I. Introduction

1.1 Background

Trauma contributes to a significant proportion of world-wide mortality. More than 5 million people die each year as a result of injuries, and it accounts for 9% of the world's deaths in the year 2000.(1) In Korea, as a result of injury, more than thirty thousand people died in the year 2010, trauma death was the third most frequent cause of death and the mortality rates is 65.4 people per 100,000 population (Figure 1).(2)

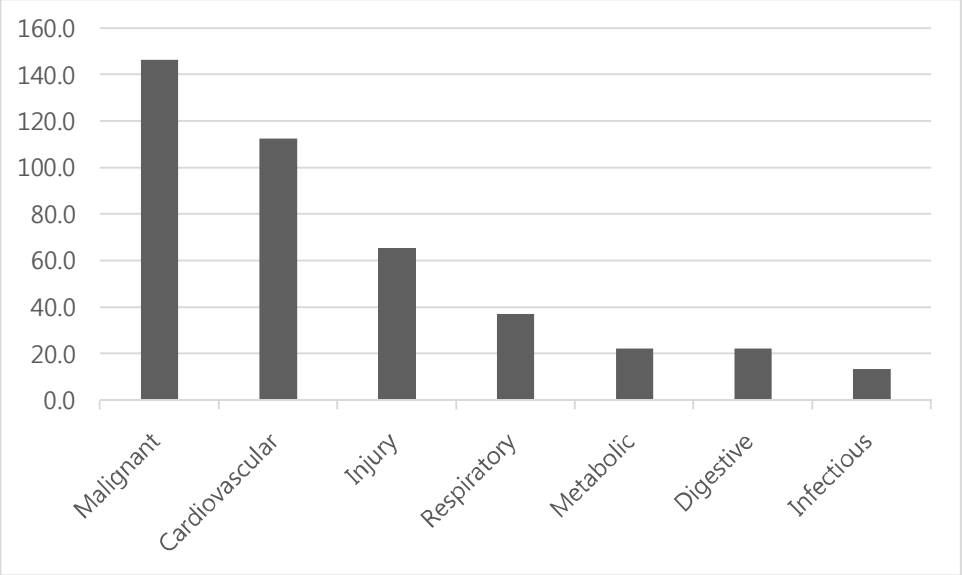

Figure 1. Death rates per 100,000 population in Korea, 2010.

Hemorrhagic shock (HS) is the main cause of death among major trauma patients. Early resuscitation and definite treatment within 1 hour post-injury (golden hour) has been emphasized to reduce preventable death from HS in life-threatening major trauma.(3) However, despite appropriate resuscitation and definite care, multiple organ failure developed in 20 - 30% of HS patients which is caused by a global ischemia-reperfusion (IR) injury.(4, 5)

IR injury is a well-known sterile inflammatory process, in which tissue damage from ischemia is exacerbated by blood flow restoration and re-oxygenation after resuscitation.(5) Usually the lung is the most frequently affected organ from the IR injury.(6) The pathophysiology of IR injury is a series of inflammatory responses that involves reactive oxygen species (ROS) production, ROS-dependent intracellular signaling pathway activation, and pro-inflammatory cytokine release.(6-8) The ROS-dependent nuclear factor (NF)-κB pathway has been known to enhance pro-inflammatory processes during IR injury.(7-9) NF-κB, DNA binding protein, is a family of transcription factors and plays a central role in inflammation and immunity.(7) NF-κB is a heterodimer consisting of 2 proteins, either p50 or p52 and p65 (Rel-A). Under a resting state, NF-κB is bound to inhibitor κB-α (IκB-α) and exists in the cytoplasm in an inactive state.(10) However, during IR injury, produced ROS stimulates IκB-α phosphorylation, ubiquitination, and proteolysis. It results in the release of NF-κB dimers and translocation into the nucleus, which triggers the transcription and release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6).(10) Thus, ROS reduction during

IR injury may contribute to the improvement of clinical outcome of trauma patients with HS.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) is a family of enzymes that generates ROS. The structure and function of Nox was first described in neutrophil. Nox is a multisubunit complex comprised of p22phox and Nox2 (gp91phox) as well as four cytoplasmic subunits, p47-phox, p67-phox, p40-phox and the small G protein, Rac. (11) Among them, Nox2 plays a key role in ROS generation and the ROS-dependent NF-κB pathway activation during IR injury.(12, 13) In pathologic conditions, the cytosolic subunits translocate to the membrane and interact with gp91phox, and activate Nox2. Activated Nox2 complex catalyzes electron transfer from NADPH to a molecular oxygen and thus generates superoxide anion ($^{\bullet}O_2^-$) and subsequent ROS.(12, 14)

Naturally occurring apocynin (4-hydroxy-3-methoxy-acetophenone) was obtained from the roots of Picrorhiza kurroa growing in the Himalaya and are used in the Ayurvedic medicine. Apocynin is metabolically activated by forming a symmetrical dimer, so called diapocynin, in myeloperoxidase-catalyzed reaction in the presence of H_2O_2. (15) It has been known as an inhibitor of the Nox activity and concomitant ROS production.(16, 17) It is assumed that activated apocynin oxidizes thiols of p47phox which are critical for the function of p47phox.(18) It suppresses the translocation and binding of cytosolic subunits to the membrane subunits and the activation of Nox2.(19, 20)

1.2 Hypothesis

We hypothesized that apocynin treatment would reduce Nox-derived ROS production, suppress ROS-mediated inflammation responses, and attenuate lung injury induced by HS.

1.3 Aims

The aim of this study was to investigate whether a Nox inhibitor, apocynin, reduces ROS production, suppresses the ROS-dependent NF-κB pathway, attenuates acute lung injury, and improves survival in a rat HS model.

II. Materials and Methods

2.1 Ethics statement

All the experimental procedures were approved by the Animal Experiment Committee of the Institutional Animal Care and Use Committee of Seoul National University Hospital (IACUC number: 15-0167-S1A1) and followed the Korea Animal Protection Laws.

2.2 Animals and Drugs

Experiments were performed on 62 male Sprague-Dawley rats (body weight, 290-340 g) purchased from the Orient Bio Inc. (Seongnam, Korea). Animals were acclimated to their environment for 1 week and allowed access to their laboratory food and tap water ad libitum. Apocynin was purchased from Sigma-Aldrich (St Louis, MO).

2.3 Experimental Procedures

Anesthetization and induction of HS in rats were performed as described previously.(8) For anesthesia, zoletil (zolazepam and tiletamine, Virbac AH, Fort Worth, TX) was injected intramuscularly with a dose of 50 mg/kg. Then the rats were intubated and mechanically ventilated using an Inspira Advanced Safety

Single Animal Pressure/Volume Controlled Ventilator (Harvard Apparatus, Holliston, MA) with pure oxygen and 2% of isoflurane. Volume-controlled mode with a tidal volume of 10 mL/kg and a rate of 45/min was applied. The venous and the arterial access were performed at the tail vein and left femoral artery with 24 gauge intravascular catheters (Jelco, Smiths Medical, Dublin, OH). For the monitoring of the mean arterial pressure (MAP) and blood retrieval, a three-way stopcock was connected to the arterial catheter. Before the induction of HS, isoflurane inhalation was discontinued. To induce HS, blood was drawn from the three-way stopcock using a 10-mL heparin-coated syringe for 10 minutes. Blood aspiration was done gently in 10 minutes with a ratio of approximately 3 mL of blood per 100 g of body weight, until the MAP dropped to a range between 20 and 25 mmHg. This MAP target was maintained until 30 minutes after induction of HS. At 30 minutes post-HS induction, the vehicle or apocynin in distilled water with the same volume (about 1 mL) was injected intraperitoneally, and the shed blood (90% of aspirated blood volume) and saline (10% of aspirated blood volume) was reinfused through the tail vein for 10 minutes for resuscitation. At 60 minutes, the arterial line was removed and the femoral artery was ligated at the proximal and distal part and the femoral wound was sutured. Then the animals were weaned from the ventilator and returned to their cages. Blood sampling was performed at baseline, 30 minutes, 60 minutes post-HS induction.

The rats were randomly allocated into four groups: (i) sham group: rats were injected with the vehicle without HS; (ii) HS group: rats were injected with the vehicle after HS; (iii) HS + low dose apocynin (LD-Apo) group: rats were injected

with 20 mg/kg of apocynin after HS; and (4) HS + high dose apocynin (HD-Apo) group: rats were injected with 40 mg/kg of apocynin after HS.

Experimental protocol was as follows (Figure 2).

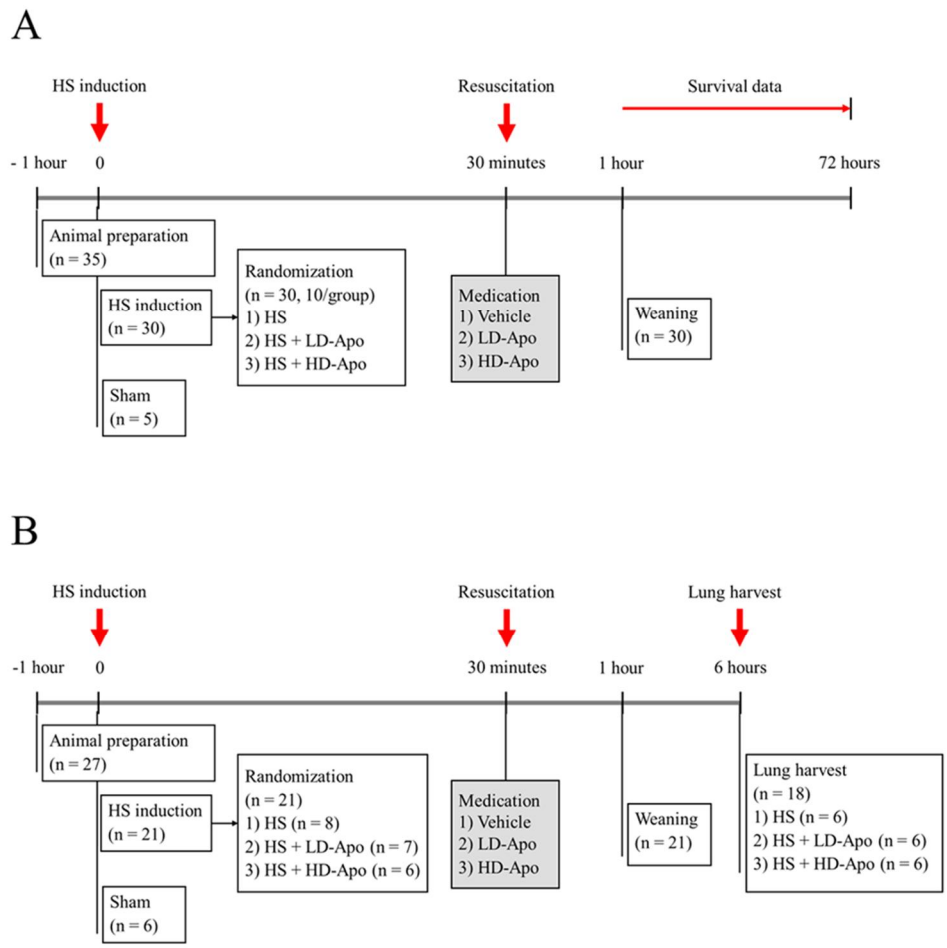

Figure 2. Experimental protocol of (A) the survival study and (B) the lung tissue analyses study to evaluate the effects of a LD-Apo and HD-Apo on a rat HS model.

HS, hemorrhagic shock; LD-Apo, low dose of apocynin; HD-Apo, high dose of apocynin.

First, for the survival study, five rats for the sham group and ten rats for each three intervention groups (N = 35) were randomly allocated and were observed for 72 hours for the occurrence of mortality. When the survival rates of HS and HS + HD-Apo groups were assumed to be 1% and 33%, respectively, which were extrapolated from previous data,(8) a sample size of at least nine per group was calculated with a power of 80% and a significance level of 0.05. Therefor, 10 rats/per group were assigned to the HS, HS + LD-Apo and HS + HD-Apo groups, respectively. Furthermore, as a normal control, 5 rats were assigned to the sham groups.

Second, for the lung tissue analyses, the other rats (n = 27) were randomly allocated to four groups based on the results of the survival study: the sham group (n=6), the HS group (n=8), the HS + LD-Apo group (n=7), and the HS + HD-Apo group (n=6). Six animals in each group were killed at 6 hours post-HS induction. The right upper lobe of the lung of each rat was harvested and fixed in 4% formaldehyde for the H & E staining and pathologic evaluation. The remaining tissues were washed in 0.1 M phosphate buffer solution and immediately frozen in liquid nitrogen and kept at – 80°C.

The hemodynamic and laboratory data of all the animals used for the survival study (n = 35) and the lung tissue analyses study (n = 27) were collected.

2.4 Experimental Analyses and Measurements

2.4.1 Nuclear Extracts

The extraction of nuclear and cytoplasmic proteins was performed using an NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL).

2.4.2 Western Blot Analysis

To confirm the lung expressions of cytoplasmic phosphorylated IκB-α (p-IκB-α) and whole tissue gp91-phox, western blotting was performed as previously described.(21) Briefly, tissue homogenates were centrifuged at 10,000 G for 20 minutes at 4℃ and the supernatants were separated. The total protein concentrations in the supernatant were determined using the bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL). Protein extracts (20 μg per lane) were run on a gradient sodium dodecyl sulfate polyacrylamide gel (Elpis biotech, Daejeon, Korea) and were transferred onto polyvinylidene difluoride membranes (Schleicher & Schuell, Dassel, Germany). Primary antibodies were anti-p-IκB-α (diluted 1:500; Cell Signaling, 2859, Beverly, CA) and anti-gp91phox (1:1000; Abcam, ab129068, Cambridge, MA). An enhanced chemiluminescence system (Amersham International, Buckinghamshire, United Kingdom) was used to detect the protein bands, and the densities of the bands were quantified by computer-assisted densitometry of the exposed films (LapWork Software, Seoulin

Bioscience, Seoul, Korea). The blots of cytoplasmic and whole tissue extracts were normalized to β-actin.

2.4.3 NF-κB p65 DNA-binding Activity

NF-κB p65 DNA-binding activities in the lung tissues were determined by the trans-AM method with an NF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA).(21, 22)

2.4.4 Real-time RT-PCR for TNF-α and IL-6 Gene Expression

For the detection and quantification of TNF-α and IL-6 messenger RNA (mRNA) expressions in the lung tissue, real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed, as previously described.(8, 21) Briefly, total RNA was extracted from the lung tissue using an RNeasy total RNA extraction kit (Qiagen, Chatsworth, CA). Next, 1 μg of RNA was used for a complementary DNA reaction in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GADPH) expression. The primers and probes used were purchased from Applied Biosystems: TNF-α (Rn99999017), IL-6 (Rn99999011), and GADPH (Rn99999916). All experiments were performed in triplicate. Complementary DNA templates were diluted 10 times and gene expressions were analyzed using the ΔCt method for TNF-α, IL-6, and GADPH genes.

2.4.5 Nox Activity

Nox activity was measured using a cytochrome *c* reductase assay kit (Sigma-Aldrich, CY0100) and was expressed as the reduced cytochrome *c* mU/mg protein as relative absorbance units at 550 nm.(23)

2.4.6 Malondialdehyde Levels

Intracellular malondialdehyde (MDA) is a biomarker for lipid peroxidation. Lung MDA levels were determined according to the Ohkawa method based on the reaction of thiobarbituric acid with the MDA.(21)

2.4.7 Myeloperoxidase Activity

Myeloperoxidase (MPO) is an active enzyme expressed in neutrophils involved in phagocytosis and a marker of neutrophil accumulation in the lung tissue. MPO activity was determined using an MPO colorimetric assay kit (BioVision, Milpitas, CA).(24, 25)

2.4.8 Histological Lung Injury

The formalin-fixed right upper lobes of the lung were washed and embedded in paraffin and cut into 4-μm sections. Next, the sections were deparaffinized and stained with hematoxylin and eosin. The histologic findings were reviewed by a pathologist blinded to the study groups. The acute lung injury (ALI) scores were calculated as previously described.(8, 21, 26) Briefly, the ALI scores have four categories with a rate from 0 (no injury) to 16 (maximal injury): alveolar congestion, hemorrhage, infiltration or aggregation of neutrophils in air spaces or vessel walls, and thickness of the alveolar wall/hyaline membrane formation.

Actually, the ALI scores were checked three times per slide and the mean value were used as a representative value for the slide.

2.5 Statistical Analysis

The survival data were analyzed using the Kaplan-Meier analysis and the log-rank test. Other data were analyzed by the Kruskal-Wallis test and the Mann-Whitney *U* post hoc test with Bonferroni correction. Adjusted $p < 0.05$ was considered statistically significant. The statistical analyses were performed using SPSS version 22.0 for Windows (SPSS, Chicago, IL).

III. Results

3.1 Hemodynamic and Laboratory Data

In the set of survival analysis, there were no statistically significant differences in most of the hemodynamic and biochemical laboratory findings between the 3 intervention groups at baseline, pre- (30 minutes post-HS induction), and post-treatment (60 minutes post-HS induction) (Table 1). The pH at baseline in HS + LD-Apo groups is numerically more alkalotic than that in the HS + HD-Apo group. The lactate level at baseline in the HS was higher than that in the HS + HD-Apo group. The hemoglobin level at 30 minutes post-HS induction in the HS + HD-Apo group was lower than that in the HS + LD-Apo groups (Table 1).

Collectively, both survival and lung tissue analysis, all the animals in each groups, there were no statistically significant differences in most of the hemodynamic and biochemical laboratory findings between the 3 intervention groups at baseline, pre- (30 minutes post-HS induction), and post-treatment (60 minutes post-HS induction) (Table 2). The hemoglobin level at 30 minutes post-HS induction in the HS + HD-Apo group was lower than that in the HS and HS + LD-Apo groups, and the lactic acid level at 60 minutes post-HS induction in the HS + LD-Apo group was lower than that in the HS and HS + HD-Apo groups (Table 2).

During the phase of HS, the MAPs in the three intervention groups were maintained within the target range (20-25 mm Hg) (Figure 3).

Table 1. Hemodynamic and Laboratory Data (the animals of survival analysis)

Survival study	Sham N=5	HS N=10	HS + LD-Apo N=10	HS + HD-Apo N=10	*P*-value
Body weight, g	331 (292-370)	302.3 (282-341)	315.5 (300-336)	295.7 (292-300)	0.214
Drawn blood volume, mL	NA	8.6 (8.1-9.4)	8.3 (7.5-9.7)	9.8 (8.9-11.0)	0.116
Baseline					
MAP, mmHg	132.6 (133-136)	124.8 (100-139)	125.9 (103-147)	121.2 (105-130)	0.713
pH	7.53 (7.50-7.58)	7.48 (7.46-7.50)	7.52 (7.50-7.53)	7.45[§] (7.41-7.49)	0.005
$PaCO_2$, mmHg	29.9 (27.4-31.3)	37.4 (32.9-43.9)	35.5 (33.0-37.5)	36.9[†] (33.6-42.8)	0.029
PaO_2, mmHg	536.8 (496.7-631.8)	561.5 (560.4-611.6)	570.0 (528.3-625.8)	606.4 (611.9-662.9)	0.133
HCO_3-, mmol/L	25.4 (21.5-29.5)	28.0 (25.1-31.4)	29.0 (27.2-30.7)	26.0 (25.4-26.8)	0.129
Hemoglobin, g/dL	11.7 (10.9-12.4)	12.2 (11.8-13.0)	13.1 (12.7-13.9)	12.3 (11.8-13.0)	0.121
Lactic acid, mmol/L	3.1 (1.5-4.5)	3.4 (2.8-4.4)	2.9 (2.6-3.4)	2.2[‡] (1.9-2.7)	0.043
30 minutes after HS induction					
MAP, mmHg	158 (138-174)	21.3[†] (21-23)	19.3[†] (18-21)	19.8[†] (19-21)	<0.001
pH	7.44 (7.40-7.50)	7.27[†] (7.20-7.34)	7.19[†] (7.21-7.25)	7.22[†] (7.19-7.28)	0.004
$PaCO_2$, mmHg	42.7 (41.9-43.9)	22.8[†] (17.7-25.7)	25.4 (17.4-23.9)	21.1[†] (18.1-23.6)	0.013
PaO_2, mmHg	532.8 (520.9-555.0)	538.9 (480.4-588.9)	522.6 (493.5-606.5)	508.9 (378.1-600.6)	0.828
HCO_3-, mmol/L	29.5 (26.6-33.0)	10.7[†] (7.3-12.4)	9.2[†] (7.6-11.0)	8.7[†] (7.9-9.4)	0.003
Hemoglobin, g/dL	12. (12.2-13.6)	9.5[†] (9.3-9.8)	10.7 (10.2-11.3)	8.9[†,§] (8.9-9.2)	<0.001
Lactic acid, mmol/L	2.4 (2.1-2.7)	13.0[†] (11.8-14.9)	12.3[†] (10.8-13.5)	12.3[†] (11.2-13.0)	0.003

60 minutes after HS induction					
MAP, mmHg	149.8 (142-161)	126.3 (115-144)	131.0 (123-138)	140.1 (138-145)	0.052
pH	7.37 (7.32-7.46)	7.17† (7.04-7.28)	7.19† (7.16-7.23)	7.13† (7.09-7.15)	0.009
$PaCO_2$, mmHg	47.5 (46.8-47.6)	50.8 (40.9-56.6)	42.0 (35.8-48.0)	44.8 (42.3-51.1)	0.536
PaO_2, mmHg	513.5 (484.9-566.7)	639.9 (594.1-679.3)	516.2 (463.1-646.9)	613.7† (599.4-725.4)	0.030
HCO_3^-, mmol/L	27.9 (24.9-29.2)	18.3† (15.5-21.0)	15.0† (12.0-16.1)	14.8† (14.8-16.0)	<0.001
Hemoglobin, g/dL	12.5 (12.0-13.0)	13.0 (12.6-13.5)	12.9 (11.3-14.3)	12.5 (12.3-13.4)	0.735
Lactic acid, mmol/L	2.2 (1.8-2.6)	8.5† (7.4-9.1)	6.4† (6.0-7.2)	6.9† (6.1-7.8)	<0.001

Data are presented as median (interquartile range) and compared using the Kruskal-Wallis test.

The animals in the survival study were included for the analysis.

†Adjusted *p*-values < 0.05 vs. the sham group. ‡Adjusted *p*-values < 0.05 vs. the HS group. §Adjusted *p*-values < 0.05 vs. the HS + LD-Apo group.

HS, hemorrhagic shock; LD-Apo, low dose of apocynin; HD-Apo, high dose of apocynin; MAP, mean arterial pressure; $PaCO_2$, arterial partial pressure of carbon dioxide; PaO_2, arterial partial pressure of oxygen; HCO_3^-, bicarbonate.

Table 2. Hemodynamic and Laboratory Data (all the animals of survival and biochemical analysis)

	Sham N=11*	HS N=18*	HS + LD-Apo N=17*	HS + HD-Apo N=16*	*P*-value
Body weight, g	313 (305-322)	334.5 (288-345)	312 (300-330)	299 (292.5-310.5)	0.233
Drawn blood volume, mL	NA	9 (7.6-9.8)	9 (8.2-9.7)	9.5 (9.1-10.1)	0.152
Baseline					
MAP, mmHg	149 (136-157)	141 (127-150)	128 (117-144)	128 (109-141)	0.107
pH	7.52 (7.49-7.55)	7.48† (7.46-7.50)	7.50 (7.48-7.52)	7.49 (7.45-7.53)	0.052
$PaCO_2$, mmHg	30.9 (27.4-33.5)	37.3† (34.8-40.8)	36.2 (34.2-37.5)	34.3 (30.5-36.4)	0.022
PaO_2, mmHg	593 (557-632)	587 (560-617)	605 (528-631)	633 (602-656)	0.168
Incidence of ALI (n, %)**	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1.000
HCO_3-, mmol/L	27.7 (23.4-29.5)	27.6 (25.4-30.9)	27.9 (27.1-30.1)	25.7 (25.4-26.5)	0.093
Hemoglobin, g/dL	12.6 (11.1-13.2)	13.1 (12.2-13.9)	12.8 (12.1-13.7)	12.2 (11.8-13.0)	0.156
Lactic acid, mmol/L	1.7 (1.5-4.5)	2.7 (2.2-3.5)	2.6 (2.0-3.0)	2.5 (2.0-2.8)	0.398
30 minutes after HS induction					
MAP, mmHg	164 (157-167)	21† (20-21)	20† (19-21)	20† (20-21)	<0.001
pH	7.46 (7.40-7.48)	7.28† (7.20-7.31)	7.22† (7.18-7.27)	7.21† (7.19-7.29)	<0.001
$PaCO_2$, mmHg	40.7 (37.3-43.9)	19.8† (17.1-26.3)	21.7† (18.0-27.9)	21.7† (17.6-23.6)	<0.001
PaO_2, mmHg	560 (521-587)	517 (385-550)	509 (412-575)	525 (414-596)	0.636
Incidence of ALI (n, %)**	1 (9.0)	2 (11.1)	1 (5.9)	1 (6.3)	0.936

HCO_3-, mmol/L	27.0 (26.1-27.8)	10.2† (8.5-11.8)	8.9† (8.1-11.0)	8.8† (7.9-9.6)	<0.001
Hemoglobin, g/dL	12.9 (12.4-13.6)	10.3† (9.4-11.3)	10.0† (9.3-10.6)	9.1†,‡,§ (8.7-9.3)	<0.001
Lactic acid, mmol/L	2.2 (1.9-2.6)	13.3† (11.8-14.7)	11.9† (11.1-13.7)	12.9† (11.3-13.8)	<0.001
60 minutes after HS induction					
MAP, mmHg	155 (142-161)	137† (122-144)	134† (125-138)	144 (138-145)	0.003
pH	7.43 (7.33-7.46)	7.15† (7.10-7.18)	7.19† (7.10-7.23)	7.13† (7.09-7.24)	<0.001
$PaCO_2$, mmHg	41.0 (38.9-47.1)	47.9 (41.1-53.4)	44.6 (36.8-53.7)	44.7 (35.1-50.0)	0.313
PaO_2, mmHg	561 (518-598)	621 (558-675)	571 (488-633)	646 (552-701)	0.105
Incidence of ALI (n, %)**	0 (0.0)	0 (0.0)	2 (11.8)	1 (6.3)	0.346
HCO_3^-, mmol/L	26.5 (25.2-29.2)	16.8† (15.3-18.9)	15.4† (14.0-17.4)	15.5† (14.8-16.2)	<0.001
Hemoglobin, g/dL	13 (12.4-13.4)	13.5 (12.8-14.3)	12.9 (12.2-13.5)	12.7 (12.3-13.3)	0.071
Lactic acid, mmol/L	2.1 (1.8-2.2)	7.9† (7.0-9.0)	6.2†,‡ (5.9-7.1)	7.3† 6.5-8.0)	<0.001

Data are presented as median (interquartile range) and compared using the Kruskal-Wallis test.

*Data of all the animals used for the survival study and the lung tissue analyses study were collected.

**Acute lung injury (ALI) was defined as the arterial partial pressure of oxygen/fraction of inspired oxygen (P/F) ratio less than 300.

†Adjusted *p*-values < 0.05 vs. the sham group. ‡Adjusted *p*-values < 0.05 vs. the HS group. §Adjusted *p*-values < 0.05 vs. the HS + LD-Apo group.

HS, hemorrhagic shock; LD-Apo, low dose of apocynin; HD-Apo, high dose of apocynin; MAP, mean arterial pressure; $PaCO_2$, arterial partial pressure of carbon dioxide; PaO_2,

arterial partial pressure of oxygen; ALI, acute lung injury; HCO_3^-, bicarbonate.

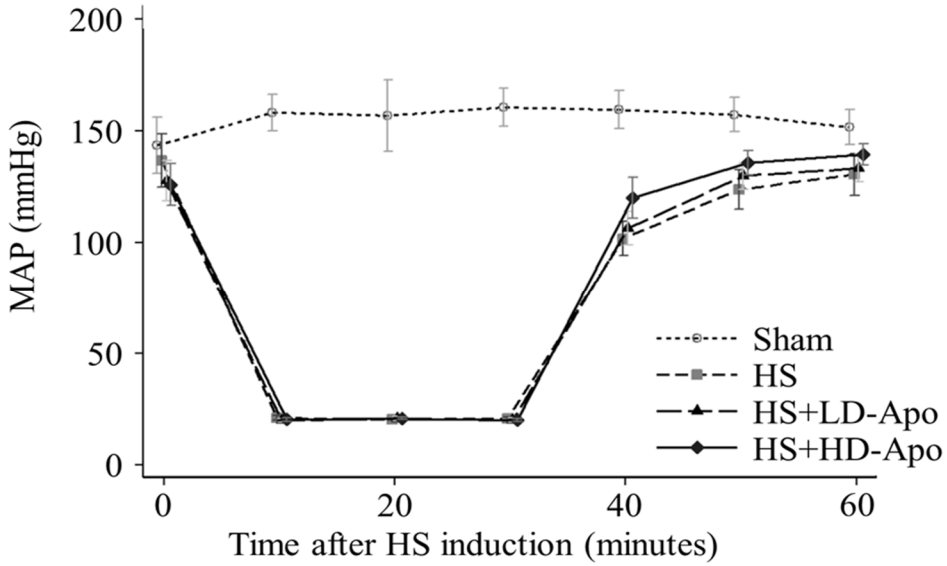

Figure 3. Changes in MAP values during HS and resuscitation in the sham, HS, HS + LD-Apo, and HS + HD-Apo groups. Data are presented as the median (range).

HS, hemorrhagic shock; LD-Apo, low dose of apocynin; HD-Apo, high dose of apocynin; MAP, mean arterial pressure.

3.2 Survival results

Two rats in the HS group and one rat in the HS + LD-Apo group were dead within 6 hours after HS induction. In the HS, HS + LD-Apo, and HS + HD-Apo groups, 3/10 rats (30%), 4/10 rats (40%) and 7/10 rats (70%) were kept alive for 72 hours after HS induction, respectively. However, no significant difference was observed between the HS and HS + HD-Apo groups ($p = 0.061$) (Figure 4). The survival data between the HS and HS + HD-Apo groups were compared using the Kaplan-Meier survival analysis and the log-rank test.

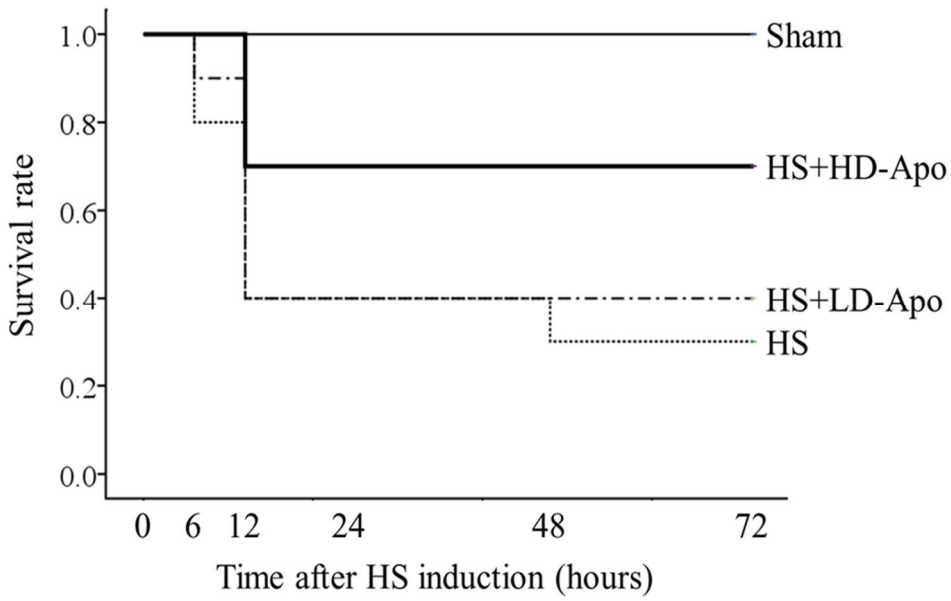

Figure 4. Survival data for 72 hours post-hemorrhagic shock induction.

HS, hemorrhagic shock; LD-Apo, low dose of apocynin; HD-Apo, high dose of apocynin.

3.3 Nox2 Expression, Nox Activity, and Lipid Peroxidation in the Lung Tissues

Lung gp91-phox expression and the Nox activity in the HS + HD-Apo group were significantly lower than those in the HS group (p = 0.002 and 0.004, respectively) (Table 3) (Figure 5A and B). Lung MDA level in the HS + HD-Apo group was lower than that in the HS group (p = 0.002) (Table 3) (Figure 5C).

Table 3. Inhibition of NADPH oxidase and lipid peroxidation by apocynin

	Sham	HS	HS + LD-Apo	HS + HD-Apo	*p*-value
gp91phox expression	16.0 (13.5-18.6)	47.8 (43.6-52.0)	32.7 (21.9-43.5)	25.5 (19.4-31.6)	0.002
Nox activity	65.6 (61.1-70.1)	107.8 (100.2-115.4)	90.9 (80.6-101.2)	83.8 (74.9-92.7)	0.004
MDA	67.3 (48.3-86.2)	220.0 (190.2-249.8)	199.8 (171.5-228.1)	165.2 (153.2-177.2)	0.002

HS, hemorrhagic shock; LD-Apo, low dose of apocynin; HD-Apo, high dose of apocynin; Nox, nicotinamide adenine dinucleotide phosphate oxidase; MDA, malondialdehyde.

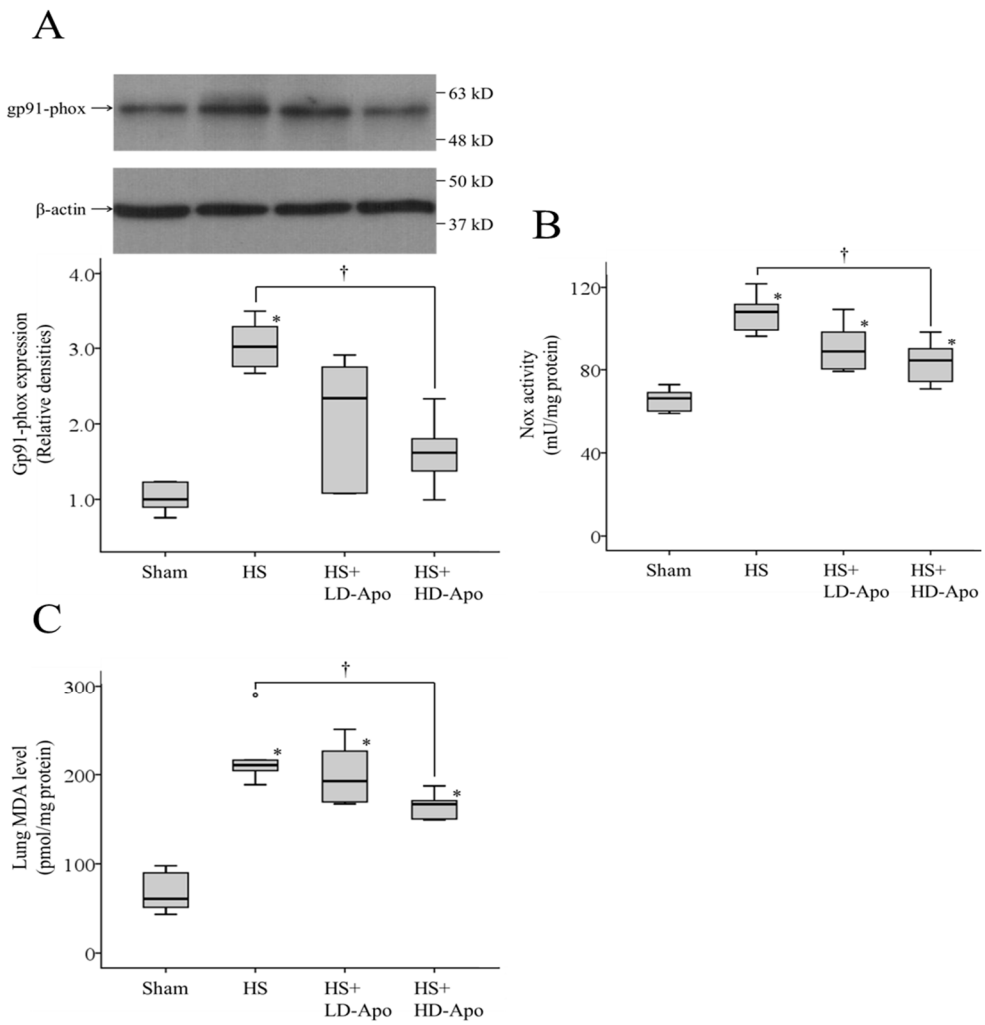

Figure 5. The effects of a LD-Apo and a HD-Apo on A) gp91-phox expression, B) Nox activity, and C) MDA level in the lung tissues during HS and resuscitation.

Blots were representative of three rats per group. Data are presented as the median (quartiles, range). *Adjusted $p < 0.05$ vs. the sham group. †Adjusted $p < 0.05$ vs. the HS group.

HS, hemorrhagic shock; LD-Apo, low dose of apocynin; HD-Apo, high dose of apocynin; Nox, nicotinamide adenine dinucleotide phosphate oxidase; MDA, malondialdehyde.

3.4 NF-κB Pathway and Pro-inflammatory Cytokine Gene Expression in the Lung Tissues

Lung cytoplasmic p-IκB-α expression and NF-κB p65 DNA-binding activity in the HS + HD-Apo group were lower than those in the HS group (p = 0.002 and 0.004, respectively) (Table 4) (Figure 6A and B). Lung TNF-α and IL-6 mRNA expressions in the HS + HD-Apo group were lower than those in the HS group (p = 0.002 and 0.004, respectively) (Table 4) (Figure 6C and D).

Table 4. Suppression of NF-κB pathway and expression of pro-inflammatory gene by apocynin.

	Sham	HS	HS + LD Apo	HS + HD Apo	*p*-value
p-IκB	15.2 (12.8-17.5)	39.8 (35.2-44.4)	24.9 (19.3-30.5)	18.6 (16.1-21.2)	0.002
NF-κB DNA binding activity	0.075 (0.038-0.112)	0.338 (0.226-0.451)	0.238 (0.175-0.302)	0.153 (0.111-0.196)	0.004
TNF-α mRNA	1.06 (0.94-1.18)	5.05 (3.42-6.68)	2.98 (2.10-3.87)	2.09 (1.76-2.41)	0.002
IL-6 mRNA	0.55 (0.43-0.66)	15.6 (4.2-27.1)	4.9 (0.8-9.1)	2.4 (1.4-3.4)	0.004

HS, hemorrhagic shock; LD-Apo, low dose of apocynin; HD-Apo, high dose of apocynin; p-IκB-α, phosphorylated inhibitor κB-α; NF-κB, nuclear factor κB; TNF-α, tumor necrosis

factor-α; IL-6, interleukin-6.

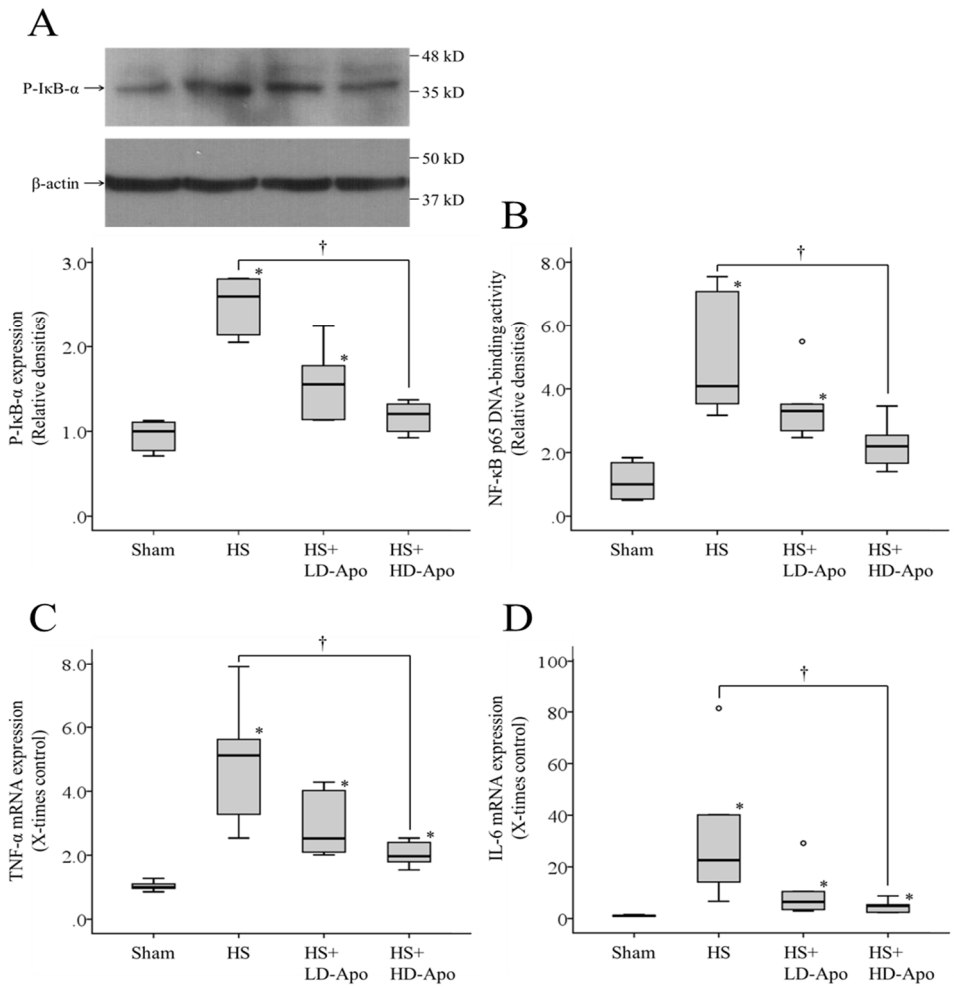

Figure 6. The effects of a LD-Apo and a HD-Apo on A) cytoplasmic p-IκB-α expression, B) NF-κB p65 DNA-binding activity, C) TNF-α mRNA expression, and D) IL-6 mRNA expression in the lung tissues during HS and resuscitation.

Blots were representative of three rats per group. Data are presented as the median (quartiles, range). *Adjusted $p < 0.05$ vs. the sham group. †Adjusted $p < 0.05$ vs. the HS group.

HS, hemorrhagic shock; LD-Apo, low dose of apocynin; HD-Apo, high dose of apocynin; p-IκB-α, phosphorylated inhibitor κB-α; NF-κB, nuclear factor κB; TNF-α, tumor necrosis

factor-α; IL-6, interleukin-6.

3.5 MPO Activity and Histological Injury in the Lung Tissues

Lung MPO activity in the HS + HD-Apo group was lower than that in the HS group (p = 0.002) (Table 5) (Figure 7A). The ALI scores in the HS + LD-Apo and HS + HD-Apo groups were found to be lower than that in the HS group (p = 0.002 and 0.002, respectively) (Table 5) (Figure 7B). Compared with the HS group, the protective effect of apocynin against histological damage to the lung parenchyma was observed in the HS + HD-Apo group (Figure 7C).

Table 5. Attenuation of acute lung injury by apocynin

	Sham	HS	HS + LD-Apo	HS + HD-Apo	*p*-value
MPO	42.9 (38.2-47.6)	101.4 (87.1-115.6)	80.1 (66.4-93.7)	71.0 (62.4-79.7)	0.002
ALI	2.2 (1.0-3.4)	9.9 (8.9-10.9)	7.2 (6.6-7.7)	5.2 (4.2-6.2)	0.002

HS, hemorrhagic shock; LD-Apo, low dose of apocynin; HD-Apo, high dose of apocynin; MPO, myeloperoxidase; ALI, acute lung injury.

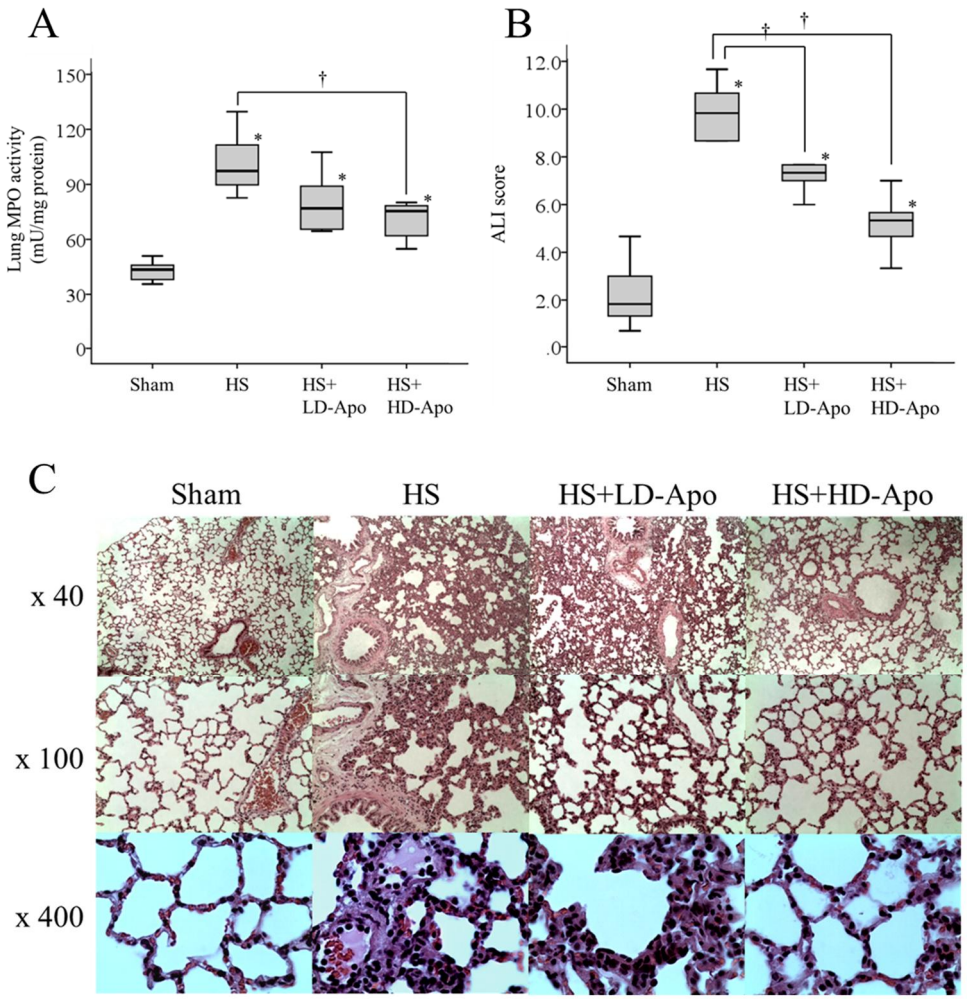

Figure 7. The effects of a LD-Apo and a HD-Apo on A) MPO activity in the lung tissues, B) the ALI scores, and C) histological lung injury shown in representative photomicrographs (hematoxylin and eosin) during HS and resuscitation.

Data are presented as the median (quartiles, range). *Adjusted $p < 0.05$ vs. the sham group. †Adjusted $p < 0.05$ vs. the HS group.

HS, hemorrhagic shock; LD-Apo, low dose of apocynin; HD-Apo, high dose of apocynin; MPO, myeloperoxidase; ALI, acute lung injury.

IV. Discussion

The main findings of the present study were as follows: during HS and resuscitation in the lung tissue, (i) administration of high dose apocynin inhibited Nox2 expression and Nox activity; (ii) administration of high dose apocynin reduced lipid peroxidation; (iii) administration of high dose apocynin suppressed the ROS-dependent NF-κB pathway and subsequent pro-inflammatory cytokines transcription; and (iv) administration of high dose apocynin decreased lung MPO activity and attenuated histological lung injury. Furthermore, these benefits of apocynin were dose-dependent. These data indicate that Nox2 at least in part mediates IR injury during HS and resuscitation and that Nox2-mediated IR injury can be reduced by Nox inhibitor administration.

Many experimental and clinical studies have shown the beneficial antioxidant effects of apocynin on atherosclerosis, neurological disorders, diabetes, and chronic obstructive pulmonary disease.(17, 27-32) However, only inhalation therapy has been tried in a clinical setting.(17, 32) Clinical trials showed that a 2 mg of apocynin inhalation therapy attenuated airway hyper-responsiveness in asthmatic patients(17) and that a 3 mg of apocynin inhalation therapy decreased oxidative stress elements, such as hydrogen peroxide and nitrite, in exhaled breath condensates in chronic obstructive pulmonary disease patients.(32) These data indicate that apocynin can be considered as a useful antioxidant agent in clinical setting. As in many previous experimental studies, the present study administered apocynin intraperitoneally.(31, 33) The low and high doses of apocynin were also

selected based on the previous experimental studies.(16) Further studies to determine the appropriate dose, route, safety and therapeutic benefit of apocynin for humans should be performed before its clinical use.

Although administration of high-dose apocynin showed a tendency to improve survival in rat HS model, the present study failed to improve survival significantly. We calculated the sample size for the survival study based on our previous data, and the survival rates of the HS and high dose apocynin groups were assumed to be 1% and 33%, respectively.(8) However, in the present study, the survival rates of the HS and high dose apocynin groups were 30% and 70%, and this might be the reason why the present study could not show a statistically significant survival improvement. In the previous study, we reinfused 100% of aspirated blood volume with the same amount of shed blood. However, in the present study, we replaced the 100% aspirated blood volume with 90% of shed blood and 10% aspirated blood volume of saline during resuscitation to prevent volume overload, which might contribute to the improvement of survival rate.

To perform the HS model that is compatible to the clinical situation, we used the previous HS rat model.(8) We used a total shock period of 30 minutes because most patients with HS have received resuscitation within an hour after HS in actual clinical settings.(34) As described earlier,(8) many studies that used MAP beyond our range failed to show severe shock-related injury and mortality or could not save enough time to get sufficient survival rates. From these data, we developed our HS model maintaining MAP of 20 - 25 mmHg and total shock period of 30 minutes, consequently, our HS model was useful to confirm survival and organ injury.

Nevertheless, our model had the limitation in the aspect of sufficient resuscitation and intensive care after HS, which might influence mortality and organ injury.

From the survival data, mortality difference between HD-Apo and HS groups was conspicuous at 6 hours. Therefore, to assess the therapeutic effects of apocynin, we performed tissue harvests and lung histologic examination at 6 hours post-HS induction.

The present study had the following limitations. First, we showed that apocynin treatment was associated with decreases in Nox2 expression, Nox activity, and Nox-derived ROS during HS and resuscitation. However, other protective effects of apocynin, such as arylamine N-acetyltransferase activity inhibition, might also contribute to its therapeutic benefits.(35) To determine the mechanistic effects of Nox inhibition on IR injury during HS and resuscitation, additional studies using Nox knockout animals are needed.

Second, because we did not use living cells but frozen lung tissue, we failed to directly measure tissue ROS level. We measured the MDA level as a marker for lipid peroxidation and observed that the MDA level was negatively correlated with Nox2 expression and Nox activity. Further studies which investigate ROS levels in apocynin-exposed living cells will be helpful to confirm the antioxidant mechanism of apocynin.

Third, we measured the effects of apocynin on tissue injuries only in the lung tissues. Although the lung has been known to be the most frequently affected organ from the IR injury,(6-8) additional studies investigating the effects of apocynin on other tissues, such as the heart, liver, kidney, or intestine, would support the use of

apocynin to improve outcomes of major trauma patients with HS.

V. Conclusion

In conclusion, the administration of high-dose apocynin inhibited Nox2 expression and Nox activity, reduced lipid peroxidation, suppressed the NF-κB pathway and subsequent pro-inflammatory cytokines transcription in the lung tissues, and attenuated lung injury during HS and resuscitation in rats.

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요약(국문초록)

백서의 출혈성 쇼크 모델에서 아포시닌의 nuclear factor-κB 경로의 억제 및 폐손상의 감소 효과

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서론: 백서의 출혈성 쇼크(hemorrhagic shock, HS) 모델에서 니코틴아미드 아데닌 디누클레오티드 포스페이트 (nicotinamide adenine dinucleotide phosphate, NADPH) 산화효소(Nox)의 저해제인 아포시닌(apocynin)을 투여함으로써 백서의 급성 폐손상을 감소시키고 생존을 향상시키는 지 알아보고자 연구를 수행하였다.

방법: 스프라그-다울리(Sprague-Dawley) 수컷 백서를 이용하여 마취 후 대퇴부 절개 및 대퇴동맥 삽관을 시행한 후 대퇴동맥에서 채혈함으로써 출혈성 쇼크를 유발하였다. 이후 평균동맥압을 20-25 mmHg으로

40분간 유지하였다. 출혈성 쇼크 유발 후 채혈된 피를 다시 주입하면서 동시에 기초제(vehicle, HS군), 저용량 아포시닌(20 mg/kg, HS + LD-Apo군), 고용량 아포시닌(40 mg/kg, HS + HD-Apo군)을 복강 내 투여하였다. 각 군에 대하여 10분마다 혈압을 측정하였고, 출혈성 쇼크 유발 전과 유발 후 30분, 60분에 각각 동맥혈을 채취하였다. 출혈성 쇼크 유발 후 72시간 동안 백서의 생존여부를 관찰하였다. 또 다른 실험에서는 장기 손상 여부 및 생화학적 측정을 위하여 출혈성 쇼크 유발 이후 6시간째 채혈 및 장기 적출을 시행하였다. 적출된 폐 조직에서 gp91-phox (Nox2) 발현, Nox 활성도, cytoplasmic phosphorylated inhibitor κB-α (*p*-IκB-α) 발현, nuclear factor-κB (NF-κB) p65 DNA 결합 활성도, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) 유전자 발현, malondialdehyde (MDA) 측정, myeloperoxidase (MPO) 활성도, 폐조직의 손상 정도를 측정하였다.

결과: Sham군, HS군, HS + LD-Apo군, HS + HD-Apo군에서의 생존률은 각각 100% (5/5), 30% (3/10), 40% (4/10), 70% (7/10)이었다. 고용량 아포시닌군의 적출된 폐조직에서 Nox2 발현, Nox 활성도, MDA 측정치가 감소하였고, 또한 p-IκB-α 발현, NF-κB p65 DNA 결합 활성도, TNF-α, IL-6 유전자 발현과 MPO 활성도가 감소하였다. 하지만 생존율의 차이는 보여주지 못했다.

결론: 백서의 출혈성 쇼크 모델에서 고용량의 아포시닌을 투여함으로써

Nox 활성도가 감소하고 전염증성 사이토카인(proinflammatory cytokine)의 생성이 줄고 폐 염증반응이 감소하여 폐손상이 감소하였다.

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주요어: 출혈성 쇼크, 아포시닌, 급성 폐손상, NADPH oxidase, NF-kappa B

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