PROTECTIVE EFFECT OF ASTAXANTHIN IN THE LUNG INJURY CAUSED BY ISCHEMIA REPERFUSION OF THE LOWER EXTREMITIES

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ABSTRACT

Pathological and biochemical alterations due to lower extremity (I/R) damage and protective effects of astaxanthine (AST) were investigated. Rats were divided into four groups. GI-Sham group (n=7): Anesthesia without (I/R)(2 hours); Group II-I/R (n=7): 2 hours of ischemia and 2 hours of reperfusion under anesthesia; Group III-AST(n=7): Rats were subchronically orally administered for 7 days at 125 mg/kg astaxanthin (AST) and then anesthetized (2 hours) without ischemia; GI-V-I/R+AST (n=7): 7 days prior to ischemia rats were subchronically orally administered 125 mg/kg astaxanthin (AST) and then 2 hours of ischemia and reperfusion under anesthesia; Then lung tissues were investigated for MDA, GSH and histopathology. An increase in MDA and a decrease in GSH was observed I/R administered group compared to control. Histopathological evaluations showed intense congestion in pulmonary veins and alveolar septum and partial alveolar macrophage and erythrocyte accumulation and edema was observed in lumens of some bronchioles and alveoli in the second and fourth group compared control. Second group (3.41) damage score had high significance compared to control (p≤0.001). Fourth group damage score (0.92) was indifferent from control but significantly different from I/R group (p<0.001). As a result; The protective effect of AST has been demonstrated by biochemical, histopathological and immunohistochemical effects.

Keywords: Lung, Astaxanthin, Rat, MDA, GSH, Histopathology.

INTRODUCTION

Lower extremity Ischemia/reperfusion (I/R) damage is a frequently encountered phenomenon in surgical interventions (Grace, P.A. 1994). Ischemia and following reperfusion triggers local and systemic damage with the involvement of free oxygen radicals and inflammatory mediators (Tekeli et al. 2001; Uysal et al. 2006). Although blood flow saves extremity from necrosis, multi organ dysfunction may progress and cause death of the patient. In general, this damage is observed in heart, kidney and lung tissue (Blaisdell et al. 2002; Bengisun et al. 1997). Following lower extremity I/R damage, lungs are targets in end-organ damage and this damage can cause very important clinical aspects. Although etiology is not clarified yet, some humoral mediators in reperfusion are blamed for this end organ damage (Fantini et al. 1995; İspir et al. 2000). Astaxanthin (ASTA; 3,30-dihidroksi-β-karoten-4,40-dion), is found in various organisms and especially in salmon, sea bream, rainbow trout, shrimp, lobster, fish eggs and algae. This lipid soluble xanthophil has strong antioxidant functions. AST scavenges single oxygen atoms as well as other free radicals and avoids or terminates peroxidation chain reactions (Gal et al. 2012; Nakagawa et al. 1997; Oshima et al. 1993). AST, is a known as a prefect antiinflammatory agent since it suppresses proinflammatory cytokine and chemokine expression (Yeh et al. 2016). AST, is used in treatment of cardiovascular disease (Pashkow et al. 2008), ischemic brain injury (Shen et al. 2009), cataract (Wu et al. 2006), diabetes (Uchiyama et al. 2002), hepatocellular injury due to ischemia ( Curek et al. 2010) and diabetic nephropathy (Manabe et al. 2008; Naito et al. 2004).

Aim of this study is to evaluate protective effect of AST in the lung injury caused by ischemia reperfusion of the lower extremities.

MATERIALS AND METHODS

Animals: Study was conducted at Bingöl University Animal Experiments Center after approval of Bingöl University Animal Experiments Local Ethical Committee (18.11.2015 -2015/08). Twenty-eight male Wistar-albino rats weighing 250-300 grams were used at 7-8 weeks of age. Animals were kept in cages in controlled rooms with 20-22°C constant temperature and 12 hour cycles of light-dark (lights were on between 07:00-19:00; dark between 19:00-07:00). Water and standard food were given ad libitum. Rats were let for adaptation for one week in their
cages and experiments started after adaptation period. Feeding was ceased 12 hours before experiments whereas water was provided throughout the experimental period.

**Experimental Design:** Commercially obtained AST was subchronically administered to Sprague-Dawley rats orally. Rats were divided into four groups each containing 7 animals; Group I (Sham Group, n=7): Rats in this group was administered with anesthesia (2 hours) without ischemia-reperfusion and tissue samples were obtained. Group II (Ischemia/Reperfusion (I/R), n=7): Rats were administered with 2 hours of ischemia and following 2 hours of reperfusion under anesthesia (Bilgiç et al. 2018), Group III (AST), n=7: Rats in this group was administered with subchronic orally AST for 7 days at 125 mg/kg dosage and following anesthesia for 2 hours without ischemia (Gross et al. 2006). Group IV (Ischemia/Reperfusion (I/R) + AST (n=7): Rats in this group was administered with subchronic orally AST for 7 days at 125 mg/kg dosage, then administered with two hours of ischemia and two hours of reperfusion under anesthesia then lung tissue samples were obtained. Rats were anesthetized with 60 mg/kg i.p. ketamine hydrochloride and 10 mg/kg Xylazine i.p. Lung tissue was removed after median laparotomy, washed with PBS, stored in deep freeze (-80°C) and biochemical (MDA, GSH) and histopathology were performed.

**Preparation of tissue extract:** Tissue samples were washed with isotonic solution, dried and weighed. They were kept at -80°C for analyses. Samples taken out from freezer were placed into tubes and Tris-buffer was added 10 times of their weight. They were disintegrated with homogenizer and placed into +4°C freezer. Following vortex, cell membrane was lysed with ultrasonic bath in 10 s intervals for 30 s. They were centrifuged at 16000 rpm for 30 min. Supernatants were transferred into Eppendorf tubes. MDA and GSH levels were determined with such supernatants of lung tissue.

**BIOCHEMICAL ASSAYS**

**Measurement of MDA Level:** 50 mg tissue sample was homogenized with 0.15 mol/L KCl solution. Homogenates were centrifuged at 1600 × g. MDA levels in tissues were determined with thiobarbituric acid (TBA) reaction according to Placer et al. (Placer et al. 1966).

**Measurement of GSH Level:** Tissue GSH levels were measured at 412 nm according to Sedlak and Lindsay (1968). Samples were precipitated with 50 % TCA and centrifuged at 1000×g for 5 minutes. Reaction mixture contained 0.5 mL supernatant, 2.0 mL EDTA (0.2 M, pH: 8.9) buffer and 0.1 mL 0.01 M DTNB. Solution was kept in room temperature for 5 minutes and then read at 412 nm with spectrophotometer.

**Total protein concentration determination:** The total protein concentration in the lung tissue homogenate was according to Lowry (1951). Total protein concentration in lung tissue homogenate was conducted by using bovine serum albumin as standard. A standard graphic was set by using standard bovine serum albumin standard solution. Measurements were performed at 695 nm wavelength.

**Histopathological and immunohistochemical investigations:** At the end of experimental procedure all rats were necropsied and lung tissues were taken. Lung tissue samples for histopathological investigation were fixed with 10 % buffered formalin solution for 48 hours. Tissue samples were passed from alcohol series for dehydration and from xylol series for clarification then blocked in paraffin. From those blocks serial cross sections were obtained with 4 micrometers thickness by using a microtome (Leica RM 2135). Samples were stained with Hematoxyline-Eozin (H.E.) and investigated and photographed under light microscope (Nikon 80i-DS-R12). Histopathological findings were partially modified and evaluated semi-quantitatively. Such criteria were; alveolar generation, thickening in interalveolar septum, capillary hyperemia, hemorrhage, inflammatory cell infiltration, terminal bronchiole structure, terminal bronchiole debris, respiratory bronchiole wall structure, respiratory wall debris, alveolar structure and edema in vascular wall. Its grading were done accordingly; 0=no alteration; 1=focal light alterations; 2=multifocal light alteration; 3=multifocal apparent alteration; 4=diffuse light alteration; 5=diffuse apparent alteration (Uysal et al. 2006). For immunohistochemical investigation slices of 4μm thickness were placed in between poly-lysinecoated slides. To show eNOS immuno reactivity, anti-eNOS primary antibodies were used and stained with Avidin-Biotin Peroxidase Complex (ABC) immunohistochemical method. Obtained slices were incubated for 2 hours at 60°C and administered with two different xylol for clarification and rehydrated with decreasing alcohol series. Finally they were remained in distilled water for 5 minutes. Slices were kept in room temperature for 20 minutes in 0.5% trypsin solution. In addition they were kept in 3% hydrogen peroxide for 5 minutes to avoid endogenous peroxidase. Slices were washed with PBS for 5 minutes 3 times and incubated at +4°C overnight after eNOS primary antibody administration. Following day, slices were washed 3 times with PBS and let for 30 min incubation with biotinylated secondary antibody. They were bound with secondary antibody Vector Elite ABC kit. They become visible by dropping diaminobenzidine (DAB chromogen) which is the coloration substance for antibody-biotin-avidin-peroxida seocomplex. Background staining was done with Mayer’s hematoxylene. Dehydration was performed with graded alcohol and clarification with xylol. Finally they were covered with entellan. Immunohistochemical staining intensity was evaluated by a double blind pathologist. eNOS staining
level was scored as none (-), low staining (+), moderate staining (+++) and intense staining (+++).

**Statistical analysis:** Statistical evaluation of the results was conducted with SPSS 20 (Statistical Program-Software System) pocket program. Data were expressed as X ± SEM. Variation analysis (ANOVA) was conducted and group comparisons were done with post-hoc Tukey’s test. Statistical significance was set as p ≤ 0.05.

**RESULTS**

**Biochemical findings:** MDA level was increased in I/R administered group compared to control as expected. MDA level was lower in I/R+AST administered group compared to I/R group significantly (p≤0.05). MDA level in AST group was found significantly lower compared to I/R group (p≤0.01) (Figure1). GSH was decreased in I/R group compared to control(p≤0.05). On the other hand in AST administered group, GSH value was increased compared to I/R group. This increase was found statistically significant (p<0.05) (Figure2).

**Histopathological and immunohistochemical findings:** No macroscopic alteration in lungs of rats was observed in groups. Microscopic evaluation of control group (Figure 3A) and AST administered group (Figure 3F) revealed no histopathological finding and lung parenchymatic tissue was in normal appearance. In histopathological investigation of rats in I/R group some intense histopathological findings were found. Intense congestion in pulmonary veins and inter alveolar septum was observed. In addition, there were macrophage and erythocyte accumulation and edema in some parts of bronchioles and alveolar lumens. Hyperemia, bleeding and inflammatory cell infiltration in capillaries in inter alveolar septums were observed. Those findings showed an interstitial pneumonia with enlarged alveolar septums. In some cases perivascular and peribronchiolar mononuclear cell infiltrations were also encountered. Partial bronchiolar and alveolar epithelial cell degeneration and necrosis as well as desquamation were also observed. In addition, there was muscular hyperplasia in smooth muscle layers of some vessels and bronchioles. Due to intense exudate accumulation, congestive atelectasis with different intensities, enlargement of some alveoli in emphysematous fashion and even destruction of morphological structure due to rupture of alveolar walls were observed (Figure 3B-3D). In group administered with AST and I/R, hyperemia, hemorrhage, edema, inflammatory cell infiltration and partial enlargement in inter alveolar septum were observed (Figure 3E). Such findings are quite light compared to I/R group and resembles control group in general appearance. In semi quantitative histopathological evaluation, lungs revealed no damage at 4th or 5th grade. Lowest calculated score was for control (0.69) and AST groups (0.72) without statistical difference in between. I/R group showed the highest damage score with 3.41. Difference of this group with control group gives high statistical significance (P≤0.001). Damage score of concomitant AST and I/R administered group was 0.92. This value was insignificant compared to control but showed statistically significant difference with I/R group (P≤0.001). Immunohistochemical evaluation of vessel endothelium showed no eNOS positive staining (-) in control group and AST administered group. In general I/R group rats showed strong eNOS staining (+++) in lung vessel endothelium (Figure 3G) whereas I/R+AST group had low eNOS staining (+) (Figure 3H).

**Figure 1. MDA levels of groups (b=p≤0.01,c=p≤0.05).**

**Figure 2. GSH levels of groups (c,c1=p≤0.05)**
Figure 3. A) Control Group; normal histological architecture of lung, H&E X 4. B) I/R Group; intense hemorrhage (stars), MNL infiltration in peribronchioles and interalveolar septum ( ), exudate in bronchial lumen (a), emphysema in alveoli ( ), H&E X10. C) I/R Group; peribronchiolar and perivascular MNL infiltration ( ), intense hemorrhage (stars) and muscular hyperplasia in bronchioles epithelial level (arrow heads), H&E X10. D) I/R Group; hemorrhage and thickening in interalveolar septum, MNL infiltration ( ), emphysema ( ) and atelectasis (arrows), H&E X10. E) I/R + AST Group; nearly normal appearance in alveoli except very weak hemorrhage and edema in some interstitial area (stars), H&E X10. F) AST group; normal histological architecture of lung, H&E X10. G) I/R Group; intense eNOS positive staining in vessel endothelial cells (arrows). Immunoperoxidase X20. H) I/R + AST Group; low eNOS positive staining in vessel endothelial cells (arrows). Immunoperoxidase X20.
Table 1. Histopathological findings of protective effect of AST on lung tissue in experimental I/R in rats.

<table>
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<tr>
<th>Parameters</th>
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<td>2/8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3/8&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Congestion and capillary hyperemia</td>
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<td>8/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3/8&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Hemorrhage in interstitial</td>
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<td>3/8&lt;sup&gt;ab&lt;/sup&gt;</td>
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**DISCUSSION**

I/R damage is due to free radical formation following re-oxygenation of the tissue after reperfusion of the tissue (Prem et al. 1999). Reactive oxygen damage may arise from more than one source and among them the most important ones are the activated neutrophils (Cetinkale et al. 1998; Pararajasingam et al. 1999; Cavanagh et al. 1998). Role of neutrophils in I/R damage is pronounced (İspir et al. 2000; Cetinkale et al. 1998; Lee et al. 1992). Most important factor for lung damage is via formation of free oxygen radicals and proteolytic enzymes released by neutrophils attached to endothelium (Berkan et al. 2001). Increased PMNL activity which is formed by I/R damage causes chemo attraction and infiltration of PMNL which is followed by their degranulation. After degranulation, free radical formation increases and proteases cause lung endothelium damage and augmented pulmonary capillary permeability (Nelson et al. 2001; Seekamp et al. 1993; Cohen et al. 1997). Recently use of substances such as superoxide dismutase, allopurinol, catalase, mannitol, vitamin C, alpha tocopherol, pentoxyphillin in treatment of patients was tested against removing untoward effects of oxygen radicals formed during I/R and they were found effective. Those antioxidant substances activate antioxidant system and exert protective effect against end organ damage (Uysal et al. 2006). AST is a naturally forming carotenoid pigment and a potent biological antioxidant (Palozza et al. 1992). It shows oxygen radical scavenging activity and protects cell membranes, cells against lipid peroxidation and oxidative damage (Lim et al. 1992). In this study, lipid peroxidation was monitored with MDA measurement which arises from damage of cell membrane structures by free radicals. As expected MDA level in lung tissue was higher in I/R group compared to control and a decrease occurred in I/R+AST administered group compared to I/R group (Figure 1). In a study protective effect of AST in a kidney I/R model was investigated. They found an increase in MDA level in I/R group compared to sham and a decrease in I/R+AST group compared to I/R group (Qiu et al. 2015).
In another study focusing on protective effect of AST on I/R induced memory loss, MDA levels were increased in I/R group compared to sham group whereas a decrease in I/R+AST group compared to lone I/R administered group was determined (Xue et al. 2017). A similar increase in MDA level in lower I/R induced lung damage and a decrease due to melatonin administration was observed in another study (Uysal et al. 2006). In a study on rat aortic I/R investigating damage of this administration on lung damage, MDA level was increased in I/R group significantly (Kapan et al. 2009). Comparison of our data with those studies reveals comparable findings. When GSH level was evaluated, a decrease in I/R group compared to control (p<0.05) and an increase in this parameter in AST group compared to I/R group was determined. This increase was found statistically significant (p<0.05) (Figure 2). A study investigating effect of AST on liver I/R model also revealed a decrease in GSH in I/R group compared to control and an increase in I/R+AST group compared to I/R group (Curek et al. 2010). GSH level was ameliorated with AST in a study focusing on memory loss due to I/R (Xue et al. 2017). In another study concerning lung damage due to I/R and protective effect of silostazol and levosimendan, GSH levels were found significantly attenuated due to I/R (Önem et al. 2012). Our results were found similar with literature. I/R is an intensely studied subject due to its incidence however its pathogenesis and molecular pathways are not clarified completely. I/R damage occurs due to acute lung damage, acute kidney failure, shock and infection with different pathophysiological alterations in cellular, tissue and organ level. Investigation of extent of I/R damage can be conducted with histopathological evaluation of samples (Tassiopoulos et al. 1997; Wagner et al. 2002). Vascular congestion, epithelial loss and hyaline membrane formation, degenerations and necrosis in bronchiolar and alveolar cell walls, thickening in interalveolar septum, congestion in capillary walls, mononuclear cell infiltrations can be observed with light microscope (Odabaşı, D.2006; Schnells et al. 1979; Pietra et al. 1981; Tanahashi et al. 1999). In addition extravascular erythrocytes, fibrinogen rich serum may fill alveolar and interstitial area thereby causing edema. Protein rich edema is caused by increased permeability in diffuse alveolar damage (Holter et al. 1986) and this permeability damage affects pulmonary surfactant system (Petty et al.1977; Aromsne et al. 1996). Alveolar surfactant is activated in the presence of plasma proteins. Anomaly or loss of this surfactant is ended up with micro atelectasis in alveoli and respiratory bronchioles (Holm et al. 1988). Such effects occurring during ischemia-reperfusion arise due to oxidative stress caused by augmented oxygen radical production in each stage of micro circulation (Colletti et al. 1995). Cells can adapt to a certain level of increased oxidative stress occurring in a slow increase fashion. However when oxidative level reaches to an intolerable level cellular function deteriorates and irreversible cell damage occurs which leads to cell death (Teoh et al. 2003). Lung tissue is one of the most vulnerable tissue against oxidative stress due to its special structure and function (Guo et al. 2007). Following I/R, formation of free oxidant radicals and inflammatory mediators initiate local and systemic damage (Grace, P.A. 1994). Although reperfusion of blood flow saves the extremity from necrosis, it may lead to multisystemic organ dysfunction and mortality. Local effects are observed in skeletal muscle and vessel endothelium whereas systemic effects are observed in especially myocardial tissue, lung and kidneys (Blaisdell et al. 2002). Lung tissue is the most vulnerable tissue for lower extremity I/R damage and due to occurred damage ventilator and inotropic support is needed in some cases which may even result in death (Rocker, G.M.1997). Researchers administered some substances with antioxidant nature to minimize or prevent damage caused by I/R. In a study including 3 hours of I/R, widespread neutrophil infiltration, alveolar array disorientation, intraalveolar edema formation in lungs of I/R administered group. This serious lung damage was alleviated with dextmedetomidin administration (Küçükebe, Ö.B. 2009). In a lower extremity I/R model dexametazon and aminoguanadion administration decreased lung PNL numbers, interstitial edema and congestion compared to I/R group (Tassiopoulos et al. 1997). Similarly in literature melatonin was given to alleviate damage following administration of different I/R models and PNL accumulation was attenuated due to melatonin administration (Celik et al. 2002; Inci et al. 2002). In histopathological investigation of protective effect of melatonin on lower extremity I/R induced lung damage; edema, alveolar congestion, presence of MNL cells, fibrin-platelet-trombus formation, chronic inflammation and intraalveolar bleeding in lungs were ameliorated by melatonin by preventing lipid peroxidation and reducing neutrophil infiltration (Uysal et al. 2006). Researchers formed ischemia in lungs by adjusting a clamp on abdominal aorta and following reperfusion congestion, mononuclear cell infiltration and interstitial edema which are frequently encountered in lung damage was avoided by using aprotin (Şirin et al. 2001). In a study investigating protective effect of caffecic acid phenylester in lower extremity I/R damage peribronchial and perivascular leucocyte infiltration was decreased compared to I/R administered group (Çalkoğlu et al. 2004). Similarly ascorbic acid was also found effective in preventing increase in MNL count, interstitial edema and congestion due to I/R (Berkan et al. 2001). Our study focuses on protective effect of AST in lung damage due to lower extremity I/R damage. Histopathological evaluation showed that I/R group had congestion in interalveolar capillaries, alveolar macrophage and erythrocyte accumulation in some
bronchiole and alveolar lumens, interstitial and alveolar edema, perivascular and peribronchiolar MNL cell infiltration, hyperemia in capillaries of inter alveolar septum, interstitial pneumonia with enlarged inter alveolar septum due to bleeding and inflammatory cellular infiltrations, degeneration and necrosis in some bronchioles and alveolar epithelial cells and desquamation with necrosis, muscular hyperplasia in vessel and bronchiolar smooth muscle layers, congestive atelectasis in different intensities due to heavy exudate accumulation in interstitial area and also disruption of normal morphological appearance of some alveolar in an emphysematous fashion was observed. However in I/R+AST group an alleviation of this damage was observed. Hyperemia in capillaries of some inter alveolar septum and inflammatory cellular infiltrations and an enlargement in some of the inter alveolar septum was observed but those findings was quite low compared to I/R group and resembling control group. Our results show similar findings with literature mentioned above when those histopathological findings and damage scores are evaluated together (Weiss et al. 1989; Faust et al. 1988; Feller et al. 1989).

**Conclusion:** The protective effect of AST has been demonstrated by biochemical, histopathological and immunohistochemical effects.

**Ethical Considerations:** Study was approved by Animal Experiments Local Ethical Committee (18.11.2015 - 2015/08, Decision number: 08/02 ).

**Conflict of Interest:** Authors declare no conflict of interest.

**REFERENCES**


