Ellagic acid ameliorates lung injury after intestinal ischemia-reperfusion

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ABSTRACT

Background: The aim of this study was to investigate the possible protective role of antioxidant treatment with ellagic acid (EA) on lung injury after intestinal ischemia-reperfusion (I/R) injury using biochemical and histopatological approaches. **Materials and Methods:** Forty rats were divided into four groups as control, control + EA, I/R, and I/R + EA. The control and control + EA groups were also anesthetized and subjected to laparotomy, but without clamp application. The control + EA and I/R + EA groups were given EA (85 mg/kg) orally prior to experiment. The I/R and I/R + EA groups underwent 30 minutes of intestinal ischemia and 1 hour of reperfusion. In all groups, serum total antioxidant capacity (TAC) and malondialdehyde (MDA) levels were determined. TAC, total oxidative status (TOS), and oxidative stress index (OSI) in lung tissue were measured. Lung tissue histopathology was also evaluated by light microscopy. **Results:** TAC levels were higher in control, EA, and I/R + EA groups while TOS, OSI, and MDA levels were lower in these groups compared with I/R group. Serum MDA levels were significantly higher in I/R + EA group than that of control group. Lung tissue TAC levels were lower in I/R + EA group while OSI values were higher in that groups compared with EA group. Histological tissue damage was milder in the EA treatment group than in the I/R group. **Conclusion:** These results suggest that EA treatment protected the rats lung tissue against intestinal I/R injury.

Key words: Ellagic acid, ıntestinal ischemia reperfusion, malondialdehyde, oxidative stress index, total antioxidant capacity, total oxidative status

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INTRODUCTION

Intestinal ischemia followed by reperfusion induces a systemic inflammatory response and releases harmful substances that may affect the function and integrity of distant organs such as lung, heart, liver, and kidney. Intestinal ischemia-reperfusion (I/R) injury is a complex and multifactorial pathophysiological process that involves the actions of reactive oxygen species (ROS), inflammatory cytokines, and polymorphonuclear neutrophils.[1-3] Reactive oxygen species (ROS), generated in the organism as byproducts of normal cellular metabolism, have been implicated in the pathogenesis of a large number of diseases such as diabetes mellitus, cancer, rheumatoid arthritis, infectious diseases, and atherosclerosis and aging.[4-9] Although ROS have several physiological functions in signal transduction, gene transcription and regulation, they are able to cause oxidation of biomolecules, thereby contributing to their structural and functional modifications. This leads to cell dysfunction and cell death, and, at the organic level, to ageing and age-related diseases. Many enzymatic and nonenzymatic antioxidants have been developed by aerobic organisms to counteract the effects of ROS on biomolecules.[10-13]

The process of lipid peroxidation is one of oxidative conversion of polyunsaturated fatty acids to products known as malondialdehyde (MDA), which is usually measured as thiobarbituric acid reactive substances (TBARS), or to lipid peroxides, which is the most studied, biologically relevant, free radical reaction.[9,14] Phenolic phytochemicals such as ellagic acid (EA) are important components of fruits and vegetables. Several studies have shown that plant-derived polyphenolic antioxidants exhibit anti-inflammatory, antimutagenic, anticarcinogenic,

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antiviral and antioxidant activities. EA contains four hydroxyl groups and two lactone groups in which hydroxyl group is known to increase antioxidant activity in lipid peroxidation and protect cells from oxidative damage. It is believed that EA functions either by countering the negative effects of oxidative stress by directly acting as an antioxidant or by activating/inducing cellular antioxidant enzyme systems.[15,16]

In recent years' the effects of intestinal I/R ınjury on distant organs became very popular. To our knowledge, there is no study that investigates simultaneously lung tissue total antioxidant capacity (TAC), total oxidative status (TOS), oxidative stress index (OSI), and serum MDA levels in lung injury induced by ischemia reperfusion. This study is the first report describing the protective effect of EA against effects of intestinal I/R injury on distant organ injury such as that affecting the lungs. Therefore, in the present study, we aimed to investigate TAC, TOS, OSI and MDA levels in this experimental model. In addition, we examined histopathological changes in the lung tissue.

MATERIALS AND METHODS

This study was conducted after approval by the Dicle University School of Medicine Ethics Committee. Forty male Wistar rats weighing 200-250 g were used. The animals were kept in single cages under standard laboratory conditions with a balanced pellet diet and water ad libitum. The animals were housed at the Center for Laboratory Animal Care of Dicle University. The animals were acclimatized for one week before the experiments. The rats were prepared for surgery with an injection of ketamin HCL (50 mg/kg intramuscular intramuscular) and xylazine (10 mg/kg intramuscular) anesthesia. The superior mesenteric artery (SMA) was exposed through a midline abdominal incision. The SMA, the collateral branches coming from the celiac axis and the inferior mesenteric artery were occluded with atraumatic microvascular clamps for 30 minutes of intestinal ischemia and 1 hour of reperfusion. Mesenteric ischemia was confirmed by cessation of the mesenteric pulsations and paling of the intestine. Following ischemia, the microvascular clamp was removed and reperfusion was confirmed by restoration of the pulsations and color.

Experimental design

The study consisted of four groups of animals and were randomly assigned to four groups, each containing 10 rats: Control, control + EA, I/R, and I/R + EA. The control + EA and I/R + EA groups were given EA (85 mg/kg) orally prior to experiment. The doses chosen for the study are the well accepted literature based doses.[17] The control and control + EA groups were also anesthetized and subjected to laparotomy, but without clamp application. The intestinal I/R and I/R + EA groups underwent 30 minutes of intestinal ischemia and 1 hour of reperfusion**.** At the end of the experiment, the animals were anesthetized with ketamin hydrochloride (20 mg/kg, intraperitoneally). Blood samples were taken from the animals for biochemical analysis. Lungs were taken out of the body after thoracotomy. Tissue specimens were fixed in 10% formalin for 48 hours, then embedded in paraffin and cut into 5 µm sections. Slides were stained with hematoxylin and eosin (H and E) and examined under a light microscope. A pathologist evaluated the slides in a blinded manner. The rest of the specimens were stored at –80 °C for biochemical examination. A piece of lung tissue (approximately 300 mg) was homogenized in 10 volumes of ice-cold phosfate buffer solution (PBS) (50 mM/L, pH 7.0) using a homogenizer (Ultra-Turrax T8 dispersing homogenizator, Staufen, Germany). Then, the homogenate was centrifuged at 10000 ×g for 15 minutes at 4 °C. The supernatant was stored at −80 °C in aliquots.

Biochemical analysis

Determination of malondialdehyde activity

Malondialdehyde levels were estimated by the double heating method of Draper and Hadley.[18] The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 ml of trichloroacetic acid solution (10%) was added to 0.5 ml plasma in each centrifuge tube, and the tubes were placed in a boiling water bath for 15 minutes. After cooling in tap water, the tubes were centrifuged at 1000 g for 10 minutes, and 2 ml of the supernatant was added to 1 ml of TBA solution (6.7 g/L) in a test tube, and the tube was placed in a boiling water bath for 15 minutes. The solution was then cooled in tap water and its absorbance was measured using a spectrophotometer (Shimadzu UV-1208, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of the MDA–TBA complex (absorbance coefficient of 1.56x105 cm^{-1} M^{-1}). Serum MDA levels were expressed as µmol /L.

Measurement of the total antioxidant capacity

Total antioxidant capacity of supernatant fractions was determined using a novel automated measurement method developed by Erel.[19] In this method, hydroxyl radical, which is the most potent biological radical, is produced. In the assay, ferrous ion solution, which is present in Reagent 1, is mixed with hydrogen peroxide, which is present in Reagent 2. The sequential produced radicals such as brown colored dianisidinyl radical cation, produced by the hydroxyl radical, are also potent radicals. Using this method, antioxidative effect of the sample against the potent-free radical reactions, which is initiated

by the produced hydroxyl radical, is measured. The assay has excellent precision values, lower than 3%. Serum and tissue TAC were expressed as mmol Trolox Equiv./L and nmol Trolox Equiv./mg protein, respectively.

Measurement of total oxidant status

Total Oxidant Status of supernatant fractions was determined using a novel automated measurement method, developed by Erel.[20] Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of nmol H_2O_2 Equiv./mg protein.

Determination of oxidative stress index

Percent ratio of TOS level to TAC level was accepted as Oxidative Stress Index (OSI). OSI value was calculated according to the following Formula:[21] OSI (Arbitrary Unit) = TOS (nmol H_2O_2 Equiv./mg protein)/TAC (nmol Trolox Equiv./mg protein). The results are expressed as Arbitrary Unit.

Histopathologic evaluation

On histopathologic examination, pulmonary injury was graded into four categories, as follows: *grade 0*, no diagnostic change; *grade 1*, mild neutrophil leukocyte infiltrations and mild to moderate interstitial congestion; *grade 2*, moderate neutrophil leukocyte infiltrations, perivascular edema formation, and partial destruction of pulmonary architecture; and *grade 3*, dense neutrophil leukocyte infiltration and complete destruction of the pulmonary architecture.[22]

Statistical analysis

All the statistical analyses were performed using the Statistical Package for Social Science 15.0 (SPSS Inc., Chicago, IL, USA) statistical package. Data was expressed as mean ± SD (standard deviation). Statistical analysis was undertaken using the one way Analysis Of Variance (ANOVA) test. For histopathologic evaluation: Differences among the groups were analyzed by the Kruskal-Wallis test. Dual comparisons among groups with significant values were evaluated with the Mann-Whitney U-test. A value of $P < 0.05$ was accepted statistically significant.

RESULTS

All parameters are shown in Table 1. As seen from the Table, TAC levels were higher in control, control + EA and I/R + EA groups while TOS, OSI and MDA levels were lower in these groups compared with I/R group. Serum MDA levels were significantly higher in I/R + EA group than that of control group. Lung tissue TAC levels were lower in I/R + EA group while OSI values were higher in that groups compared with control + EA group.

Histological grading of lung tissues is summarized in Table 2. The lung damage score was significantly higher in I/R group than in the groups control, control + EA, and I/R + EA. In the I/R group, the lung microscopic examination revealed alveolar, perivascular, and interstitial edema, massive infiltration of the alveolar wall by inflammatory cells, dilated alveolar ducts and destruction of the interstisium with focal bleeding. Whereas in the I/R + EA group, tissue showed almost normal alveolar architecture without bleeding and lung tissue destruction. Representative histological samples of lungs from the four groups are shown in Figure 1.

DISCUSSION

Many studies have been designed to reveal the pathogenesis of intestinal ischemia reperfusion injury and to prevent local and distant tissue damage induced by this process. Ischemia induced by vascular occlusion also causes intestinal damage, but major damage is caused by reperfusion.[23-25] Intestinal I/R injury is a condition resulting from necrotizing enterocolitis, midgut volvulus, acute mesenteric ischemia,

Table 1: Oxidative and antioxidative parameters in control, control + ellagic acid, ischemia-reperfusion, and ischemia-reperfusion + ellagic acid

Groups (n=10)	Serum TAC	Serum MDA	Lung TAC	Lung TOS	Lung OSI
Control	2.45 ± 0.43*	0.60 ± 0.35 #	3.45 ± 0.38#	138.97 ± 20.9§	4.03 ± 0.69#
Control + EA	2.50 ± 0.58#	0.77 ± 0.42#	3.76 ± 0.45#	137.33 ± 12.23§	3.65 ± 0.56#
I/R	1.65 ± 0.39	2.73 ± 0.72	2.53 ± 0.29	184.44 ± 31.77	7.29 ± 1.15
I/R + EA	2.23 ± 0.63§	1.14 ± 0.49#,‡	3.15 ± 0.58*,†	152.95 ± 32.19µ	4.85 ± 1.75#,¶

Values are mean ± SD. MDA: Malondialdehyde (µmol /L), TAC: Total antioxidant capacity (nmol Trolox Equiv./mg protein), TOS: Total oxidative status (nmol H_2O_2 Equiv./mg protein), OSI: Oxidative stress index (Arbitrary Unit), I/R: Ischemia-reperfusion, EA: Ellagic acid, §: $P < 0.05$, µ: $P < 0.01$, *: $P < 0.005$, #: $P < 0.001$ versus I/R, ‡: $P < 0.05$, versus control, †: $P < 0.05$, ¶: $P < 0.01$ versus control + EA

Table 2: Histopathologic evaluation of lung tissue for each group, that is, in control, control + ellagic acid, ischemia-reperfusion, and ischemia-reperfusion + ellagic acid

Groups (n=10)	Pulmonary injury score
Control	0.20 ± 0.42
Control + EA	0.20 ± 0.42
I/R	1.50± 0.71*,#
I/R + EA	0.50 ± 0.53

Results are expressed as mean ± SD, *: P = 0.001 versus control and control + EA, #: P < 0.05 versus IR + EA, I/R: Ischemia-reperfusion, EA: Ellagic acid,

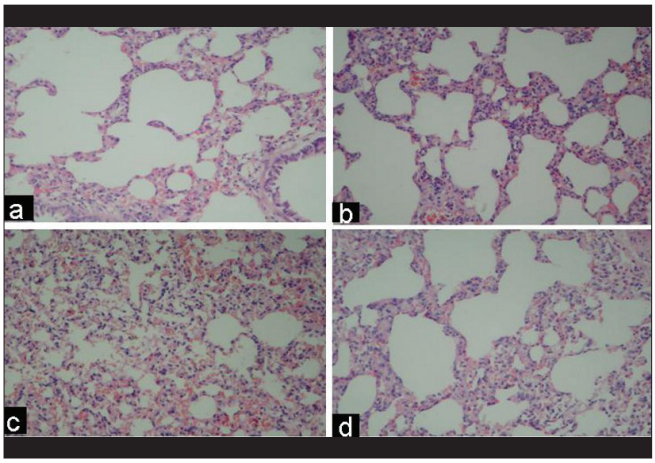

Figure 1: (a) Control group, lung: Mild polymorph nuclear leukocyte infiltration and mild to moderate interstitial congestion in the lung tissue (H and E, ×200). (b) Ellagic Acid+Control group, lung: Mild polymorph nuclear leukocyte infiltration and moderate interstitial congestion in the lung tissue (H and E, ×200). (c) Ischemia-reperfusion group, lung: Interstitial inflammation, perivascular edema and haemorrhage with disintegration of the parenchimal lung architecture (H and E, ×200). (d) Ischemia-reperfusion+ Ellagic Acid group, lung: Mild to moderate PNL infiltration and interstitial congestion in the lung tissue (H and E, ×200)

multiple traumas, shock, sepsis, small bowel transplantation, and incarcerated hernia.[2,3] I/R injury is complex and multifactorial pathophysiological process. ROS produced upon reperfusion play a critical role in the injury caused by I/R. ROS lead to an inflammatory response and tissue damage by activating certain mediators; they can also directly damage cell components.[26]

It has been reported that ROS are associated with the pathogenesis of a number of diseases. ROS are capable of reversibly or irreversibly damaging compounds of all biochemical classes, including nucleic acids, protein, free amino acids, lipids and lipoproteins, carbohydrates, and connective tissue macromolecules. These species may impair cell activities such as membrane function, metabolism, and gene expression. Propagation of damage results in a repeated chain reaction. When the balance between ROS production and the antioxidative defense mechanisms is impaired, ROS levels may increase. When ROS are not removed by natural scavengers, damage occurs through peroxidation of structurally important polyunsaturated fatty acid within the phospholipid structure of the membranes. Lipid peroxidation decreases both the fluidity and the barrier function of membranes, resulting in disturbances in structural organization, enzymic inhibition, and possible cell death. In addition, lipid peroxides may inhibit protein synthesis, block macrophage function, and alter chemotactic activity.[4,11,26,27]

In the study, our data confirm that intestinal ischemia-reperfusion increases oxidative stress, an effect that not only produces direct tissue damage, but also modulates production of toxic cytokines leading to inflammation and leukocyte infiltration, consistent with previous studies. In addition, EA treatment alleviated pathological structural changes.

In the study, we observed increased TOS, MDA levels, an indicator of lipid peroxidation, and decreased TAC levels in the ischemia reperfusion group as compared to those in control group. In addition, we detected significantly decreased TOS, MDA levels and increased TAC levels with the administration of EA, which could be related to its antioxidant and free radical scavenging effect. Ischemia reperfusion-induced increase in lipid peroxidation was prevented by EA, which was reported for the first time in this study. In agreement with the results of Atessahin *et al*.,[15] EA prevented ischemia reperfusion-induced increase in oxidative stress parameters in our study, also. Increased TBARS, conjugated dienes and hydroperoxides' levels have been reported in heart, liver, and lung tissues in rats.[4,11,27] The possible mechanisms by which ischemia reperfusion increases oxidative stress include disruption of the mitochondrial respiratory chain leading to leakage from the electron transport chain in rats, depletion of cellular glutathione level, and decreased activities of antioxidant enzyme and increased activity of oxidant enzyme in lung tissue of rats. It seems that superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are the main source of ischemia reperfusion-induced free radical production, which depletes the cellular glutathione level, which has central role in the antioxidant defense in the cell.[27,28]

CONCLUSİON

In conclusion, this is the first study that investigates simultaneously TAC, TOS and MDA levels in lung injury after intestinal ischemia-reperfusion in rats. We found the increased TOS, MDA levels and decreased TAC levels in the I/R group as compared to those in control group. These results suggest that reactive oxygen species play a

role in this experimental model in rats and the antioxidant properties of EA seem to be effective in preserving lung tissue against ischemia reperfusion oxidative injury. Therapy with antioxidants may lead to the increase in the antioxidant defense system. However, more investigations are required to evaluate the protective effects of EA on lung tissue damage in clinical and experimental models.

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