The Effects of Pentoxifylline Administration on TGF-β1 Expression in Rats with Radiation-Induced Muscle Fibrosis

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ABSTRACT

The aim of this study was to investigate the potential influence of pentoxifylline (PTX) administration on transforming growth factor beta 1 (TGF-β1) expression and the development of radiation-induced fibrosis (RIF) in Sprague-Dawley rats. Seventeen rats (n= 17) were randomly divided into four groups: The first one received a single dose equivalent to 90 Gy of radiotherapy (RT) to the rectus femoris muscle, the second received PTX administration (25 mg/kg/day) via gavage in addition to irradiation (RT+PTX), the third group received only PTX, and the fourth group served as the controls. At the end of 24 days, fibrosis formation was assessed by light microscopy. The TGF-β1serum and tissue levels were assessed with enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction methods. Immunohistochemical staining revealed focal punctate cytoplasmic TGF-β1 staining in the irradiated group compared with the control group. Morphological changes, including increased oedema and mild collagen thickening, were observed in both the RT and RT+PTX groups. Reverse transcription polymerase chain reaction results revealed no significant differences between the RT, PT+PTX, PTX, and Control group. ELISA results showed substantial differences between the RT, RT+PTX, PTX, and Control group (p= 0.125). The Control group without therapy exhibited higher ELISA results. PTX administration did not demonstrate a positive effect on the serum and muscle expression of TGF-β1 in rats with fibrosis induced by a single dose of radiation. Additionally, TGF-β1 may not be a reliable marker of fibrosis that can be routinely used in the early stages of similar radiotherapy-related tissue damage.

Keywords: Pentoxifylline, Fibrosis, Radiation therapy, TGF-β1 expression

INTRODUCTION

Head and neck cancers are the seventh most common type of cancer worldwide. However, recent advancements in cancer treatment and early detection methods have contributed to extending patient survival rates.[1] External beam ionizing radiation is frequently used in cancer treatment, alone or in conjunction with chemotherapy and/or surgery. However, this therapy may damage normal tissue alongside tumor cells.[2] Radiation therapy (RT) can induce adverse effects – classified as either acute or chronic – on different tissues. Acute effects manifest within the initial three months following RT, while late sequelae may emerge months or even years after the conclusion of the RT regimen. One of the delayed adverse effects, with a major impact on quality of life, is radiation-induced fibrosis (RIF).[1] This condition can manifest in various anatomical regions, including the skin and subcutaneous tissue, the lungs, gastrointestinal and genitourinary tracts, and any other organs exposed to RT radiation fields.[2,3] Presently, therapeutic options for RIF remain limited, although they demonstrate moderate success.

These options include stem cell therapies, anti-inflammatory interventions, vascular-directed treatments, and antioxidant modalities.[4]

Pentoxifylline (PTX) is conventionally used in the treatment of peripheral vascular disease.[5] PTX is being explored for its potential benefits in oncology. PTX may ameliorate tumor perfusion, thereby enhancing oxygenation and, consequently, bolstering the radiosensitivity and delivery efficacy of anticancer agents.[6] Functioning as an inhibitor of tumor necrosis factor-alpha, interleukin-1, and fibroblast growth factor, PTX also exhibits mitigating effects on fibrosis via its modulation of transforming growth factor beta (TGF-β).[7]

TGF-β1 belongs to a ligand superfamily and is classified as a cytokine and growth factor.[8] TGF-β1 is a key player in both normal and abnormal tissue repair, influencing processes such as cell differentiation, survival, proliferation, and behavior induction.[9] Activation of the TGF-β signaling pathway is essential for the beginning of collagen accumulation during the healing process. TGF-β1 exerts regulatory control over fibroblast phenotypes, myofibroblast transdifferentiation, and extracellular matrix preservation. In addition, TGF-β inhibits the breakdown of the extracellular matrix via enzymes such as matrix metalloproteinases. Excessive expression of TGF-β1 leads to the development of fibrotic tissue.[10]

The principal aim of this study is to provide evidence of the involvement of TGF-β1 in muscle fibrosis and investigate the molecular mechanisms responsible for the fibrotic effects of TGF-β1's in muscle. Another objective of this study is to evaluate the efficacy of administered PTX in mitigating RIF. The null hypothesis tested in this study is as follows: PTX administration will not cause any differences in TGF-β1 expression in the serum or muscle tissue of rats that have undergone RT.

MATERIALS AND METHODS

Sample Size Determination

G*Power software, version 3.1.9.7, was utilized for a priori sample size determination based on the potential variance difference in serum enzyme-linked immunosorbent assay (ELISA) levels among the study groups. The interface selected a one-way analysis of variance (ANOVA) design from the F-test family. Because there was no prior research available on this subject, a pilot study was conducted to calculate the effect size, yielding a value of 0.97. The α error was set at 0.05, power (1-β) at 0.8, and the number of groups at four. The noncentrality parameter estimate was 15.31, and the total sample size calculated by the software was 16. This number was increased to 17 for non-parametric analysis (n= 17).[11]

Experimental Animals and Housing Conditions

Sixteen- to twenty-week-old male Sprague Dawley rats were used in this study. The rats were housed in vented cages at a room temperature of 22^0C with a regular 12-hour light/dark cycle and had ad libitum access to food and water.

Experimental Groups

After several days of acclimatization, the rats were randomly assigned to four groups: the RT group (n= 6), the PTX and RT group (RT+PTX, n= 6), the PTX group (n= 3), and a control group (C, n= 2). The RT group received a single dose of radiation targeting either the left or right hind leg, including the musculus rectus femoris. The animals in the RT+PTX group received the same radiation dose as those in the RT group and were also administered 25 mg/kg/day of PTX dissolved in 2 ml of physiological saline for 24 consecutive days. The PTX group received the same dose of PTX as the RT+PTX group but underwent no radiotherapy. The C group received no treatment.

Radiotherapy Session

The RT was conducted at the Radiation Oncology Clinic, Istanbul University Oncology Institute, and the radiation dose was determined based on the findings of an animal model study conducted by Zhou et al.[12] In their animal model study, a single dose of 90 Gy radiation was administered using the Nucletron Microselectron-HDR Ir-192 Afterloading System (Nucletron Operations BV, Netherlands), with the applicator tube fixed at a mark on the left thigh. The dose normalization point

was 0.5 cm below the source center, and the irradiation area had a 0.5 cm radius around the mark point. However, the radiation device utilized in our study lacked the capability for focal irradiation as described in Zhou's research. Consequently, the radiation was administered to the entire leg rather than being confined to a specific area. The rats were subjected to an intraperitoneal injection of 35 mg/kg of ketamine hydrochloride and 5 mg/kg of xylazine hydrochloride before receiving radiation in a deeply sedated state. The radiation field was set to 20 × 20 cm^2, and the right/left legs of the rats were placed and fixed within this area. To ensure that the maximum dose reached the skin, a 1.5 cm-thick tissue-equivalent material was placed on the rats within the irradiation area. A 6-MV Varian linear accelerator (Varian, Palo Alto, CA, USA) was employed to administer an equivalent radiation dosage of 90 Gy at a dose rate of 400 MU/min, positioned 100 cm away from the source skin. Varian linear accelerators generate high-energy X-rays or gamma rays by accelerating electrons and then directing them onto a target.

Pentoxifylline Administration

After irradiation, PTX was administered once daily via gavage to three rats in the PTX group and all experimental animals in the RT+PTX group. A total daily dosage of 25 mg/kg was administered. PTX administration continued for 24 days after radiation. The experimental animals were sacrificed alongside those in the other groups at the end of the 24th day.

Sample Collection

All rats were anesthetized with a high dose, and 10 ml of blood was collected from their hearts using a 25-gauge syringe. Muscle tissue samples were taken from the RT-treated legs in the RT and PTX+RT groups, and from a randomly selected leg in the C and PTX groups.

The sample legs of the rats were fixed, and after making a skin incision approximately 2 cm in length on the medial surfaces of the femurs, the Musculus rectus femoris was exposed. The excised muscle tissues were divided into two parts for genetic and histopathological analyses.

Immunohistochemistry

For immunohistochemistry, paraffin blocks were cut serially into approximately 5-μm-thick sections on charged slides. Sections were penetrated overnight in an autoclave (56^{0}C). The sections were deparaffinized, and a Histostain-Plus Bulk Kit (Zymed 2nd Generation; LAB-SA Detection System, 85-9043, Camarillo, USA) was used. For antigen retrieval, the sections were microwaved four times for 5 min in a citrate buffer solution (pH= 6.0). Endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2. TGF-β1 was used as the primary antibody and was incubated for 60 min. Negative control sections treated with phosphate-buffered antibodies were confirmed to be unstained. The secondary antibody was reacted for 25 min. AEC chromogen (ScyTek Laboratories, Inc., Logan, UT, USA) was used to visualize the reaction. Finally, the sections were counterstained with Mayer's haematoxylin, coverslipped, and then evaluated using a light microscope.

Serum Transforming Growth Factor Beta 1 Levels

TGF-β1 protein levels in the serum were assessed using an EliKine Rat TGF-β1 ELISA Kit (Abbkine, Atlanta, GA, USA). The assay was conducted in accordance with the manufacturer's instructions. Following centrifugation, the serum parts of the blood samples were moved to storage tubes and kept in −80^{0}C refrigerators. Frequent cycles of freezing and thawing were prevented. Throughout the experimentation, the required quantity of serum samples was determined in compliance with the ELISA kit's suggested protocol following dilution, and variations in TGF-β1 serum levels among the four groups were analyzed independently.

RNA Isolation, cDNA Synthesis, and Reverse Transcription Polymerase Chain Reaction

Following tissue homogenization, RNA samples were isolated from muscle tissues obtained from each of the four experimental groups using a NucleoSpin isolation kit (Macherey-Nagel, Düeren, Germany). A NanoDrop instrument was utilized to determine the concentration and purity of the RNA samples. The RNA samples were then stored at

Table 1. Comparison of the serum level of TGF-β1 between groups

Group	Mean ± SD (pg/ml)	F	p
RT	56.018 ± 37.996[a]		
RT+PTX	58.562 ± 52.732[b]		
PTX	145.040 ± 56.418[abc]	15.72	0.001
C	273.790 ± 29.731[abc]		

ELISA= enzyme-linked immunosorbent assay; RT= radiotherapy group; RT+PTX= radiotherapy and pentoxifylline group; PTX= pentoxifylline group; C= control group; SD= standard deviation.
The difference between the values followed by the same lowercase letter in the same column is significant

Table 2. Real-time PCR values of TGF-β1 mRNA expression

Group	Mean ± SD	F	p
RT	2.17 ± 1.49	2.301	0.125
RT+PTX	3.25 ± 1.89		
PTX	0.73 ± 0.70		
C	1.01 ± 0.18		

TGF-β1= transforming growth factor type beta1; F= variance

–80^{0}C until the next experimental step. For cDNA synthesis, approximately 200 ng of RNA samples were employed using the FIREScript RT cDNA synthesis kit (Solis BioDyne, Tartu, Estonia). EvaGreen qPCR Mix and the TGF-β1 TaqMan probe set were used to amplify the cDNA samples. The ACTB housekeeping gene was chosen as a control, and the TGF-β1 gene expression levels were calculated using the $2^{-\triangle\triangle CT}$ technique.

Ethical approval: The study protocol was reviewed and approved by the Bezmialem Vakıf University Local Ethics Committee for Animal Experiments (No: 2020/137; November, 23, 2020).

Statistical Analysis

The data set was analyzed using IBM SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). The mean, median, and standard deviation values for the experimental groups were used for descriptive statistics. The normality of the continuous variables was confirmed using graphic methods and the Shapiro–Wilk test. For variables that met the requirements for normal distribution, ANOVA and post hoc Tukey's honestly significant difference tests were used for multiple and pairwise comparisons, respectively. The Kruskal–Wallis and Mann–Whitney tests were employed for the same purposes for non-normally distributed variables. The confidence interval was set to 95%, and $p < 0.05$ was considered statistically significant.

RESULTS

Mortality Rate

In this study, 3 (17.64%) of the 17 rats did not survive, resulting in the unavailability of blood for ELISA analysis. Due to the absence of focal irradiation capability in our radiation device, the radiation applied to the entire leg likely caused damage to surrounding healthy tissues and potential organ injury. All the rats that were lost during the study belonged to the RT group. However, muscle tissue samples were successfully obtained from all rats for reverse transcription polymerase chain reaction (PCR) procedures and a subsequent histopathological examination.

Enzyme-linked Immunosorbent Assay

The C group, which did not receive any therapy, had higher ELISA values than all the other groups. The ELISA value for the PTX group was higher than that of the RT+PTX group (p= 0.007). The ELISA value for the RT+PTX group was lower than that of the C group (p= 0.001). The ELISA values for the RT group were found to be significantly lower than those for the PTX and C groups (p= 0.025 and p= 0.01, respectively), while the ELISA values for the C group were significantly higher than those of the PTX group (p= 0.01). There was no significant difference between the ELISA values for the RT and RT+PTX groups. Regarding the ELISA data, there were significant differences between the RT, RT+PTX, PTX, and C groups (p= 0.001, Table 1).

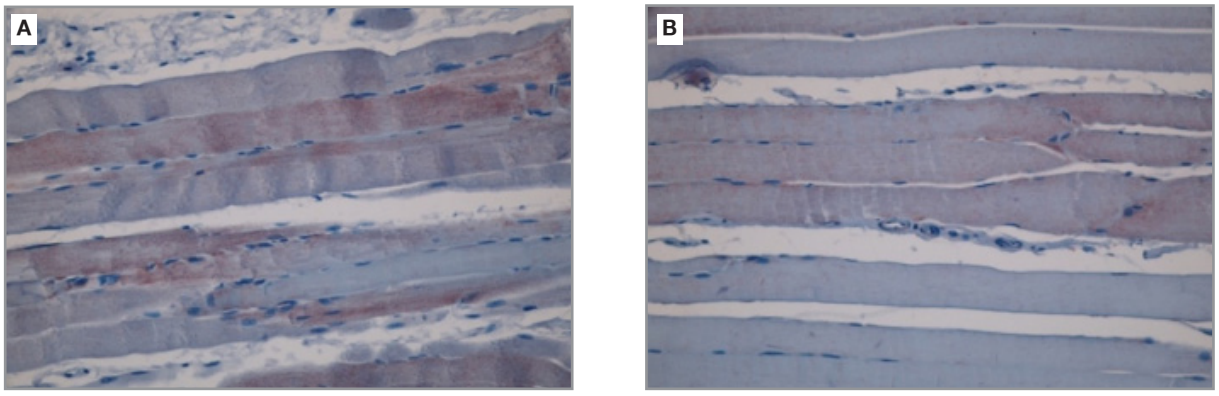

Figure 1 A-B. Focal punctate cytoplasmatic staining observed in the RT and RT+PTX groups (A–B)

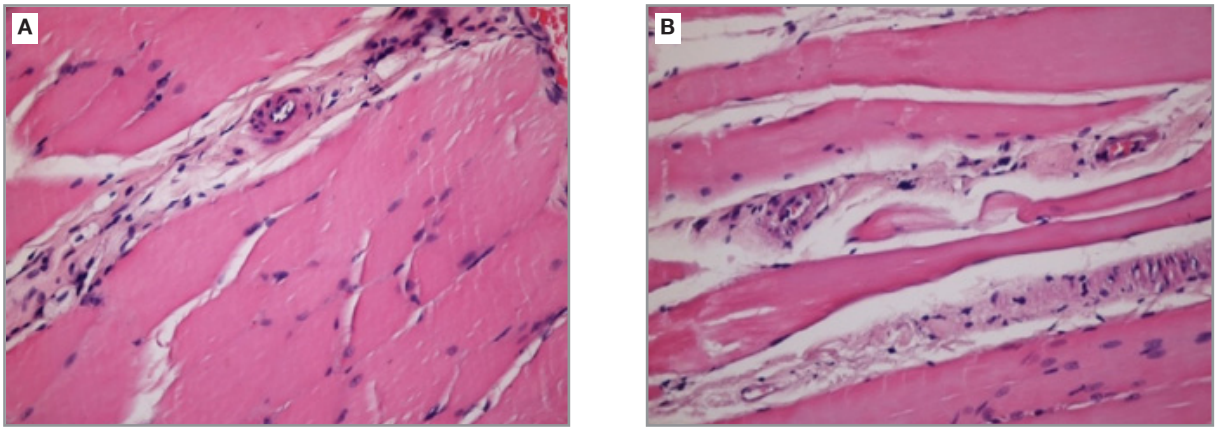

Figure 2 A-B. Increased edema and mild thickening of both collagen and vessels were observed in some areas of the specimens from the RT and PT+PTX groups. Muscle tissues with a natural histology observed in the PTX and C groups (A–B).

Reverse Transcription Polymerase Chain Reaction

The real-time PCR values for the RT+PTX group was higher than those for the other groups. Nevertheless, the minimum level of TGF-β1 mRNA expression was specifically detected in the specimens from rats in the PTX group—to which only PTX was administered. No statistically significant relationship was found between the RT, RT+PTX, PTX, and C groups (p= 0.125, Table 2).

Immunohistochemical Staining

Immunohistochemical results were examined under a light microscope at between 40× and 400× magnification. No distinct staining of TGF-β1 was detected in the specimens from any of the experimental groups. Focal punctate cytoplasmatic staining was observed in the specimens from the RT and RT+PTX groups (Figure 1 A-B).

Haematoxylin and Eosin Staining

Increased edema and mild thickening of both collagen and vessels were observed in some areas of the specimens from the RT and PT+PTX groups. Muscle tissues with natural histology were observed in the specimens from the PTX and C groups (Figure 2 A-B).

DISCUSSION

This study investigates the impact of pentoxifylline (PTX) on early-stage muscle fibrosis by quantifying TGF-β1 levels in rat serum and muscle tissue after RT. Previous studies have not measured TGF-β1 concurrently in both serum and muscle tissue despite its known increase following RT.

RT is widely used in treating various cancer types, including head and neck cancers; however, it pro-

duces significant adverse effects.[13] Fibrosis, which brings about muscle tissue degeneration, significantly impacts quality of life. The role of agents that enhance blood circulation in mitigating fibrosis, especially in the trismus of the head and neck, has been studied previously.[1] PTX is one of the few agents proven to be effective in this context [14]. Animal models are preferred for studying fibrosis due to the invasive techniques required. In this study, a rat model was chosen for its availability, ease of care, and low cost, and we focused on the musculus rectus femoris to minimize tissue loss. TGF-β1, a key fibrosis marker, is present in both serum and muscle tissue.[15,16] Due to the limited number of studies that simultaneously evaluate serum and muscle tissue, our experimental design involved taking separate measurements for serum and muscle TGF-β1 values.

This study aimed to observe an RIF model using non-fractionated and single-dose RT in the early stage of fibrosis. Numerous studies in the current literature have investigated the development of RIF using different animal species. Some researchers have administered radiation in fractions, while others have used lower doses or employed other strategies to minimize the radiation field generated by the technique employed.[15,17-19]

In their research on Wistar rats, Hsu et al. found mild smooth muscle fiber degeneration after 80 Gy fractional irradiation, leading to severe symptoms after 12 months, including muscle fiber loss and thickening of the vascular wall.[18] Andrade et al. observed aggravated skin damage with high radiation exposure in Wistar rats; their 60 Gy group exhibited thicker collagen fibers than the other groups during the initial stages of fibrosis, while the 10 Gy group completely healed.[19] Sonstevold et al. observed morphological changes in Sprague Dawley rats 6 weeks after exposure to 15 Gy radiation. The changes included reduced saliva production, impaired tooth development, and decreased vascular density in the epidermis and chewing muscles. However, no significant change in collagen density was observed.[20] Similar to the aforementioned studies, in this study, haematoxylin and eosin-stained sections of specimens from the RT and PT+PTX groups exhibited increased edema, mild collagen thickening, and vascular changes under a light microscope. Although no significant difference was observed between the experimental groups in the immunohistochemical staining results, focal punctate staining of TGF-β1 was noted in the irradiated tissues. The C group showed a normal histologic appearance of muscle tissue. The findings of this study indicate that fibrosis initiates within 24 days but requires a more extended period for full maturation. Considering its ability to yield successful results in a shorter time frame, and taking into account financial and animal care costs, we adopted the approach employed in the Zhou et al. study. Zhou et al. show that by delivering a single equivalent dose of 90 Gy radiation to the tissues, RIF can be induced in Sprague Dawley rat models within 4 weeks. In the fourth week after irradiation, TGF-β1 mRNA expression was shown to be statistically higher than in the control group. Other findings of the Zhou et al. study include an increase in the number of genes associated with muscle regeneration, swelling in endothelial cells, irregularities in muscle fibers, increase in vascular permeability, and accumulation of fibrous tissue surrounding the vessel. Their study demonstrates that a single equivalent dose of 90 Gy can successfully create a fibrosis model.[12] However, we expedited the completion of our study to within 24 days because of the observed constraints in locomotion and feeding patterns in rats, as these hinted at potential organ damage.

RIF involves radiation-induced DNA damage and the generation of reactive oxygen species. M2 macrophages release TGF-β1 following immune cell migration, which triggers fibroblast production and differentiation into myofibroblasts. In RIF, excessive collagen production by myofibroblasts reduces vascularity, potentially causing necrosis, tissue atrophy, and a loss of function.[2] TGF-β1 is an essential cytokine that regulates skeletal muscle function by activating satellite cells in response to various stimuli, such as acute or chronic damage, thereby inhibiting myogenesis and regeneration and thus affecting the overall function of skeletal muscles.[8] The objective of this study is to assess TGF-β1 gene expression in muscle tissue using real-time PCR. Several studies have reported that RT has resulted in an increase in real-time PCR results.[12,21,22] In a study of cervical strap muscles from

30 patients, it was found that the group of patients receiving RT alone – without neuromuscular electrical stimulation therapy or conventional swallow therapy – had significantly greater TGF-β1 expression levels than the other groups.[23] Tangami et al. delivered a single 20 Gy radiation dose in a mouse study to establish a vocal cord fibrosis model. The vocal cords were histologically and genetically examined at 1, 2, and 6 months, revealing an increase in TGF-β1 expression 6 months after RT.[21] According to Liu et al., TGF-β1 reached its highest levels at 2 and 12 weeks after RT in rats exposed to a single dose of 20 Gy.[22] In a study by Andrade et al., TGF-β1 expression was distinct in the 60 Gy radiation group on day 10.[19] Another study shows that injecting TGF-β1 significantly increases scar formation in the central nervous system of rats, while neutralizing TGF-β1 reduces fibrotic tissue accumulation.[24] In our study, the RT group exhibited higher TGF-β1 expression levels than the C group, implying a correlation between TGF-β1 gene expression and tissue fibrosis development. TGF-β1 expression was higher in the RT+PTX group than in the RT group, contrary to expectations.

PTX, a methylxanthine derivative, treats peripheral vascular diseases by reducing blood viscosity, improving vasodilation, enhancing erythrocyte flexibility, and inhibiting fibroblast proliferation and extracellular matrix synthesis.[25,26] The intent behind this research was to determine how PTX can facilitate the prevention of early-stage fibrosis and whether TGF-β1 could be useful in monitoring this effect. The effect of PTX on RIF has been examined in a human study of 30 patients. After 8 weeks of PTX use, improvements in muscle strength were observed, including improvements in active and passive range of motion, as well as a decrease in edema and pain in the extremities.[14]]. In another study, the impact of PTX on radiation-induced lung fibrosis in Wistar rats exposed to 20 Gy of radiation was investigated. Based on the micro-CT and histological results, the fibrotic changes in the Wistar rats were reduced by the intraperitoneal PTX injections administered. Therefore, PTX significantly reduced the expression of fibronectin and plasminogen activator inhibitor-1 following RT. However, an observable effect of PTX on TGF-β/Smad in radiation-induced lung fibrosis has not been reported.[27] It has been found that delivering PTX combined with vitamin E to irradiated Sprague Dawley rats significantly reduces TGF-β1 mRNA expression and collagen accumulation.[22] In their in vitro study, Kumar et al. observed a substantial reduction in collagen deposition within irradiated fibroblast cultures after the application of antifibrotic drugs, including PTX and curcumin.[28]

Our study investigates the relationship between TGF-β1serum levels and RIF using ELISAs. Statistically significant results were obtained. At the outset of this study, it was anticipated that the RT group would have higher ELISA values than the other groups, as a previous study involving 38 patients reported a positive correlation between TGF-β1 serum levels and RIF.[16] However, the untreated C group had higher values, contrary to expectations. Furthermore, similar results have been reported by Zhang et al. regarding TGF-β1 serum levels. In another study, no significant differences were observed in terms of inflammation, liver fibrosis, or disease severity, especially when compared with the control group.[29] In a clinical study involving 20 patients with early-stage breast cancer, it was reported that the TGF-β1 serum levels significantly decreased after both whole-breast RT and accelerated partial breast irradiation. However, in the group treated with whole-breast RT, the values of the TGF-β1 serum levels showed a statistically significant increase 3 months later.[30] Similarly, in our study, the ELISA values decreased significantly following RT. However, there was insufficient time for a substantial increase in these values. These results indicate that serum TGF-β1 might not possess the necessary sensitivity to function as a reliable diagnostic marker. However, our study is constrained by several limitations, notably the limited sample size, dependence on a single marker for result assessment, and a short study duration.

Conclusion

Within the limits of this experimental study, it can be concluded that 24 days of PTX administration does not have a positive effect on the serum and muscle expression of TGF-β1 in rats with fibrosis induced by a single dose of radiation. Furthermore,

TGF-β1 may not be a reliable marker of fibrosis that can be routinely used in the early stages of similar RT-related tissue damage.

Acknowledgments: *The authors would like to thank Dr. Selahattin Yiğit Şirin for his observations.*

Funding: *This study was supported by the Istanbul University Scientific Research Projects Coordination Unit (Project ID: 37539; April, 26, 2021).*

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