

Astaxanthin-Mediated Returning the Aquaporin-1 to Basal Level May Be an Alternative Treatment Approach in Radiotherapy-Induced Lung Injury

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Abstract

Background: Radiotherapy (12-RAD), which has an important place in cancer treatment, destroys cancer cells, but also negatively affects healthy tissues in that area. Astaxanthin (ATX) is known to have powerful antioxidant, anti-inflammatory and antiapoptotic properties. In this study, we aimed to evaluate the effects of ATX on lung injury after radiotherapy.

Methods and Results: 32 rats were divided into 4 groups as control, 12-RAD (a single dose of 10 Gy x-ray was given at a rate of 0.62 Gy/min), 12-RAD+ATX, + ATX (10mg/kg ATX was administered by oral gavage for 7 days). Twenty-four hours after the last ATX application, the lung tissues of the rats were taken for biochemical, genetic, histopathological and immunohistochemical examinations. 12-RAD increased the oxidative stress markers TOS, OSI levels, the expression of VCAM-1, VEGF-A, which increases in inflammation, and the apoptotic marker, caspase-3. It also significantly reduced Aquaporin-1 and TAS levels. On the other hand, ATX has returned all these parameters.

Conclusion: All evidence suggests that the use of ATX may be a potential therapeutic agent against radiotherapy-induced lung injury.

Introduction

A hundred years ago, Marie Curie won the Nobel Prize for her research into radium, which established Marie Curie's position as a pioneer in the field of radiation therapy, her position as a pioneer in radiation therapy. After the discovery of X-rays in 1895, its clinical use from Germany by Wilhelm Conrad Röntgen became involved in the process of gaining acceptance as a means of cancer treatment. In the process since then, radiation therapy has become a recognized medical specialty with radiation oncology, a discipline in which various health and scientific professionals from many disciplines work together [1]. Along with surgery and systemic treatments, radiotherapy is an important component of cancer treatment. According to evidence-based radiotherapy indications, more than half of cancer patients require at least one radiation therapy throughout the course of their disease, either alone or in combination with other treatment modalities [2]. This treatment destroys cancer cells but also affects healthy tissues around the irradiated area and causes vascular lesions. These lesions may develop into fibrosis and adhesions between the skin and the chest wall, shoulder, and muscles of the supraclavicular and axillary spaces [3]. Radiation can disrupt epithelial and endothelial integrity leading to a balance of molecular events, leukocytes, angiogenesis, and molecular events that alter the microenvironment of edema, leukocytes, angiogenesis, and a self-sustaining inflammation cycle and chronic oxidative stress in lung tissue. In radiation-induced lung stages, a dose-dependent leakage of proteins into the alveolar space, thickening of the alveolar septa, Interstitium edema, replacement of capillaries, and changes in Type II pneumocytes and alveolar macrophages occur [4]. Radiation-induced cell death is modulated by several factors related to molecular physicochemical effects that induce cellular stress. Depending on the dose and irradiation programs used, cellular senescence or death will occur as a result of the imbalance

caused by stress [5]. Induction of cell apoptosis and arrest of the cell cycle in cancer cells are strategies of radiotherapy [6].

Apoptosis is a highly regulated cell death process [7]. The appearance of discontinuous uncontrolled growth, meaning cancer, means that homeostasis is no longer maintained and a subpopulation of cells called "malignant" has, by genetic or epigenetic means, escaped the body's natural oversight. This apoptosis resistance is one of the defining features of cancer. The therapeutic intervention basically tries to restart this intrinsic property of the cells. The result is that high doses of radiation or chemotoxic agents to overcome the block in apoptosis cause collateral damage to healthy tissue. To maximize apoptosis of malignant cells, alternative foci of attack should be considered while simultaneously minimizing damage sustained by normal cells [8]. A notable feature of apoptosis exerts its effects mainly by the action of a type of serine protease known as caspases (cas). Death signals are transmitted via signaling pathways that ultimately lead to the activation of cas responsible for carrying out cell destruction. Both external and internal stimuli that bind to external and internal apoptosis pathways can initiate apoptosis. Despite the many differences in different pathways, they eventually coalesce to form apoptotic bodies that are inserted by phagocytes in a process known as efferocytosis [7]. As a result, the cell is involved in the apoptosis process.

Carotenoids are a class of bioactive natural products synthesized by plants and some photosynthetic microorganisms. Many carotenoids directly participate in the photosynthesis process, while others protect the host from photooxidation and related damage. Astaxanthin (ATX) is a carotenoid that has received great attention in recent years. In addition, ATX (3,3'-dihydroxy- β , β '-carotene-4,4'-dione), the red carotenoid pigment classified as a xanthophyll, is known to have the strong antioxidant ability. The beneficial effects of astaxanthin are associated with its antioxidant, anti-inflammatory, and antiapoptotic properties. ATX is one of the promising agents in the prevention of various diseases, and both basic and clinical research on the health benefits of ATX has developed rapidly over the past few years [9, 10]. In this study, we aimed to evaluate the effects of ATX on lung injury after radiotherapy.

Materials And Methods

Study animals and design of experiment

The animal experiments were approved by the local animal ethics committee of Suleyman Demirel University (Ethic No: 07.01.2021/01–05) This study was supported by the Scientific Research Projects Coordination Unit of Suleyman Demirel University with project code TSG-2020-8134. We used 32 adult female Wistar albino rats weighing 300–350 g that were housed at 21–22°C and 60 ± 5% humidity with a 12 h light:12 h dark cycle. All rats were fed a standard commercial chow (Korkuteli Yem, Antalya, Turkey) with food and water available ad libitum. The rats were distributed into four groups of ten: control, 12-RAD, 12-RAD + ATX, and ATX groups.

1- Control group (n = 10); On the first day of the study, all experimental animals will be given the same cage conditions without being exposed to X-rays after anesthesia with 90 mg/kg ketamine (Alfamin, Alfasan IBV, Turkey) and 10 mg/kg xylazine (Alfazin, Alfasan IBV, Turkey) and 1 ml saline orally. It was applied for 7 days by the gavage method.

2- 12-RAD group (n = 10); On the first day of the study, after anesthesia of 90 mg/kg ketamine (Alfamin, Alfasan IBV, Turkey) and 10 mg/kg xylazine (Alfazin, Alfasan IBV, Turkey) i.p, all experimental animals were given a single dose of 10 Gy X-ray at a rate of 0.62 Gy/min. was applied to the body (600C linear accelerator, Varian, USA) and 1 ml of saline was applied by oral gavage method for 7 days.

3- 12-RAD + ATX group (n = 10); On the first day of the study, all experimental animals were given 90 mg/kg ketamine (Alfamin, Alfasan IBV, Turkey) and 10 mg/kg xylazine (Alfazin, Alfasan IBV, Turkey) ip as a single dose of 10 Gy X-ray at a rate of 0.62 Gy/min. was applied to the body (600C linear accelerator, Varian, USA) and 1 ml of 10mg/kg ATX was applied by oral gavage method for 7 days.

4- ATX group (n = 10); On the first day of the study, all experimental animals were administered i.p with 90 mg/kg ketamine (Alfamin, Alfasan IBV, Turkey) and 10 mg/kg xylazine (Alfazin, Alfasan IBV, Turkey) for 7 days with 1 ml 10mg/kg ATX oral gavage method.

All rats were anesthetized with 80–100 mg/kg Ketamine (Alfamine, Alfasan IBV) and 8–10 mg/kg Xylazine (Alfazin, Alfasan IBV) 24 hours after the last drug administration. Blood was taken from the Vena Cava Inferior from the rats who underwent abdominal incision following anesthesia. Then, half of the tissues taken were wrapped with aluminum foil in a cold environment and brought to -20°C for biochemical and genetic analysis.

Histopathology analysis

Lung samples were collected during autopsy and fixed in 10% formaldehyde. The samples were then routinely processed by an automated tissue processing equipment (Leica ASP300S, Wetzlar, Germany) and embedded in paraffin wax. Tissue sections were cut at a thickness of 4 µm with a rotary microtome (Leica RM2155, Leica Microsystems, Wetzlar, Germany). It was then stained with Hematoxylin-Eosin (HE), covered with a coverslip with a mounting medium, and examined under a Nikon Eclipse light microscope.

Immunohistochemistry analysis

For immunohistochemistry, lung samples by streptavidin-biotin technique cas-3 (Anti cas-3 antibody (31A1067):sc-56053) vascular cell adhesion molecule- 1 (VCAM-1). 1 (Anti VCAM-1 antibody (M/K-2):sc-18864) all primary sera and secondary antibodies were purchased from Abcam (Cambridge, UK) and all primary antibodies were used at 1/100 dilution. Primary antibodies were incubated for a period of 60 min and immunohistochemistry was performed using biotinylated secondary antibody and streptavidin-alkaline phosphatase conjugate. The ready-to-use kit [EXPOSE Mouse and Rabbit Specific HRP / DAB Detection IHC kit (ab80436)] was used as the secondary antibody and 3,3-diaminobenzidine (DAB) as the chromogen for 5 minutes. The primary antiserum step was skipped for negative controls. All

examinations were performed on blind samples. To assess the percentage of positive cells for each marker, statistical analyzes of the results from the Image analyzer of 100 cells counted in 10 different fields for each section at a magnification under the X40 objective for all groups were performed. Morphometric analyzes were performed using the Database Manual Cell Sens Life Science Imaging Software System (Olympus Co., Tokyo, Japan).

Biochemical analysis

Oxidative stress was analyzed in lung tissue. Lung tissues were diluted with 5x (w/v) phosphate-buffered saline (10 mM pH 7.4) and homogenized using Janke&Kunkel IKA Ultra Turrax T25 (Germany) tissue homogenizator. After the homogenization process, the samples were centrifuged at + 4 ° C at 2000 rpm for 20 minutes (Nüve NF 1200 R, Turkey). From the supernatants, tissue TAS (total antioxidant status) and TOS (total oxidant status) levels were measured on Beckman Coulter AU 5800 biochemistry autoanalyzer (USA), and also OSI (oxidative stress index) levels of the samples were calculated [11, 14].

TAS was measured using an automated colorimetric measurement method developed by Erel [12]. Antioxidants in the sample reduce dark blue-green colored 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical to colorless reduced ABTS. The change of absorbance at 660 nm is related to the total antioxidant level of the sample. In this way, the antioxidative effect of the sample against the free radical reactions initiated by hydroxyl radicals can be measured. The tissue TAS levels were expressed as $\mu\text{mol trolox equiv/l}$.

TOS of lung tissue was measured using an automated colorimetric method [13]. Oxidants in the sample oxidize the ferrous ion-dianisidine complex to the ferric ion. The oxidation reactions are enhanced by glycerol, which is abundant in the reaction medium. The ferric ion forms 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 already present in the sample. The assay was calibrated using H_2O_2 and the results were expressed as $\mu\text{mol H}_2\text{O}_2 \text{ equiv/l}$ [13]. OSI was calculated using the formula: $\text{OSI} = \text{TOS}/\text{TAS}$ [14].

Reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was extracted from rat lung tissues by using AurumTM Total RNA mini kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. The concentration and purity of isolated RNAs were determined by Allseng Nano 400A microvolume spectrophotometer (Allsheng, Hanngzou, China). 1 μg RNA was reverse transcribed with the iScript cDNA Synthesis kit by using oligo dT primers (Bio-Rad Laboratories, Hercules, CA). The reaction mix was incubated for 5 min at 25°C, for 20 min at 46°C, and for 1 min at 95°C. Real-time PCR amplification was performed using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions, and a fluorescence signal was detected on a CFX Connect instrument (Bio-Rad Laboratories, Hercules, CA). Specific primers were designed to amplify VEGF-A (Forward 5'-GGAAGAGAGAGAGAGAGAGAC-3', Reverse 5'-GACTGGTCCGATGAAAGATCC-3'), Aquaporin-1 (Forward 5'-CTGAGGAAAGAAGCAGCTAGAC-3',

Reverse 5'-CTCATAAGCCAGGACAAGAGTG-3') and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Forward 5'-CAAGGTCATCCCAGAGCTGAA-3', Reverse 5'-CATGTAGGCCATGAGGTCCAC-3'). cDNA samples were analyzed in triplicates for each PCR. The expression of VEGF-A and Aquaporin-1 were detected, and GAPDH was used as a housekeeping gene for normalization. The following PCR conditions were used: denaturation at 95°C for 10 min, followed by 40 cycles of: 10 s at 95°C, 30 s at 60°C. The total reaction volume was 25µl, and 100ng cDNA was used as the template. Relative quantification of gene expression was performed using the comparative $2\Delta\Delta C_t$ analysis method. All results were presented in the graph as a fold change.

Statistical analysis

The biochemical scores of the groups were compared between groups for statistical analysis. One-way ANOVA post hoc LSD test was used. Data are mean $\pm$ SD. Statistical calculations were performed using the SPSS 18.0 program package (SPSS Inc., Chicago, IL). Morphometric analyzes were performed using the Database Manual Cell Sens Life Science Imaging Software System (Olympus Co., Tokyo, Japan). Values for $p < 0.05$ were considered significant.

Results

Histopathological Lung Findings

In the microscopic examination, no pathological findings were observed in the lung tissues of the control group. In the 12-RAD group, alveolar hemorrhage was observed in one, while congestion was observed in all animals. It was observed that the treatment groups treated with 12-RAD + ATX decreased histopathologically compared to the 12-RAD group. No pathological findings were observed in the groups that were administered only ATX(Fig. 1).

Expression with cas-3 was not observed in the control group. In the 12-RAD group, Cas – 3 positive (+) accumulation was observed in the alveoli. It was observed that cas-3 positivity decreased in the 12-RAD + ATX applied group. Expression with cas-3 was not observed in the ATX administered group(Fig. 2). Expression with VCAM-1 was not observed in the control group. In the 12-RAD group, there is weak accumulation positivity around the bronchioles and on the alveolar walls. Its positivity was VCAM-1. No expression was observed in the 12-RAD + ATX administered group(Fig. 3). Expression of VCAM-1 was not observed in the ATX group(Table 1).

Table 1
Histopathological changes in lung of the experiment groups.

GROUPS	VCAM-1		Cas-3	
	Mean $\pm$ SD	p value	Mean $\pm$ SD	p value
Control	0.00 $\pm$ 0.00		0.00 $\pm$ 0.00	
12-RAD	1.25 $\pm$ 0.70 ^a	a: 0.000	1.12 $\pm$ 0.83 ^a	a: 0.000
12-RAD + ATX	0.25 $\pm$ 0.46 ^b	b: 0.000	0.37 $\pm$ 0.51 ^b	b: 0.005
ATX	0.00 $\pm$ 0.00 ^b	b: 0.000	0.00 $\pm$ 0.00 ^b	b: 0.000
<i>Values were presented as means $\pm$ SD. 12-RAD: Radiation; 12-RAD + ATX: Radiation + Astaxanthin; ATX: Astaxanthin, a: Control and others; b: 12-RAD and others</i>				

TAS, TOS and OSI Results

TAS level decreased significantly in the 12-RAD group compared to the Control group ($p = 0.002$). On the other hand, it was found to be significantly higher in the 12-RAD + ATX group than in the 12-RAD group ($p = 0.019$).

There was a significant increase in TOS and OSI levels in the 12-RAD group compared to the Control group ($p = 0.004$ and $p < 0.001$ respectively). There was a significant decrease in TOS and OSI levels in the 12-RAD + ATX group compared to the 12-RAD group ($p = 0.025$ and $p < 0.001$, respectively) (Fig. 4).

Relative mRNA expression level of VEGF-A and Aquaporin-1 in Lung Tissue

Relative VEGF-A mRNA levels were found to be significantly higher in the 12-RAD group than in the control and ATX groups ($p < 0.001$). In 12-RAD + ATX group, mRNA expression of VEGF-A decreased compared with the 12-RAD group (not significant)(Fig. 5).

Aquaporin (AQP)-1 level was also found to be significantly lower in the 12-RAD group compared to the control group ($p = 0.006$). A significant increase in the AQP-1 level was observed in the 12-RAD + ATX group compared to the 12-RAD group ($p = 0.045$)(Fig. 5).

Discussion

Ionizing radiation damages not only rapidly proliferating tumor cells but also normal tissue in the radiation area. Radiation injury triggers inflammation [15]. Inflammation is a vital response of the human immune system. Inflammation is a comprehensive set of physiological responses to a foreign organism, including human pathogens, dust particles, and viruses. Inflammations are basically divided into acute and chronic inflammation depending on various inflammatory processes and cellular mechanisms. Acute

inflammation is a short process lasting from minutes to days, and its main features are infiltration of plasma proteins or fluids and movement of leukocytes into the extravascular space. Chronic inflammation arises either through infections that are not resolved within the endogenous defense mechanisms or through another resistance mechanism from the host defense [16]. A typical inflammatory response consists of four components: inflammatory inducers, sensors that detect them, inflammatory mediators induced by sensors, and target tissues affected by inflammatory mediators. Each ingredient comes in multiple forms, and their combinations function in different inflammatory pathways. The type of pathway induced under certain conditions depends on the nature of the inflammatory trigger [17].

The vascular endothelium forms the interface between blood and tissues and plays an important role in promoting the inflammatory response, including recruitment of circulating leukocytes to the vessel wall and surrounding tissues at sites of inflammation. One of the first and key events in the response of the endothelium to inflammatory stimuli is the expression of VCAM-1. This protein mediates early leukocyte binding and rolling on the endothelial surface. Subsequent events, such as tight adhesion and migration along the endothelial lining, set the stage for an inflammatory response in the tissues [18]. As we expected in the findings we obtained in the histopathological results, an increase in VCAM-1 levels was detected in the 12-RAD group. Considering that the increase in VCAM-1 levels is associated with inflammation, we can conclude that radiotherapy contributes to inflammation by increasing VCAM-1 levels. ATX treatment decreased the VCAM-1 levels and regressed the severity of inflammation and regressed the damage in the lung tissue.

Alveolar damage occurs after radiotherapy secondary to inflammation. This causes edema of the alveolar walls due to increased vascular permeability and exudation of proteins. A number of pathological conditions (such as respiratory distress syndrome and pulmonary edema from injury or infection) can severely affect the lungs, resulting in impaired fluid transport of AQPs [19]. AQPs are small, intrinsic membrane proteins that facilitate water flow across cell membranes and provide an intercellular pathway for water transport across the epithelium [20, 21]. AQPs are found in many tissues where rapid water transport is important and necessary, such as the lungs, eyes, and kidneys. Major AQPs in peripheral lung tissues are AQP-1 and AQP-5 [22]. It has been shown that AQP-1 plays an important role in the water balance of the respiratory system [23]. In a study, a decrease in AQP-1 and AQP-5 levels was found in mouse lung after acute adenoviral infection. In addition, deletion of AQP-1 in mice has been shown to result in a 10-fold reduction in osmotic water transport between the airspace and capillary compartments [19]. In this study, the level of AQP-1 in the lung tissue was decreased in the 12-RAD group. From this point of view, radiotherapy caused serious damage by decreasing the level of AQP-1 in the lung tissue and disrupting the intercellular water transport function. This damage has been revealed by both genetic analysis and histopathological findings. Administration of ATX caused a significant increase in the AQP-1 level, preventing the decrease caused by radiotherapy. It also significantly reduced the damage caused by the change in the AQP-1 level.

Vascular endothelial growth factor-A (VEGF) is one of the most important growth factors for the regulation of vascular development and angiogenesis [24]. In contrast, increased expression of VEGF is a potent mitogen and permogen that increases in plasma and decreases in the alveolar space in respiratory diseases. It is also known that an increase in VEGF causes an increase in lung damage [25]. In this study, it was shown by genetic analysis that VEGF level increased in lung tissue in the 12-RAD group. Based on this result, we can say that radiotherapy contributes negatively to lung damage by causing an increase in VEGF level. On the other hand, ATX treatment regressed the damage in the lung tissue by reducing the abnormally increased VEGF levels with radiotherapy.

Oxidative stress and inflammation are closely linked. One of the most important sources of damage caused by inflammation is free oxygen radicals. Oxidative stress causes cells such as leukocytes to accumulate significantly in lung tissue, causing inflammation [26, 27]. In this study, decreases in TAS levels were found in the injury group treated with 12-RAD. From this point of view, the use of too many antioxidants was needed to cope with oxidative stress in the case of 12-RAD. Accordingly, the lowest level was found in the 12-RAD group in our analysis. On the other hand, the significant increase in TOS and OSI levels in the 12-RAD group indicates that the increase in oxidant levels in case of damage causes oxidative stress. Reversing these negative outcomes in ATX treatment; It reveals that ATX has antioxidant properties.

Radiation-induced cell death is modulated by several factors related to molecular physicochemical effects that induce cellular stress. Depending on the dose and irradiation programs used, cellular aging or death occurs as a result of the imbalance caused by stress [5]. Apoptosis, or programmed cell death, plays an important role in various disease states and is regulated in a variety of ways [28]. Excessive or insufficient apoptosis may contribute to the pathogenesis of various human diseases. Apoptosis is triggered by external stressors (eg, death ligands, ultraviolet, and γ radiation) and/or internal stimuli (eg, oxidants, DNA damage, increased Ca^{2+}) [29]. Regardless of how apoptosis is initiated, a family of cysteine aspartate-directed proteases known as cas are systematically activated from inactive zymogen-like states and target a number of cellular proteins for hydrolysis upon maturation. Induction of apoptosis leads to both protein substrate degradation and activation and ultimately cell death. Cas are heavily regulated proteins by the nature of their activity, as inappropriate activation can have destructive effects [30]. Apoptotic cas exist in inactive preforms and each cleaves to induce its activation. Following activation of intrinsic or extrinsic pathways of the apoptotic cascade, initiator cas cleave and activate executioner cas-3, 6, or 7. Pro-cas-3 becomes an active enzyme when two cleaved monomers combine to form an active dimer; it has potent activity, including the ability to autocatalytically activate it, which contributes to the observed cascade effect of cas-3 activity, which increases as apoptosis progresses. Active cas-3 recognizes a specific short peptide cleavage motif and cleaves cellular proteins where this motif is present and accessible [31]. In addition, the data we obtained from the histopathological results show that cas-3 levels increased in the 12-RAD group. In conclusion, it can be said that 12-RAD causes an increase in cas-3 levels and drives the cell to apoptosis. There was a decrease in cas-3 levels in the 12-RAD + ATX group treated with ATX. Based on these data, it can be said that ATX regresses apoptosis by

causing a decrease in cas-3 levels. In other words, we can conclude that ATX significantly regresses lung tissue damage by showing anti-apoptotic activity.

Conclusion

In our study, we showed with our analyzes that ATX regresses the lung damage caused by 12-RAD. Our results proved the effectiveness of ATX in our study. The damage caused by 12-RAD was significantly reduced by ATX by showing anti-inflammatory, antioxidant, and antiapoptotic effects. All the evidences indicated that ATX usage could be a potential therapy to against radiotherapy-induced lung injury.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Authors' Contrubutions

Study was designed by Nursel Hasseyid, Suleyman Emre Akin and Halil Asci. Material preparation, data collection and analysis were performed by Yalcin Erzurumlu, Halil Ibrahim Buyukbayram, Hasan Ekrem Camas, Nursel Hasseyid and Ozlem Ozmen. The manuscript was written by Nursel Hasseyid and Suleyman Emre Akin and edited by Halil Asci. All authors read and approved the final manuscript.

Ethical Approval

The animal experiments were approved by the local animal ethics committee of Suleyman Demirel University (Ethic No: 07.01.2021/01-05). This study has been carried out in accordance with the standards set out in the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgements

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Figures

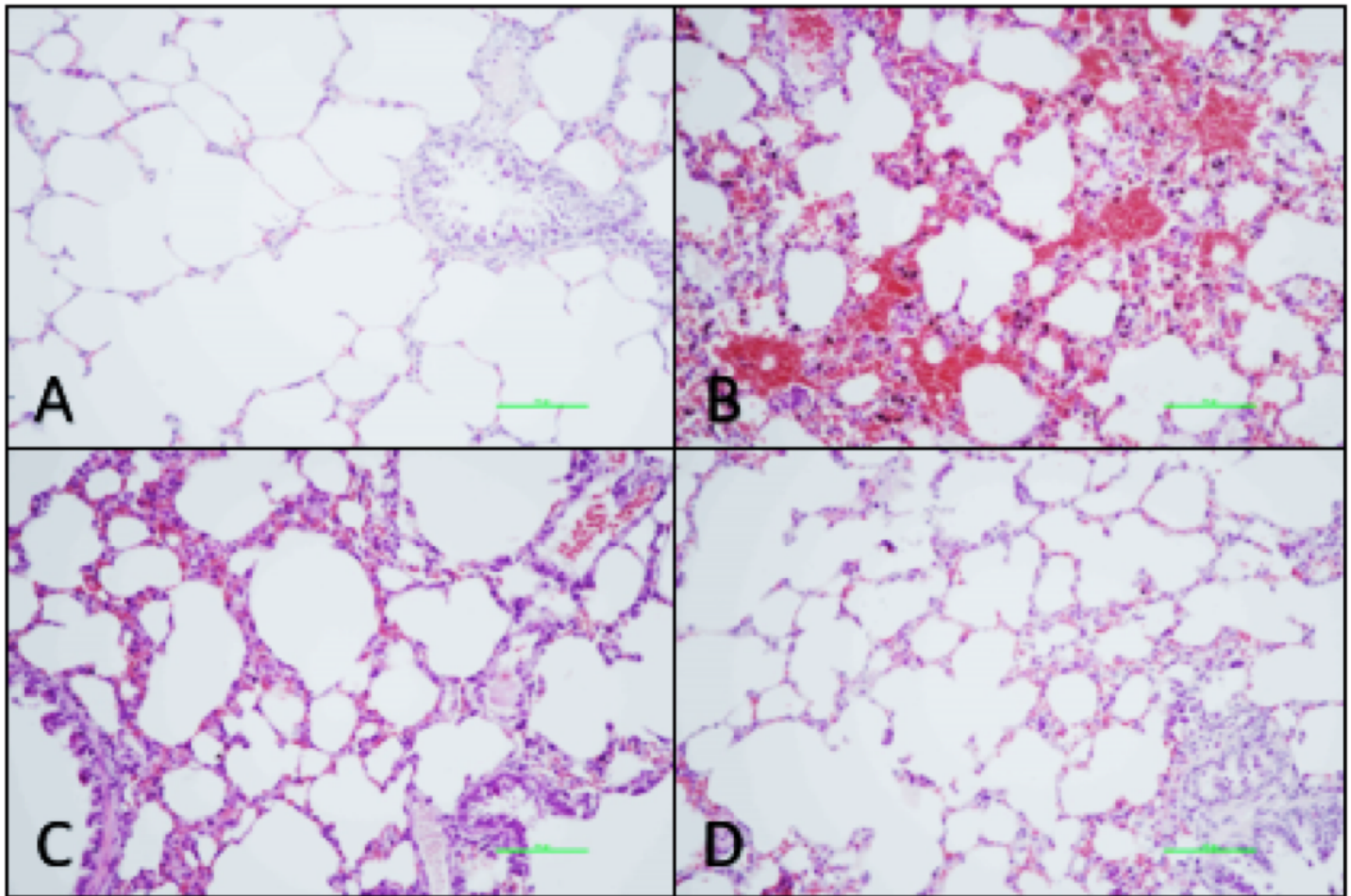


Figure 1

Representative histopathological microphotos between the groups.

(A) No expression was observed in the control group, (B) Congestion and bleeding in the alveoli were observed in the 12-RAD group (C) In the 12-RAD+ATX group, there was a decrease in the findings (D) No expression was observed in the ATX group (HE, 200X).

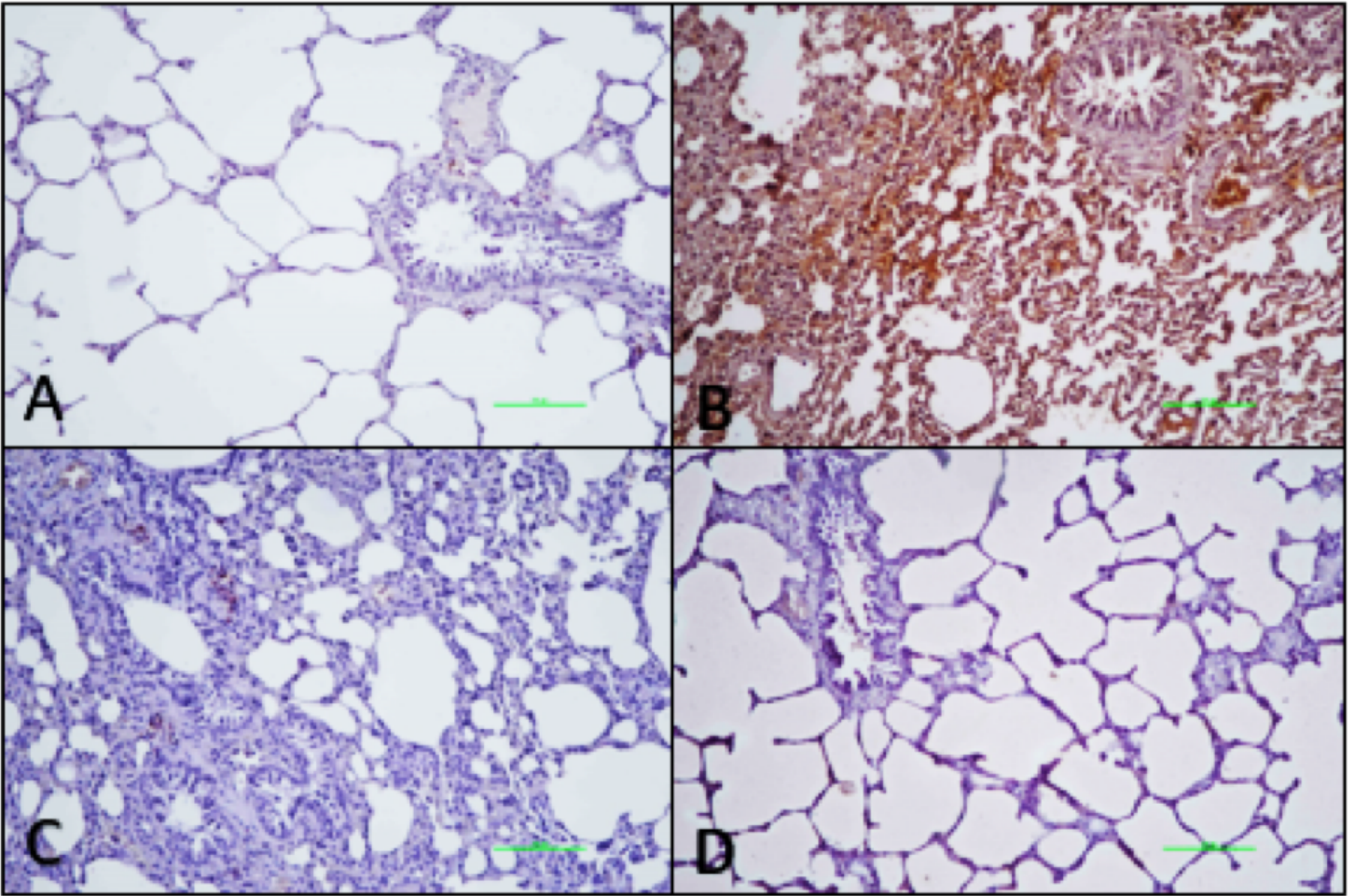


Figure 2

Cas-3 immunohistochemical finding between the groups.

(A) Cas-3 expression was not observed in the control group, (B) Cas-3 positivity in the alveolar walls of the 12-RAD group (+), (C) In the 12-RAD+ATX group, no expression was observed in the alveoli except positivity in the focal vessel wall (D) No expression was observed in Cas-3 in the ATX group (Cas-3, DAB, 200X).

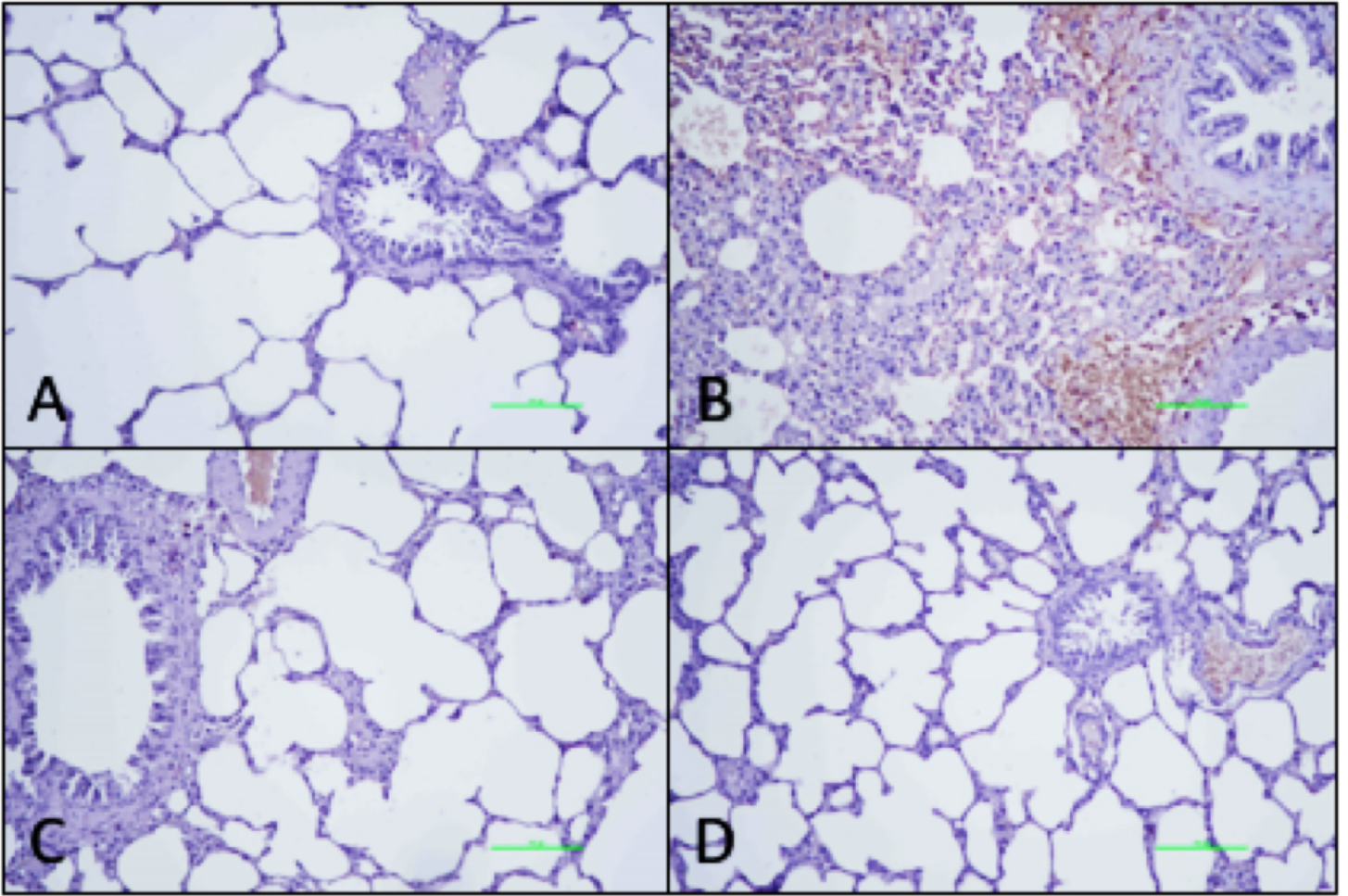


Figure 3

VCAM-1 immunohistochemical finding between the groups.

(A) No expression was observed in VCAM-1 in the control group, (B) Weak accumulation of VCAM-1 positivity around the bronchiole and alveolar walls in the 12-RAD group (+), (C) No expression was observed in the 12-RAD+ATX group (D) In the ATX group No expression was observed in VCAM-1 (VCAM-1, DAB, 200X).

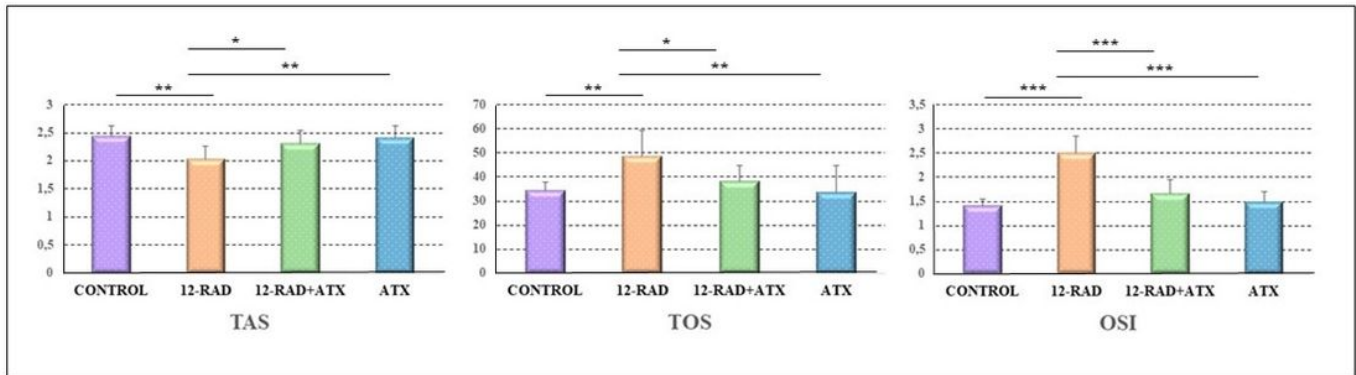


Figure 4

TAS, TOS and OSI Levels in Lung Tissue

Values were presented as means $\pm$ SD. 12-RAD: Radiation; 12-RAD+ATX: Radiation + Astaxanthin; ATX: Astaxanthin; TAS: Total Antioxidant Status; TOS: Total Oxidant Status; OSI: Oxidative Stress Index *: $p < 0,05$, **: $p < 0,01$, ***: $p < 0,001$.

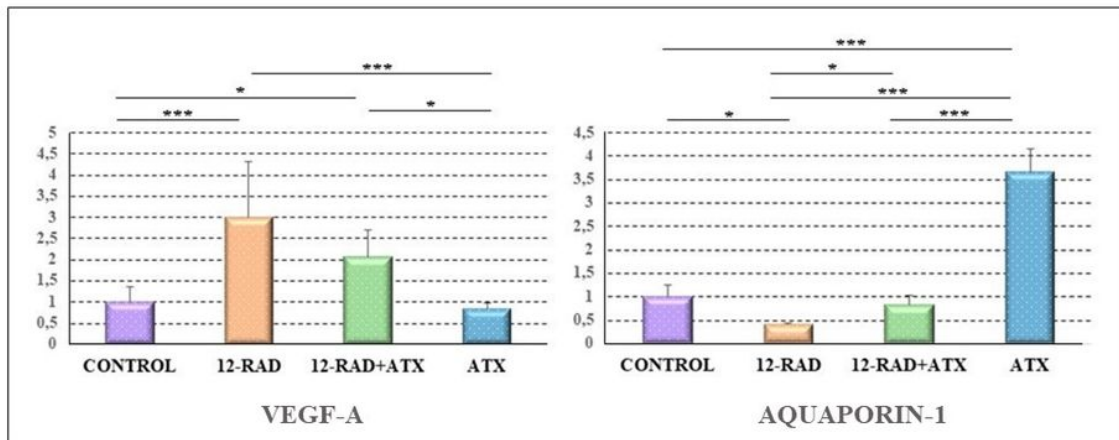


Figure 5

VEGF-A and Aquaporin-1 Levels in Lung Tissue

Values were presented as means $\pm$ SD. 12-RAD: Radiation; 12-RAD+ATX: Radiation + Astaxanthin; ATX: Astaxanthin; *: $p<0,05$, **: $p<0,01$, ***: $p<0,001$