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# H2 therapy protects myocardial cell injury induce by sepsis via attenuated expression the level of HMGB1

Amy A Bravo; Krneta Kumm; Catalina Lamy; Jorge Jeanneau; Gisèle Cuello; Jack Lefer [*1]

## Abstract

Sepsis involves a robust inflammatory response, involving up-regulated chemokine expression and leukocyte accumulation, contributes to the mechanism of myocardial injury and cardiac dysfunction. Currently, it is unknown whether H2 suppresses myocardial inflammatory response to sepsis. We tested the hypothesis that treated mouse by H2 protects the heart against sepsis and LV dysfunction through suppression of the high-mobility group box 1 protein (HMGB1) expression. Cecal ligation and puncture (CLP) was used to induce sepsis. By inhaled mice with 2% H2 for 1 h followed CLP in both sham and sepsis model mice was associated with reduced myocardial cell injury mainly associated with reduced expression of HMGB1. Furthermore, H2 treated animals associated with decreased numbers of monocyte and improved LV function. These findings suggest that this novel H2 has a therapeutic potential for the regulation of myocardial inflammatory response to sepsis.

**Keywords**: Sepsis; Hydrogen; Inflammatory response; HMGB1; CLP

[*]Corresponding Author: Jack Lefer, MD, PhD: jack.lefer@inserm.fr
Author Affiliations:
[1]Department of Clinical Pharmacology, Toulouse University Hospital, Toulouse France
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## Introduction

Sepsis is defined as organ dysfunction, hypotension, elevated lactate, or oliguria [1]. Sepsis and its various adverse squeal, such as septic shock, acute respiratory distress syndrome (ARDS), and multiple organ dysfunction syndrome (MODS), continue to be a leading cause of mortality in intensive care unit (ICU) and a major public health burden throughout the world [2]. With recent dramatic advances in powerful antibiotics and monitoring devices, the mortality rate has been decreased over the past half century [3]. The pathogenesis and mechanisms of sepsis are complex and not fully understood,

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which include the excessive release of inflammatory cytokines, the action of oxidative stress (excessive release of reactive oxygen species, ROS) [4].

Furthermore, trauma and stress associated with major surgery can cause gut bacteria translocation, which leads to myocardial endotoxemia and the systemic inflammatory response [5]. The systemic inflammatory response associated with major surgery has a significant impact on the post-surgery outcome in the population [6].

HMGB1 is a member of the high-mobility group protein superfamily that has been widely studied as nuclear proteins that bind DNA, stabilize nucleosomes, and facilitate gene transcription [7]. HMGB-1 has been implicated in the pathogenesis of inflammatory diseases and proposed to be a crucial mediator in the pathogenesis of many diseases including sepsis, arthritis, cancer, autoimmunity diseases, and diabetes [8]. HMGB1 can interact with various receptors including RAGE, Toll-like receptor-(TLR-) 2, and TLR-4 to mediate chemotaxis and release of proinflammatory cytokines in monocytes/macrophages and delayed endotoxin lethality, which is required for the full expression of inflammation in animal models of endotoxemia, sepsis, and arthritis [9]. Furthermore, targeting of HMGB1 with antibodies or specific antagonists has been found to have protective effects in established preclinical inflammatory disease models, including lethal endotoxemia and sepsis [10].

Hydrogen is electronically neutral and has favorable distribution characteristics: it can penetrate biomembranes and diffuse into the cytosol, mitochondria, and nucleus [11]. Despite the moderate reduction activity of H2, its rapid gaseous diffusion might make it highly effective for reducing cytotoxic radicals. Besides, it stands to reason that H2 will react with only the strongest oxidants [12]. H2 is mild enough not to disturb metabolic oxidation reduction reactions or to disrupt ROS involved in cell signalingunlike some antioxidant supplements with strong reductive reactivity, which increase mortality, possibly by affecting essential defensive mechanisms. Thus, H2 treatment is advantageous for medical procedures without serious unwanted side effects [13].

The purpose of this study was to investigate the mechanisms of H2 in myocardial protective effects through down-regulation of HMGB1.

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**Method**

*Animal*

Male wild type mice, body weight 22-30 g, were obtained from Jackson Laboratory acclimatized in a quarantine room for 2 weeks, and their age range from 8 to 12 weeks. All animal experiments were approved by the Animal Care and Research Committee, Toulouse University Hospital. Animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" [DHEW Publication No. (NIH) 85-23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205]. The animals were randomized into 5 groups: sham; sham+H2; sepsis+H2; eight animals in each group.

*Protocol*

Mouse model for human sepsis by cecal ligation and puncture (CLP) protocol as described previously [14]. In briefly, the CLP model consists of the perforation of the cecum allowing the release of fecal material into the peritoneal cavity to generate an exacerbated immune response induced by polymicrobial infection. This model fulfills the human condition that is clinically relevant.

*Pressure-volume loop and hemodynamic analysis*

Pressure-volume loop and hemodynamic analysis was did after 6hs of post cecal puncture, just before the animal scarification, the mouse anesthetized with tribromoethnaol (avertine) in dose of 250mg/kg injected i.p and when the mouse sleeps well, it will be positioned over the heating pad in supine position and four limbs are taped, in orientation that the hind limb in front of operating researcher. Neck was opened longitudinally and right common carotid artery exposed and freed, ligated distally and stay suture placed proximally, then small opening was made in artery and size 1 F-micro tipped pressure transducer catheter (Millar Instruments, Houston, TX, USA) was inserted into the LV lumen via the right carotid artery for measurement of LV pressure, volume, function and related parameters. Then after about 20 minutes of data recording, the abdomen is opened by right sub costal incision to reach the inferior vena cava. To acutely change the cardiac preload, caval occlusion was produced over a 3-s period using a nonmetallic occluder applied to the IVC. The data were recorded as a series of pressure-volume loops.

P-van software (Conductance Technologies, San Antonio, TX, and Millar, Houston, TX) was used to analyze all pressure-volume loop data. Regression analyses of multiple isochronal pressure-volume loop data were produced by IVC compression. From the baseline and IVC compression loops, comprehensive sets of hemodynamic parameters were calculated. All steady-state and caval occlusion pressure-volume loops were acquired with the computer data acquisition system. From these data we selected the following parameter: LV diameters were measured at end diastole (LVEDD) and end systole (LVESD), ejection fraction (EF), heart rate (HR), LV systolic pressure in the ends of both systole and diastole (LVESP, LVEDP). Computations were performed according to recently reported method by [15].

*Animal scarification*

Immediately after finishing the pressure volume loop measurement, the mouse was sacrificed, starting by injection of equal volume of thiopental and heparin intraperitoneal in doses ranging from 100µl to 200 µl for each one, after giving good time for the animal to go into deep anesthesia, the mouse is positioned and taped and the chest is opened in flap like manner revealing the heart then a needle of the syringe is introduced into right ventricle to aspirate around 0.5 ml of blood for plasma analysis. After that the heart is cut from the great vessels and mediastinum.

*Immunofluorescent Staining*

Myocardial sections (5 µm thick) were fixed in 4% paraformaldehyde, incubated with a rabbit polyclonal antibody against PMNs and monocytes, and then incubated with Cy3-tagged secondary goat anti-rabbit IgG (imaged on the red channel). Nuclei were stained with bis-benzimide (DAPI, imaged on the blue channel), and glycoproteins on cell surfaces with Alexa 488-tagged wheat germ agglutinin (imaged on the green channel). Microscopy was performed with a Leica DMRXA digital microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany). Immunoflourecent antibodies were used to target the monocytes.

*Western immunoblotting assay*

Myocardial tissue was homogenized with a rotor-stator homogenizer and treated in PBS containing 0.5% Triton X-100 and a protease inhibitor cocktail. Size fraction of crude protein (20 µg) was performed by electrophoresis. After transfer, the membrane was incubated in PBS 5% nonfat dry milk to block nonspecific binding. The membrane was

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then incubated for 60 minutes with an antibody against HMGB1 at 1:1000 to 1:2000 dilutions with PBS containing 0.05% Tween 20 and 5% dry milk. After thorough washes, the membrane was treated with peroxidase-labeled secondary antibody (1:5000 dilutions with phosphate-buffered saline containing 0.05% Tween 20 and 5% dry milk) for 45 minutes. Protein bands were developed using enhanced chemiluminescence technique. Densitometry was performed using a computerized densitometer (Molecular Dynamics, Sunnyvale, CA).

*Statistical analysis*

Data are presented as the means ± standard error of the mean (SEM). All statistical analyses were carried out using the SPSS 16.0 statistical software package. The $\chi 2$ test or Fisher's exact test were used to analyze the relationship between myocardial injury and H2 treatment.

**Results**

*H2 reduces HMGB1 expression after sepsis*

Septic shock is a common complication of infection, severe trauma, and major operation in patients. Septic shock is an autoimmune injury of the body, and the pathogenesis is very complicated. To approved the role of HMGB1 in sepsis we analysis the HMGB1 expression by western blotting. The expression level of high-mobility group box 1 protein (HMGB1) is reduced in treated mice with H2 after ceacal puncture compared with the untreated wild type, as shown in (Fig. 1).

*Myocardial NF-kB intranuclear translocation is attenuated in H2 treated mice*

Immunoblotting analyses revealed low and consistent levels of NF-κB were detected in sham and treated mice with H2, while significantly higher levels of NF-κB were detected in H2 untreated mice, indicating H2 regulated NF-κB after sepsis, as in (Fig. 2).

*H2 have a reduced myocardial monocytes infiltration*

Monocytes are key regulators of the early LV injury process after sepsis, and by using red–blue–green and red-blue light under fluorescent microscope, there is significant difference in monocytes infiltration among animal, being significantly reduced in the

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monocytes accumulation in H2 treated mice in comparison with untreated wild type group, as shown in (Fig. 3).

*Improved LV function in H2 treated mice*

Average parameters from pressure-volume loop study in the in treated and untreated wild type mice groups were shown in (Fig.4), H2 has significantly better LV function and ventricular elasticity than the untreated group in form of EF%, and CO, with P value for both first two variables = 0.01.

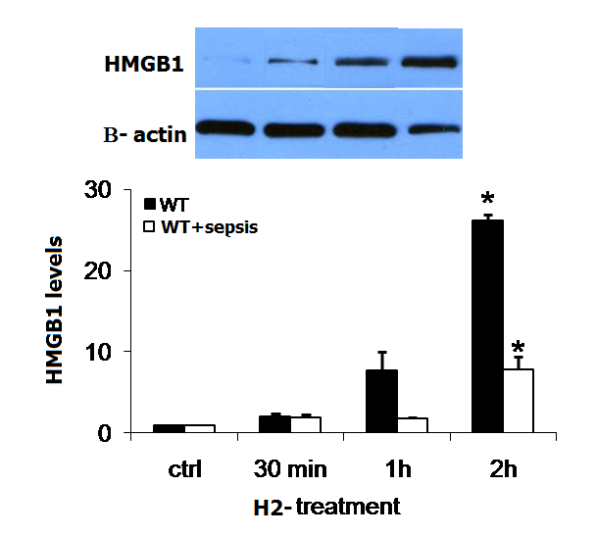


**Figure 1.**

Levels of HMGB1 proteins in myocardial tissues quantified by Western blotting. β-actin was used as a loading control. Data were considered significant at $P < 0.05$ and highly significant vs the control group.

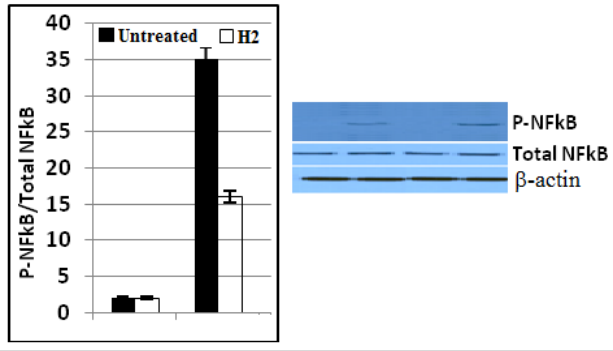


**Figure 2.**

**Activation of phosphorylated NF-KB levels**

Activation of phosphorylated NF-κB levels was analyses by western blot analysis. β-actin used as a loading control elevated in the wild type hearts mainly at 6h following ceacal puncture compared with H2 treated mice. Data are expressed as mean±SEM. *P<0.05.

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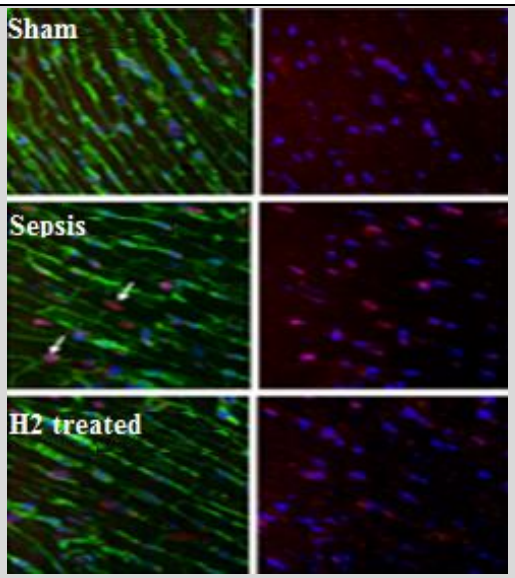


**Figure 3.**

**Myocardial mononuclear cell accumulation are enhanced after sepsis.**
Adult mice were subjected to ceacal ligation or normal saline. Mononuclear cells in the myocardium were detected by immunofluorescence staining using a polyclonal antibody against murine CD68 (red). The myocardium of old mice displayed higher densities of mononuclear cells (arrow) after ceacal puncture. The data expressed as mean±SEM; P<0.05 versus untreated group.

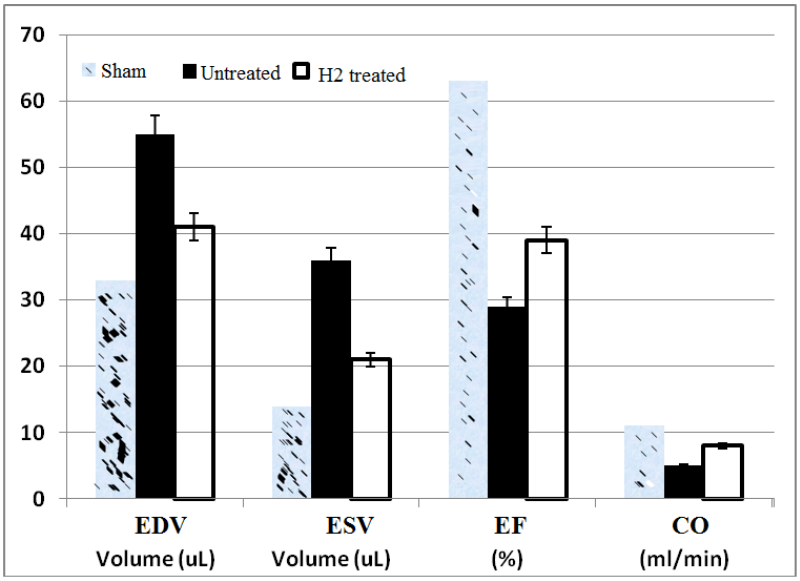


**Figure 4.**

**Pressure-volume loop study**
H2 treated group has significantly better LV function and ventricular elasticity than the untreated WT group in form of EF%, CO, EDV and ESV. The data expressed as mean±SEM; P<0.05 versus untreated group.

**Discussion**

Endotoxemia depresses cardiac function via up-regulation of the expression of cardio-depressant cytokines, and HMGB1 [16]. Thus, it is likely that HMGB1 upregulates the myocardial inflammatory responses to endotoxin and exaggerates endotoxemic cardiac depression [17].

Mononuclear cells are major sources of tissue pro-inflammatory cytokines [18]. While endotoxin induces mononuclear cell infiltration to the myocardium and other tissues [19], the effect of H2 on mononuclear cell accumulation in the myocardium during endotoxemia is unclear. Further, the impacts of myocardial mononuclear cell accumulation and associated cytokine production on cardiac functional performance in the heart remain to be determined.

Some studies have shown that neuro inflammation in the central nervous system can cause brain damage in sepsis. The vast release of HMGB1, leads to alterations of cell function, the blood-brain barrier disruption, and brain dysfunction [20]. The HMGB1, released from necrotic neurons via a NR2B-mediated mechanism, promotes cerebral edema via activation of microglial TLR4 and the subsequent expression of the astrocytic water channel, aquaporin-4 in traumatic brain injury [21]. Furthermore, targeting of HMGB1 with antibodies or specific antagonists has been found to have protective effects in established preclinical inflammatory disease models, including lethal endotoxemia and sepsis [22]. As the late inflammatory cytokine, HMGB1 plays a central role in the inflammatory response, becoming a key therapy to resolve inflammation [23].

Our studies show that H2 inhalation can decrease the proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and (HMGB1) in serum and myocardial tissue mice models of sepsis. Furthermore, H2 treatment reduces the levels of chemokines (KC, MIP-1α, MIP-2, and MCP-1) in the serum. In addition, H2 treatment decreases monocyte recruitment into the myocardial cells [24]. All results demonstrate that H2 treatment down-regulates the HMGB1in the mouse models of sepsis.

**In conclusion**, it has been suggested to use molecular hydrogen as a new anti-inflammatory strategy.

## Competing interests

The authors declare that there is no conflict of interest.

## Authors' contributions

AB, KK, YW, GC and JL are designed the study and wrote the manuscript. CL, JJ carried out the western blotting procedure. All authors read and approved the final manuscript.

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