Effects of Various Antioxidants on Rat Lung Tissue During Chemotherapy: Electron Microscopic Study

Kemoterapi Uygulamasında Çeşitli Antioksidanların Sıçan Akciğer Dokusu Üzerindeki Etkileri: Elektron Mikroskobik Çalışma

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ABSTRACT

Objectives: This study uses transmission electron microscopy technique to investigate the efficacy of different antioxidants (such as ascorbic acid, alpha-tocopherol and selenium) in repairing or reversing lung damage caused by the possible adverse effects of the chemotherapy (cyclophosphamide) application on the lung tissue of the subjects.
Materials and Methods: Thirty female Wistar rats were divided into five groups of six rats each: (I) control, (II) cyclophosphamide only (75 µg/kg), (III) cyclophosphamide (75 µg/kg) + ascorbic acid (200 µg/kg/day), (IV) cyclophosphamide (75 µg/kg) + α-tocopherol (150 µg/kg/day) and (V) cyclophosphamide (75 µg/kg) + selenium (40 ppm/kg/day). At the end of the experimental period the rats were sacrificed and the left lung of the subjects was removed and placed in a 2.5% glutaraldehyde solution in a 1/15 µ phosphate buffer (pH 7.4). The tissues were then stained with uranyl acetate and lead citrate to enhance the contrast, and examined and photographed with an electron microscope (Carl Zeiss 900 EM).
Results: Alveolar type II cells were found to have degenerated in the cyclophosphamide-treated lung tissues. Vacuolization and crystolisis of mitochondria, disruption of the lamellar order and indications of apoptosis were observed. In the α-tocopherol group, mitochondria were normal and fibrosis was reduced. In this group, damage to the cell membrane and defects of lamellar bodies were present. Other groups produced similar results to the cyclophosphamide group.
Conclusion: The results of our study showed that from all the antioxidants administered to rats during chemotherapy, only α-tocopherol was efficient in healing the tissue damage caused by cyclophosphamide.

Keywords: Cyclophosphamide, lung, chemotherapy, antioxidant, electron microscopy

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ÖZET

Amaç: Çalışmamızda, siklofosfamid uygulamasının sağlıklı sıçan akciğer dokusu üzerindeki olumsuz etkilerinin neden olduğu akciğer hasarını onarmada ya da geri çevirmede, farklı antioksidanların (askorbik asit, alfa-tokoferol ve selenyum gibi) etkinliğini, geçirmeli elektron mikroskobu tekniği ile araştırmayı amaçladık.
Yöntem: 30 adet dişi Wistar sıçanı, her biri altı sıçandan oluşan beş gruba ayrıldı: (I) kontrol, (II) yalnızca siklofosfamid (75 µg / kg), (III) siklofosfamid (75 µg / kg) + askorbik asit (200 µg / kg / gün), (IV) siklofosfamid (75 ug / kg) + a-tokoferol (150 ug / kg / gün) ve (V) siklofosfamid (75 ug / kg) + selenyum (40 ppm / kg / gün). Deney süresi sonunda sıçanlar sakrifiye edilerek sol akciğerleri çıkarıldı ve 1/15 u fosfat tamponu (pH 7.4) içinde % 2.5'lik glutaraldehit çözeltisine yerleştirildi. Dokular daha sonra kontrastı arttırmak için uranil asetat ve kurşun sitrat ile boyandı ve Carl Zeiss 900 EM ile incelendi ve fotoğraflandı.
Bulgular: Siklofosfamid uygulanan grupta; tip II alveolar hücrelerinde dejenerasyon tespit edildi. Mitokondri vakuolizasyonu ve kristolizi, lameller düzenin bozulması ve apopitoz belirtileri gözlendi. A-tokoferol grubunda ise mitokondriyonlar normaldi ve fibroz azalmıştı. Bu grupta, hücre zarı hasarlanması ve lamelli cisimlerin kusurları dikkati çekti. Diğer gruplarda, siklofosfamid grubuna benzer sonuçlar gözlemlendi.
Sonuç: Çalışmamızda, kemoterapi sırasında sıçanlara uygulanan çeşitli antioksidanlardan yalnızca α-tokoferolün ince yapı düzeyinde siklofosfamidin neden olduğu doku hasarını iyileştirmede etkili olduğu sonucuna varılmıştır

Anahtar Sözcükler: Siklofosfamid, akciğer, kemoterapi, antioksidan, elektron mikroskobu

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INTRODUCTION

In the last decade, there have been considerable developments in the treatment of cancer which is one of the most important health problems of our time. The conventional treatments for cancer include chemotherapy, radiotherapy, surgery and immunotherapy. These methods are used alone or in combination based on the individual characteristics of the patients diagnosed with cancer and the stage of the disease. These treatment methods aim to help cancer patients have a higher quality of life and increase their life span. Radiotherapy and chemotherapy methods used in the treatment of cancer have known toxic effects (1). One of the chemotherapy drugs commonly used in clinical studies is cyclophosphamide which is an alkylating oxazaphosphorine agent that is effective when taken orally. In malign and inflammatory cases, it is used to suppress the immune system. On its own, cyclophosphamide is an inactive molecule activated by the cytochrome P450 mixed function oxidase system, which metabolizes cyclophosphamide in hepatic microsomal enzymes and reduces it to its metabolite, acrolein (2).

In the literature, lung toxicity caused by cyclophosphamide has been described; however, the individual mechanism of the lung has not been fully elucidated. In addition, the functioning of pulmonary metabolism through the oxidase system has not been explained in detail (2).

Acrolein is generally considered to be the molecule responsible for lung injury. However, it is not clear whether acrolein is responsible for the metabolic pathways in the lungs of mice, and the role it has in the damage caused by cyclophosphamide. The possibility of the formation of nicotinamide adenine dinucleotide phosphate (NADPH)-supported alkylating metabolites in pulmonary microsomes is lower in mice compared to rats. This indicates that the therapeutic effects and toxicity differ according to the species (3).

The alveolar regions of the lung are exposed to reactive oxygen and nitrogen molecules from the inhaled air. The antioxidant defense system contains several low-molecule agents including lipophilic antioxidants such as glutathione, ascorbic acid (vitamin C), uric acid, alpha tocopherol (vitamin E), retinol and plasmalogens, and enzymes such as superoxide dismutase, catalase and glutathione peroxidase. These antioxidants protect the lung from complex pro-oxidant effects (4). Maintaining the balance between the antioxidant and oxidant concentrations is effective in preventing lung injury that can result in surfactant metabolism dysfunction as well as increased capillary leak. When free radicals attack the cell membrane, the thickness of the membrane is reduced resulting in rapid cellular and tissue degeneration. Furthermore, DNA mutations known as oxidative stress induce DNA fragmentation and apoptosis (5).

The current study investigated the efficacy of different antioxidants in repairing or reversing lung injury caused by the possible adverse effects of chemotherapy on the lung tissue of rats. Cyclophosphamide was used as the chemotherapy drug. Transmission electron microscopic examination was performed on the ultrastructure of pulmonary alveoli to determine whether the antioxidants administered during chemotherapy reduce the damage to cancer-free normal tissues caused by chemotherapeutic agents.

MATERIALS and METHODS

Animals

Thirty female Wistar albino rats (weighing between 100 g and 130 g) from the Hacettepe University Laboratory Animals Breeding and Experimental Research Center, Turkey, were used in this experimental study. All animals were housed in groups in plastic cages at a constant room temperature (21-22 °C) and humidity (55-60%) and in a 12 h light/ 12 h dark cycle (lights on at 07:00 a.m.) in the Gazi University Laboratory Animals Breeding and Experimental Research Center. Rats were given free access to food and water. All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Animal Care Committee of Gazi University.

Experimental procedure and dosing

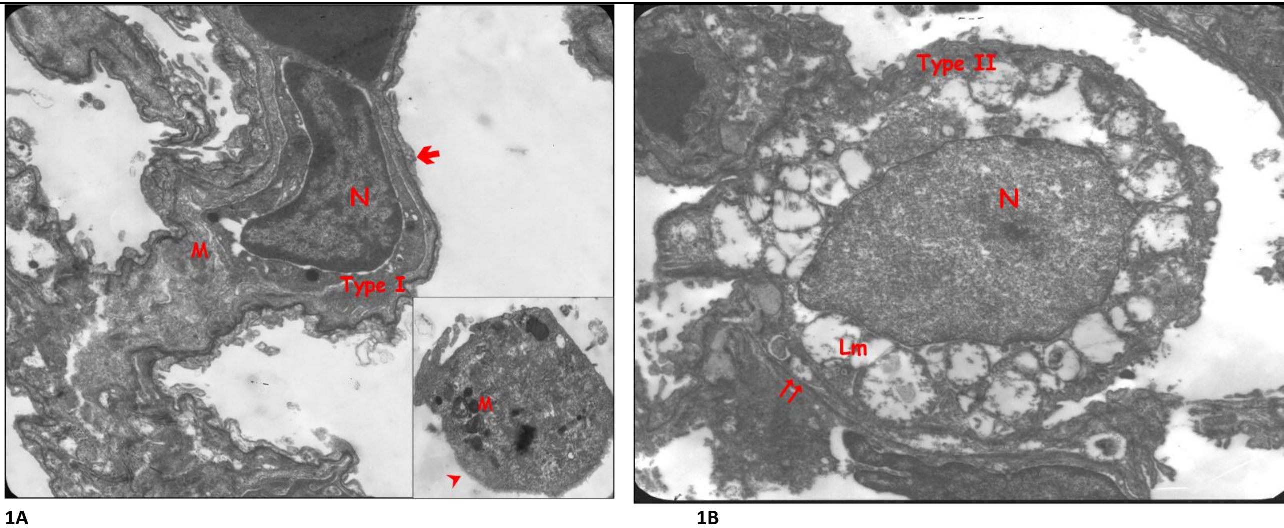
Rats were divided into five groups of six animals each. Group I served as a control, receiving saline throughout the experimental period (n = 6). Group II received intraperitoneal CP (Sigma, St. Louis, MO, USA) (75 mg/kg body weight) dissolved in saline, once a week for 3 weeks (6). Group III rats received intraperitoneal CP (75 mg/kg body weight) once a week for 3 weeks plus ascorbic acid (Sigma, St. Louis, MO, USA) (200 mg/kg) dissolved in distilled water and taken daily by oral gavage for 3 weeks (7). Group IV received intraperitoneal CP (75 mg/kg body weight) once a week for 3 weeks plus α-tocopherol (Sigma, St. Louis, MO, USA) (150 mg/kg) dissolved in olive oil and taken daily by oral gavage for 3 weeks (8). Group V received intraperitoneal CP (75 mg/kg body weight) once a week for 3 weeks plus selenium (Sigma, St. Louis, MO, USA) (40 ppm/day/rat) dissolved in distilled water and taken daily by oral gavage for 3 weeks (9). The extracted tissues were placed in a 2.5% glutaraldehyde solution in a 1/15 μ phosphate buffer (pH 7.4) for transmission electron microscopic examination.

Transmission electron microscopy

The tissues that were placed in the glutaraldehyde solution for 30 minutes were cut into 1-mm^3 pieces, which were then fixed with the glutaraldehyde solution for another hour. After the secondary fixation and staining in buffered osmium tetroxide, the tissues were passed through a series of alcohol concentrations. After being kept in propylene oxide, tissues were transitioned in propylene oxide for another 30 minutes to be implanted in the embedding material. The embedded materials were subsequently kept in a rotating oven at 40°C for 2 hours. Finally, the tissues were embedded in a size 00 gelatin capsule using the same mixture. From the prepared blocks, 1μ sections were cut with an LKB Leica ultramicrotome and stained with toluidine blue. Ultra-thick sections were examined under light microscopy (DCM 4000, Leica, Germany) and the identified ultra-thin sections of 0.2–0.5μ were cut on the same microtome mounted on formvar-coated copper grids. For the enhancement of contrast, these sections were stained with a solution of uranyl acetate and lead citrate, then examined and photographed with an electron microscope (Carl Zeiss EM 900).

RESULTS

In the pulmonary tissue of the control group, type I and type II alveolar cells in the alveolar wall, macrophages and vessels displayed a normal, ultra-thin structure. At high magnification, the cytoplasmic structure of type I alveolar cells were found to be normal and organelles were present. In the type II alveolar cells, lamellar bodies were frequently seen, the nucleus was of a normal structure and the basal lamina was distinct (Figures 1A, 1B).

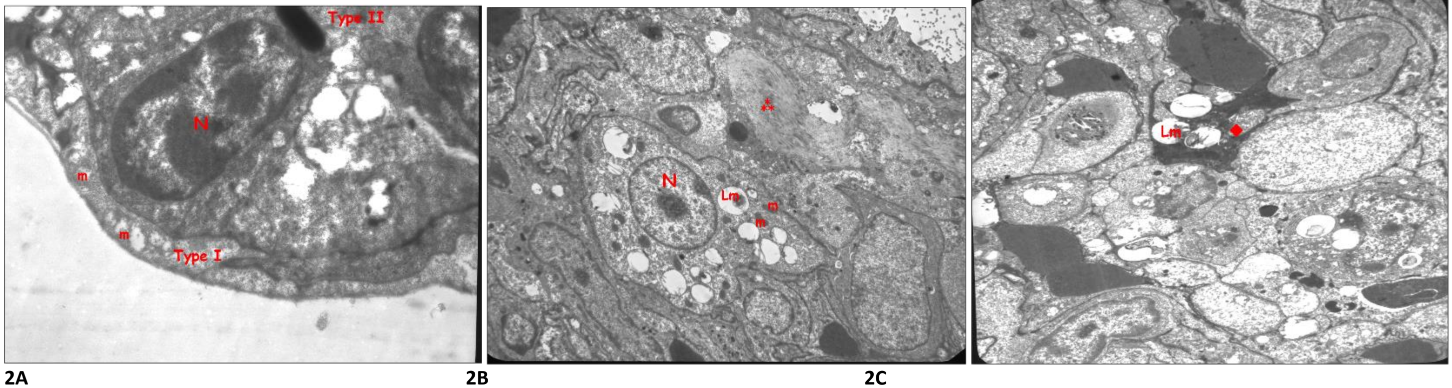


1A **1B**

Figure 1. TEM image lung tissue in the control group (A - B), (Type I): type I alveolar cell, (Type II): type II alveolar cell, (N): nucleus, (m): mitochondria, (⇉): basal lamina, (⋏): alveolar macrophage, (Lm): lamellar body. A, B x7000. (Uranyl acetate – lead citrate)

In the cyclophosphamide group, the type I cell structure in the alveolar wall was generally intact; however, in type II alveolar cell cytoplasm, vacuolization and crystolisis of mitochondria was commonly seen, lamellar bodies acquired a vacuolar structure and the lamellar order was disrupted (Figures 2A, 2B).

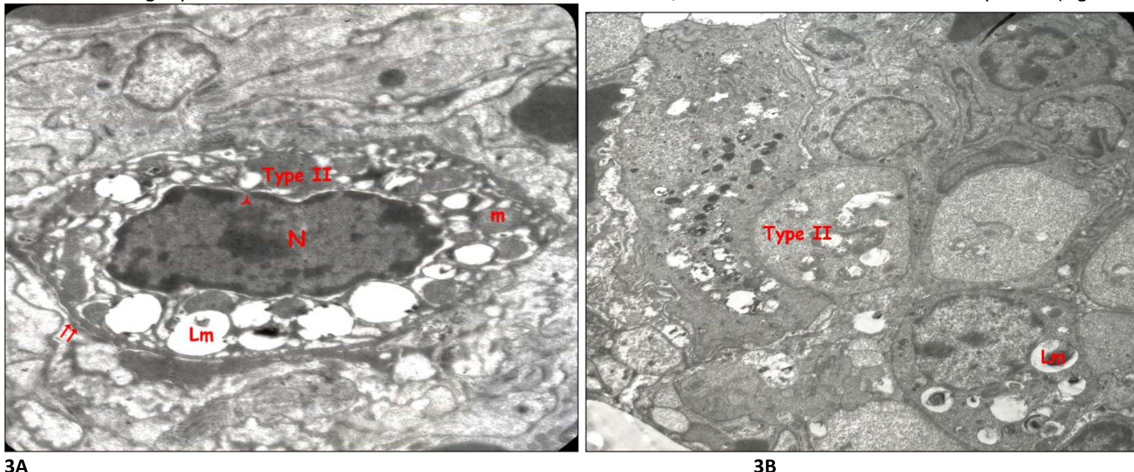
Some of the type II alveolar cells were observed to have undergone apoptosis, the alveolar lumen partially obliterated and fibrosis was commonly seen in the connective tissue (Figure 2C).



2A **2B** **2C**

Figure 2. TEM image lung tissue in cyclophoshamide group (A - B - C), (Type I): type I alveolar cell, (Type II): type II alveolar cell, (N): nucleus, (m): mitochondria, (Lm): lamellar body, (**⁂**): fibrosis, (◆): apoptozis. A x7000, B, C x3000. (Uranyl acetate – lead citrate)

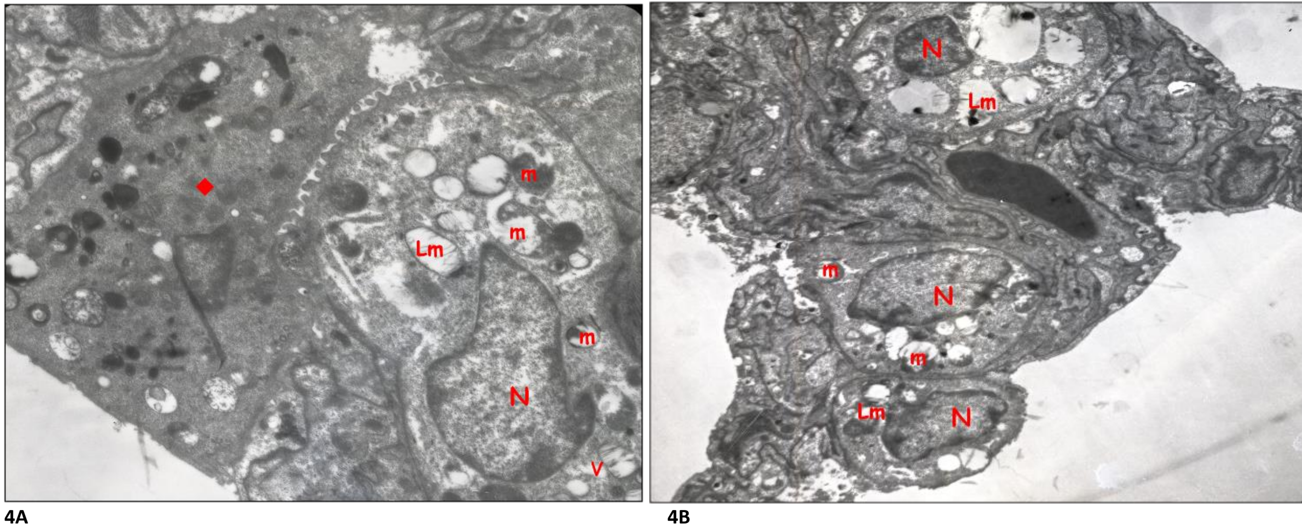
In the cyclophosphamide and �-tocopherol group, the frequency of fibrosis was found to be slightly lower. The mitochondrial structure was in a better state; however, the nuclear membrane was punctured in parts and the lamellar body defects continued to be present (Figures 3A, 3B).



3A **3B**

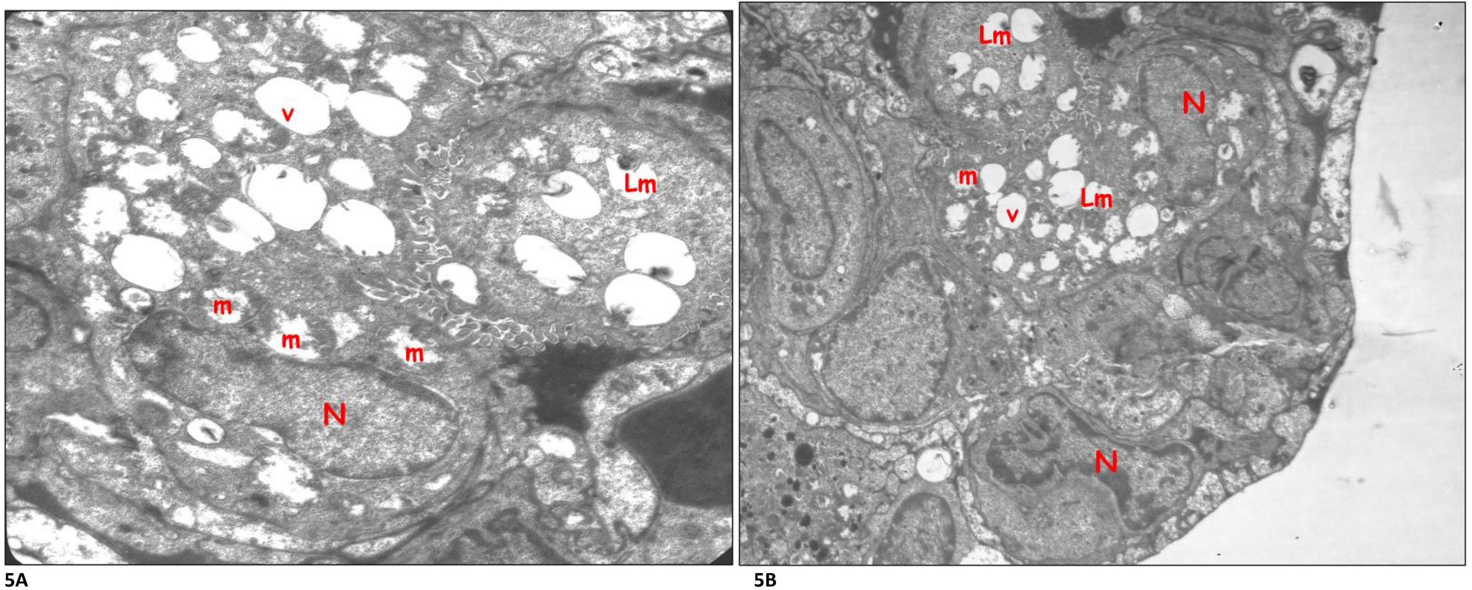
Figure 3. TEM image lung tissue in α-tocopherol group (A - B), (Type II): type II alveolar cell, (N): nucleus, (m): mitochondria, (Lm): lamellar body, (⇉): basal lamina, (⅄): separation in nuclear membrane. A x7000, B, x3000. (Uranyl acetate – lead citrate)

In the cyclophosphamide and ascorbic acid group, the ultrastructure was similar to that observed in the cyclophosphamide group (Figures 4A, 4B) in which the cyclophosphamide and selenium was not found to have a positive effect in terms of this structure (Figures 5A, 5B).



4A 4B

Figure 4. TEM image lung tissue in ascorbic acid group (A - B), (N): nucleus, (m): mitochondria, (Lm): lamellar body, (◆): apoptozis, (v): vacuoles. A x7000, B, x3000. (Uranyl acetate – lead citrate)



5A 5B

Figure 5. TEM image lung tissue in selenium group (A - B), (N): nucleus, (m): mitochondria, (Lm): lamellar body, (v): vacuoles. A x7000, B x3000. (Uranyl acetate – lead citrate)

DISCUSSION

Cancer increasingly affects human health and life span. The common treatment options for cancer include; chemotherapy, radiotherapy and immunotherapy. These methods are used alone or in combination based on the individual characteristics of the patients diagnosed with cancer and the stage of the disease. In many severe cases, IP chemotherapy not only has a significant role in the rapid recovery of general health but also in the treatment of tumors that metastasize to the peritoneum (10). However, several chemical agents, in particular the chemotherapeutical drugs that have a cytotoxic effect, cause systematic damage to the lungs (11).

Cyclophosphamide is an antineoplastic agent that has been shown in vivo and in vitro experiments to have a toxic effect on the lungs and therefore, it is commonly used in research. Cyclophosphamide is generally used in a single dose. Patel et al. conducted a study on rats and demonstrated the toxic effect of cyclophosphamide comprising alveolar pneumonia, damaged type I pneumocytes, abnormal formation of type II cells and increased collagen content characterized by fibrosis and loss of alveolar elasticity (12). An important result reported by similar studies is that such histopathologic findings are obtained even following the administration of a single dose of cyclophosphamide (13).

In the current study, the results of the transmission electron microscopic examination in the cyclophosphamide-treated groups showed that the degeneration of the alveolar structure reduced the alveolar tension. At the ultrastructural level, the type II alveolar cells started to lose their normal cytoplasmic features, the lamellae in the lamellar bodies vanished by being converted into vacuoles. Crystolisis of mitochondria was frequently observed. In certain parts of the lung tissue, there were distinct deposits of collagen fibers indicating the presence of fibrosis.

Despite not being the only option, multi-functional molecules of cell oxidation are an important way of metabolizing cyclophosphamide. Molecules oxidized and alkylated through hydroperoxide-independent oxidation (cooxidation) are recognized, thus preventing tissue damage and carcinogenesis. Two oxidant agents, prostaglandin-H synthase and lipoxygenase, are present in the lungs. Using these molecules, the lungs have the ability to alkylate and metabolize cyclophosphamide (14). In the lung, cell damage and fibrosis are significantly suppressed by prostaglandin-H synthase. The in vitro alkylation metabolism

occurs in an NADPH-independent manner in the hepatic microsome and in an arachidonic acid-independent manner in the pulmonary microsome (2).

Radiolabeled thymidine enters and damages the DNA of the pulmonary cell at the latest seven days after the cyclophosphamide treatment. Preliminary treatment with antioxidants that suppress the oxidation activity can prevent damage during the use of the drug (15). Several in vitro and in vivo studies have reported that antioxidants such as ascorbic acid, α - tocopherol and selenium (β-carotene) prevent oxidation caused by free radicals (16).

Ascorbic acid has intracellular antioxidant properties. Jain et al. investigated the effect of ascorbic acid on oxidative stress by administering ascorbic acid to glutathione-deficient mice and evaluated the findings with transmission electron microscopy. The results showed that glutathione deficiency damaged the lamellar bodies and mitochondria of type II cells in the lungs of adult mice. The researchers attributed this result to the reduced phosphatidylcholine level in the lungs and the bronchoalveolar lining. Furthermore, the authors explained that glutathione deficiency was due to the depletion of the lung surfactant. Adult mice can synthesize ascorbate in their tissue while newborns cannot. The results of several studies showed that the administration of high doses of ascorbic acid have a protective effect on glutathione-deficient newborn mice, newborn rats and guinea pigs while ascorbate treatment in glutathione-deficient adult mice prevents lung injury. In the antioxidant system, ascorbate and glutathione have a similar effect. In the absence of glutathione, ascorbate catalyzes glutathione peroxidase. Many researchers reporting the protective effect of ascorbic acid suggested that it could provide clinical benefits in practice (17).

The external administration of ascorbic acid to mice and rats reduces the level of ascorbic acid in the body and stops synthesis by raising the feedback mechanism. In this case, adding 50 mg daily ascorbic acid to diet reduces the ascorbic acid concentration in the liver and kidneys. All results suggest that the ascorbic acid concentration in the blood or culture affect the biosynthesis in tissues (18). Similarly, in the current study, the external administration of ascorbic acid did not have a positive effect on the pulmonary tissue. The ultrastructure in the cyclophosphamide and ascorbic acid group was found to be similar to that in the cyclophosphamide group.

The experimental results demonstrated the presence of an interaction between antioxidants that results in their regeneration. α-tocopherol is an effective chain-breaking antioxidant suppressing lipid peroxidation (19). Ascorbic acid renews the fragmented tocopherol radicals of α-tocopherol and organizes the selenium radicals (20, 21). α-tocopherol protects selenium from autoxidation. In contrast, selenium converts oxidized α-tocopherol to reduced α-tocopherol. However, the metabolism of selenium in the lung is still not clear. Ascorbic acid and α-tocopherol work together to prevent the decomposition of selenium by facilitating the conversion of retinoic acid (vitamin A) to retinol (19).

There are many protective agents, known as antioxidants, against oxidative damage in the cell caused by free oxygen radicals. These prevent lipid peroxidation by blocking peroxidation chain reaction or collecting reactive oxygen species. When free radicals attack the cell membrane, the membrane loses stability and cell and tissue degeneration occurs quickly due to lipid peroxidation. In addition, free radicals elicit DNA mutations, DNA chain breakage and apoptosis. These factors are also known as oxidative stress (22).

Selenium is a basic element in the structure of mRNA that regulates the function of the cell. Studies have shown that taking selenium reduces the risk of cancer development in the colon, lungs and prostate (23). In addition, in the literature, it has been reported that selenium also decreases the prevalence and mortality rate of liver, prostate, lung and colon cancer (24). Selenium is present in the catalytic site of the glutathione peroxidase enzyme. This enzyme uses glutathione to reduce hydroperoxides and protect proteins, nucleic acids and membrane lipids from the harmful effects of peroxides. Selenium also plays a significant role in the metabolism of platelet fatty acids. It increases the lipoxygenase activity in the arachidonic acid metabolism; metabolites formed in this process reduce prostaglandin production, increase vasodilation and reduce the accumulation of platelets. Furthermore, selenium is considered to be important in destroying microorganisms after phagocytosis in neutrophils. It is also known to reduce the mutagenic effects of several carcinogenic substances (25).

Inspired by the reports of the in vitro and animal studies that the chemotherapeutic agents are more effective when supported by antioxidants. Pathak et al. conducted a similar study on patients with lung cancer.

The researchers divided the patients into two groups; one only receiving chemotherapy every three weeks for no more than six cycles while the other was administered a high dose of antioxidants (ascorbic acid, α-tocopherol and selenium) two days before the chemotherapy application of the same duration. Chest radiographs of the patients were evaluated with physiological toxicity tests and statistical analyses. The result showed that was no sufficient evidence suggesting that antioxidants provide full protection against chemotherapy-induced lung tissue injury. The authors also concluded that there was concern among researchers about the possibility that the combined use of antioxidants might reduce the effect of chemotherapy (26).

It has been suggested that preliminary treatment with antioxidants such as ascorbic acid, α-tocopherol and selenium in patients with lung melanoma and carcinoma increase the growth-inhibition effects of chemotherapy and other cancer agents. Furthermore, the combined use of α – tocopherol, radiotherapy and cyclophosphamide was reported to increase patient recovery rate (27).

Ascorbic acid and α-tocopherol, when present in the body together, return the reduced selenium back to normal levels (28). Ascorbic acid, α-tocopherol and selenium synergistically act synergistically as an antioxidant in vivo and in vitro (29). In our study, the transmission electron microscopic examination showed that when the cyclophosphamide–treated pulmonary tissue was compared with the control group, in the type II alveolar cell cytoplasm, mitochondrial vacuolization and crystolisis was present; lamellar bodies acquired a vacuolar structure; the lamellar order was disrupted, the cell underwent apoptosis and the alveolar lumen was narrowed and closed. In the α–tocopherol group, fibrosis was reduced; intact mitochondria were still present but decreased in number, and lamellar body defects were observed. The other groups were found to provide similar results to the cyclophosphamide group.

Based on the results of our study, it can be concluded that among the antioxidants administered to rats during chemotherapy, only α-tocopherol reduced the free radicals to a minimum level and thus can effectively reverse tissue damage.

Conflict of interest
No conflict of interest was declared by the authors.

Acknowledgments
We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

REFERENCES

1. Pouzet MT, Travis EL. No change in repair capacity of mouse lung irradiated three mounths after a single dose of cyclophosphamide. Cancer Res. 1992; 52: 1096-100.
2. Smith RD, Kehrer JP. Cooxidation of cyclophosphamide as an alternative pathway for its bioactivation and lung toxidity. Cancer Res. 1991; 51: 542-48.
3. Patel JM. Metabolism and pulmonary toxidity of cyclophosphamide. Pharmacol. Ther. 1990; 47: 137-46.
4. Schmidt R, Luboenski T, Markart P, Ruppert C, Daum C, Grimminger F, et al. Alveolar antioxidant status in patients with acute respiratory distress syndrome. Eur. Respir. J. 2004; 24: 994-99.
5. Messent M, Sinclair DG, Quinlan GJ, Mumby SE, Gutteridge JM, Evans TW, et al. Pulmonary vascular permeability after cardiopulmonary bypass and its relationship to oxidative stress. Crit. Cor. Med. 1997; 25: 425-29.
6. Gurgen SG, Erdoğan D, Kaplanoğlu GT. The effect of histamine on kidney by fasting in rats. Bratisl Le. Listy. 2013; 114: 251-7.
7. Muralikrishnan G, Amalan SV, Sadasivan PK. Dual role of vitamin C on lipid profile and combined application of cyclophosphamide, methotrexate and 5-fluorouracil treatment in fibrosarcoma-bearing rats. Cancer Lett. 2001; 169: 115-120.
8. Viana M, Herrera E, Bonet B. Teratogenic effects of diabetes mellitus in the rat. Prevention by vitamin E. Diabetologia. 1996; 39: 1041- 46.
9. Dias MF, Sousa E, Cabrita S, Patrício J, Oliveira CF. Chemoprevention of DMBA-induced mammary tumors in rats by a combined regimen of alpha-tocopherol, selenium, and ascorbic Acid. Breast. J. 2000; 6: 14-19.

10. Markman M, Bundy BN, Alberts DS, et al. Phase III Trial of standard-dose intravenous cisplatin plus paclitaxel versus moderately high-dose carboplatin followed by intravenous paclitaxel and intraperitoneal cisplatin in small-volume stage III ovarian carcinoma: An Intergroup Study of Gynaecologic Oncology Group, Southwestern Oncology Group, and Eastern Cooperative Oncology Group. J. Clin. Oncol. 2001; 19: (4) 1001-7.
11. Kehrer JP, Kacew S, et al. Systemically applied chemicals that damage lung tissue. Toxicology. 1985; 35: 251-93.
12. Patel JW. Stimulation of cyclophosphamide-induced pulmonary microsomal lipid peroxidation by oxygen. Toxicology. 1987; 45: 79-91.
13. Kumar RK, Truscoll JY, Rhodes GC, Lykke AWJ. Type 2 pneumocyte responses to cyclophosphamide-induced pulmonary injury: functional and morphological correlation. Br. J. Exp. Pathol. 1988; 69: 69-80.
14. Eling TE, Thompson DC, Foureman GL, Curtis JF, Hughs MF, et al. Prostaglandin H synthase and xenobiotic oxidation. Annu. Rev. Pharmacol. Toxicol. 1990; 30: 1-45.
15. Martin FM, Witschi HP, et al. Cadmium-induced lung injury: cell kinetics and long-term effects. Toxicol. Appi. Pharmacol. 1985; 81: 215-27.
16. Chen FP, Gong LK, Zhang L, Wang H, Qi XM, Xiao Y, et al. Early lung injury contributes to lung fibrosis via AT_1, receptor in rats. Acta Pharma. Col. Sin. 2007; 28: 227-37.
17. Jain A, Martensson J, Mehta T, Krauss AN, Auld PAM, Meister A, et al. Ascorbic acid prevents oxidative stres in glutathione-deficient mice: Effects on lung type 2 cell lamellar bodies, lung surfactant and skeletal muscle. Proc. Natl. Acad. Sci. 1992; 89: 5093-97.
18. Liotti FS, Tasela V, Menghini AR, et al. Absence of accumulation phenomena in normal and tumoral tissues of mice treated with ascorbic acid. Int. J. Vit. Nutr. Res. 1983; 53: 251-57.
19. Frei B. Reactive oxygen species and antioxidant vitamins: mechanisms of action. Am. J. Med. 1994; 97: 5–13; discussion, 22–28.
20. Black HS. Radical interception by carotenoids and effects on UV carcinogenesis. Nutr. Cancer. 1998; 31: 212–17.
21. Edge R. Relative one-electron reduction potentials of carotenoid radical cations and the interactions of carotenoids with the vitamin E radical cation. J. Am. Chem. Soc. 1998; 120: 4087–90.
22. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem. Biol. Interact. 2006; 10: 1-40.
23. Reid ME, Duffield-Lillico AJ, Sunga A, et al. Selenium suplementation and colorectal adenomas: An analysis of the nutritional prevention of cancer trial. Int. J. Cancer. 2006; 118: (7) 1777-81.
24. Rayman MP. Selenium in cancer prevention: A review of the evidence and mechanism of action. Prog. Nutr. Soc. 2005; 64: (4) 527-42.
25. Kao JT, Chuah SK, Huang CC, Chen CL, Wang CC, Hung CH, et al. P21/WAF1 is an independent survival prognostic factor for patiens with hepatocellular carcinoma after resection. Liver Int. 2007; 27: 772-81.
26. Pathak AK, Bhutani M, Guleira R, Bal S, Mohan A, Mohanti BK, et al. Chemotherapy alone *vs.* chemotherapy plus high dose multiple antioxidants in patients with advanced non-small cell lung cancer. J. Ame. Col. Nutr. 2005; 24: (1) 16-21.
27. Prasad K, Kumar R, et al. Effect of individual antioxidant vitamins alone and in combination on growth and differentiation of human non-tumorigenic and tumorigenic parotid acinar cells in culture. Nutr. Cancer. 1996; 26: 11–19.
28. Kim Y, Chongviriyaphan N, Liu C, Russell RM, Wang XD, et al. Combined antioxidant (β-carotene, α-tocopherol and ascorbic acid) supplementation increases the levels of lung retinoic acid and inhibits the activation of mitogen-activated protein kinase in the ferret lung cancer model. Carcinogenesis. 2006; 27: 1410-19.
29. Liu C, Russell RM, Wang XD, et al. A-Tocopherol and ascorbic acid decrease the production of β-apo-carotenals and increase the formation of retinoids from β-carotene in the lung tissues of cigarette smoke-exposed ferrets in vitro. J. Nutr. 2003; 134: 426-30.