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Effects of upadacitinib and PD29 on oxidative damage and inflammation in bleomycin-induced scleroderma model kidney tissues

Ayse KOCAK[1], Meliha KOLDEMIR GUNDUZ[2], Gullu KAYMAK[3], Elif AYDIN[4]

[1] Department of Medical Biochemistry, Faculty of Medicine, Kutahya Health Sciences University, Kutahya, Turkey
[2] Department of Basic Engineering Sciences, Faculty of Engineering and Natural Sciences, Kutahya Health Sciences University, Kutahya, Turkey
[3] Department of Medical Services and Techniques, Vocational School of Simav Health Services, Kutahya Health Sciences University, Kutahya, Turkey
[4] Department of Medical Services and Techniques, Vocational School of Tavsanlı Health Services, Kutahya Health Sciences University, Kutahya, Turkey

Corresponding Author: Ayse KOCAK
E-mail: kocak.ayse@gmail.com

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ABSTRACT

Objective: Scleroderma (SSc) is a rare autoimmune tissue disease. There is currently no effective treatment for SSc. The aim of this study was to investigate the antioxidant and anti-inflammatory effects of upadacitinib and PD29 on total oxidant status (TOS), total antioxidant status (TAS), *malondialdehyde* (MDA), catalase (CAT), glutathione (GSH) peroxidase levels, and interleukin-6 (IL-6) and interleukin-13 (IL-13) in kidney tissues of an experimental SSc model.

Materials and Methods: The experimental design was established with five groups of eight mice: Control, bleomycin (BLM) (5 µg/kg), BLM + upadacitinib (3mg/kg), BLM + PD29 (5 mg/kg) and BLM + PD29 + upadacitinib group. BLM was administered subcutaneously once a day for 21 days. PD29 was administered subcutaneously and upadacitinib (gavage) were injected for 21 days. Renal tissues were collected at the end of the experiment. Renal TOS, TAS, MDA, CAT, GSH levels, and IL-6 and IL-13 gene expressions were evaluated.

Results: Upadacitinib and PD29 affected oxidant status and TOS. MDA levels decreased, and GSH, CAT, and TAS levels increased. Also, upadacitinib and PD29 decreased inflammation via IL-6 and IL-13 cytokines.

Conclusion: Upadacitinib and PD29 may have therapeutic roles for SSc renal crisis.

Keywords: Scleroderma, Upadacitinib, PD29, Renal tissue

1. INTRODUCTION

Scleroderma (SSc) is an autoimmune disease characterized by fibrosis involving internal organs and the skin. The pathogenesis of SSc has not been fully elucidated, yet, dermis thickening, inflammation, and uncontrollable extracellular matrix (ECM) increase are observed [1, 2]. Clinically, systemic sclerosis is a heterogeneous disease that is measured by the presence of different antibodies, progresses with internal organ involvement, and is divided into different subgroups according to the severity of the disease [3-5]. SSc is rare, with a prevalence ranging from 50-to-300 per million. As many other autoimmune diseases, women are at higher risk than men [6, 7] and SSc is not genetically transmitted [8]. SSc pathogenesis is complex and is associated with dysregulation of type I interferon (IFN), type II IFN, interleukin-6 (IL-6), IL-13 IL-2, and IL-23 regulated by JAK-STAT pathways [9]. Increased activation of Janus-kinase-2 (JAK-2) is detected in the skin of patients with SSc, especially in fibroblasts [10], due to increased levels of transforming growth factor-β (TGF-β). In this study, it is emphasized that JAK signaling pathways can be intracellular targets for the treatment of SSc [10]. The distribution of SSc patients also shows heterogeneity in clinics [10, 11].

In this study, the *Balb/c* mouse strain was used. The substance bleomycin (BLM) was used for the SSc model. BLM is an anti-tumor antibiotic isolated from the fungus *Streptomyces verticillus* [12-14]. This model is still assumed to be the best experimental model. In the previous work of the project team, the SSc model was successfully applied [15, 16].

Today, there is no approved drug used in the treatment of SSc [17-19]. Further studies, molecular and clinical data are needed primarily for the treatment of the disease [18].

Janus-kinase/signal transducer and its activators of the transcription pathway (JAK / STAT) is a conserved pleiotropic cascade involved in development and homeostasis from humans to flies [19]. JAKs are tyrosine kinases that play a key role in many cytokines, especially in IL-6 and IL13 and cellular signaling pathways (type 1 IFNs), type I/II cytokines such as IL-12 and

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IL-23, etc. [20]. Dysregulation of the JAK-STAT pathways is associated with various immune disorders, diseases, and inflammation [21]. JAK inhibitors (Jakinibs) act on competitive ATP binding and block phosphorylation of cytokine receptors. Thus, it leads to decreased production of cytokines and impaired differentiation of Th1, Th2 and Th17 cells [22]. First generation pan-JAK inhibitors (tofacitinib, baricitinib, ruxolitinib, peficitinib) and second-generation selective JAK inhibitors (decernotinib, filgotinib, upadacitinib) have been developed for the treatment of various autoimmune and malignant diseases [23]. Upadacitinib is the third selective JAK1 inhibitor approved for the rheumatoid arthritis (RA). Upadacitinib is effective in the treatment of RA and has been approved by the FDA (2019, August) and EMA (2019, December) for use in the treatment of moderate to severe RA in adults, if other treatments fail [24].

Molecular mechanisms of SSc renal involvement are not fully known, the role of oxidative stress has been demonstrated in many studies [25-29]. Increased reactive oxygen species (ROS) production leads to lipid peroxidation *malondialdehyde* (MDA) and oxidative damage in important cellular macromolecules such as proteins and even DNA. Also, increased ROS production, decreases the activity of cellular antioxidant enzymes, such as catalase (CAT), and glutathione (GSH) peroxidase.

SSc is characterized by accumulation of ROS with microvascular and immune dysregulation that causes multi-organ fibrosis [30-33]. Activation of the TGF-β pathway is known by transcriptome analysis in SSc skin biopsies [34]. TGF-β plays a central role in the pathogenesis of SSc by regulating fibrotic responses, including myofibroblast differentiation, ROS production, ECM synthesis, and hardening [33]. In addition, the role of oxidative stress in SSc has been emphasized in many researches conducted on patients and animal models [35-37]. High ROS rate in SSc has been found to be directly proportional to increased activation of dermal fibroblasts as well as collagen synthesis [37]. In short, tissue fibrosis and oxidative stress in SSc feed each other [38-40]. In SSc dermal fibroblasts, high levels of intracellular and mitochondrial ROS have been shown to induce oxidative stress and the expression of genes involved in the fibrotic process [41]. There is a significant imbalance between high oxidative stress level and insufficient antioxidant defense in SSc [42]. Reduction of ROS production in fibroblast, immune and endothelial cells is necessary for clinical recovery of the disease [43, 44]. In SSc, antioxidant defense is important in regulating ROS production and plays an important role in the pathogenesis of the disease [45].

The PD29 peptide, with its 29 amino acid sequence, is designed to target pulmonary fibrosis (PF). It has been shown that PD29 is responsible for anti-angiogenesis, inhibition of matrix metalloproteinase activities and inhibition of integrins in PF [46]. Again, in the same study, it is suggested that PD29 can treat PF by partially regulating the expression of TGF-ß1, Smad3 and Smad7 [46].

In this study, the effects of upadacitinib and PD29 on oxidative damage and inflammation in BLM-induced SSc model kidney tissues were investigated. TOS, TAS, MDA, CAT and GSH parameters and IL-6 and IL-13 gene expressions were examined in scleroderma kidney tissues.

2. MATERIALS and METHODS

SSc animal model

SSc animal model studies were approved by Kutahya Health Sciences University (KSBU), Faculty of Medicine, Experimental Animals Local Ethics Committee (KSBU-DEHYUB) (23.03.2022, 46959). The SSc experimental animal model was used in our previous projects [15, 16].

Experimental design:

Mice were randomly divided into 5 groups:

Group 1: Control group (n = 8): Mice in this group were given 0.9% saline subcutaneously and as oral gavage for 21 days.

Group 2: Bleomycin group (SSc group, n=8): Mice in this group were given 5 mg/kg BLM (Onko, Koçsel, Turkey) subcutaneously for 21 days and 0.9% saline by oral gavage [17, 18].

Group 3: Bleomycin + upadacitinib (ABT-494) group (n=8): Mice in this group were given 5 mg/kg BLM subcutaneously and 3mg/kg [47] upadacitinib (ABT-494) as oral gavage for 21 days.

Group 4: Bleomycin + peptide PD29 group (n=8): Mice in this group were given 5 mg/kg BLM (Onko, Koçsel, Turkey) and 5 mg/kg peptide PD29 subcutaneously for 21 days [48].

Group 5: Bleomycin + peptide PD29 + upadacitinib (ABT-494) group (n=8): Mice in this group were given 5 mg/kg BLM subcutaneously for 21 days, 3mg/kg [35] gavage upadacitinib (ABT-494) and 5 mg/kg peptide PD29 were administered subcutaneously [48].

Mice were sacrificed at the end of day 21 under 90 mg/kg ketamine and 10 mg/kg Xylazine anesthesia. Kidney samples were stored at – 150^0 C until the relevant experimental step.

Homogenization method

All tissue samples were washed twice with cold saline and homogenized using a Tissue-Lyser (TissueLyser, Qiagen, West Sussex, UK). 0.1 g of each tissue was homogenized at a ratio of 1:8 (w/v) with 50 mM Tris-HCl, pH 7.0, that included 0.15 M NaCl, 10 mM CaCl 2 and 0.05% Brij 35, 20 µ g/ml pepstatin and 20 µ g/ml leupeptin. The homogenates then were centrifuged at 15000 g for 15 min. The supernatants were used for analysis. All procedures were performed at +4 ° C. All homogenates were stored at – 150 ° C until the experimental step.

TOS and TAS determination

TAS and TOS were determined with kits (Rel Assay Diagnostics kit; Mega Tıp, Gaziantep, Turkey) developed by Erel. Measurement of the TAS level was determined using an Erel method. In this method, the antioxidative effect of the sample against the potent free radical reactions, which are initiated by the produced hydroxyl radical, is measured [49]. The results are expressed as µmol Trolox Eq/L.

Measurement of the TOS value was determined using a novel method, such as TAS, developed by Erel [50]. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the experimental

sample. The assay was calibrated with hydrogen peroxide (H2O2) and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H2O2 Eq/L).

Oxidative stress index

The OSI is defined as the ratio of the TOS to TAS level, expressed as a percentage. For this calculation, TAS units were changed to mmol/L, and the OSI value was calculated according to the following formula:

OSI (arbitrary unit) = TOS (μmol H2O2 Eq/L)/TAS (μmol Trolox Eq/L).

MDA level assay

MDA, one of the peroxidation products formed by the reaction of fatty acids with free radicals, was measured as the formation of its colored form with thio-barbituric acid. For each renal tissue, 200 μl homogenate was transferred to a tube, and 800 μl phosphate buffer, 25 μl butylhydroxytoluene solution, and 500 μl 30% trichloroacetic acid were added. The tubes were mixed and incubated on ice for 2 h. They were then centrifuged at 2000 rpm for 15 min, and 1 ml of each supernatant was transferred to a new tube, after which 75 μl ethylene-diamine-tetraacetic acid and 25 μl thio-barbituric acid was added. The tubes were mixed and incubated in a hot water bath for 15 min. They were then brought to room temperature, and the absorbance at 532 nm was read on a UV/Vis spectrophotometer.

CAT activity assay

CAT activity in renal tissue was determined using the method described by Aebi et al., previously [51]. First of all, 2.8 mL 30 mM H2O2 was placed in a blind tube, and 0.2 mL phosphate buffer was added to it. Then 2.8 mL 30 mM H2O2 was added to the sample tube. For both tubes, 0.2 ml enzyme was added. The absorbances at 240 nm were read twice at 30 s intervals to determine the catalase activity.

GSH assay

The GSH level was determined using the method described by Beutler et al. [52]. First, 200 μl renal tissue homogenate was diluted in an 800 μl phosphate buffer, and the first absorbance (OD1) was measured at 412 nm. Then 100 μl Ellman's reagent was added to the same tube, and the second absorbance value (OD2) was recorded.

The levels of MDA and activities of CAT were standardized according to the protein level. Protein levels determination of inappropriately prepared tissue homogenates was spectrophotometrically performed according to the Bradford method.

IL-6 and IL-13 gene expression experiments (qPCR)

cDNA synthesis was achieved from isolated RNA by using a reverse transcription kit (Prime Script RT, Qiagen, USA). qPCR was established with SYBR Green PCR master mix (RT2 SYBR Green qPCR Mastermix, Qiagen, USA) using the qPCR instrument (Rotor-Gene, Qiagen, USA). The used primer sequences (Oligomer Biotechnology, Turkey) are presented in Table I. ß-actin, which is a housekeeping gene, was used for the normalization of the results. Gene expression fold changes were calculated according to the 2ΔΔ Ct method.

Statistical Analysis

All statistical analyses were done by Prism version 9 (GraphPad Software, California, US). The Kruskal–Wallis test was performed to determine the differences between multiple independent groups. The Mann–Whitney *U* test was used to analyze the difference between two independent groups. For correlation analysis, Pearson's test was used. All data were presented as mean ± SD. P< 0.05 was considered statistically significant.

3. RESULTS

TOS and TAS determination

SSc renal tissue TOS level was detected to be significantly higher in the BLM group (426.0 ±40.35 μmol/L) compared to the control group (189.6 ± 27.73 μmol/L) (P = 0.02). However, upadacitinib (227.7± 42.30 μmol/L) (P= 0.03), PD29 (332.8±40.97 μmol/L) (P = 0.04) and upadacitinib + PD29 (319.50±1.78 μmol/L) (P = 0.04) administration significantly decreased tissue TOS level compared to BLM group (Figure 1(a)). In addition, SSc renal tissue TAS level was detected to be significantly lower in the BLM group (0.07 ± 0.05 μmol/L) compared to that of the control group (0.23 ± 0.03 μmol/L) (P= 0.02). However, upadacitinib (0.22 ± 0.06 μmol/L) (P = 0.02), PD29 (0.217 ± 0.10 μmol/L) (P = 0.02) and upadacitinib + PD29 (0.18± 0.07 μmol/L) (P = 0.02) administration significantly increased tissue TAS level (P< 0.05) compared to BLM group (Figure 1(b)).

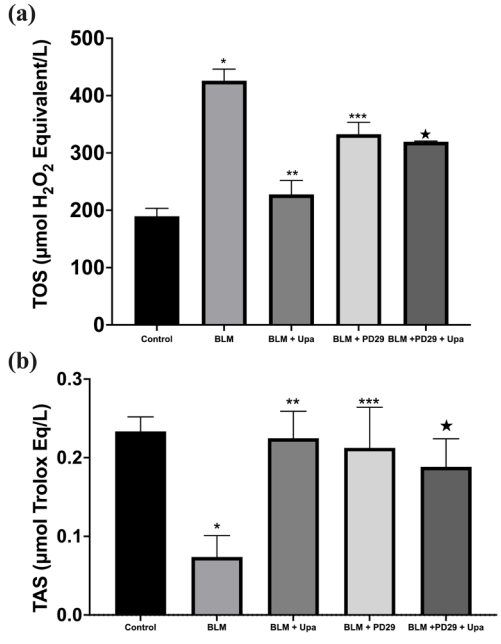

Figure 1. *(a) TOS and (b) TAS levels in SSc renal tissue. Upadacitinib, PD29 and PD29 + Upadacitinib decreased TOS and increased TAS statistically *. Control vs BLM, ** BLM vs BLM + Upa, *** BLM vs BLM + PD29, ★ BLM vs BLM + PD29 + Upa*

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OSI level of BLM group was found to be significantly higher in the BLM group (10.262 ± 8.126) compared to control group (816.8 ± 92.64) (P = 0.02). Upadacitinib (1162±287.3) (P = 0.02), and PD29 (1727 ± 449.3) (P= 0.02) administration significantly decreased compared to BLM group (Figure 2).

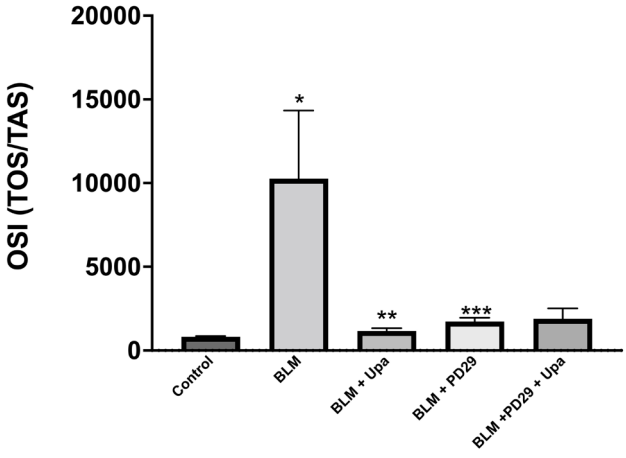

Figure 2. *OSI levels on SSc renal tissue. * Control vs BLM, ** BLM vs BLM + Upadacitinib, *** BLM vs BLM + PD29*

MDA level assay

SSc renal tissue MDA levels were detected to be significantly higher in the BLM group (0.244± 0.02 nmol/mg protein) compared to that of the control group (0.10 ± 0.009 nmol/mg protein) (P= 0.02). However, upadacitinib (0.11 ± 0.03 nmol/mg protein) (P= 0.03), PD29 (0.08 ± 0.009 nmol/mg protein) (P = 0.01) and upadacitinib + PD29 (0.08 ± 0.01 nmol/mg protein) (p = 0.02) administration significantly decreased tissue MDA level compared to BLM group (Figure 3).

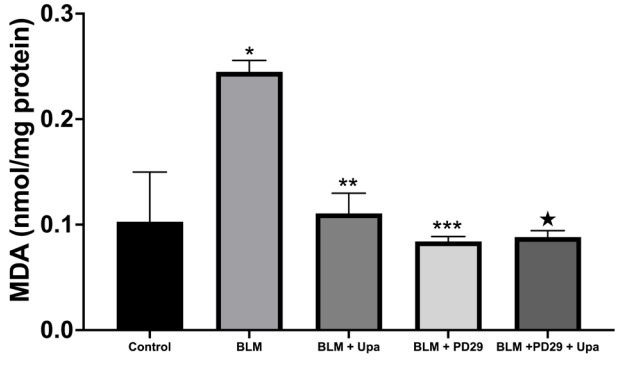

Figure 3. *MDA levels in SSc renal tissue. Upadacitinib, PD29 and PD29 + Upadacitinib, decreased MDA levels statistically. * Control vs BLM, ** BLM vs BLM + Upadacitinib,, *** BLM vs BLM+PD29, ★ BLM vs BLM + PD29 + Upadacitinib*

CAT activity assay

SSc renal tissue CAT levels were detected to be significantly lower in the BLM group (2.34 ± 0.94 U/ mg protein) compared to that of the control group (4.65± 0.99 U/ mg protein) (P = 0.02). However, just upadacitinib administration significantly increased tissue CAT levels compared to levels of BLM group (5.32 ± 1.62 U/ mg protein) (P= 0.04) (Figure 4).

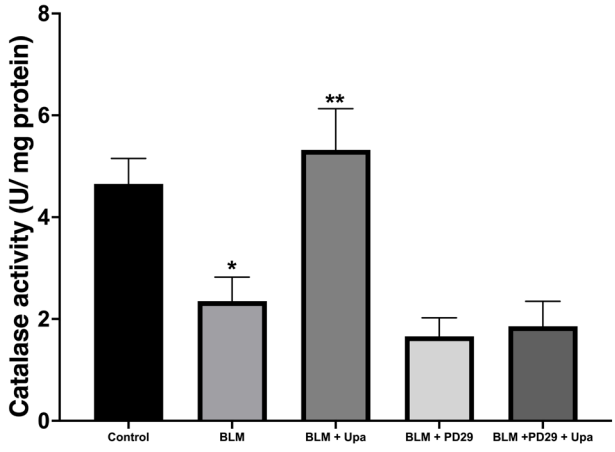

Figure 4. *CAT activity in SSc renal tissue. Upadacitinib, increased CAT activity statistically. * Control vs BLM, ** BLM vs BLM + Upadacitinib*

GSH assay

SSc renal tissue GSH levels were detected to be significantly lower in the BLM group (0.22 ± 0.003 nmol/g protein) compared to that of the control group (0.27 ± 0.06 nmol/g protein) (P = 0.00). However, just PD29 administration significantly increased tissue GSH level (0.38 ± 0.11 nmol/g protein) (P= 0.01) compared to BLM group (Figure 5).

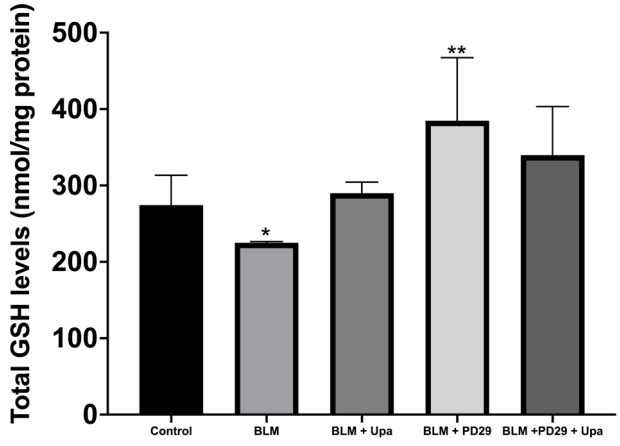

Figure 5. *Total GSH levels in SSc renal tissue. PD29 increased GSH levels statistically. * Control vs BLM, ** BLM vs BLM + PD29*

IL-6 and IL-13 gene expression (qPCR)

IL-6 and IL-13 gene expression in the renal tissue were found to be significantly higher in the BLM group compared to the control group (P= 0.02). However, upadacitinib and PD29 led to a significant decline in the IL-6 and IL-13 gene expression in the BLM + upadacitinib and BLM + PD29 group (P= 0.02) compared to BLM group (Figure 6).

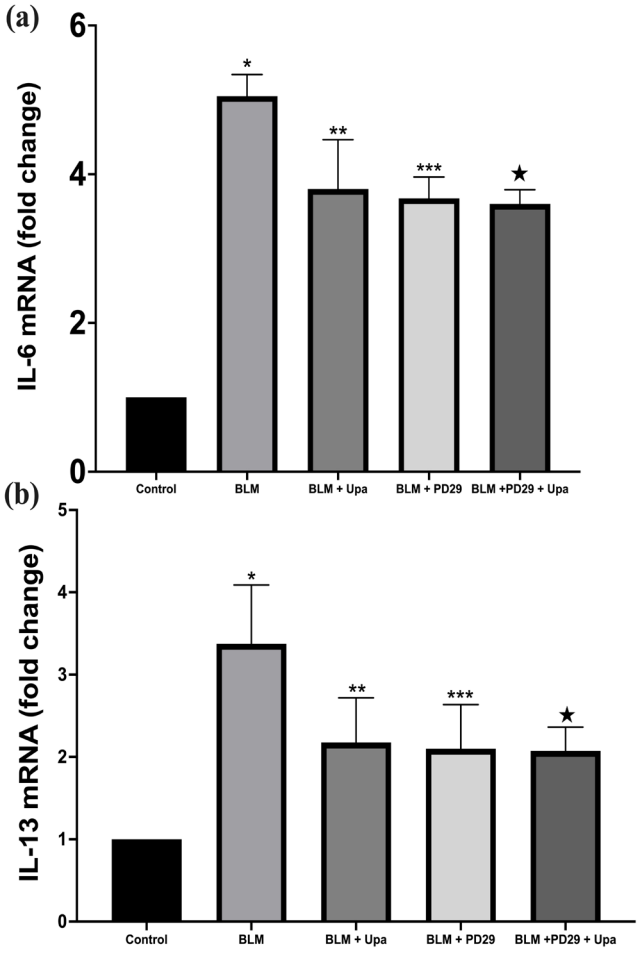

Figure 6. *(a) IL-6 and (b) IL-13 gene expression levels in SSc renal tissue. Upadacitinib,, PD29 and PD29 + Upadacitinib, decreased IL-6 and IL-13 statistically. * Control vs BLM, ** BLM vs BLM + Upa, *** BLM vs BLM + PD29, ★ BLM vs BLM + PD29 + Upadacitinib,Also, in correlation analyses, MDA and TAS, IL-13 and TOS, IL-13 and IL-6 pairs were significant and they had positive correlations (P= 0.024, 0.048 and 0.011 respectively).*

4. DISCUSSION

The present study showed that upadacitinib and PD29 decreased oxidative stress and inflammation in SSc. This is the first study to reveal that upadacitinib and PD29 decreased MDA, TOS levels and IL-6 and IL-13 gene expression and increased GSH, CAT and TAS levels in SSc model renal tissue.

Li et al., in 2018 noted that JAK signal transducers and activators of the transcription (JAK-STAT) signaling pathway contribute to injury as well as inflammation in liver cells [53]. This may explain the potential beneficial effects of upadacitinib to reverse the oxidative stress in tissue TAS, TOS, MDA, CAT activation levels. In addition, upadacitinib significantly improved IL-6 and IL-13 expression levels which were elevated due to JAK-1 inhibition effects.

In SSc, the molecular mechanisms of ROS stimulation of the fibrotic process are highly complex and involve many molecular pathways. The newly produced myofibroblasts are highly active mesenchymal cells capable of producing large quantities of interstitial fibrillar collagens and other fibrotic proteins. The high production of these proteins by activated myofibroblasts and their exaggerated accumulation in the interstitial space of the affected organs result in the severe and often progressive fibrotic changes that are characteristic of SSc.

Animal models are critical for several disease pathogenesis, disease duration research and drug exploration. BLM induced mice are the most widely used for SSc. In 1993, oxidative stress was proposed as the etiology of SSc [54]. Many subsequent studies confirmed this hypothesis. High levels of oxidative stress markers and decreased antioxidant components were found in SSc [55]. Some of them were correlated with disease duration, modified Rodnan skin score (mRSS), cardiovascular events, renal vascular damage, the severity of pulmonary fibrosis and immunological abnormalities [56-59]. We confirmed that OSI and TOS levels are increased in the SSc group as BLM group. Also, we showed that there is a correlation between oxidative stress and inflammation parameters in the renal tissue. Regarding to our results, especially JAK-STAT signaling leads to inflammation and oxidative stress. The important role of oxidative stress was also confirmed in other experimental SSc mouse models [14, 15].

The peptide PD29 is new and 29 amino acid peptide that can interfere with the pathogenesis of PF through three mechanisms that are anti-angiogenesis, inhibition of collagen degradation, and integrin inhibition. In addition, PD29 has high biological activity, relatively low toxicity, and does not easily accumulate in the body [46]. In the same research, PD29 reduced oxidative damage in the lungs reduced the release of inflammatory and profibrotic factors in BLM affected mice. In our results, we showed that, PD29 reduced TOS, TAS, MDA, IL-6 and IL-1.

PD29 may be by downregulating the TGF-β/Smad signaling pathway, also its mechanism directly inhibited TGF-β downstream proteins such as smad2/3 and promoting smad7 expression [46]. In SSc, TGF-β/ Smad signaling has a pivotal role in the disease progression.

ROS and TGF-β have important roles in cell metabolism. TGF-β can stimulate ROS production in cells [60] and several studies have shown that TGF-β can induce ROS production in different cellular compartments. TGF-β reduces ROS production in mitochondria [61, 62]. A mitochondrial thioredoxin (TXN2)-responsive mechanism that regulates TGF-β-induced ROS production in mammary epithelial cells has been recently

described. These data suggest that a cysteine thiol-disulfide exchange reaction in mitochondria may be involved in TGF-β-mediated regulation of ROS and gene expression [63]. ROS production in SSc also increases due to TGF-β, but as we have shown in our study, oxidative stress and markers are reduced with PD29 application.

Qingbo et al., showed that in PD29 group, IL-6 and MDA were decreased. Also, they showed that GSH was increased in PD29 dose dependent application compared to BLM stimulation [46]. We showed that GSH levels were increased in the PD29 group. Researches related to ROS/RNS production during metabolic processes, open another branch of research focusing on the role of GSH ranging from antioxidant/radical scavenger to redox signal modulator [64]. GSH effectively scavenges free radicals and other ROS and RNS (e.g., hydroxyl radical, lipid peroxyl radical, superoxide anion and hydrogen peroxide) directly and indirectly through enzymatic reactions. The chemical structure of GSH determines its functions, and its wide distribution among all living organisms reflects its important biological role [64]. The PD29 may direct stimulating GSH levels in metabolism because we showed just significant effect on PD29 group.

The expression levels of protective antioxidants were reduced in the skin of the tight-skin (TSK-1/+) mouse fibrosis model [65]. It is another mouse model of SSc. Increased total protein excretion was detected in 17.5% of SSc patients and albuminuria was identified in 25% [66].

The effect of the synergistic effect of upadacitinib and PD292 together on oxidative stress should be repeated with more detailed studies. This research shows that upadacitinib and PD29 may effectively be used for the treatment of SSc.

Conclusion

In conclusion, our findings show that upadacitinib and PD29 have notably protective antioxidative effects on SSc renal tissue induced by BLM.

Compliance with the Ethical Standards

Ethics Committee approval: This study was approved by Kutahya Health Sciences University, Faculty of Medicine, Experimental Animals Local Ethics Committee (approval number: 23.03.2022, 46959).

Conflicts of interest: Authors declare no conflicts of interest.

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Authors contributions: AK: Designed the research and also conceived and planned the experiments, AK, MKG, G.K., and EA: Carried out the experiments. AK, MKG, GK, EA: Contributed to the interpretation of the results, AK: Took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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