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Carboxyfullerene nanoparticles alleviate acute hepatic injury in severe hemorrhagic shock

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ABSTRACT

Hemorrhagic shock/resuscitation involves overwhelming reactive oxygen species (ROS) that cause oxidative stress, inflammation, and subsequent tissue injury. We investigated the effects of the potent antioxidant carboxyfullerene (C_3) on acute liver injury during hemorrhage shock/resuscitation. C_3 infusion reduced the alanine aminotransferase (ALT) activity, methemoglobin content, malondialdehyde content, myeloperoxidase activity and expression levels of tumor necrosis factor -α and interleukin-6; it increased superoxide dismutase activity in the liver. The histologic injury score and apoptotic index were also markedly decreased after C_3 treatment compared with the vehicle group. Additionally, C_3-treated rats showed a significant decrease in nuclear factor-κB DNA binding capacity, which was preceded by reduced phosphorylation of the nuclear factor κB (NF-κB) p65 subunit in the liver. C_3 nanoparticles ameliorate oxidative stress, the inflammatory response, and subsequent acute liver injury after hemorrhagic shock/resuscitation. These protective effects appear to be mediated through the inhibition of the nuclear factor-κB pathway. C_3 treatment may be a promising strategy to improve tissue injury in hemorrhagic shock/resuscitation.

1. Introduction

Hemorrhagic shock is the leading cause of potentially preventable deaths in civilian and military trauma, accounting for up to 40% of the deaths associated with traumatic injuries [1]. Patients who initially survive hemorrhagic shock are particularly susceptible to systemic inflammatory response syndrome (SIRS), which subsequently triggers multiple organ failure (MOF) and post-traumatic death [2,3].

Among the major biochemical mediators implicated in MOF, the overwhelming reactive oxygen species (ROS) generated during hemorrhagic shock/resuscitation induce tissue injury through the consumption of intrinsic antioxidant activity, oxidation of plasma membranes and critical proteins, and initiation of systemic inflammation cascades [4]. The importance of ROS in mediating tissue injury suggests that antioxidative therapies aimed at reducing the oxidant load may be beneficial in alleviating tissue injury in hemorrhagic shock/resuscitation.

Nanoparticle-based systems are an emerging class of powerful antioxidants with potential advantages over currently available antioxidants due to the possibility that they can fully quench radicals without the need for assistance from other detoxifying molecules [5,6]. The *tris*-malonic acid derivative C_{60} (C_3), a fullerene derivative, has been investigated as a powerful ROS scavenger with high aqueous solubility for reducing tissue injury in several disease models [7–9]. However, the direct effects of C_3 treatment on tissue injury in hemorrhagic shock/resuscitation or of the molecular mechanism underlying these effects are still unknown and in need of investigation.

Nuclear factor κB (NF-κB) is a ubiquitous rapid response transcription factor involved in inflammatory reactions [10]. A previous study indicated that the NF-κB pathway becomes activated early on during acute hemorrhage [11], leading to the induction of genes critical to the initiation and perpetuation of SIRS and MOF. Therefore, the main aims of this study are to investigate the effect of C_3 nanoparticles infusion on acute liver injury in a hemorrhagic shock model and to reveal a possible protective mechanism involving the NF-κB pathway.

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In this study, we first demonstrate that C_3 intravenous administration attenuates oxidative stress, inflammation, and subsequent hepatic injury in a rat hemorrhagic shock model, which involves the inhibition of the NF-κB pathway.

2. Materials and methods

2.1. Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Academy of Military Medical Sciences (AMMS-13-2015-003) and conformed to the Guide for the Care and Use of Laboratory Animals. Adult male Wistar rats weighing 250–300 g (Vital River Laboratories, Beijing, China) were used after a minimum 5–7-day acclimation period in a specific pathogen-free animal facility according to standard laboratory procedures.

2.2. Preparation and characterization of carboxyfullerene nanoparticles

The *tris*-malonic acid derivative C_{60} (>99.0% purity) was purchased from MTR Ltd. (Cleveland, USA). A stock solution (25 mg/mL) was prepared in normal saline (pH 6.05 ± 0.07; osmolarity 283 ± 0.58 mOsm/kg) by sonication at room temperature and characterized. Zeta potential and dynamic light scattering (DLS) measurements were performed to determine the particle size distribution using a ZetaSizer Nano series Nano-ZS (Malvern Instruments Ltd., Malvern, UK) as previously described [12].

The morphology of the C_3 nanoparticles was characterized using transmission electron microscopy (EM-200CX, JEOL Ltd., Tokyo, Japan) and scanning electronic microscopy (Quanta 200 FEG, FEI Co., USA).

2.3. Antibodies

Antibodies against NF-κB p65 and phospho–NF–κB p65 (Ser536) were purchased from Cell Signaling Technology (Beverly, MA, USA). An anti-caspase-3 antibody was purchased from Proteintech (Chicago, IL, USA). Rabbit anti-TNF-α and IL-6 antibodies were purchased from Abcam (Cambridge, MA, USA).

2.4. Surgical procedures

The rats were anesthetized with intraperitoneal injections of sodium pentobarbital (50 mg/kg) and allowed to breathe spontaneously on a heating pad (SOFTRON, TMS-201, China) that was maintained at 37 ± 0.1 °C throughout the study. The two femoral arteries and the right femoral vein were catheterized with heparinized polyethylene catheters (PE-50). The mean arterial pressure (MAP) was monitored in the left femoral artery using a monitor (MP150, BIOPAC Systems Inc., Santa Barbara, CA, USA). Rats were heparinized with 400 U/kg heparin (IV). Supplementary doses of pentobarbital were administered when necessary.

2.5. Hemorrhagic shock and resuscitation model

A pressure-controlled hemorrhagic model was generated using the right femoral arterial catheter after surgical preparation and 10 min of stabilization as described previously [13] with modifications. In brief, after measuring the baseline blood pressure, hemorrhage was initiated by bleeding at the rate of 0.4 mL/min to achieve a MAP of 30–35 mmHg within 10 min using syringe pumps (Softron Beijing, Inc., Beijing, China). This blood pressure was maintained for 50 min by further blood withdrawal.

At the end of the hemorrhagic shock, the animals were randomized into two groups. C_3 solution (10 mg/kg) or vehicle (the same volume of saline) was administered intravenously. The animals were then resuscitated with 1.5 times the volume of shed blood with normal saline (Shijiazhuang SiYao Ltd., Hebei, China) at 70 min after hemorrhage. Rats in the sham group underwent surgical procedure without blood withdrawal. The C_3 dosage refers to the previous studies [14–16]. All infusions were performed using a pump driven at a constant rate of 0.3 mL/min.

2.6. Blood and tissue sampling

Arterial blood gas analysis was performed at baseline, after blood withdrawal, and 2 h post-resuscitation using a blood gas analyzer (ABL90 FLEX, Radiometer Copenhagen, Denmark). All of the animals were euthanized by exsanguination under anesthesia at 2 h post-resuscitation. The livers were quickly removed and washed with cold saline, snap-frozen and stored in liquid nitrogen until assayed.

2.7. Plasma biochemistry

Plasma aspartate aminotransferase (ALT) activity was evaluated using the Hitachi 7180 autoanalyzer (Hitachi High-Technologies Corp., Tokyo, Japan).

2.8. Measurements

The liver tissues were homogenized and sonicated on ice in 0.9% normal saline containing a protease inhibitor cocktail (Roche, Mannheim, Germany), and then the homogenates were centrifuged at 1000 × g for 6 min at 4 °C. The supernatants were assayed for malondialdehyde (MDA) content, myeloperoxidase (MPO) activity, catalase activity, and superoxide dismutase (SOD) activity (Jiancheng Biological Institute, Nanjing, China) using a colorimetric determination according to the manufacturer's recommendations as described previously [2]. Total hepatic protein levels were measured using a BCA protein assay kit (Pierce, Rockford, IL, USA).

2.9. Immunohistochemistry

The hepatic tumor necrosis factor (TNF)-α and interleukin (IL)-6 expression levels were evaluated by immunohistochemistry as previously reported [17]. In brief, sections (4 μm thick) were deparaffinized and hydrated by standard methods and then placed in citrate buffer and microwaved for antigen retrieval. After the endogenous peroxidase activity and nonspecific proteins were blocked, the sections were incubated with anti-TNF-α antibody (1:50) and anti-IL-6 antibody (1:50) as the primary antibodies. Then, the sections were incubated with a biotinylated secondary antibody solution and detected with diaminobenzidine. The nuclei were counterstained with Mayer's hematoxylin. Positive and negative immunohistochemical controls were routinely used. After staining, ten random fields were observed for each slide in a blinded fashion. The average proportion of positive cells in each field was calculated.

2.10. NF-κB activation assay

The DNA-binding capacity of NF-κB was measured using an enzyme-linked immunosorbent colorimetric oligonucleotide binding assay (TransAM NF-κB p65, Active Mitif, Carlsbad, CA, USA) as previously reported [18]. Briefly, after the NF-κB-specific oligonucleotide was immobilized in a 96-well plate, the whole tissue extract (20 μg) was added to the plate and incubated for 1 h with