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Protective effects of apocynin on "two-hit" injury induced by hemorrhagic shock and lipopolysaccharide

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KEY WORDS apocynin; hemorrhagic shock; lipopolysaccharides; malondialdehyde; myeloperoxidase

ABSTRACT

AIM: To evaluate the protective effects of apocynin on "two-hit" injury in rats. **METHODS:** "Two-hit" injury model of rat was induced by hemorrhagic shock (40 mmHg for 45 min) followed by iv administration of lipopolysaccharide (LPS, 150 μg/kg). Rats were randomized into seven groups: Sham, LPS, hemorrhage, hemorrhage/LPS, and hemorrhage/LPS+apocynin (2.5, 5.0, and 10.0 mg/kg). Apocynin was dissolved in the resuscitation fluid (normal saline, NS) and administered iv for 2 h. After LPS or NS administration, the survival rates at 8 h, 16 h, 24 h, and 48 h were monitored. The content of malondialdehyde (MDA) was measured in lung at 3 h and 6 h after iv LPS and in serum before hemorrhage, after hemorrhage, and at 0, 0.5, 1, 2, 4, and 6 h after iv LPS. Myeloperoxidase (MPO) activity in lung and liver was examined at 3 h and 6 h after iv LPS/NS. **RESULTS:** After "two-hit" injury, the survival rates of rats at 8 h, 16 h, 24 h, and 48 h were 64.3 % , 35.7 % , 28.6 %, and 14.3 % respectively, there were significant differences as compared to sham group ($P<0.05$ or $P<0.01$, respectively), the MDA level in lung and serum were significantly enhanced ($P<0.01$) as compared to sham group, and MPO activity in lung and liver after "two-hit" injury was also significantly increased ($P<0.01$). Apocynin treatment enhanced the mean arterial pressure (MAP) of hemorrhagic shock rats dose-dependently ($P<0.05$), increased the survival rate of "two-hit" injury rats, decreased the serum and lung MDA content, and downregulated MPO activity in lung and liver. **CONCLUSION:** Apocynin could preventively ameliorate "two-hit" injury in rats induced by hemorrhagic shock and LPS insult.

INTRODUCTION

The systemic inflammatory response syndrome (SIRS) is a common cause of death in patients with severe trauma. Hemorrhage followed by infection plays an important role in the occurrence of SIRS and development of multiple organ dysfunction syndrome (MODS). The initial hemorrhagic shock, as the first-hit, "primes" the inflammatory cell including polymorphonuclear leukocyte (PMN), and makes patients more susceptible to a second, seemingly trivial, and inflammatory stimulus, such as lipopolysaccharide (LPS). This so-called "two-hit" model, characterized with excessive release of mediators (superoxidants, TNF-α, and ILs, *etc*), is widely accepted to play important roles in the occurrence of SIRS in recent years. Studies showed that ischemia-reperfusion could augment PMN-mediated tissue injury *via* the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system[1].

Apocynin (4-hydroxy-3-methoxy-acetophenone),

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a constituent of root extracts of the medicinal herb *Picrorrhiza Kurroa*, is an effective and selective inhibitor of NADPH oxidase in PMN[2]. Research showed that apocynin inhibited peroxynitrite formation in murine macrophages[3] and vascular cell adhension molecule 1 (VCAM-1) expression in endothelial cell[4], increased glutathione synthesis, activized AP-1 in alveolar epithelial cells[5], and decreased the LPS-induced TNF-α formation in human monocytes, but interestingly, PMN's chemotaxis, infiltration, phago-cytolysis, and intracellular killing of Staphylococcus aureus were not affected by apocynin[6]. All these properties of apocynin make it a promising anti-inflammatory agent *in vivo*.

We developed a "two-hit" injury model with rats to evaluate its protective effects and probe for a preventive way to reduce the occurrence of MODS in traumatic patients in clinic.

MATERIALS AND METHODS

Animal preparation Wistar rats of either sex weighing 220 g±30 g (n=159, Grade II, Certificate No 310303002) were purchased from the Laboratory Animal Center of the Third Military Medical University. Apocynin (498-02-2) and LPS (*Escherichia coli*, O_{111}: B_4) were purchased from Sigma Co. Rats were anesthetized by ip injection of pentobarbital sodium 50 mg/kg (Shanghai Second Chemical Company, F20000522). The left femoral artery was cannulated and connected to a pressure recorder for the measurement of mean arterial pressure (MAP) and collecting shed blood. The left femoral vein was cannulated for fluid infusion and drug administration.

"Two-hit" injury model of rat was induced by hemorrhage followed by iv administration of lipopolysaccharide. Hemorrhage was initiated by blood withdrawal. MAP was reduced to and maintained at 40 mmHg for 45 min. Shed blood was collected in the heparinized tube to prevent clotting. After a hypotensive period of 45 min, animals were resuscitated by reinfusion of the shed blood and NS of 1.5 fold volumes of shed blood over 2 h. Apocynin was dissolved in resuscitation fluid (NS) and administered *via* the vein catheter. At 1 h after resuscitation, either LPS (150 μg/kg) or the equal volume of NS alone was administered.

Effect of apocynin on MAP of hemorrhaged rats Forty rats were randomized into four groups: hemorrhage+resuscitation (n=17), hemorrhage+apocynin 2.5 mg/kg (n=6), hemorrhage+apocynin 5.0 mg/kg (n=7), hemorrhage+ apocynin 10.0 mg/kg (n=10). After hemorrhage was induced, shed blood plus NS of 1.5 fold volumes of shed blood or apocynin dissolved in NS was administered intravenously, and the MAP was measured 1 h after resuscitation.

Effect of apocynin on survival rate of "two-hit" injury in rats Fifty-nine rats were randomized into seven groups: Sham (n=8), LPS (n=6), hemorrhage (n=9), hemorrhage/LPS (n=14), hemorrhage/LPS+apocynin 2.5 mg/kg (n=6), hemorrhage/LPS+apocynin 5.0 mg/kg (n=9), and hemorrhage/LPS+apocynin 10.0 mg/kg (n=7). Sham group animals underwent the same surgical procedures but without hemorrhage or LPS. After the injection of LPS or NS, the catheters were removed, the femoral vessels were ligated, the incision was closed, and the survival rates were monitored at 8 h, 16 h, 24 h, and 48 h. All rats received the normal food and water and the environmental temperature was maintained at 24 °C with sufficient light and ventilation.

Effect of apocynin on plasma and tissue MDA and MPO following "two-hit" injury in rats Thirty rats were randomized into five groups: Sham (n=6), LPS (n=6), hemorrhage (n=6), hemorrhage/LPS (n=6), and hemorrhage/LPS+apocynin 5.0 mg/kg (n=6). Thiobarbituric acid reaction (TBAR) method[7] was used to determine the malondialdehyde (MDA) content. Blood samples (1.0 mL) were obtained before hemorrhage, after hemorrhage, and at 0, 0.5, 1, 2, 4, and 6 h after iv LPS/NS and the MDA level was measured by fluorescence spectrophotometer (Beckman 7500). MDA in lung at 3 h and 6 h was measured as above using 10 % tissue homogenate. Myeloperoxidase (MPO) activity of lung and liver was measured with a biochemistry kit (Nanjing Jiancheng Agent Co, China) following the illustration.

Statistical analysis All data were expressed as mean ± SD. Statistical difference was analyzed by t test except the comparison of survival rate among different groups, in which the *Chi*-square test was used for determining the statistical differences. $P<0.05$ was considered statistically significant.

RESULTS

Effects of apocynin on MAP Reinfusion of the shed blood and NS of 1.5-fold volumes of the shed

blood could not restore the MAP to the baseline level in hemorrhage+resuscitation group. Apocynin companied with reinfusion could enhance MAP in hemorrhage rat in a dose-dependent manner, especially in 10 mg/kg group ($P<0.05$, Tab 1).

Tab 1. Effects of apocynin on MAP following hemorrhage in rats. Mean±SD. $^cP<0.01$ *vs* before hemorrhage. $^eP<0.05$ *vs* hemorrhage+resuscitation group.

Group/mg·kg^{-1}	*n*	MAP/mmHg	
		Before hemorrhage	1 h after reperfusion
Hemorrhage+resuscitation	17	117±13	96±15^c
Hemorrhage+apocynin 2.5	6	100±16	96±16
Hemorrhage+apocynin 5.0	7	104±19	96±20
Hemorrhage+apocynin 10.0	10	118±14	107±7^e

Survival rate After "two-hit" injury, the survival rate of rats at 8 h (64.3 %), 16 h (35.7 %), 24 h (28.6 %), and 48 h (14.3 %) were decreased significantly *vs* sham group ($P<0.05$ or $P<0.01$, respectively). Apocynin (5.0 mg/kg) increased the survival rate significantly at 8 h ($P<0.05$). The 24 h and 48 h survival rate in apocynin (5.0 mg/kg) group and the 8 h survival rate in apocynin (2.5 mg/kg) group were enhanced, but there was no significant difference as compared to hemorrhage/LPS group. Apocynin (10.0 mg/kg) decreased the survival rate at 8 h *vs* hemorrhage/LPS group (Tab 2).

MPO activity in tissue PMN inflitration into lung and liver was evaluated by measurement of tissue MPO activity. In hemorrhage/LPS group, MPO activity in liver at 3 h (2.4 U/g) and 6 h (2.3 U/g) was increased *vs* sham group ($P<0.01$, respectively) and LPS or hemorrhage group. MPO activity in lung at 3 h (7.6 U/g) and 6 h (4.2 U/g) was increased significantly as compared with sham group ($P<0.01$) and LPS or hemorrhage group. There was a tendency of declination in MPO activity in lung at 6 h, which means that the summit of MPO activity in lung was earlier than in liver, and lung was the earlier organ involved in "two-hit" injury. Apocynin (5.0 mg/kg) deceased the MPO activity in lung and liver at 6 h after LPS injection *vs* hemorrhage/LPS group ($P<0.05$, Fig 1).

MDA level in serum In hemorrhage/LPS group, the MDA level in serum at 0.5 h (5.1 mmol/L), 1 h (5.7 mmol/L), 2 h (4.8 mmol/L), 4 h (6.3 mmol/L), 6 h (6.8 mmol/L) was significantly enhanced. Apocynin (5.0 mg/kg) lowered MDA content at 4 h and 6 h significantly as compared with hemorrhage/LPS group ($P<0.01$ or $P<0.05$, Tab 3).

MDA level in lung In hemorrhage/LPS group, the MDA level in lung at 3 h (20 mmol/g) and 6 h (29 mmol/g) were enhanced significantly ($P<0.01$) *vs* sham group after the injection of LPS, which was consistent with the results above. Although the MDA content in LPS group at 6 h and in hemorrhage group at 3 h and 6 h increased *vs* sham group, the extent was not as serious as in hemorrhage/LPS group. Compared with hemorrhage/LPS group, apocynin (5.0 mg/kg) could effectively diminish the production of MDA in lung at 3 h and 6 h after the injection of LPS ($P<0.05$ or $P<0.01$, Tab 4).

DISCUSSION

Hemorrhagic shock (40 mmHg, 45 min) followed

Tab 2. Effects of apocynin on survival rate in "two-hit" rats. $^bP<0.05$, $^cP<0.01$ *vs* sham group. $^eP<0.05$ *vs* hemorrhage /LPS group.

Group	*n*	Survival rate after injection of LPS/%			
		8 h	16 h	24 h	48 h
Sham	8	100	100	100	100
LPS	6	83.3	83.3	83.3	66.7
Hemorrahage	9	77.8	66.7^b	33.3^c	22.2^c
Hemorrahage/LPS	14	64.3^b	35.7^c	28.6^c	14.3^c
Hemorrahage/LPS+apocynin 2.5 mg/kg	6	83.3	66.7	16.7	0
Hemorrahage/LPS+apocynin 5.0 mg/kg	9	100^e	66.7	66.7	44.4
Hemorrahage/LPS+apocynin 10.0 mg/kg	7	57.1	42.9	28.6	0

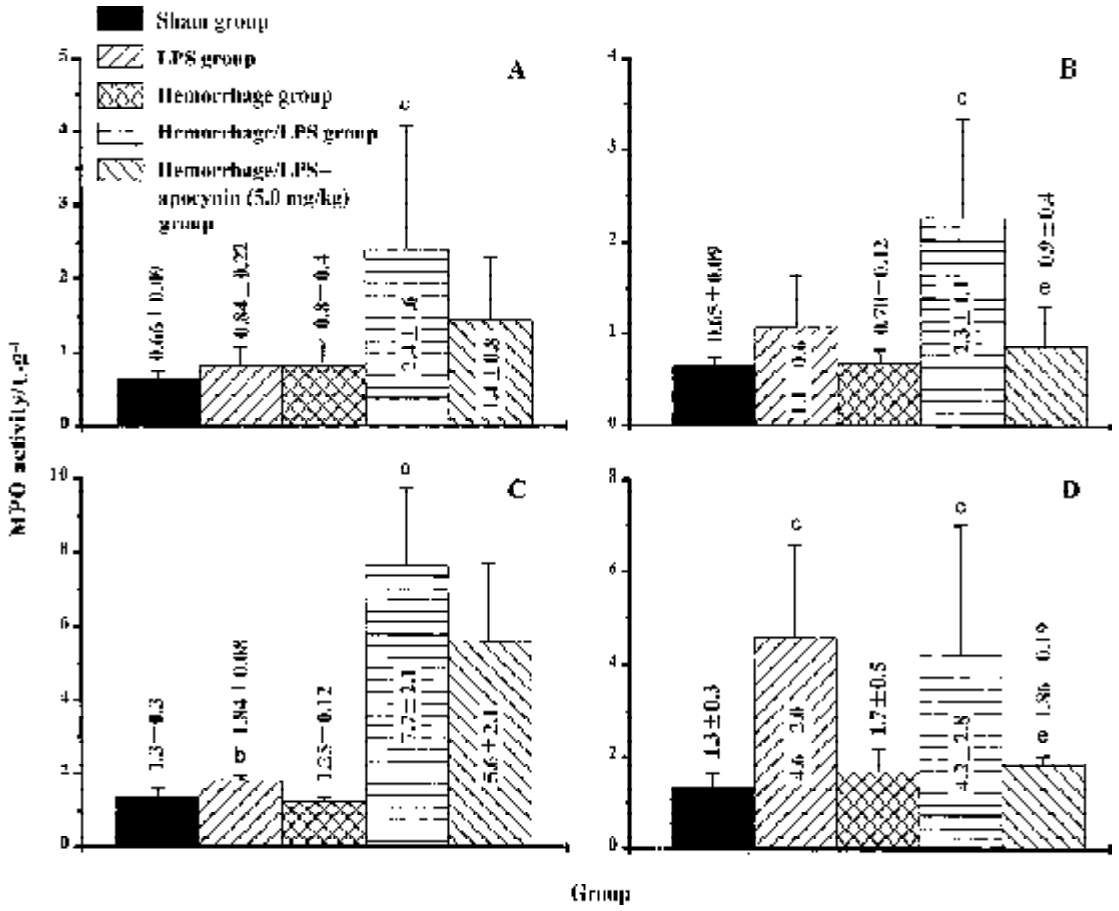

Fig 1. MPO activity (U/g) at 3 h after LPS/NS injection in liver (A) and 6 h (B), MPO activity in lung after LPS/NS injection 3 h (C) and 6 h (D). *n*=6. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* sham group. $^{e}P<0.05$ *vs* hemorrhage/LPS group.

Tab 3. MDA level in serum after LPS injection in rats. *n*=6. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* sham group. $^{e}P<0.05$ *vs* hemorrhage/LPS group. $^{h}P<0.05$, $^{i}P<0.01$ *vs* itself before hemorrhage.

Group	Before hemorrhage	After hemorrhage	MDA level/mmol·L^{-1} 0 h	0.5 h	1 h	2 h	4 h	6 h
Sham	2.6±0.5	2.7±0.8	2.56±0.22	1.6±0.3	2.6±0.3	2.7±0.3	3.1±0.5	3.3±0.4
LPS			2.6±0.5	2.8±0.4	3.0±1.2	2.6±1.1	2.9±0.9	2.6±0.7
Hemorrhage	3.0±0.8	3.2±0.5	4.2±1.5	2.7±1.3	3.8±0.6	4.2±1.2^{b}	3.8±1.6	3.7±1.5
Hemorrhage/LPS	3.3±1.1	4.2±1.0	4.3±0.6	5.1±0.5^{ci}	5.7±0.6^{ci}	4.8±0.7^{bi}	6.3±1.3^{ci}	6.8±1.2^{ci}
Hemorrhage/LPS+apocynin	2.9±0.7	3.6±0.9	3.9±1.0^{eh}	3.2±0.7^{i}	3.5±0.8^{i}	3.1±1.9^{h}	3.8±0.7^{ei}	4.2±1.0^{eh}

with intravenation of LPS (150 μg/kg) increased the rat mortality at 8, 16, 24, and 48 h after the injection of LPS, shortened the survival time, upregulated tissue PMN chemotaxis during early time, and augmented the tissue oxidative injury, especially in lung. These results are consistent with others[8,9].

Traumatic hemorrhage could increase the susceptibility to LPS by inhibiting immune function when "priming" inflammatory cells to excessively release inflammatory mediators. The traditional medical treatments, such as application of antibiotics, steroidal anti-inflammatory drugs (SAID), and monoclonal antibody (anti-LPS McAb) manifested that the curative effects were not as the suspected.

MDA is the direct product of lipid peroxidation in tissue, and the concentration of MDA in plasma and

Tab 4. MDA level in lung after LPS injection in rats. Mean±SD. *n*=6. [b]*P*<0.05, [c]*P*<0.01 *vs* sham group. [e]*P*<0.05, [f]*P*<0.01 *vs* hemorrhage/LPS group.

Group	MDA level/mmol·g^{-1} 3 h	6 h
Sham	10± 6	10±6
LPS	8±4	20±4[c]
Hemorrhage	17±3[b]	23±8[c]
Hemorrhage/LPS	20±3[c]	29±3[c]
Hemorrhage/LPS+apocynin 5.0 mg·kg^{-1}	12±5[e]	10±3[f]

tissue reflects the extent of damage in tissue. MPO is one of the special oxidase in PMN, and MPO activity in tissue reflects the PMN chemotaxis and infiltration. PMN infiltration and activation could produce large amount of oxidants and cause tissue oxidative injury, which contributes to the organ functional damage in MODS. The infiltration and sequestration of circulating PMN in lung cause lung edema, ARDS, and respiratory failure. Adhesion molecule is responsible for PMN chemotaxis and infiltration, and the expression of adhesion molecule is the first step of inflammatory activation. Apocynin could decrease the expression of VCAM-1[5] in endothelium which may be one of the most major mechanism by which apocynin down-regulated the PMN infiltration in lung and liver effectively in our studies. NADPH oxidase system in PMN is the key enzyme involved in the formation of oxides, and it has been implicated as an early pivotal player in the occurrence of MODS[1]. Apocynin could decrease the tissue oxidative damage in "two-hit" rats by selectively inhibiting NADPH oxidase, lower the production of inflammatory mediators (such as TNF-α), and prevent oxidative injury in tissue. SAID is the widely accepted anti-inflammatory drug in clinic, but it has broad side effects which limits the application in traumatic patients, such as immunodepression which is unfavorable to the latter infection, while apocynin does not affect PMN's defensive function[6]. To this point, apocynin is a more acceptable/prospective agent as a preventive treament in MODS.

Our studies found that apocynin (2.5 and 5.0 mg/kg) dissolved in resuscitation fluid (NS) could improve survival rate of hemorrhaged and LPS insulted rats, and gain precious time for the further treatment. Apocynin 10 mg/kg enhanced the mortality in rat at 8 h after the injection of LPS, and also significantly increased the MAP after resuscitation. Although the correlation analysis between the survival time and the MAP after using apocynin (10 mg/kg) did not show statistical negative relationship (r= –0.2127, P>0.05), it is rational to consider that whether the usage of agents (such as cardiotonic agent) affecting hemodynamic parameters vigorously during the early phase in "two-hit" patient may cause dangerous/unfavorable consequence. Our studies also point out that it is a reliable and prospective way to use anti-oxidative or anti-inflammatory agent during resuscitation after hemorrhagic shock to avoid "two-hit" injury no matter in traumatic patient or in serious operation in clinic.

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