DOI: 10.4149/BLL_2022_132

Bratisl Med J 2022; 123 (11)

828 – 832

EXPERIMENTAL STUDY

Vitamins C and E protect from sepsis-induced lung damage in rat and CT correlation

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ABSTRACT

BACKGROUND: Sepsis is one of the leading causes of death in intensive care units worldwide. Vitamins C and E are natural antioxidants and anti-inflammatory agents. Suppressing the inflammation is an important treatment target because it plays a role in the pathophysiology of sepsis. The purpose of this study was to investigate the effect of vitamins C and E treatment in rats with sepsis-induced lung damage.

METHODS: In this animal study, fecal intraperitoneal injection procedure (FIP) was performed on 30 of 40 rats included for creating a sepsis model. Rats were randomly assigned into four groups: Group 1, control group (no procedure was applied, n = 10), Group 2, FIP (untreated septic group n = 10), Group 3, FIP+vitC (treated with 500 mg/kg/day ascorbic acid, n = 10), and Group 4, FIP+vitE (treated with 300 mg/kg/day alpha-tocopherol, n = 10). Chest CT was performed in all rats and density of the lungs was measured by using Hounsfield unit (HU). Histopathological examination of lung damage was performed, and blood samples were collected for biochemical analysis.

RESULTS: TNF-α, CRP, IL 1-β, IL-6, and MDA plasma levels in groups treated with vitamin C or vitamin E were lower than in the FIP group. Histological scores in groups treated either with vitamin C or vitamin E were significantly lower as compared to those in the FIP group. The HU value of lung in groups treated wither with vitamin C or vitamin E were lower than that in the FIP group ($p < 0.05$).

CONCLUSION: The rats treated either with vitamin C or E showed improved results for sepsis. We think that they can be used as adjuvant therapy for septic patients because of their effectivity and low costs *(Tab. 3, Fig. 2, Ref. 27)*. Text in PDF *www.elis.sk*

KEY WORDS: acute lung injury, sepsis, vitamin C, vitamin E, antioxidant.

Introduction

Sepsis is one of the leading causes of death in intensive care units worldwide and is an important public health problem (1). Physiological, pathological and biochemical abnormalities caused by infection in sepsis result in organ dysfunction (1, 2).

Sepsis-induced acute lung injury (ALI) is an acute and persistent lung inflammation characterized by increased microvascular permeability, hypoxemia, hypercapnia, diffuse infiltration on the chest X-ray, and decreased pulmonary compliance (1,2). As a result of inflammatory reactions in the alveolo-capillary membrane in the lung, cellular and biochemical mediators are activated, proteolytic enzymes and free oxygen radicals (ROS) appear (3).

Ascorbic acid (vitamin C) and a-tocopherol (vitamin E) exert antioxidant effects by preventing lipid peroxidation in cells and regulating the activity of NADPH oxidase and superoxide dismutase (4, 5). Prolyl hydroxylases are involved in the regulation of hypoxia-inducible factor (HIF)-1, a transcription factor that regulates many genes responsible for apoptosis (6). Inhibition of the HIF pathway with vitamin C may be beneficial in controlling infections and inflammation (6). Vitamin E is an essential fat-soluble antioxidant that scavenges hydroperoxyl radicals in the lipid environment (7). As a water-soluble antioxidant, vitamin C protects against cellular damage caused by oxidative stress through ROS scavenging, vitamin E-dependent neutralization of lipid hydroperoxyl radicals, and protection of electrophilic lipid peroxidation products and proteins from alkylation (7, 8). Therefore, septic patients may benefit from antioxidant therapy aimed at reducing microvascular inflammation (9, 10, 11).

Our hypothesis was that vitamins C and E would decrease malondialdehyde (MDA) levels as a parameter of lipid peroxida-

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tion (12), systemic proinflammatory cytokines, interleukin-6 (IL-6), interleukin 1-beta (IL 1-β), tumor necrosis factor alpha (TNF-α), c-reactive protein (CRP) and lactic acid (LA). The purpose of this study was to investigate the effect of treatment with vitamins C and E in rats with sepsis-induced lung damage.

Materials and methods

Animals

In this study, 40 male Wistar albino mature rats weighing 200–250 g were used. The experiments performed in this study have been carried out according to the rules in the Guidelines for the Care and Use of Laboratory Animals adopted by National Institutes of Health (U.S.A). The present study received Animal Ethics Committee's consent (Science University, Ethical number: 22210902). The rats used in the experiment were obtained from Experimental Animal laboratory of Science University. Rats were fed *ad libitum* and housed in pairs in steel cages in a temperature-controlled environment (22 ± 2 °C) with 12/12-h light/dark cycles.

Experimental procedures

Rats were randomly assigned into 3 groups and an intraperitoneal injection of feces (FIP procedure) was administered to 30 rats to induce a sepsis model, of whom 10 rats had no other procedure. The FIP rat model was established according to a method previously described by Shrum et al and Tyml et al (13,14). The feces were collected and suspended in saline to prepare a fecal saline solution which was then injected intraperitoneally at a dose of 1 g per kg body weight. Study groups were designed as follows: Group 1: normal control (non-operative and orally fed control, n = 10); Group 2: FIP group, Group 3: FIP and 500 mg/kg/day ascorbic acid (vitamin C; Redox-C Ampul, 500 mg/5 ml, Bayer) given i.p. (n = 10), Group 4: FIP and 300 mg/kg/day alpha-tocopherol (vitamin E; Evin Ampul, 300 mg/2 ml, Farmalas) given i.p. (n = 10). All treatments were administered after a one-hour FIP procedure. The study was finished after 24 hours. During the first 24 hours following the procedure, 5 rats died (3 rats from the placebo group, 1 rat from the vitamin E group and 1 rat from the vitamin C group) and were excluded from the study. At the end of the study, all animals were sacrificed (cervical dislocation under anesthesia; 100 mg/kg, Ketasol, Richterpharma AG Austria)/ xylazine (50 mg/kg, Rompun, Bayer, Germany) and their blood samples were collected by cardiac puncture for biochemical analysis.

Determination of TNF-α, CRP, IL-6, IL 1-β, lactic acid levels in plasma

With the use of commercially available enzyme-linked immunosorbent assay (ELISA) kits (Biosciences, Abcam) plasma TNF-α, CRP, IL 1-β, and IL-6 levels were measured. The measurements were made according to the user's guide. The plasma samples were diluted in a ratio of 1:2; CRP and TNF-α were duplicated. Lactic acid values were recorded from blood gas.

Measurement of lipid peroxidation

The end product of lipid peroxidation, malondialdehyde, was determined by the reaction of thiobarbituric acid in plasma specimens. Trichloroacetic acid and TBARS were added to the plasma specimens and incubated at 100 °C for 60 minutes. Then they were cooled on ice. The specimens were centrifuged at 3,000 rpm for 20 minutes. The absorbance of the supernatant was read at 535 nm.

Histopathological examination of the lung

All animals were anesthetized by i.p. 100 mg/kg ketamine and 50 mg/kg xylazine and perfused with 200 ml of 4 % formaldehyde in 0.1 M phosphate-buffer saline for histological study. Formalin-fixed kidney sections (5 μm) were stained with hematoxylin and eosin (H&E). All sections were photographed with Olympus C-5050 digital camera mounted on Olympus BX51 microscope. The severity of histopathological lung injury was graded between 1 (0–25 %), 2 (25–50 %), 3 (50–75 %), and 4 (75–100 %). The thickness of the alveolar wall (TA), perivascular edema (PE), infiltration of leukocytes in vessel walls (AL), hemorrhage (H) and alveolar congestion (AC) were determined (15).

CT examination of lung

Rats were anesthetized by i.p 80 mg/kg ketamine and 10 mg/kg xylazine. Radiological images were performed by 16-slice multi-detector row CT scanner without using contrast media in the supine position. Scanning was performed between the diaphragm and C3 vertebra, including the apex and base of the lung. According to the automatic system, 120 kV, variable mAs, 1-mm sections were taken. All images were reconstructed at a 512 x 512 matrix size. The images were evaluated by two radiologists who were blinded to animals. Six regions of interests (ROI) were placed on axial images with parenchymal window at the level of near heart apex for all animals.

Tab. 1. Plasma biomarker levels of all groups.

	Normal control	FIP	FIP and 500 mg/kg Vitamin C	FIP and 300 mg/kg Vitamin E
MDA (nM/mg protein)	7.5±1.3	24.6±1.3*	11.4±2.5#	13.9±1.7#
IL-6 (pg/ml)	5.5±0.9	24565.8±1895.6**	9856.8±945.7##	10212.9±1015.8##
IL 1-Beta (pg/ml)	2.4±0.08	2145.7±101.4**	645.2±58.5##	842.5±39.4#
TNF alfa (pg/ml)	11.7±1.1	415.7±14.4**	105.1±13.5##	145.9±10.03#
CRP (mg/dl)	0.41±0.1	1.39±0.2*	0.87±0.16##	1.1±0.25#
Lactic acid (mmol/L)	1.3±0.2	3.8±0.4**	1.9±0.2#	2.02±0.3#

Results were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA.* $p < 0.05$,** $p < 0.001$ different from normal groups; # $p < 0.01$, ## $p < 0.001$ different from FIP group. FIP – fecal intraperitoneal injection procedure; MDA – malondialdehyde; IL – interleukin; TNF – tumor necrosis factor; CRP – c-reactive protein

Tab. 2. Comparison of histopathologic scores and Hounsfield unit (HU) values.

	Normal control	FIP	FIP and 500 mg/kg Vitamin C	FIP and 300 mg/kg Vitamin E
AC	0.2±0.1	3.5±0.2**	1.2±0.4#	1.8±0.2##
H	0.4±0.2	1.4±0.2*	0.5±0.1##	0.6±0.3##
AL	0.2±0.1	2.8±0.1**	1.1± 0.3#	1.7±0.4##
PE	0.3±0.2	3.4±0.3**	1.4±0.1#	1.9±0.3##
TA	0.2±0.1	2.8±0.4**	1.2±0.2#	1.1±0.3#
CT Hounsfield unit (HU)	–638.5±13.5	–517.2±8.8**	–612.7±12.5##	–598.2±14.6##

Results were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA. * $p < 0.05$, ** $p < 0.001$ different from normal groups; # $p < 0.05$, ## $p < 0.001$ different from FIP group. AC – alveolar congestion; H – hemorrhage; AL – infiltration or aggregation of leukocytes in air spaces/vessel walls; PE – perivascular/interstitial edema; TA – thickness of the alveolar wall/hyaline membrane formation

Tab. 3. Comparison of arterial blood paO_2 and $paCO_2$ values.

	Normal control	FIP	FIP and 500 mg/kg vitamin C	FIP and 300 mg/kg vitamin E
paO_2 (mmHg)	104.2±13.5	78.1±10.6*	101.2±9.9#	89.2±7.5#
$paCO_2$ (mmHg)	39.1±4.4	33.2±3.8*	34.1±5.3	37.9±6.7

Results were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA and post-hoc Bonferroni test. * $p < 0.05$, different from normal groups; # $p < 0.05$ different from FIP group

Arterial blood gas analysis

The carotid artery blood of rats in each group was sampled (0.2 mL) at 24 h after the operation and paO_2, and $paCO_2$ in the blood samples were assayed using a blood gas analyzer.

Statistical analysis

Data are presented as mean values ± standard error of the mean (SEM). Data analyses were performed using SPSS version 15.0 for Windows. All data were analyzed by non-parametric Mann-Whitney U test; p values of 0.05 or lower were regarded as statistically significant.

Results

Biochemical findings

TNF-α, CRP, IL 1-β, IL-6, and MDA plasma levels were significantly higher in the FIP group as compared to those in the control group. On the other hand, TNF-α, CRP, IL 1-β, IL-6, and MDA plasma levels in groups treated with vitamin C or vitamin E were lower than in the FIP group (Tab. 1).

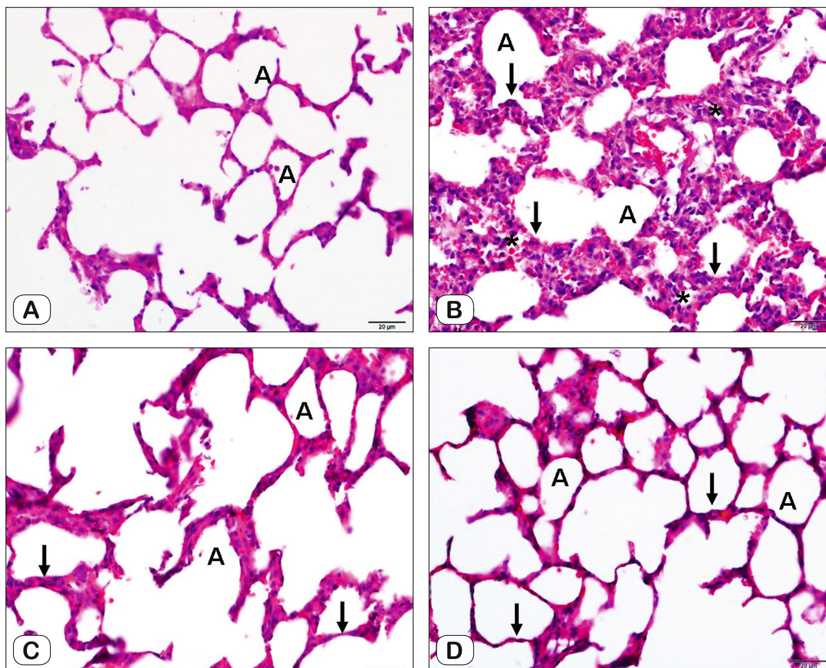

Fig. 1. Lung histopathology x40 magnification H&E staining. A: normal control group lung, (A) alveolus, B: FIP group showed severe histopathologic alteration related to increased alveolar inflammation (*) and septal thickness (arrow), C: FIP and 500 mg/kg/day ascorbic acid (vitamin C) group showed a decrease in inflammation and septal thickening (arrow), D: FIP and 300 mg/kg/day alpha-tocopherol (vitamin E) group showed a decrease in inflammation and septal thickening (arrow)

Histological score and CT findings

AC, H, AL, PE and TA scores, which are representative markers of lung damage, were significantly higher in the FIP group than in the control group. All scores in groups treated either with vitamin C or vitamin E were significantly lower as compared to those in the FIP group (Tab. 2, Fig. 1).

The HU value of lung in the FIP group was significantly higher than that in the control group. The HU value of lung in groups treated either with vitamin C or vitamin E were significantly lower than in the FIP group (Tab. 2, Fig. 2).

Arterial paO_2 and $paCO_2$

The arterial blood paO_2 in groups treated either with vitamin C or vitamin E were significantly higher as compared to that in the FIP group (Tab. 3).

Discussion

In this animal study, we showed low levels of plasma inflammatory markers, his-

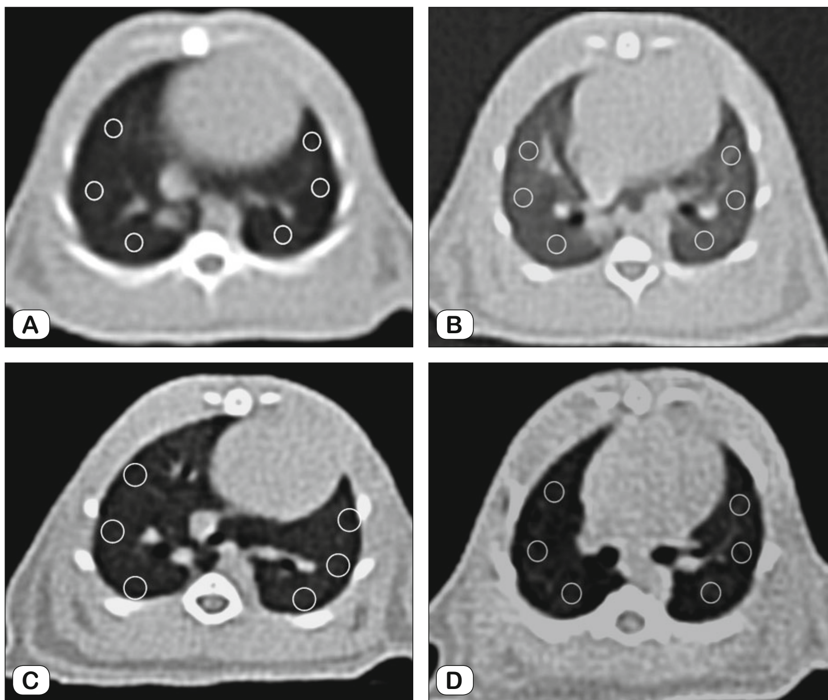

Fig. 2. Axial CT images of lung at the level of the heart; six ROI with the same size placed at the same location a: normal control group lung, b: FIP group shows an increase in the density of lung, C: FIP and 500 mg/kg/day ascorbic acid (vitamin C) group shows a decrease in the density of lung, D: FIP and 300 mg/kg/day alpha-tocopherol (vitamin E) group showed a decrease in the density of lung.

tological scores, HU values, as well as high paO_2 values in the FIP group of rats treated with vitamin C or vitamin E.

In sepsis, cytokines activated by the stimulation of complement-mediated neutrophils play a major role in the inflammatory response (16). In response to infection, proinflammatory cytokines such as TNF-α, IL 1-β, and IL-6 are produced from lung endothelium and fibroblasts. TNF activates adhesion molecules on the leukocyte surface, causing neutrophils to adhere to endothelial cells (17). Proteases and ROS released as a result of the accumulation of activated neutrophils facilitate endothelial cell injury. In sepsis-induced ALI, pulmonary gas exchange is impaired because the endothelium is damaged by hypoxia, ROS, and cytokines (18). An increase in CRP produced under the control of IL-6 is seen in the plasma (19).

Although vitamin E and C deficiencies are rare, septic patients followed in the intensive care unit are known to have low plasma concentrations (20, 21). The antioxidant activity and immunomodulatory effects of vitamins C and E are important in the functioning of the immune system and inflammation mechanisms (22, 23). We created a sepsis model in our study animals by intraperitoneal injection of feces. We showed a decrease in inflammation and septal thickening (Figure 1) in lung tissue sections in the groups treated with Vitamin C or Vitamin In their studies, Fisher et al (24) showed that vitamin C reduces pulmonary vascular inflammation. In this paper, it has been shown that vitamins C and E reduce the plasma values of MDA, which is the end-product of lipid peroxidation, with both histopathological effects and antioxidant activities in the lung. Consistent with previous studies, capillary-damaging cytokine activities in the lung was prevented with vitamins C and E and plasma TNF-α, IL 1-β, and IL-6 values were decreased (24, 25). We clinically supported these results with a significant increase in paO_2 values in arterial blood gas and decreased density of lung in CT images (Fig. 2). We think that confirming the improvement in laboratory blood values with CT findings included in the definition of sepsis-induced ALI in our study is important and different from previous studies.

There are some limitations to this study. In animal models of sepsis, rats are highly resistant to the toxic effects of bacterial lipopolysaccharide, whereas humans are more susceptible (26). Different from rats, patients hospitalized in the intensive care unit due to sepsis have various comorbidities. Therefore, there is a need to examine the clinical effects in humans.

Conclusion

Vitamins C and E are safe low-cost nutrients. They are natural antioxidants and anti-inflammatory agents (22, 27). Therefore, it is interesting that they are effective as an adjuvant in the treatment of sepsis, a worldwide disease with high mortality and cost.

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Received May 22, 2022.
Accepted July 6, 2022.