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EXPERIMENTAL STUDY

Does tamoxifen citrate prevent pulmonary fibrosis due to silica inhalation?

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ABSTRACT

BACKGROUND: As shown in several studies, besides being used in breast cancer, tamoxifen is also known for its antifibrotic effects via reducing the serum TGF-beta levels. We investigated the possible preventive effect of tamoxifen in rats exposed to silica particles depending on the antifibrotic effect.

MATERIALS AND METHODS: A total of 102 adult female Wistar Albino rats were divided into five groups. First two groups (control and tmx) were free of silica and the last three groups (slc, tmx1 and tmx 10) were exposed to crystalline silica. The rats in tmx, tmx1 and tmx10 groups received 10 mg/kg, 1 mg/kg and 10 mg/kg of body weight tamoxifen, respectively. On day 84, all rats were sacrified and tissue samples were obtained together with blood samples. The differences in serum TGF-β levels, histological grades of fibrosis and inflammation in the lung and liver tissues together with addional biochemical markers were calculated between the groups.

RESULTS: Silicosis occurred in slc, tmx1 and tmx10 groups in 100 %, 91.7 % and 52.1 %, respectively. Liver fibrosis did not occur. The highest mean lung fibrosis scores were obtained in slc group while the scores were lower in tmx1 group and the lowest in tmx10 within silica-exposed rats. Nevertheless, the inflammation scores were higher in tamoxifen-administered rats in a dose-dependent pattern.

CONCLUSION: Silica inhalation did not result in liver fibrosis. Tamoxifen is found to prevent lung fibrosis and reduce serum TGFβ-1 levels while increasing lung inflammation *(Tab. 3, Fig. 3, Ref. 27)*. Text in PDF *www.elis.sk.*

KEY WORDS: tamoxifen, silicosis, TGF-beta, pulmonary fibrosis.

Introduction

Crystalline silica is silicon dioxide (SiO_2) arranged in a three-dimensional crystal lattice (1, 2). Silica nanoparticles have extensive applications in chemical and mechanical polishing and as additives to drugs, cosmetics, printer toners, varnishes and food (3–7). Inhalation of crystalline silica during exposure within industrial environment was shown to result in silicosis, and believed to result from pulmonary damage that brings about inflammation, lung scarring and fibrosis (3–5, 8–10). Like other occupational diseases, the condition is preventable with several precautions like the constitution of optional workplace and usage of personal protection equipments.

To make the fight for elimination of such disaeases efficient, most of the effort should be focused on exposure prevention. Nevertheless, at the same time the researchers study also the effective treatment for an established disease status.

Tamoxifen citrate is a selective estrogen receptor modulator which has been approved for the treatment of breast cancer. It inhibits keloid fibroblast proliferation and reduces collagen production. Effects of tamoxifen include altering transcriptional synthesis, reducing cellular proliferation, and modulating production of multiple polypeptide growth factors (11–15). Tamoxifen is thought to reduce the production of transforming growth factor-beta (TGF-β) and insülin-like growth-facor (IGF-1) (12, 16). TGF-β has a key role in modulation of inflammation, wound repair, and immunity by supressing lymphocyte proliferation and promoting anchorage-independent growth of fibroblasts (17).

Our study aims to investigate the possible preventive effect of tamoxifen citrate on pulmonary fibrosis in relation to serum TGFβ-1 levels. The study will be a preliminary finding shedding light on the way of finding a possible curative agent for this highly disabling disaease. Additionally, we search for the presence of liver fibrosis after silica inhalation.

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Tab. 1. Charactesitics of exposure and preventive medication within groups.

Silica free		Silicae xposed	
Group 1 (control)	Control group	Group 3 (slc)	Not received tamoxifen
Group 2 (tmx)	10mg/kg tamoxifen	Group 4 (tmx1)	1 mg/kg tamoxifen
		Group 5 (tmx10)	10 mg/kg tamoxifen

Abbreviation of group names were given in parantheses

Tab. 2. Comparison of several characteristics between groups.

	Group1 (n=10)	Group2 (n=22)	Group3 (n=23)	Group4 (n= 4)	Group5 (n=23)	p
Number of silicosis [n (%)]	0 (0)	0 (0)	23 (100)	22 (91.7)	12 (52.1)	< 0.001**
Fibrosis score	0	0	1.65	0.9	0.5	< 0.001*
Inflammation score	0.3	1.56	1.17	1.16	1.87	< 0.001**
TGF β-1 (pg/ml)	38.6	38.2	65.3	57.2	44.9	< 0.001**
AST (U/L)	59.3	54.09	63.73	39.28	46.27	NS**
ALT (U/L)	24.25	54.28	43.61	49.27	67.35	0.001**
Alb (g/dl)	58.5	57.2	48.2	47.9	54.1	NS**
T.Bil (mg/dl)	61	59.8	58.3	42.4	40.8	0.04**
D.Bil (mg/dl)	54.9	52.9	62	51.3	37.5	0.07**

*One-Way ANOVA **Kruskal–Wallis analysis, NS – not significant

Materials and methods

A total of 110 adult female Wistar Albino rats (200–250 g) were housed in wire cages under stable temperature (21 ± 2 °C) and 12 h light/dark cycle. The animals were allowed free access to water and standard rat chow. The rats were divided into five groups as shown in Table 1. Group 1 (control) was composed of 10 rats. In group 2 (tmx) there were 25 rats and they were fed tamoxifen at 10 mg/kg of body weight. Other 75 rats were exposed to silica. In group 3 (slc) there were 25 rats and they were exposed to silica. In group 4 (tmx1) there were 25 rats and they were exposed to silica and fed tamoxifen at 1 mg/kg of body weight. In group 5 (tmx10), there were 25 rats that were exposed to silica and fed tamoxifen at 10 mg/kg of body weight with oral gavage.

Crystalline silica (approx. 98 % between 40 and 100 nm) was purchased from MMR Refrakter Co (Konya, Turkey). Rats were exposed to silica via dry air compressor released to the cage in 10m3 volume while inhalation took 6 hours/day and 5 days/week for 12 weeks and the rats were followed up for 84 days. On days 1, 42 and 84, non-specific respirable dust concentrations and respirable crystalline silica concentrations were measured with gravimetric method (18).

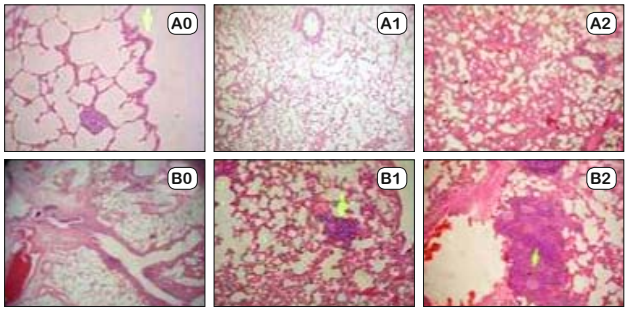

Fig. 1. Pathological classification of lung specimens. Classification for fibrosis: A0: No fibrosis (arrow shows mesothelial layer of pleura), A1: Moderate increase of fibroblasts in central septa, A2: Interalveolar septal thickening extending to the periphery, significant fibrosis with Masson trichrome. Classification for inflammation: B0: No inflammation, B1: Mild peribronchial lymphocytic inflammation (arrow), B2: Severe peribronchial and peri-alveolar inflammation, active-chronic or dense eosinophilic polymorphonuclear leukocyte influx or subpleural granulomatous inflammation and/or hilar lymphadenopathy and granulomatous inflammation (arrow).

Characteristics of the exposure

Exposure concentration: 23.375 mg/m^3

Respirable crystalline silica concentration levels: 0.268 mg/m^3 – 2.39 %

All animals were anesthetized by intramuscular injection of 30 mg/kg ketamine hydrochloride (Ketalar®, Parke-Davis, Istanbul) and 5 mg/kg xylazine (Rompun®, Bayer, Istanbul). After the rats had been anesthetized, the abdomen and thorax were shaved and povidone iodine (Repithel ®Mundipharma GmbH -Limburg, Germany) was applied. Under sterile conditions, a midline laparotomy and thoracotomy were performed. Pathologic samples were obtained from the lower lobe of the right lung together with hilar lymphoid structures and right lobe of the liver. Blood samples were centrifuged at 30,000 rpm for 5 min at 4 °C. Then the serum was aspirated and stored at –80 °C until analyzed. The animals were sacrified with a high dose of anesthesics.

The tissue samples from lung and liver were evaluated histopathologically for fibrosis and also for the presence of inflammation. The histopathological analyses were carried out at the Pathology Department of Kecioren Research and Training Hospital. Histopathological examination was performed by using light microscopic analysis. The samples obtained from the lower lobe of the right lung and right lobe of the liver were fixed in 10 % neutral buffered formalin solution for 2 days (d).

Tissues were washed in running water, and were dehydrated with increasing concentrations of ethanol (50 %, 75 %, 96 % and 100 %). After dehydration, specimens were placed into xylene to obtain transparency, and embedded in paraffin. Embedded tissues

were cut into 5 μm-thick sections and were stained with hematoxylin and eosin and trichrome. Histopathologic examinations were performed by two pathologists blinded to the exposure status, and scored for fibrosis and inflammation using a classification system as shown in Figure 1.

Serum TGFβ-1 protein levels were assessed at the Biochemistry Department of Hacettepe University by enzyme-linked immunsorbent assay (ELISA) using a kit spesific for TGFβ-1. The kit was purchased from Invitrogen Corporation (Camarillo, CA, USA). Total protein concentrations were determined by spectrophotometry and specimens were subsequently brought to equal concentrations by dilution with an appropriate amount of mild lysis buffer. All specimens were acidified for 1 hour to facilitate the quantification of total TGFβ-1 level, followed by neutralization immediately prior to conducting the ELISA. The samples were then analyzed by ELISA with serial dilutions of known quantities of recombinant TGFβ-1, and used as a positive control and standard to quantify the absolute TGFβ-1 protein content of each sample. All specimens were analyzed in duplicate and the assay was repeated at least three times. All TGFβ-1 results were given as picograms per microliter.

Serum aspartate amino transferase, alanine amino transferase, and total and direct bilirubin levels were measured with the Roche integra 800 device by photometric method. The kit was purchased from Thermo Fisher Scientific Oy (Vantaa, Finland).

Data analysis was performed using the SPSS 16.0 program. Shapiro Wilk test was used for normality analyses. One-way Anova and Kruskal–Wallis statistic analyses were used to determine differences between groups. Tukey and Mann–Whitney U tests were used in pairwise analyses. A value < 0.05 was considered to be statistically significant.

The procedures in this experimental study were performed in accordance with the National Guidelines for the Care and Use of Laboratory Animals, and approved by the Animal Ethics Committee of Ankara Research and Training Hospital on 26.5.2011 under number 65.

Results

There were 10 rats in group 1 (control), 25 rats in group 2 (tmx), 25 rats in group 3(slc), 25 rats in group 4 (tmx1) and 25 rats in group 5 (tmx10). Eight rats died during the research period (3 rats from second group due to stres and gastrointestinal infection, 2 rats from third group, 1 rat from fourth group and 2 rats from fifth group due to traumatic gavage and fighting with each other). A total of 102 rats were sacrified on day 84 day.

The number of silicotic cases, mean of lung fibrosis inflammation scores, and serum TGFβ-1, AST, ALT, total and direct bilirubin levels are summarized in Table 2.

Lung fibrosis was not detected in control group and tmx group. Silicosis occurred in all of the rats in slc group (100 %) while it was found in 22 of 24 (91.7 %) rats in tmx1 group and 12 of 23 (52.1 %) rats in tmx10 group (Tab. 3). The highest mean lung fibrosis scores were obtained in slc group. In this group, septal thickening was significant in the central part and it was extending to the periphery of the lung. We also identified the formation of fibrotic lung parenchymal bands and subpleural thickening (Fig. 2) in the slc group. In group tmx1, peripheral septal thickness was less present than in group slc but it was persistent in the central part. In group tmx10, peripheral septal thickness was least present but it was persistent in the central part. Lung fibrosis was not detected in some samples in tmx10 group. The mean fibrosis score was significantly different between all groups except for control and tmx groups.

The highest lung inflammation scores were observed in tmx10 group. In tmx and tmx10 groups, hilar lymphadenopathy and granulomatous inflammation of lymph nodes eventuated; vascular and peribronchial polymorphonuclear leukocytes and eosinophilic leukocytic influx were highlighted (Fig. 2). The mean inflammation score was significantly different between all groups except for tmx and tmx10 groups as well as slc and tmx1 groups. This shows that tamoxifen reduced the fibrosis while stimulating inflammation in a dose-dependent manner (Fig. 3).

Serum TGFβ-1 levels were highest in slc and tmx1 groups and lower in tmx 10 group. The levels were not statistically different between control and tmx10 group as well as slc and tmx1 groups, thus demonstrating the dose-dependent reducing effect of tamoxifen on serum TGFβ-1 levels.

Liver fibrosis developed in no groups. Serum AST, ALT, total bilirubin and direct bilirubin are shown in Table 3.

Discussion

Silicosis is a fibrotic pulmonary disease associated with inhalation of crystalline silica and it has no successful treatment16. There

Tab. 3. Differences in pairwise comparisons.

	*Fibrosis score	**Inflammation score	**ALT	**T.Bil	**D.Bil	**TGFβ-1
Group 1-2	NS	< 0.001	0.004	NS	NS	NS
Group 1-3	< 0.001	< 0.001	NS	NS	NS	0.03
Group 1-4	< 0.001	< 0.001	0.005	NS	NS	0.003
Group 1-5	0.004	< 0.001	<0.001	NS	NS	NS
Group 2-3	< 0.001	0.015	NS	NS	NS	0.007
Group 2-4	< 0.001	0.012	NS	0.03	NS	< 0.001
Group 2-5	< 0.001	NS	NS	0.016	0.033	NS
Group 3-4	< 0.001	NS	NS	0.005	NS	NS
Group 3-5	< 0.001	< 0.001	0.020	NS	0.037	0.045
Group 4-5	= 0.001	< 0.001	0.012	NS	NS	0.009

*Turkey analysis. ** Mann–Whitney U test. NS – not significant. T.Bil – total bilirubin. D.Bil – direct bilirubin

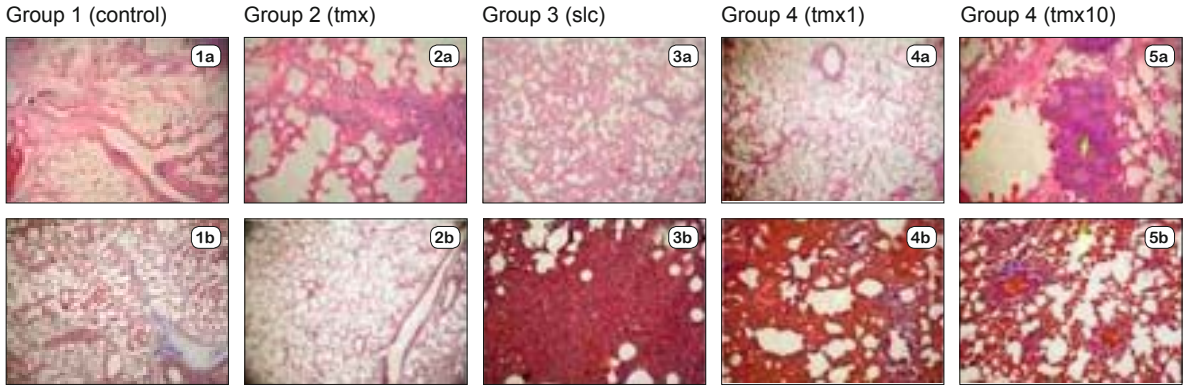

Fig. 2. Histological analysis of lungs under a light microscop in five groups. 1a. Lung microscopic section of control group, haemotoxylin–eosin staining (H&E x4), 1b. Lung microscopic section of control group, mason trichrome staining (Masson x4), 2a. Lung microscopic section of tmx group, haemotoxylin–eosin staining (H&E x4), 2b. Lung microscopic section of tmx group, mason trichrome staining (Masson x4), 3a. Lung microscopic section of slc group, haemotoxylin–eosin staining (H&E x4), 3b. Lung microscopic section of slc group, mason trichrome staining (Masson x10), 4a. Lung microscopic section of tmx1 group, haemotoxylin–eosin staining (H&E x4), 4b. Lung microscopic section of tmx1 group, mason trichrome staining (Masson x10), 5a. Lung microscopic section of tmx10 group, haemotoxylin–eosin staining (H&E x4) (Arrow shows granulomatous lymphadenitis), 5b. Lung microscopic section of tmx10 group, mason trichrome staining (Masson x10).

are several clinical and pathologic varieties of silicosis, including simple silicosis, acute silicosis, complicated pneumoconiosis and true diffuse interstitial fibrosis18.

In a research that examined sand and quarry workers, nonspecific respirable dust concentrations were measured as 16, 13 and 15 mg/m^3, respectively and respirable quartz concentrations were 0.13, 0.15, and 0.17 mg/m^3, respectively. They founded that the prevelance of silicosis was 32.2 % in directly exposed quartz workers19. According to the United States National Instute for Occupational Safety and Health (NIOSH) the recommended exposure limit (REL) is 0.05 mg/m^3 (NIOSH 2002). In our study, periodic air samplings were performed on days 1, 42, and 84. Mean exposure concentration was 23.375 mg/m^3 and respirable crystalline silica concentration level was 0.268 mg/m^3. We showed that this model was highly capable of developing silicosis as all of rats in slc group were affected.

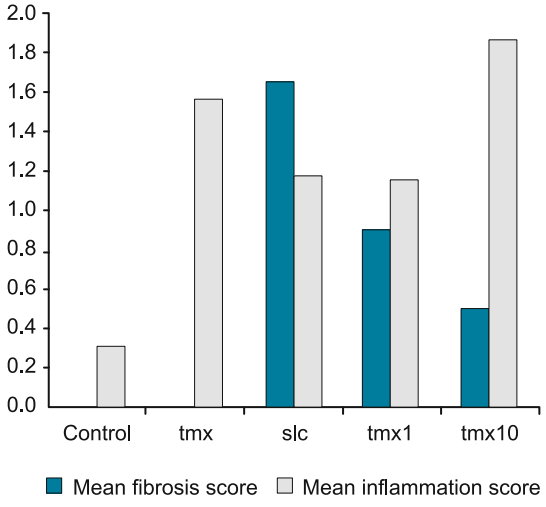

Fig. 3. Comparison of lung fibrosis and inflammation scores between groups.

Tamoxifen citrate is a synthetic nonsteroidal antiestrogen agent mainly used in the treatment of breast cancer. A seven-day tamoxifen treatment decreased the secreted levels of TGFβ-1 whereas estradiol increased these levels (20). It may also be effective in the treatment of abnormal proliferative healing disorders. *In vitro* studies have shown that tamoxifen inhibits the proliferation of keloid fibroblasts, decreases the rate of collagen synthesis, decreases the production of TGF β and decreases the ability to contract fibroblast-populated collagen lattices (11). Tamoxifen has been demonstrated to inhibit fibroblast-populated collagen lattice contraction containing normal fibroblast, keloid fibroblasts, Dupuytren affected palmar fascia fibroblasts and fibroblasts derived from rhinophymas (11–13). Tamoxifen was administered in the treatment of Riedel's thyroiditis. In their study, all four patients responded to the treatment with tamoxifen (21). Tamoxifen is known to show the antifibrotic effect via reducing TGF β-1 levels (12, 17).

Considering the antifibrotic properties of tamoxifen, we examined the possible preventive effect of tamoxifen in silica-exposed rats. We found that within silica-exposed rats, tamoxifen reduced the number of silicosis cases and severity of fibrosis (mean fibrosis score) in silica-exposed rats. This effect was dose-dependent.

The exposure to crystalline silica induces the production of chemokines, inflammatory cytokines, and growth factors from alveolar macrophages and alveolar type II cells, which have been linked to the initation and progression of silica-induced lung disease.

The production of chemokines, inflammatory cytokines, and growth factors is believed to be a key event in the initation and progression of silica-induced lung disease1. A variety of cytokines with potential impact on immunoglobulin production, including IL-1b, TNF-a, IL-6, TGF-β, interferon-g, and IL-10, have been

reported to play important roles in responses to intrapulmonary deposition of silica (22). TGF-β is a multifunctional cytokine that regulates the development, cell proliferation and matrix protein synthesis (23), and it is functional in stimulating fibroblast proliferation and collagen synthesis in silicosis (24). The alveolar macrophages stimulated by quartz dust were able to synthesize TGFβ-1 which was responsible for collagen synthesis of human lung fibroblasts and proliferation (25). Using a quartz-induced silicosis model, TGFβ-1expression was increased in the lung (26). The anti-fibrotic effect of n-acetyl-seryl-aspartyl-lysyl-proline in silicosis was mediated by inhibiting chronic inflammation, TGFβ-1 production, and TGFβ-1-induced pulmonary fibroblast proliferation and collagen synthesis (5).

Among all silica-exposed rats, we detected that the highest mean serum TGFβ-1 levels were in group slc. There was a statistically significant difference for TGFβ-1 levels between slc and tmx10 groups and between slc and control groups. We showed that tamoxifen reduced TGFβ-1 levels in silica-exposed groups in a dose-dependent manner.

The highest lung inflammation scores were observed in tmx group. In tmx and tmx10 groups, we detected higher lung inflammation scores than in other groups. We thought that this was a side effect of a high dose of tamoxifen. Although the high dose of tamoxifen reduced the development of fibrosis, the inflammation was elevated in the lung, which was possibly due to the decreased serum level of TGF-β, which is a lymphocyte proliferation inhibitor. We did not find any experimental studies about the inflammation-promoting effect of tamoxifen in the lung. Further studies are necessary to explore this side effect of tamoxifen.

Hepatic silicosis developed in three months as a result of subcutaneous and intraperitoneal exposure to quartz. TGFβ-1 was implicated in the induction of synthesis and accumulation of extracellular matrix in the liver, which may lead to hepatic fibrosis in the regulation of liver cell proliferation by subcutaneous and intraperitoneal silica administration9. In a study of antifibrotic effects of tamoxifen in a rat model of periportal hepatic fibrosis, tamoxifen was given orally to rats at 1, 5, 10 mg/kg doses while tamoxifen inhibited the process of hepatic fibrosis dose dependently11. In our experimental study, liver fibrosis developed in no groups although we administered 40-100nm-diameter silica via inhalation. We thought that crystalline silica did not reach the liver in sufficient amounts for developing hepatic fibrosis.

In a research, 10 mg/kg tamoxifen citrate elevated the serum levels of AST and ALT (27). In our study, serum AST levels did not differ between groups. Serum ALT, direct and total bilirubin levels were not significantly different between control group and slc group but higher in tmx-administered rats. This indicates that silica exposure caused no hepatic abnormality but tamoxifen showed a hepatotoxic effect, especially in silica-free rats. Serum ALT levels in groups tmx group and tmx10 were higher than in other groups. This may be explained by the possible hepatotoxic effect of tamoxifen. In contrast, direct and total bilirubin levels were significantly lower in silica-exposed and tamoxifen-given rats compared with other groups.

Conclusion

While stimulating inflammation and increasing ALT levels, tamoxifen reduces serum TGFβ-1 levels, which can possibly result in dose-dependent prevention of lung fibrosis. Further studies are needed to explore the curative effect of tamoxifen on silicosis.

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