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Vitamin B12 alleviates methotrexate-induced lung injury in rat: A histopathological, immunohistochemical and biochemical study

B12 vitamini sıçanlarda metotreksatın neden olduğu akciğer hasarını azaltır: Histopatolojik, immünohistokimyasal ve biyokimyasal bir çalışma

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ABSTRACT

Objective: Methotrexate (MTX) is an important chemotherapeutic and an important anticarcinogen used in cancer patients, but it causes side effects in other tissues. Among these side effects is lung toxicity. Vitamin B12 is a powerful antioxidant and reduces reactive oxygen species. This study was designed to determine whether vitamin B12 could protect against methotrexate-induced damage in rat lungs.

Methods: A total of 32 male Wistar albino rats were divided into four groups: The control group (n=8) received intraperitoneal (i.p.) saline throughout the experiment. Vitamin B12 group (B12) (n=8) 3 µg/kg/i.p. Vitamin B12 was administered for 14 days. The methotrexate (MTX) group received a single dose MTX injection at 20 mg/kg/i.p. on the 8th day of the experiment. On the 8th day of the experiment, a single dose of MTX 20 mg/kg/i.p. was given to the MTX+B12 group. + 3 µg/kg/i.p. Vitamin B12 was administered daily throughout the experiment. Histopathological, immunohistochemical, and biochemical methods were

ÖZET

Amaç: Metotreksat (MTX) önemli bir kemoterapötik olup kanser hastalarında kullanılan önemli bir antikanserojen olmakla birlikte diğer dokularda yan etkilere yol açmaktadır. Bu yan etkiler arasında akciğer toksisitesi de yer almaktadır. B12 Vitamini güçlü bir antioksidandır ve reaktif oksijen türlerinin azalmasını sağlar. Bu çalışma, B12 vitamininin sıçan akciğerinde metotreksat kaynaklı hasara karşı koruma sağlayıp sağlayamayacağını belirlemek için tasarlanmıştır.

Yöntem: Toplam 32 erkek Wistar albino sıçanı dört gruba ayrılmıştır: Kontrol grubuna (n=8) deney boyunca intraperitoneal (i.p.) salin uygulanmıştır. B12 vitamini (B12) grubuna (n=8) 3 µg/kg/i.p. 14 gün boyunca B12 vitamini verilmiştir. Metotreksat (MTX) grubuna 20 mg/kg/i.p. tek doz (MTX) enjeksiyonu deneyin sekizinci gününde yapılmıştır. MTX+B12 grubuna deneyin sekizinci gününde MTX tek doz 20 mg/kg/ i.p. + 3 µg/kg/i.p. B12 vitamini günlük deney boyunca uygulandı. Elde edilen akciğer dokularına histopatolojik, immünohistokimyasal ve biyokimyasal yöntemler uygulanmıştır. Akciğer dokuları Masson's Trikrom (MT) ile boyanmıştır. Ayrıca α-Sma,

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applied to the obtained lung tissues. Lung tissues were stained with Masson's Trichrome (MT). In addition, α-Sma, Laminin, PCNA, and TNF-α antibodies were stained by immunohistochemistry. Superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels were evaluated in lung tissue homogenates.

Results: MTX caused an increase in MDA and IL-6 levels and expression of α-Sma, Laminin, and TNF-α, and the number of apoptotic cells in lung tissue. It also caused a decrease in PCNA expression and SOD and CAT levels in the MTX group. Vitamin B12 inhibited the increase in MDA and IL-6 levels and the expression of α-Sma, Laminin, and TNF-α. Vitamin B12 was found to increase antioxidant capacity.

Conclusion: Vitamin B12 has been shown to be effective on factors such as oxidative stress, inflammation, fibrosis, and apoptosis in MTX-induced lung toxicity and reduce damage. We observed that MTX stimulated lung injury decreased with vitamin B12 treatment It suggests that vitamin B12 should not be ignored in reducing side effects in cancer drug use.

Key Words: Methotrexate, vitamin B12, fibrosis, antioxidant capacity

Laminin, PCNA ve TNF-α antikorları immünohistokimya ile boyandı. Akciğer doku homojenatlarında ise süperoksit dismutaz (SOD), katalaz (CAT) ve malondialdehit (MDA) seviyeleri değerlendirilmiştir.

Bulgular: Metotreksat, MDA ve IL-6 seviyelerinde ve α-Sma, Laminin ve TNF-α ekspresyonunda ve akciğer dokusunda apoptotik hücre sayısında artışa neden olmuştur. Ayrıca MTX grubunda PCNA ekspresyonu ve SOD ve CAT seviyelerinde azalma görülmüştür. B12 vitamini, MDA ve IL-6 düzeylerindeki artışı ve α-Sma, Laminin ve TNF-α ekspresyonunu engellemiştir. B12 vitamininin antioksidan kapasiteyi artırdığı tespit edilmiştir.

Sonuç: B12 vitamininin metotreksat kaynaklı akciğer toksisitesinde oksidatif stress, inflamasyon, fibrosis ve apoptosis gibi faktörler üzerinde etkili olduğu ve hasarı azalttığı görülmüştür. Bu çalışma, metotreksatın neden olduğu akciğer hasarı için B12 vitamini kullanımının faydalı olacağını göstermiştir. Kanser ilacı kullanımında yan etkileri azaltmada B12 vitamininin gözardı edilmemesi gerektiğini düşündürmektedir.

Anahtar Kelimeler: Metotreksat, vitamin B12, fibrozis, antioksidan kapasite

INTRODUCTION

Methotrexate (MTX) is a folic acid analog chemotherapeutic commonly used in many cancer diseases. MTX can inhibit the synthesis of DNA, RNA, and proteins (1). Although it is used in many cancer treatments, it also has side effects in many tissues. These side effects include nephrotoxicity, hepatotoxicity, bone marrow toxicity, and lung toxicity induced by MTX (2-4). It has been shown in studies that MTX will cause significant pulmonary toxicity (4, 5). One of the mechanisms by which MTX causes lung damage is inducing oxidative stress in lung epithelial cells (6).

It has been reported that MTX causes injury to the alveolar epithelium, apoptosis, and fibrosis in the lung (7). MTX stimulates oxidative stress, leading to increased reactive oxygen species and later cellular apoptosis (8). MTX increases apoptosis and decreases cell regeneration (7). The proliferating cell nuclear antigen (PCNA) is considered a hub protein and is an important regulator of DNA replication, repair, cell cycle control, and apoptosis (9). It has been reported that MTX increases lipid peroxidation in different tissues, increases inflammation, and decreases antioxidant capacity by increasing oxidative stress (10-12).

Inflammation and fibrosis stages are important in the development of pulmonary fibrosis. Proinflammatory cytokine production is stimulated by the increase of reactive oxygen species by MTX (13). Cytokines such as TNF-α and IL-6 play a role in inflammation (14). Such cytokines have been shown to increase and play an important role in MTX-induced lung toxicity (15). As MTX increases reactive oxygen species, it will lead to the development of pulmonary fibrosis (6). Alpha-smooth muscle actin (α-SMA) is a protein that plays an important role in fibrogenesis (16). Laminin, a heterotrimeric protein, plays an important role in maintaining tissue integrity (17). Laminin, an important component of the basement membrane, also contributes to pulmonary fibrosis (18).

Reactive oxygen species are controlled by the antioxidant defense system, which includes enzymes such as SOD, CAT. MTX breaks down the enzymes of the antioxidant system and reduces their amount (19, 20). MTX produces reactive oxygen species and increases lipid peroxidation (21).

Alternative or complementary therapies play an important role in reducing the side effects of cancer drugs. Vitamin B12 (B12, cobalamin) is a water-soluble molecule found in foods of animal origin (22). Vitamin B12 is required as a cofactor in the metabolic process of remethylation of homocysteine to methionine (23). Vitamin B12 plays an important role in oxidative stress and increases antioxidant capacity. It provides a reduction of reactive oxygen species. Moreover, it has been shown in studies that it reduces inflammation, fibrosis, and apoptosis (24-26). The antioxidant property of vitamin B12 is achieved by removing reactive oxygen species and protecting the enzymes of the antioxidant system. It also provides modulation of cytokines (27).

The effects of vitamin B12 on damages such as fibrosis, inflammation, and apoptosis in MTX-induced lung injury has not been studied before. Therefore, the aim of this study was to investigate the protective effects and to reveal possible mechanisms of vitamin B12 against MTX-induced lung toxicity.

MATERIAL and METHOD

Experimental procedure

This study was planned and performed in Erciyes University Faculty of Medicine Histology-Embryology Department in line with the approval of the Ethics Committee of Erciyes University Experiments Local Ethics Committee. All procedures were carried out in accordance with the Universal Declaration of Animal Rights, with the approval of the Ethical Committee (Date: 12.09.2018, Decision no: 18/116 and subject for lung tissue: 21/131) of Erciyes University Experimental Animals. In this study, all the animals received human care according to standart guidelines. The experimental stage of the study was carried out in the Erciyes University Experimental Research Application and Research Center (DEKAM).

In this study, eight weeks old 150-200 g adult 32 Wistar albino type male rats produced in DEKAM were used. Rats kept in cages were kept in the normal order of the day at 21^{0}C and 12 hours of light / dark environment and water and nutrient needs were provided. The experimental groups were formed by weighing the subjects and putting them together so that their weights were close to each other.

Group I: the control group (n = 8) was administered intraperitoneal (i.p.) saline throughout the experiment.

Group II: Vitamin B12 (B12) (n = 8) 3 µg / kg / i.p. Vitamin B12 (Vitamin B12 Rubranova, 5,000 µg; Bristol-Myers Squibb) was administered throughout the experiment.

Group III: On the 8th day of the experiment, the Methotrexate (MTX) (n = 8) group received a single dose of 20 mg/kg / i.p. Methotrexate (Methotrexate, 500mg / 20ml, Kocak Farma, Turkey).

Group IV: MTX + Vitamin B12 (B12) (n = 8) group single dose 20 mg / kg i.p. MTX + daily Vitamin B12 3µg / kg / i.p.

The experiment continued for 15 days. At the end of the experiment, animals were anesthetized with 30 mg/kg ketamine and 4 mg/kg xylazine and

they were sacrificed. At the end of the experiment, lung tissues were taken for histopathological, immunohistochemical, and biochemical evaluation.

Histopathological evaluation

Lung sections of 5 µm from paraffin blocks were left in the oven for a certain period of time using histological methods, then paraffin was removed with xylene and passed through graduated alcohol series and diluted. Masson's Trichrome (MT) staining was performed to see the general histological structure and fibrotic areas.

Immunohistochemistry

The immunohistochemistry method was used to investigate PCNA, α-Sma, Laminin, and TNF-α antibodies in lung tissues. Avidin biotin peroxidase method was used to determine the difference in expression of PCNA (PC10, sc-56, Santa Cruz Biotechnology), α-Sma (BSM-33188M, Bioss), laminin (ab128053, Abcam), and TNF-α(bs-2081R, Bioss) in lung sections. Paraffin sections were deparaffinized in xylene. For antigen recovery, 0.01 M 10% citrate buffer was applied in the microwave for seven minutes at 600 w and then allowed to cool at room temperature for 10 minutes. Sections washed with phosphate buffer (PBS) were treated with 3% hydrogen peroxide (H2O2) for 12 minutes to prevent endogenous peroxidase activity. It was washed again with PBS three times for five minutes. The staining kit (TA-125-HDX, Thermo Fisher Scientific, Waltham, MA, USA) was used for the next steps. After washing again 3 times in PBS, ultra v block solution was added to the tissues and kept in the tank for 10 minutes. After then PCNA, α-Sma, Laminin, and TNF-α antibodies were added to the tissues and incubated overnight at 4°C. After re-washing, the peroxidase in the kit, which exhibits diaminobenzidine (DAB) (TA-060-HDX, Thermo Fisher Scientific, Waltham, MA, USA), was treated with the peroxidase substrate for 1.5 minutes to make its immunoreactivity visible. Image J program was used to evaluate antibody expressions (28, 29). 20 fields were examined and counted at 200 X magnification.

Apoptosis (TUNEL) staining

Apoptotic cells in the lung sections taken from the subjects were determined using the Roche brand In Situ Cell Detection Apoptosis Fluorescein Kit. The staining was performed according to the kit procedure. Lung sections taken at a thickness of 5 µm were deparaffinized and then rehydrated and washed twice with PBS for five minutes. Then, 270°C in a microwave oven in 0.01 M 5% sodium citrate buffer for antigen recovery was left for 5 minutes, then allowed to cool at room temperature for 10 minutes. Tissues washed with PBS for 3x5 minutes were then placed in the moisture chamber at 37 0C with the TUNEL reaction mixture coming out of the kit and incubated in the oven for 75 minutes. Tissues washed two times for five minutes with PBS were contrasted with DAPI (4', 6-diamidino-2-phenylindole). Tissues sealed with glycerol closure solution were visualized on the Olympus BX51 fluorescent microscope at a wavelength of 450-500 nm. For the apoptotic index, apoptotic cells in 25 different areas (each group: total 200 area) were counted in 40X objective from each section (28).

Biochemical Analysis

Lung tissues from subjects were raised to -80°C. Tissues were homogenized before the study. Then, centrifugation was applied and supernatants were transferred to Eppendorf tubes for use. By ELISA method SOD (Cat. No: 201-11-0169, Sun Red Biological Technology), CAT (Cat. No: 201-11-5106, Sun Red Biological Technology), Malondialdehyde (MDA) (Cat. No: Cat. No: 201-11-0157, Sun Red Biological Technology), Interleukin-6 (IL-6) (Cat. No: Cat. No: 201-11-0136, Sun Red Biological Technology) kits were used and quantities were determined in ng/ml at 450 nm in ELISA reader.

Statistical Analysis

The Kolmogorov-Smirnov test was used to identify the normal distribution of the data. One-way analysis of variance and posthoc Tukey test were used to determine differences between groups. Results are

presented as mean ± Standart deviation (SD). The SPSS/PC program (Version 20.0; SPSS, Chicago, IL) and Graphpad Prism 8.0 software was used for statistical analysis. P < 0.05.were considered as statistically significant.

RESULTS

Histopathological results

In lung tissue was performed Masson's trichrome (MT) to investigate the histopathological changes. Healthy lung tissue was observed in the control, vitamin B12, and MTX+ vitamin B12 groups. In the MTX group, in interalveolar areas of the lung tissues were observed fibrotic areas compared to the other groups. With Masson trichrome staining, we saw increased fibers in the connective tissue in the lung of the MTX group (Figure 1 A-B-C-D).

Immunohistochemistry results

Immunohistochemical staining was performed by using the avidin-biotin method to determine the lung tissue expressions of PCNA, α-Sma, Laminin, and TNF-α (Table 1, Figure 2). In the MTX group, PCNA positive cell counts significantly decreased compared to the control group (p=0.0001). In the MTX+B12 group, PCNA positive cell counts significantly increased compared to the MTX group (p=0.0001). In the MTX group, α-Sma, Laminin, and TNF-α staining areas significantly increased compared to the control group (p=0.0001). There was a statistically significant decrease in α-Sma, Laminin, and TNF-α staining area in the group MTX+B12 compared to the MTX group (p=0.0001).

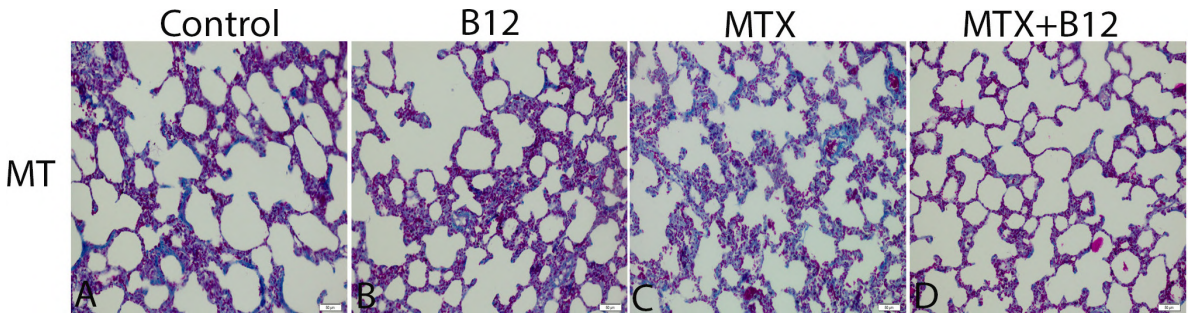

Figure 1. MT staining in lung tissues. In the Control group (A), B12 group (B), MTX+B12 group (D) is seen healthy lung tissue. In the MTX group (C) were seen pathologically increased fibrotic areas.
Fibrotic areas are shown with a black arrow. Blue stained areas show fibrosis. X200
MT: masson'trichrome,
MTX: methotrexate,
B12: B12 vitamin

Table 1. Immunohistochemistry data on the lung tissues

Groups	Control	B12	MTX	MTX+B12	*p*
PCNA Positive cell count	4.90±2.11[a]	5.25±1.18[a]	1.45±1.17[b]	4.72±1.86[a]	0.0001
α-SMA % staining area	12.69±3.39[a]	8.64±2.48[b]	31.84±5.41[c]	18.94±4.73[d]	0.0001
Laminin % staining area	4.20±2.11[a]	4.44±1.15[a]	7.52±1.88[b]	3.51±1.97[a]	0.0001
TNF α % Staining area	0.40±0.25[a]	0.42±0.27[a]	5.16±3.72[b]	1.21±0.69[a]	0.0001

The data are expressed as mean + standard deviation. $p < 0.05$ was accepted as significant. There were no significant differences between the groups expressed with the same letter (a, b, c, d).
MTX: methotrexate, B12: vitamin B12, PCNA: Proliferating cell nuclear antigen,
α-SMA: Alpha-smooth muscle actin, TNF α: Tumor necrosis factor-alpha

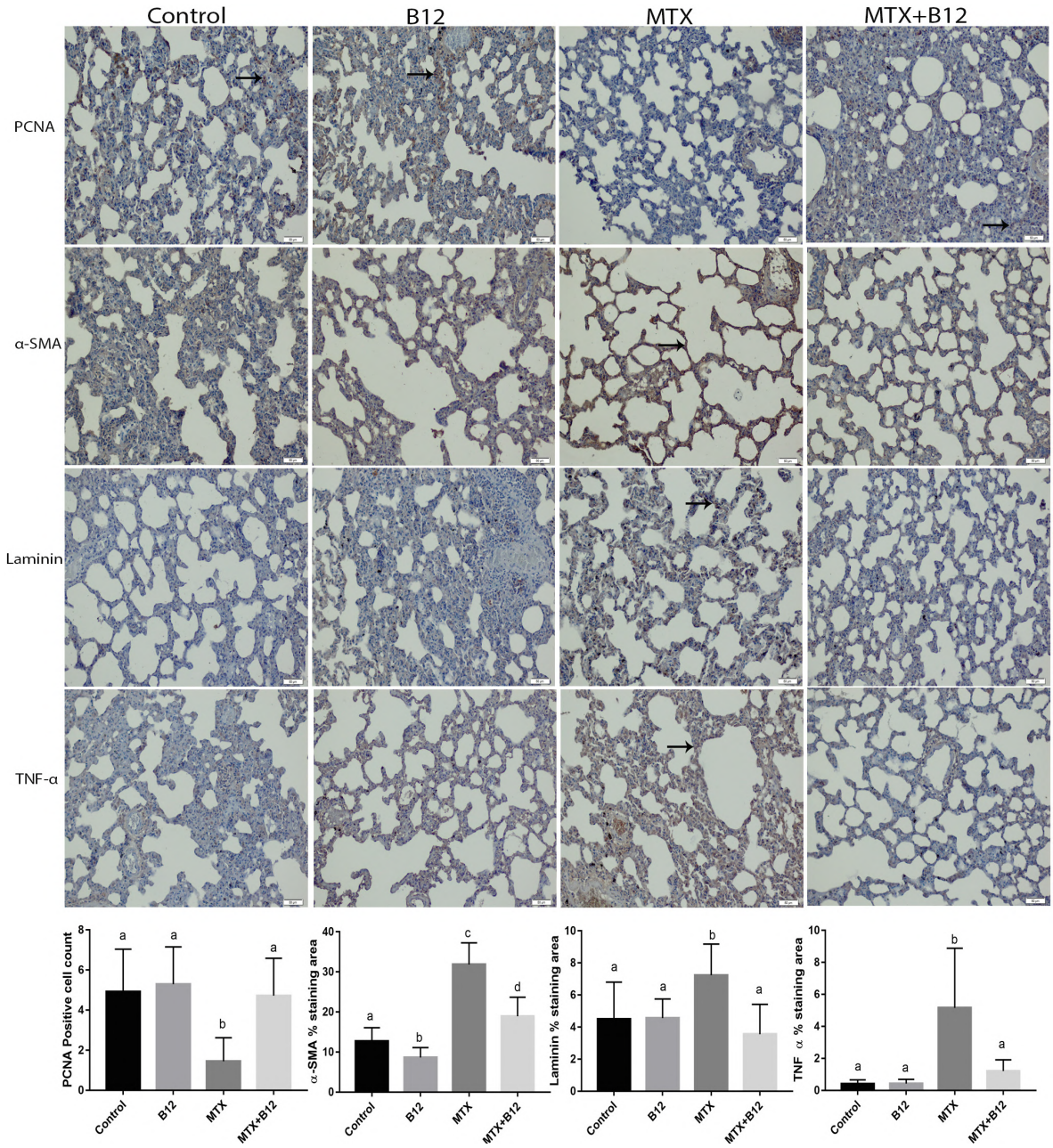

Figure 2. Results of MAP 2, eNOS, iNOS and nNOS immunohistochemical staining of lung tissues in experimental groups. Immunoreactive areas are indicated by arrows. X200.
Values are given as mean ± standard deviation. P < 0.05 was considered significant.
There is no significant difference between groups containing the same letter (a, b, c, d).
MTX: methotrexate, B12: vitamin B12, PCNA: Proliferating cell nuclear antigen,
α-SMA: Alpha-smooth muscle actin, TNF α: Tumor necrosis factor-alpha

TUNEL Results

TUNEL staining was performed to determine apoptotic cells in lung tissue. In the MTX group, TUNEL positive cell counts increased statistically significantly compared to the control group (p=0.0001). There was a statistically significant decrease in TUNEL positive cell counts in the MTX+B12 group compared to the DOX group (p=0.0001). (Table2, Figure 3).

Table 2. TUNEL positive cell numbers belonging to the groups

Groups	Control	B12	MTX	MTX+B12	*p*
TUNEL positive cell count	3.24±2.65[a]	2.51±2.16[b]	3.70±2.15[c]	3.04±1.78[a]	0.0001

The data are expressed as mean + standard deviation. $p < 0.05$ was accepted as significant.
There were no significant differences between the groups expressed with the same letter (a, b, c).
MTX: methotrexate, B12: vitamin B12

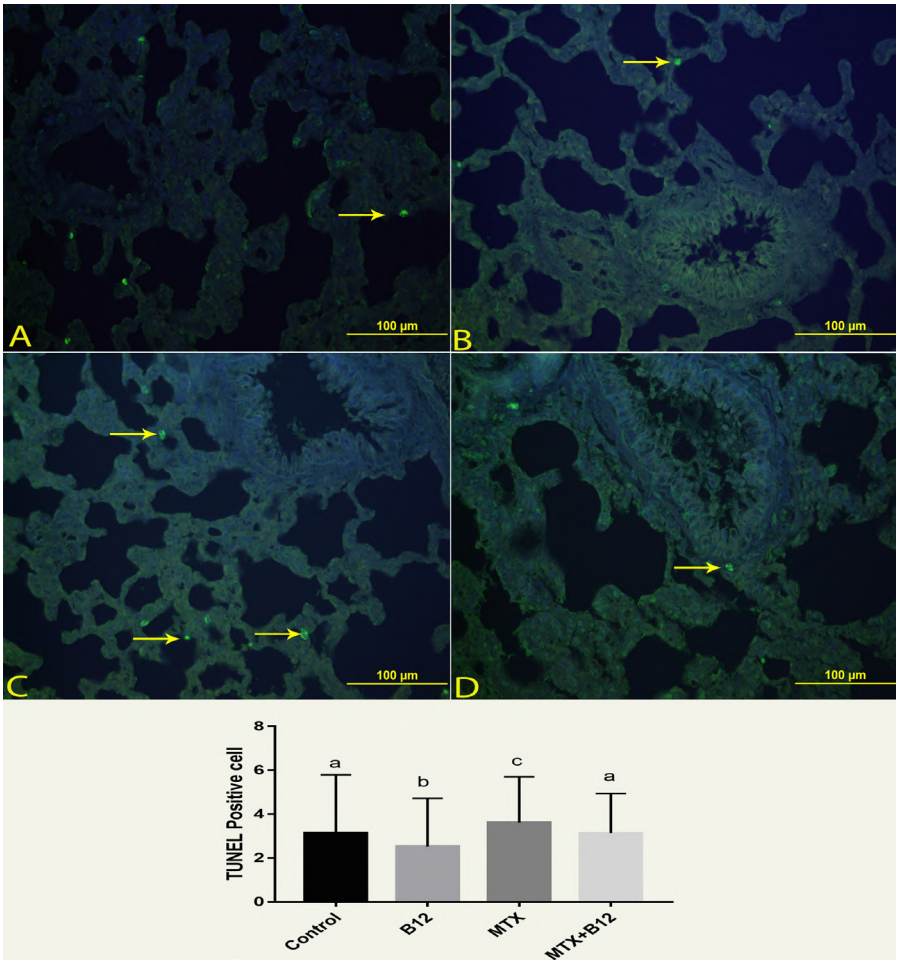

Figure 3. TUNEL data of the groups.
(A) Control group, (B) B12 group, (C) MTX group, (D) MTX+B12 group.
Apoptotic cells are indicated by yellow arrows. X400. P < 0.05 was considered significant.
There is no significant difference between groups containing the same letter (a, b, c).

Biochemical Results

Lung Tissue Antioxidant Enzymes Activities

The results of the antioxidant enzymes are shown in Table 3. SOD and CAT levels significantly decreased in the MTX group compared to the Control group (p=0.005). SOD and CAT levels significantly increased in the MTX+B12 group compared to the MTX group (p=0.005). MDA level significantly increased in the MTX group compared to the Control group (p=0.005). MDA level was significantly decreased in the MTX+B12 group compared to the MTX group (p=0.0001). (Figure 4).

Lung Tissue inflammatory marker IL-6 Activity

The results of the inflammatory IL-6 are shown in Table 3. IL-6 level significantly increased in the MTX group compared to the control group (p=0.005). IL-6 level significantly decreased in the MTX+B12 group compared to the MTX group (p=0.005). (Figure 4).

Table 3. Biochemical data of the experimental groups

Groups	Control	B12	MTX	MTX+B12	*p*
SOD ng/ml	33.13±9.73[a]	34.9±8.49[a]	21.41±2.09[b]	32.44±2.09[a]	0.0086
CAT ng/ml	96.9±28.4[a]	101.4±24.99[a]	56.32±22.65[b]	96.06±12.55[a]	0.0091
MDA ng/ml	10.31±2.20[a]	10.39±1.21[a]	18.56±2.76[b]	12.31±1.25[a]	0.0001
IL-6 ng/ml	99.73±12.56[a]	98.67±10.10[a]	148.10±20.35[b]	119.8±9.66[a]	0.0001

The data are expressed as mean + standard deviation. $p < 0.05$ was accepted as significant.
There were no significant differences between the groups expressed with the same letter (a, b).
MTX: methotrexate, B12: vitamin B12, SOD: Superoxide dismutase,
CAT: Catalase, MDA: Malondialdehyde, IL-6: Interleukin 6.

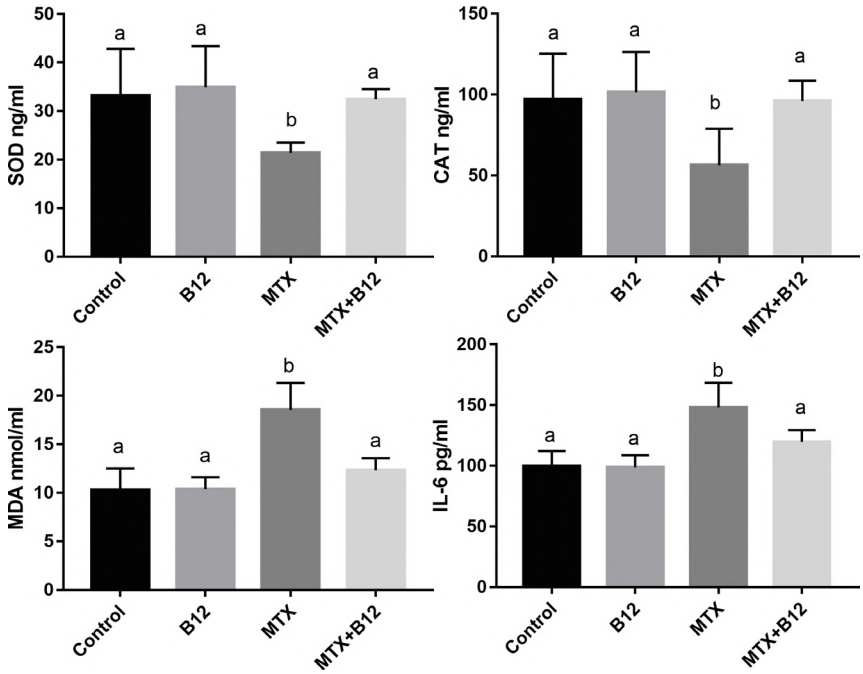

Figure 4. Graphics of biochemical results.
There were no significant differences between the groups expressed with the same letter (a, b).
MTX: methotrexate, B12: vitamin B12, SOD: Superoxide dismutase,
CAT: Catalase, MDA: Malondialdehyde, IL-6: Interleukin 6

DISCUSSION and CONCLUSION

Methotrexate (MTX) is a chemotherapeutic drug that is generally used in diseases such as rheumatoid arthritis and psoriasis (30, 31). MTX also causes neurotoxicity, hepatotoxicity, nephrotoxicity, and lung damage (32-35). In our study, when the lung tissue is examined histopathologically, we show that MTX stimulates fibrosis by increasing the amount of collagen around the alveoli. However, in the group treated with vitamin B12, lung tissue was observed similar to the control group. MTX has been shown to cause fibrosis in studies (20, 36). Pulmonary fibrosis is an important life-threatening disease. It is a fatal disease that causes excessive collagen accumulation and lung dysfunction in the lung parenchyma due to excessive activation of the fibroblast (37). Myofibroblasts provide an increase in the extracellular matrix such as collagen in the development of fibrosis. α-sma is synthesized by myofibroblasts (38, 39). It has been observed that the amount of α-sma and laminin increases in pulmonary fibrosis (40, 41). MTX has been shown to be associated with pulmonary fibrosis (42, 43). In our study, the results of immunohistochemistry staining, it was observed that MTX significantly increased the amount of α-sma and laminin around the alveoli in the lung compared to the control group. In the group treated with vitamin B12, there was a significant decrease in the amount of immunoreactivity of α-sma and laminin compared to the MTX group. This result shows that vitamin B12 reduces fibrosis by decreasing collagen accumulation similar to other studies (44, 45).

MTX-induced pulmonary toxicity has been shown to increase inflammation. In this case, the increase in pro-inflammatory cytokines occurs (46). In our study, TNF-α immunoreactivity in lung tissue significantly increased in the MTX group. IL-6 level, another cytokine, showed a significant increase in the MTX group when evaluated biochemically. Our results confirm the increase in MTX-induced TNF-α and IL-6 levels and comply with other studies (46, 47). In the current study, TNF-α and IL-6 levels significantly decreased in the group treated with vitamin B12. It has been noted that increasing the level of cytokines will significantly increase pulmonary profibrotic activity by activation of fibroblast, macrophage, and myofibroblast cells (48). These results confirm the anti-inflammatory presentation of vitamin B12 (49, 50).

Although chemotherapeutic drugs are used in cancer, they cause apoptosis in healthy tissues. MTX application also causes apoptosis in different tissues (7, 51). In our study While the number of apoptotic cells increased in the lung tissue in the MTX group, the number of PCNA positive cells decreased. In the group treated with vitamin B12, we showed that the number of TUNEL positive cells in the lung tissue decreased and the number of PCNA positive cells increased. As in other studies, it shows the positive effects of vitamin B12 on cell proliferation (52, 53).

Oxidative stress plays an important role in lung toxicity caused by MTX, and oxidants and antioxidants play a role in this process. Malondialdehyde (MDA) from oxidants and superoxide dismutase (SOD) and catalase (CAT) from antioxidants play an important role in this process (46, 47). MTX causes oxidative damage and increases lipid peroxidation. Excessive production of reactive oxygen species disrupts the antioxidant system (54). In the current study, we showed that in the MTX applied group, the level of MDA showing lipid peroxidation increased significantly as a result of the biochemistry of lung tissue. The level of enzymes such as SOD and CAT, which play a role in the antioxidant system, has decreased significantly in the MTX treated group. This decreased antioxidant capacity and increased lipid peroxidation amount are compatible with other studies in the literature (33, 54). In the group treated with B12, which we use as an antioxidant, lipid peroxidation significantly decreased, while a significant increase in the enzymes of the antioxidant system contributed to the preservation of lung tissue. Vitamin B12, which has functions such as removing reactive oxygen species and reducing oxidative stress, demonstrated this feature in our study in MTX-induced lung cytotoxicity (27, 55).

In conclusion, we found that vitamin B12 has positive effects by reducing factors such as oxidative stress, fibrosis, apoptosis, and inflammation in methotrexate-induced lung damage. We observed that MTX stimulated lung injury decreased with vitamin B12 treatment. The administration of vitamin B12 together with chemotherapeutics used in cancer draws attention as an important agent in minimizing the damage to other tissues.

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ETHICS COMITTEE APPROVAL

* The study was approved by the Erciyes University's Experimental Animal and Local Ethics' Committee (Date: 12.09.2018 and Number: 18/116).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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