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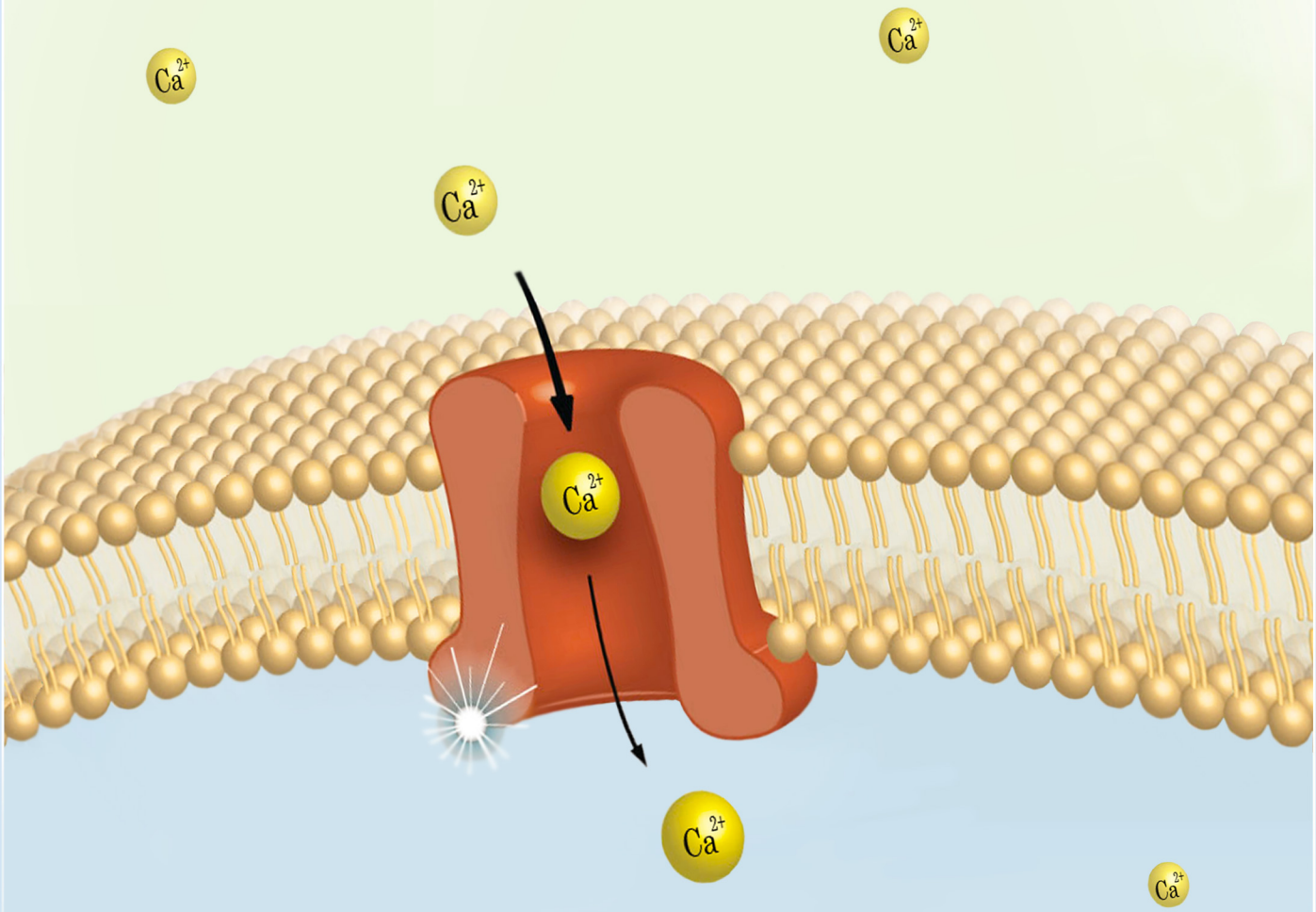

Neuroscience Research Center

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AIM AND SCOPES

Journal of Cellular Neuroscience and Oxidative Stress is an online journal that publishes original research articles, reviews and short reviews on the molecular basis of biophysical, physiological and pharmacological processes that regulate cellular function, and the control or alteration of these processes by the action of receptors, neurotransmitters, second messengers, cation, anions, drugs or disease.

Areas of particular interest are four topics. They are;

A- Ion Channels (Na^+- K^+ Channels, Cl^- channels, Ca^{2+} channels, ADP-Ribose and metabolism of NAD^+, Patch- Clamp applications)

B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals)

C- Interaction Between Oxidative Stress and Ion Channels in Neuroscience
(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels, role of TRPM2 channels in neurodegenerative diseases such Parkinson's and Alzheimer's diseases)

D- Gene and Oxidative Stress
(Gene abnormalities. Interaction between gene and free radicals. Gene anomalies and iron. Role of radiation and cancer on gene polymorphism)

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Biophysics	Biochemistry
Biology	Biomedical Engineering
Pharmacology	PhysiologyGenetics
Cardiology	Neurology
Oncology	Psychiatry
Neuroscience	Neuropharmacology

Keywords
Ion channels, cell biochemistry, biophysics, calcium signaling, cellular function, cellular physiology, metabolism, apoptosis, lipid peroxidation, nitric oxide synthase, ageing, antioxidants, neuropathy, traumatic brain injury, spinal cord injury, Alzheimer' s Disease, Parkinson's Disease.

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The Effects of Delayed Fluid Resuscitation on Lung Oxidative Stress and Antioxidant Vitamin Levels in Controlled Hemorrhagic Shock: An Experimental Study

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List of Abbreviations

BAL, bronchoalveolar lavage
BALF, bronchoalveolar lavage fluid
CAT, catalase
DR, delayed resuscitation
ER, early resuscitation
GSH, reduced glutathione
GSH-Px, glutathione peroxidase
HS, hemorrhagic shock
LP, lipid peroxidation
MDA, Malondialdehyde
PMNL, polymorphonuclear leukocyte
ROS, reactive oxygen species
SOD, superoxide dismutase

ABSTRACT

We aimed to determine the effects of delayed fluid resuscitation on the lung oxidative stress and antioxidant vitamin levels in a rat model of controlled hemorrhagic shock (HS). Male Wistar rats were exposed to controlled HS via arterial catheterization to reduce mean arterial pressure (MAP) to 40 mmHg over 10 minutes. Two groups were constituted according to resuscitation time: early (n=6) and delayed (n=5), respectively resuscitated 30 or 90 minutes after HS. A control group (n=5) was subjected to catheterization only. Intravenous fluid resuscitation was done with Ringer lactate solution. After 24 hours, bronchoalveolar lavage (BAL) was performed and the lungs were harvested for biochemical, cytological and histopathological analyses. Lipid peroxidation (as MDA), reduced glutathione (GSH), glutathione peroxidase (GSH-Px) and vitamin A, vitamin C and vitamin E levels were measured in both BAL fluid (BALF) and lung homogenate. Lung tissue GSH-Px and vitamin E levels are increased in both HS groups compared to the control group. No significant differences were found in MDA, GSH, vitamin A and vitamin C BALF levels among all groups, except for GSH-Px ($p=0.007$). Intracellular antioxidants, especially GSH-Px and vitamin E, increase in the lungs of rats (in both HS groups), possibly due to increased oxidative stress and increased physiological requirements after HS and resuscitation.

Keywords: Antioxidants; Hemorrhagic shock; Lung; Oxidative stress; Vitamin

Introduction

Acute lung injury (ALI) and subsequent acute respiratory distress syndrome (ARDS) are major causes of acute respiratory failure with significant morbidity and mortality. Major trauma itself and subsequent major hemorrhage and hemorrhagic shock (HS) are the main reasons of trauma-related multiple organ failure in, ALI and ARDS. Lung ischemia induces the generation of oxidants by the pulmonary endothelium, and the release of elastases and proteases from neutrophils leading to endothelial damage and alveolo-capillary barrier leakage. This leads to further sequestration of neutrophils and increased interstitial and alveolar edema. Increased alveolar cytokine expression, cell injury and lipid peroxidation in the lung are other prominent features of ALI (Conrad and Bidani, 2002; Matthay et al., 2003; Sharp et al., 2015). Clinically, it is characterized by impaired gas exchange, dyspnea, decreased static compliance and non-hydrostatic pulmonary edema (Bernard, 1991; Sharp et al., 2015).

HS is characterized by a loss in circulatory volume which leads to early death in humans from multiple organ failure, especially in the cardiovascular system, liver, kidney, lung and brain (Sauaia et al., 1995). Although traditional management of HS involves rapid fluid infusion to restore blood pressure, it has been found that aggressive early volume resuscitation without surgical control is associated with increased bleeding due to dilution of clotting factors (Lee et al., 2007; Santibanez-Gallerani et al., 2001). Nevertheless, despite these detrimental effects of early fluid resuscitation, little is known about different resuscitation speeds used during the treatment of HS. In one study, it has been found that rapid fluid resuscitation ameliorates hyperglycemia and inflammatory response after HS (Subeq et al., 2009). In contrast to this, new studies have shown that initially slow rate resuscitation with limited-volume significantly decreases body temperature resulting decreased organ damage caused by HS (Subeq et al., 2012; Yu et al., 2014).

Severe HS is believed to render the host vulnerable to a second minor inflammatory stimulus, the so called ‘second hit’, contributing to the inappropriate activation of neutrophils and endothelium and leading to the development of systemic organ dysfunction (Moore et al., 1993; Swank and Moore, 1989). After resuscitation following HS, aberrant and unbridled neutrophil-endothelial interactions are believed to contribute to inappropriate neutrophil activation with the release of toxic reactive oxygen species (ROS) and proteinases that promote organ injury (Botha et al., 1997; Botha et al., 1995). Some concerns also remain about the consequences of prolonged shock in patients receiving delayed fluid resuscitation (Lee et al., 2007). Lee *et al.* showed that delaying fluid resuscitation for 30 - 60 minutes after HS increased the production of proinflammatory cytokines while cytokine release correlated with the length of resuscitation delay in a rat model of volume-controlled HS (Lee et al., 2007). For uncontrolled HS, 90 minutes of hypotensive resuscitation was the maximum tolerance limit for the body; more than 90 minutes of hypotensive resuscitation caused severe organ damage and worse outcomes (Li et al., 2011; Yang et al., 2015).

ROS generated from hypoxia after blood loss play an important role in disease progression. If generated excessively or when enzymatic and non-enzymatic defense systems are impaired, ROS act as subcellular messengers in complex processes, such as mitogenic signal transduction, gene expression, and regulation of cell proliferation (Naziroglu, 2007; Naziroglu et al., 2013). Enzymatic antioxidants include superoxide dismutase (SOD), reduced glutathione peroxidase (GSH-Px), and catalase (CAT), while non-enzymatic antioxidants include thiol antioxidants (thioredoxin, glutathione (GSH) and lipoic acid), vitamin E and vitamin C.

As the first line of defense against ROS, SOD specifically converts superoxide radicals to hydrogen peroxide which can be later transformed into water and oxygen. GSH-Px, the major intracellular antioxidant enzyme, detoxifies hydrogen peroxide into water while also removing organic hydroperoxides. As a hydroxyl radical and singlet oxygen scavenger, GSH participates in a wide range of cellular functions (Whanger, 2001). One of the most important degradation products of lipid oxidation and oxygen-derived free radicals is malonyl dialdehyde (MDA). Vitamin E (α-tocopherol) and vitamin C are well studied antioxidant vitamins. Vitamin E is a lipophilic powerful membrane-bound antioxidant found in the lung and thought to play a role against oxidative lung injury (Heffner and Repine, 1989; Hensley et al., 2004). Vitamin C is an aqueous phase hydrophilic antioxidant molecule that can

scavenge several hydroxyl radical species. There is a close interaction between vitamin C, GSH and vitamin E (Hensley et al., 2004; Kojo, 2004), because vitamin C not only functions directly as an antioxidant, but also transforms oxidized vitamin E into its active form by reducing tocopherol radicals (Chan, 1993; Kojo, 2004). Vitamin A, which serves as a prohormone for retinoids, is an important factor in promoting the normal respiratory epithelial differentiation and growth (Georgieff et al., 1991). The protective and regenerative role of retinoids for lung cells against oxidative damage has been shown in multiple mouse models (Hind and Maden, 2004; Maden and Hind, 2004; Massaro and Massaro, 2000).

In the light of this background, we aimed to evaluate the effect of resuscitation time (early resuscitation (ER) vs delayed resuscitation (DR)) on inflammatory responses, especially in terms of lung oxidative stress and antioxidant vitamin levels, without any protective treatment for ALI in the pressure controlled HS rat model.

Materials and Methods

Animal care:

Adult male Wistar albino rats weighing 240±20 gr were housed in a temperature controlled room (20 - 25°C; humidity 55 - 60%) on a 12h light:dark cycle. Standard rat pellet food and tap water were available *ad libitum* for the duration of the experiments unless otherwise noted. This study was approved by the Institutional Animal Care and Use Committee (26.6.2008/05), and was conducted in compliance with the Animal Welfare Act and principles stated in the *Guide for the Care and Use of Laboratory Animals* prepared by Suleyman Demirel University.

Group definition:

Seventeen rats were used, randomized into three groups. Group I (n=5) formed the control group, while groups II, (n=6) and III (n=6) were taken as the controlled HS groups, which respectively resuscitated after 30 (ER) or 90 minutes (DR) after the shock period. These time points simulate clinically relevant times for transferring of human victims to the nearest hospital in suburban and urban contexts (Sukumaran et al., 2005).

Anesthesia and controlled hemorrhagic shock model:

The animals were anesthetized with 100 mg/kg ketamine and 4 mg/kg xylazine, intraperitoneally. The right carotid artery of each animal, including the controls, was cannulated with a 24-gauge angio-catheter. Mean arterial pressure (MAP) was monitored continuously with a pressure transducer (Bicakcilar, Turkey). In the HS groups, hemorrhage was carried out via carotid arterial catheter to reduce MAP to 40 mmHg over 10 minutes. Hypotension was maintained for 30 minutes simulating field medical response times in group II (HS/ER) and 90 minutes in group III (HS/DR) by further blood withdrawals if MAP rose above 45 mmHg or by reinfusion of withdrawn blood if MAP fell below 35 mmHg. At the end of the shock period, intravenous fluid resuscitation was applied with Ringer lactate at double the blood volume. After resuscitation, the catheter was removed and the artery was ligated. The animals were allowed to survive the next 24 hours following their daily biological rhythm. Paracetamol was given for postsurgical analgesia at 15 mg/kg twice daily, intraperitoneally.

Bronchoalveolar lavage (BAL):

One rat in group III was died before 24 hours. In the remaining 16 rats, a midline cervical incision was made to isolate the trachea. A feeding tube (No: 8) was inserted in to the trachea and stabilized with 4/0 silk suture to perform bronchoalveolar lavage (BAL), after which all rats were sacrificed by decapitation. Both lungs were harvested for histopatological and biochemical analyses.

Bronchoalveolar lavage fluid and its analysis:

The lungs were lavaged using the feeding tube with a solution containing cold phosphate buffered saline (PBS), 8 mmol/L sodium phosphate, 2 mmol/L potassium phosphate, 0.14 mol/L sodium chloride, 0.01 mol/L potassium chloride (pH, 7.4) and 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA). Twenty mL of fluid was used for lavage in each animal with about 90% being re-collected. The BALF was centrifuged at 200 x g for 15 min with brakeless stopping. Cytospin slides were prepared for each animal (Shandon Southern Instruments, PA, USA). Each slide was dried on air and stained with Wright-Giemsa. A total of 400 cells (neutrophils, lymphocytes and alveolar macrophages)

were counted from each slide under high-power magnification (400 ×)(Olympus CX41, Japan). The cells counted under the microscope were expressed as a percentage. The BALF supernatant of was collected and stored at -80^{0}C for biochemical analysis until analyzed.

MDA and GSH levels in lung tissue homogenates:

Lung tissue homogenates were stored at –80^{0}C until assay (Sanyo, Japan). The lung tissues were homogenized with a mechanic homogenizator (Ultra-Turrax T25, IKA, Germany) and sonicated (Bandelin D.12207, Germany) in phosphate buffered saline (pH, 7.4). The homogenate was centrifuged (Kubata, Japan) at 16.700 x g for 15 min at 4 ^{0}C. The lung homogenate supernatant was used for determination of the MDA, GSH and GSH-Px values.

Lipid peroxidation level determinations:

Lipid peroxidation levels in BALF and lung homogenate were determined using the thiobarbituric-acid reaction by the method of Placer *et al.*(Placer et al., 1966). Quantification of thiobarbituric acid reactive substances in each sample was done by comparing absorption to the standard curve of MDA equivalents generated by acid catalyzed hydrolysis of 1,1,3,3 tetramethoxypropane. The values of lipid peroxidation in BALF and lung homogenate were expressed as nmol/ml or μmol/g protein**,** respectively.

Reduced glutathione (GSH), glutathione peroxidase (GSH-Px) and protein assay:

The GSH contents and GSH-Px activities of lung homogenate and BALF were measured spectrophotometrically at 412 nm (Lawrence and Burk, 1976; Sedlak and Lindsay, 1968). GSH oxidation was catalyzed by cumene hydroperoxide. In the presence of GSH reductase and NADPH, the oxidized glutathione was immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The enzymatic reaction was stopped on 10 minutes using trichloroacetic acid. The decrease in absorbance at 412 nm against the blank was measured spectrophotometrically. Lung homogenate protein amount was also determined with the fonil fenol reagent according to Lowry *et al.* (Lowry et al., 1951). Protein level in the sample was estimated against a standard curve for bovine serum albumin.

Vitamin A, vitamin E and vitamin C analyses:

A modification of the method of Desai (Desai, 1984) and Suzuki and Katoh (Suzuki and Katoh, 1990) was used to determined Vitamin A (retinol) and vitamin E (a-tocopherol) levels in the BALF and lung homogenate. Approximately 0.5 ml plasma and 0.5 g lung samples were saponified by adding of 0.3 ml of 60% (w/v in water) KOH and 2 ml of 1% (w/v in ethanol) ascorbic acid, followed by heating at 70 ^{0}C for 30 minutes. After cooling the samples on ice, 2 ml of water and 1ml of n-hexane were mixed with the samples, which were then rested for 10 minutes to allow phase separation. A 0.5 ml sample of n-hexane extract was removed and vitamin A levels were measured at 325 nm. Ferrichloride and bathophenetroline reagents were added to the remainder; with the pink color of the hexane extract was measured spectrophotometrically at 535 nm. Calibration was performed using standard solutions of all-trans retinol and α-tocopherol in hexane, respectively. Quantification of ascorbic acid in BALF and lung samples was measured using the method of Jagota and Dani (Jagota and Dani, 1982). Vitamin C levels were determined by redox titration between ascorbic acid and 2,6-dichloroindophenol. Homogenate was precipitated on ice with trichloroacetic acid centrifuged at 12,000 × *g* for 5 min. Supernatant was subsequently diluted with distilled water. Folin-Ciocalteau's solution was added to the samples, which were then immediately mixed. After 10 min., the absorbance at 760 nm of the blue color developed was measured spectrophotometrically. The sample values were compared with values of standard samples of ascorbic acid prepared in distilled water. All chemicals used were analytical grade (Sigma –Aldrich, Germany).

Histopathological Examination:

The entire left lung was used for histopathological examination. Sections in 5 μm thickness was obtained from formalin fixed paraffin embedded tissues and stained with hematoxylin-eosin. Slides were examined by a pathologist in a blinded manner. For each animal, inflammatory cell infiltration (interstitial and alveolar neutrophils) and interalveolar septum thickness were graded as 0 (none), 1 (scant), 2 (moderate), or 3 (extensive) points to compose a total lung histology score which was the sum of the individual parameter scores (Bachofen and Weibel, 1982).

Statistical Analysis

Data were analyzed using Statistical Package for the Social Sciences version 15.0 for Windows (SPSS-15.0, Chicago, IL, USA). Since there were few animals in each group, histopathological and biochemical results were evaluated using the Kruskal–Wallis non-parametric test for any differences between groups. In the presence of statistical significance we proceed to Mann-Whitney U-test for between-groups comparisons with Bonferroni correction for multiple comparisons. Results were presented as means ± SD. *P* values less than 0.05 for the Kruskal-Wallis test and less than 0.0167 for the Mann-Whitney U test were regarded as statistically significant.

Results

Biochemical results:

Table 1 shows lung homogenate MDA, GSH, GSH-Px, antioxidant vitamin A, vitamin C and vitamin E levels. Lung homogenate GSH-Px and vitamin E levels varied significantly among the groups (p=0.027 and p=0.001, respectively). Lung tissue GSH-Px activity was significantly higher in HS/DR group than the control group (p=0.009), but there was no significant difference between HS/DR and HS/ER or between HS/ER and control groups. Both HS groups (HS/ER and HS/DR) had higher lung tissue vitamin E levels than the control group (p=0.009 and p=0.006, respectively).

Table 1
Effects of delayed fluid resuscitation on lipid peroxidation (MDA), reduced glutathione (GSH) and glutathione peroxidation (GSH-Px) levels and antioxidant vitamin values in the lung homogenate of rats.

	MDA (µmol/g prot)	GSH (µmol/g prot)	GSH-Px (IU/g prot)	Vit-A (µmol/gr tissue)	Vit-C (µmol/g tissue)	Vit-E (µmol/g tissue)
I. Control (n=5)	57.9 ± 13.3	7.4 ± 0.9	8.0 ± 0.9	4.4 ± 0.2	43.1 ± 10.3	5.0 ± 0.3
II. HS/ER (n=6)	58.4 ± 6.7	8.5 ± 1.0	8.1 ± 1.2	4.0 ± 0.7	35.2 ± 9.3	8.6 ± 0.4
III.HS/DR (n=5)	61.0 ± 13.3	9.1 ± 1.1	10.4 ± 1.5	4.0 ± 0.4	34.1 ± 9.5	10.6 ± 0.7
P VALUES ✝						
I-II	NS	NS	NS	NS	NS	0.004
I-III	NS	0.047	0.009	NS	NS	0.008
II-III	NS	NS	0.052	NS	NS	0.004

GSH; reduced glutathione, GSH-Px; glutathione peroxidase, HS/DR; hemorrhagic shock and delayed resuscitation after 90 min, HS/ER; hemorrhagic shock and early resuscitation after 30 min, MDA; malondialdehyde, ✝; Mann Whitney-U and Kruskall Wallis tests, NS; not significant, Vit-A; vitamin A, Vit-C; vitamin C, Vit-E; vitamin E. All samples were read six times.

Table 2
Effects of delayed fluid resuscitation on BALF lipid peroxidation (MDA), reduced glutathione (GSH) and glutathione peroxidation (GSH-Px) values and antioxidant vitamin levels.

	MDA (pmol/ml)	GSH (nmol/ml)	GSH-Px (IU/L)	Vit-A (nmol/L)	Vit-C (nmol/L)
I. Control (n=5)	32.4 ± 5.0	222 ± 30	44.03±10.27	3.6 ± 1	19 ± 6
II.HS/ER (n=6)	36.8 ± 5.1	203 ± 8	135.75±12.99	3.8 ± 1	20 ± 6
III.HS/DR (n=5)	37.6 ± 8.0	212 ± 8	134.11±7.89	3 ± 0.7	16 ± 4
P VALUES					
I-II	NS	NS	0.004	NS	NS
I-III	NS	NS	0.008	NS	NS
II-III	NS	NS	NS	NS	NS

GSH; reduced glutathione, GSH-Px; glutathione peroxidase, HS/DR; hemorrhagic shock and delayed resuscitation after 90 min, HS/ER; hemorrhagic shock and early resuscitation after 30 min, MDA; malondialdehyde, NS; not significant, Vit-A; vitamin A, Vit-C; vitamin C.

Table 3
Analysis of total lung tissue histological score, comprising interstitial septal thickening and alveolar/interstitial leukocyte infiltration.

	Alveolar leukocyte Infiltration†	Interstitial leukocyte infiltration†	Interstitial septum Thickening†	Total histologic score †
Control (n = 5)	0.1 ± 0.1	0.5 ± 0.1	0.0 ± 0.0	0.6 ± 0.1
HS/ER (n = 6)	1.0 ± 0.1	1.7 ± 0.2	1.3 ± 0.2	4.0 ± 0.5
HS/DR (n = 5)	1.6 ± 0.2	2.2 ± 0.2	1.8 ± 0.3	5.6 ± 0.7
P$^{\Psi}$	0.004*, 0.008**, 0.01***			

HS/DR; hemorrhagic shock and delayed resuscitation after 90 min, HS/ER; hemorrhagic shock and early resuscitation after 30 min, †; mean ± SD, $^{\Psi}$; for total histologic scores, * in comparison with control vs HS/ER group; ** control vs HS/DR group and *** HS/ER vs HS/DR.

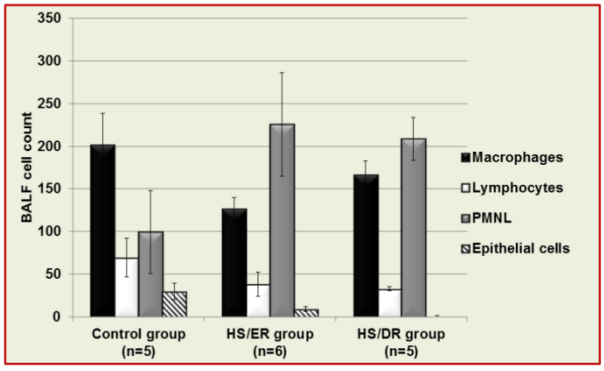
In the HS/DR group lung tissue mean vitamin E level was also higher than in the HS/ER group (p=0.006). However, lung homogenate MDA, GSH, vitamin C and vitamin A levels did not differ significantly among the three groups.

Table 2 shows mean MDA, GSH, GSH-Px, vitamin A and vitamin C levels in the BALF. No data on vitamin E in BALF are reported since BALF vitamin E levels were too low to detect with spectrophotometrically. BALF MDA, GSH, vitamin A and vitamin C levels did not differ significantly among the three groups. However, HS/DR and HS/ER groups had higher BALF GSH-Px activity than the control group (p=0.009 and p=0.006, respectively), but GSH-Px activity did not differ between HS/ER and HS/DR groups.

BAL fluid cell differentiation:

BALF cell count was affected by resuscitation time in the HS groups, with increased neutrophil counts and decreased macrophage and lymphocyte counts in the BALFs of HS groups compared to the control group. However, these differences were not statistically significant (Figure 1).

Figure 1
Delayed fluid resuscitation causes increase in neutrophils and decrease in macrophages and lymphocytes in BALF of rats (p>0.05).

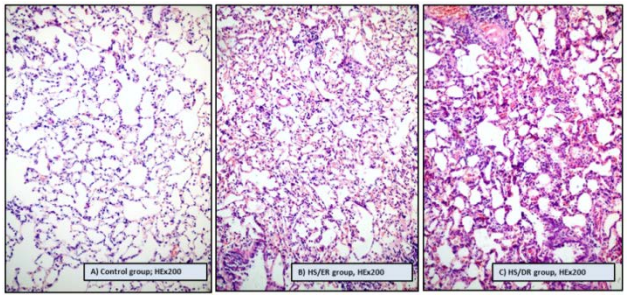


BAL; bronchoalveolar lavage, BALF; bronchoalveolar lavage fluid, HS/DR; hemorrhagic shock and delayed resuscitation after 90 min, HS/ER; hemorrhagic shock and early resuscitation after 30 min

Histopathological results:

Histopathological examination of the lungs showed evidence of prominent inflammatory reaction and destruction of normal alveolar structure in both HS groups, but no signs of lung damage in the control rats (Figure 2). Interstitial septum thickening, alveolar polymorphonuclear leukocytes (PMNL), interstitial PMNL and total histological scores were significantly different among the study groups (p=0.004, p=0.002, p=0.003, p=0.002, respectively). Both HS groups induced more prominent PMNL accumulation in the alveolar and interstitial spaces than the control group. In addition, the HS/DR group had a higher total histological score than the HS/ER group (p=0.01), while interstitial thickening and PMNL accumulation were non-significantly higher in the HS/DR group than the HS/ER group (Table 3 and Figure 2).

Figure 2
Histopathological examination of lung tissues showed hallmarks of ARDS damage in hemorrhagic shock groups, whereas A) no damage in the control group (HEx200); B) minimum alveolar / interstitiel PMNL infiltration and septal thickening in the lungs of rats subjected to pressure-controlled hemorrhagic shock and early fluid resuscitation begun 30 min after shock (HS/ER group) (HEx200); C) more prominent PMNL infiltration and alveolar septal thickening in pressure-controlled hemorrhagic shock and delayed fluid resuscitation begun 90 min after shock (HS/DR group) (HEx200)



Discussion

The clinical syndrome of acute lung injury (ALI) occurs as a result of an initial acute systemic inflammatory response. This can be consequent to a plethora of insults, either direct to the lung or indirect. The insult results in increased alveolar cytokine expression and epithelial permeability, leading to neutrophil and monocyte recruitment, oxidative stress, alveolar cell apoptosis and alveolar flooding with a protein-rich oedema fluid. The resulting loss of gas exchange leads to acute respiratory failure and typically catastrophic illness, termed acute respiratory distress syndrome (ARDS), requiring ventilatory and critical care support (Matthay et al., 2003; Sharp et al., 2015). As presented before (Alkan et al., 2006; Keller et al., 2003; Zheng et al., 2008), and also in this study, we once again found that the lung, as a remote organ, was affected by controlled HS, resulting in increased intra-alveolar and interstitial neutrophil infiltration characteristic of ALI. Zakaria et al. (Zakaria el et al., 2007) showed that HS induced neutrophil sequestration

as measured with myeloperoxidase, a marker of neutrophil infiltration, has both time dependent and organ specific trends. For lung tissue, neutrophil sequestration begins as early as one hour after resuscitation before peaking four hours after shock and resuscitation (Zakaria el et al., 2007). Similar findings were obtained in our study, namely that excessive neutrophil infiltration and more severe lung injury occurred when resuscitation was delayed for 90 minutes.

Delayed fluid resuscitation after HS potentially leads to multiple-organ dysfunction syndrome and systemic inflammatory response as a consequence of ALI. Lee *et al.* found markedly different profiles of cytokine activation in animals depending on the delay in resuscitation, even if they were subjected to similar degree of shock and resuscitation. Higher circulating TNF-α and IL-6 levels and lower IL-10 concentrations have been observed in both immediate and delayed resuscitation groups compared to the unresuscitated group. Moreover, cytokine release was correlated with the length of delay before resuscitation in animals subject to HS (Lee et al., 2007)

Free radical-induced reactions play an important role in the pathophysiology of circulatory shock. Increased production of ROS including superoxide radical, hydroxyl radical, (hydrogen peroxide (H_2O_2), peroxynitrite, (ROOH) hydroperoxide and lipid peroxidation has been shown to be implicated in ischemic and hemorrhagic tissue injury (Zheng et al., 2008). In this study, we showed that animals resuscitated at two different delayed times had markedly impaired oxidant/antioxidant balance. Animals receiving delayed fluid resuscitation had increased MDA levels, a marker of lipid peroxidation, compared to controls, although this difference was not statistically significant. This might be due to small sample size and/or the weakness of the assay used to determine lipid peroxidation. It should be kept in mind that this assay is not regarded as specific or sensitive. Antioxidant systems are generally augmented by the accumulation of free radicals. GSH-Px, a well-known selenium-containing enzyme, is the main enzyme of the enzymatic antioxidant defense system, responsible for protection against increased ROS production (Naziroglu and Ozgul, 2013). The integrity of both cellular and subcellular membranes depends heavily on its functions, which are reducing lipid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water.

In the current study, higher levels of reduced-GSH, although it was not statistically significant, and higher GSH-Px in the lung homogenates were found in the HS/DR group than the control group. Parallel to this, BALF GSH-Px was also significantly higher in both HS groups than the control group. In this study, characteristic features of ALI, such as pulmonary edema, intraalveolar/interstitial PMNL infiltration and interalveolar septal thickening were all clearly expressed in animals resuscitated at both resuscitation times. These results are consistent with increased demand for antioxidant activity in the lungs of rats in response to oxidative stress in ALI due to HS and resuscitation.

Most vitamins are known to have a variable degree of antioxidant activity; the highest antioxidant potential among all known vitamins is vitamin A (Olson, 1996). Supplementing antioxidant vitamins to protect cells from oxidative stress is a well-studied topic. However, to the best of our knowledge, ours is the first study to investigate the oxidant/antioxidant balance and antioxidant vitamin levels in the lung tissue of animals subjected to HS and resuscitation without any protective administration of antioxidants.

Vitamin A has multiple functions in cell regulation, maintenance of epithelial cell integrity, resistance to infection and antioxidant roles. It regulates the endogenous activities of scavenging enzymes, which are usually elevated under oxidative stress (Keller et al., 2003). Retinol also protects mitochondria via chelation of ROS and serves as a more effective lipoperoxyl radical scavenger at low partial pressures of oxygen (Jagota and Dani, 1982). ALI is one of the major reasons for hypoxemia. However, contrary to expectations, retinol levels in the lungs and BALFs did not differ among groups in our study. While retinol levels slightly lower in the HS groups, we had predicted decreased larger fall because retinol is consumed during lipid oxidation through self-oxidation reactions, being oxidized to 5,6-retinol epoxide, thereby removing the excess free radicals (Bachofen and Weibel, 1982).

Vitamin C is the strongest physiological antioxidant vitamin acting in an organism's aqueous environment (Frei et al., 1989). It protects cells against oxidative stress by transforming vitamin E to its active

form and raising intracellular glutathione levels. In physiological conditions, vitamin E molar concentration in membranes is quite low, but with its lipophilic properties it acts in the cellular membrane to protect cells against the effects of free radicals which are potentially damaging by products of the body's metabolism (Naziroglu and Ozgul, 2013).

Consistent with this, we found significantly higher lung homogenate vitamin E content in the HS/DR and HS/ER groups than the control group. Although there were no statistically significant differences in vitamin C content in both the rat lung homogenate and BALF, lung homogenate vitamin C levels were lower in the HS groups than control group. Our results may therefore indicate that vitamin E content in the lung tissues of animals may be increased to protect against oxidative stress whereas vitamin C may be used as a scavenger of free radicals and lipid peroxidation in the rat lungs after HS and resuscitation. In contrast to our study, Schmidt *et al.* (Schmidt et al., 2004b) found a significant increase in antioxidant compounds [GSH, vitamin E, vitamin A, vitamin C, uric acid, and plasmalogens (1-O-alkenyl-2-acyl-phospholipids)] in BALF of isolated rabbit lung under both anoxic and hyperoxic ventilation, with maximum levels occurring after 3 hours of ischemia (Schmidt et al., 2004b).

In the literature, the protective effect of vitamin C in HS experimental models is debatable. Some previous studies have shown that vitamin C does not have a protective effect against free radical generation and does not improve survival in HS conditions in rats (Daughters et al., 1996; Minor et al., 1996). On the other hand, recent studies have shown that resuscitation with lyophilized plasma reconstituted with ascorbic acid reduces inflammation in HS (Hamilton et al., 2011; Van et al., 2011). New animal studies have also found that pharmacological preconditioning or treatment with vitamin C attenuates HS related multiple organ injury via the induction of heme oxygenase-1 (Zhao et al., 2014a; Zhao et al., 2014b).

Turning to human studies, an adaptive response of antioxidants to oxidative stress is an increase in the majority of antioxidants, including uric acid, ascorbic acid, retinol, and alpha-tocopherol whereas unchanged glutathione levels in the BALF of patients with interstitial lung diseases or ARDS (Markart et al., 2009; Schmidt et al., 2004a). In these studies, alveolar oxidative stress is associated with elevated levels of nonenzymatic low-molecular-weight antioxidants.

Our study has several limitations. First, we used pressure-controlled HS in a rat model, which is not so similar to the clinical situation. In an uncontrolled HS model, the volume of blood loss and the severity of shock may not be controlled. Our primary aim was to evaluate the effect of delayed fluid resuscitation on the antioxidant status of lung after HS. Second, the study had a small size in order to minimize the number of required animals. Unfortunately, one of them also died during experiment. Third, we used spectrofotometric methods for the biochemical analysis instead of High Performance Liquid Chromatography (HPLC), which produces more precise results. Fourth, the effect of the rapidity of resuscitation fluid on the development of ALI was not evaluated in this study, although it may have a potential role.

In conclusion, delayed fluid resuscitation after HS induces more prominent lung injury than early resuscitation. In response to lung injury, intracellular antioxidants, especially GSH-Px and vitamin E, increase in a time dependent manner both BALF and lung homogenate of rats exposed to resuscitation after HS.

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Authors' roles: AB formulated the hypothesis and was responsible for writing the report. MN was responsible for the antioxidant analyses. SB performed the pathological analysis. NS made critical revisions to the manuscript. SY, AB and EE did animal controlled hemorrhagic shock/resuscitation experiment.

Declaration of interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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