

not significantly increase hemoglobin level compared with that in the HS group after induction of HS (Fig. 1B).
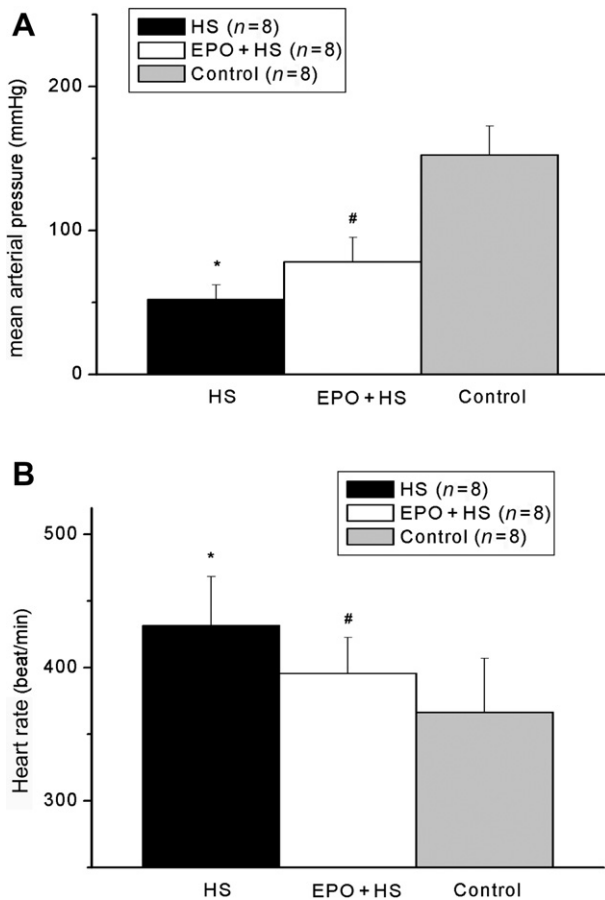
3.2. Mean arterial pressure and heart rate

Mean arterial pressure (MAP) decreased rapidly after the withdrawal of 60% of total blood volume from the femoral arterial catheter in conscious SHRs ($^*p < 0.05$; Fig. 2A). Pretreatment with EPO increased MAP compared with that in the HS group at 1 hour after HS ($^\#p < 0.05$; Fig. 2A). HR was significantly increased at 1 hour after HS ($^*p < 0.05$; Fig. 2B). Tachycardia decreased in the EPO + HS group compared with the HS group at 1 hour after HS ($^\#p < 0.05$; Fig. 2B).
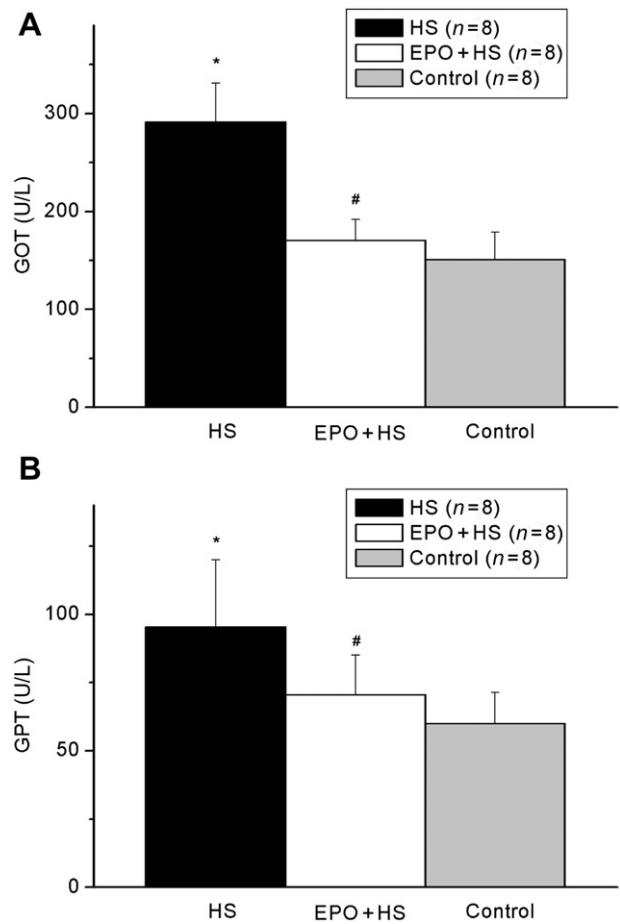
3.3. GOT and GPT

GOT was increased at 1 hour after induction of HS in SHRs ($^*p < 0.05$; Fig. 3A). Pretreatment with EPO decreased GOT compared with the HS group at 1 hour after induction of HS ($^\#p < 0.05$; Fig. 3A). Serum GPT increased at 1 hour after HS compared with the control group ($^* p < 0.05$; Fig. 3B). Pretreatment with EPO decreased GPT compared with the HS group at 1 hour in SHRs ($^\#p < 0.05$; Fig. 3B).

3.4. BUN and Cre

HS increased blood BUN at 1 hour in SHRs compared with the control group ($^*p < 0.05$; Fig. 4A). Pretreatment with EPO decreased

Fig. 2. Changes in (A) mean arterial pressure and (B) heart rate after hemorrhagic shock (HS) in spontaneously hypertensive rats. $^*p < 0.05$ for the HS group compared with the control group. $^\#p < 0.05$ for the erythropoietin (EPO) + HS group compared with the HS group.

Fig. 3. Changes in (A) serum glutamic oxaloacetic transaminase (GOT) and (B) serum glutamic pyruvic transaminase (GPT) after hemorrhagic shock (HS) in spontaneously hypertensive rats. $^*p < 0.05$ for the HS group compared with the control group. $^\#p < 0.05$ for the erythropoietin (EPO) + HS group compared with the HS group.
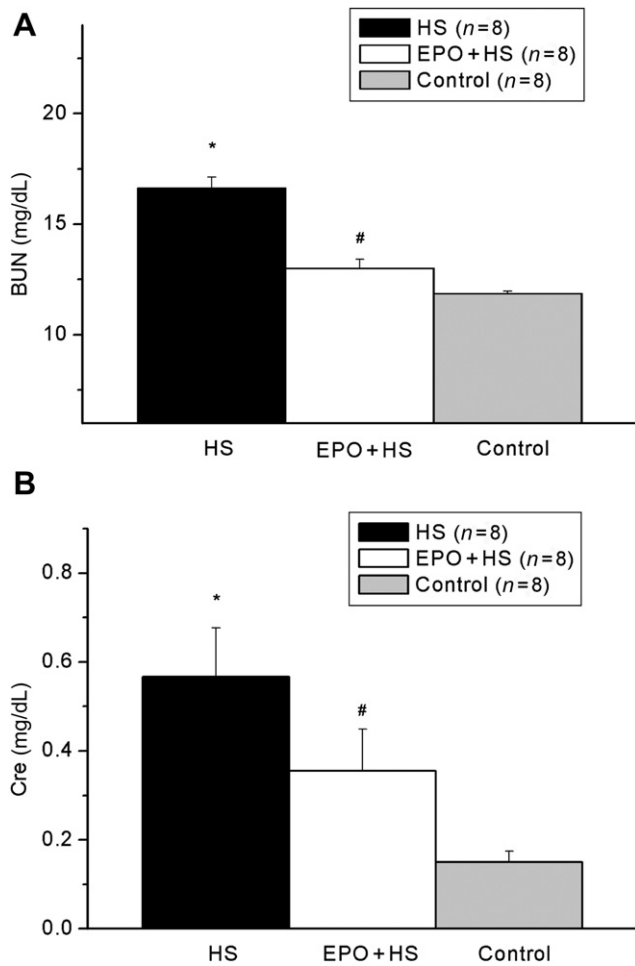
BUN at 1 hour compared with the HS group ($^\#p < 0.05$; Fig. 4A). Serum Cre increased at 1 hour after induction of HS compared with the control group ($^*p < 0.05$; Fig. 4B). Pretreatment with EPO decreased serum Cre compared with the HS group at 1 hour after HS in SHRs ($^\#p < 0.05$; Fig. 4B).

3.5. CPK and lactate

Blood CPK increased 1 hour after induction of HS (Fig. 5A). Pretreatment with EPO decreased CPK compared with the HS group at 1 hour after induction of HS ($^\#p < 0.05$; Fig. 5A). Serum lactate increased at 1 hour after HS compared with the control group ($^*p < 0.05$; Fig. 5B). Pretreatment with EPO decreased the serum lactate compared with the HS group at 1 hour after HS ($^\#p < 0.05$; Fig. 5B).

3.6. TNF-α and interleukin-6

HS greatly elevated serum TNF-α compared with the control group at 1 hour after induction of HS ($^*p < 0.05$; Fig. 6A). Pretreatment with EPO significantly decreased serum TNF-α at 1 hour after induction of HS ($^\#p < 0.05$; Fig. 6A). HS increased serum IL-6 compared with the control group at 1 hour after induction of HS ($^*p < 0.05$; Fig. 6B). EPO decreased serum IL-6 compared with the HS group at 1 hour after induction of HS ($^\#p < 0.05$; Fig. 6B).

Fig. 4. Changes in (A) serum blood urea nitrogen (BUN) and (B) serum creatinine (Cre) after hemorrhagic shock (HS) in spontaneously hypertensive rats. $*p < 0.05$ for the HS group compared with the control group. $\#p < 0.05$ for the erythropoietin (EPO) + HS group compared with the HS group.
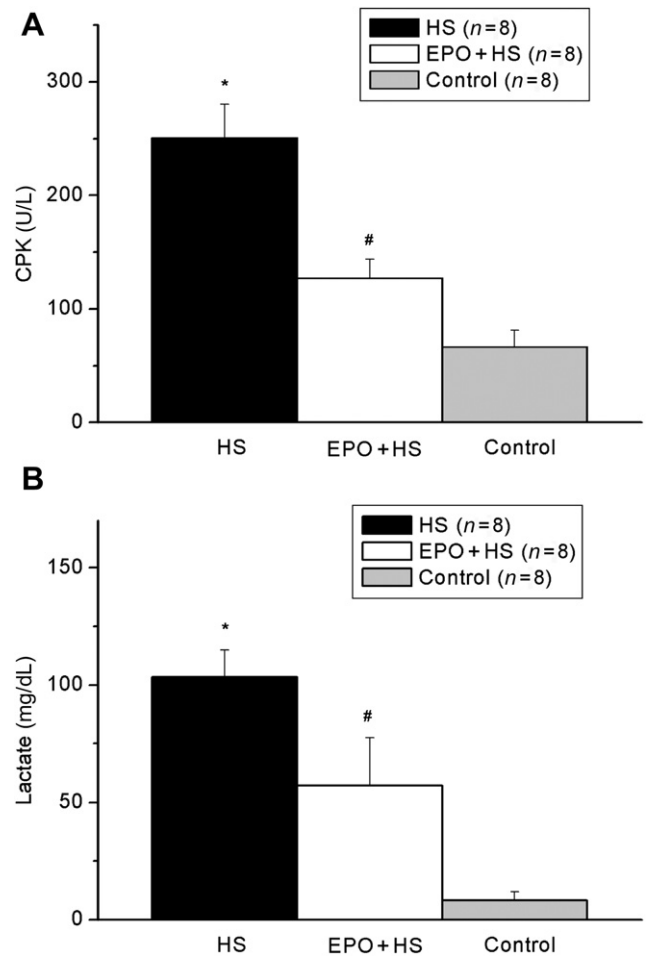
Fig. 5. Changes in serum (A) creatine phosphokinase (CPK) and (B) lactate after hemorrhagic shock (HS) in spontaneously hypertensive rats. $*p < 0.05$ for the HS group compared with the control group. $\#p < 0.05$ for the erythropoietin (EPO) + HS group compared with the HS group.

4. Discussion

This study found that pretreatment of test rats with EPO improved survival, decreased hypotension after induction of hemorrhage and ameliorated severe HS-induced organ damage (GOT, GPT, BUN, Cre, CPK, lactate) and possible effects by decreasing serum TNF-α, IL-6 levels in SHRs.

Hypertensive patients have a higher mortality rate from HS than normotensive individuals [10,11]. Endothelial cell damage, accumulation of leukocytes, disseminated intravascular coagulation, and microcirculatory dysfunction finally lead to programmed cell death (apoptosis) and necrosis of parenchymal cells with the development of multiple organ damage in HS [3].

Endothelial dysfunction in conduit and resistance arteries is common in SHRs [18]. EPO raises blood pressure in SHRs [19] and stabilizes endothelial structures and vascular integrity such as cell–cell and cell–matrix contacts, and is also a potent regulator of proliferation and differentiation in endothelial progenitor cells [20]. These actions show that endothelial progenitor cells have vascular protection/repair properties through the mobilization and recruitment of endothelial progenitor cells to sites of endothelial injury [21].

A previous study noted that conscious SHRs had a higher mortality rate than conscious Wistar-Kyoto rats after the same degree of HS [12]. In this study, pretreatment with EPO improved survival, and decreased hypotension and tachycardia, after induction of severe HS in conscious SHRs.

EPO has for nearly 20 years been widely used for the treatment of anemia associated with chronic kidney disease and cancer chemotherapy [9]. EPO and its receptor are widely expressed in embryonic and adult tissues, including the liver and kidney [9,21]. EPO protects renal tissues from different models of acute renal failure and ameliorates ischemia/reperfusion liver injury in rats [22,23]. EPO has also been shown to improve liver and kidney injury after HS in rats [17,24]. Our results show that treatment with EPO reduced the severe HS-induced increases in serum GOT, GPT, BUN, and Cre levels in SHRs.

Hyperlactatemia is a common complication during HS, and serum lactate has been shown to be a predictor of the outcome of HS [25,26]. Our results noted that hypotension and hyperlactatemia were observed after severe HS in SHRs. The increase in serum lactate generally originates from both increased lactate production and decreased hepatic clearance [27]. The kidneys also contribute to lactic acid removal [27]. It was found that EPO decreased severe HS-induced liver and kidney damage, preserved blood pressure, decreased lactic acid production, and increased the survival of SHRs after HS.
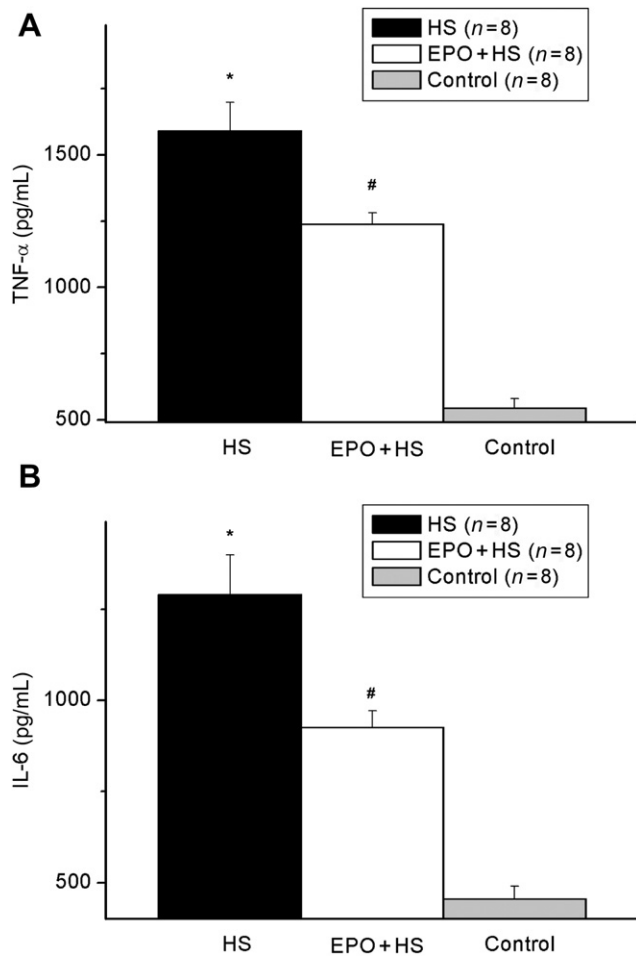
Fig. 6. Changes in serum (A) tumor necrosis factor-α (TNF-α) and (B) interleukin-6 (IL-6) after hemorrhagic shock (HS) in spontaneously hypertensive rats. *$p < 0.05$ for the HS group compared with the control group. #$p < 0.05$ for the erythropoietin (EPO) + HS group compared with the HS group.

During hemorrhage, NF-κB is involved and further increases the inflammatory cascade, such as TNF-α and IL-6 levels [3,4]. TNF-α and IL-6 have been shown to peak early after HS [12–14]. Treatment with monoclonal antibodies to TNF-α can reduce HS-induced acute lung injury [28]. Moreover, administration of recombinant IL-6 blunts lung mRNA levels of proinflammatory cytokine TNF-α after HS in swine [29]. EPO promotes degradation of IκB with subsequent translocation of NF-κB to the nucleus [9]. EPO decreased TNF-α and IL-6 production in a chronic post-myocardial infarction heart failure model in mice and a stroke model in rats [30,31]. In this study, pretreatment with EPO reduced the severe HS-induced serum TNF-α and IL-6 production. These results suggest that EPO prevents HS-induced organ damage by decreasing the production of proinflammatory cytokines and improves the outcome after induction of HS in SHRs. Further studies are needed to show the effects of EPO on HS.

In conclusion, pretreatment with EPO suppressed the release of TNF-α and the production of IL-6, and decreased the levels of markers of organ injury after induction of HS in conscious SHRs.

Acknowledgments

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