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The safety and efficacy of light emitting diodes-based ultraviolet A1 phototherapy in bleomycin-induced scleroderma in mice

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ABSTRACT

Purpose: To define the efficacy and safety of narrowband ultraviolet A1 (UVA1) for the treatment of dermal fibrosis in bleomycin-induced mouse model of scleroderma.
Materials and methods: 42 DBA/2 strain mice were included in the study: healthy mice and mice with established scleroderma, treated with high or medium dose of UVA1. Non-treated groups served as control. The equipment emitting 365 ± 5 nm UVA1 radiation was used in the study. The average cumulative doses were 1200 J/cm^2 for high and 600 J/cm^2 for medium dose course. Histological analysis was performed for the evaluation of the dermal thickness and mast cells density. The expressions of p53 and Ki-67 proteins were assessed by immunohistochemical analyses.
Results: Skin thickness of mice with scleroderma, treated with high and medium dose of UVA1, were lower (272.9 ± 113.2 μm and 394 ± 125.9 μm, respectively) in comparison to the dermal thickness of non-treated animals (599 ± 55.7 μm). The dermal mast cells count in mice with scleroderma was reduced after high and medium dose treatment to 11 ± 1.7 and 13 ± 2.2, respectively, as compared to that in non-treated mice (23 ± 3.0). No significant upregulation of p53 nor Ki-67 proteins was observed in the skin of healthy mice and mice with scleroderma after high- and medium-dose of UVA1.
Conclusions: The results of this study indicate that 365 nm UVA1 with the cumulative doses of 1200 J/cm^2 and 600 J/cm^2 is safe and effective for the dermal fibrosis treatment.

1. Introduction

Systemic sclerosis (SSc) is a rare chronic autoimmune disease of unknown etiology. The pathogenesis of SSc includes vasculopathy, progressive fibrosis and alterations in the immune system [1–4]. Imbalance between collagen synthesis and degradation is the consequence of specific fibroblasts activation [3]. Increased accumulation of extracellular matrix leads to the progression of fibrosis [5,6]. The therapeutic SSc strategies include the reduction of fibroblasts hyperactivity in extracellular matrix synthesis and the degradation of excessive collagen amount.

Over the last few decades phototherapy has been implied as a new possibility for the treatment of many skin diseases (atopic dermatitis, urticaria pigmentosa, psoriasis, prurigo, sclerotic skin conditions, etc.) [7]. Commonly used radiation modalities consist of broadband ultraviolet B (UVB, 290–320 nm), narrowband UVB (311–313 nm), ultraviolet A2 (UVA2, 320–340 nm) and ultraviolet A1 (UVA1, 340–400 nm). The most common minor side effects of UVA1 phototherapy are hyperpigmentation, redness, dryness, pruritus and recrudescence of a herpes simplex infection [8,9]. Most notably, the frequency of UV induced burning and erythema seems to be lower for UVA1 than for the conventional UVB or PUVA [10]. Whereas the major side effects of UVB phototherapy include the skin aging, local and systemic immunosuppression as well as development of melanoma and non-melanoma skin cancer [11,12]. The minor side effects of UVA1 and the major side effects of UVB phototherapy demonstrate the superiority of UVA irradiation over broadband-UVB or narrowband-UVB [8,10]. UVB and UVA2 have shorter wavelengths but higher energy than UVA1. These shorter wavelengths mainly affect the epidermal cells and target nuclear DNA, causing the direct damage of DNA. The harm is caused through generation of photoproducts including dimers, strand

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breaks and molecular crosslinks of DNA and proteins [13,14]. Meanwhile, UVA1 has been shown to be more effective due to its deeper penetration into skin, causing less side effects, such as erythema and immunosuppression, as compared to UVA2 [15]. Some of the actions of UVA1 include the induction of skin-infiltrating T cells apoptosis and expression of matrix metalloproteinase-1 (MMP-1) in the human dermal fibroblasts [16]. Furthermore, UVA1 light, especially the long wave UVA1, induces the formation of reactive oxygen species (ROS) which leads to a high expression of heme oxygenase-1 (HO-1) in the dermal fibroblasts [17]. HO-1 is known to reduce the levels of pro-inflammatory cytokines and it may boost the expression of matrix metalloproteinases (MMPs) in skin cells [18].

There are no randomized, double blind, placebo-controlled studies concerning the phototherapy in dermal fibrosis [9]. Clinical trials of SSc are difficult to carry due to severe conditions of the patients, variety of symptoms, small patients' cohort size and probable disease-related complications. Thus, the animal models are essential tools to investigate in depth the pathogenesis of SSc and search of new therapeutic modalities. In the study with bleomycin-induced BALB/c mice model of scleroderma it has been shown that UVA1 (spectral range 340–400 nm) reduced the skin thickness in a dose–dependent manner [19]. None of the previously conducted studies evaluated the safety and effectiveness of narrowband UVA1 on dermal fibrosis. However, Inada et al. [20] compared the properties of conventional UVA1 lamp and the UVA1 light emitting diodes (LED)-based (UVA1 LED) device. The latter source consumed less power and produced uniform intensity compared with the features of the conventional UVA1 lamp. The body and skin surface temperature of mice, irradiated with UVA1 LED device, did not change, while it increased sharply after irradiation using UVA1 lamp. The authors previously showed that apoptosis and necrosis were successfully induced in Jurkat T cells irradiated with the UVA1 LED and conventional UVA1 lamp devices [21]. According to the physical properties of UVA1 and optical features of the skin, the narrowband UVA1 would influence collagen metabolism with reduced chance of side effects. Furthermore, United States Food and Drug Administration (USFDA) approved UVA1 (340–400 nm) phototherapy device (*Daavlin©*) with the peak of 365–375 nm for the treatment for psoriasis, vitiligo and various sclerosing disorders [7]. The wide spectral waveband of UVA (320–400 nm) could react with the different chromophores in the skin, leading to different effects on the immune system of skin [22]. In our study we used the narrowband UVA1 LED-based device (narrowband UVA1 LED) which emits the UV rays with a peak wavelength of 365 nm. Only a few studies were performed by using the narrowband UVA radiation for the skin diseases treatment. For instance, Nistico and coauthors [23] proved that monochromatic UVA1 laser (355 nm) was effective for the treatment of palmoplantar pustular psoriasis. Though the action spectrum for scleroderma treatment is not determined yet. The main pathways that lead to the development of scleroderma include collagen metabolism disturbance, autoimmune activity and vascular dysregulation [10]. UVA1 photons are the most deeply penetrating form of UV therapy, so narrowband UVA1 exhibits selectively its' effects in the all above mentioned pathways. The effective treatment for scleroderma is defined by the anti-fibrotic and anti-inflammatory features of the phototherapy resulting in the decreased dermal thickness. The safe treatment is defined by the low carcinogenic risk of phototherapy.

The study purpose was to evaluate the impact of narrowband UVA1 (the cumulative doses of 1200 J/cm^2 and 600 J/cm^2) on the dermal fibrosis in bleomycin-induced mouse model of scleroderma. The dermal thickness, mast cells count, the expression of cell apoptosis and proliferation markers were assessed in the healthy and sclerotic skin.

2. Materials and methods

2.1. Animals

Forty two 6-weeks-old female mice (each weighing 17–20 g) of DBA/2 strain were involved in the study and kept under standard housing conditions with unlimited food and water access. The use of animals in this study was in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The study was approved by the Lithuanian Laboratory Animal Use Ethical Committee under the State Food and Veterinary Service (No G2-15).

2.2. Establishment of the mouse model of scleroderma

We selected DBA/2 mice due its high susceptibility to bleomycin-induced fibrosis model. Yamamoto with colleagues [24] showed that C3H/He, DBA/2, B10.D2, and B10.A mice developed more intense dermal sclerosis characterized by deposition of homogeneous material in the dermis and thickened collagen bundles following bleomycin treatment. Subsequently several authors also successfully induced the dermal fibrosis in DBA/2 mice or proved its superiority to other mice strains concerning the susceptibility to bleomycin-induced dermal fibrosis [25,26]. At the present study the skin fibrosis was induced in 21 DBA/2 strain female mice by subcutaneous injections of bleomycin (Bleo) (Bleomycin sulfate from *Streptomyces verticillus,* Sigma Aldrich, Buchs, Switzerland) [27]. At first, the hair from the mouse back was removed using the depilation cream (*Veet*®, Reckit Benckiser, England) and the square area of 1 cm^2 was marked. The injection site included 5 points – every corner and the center of the marked square. Bleo was dissolved in 0.9% sodium chloride (*Sanitas*, Lithuania) at a concentration of 0.5 mg/ml and subcutaneous injection of 100 μl in total for 5 points was performed every other day for 25 days. Other 21 mice received 100 μl of sodium chloride injections at the matched area of the back and served as the negative control groups.

2.3. UVA1 source

Custom made narrowband UVA1 LED device was used as a PT source in the study. The equipment consisted of 20 built-in UVA1 LEDs (UVLED-365-330–surface mounted devices (SMD), *Roithner LaserTechnik,* Austria) emitting 365 ± 5 nm radiation. LEDs were laid out to ensure the uniform power density of 21 mW/cm^2 at the surface distant 4 cm from the device. The PM200 power and energy meter (*Thorlabs GmbH*, Germany) with loaded the spectral emission curve of used LED source as well as S302C thermal power sensor (*Thorlabs GmbH*, Germany) were used for the power density measurements. The applied doses per session were 80 J/cm^2 for high and 40 J/cm^2 for medium dose of UVA1. The dosage regimen was selected according to previously conducted studies with scleroderma patients. Based on the literature review, 20–50 J/cm^2 of broadband UVA1 therapy 3–4 times a week for 30 procedures is recommended [9].

2.4. Study design

Forty two DBA/2 line mice were randomly assigned to 6 groups (seven mice in each):

- Group I (Healthy) – healthy animals;
- Group II (Bleo) – control group with bleomycin-induced scleroderma;

- Group III (BleoHighPT) – mice with established scleroderma treated with high dose of UVA1;
- Group IV (BleoMediumPT) – mice with established scleroderma treated with medium dose of UVA1;
- Group V (HealthyHighPT) – healthy mice treated with high dose of UVA1;
- Group VI (HealthyMediumPT) – healthy mice treated with medium dose of UVA1.

Narrowband UVA1 PT was performed in a black metal box with separate cages for each mouse. Mice were irradiated 3 times per week for 5 weeks. The average cumulative doses over five weeks were 1200 J/cm^2 for high and 600 J/cm^2 for medium dose treatments. The non-irradiated groups (I and II) were left untreated. All mice were sacrificed at the end of experiment on week 8. Graphical illustration of study design is summarized in Fig. 1.

2.5. Histological analysis

Skin samples were stained according to the standard protocols: a) Hematoxylin and eosin (H&E), for the analysis of nuclear, cytoplasmic and extracellular matrix features; b) Toluidine blue (TB), pH 2.0 for the analysis of glycosaminoglycans in the mast cells and connective tissue matrix. The histological preparations were photographed using an Olympus BX51 microscope (Olympus Corporation, Inc.,Tokyo, Japan) with a Nikon DXM 1200 camera (Nikon Instruments, Inc., New York, USA). Dermal thickness was evaluated by measuring the distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction at four different points of H&E stained skin sections. The quantitative analysis was made using NIS ELEMENTS software v.BR 2.30 (Nikon instruments, Netherlands). Violet (red-purple) color cells, containing metachromatic cytoplasmic granules or degranulated cells in TB stained sections, were counted in four random grids under magnification of ×200 by using the light microscope. The mean number was calculated. The mast cell density was expressed as the average of mast cell count/high power field (HPF).

2.6. Immunohistochemical (IHC) analysis

Immunohistochemical analysis was performed manually by using DAKO Envision + System-Peroxidase Kit (Glostrup, Denmark) following the protocols of the manufacturer. The following primary antibodies were used: anti-Ki-67 (1:200, rabbit anti-human monoclonal [Sp6], *Spring Bioscience*, Pleasanton, CA, USA) and anti-p53 (1:200, rabbit polyclonal, *Abcam*, Cambridge, UK). The preparations were photographed using a Nikon "Eclipse TE2000" microscope (Nikon, Tokyo, Japan) with a Nikon DS-Fi2 high-definition color camera head (Nikon, Tokyo, Japan). For the semi-quantitative analysis of p53, the immunohistochemical staining was evaluated by using a four point score system: 0–no immunoreactive profiles, 1–only few immunoreactive profiles, 2–moderate numbers of immunoreactive profiles, 3–many immnoreactive profiles. For the semi-quantitative analysis of Ki-67, four high magnification fields of epidermis were randomly chosen and the percentage of Ki-67-positive cells/total cells population was scored. All slides were evaluated blindly by two independent examiners.

2.7. Statistics

SPSS Statistics software (version SPSS17) system package for Windows software was used for the statistical analysis. Continuous data were expressed as mean ± SD. Continuous variables (dermal thickness (μm), mast cells count and Ki-67 percentage) were compared by non-parametric Mann-Whitney U (Wilcoxon rank sum) test. Categorical data (p53 expression profile) were analyzed by Fisher's exact test. Statistical significance was expressed by a P-value < 0.05.

3. Results

3.1. Dermal thickness

The mean dermal thickness was 599.0 ± 55.7 μm after three weeks of bleomycin injections (group II), whereas it was only 179.3 ± 25.3 μm in the group of healthy mice ($P < 0.05$). After

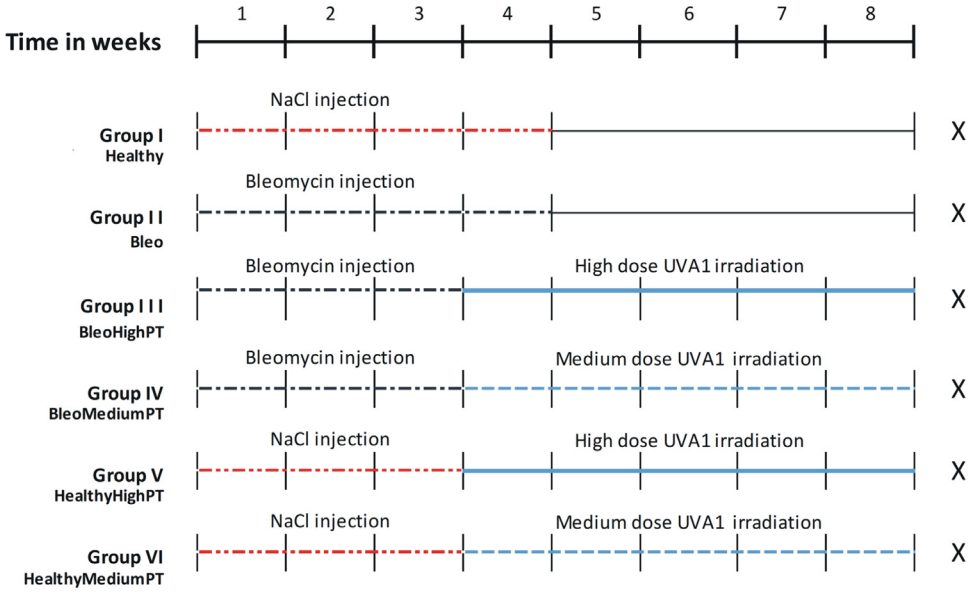

Fig. 1. Design of the study. NaCl – sodium chloride; bleo – bleomycin; PT – phototherapy; high dose of UVA1–80 J/cm^2 (3 times per week for 5 weeks); medium dose of UVA1–40 J/cm^2 (3 times per week for 5 weeks); X – sacrifice of mice.

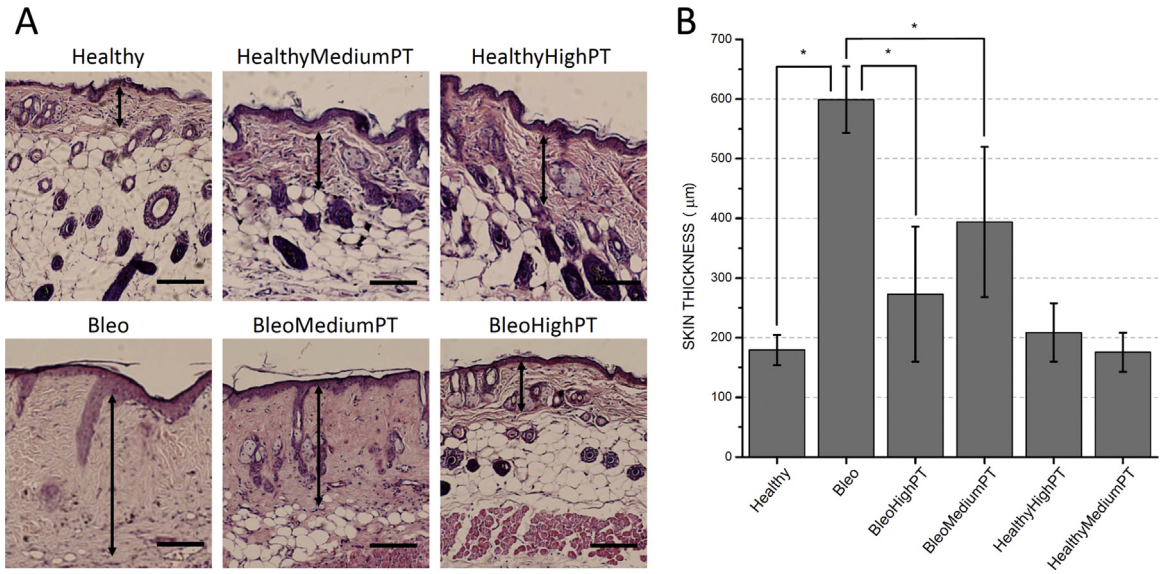

Fig. 2. The evaluation of dermal thickness. a) H&E-stained sections of the skin; magnification ×40; scale bar – 100 μm. The arrow indicates the length between epidermal-dermal and dermal-subcutaneous fat layers; b) Comparison of changes in the skin thickness. The graph shows the mean (±SD) results obtained from 7 mice of each group. *P < 0.05; bleo – mice with bleomycin-induced scleroderma group; healthy – healthy mice control group; highPT – high-dose phototherapy; mediumPT – medium-dose phototherapy; SD – standard deviation.

UVA1 PT (groups III and IV) the histopathological skin examination showed both the thinning of skin layers and the reduced number of irregular fibroblasts with less cellular infiltration between the collagen bundles. Also vascular infiltration with neutrophils and lymphocytes was less expressed in these groups compared to the vascular changes of the control group II that received bleomycin. Skin thickness of mice with scleroderma, treated with high and medium dose of UVA, was lower (group III – 272.9 ± 113.2 μm; group IV – 394.0 ± 125.9 μm; $P < 0.05$) in comparison to the thickness of non-irradiated animals (group II – 599 ± 55.7 μm). Thus, the skin thickness reduction by 54% and 34% after irradiation with the cumulative doses of 1200 J/cm^2 and 600 J/cm^2, respectively, was observed. There were no statistically significant differences in the mean dermal thickness between healthy mice (group I – 179.3 ± 25.3 μm) and UVA1-treated healthy mice (group V – 208.7 ± 49.0 μm; group VI – 175.5 ± 32.8 μm). The evaluation of the skin thickness is summarized in Fig. 2 and Table 1.

3.2. Mast cells

Histological examination after toluidine blue stain (pH 2.0) highlighted the mast cells, which were distinguished by a violet/red-purple color (metachromasia) with a background of blue. The dermal mast cells density (cells count/HPF) was significantly higher in mice with scleroderma as compared to that in the healthy mice group (23 ± 3.0 and 7 ± 1.9, respectively; $P < 0.05$). After irradiation with high and medium dose of UVA1 on mice with scleroderma, the density of mast cells was reduced to 11 ± 1.7 and 13 ± 2.2, respectively ($P < 0.05$). The infiltration of mast cells did not differ between non-irradiated and UVA1-treated healthy mice groups (group I – 7 ± 1.97; groups V and VI – 6 ± 1.0 and 7 ± 1.13, respectively). Results concerning mast cells infiltration are summarized in Fig. 3 and Table 1.

3.3. Ki-67 expression

Nuclear expression of Ki-67 in proliferating keratinocytes is stained in a brown color in the immunoperoxidase histochemical reaction. In the healthy skin (group I), the percentage of Ki-67-positive cells was 50.4 ± 2.6%. The number of these positive cells was reduced to 36.1 ± 3% in the skin of the control group II after bleomycin injections, reflecting reduced proliferation of keratinocytes ($P < 0.05$). The percentage of Ki-67 positive cells after high and medium dose of UVA1 treatment in bleomycin-induced scleroderma (groups III and IV) were 35.4 ± 3.2% and 37.2 ± 2.8%, respectively. The results did not differ from the control group II that

Table 1
The values of dermal thickness, mast cells infiltration, expressions of Ki-67 and p53 in all the experimental groups.

Groups	Mean dermal thickness (μm) ± SD	Mast cells count/HPF ± SD	Ki-67 expression (%) ± SD	p53 expression profile[1]
Healthy (N7)	179.3 ± 25.3	7 ± 1.9	50.4 ± 2.6	(0) and (1)
Bleo (N7)	599.0 ± 55.7 $^{+}$	23 ± 3.0 $^{+}$	36.1 ± 3.0 $^{+}$	(2) and (3) $^{+}$
BleoHighPT (N7)	272.9 ± 113.2 *	11 ± 1.8 *	35.4 ± 3.2 *	(2) and (3) $^{+}$
BleoMediumPT (N7)	394.0 ± 125.9 *	13 ± 2.2 *	37.2 ± 2.8 *	(2) and (3) $^{+}$
HealthyHighPT (N7)	208.7 ± 49.0	6 ± 1.0	50.1 ± 1.9	(0) and (1)
HealthyMediumPT (N7)	175.5 ± 32.8	7 ± 1.1	49.4 ± 1.4	(0) and (1)

[1]Score in staining profile of p53 expression: (0) – no immunoreactive profiles, (1) – only few immunoreactive profiles, (2) – moderate numbers of immunoreactive profiles, (3) – many immnoreactive profiles. Bleo – mice with bleomycin-induced scleroderma; healthy – healthy mice control group; highPT – high-dose (1200 J/cm^2) phototherapy; mediumPT – medium-dose (600 J/cm^2) phototherapy; SD – standard deviation; HPF – high power field.

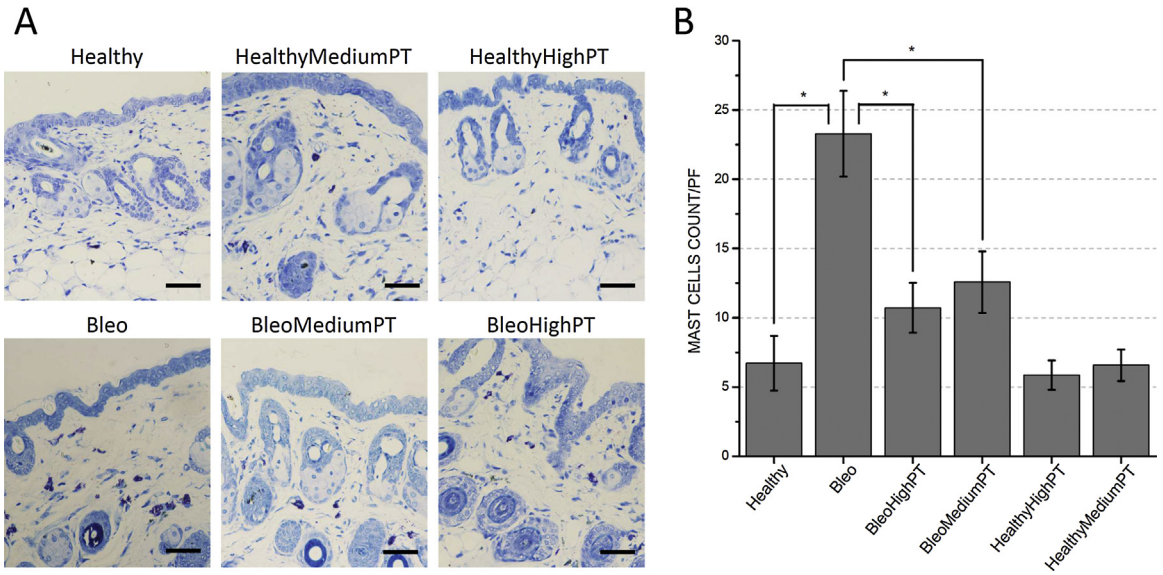

Fig. 3. The infiltration of mast cells. a) Histopathological views representing infiltration with dermal mast cells; toluidine blue staining; magnification ×200; scale bar – 50 μm; b) Count of mast cells. The number of cells counted under magnification of ×200 high power field in 4 random grids. The graph shows the mean (±SD) results obtained from 7 mice of each group. *P < 0.05; bleo – mice with bleomycin-induced scleroderma group; healthy – healthy mice control group; highPT – high-dose phototherapy; mediumPT – medium-dose phototherapy; SD – standard deviation.

received bleomycin without phototherapy procedures. The effect of high and medium dose of UVA1 on Ki-67 expression in the skin of healthy mice (groups V and VI; 50.1 ± 1.95% and 49.4 ± 1.3%, respectively) did not differ compared to the expression of protein in the skin of healthy mice without PT procedures (group I; 50.4 ± 2.6%). Results are summarized in Fig. 4 and Table 1.

3.4. p53 expression

TP53 or p53 is a tumor suppressor gene and its mutations are involved in the development of cancer. The protein expression is nuclear and cytoplasmic. Many immunoreactive profiles of p53 staining were observed in the skin of bleomycin-induced scleroderma group II. Thus, the expression of p53 was higher in the skin of the control group (group II) compared to the skin of healthy mice (group I) (P < 0.05). Moderate numbers of p53 staining were also present after high and medium dose of UVA1 PT in the groups of mice with bleomycin-induced scleroderma (groups III and IV). However, these observations did not significantly differ from the control group II. Only few or no immunoreactive profiles of p53 expression in epidermis and dermal cells were observed in the skin of healthy mice (group I).

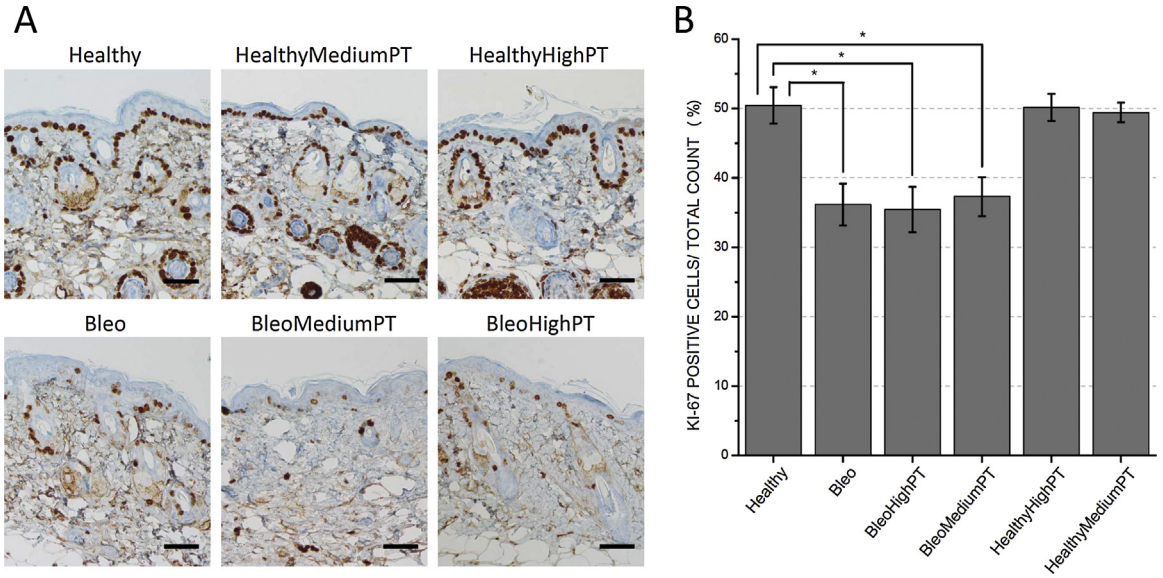

Fig. 4. Ki-67 immunohistochemistry. a) Nuclear expression of Ki-67 in proliferating keratinocytes; IHC staining; ×200 magnification; scale bar – 50 μm; b) Percentage of Ki-67-positive cells to total cell population/microscope field; ×200 magnification. The graph shows the mean (±SD) results obtained from 7 mice of each group. *P < 0.05; bleo – mice with bleomycin-induced scleroderma group; healthy – healthy mice control group; highPT – high-dose phototherapy; mediumPT – medium-dose phototherapy; SD – standard deviation.

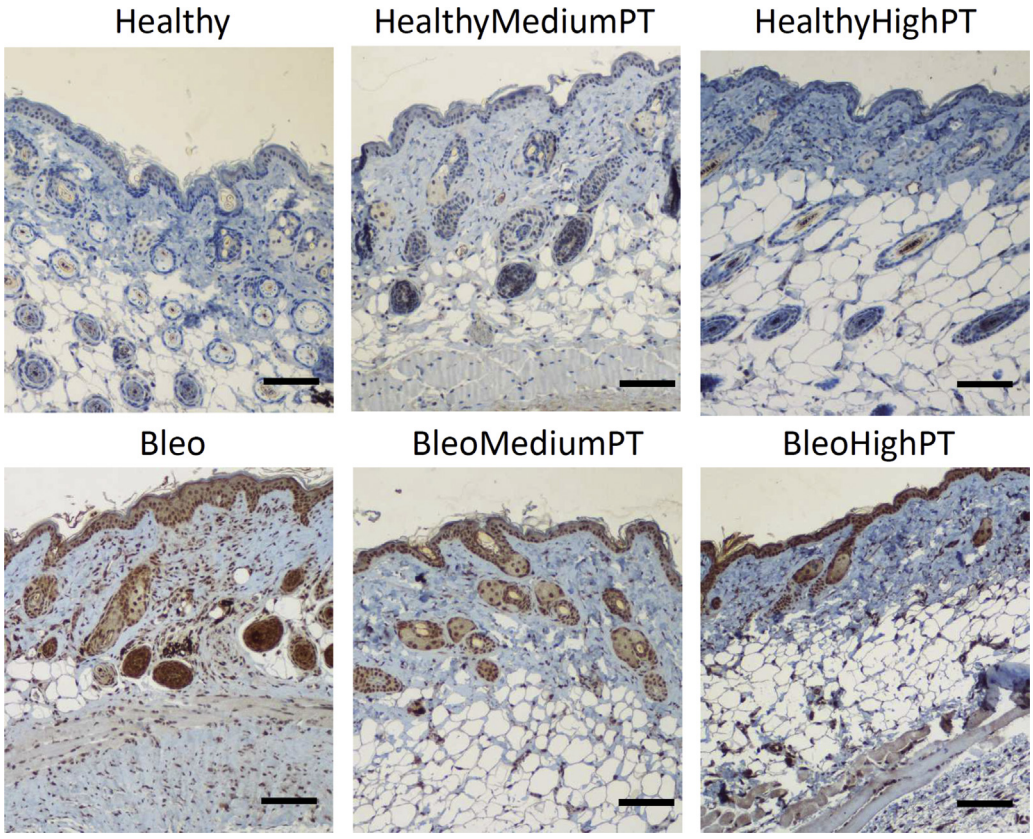

Fig. 5. p53 immunohistochemistry. p53 expression in epidermis and dermal cells in all the groups of experiment; IHC staining; ×100 magnification; scale bar – 100 μm; bleo – mice with bleomycin-induced scleroderma group; healthy – healthy mice control group; highPT – high-dose phototherapy; mediumPT – medium-dose phototherapy; SD – standard deviation.

Protein expression profile in skin samples also did not differ between healthy (group I) and UVA1–treated healthy mice (groups V and VI) (Fig. 5 and Table 1).

4. Discussion

The main aim of our study was to evaluate the impact of 365 nm UVA1 on the dermal fibrosis in the mouse model of scleroderma. We assessed the narrowband UVA1 effect on the dermal thickness, masts cells density, and the expression of carcinogenic proteins both in scleroderma and normal mice skin.

Fibroblasts are one of the targets in the dermis for the treatment of sclerosing skin conditions. Two decades ago *in vivo* and *in vitro* conducted studies proved that UVA1 could induce the activity of matrix metalloproteinase and decrease the level of collagen I and III in the various skin conditions [28,29]. Our histological analysis revealed that after irradiation with the dose of 80 J/cm^2 for 5 weeks (cumulative dose – 1200 J/cm^2), the dermal thickness was reduced by 54% compared to the skin of the control mice group (II). The cumulative dose of 600 J/cm^2 UVA1 also diminished the dermal thickness by 34% in comparison to that of the control group. Ju and coauthors [30] evaluated the effects of different doses of broadband UVA1 (340–400 nm) on the skin fibrosis in the BALB/c mouse model of scleroderma. Mice were irradiated with the low dose (20 J/cm^2), medium dose (60 J/cm^2) or high dose (100 J/cm^2) 3 times weekly for 10 weeks resulting in the cumulative doses of 600 J/cm^2, 1800 J/cm^2 or 3000 J/cm^2 of UVA1. The dermal thickness was reduced by 4% in the low-dose and by 13% in the medium-dose UVA1 groups after the phototherapy as compared to the skin thickness of the control group without irradiation. The treatment with high dose of UVA1 reduced the skin thickness by 36%. In our experiment, even 5 times lower cumulative dose of UVA1 using narrowband UVA1 LED device had almost the same effect on the decrease of dermal thickness as compared with conventional broadband UVA1 lamp (skin thickness reduction by 34% and 36% after irradiation with cumulative doses of 600 J/cm^2 and 3000 J/cm^2, respectively, was observed). Furthermore, the phototherapy course in our study did not induce any local skin reactions (ulceration, itching signs, etc.). Antifibrotic effect of narrowband UVA1 phototherapy might be also related to the enzyme HO-1, but further experiments are needed to confirm this hypothesis.

Previous studies revealed that UVA1 decreased the number of mast cells in patients with atopic dermatitis [31] and cutaneous mastocytosis [32]. It has been shown that high dose of UVA1 inhibited the release of histamine *in vitro* in human mast cells [33]. Abundant dermal mast cells as well as their degranulation in bleomycin-induced mice scleroderma group (II) indicate the important role of mast celss in the pathogenesis of systemic sclerosis (promoting both inflammation and fibrosis) as was also described in the previous studies [34]. We observed the decrease of dermal mast cells infiltration in mice with scleroderma groups after UVA1-treatment. It proves the direct effect of narrowband UVA1 on the mast cells which are one of the pathogenetic factors in systemic sclerosis, especially in the early phase of the disease. Histological analysis of skin samples in UVA1-exposed healthy mice (groups V and VI) did not show any changes in the count of mast cells. This phenomenon can be explained as the resistance of the normal mast cells to UVA1-induced apoptosis. The apoptosis of inflammatory infiltrating cells has been recognized as one of the main mechanisms responsible for UVA1 treatment [35]. Mikita and

coauthors [36] investigated the effects of ultraviolet A1 (UVA1) irradiation on spontaneous lupus erythematosus- (LE-) like skin lesions of MRL/lpr mice. Authors found that UVA1 irradiation significantly inhibited the development of LE-like skin lesions. UVA1 exerted its effects, at least in part, through the induction of the apoptosis of pathogenic mast cells. In consistent with their results, apoptosis of proliferating mast cells was reported to be induced by UV *in vitro*, while resting skin mast cells were resistant to UV light-induced apoptosis [37].

Ki-67 antigen is a high molecular weight protein and a marker of proliferation. Increased epidermal Ki-67 expression is a sign of hyperproliferation and altered cells differentiation which might play a role in the cutaneous carcinogenesis. The aim of Ki-67 immunohistochemical staining in our study was to evaluate narrowband UVA1 effect on the proliferation of epithelial cells. The number of Ki-67-positive keratinocytes was significantly lower in the epidermis of mice with scleroderma as compared to the expression in the skin of healthy mice. It reflects the anti-proliferative mechanism of bleomycin action [38]. After high and medium dose of UVA1 irradiation on mice with scleroderma (groups III and IV), the Ki-67 proliferation index did not differ from the control group (group II). As well as UVA1-treated healthy mice epidermis (groups V and VI) did not show higher expression of keratinocytes proliferation compared with the group of healthy mice (group I). Gambichler et al. [39] also showed that 3 minimal erythema doses of broadband UVA1 did not cause the increase of Ki-67 expression in the skin of healthy volunteers. On the contrary, Edstrom et al. [40] noted that repetitive low doses of broadband UVA1 (340–400 nm, cumulative dose – 240 J/cm^2) induced a significant increase of Ki-67 positive cells in the skin of healthy volunteers. Meanwhile our results confirmed that even therapeutic doses of narrowband UVA1 (1200 J/cm^2 and 600 J/cm^2) did not induce hyperproliferation of epidermal cells in the skin of healthy mice and mice with scleroderma.

Protein p53 is a tumor suppressor gene that takes a role in the control of cell cycle, initiation of apoptosis and genesis of the skin cancer. In normal cells, p53 protein level is low, whereas it is found mutated in 50–90% of human malignant tumors including the skin epithelial cancers [41]. Expression of p53 protein has also been detected in the normal sun exposed epidermis and normal epidermis near basal cell carcinoma [42]. Hence, ultraviolet light exposure is one of the main cause of p53 mutation. In our experiment we evaluated the narrowband UVA1 effect on this tumor suppressor gene. Immunohistochemical staining of p53 revealed that only few or no immunoreactive profiles of p53 expression were observed in the skin of healthy (group I) and UVA1-treated groups of healthy mice (groups V and VI). Meanwhile Burren et al. [43] performed a study on the healthy volunteers, irradiating the skin with 0.5, 1 or 2 minimal erythema doses of broadband UVA1 (360–450 nm). They noted a dose-dependent increase of p53 in keratinocytes throughout all layers of epidermis [43]. Thereafter, Seite et al. [44] proved that even 8 exposures of 25 J/cm^2 broadband UVA1 (340–400 nm, cumulative dose – 200 J/cm^2) induced a significant increase in p53 expression in human epidermis cells. Thus, the difference in radiation spectra could influence the expression of p53. Shorter wavelengths exhibit higher energy and might increase the expression of apoptotic proteins, especially in the upper layers of the skin. Actually, there were numerous signs of protein p53 expression in the skin of mice with scleroderma in our experiment. Yamamoto et al. [45] confirmed that apoptotic processes were involved in the pathophysiology of bleomycin-induced model of scleroderma. So, overexpression of p53 might play a role in the skin inflammation, remodeling and fibrosis [46]. In the present study we found the same expression profile of p53 staining in the UVA1-treated and non-treated groups of mice with scleroderma. Overall studies of chronic UVA1 effects in scleroderma patients are still limited. Three retrospective studies involving 423 patients, who received between 4 and 116 broadband UVA1 treatments in total, reported no chronic effects, such as photocarcinogenesis [47–49]. Case reports of skin cancer in patients treated with UVA1 are usually confounded with the use of other therapies known to increase the risk of cutaneous malignancies [10]. Whereas the carcinogenic properties of short-wavelength UV light (UVB) are well established [12]. UVB is 1000–10000 times more carcinogenic than UVA, even though less penetrating [50]. In a murine study, the tumor overgrowth was enhanced by broadband UVB but not by narrowband UVB or UVA1 [51]. In a mutant mice model, UVB, but not UVA, was observed to induce malignant melanoma [52]. Whereas the results of our study showed that the cumulative doses of 1200 J/cm^2 and 600 J/cm^2 of narrowband UVA1 did not increase the carcinogenic risk in the skin of healthy mice and mice with scleroderma.

Our experiment has its limitations. We evaluated the impact of narrowband UVA1 on the bleomycin-induced scleroderma model. The main features of the fibrosis model induced by bleomycin include inflammation, autoimmunity and fibrosis. Typical generalized vasculopathy that precedes fibrosis in human SSc is usually absent in the bleomycin-induced model. Spontaneous genetic model, such as tight-skin mice (Tsk-1) model is less dependent from inflammation and mimics the non-inflammatory stage of SSc disease. Dooley with colleagues [53] found that the expression of endothelial nitric oxide was significantly reduced in Tsk-1 skin tissue. Thus, Tsk-1 mice should be considered as a choice of strain for testing treatments that target vascular endothelial cell function in patients with SSc. To prove the efficacy of antifibrotic agents in the different stages of SSc, the antifibrotic effect should be confirmed also in non-inflammatory models of SSc. Therefore, we consider the future research by the use of Tsk-1 model for the evaluation of 365 nm UVA1 on dermal fibrosis.

5. Conclusions

In the present study we demonstrated that the cumulative doses of 1200 J/cm^2 and 600 J/cm^2 of narrowband UVA1 effectively reduced the dermal thickness, and the impact was dose-dependent. High and medium dose regimens achieved similar responses in reduction of the dermal mast cells count in the skin of mice with scleroderma. On the contrary, irradiation of healthy mice with UVA1 did not affect the skin thickness neither the density of mast cells. We found that high and medium doses of narrowband UVA1 phototherapy did not upregulate p53 nor Ki-67 proteins in the healthy mice and mice with scleroderma skin. The results of this study indicate that 365 nm UVA1 phototherapy is safe and effective for the dermal fibrosis treatment.

Conflict of interests

The authors declare no conflict of interests.

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References

[1] Kahaleh MB, Sherer GK, LeRoy EC. Endothelial injury in scleroderma. J Exp Med 1979;149:1326–35.
[2] Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Invest 2007;117:557–67.
[3] Abraham DJ, Eckes B, Rajkumar V, Krieg T. New developments in fibroblast and myofibroblast biology: implications for fibrosis and scleroderma. Curr Rheumatol Rep 2007;9:136–43.
[4] Altorok N, Wang Y, Kahaleh B. Endothelial dysfunction in systemic sclerosis. Curr Opin Rheumatol 2014;26:615–20.
[5] Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol 2008;214:199–210.
[6] Ho YY, Lagares D, Tager AM, Kapoor M. Fibrosis – a lethal component of systemic sclerosis. Nat Rev Rheumatol 2014;10:390–402.
[7] Connolly KL, Griffith JL, McEvoy M, Lim HW. Ultraviolet A1 phototherapy beyond morphea: experience in 83 patients. Photodermatol Photoimmunol Photomed 2015;31:289–95.
[8] York NR, Jacobe HT. UVA1 phototherapy: a review of mechanism and therapeutic application. Int J Dermatol 2010;49:623–30.
[9] Hassani J, Feldman SR. Phototherapy in scleroderma. Dermatol Ther (Heidelb) 2016;6:519–53.
[10] Zandi S, Kalia S, Lui H. UVA1 phototherapy: a concise and practical review. Skin Therapy Lett 2012;17:1–4.
[11] Reich A, Medrek K. Effects of narrow band UVB (311 nm) irradiation on epidermal cells. Int J Mol Sci 2013;14:8456–66.
[12] Moura Valejo Coelho M, Matos TR, Apetato M. The dark side of the light: mechanisms of photocarcinogenesis. Clin Dermatol 2016;34:563–70.
[13] Yarosh DB, Kibitel J, Ullrich SE, Kim TH, Ananthaswamy HN, Krien P, et al. Direct comparison of DNA damage, isomerization of urocanic acid and edema in the mouse produced by three commonly used artificial UV light sources. Photochem Photobiol 1999;69:571–4.
[14] Goukassian DA, Eller MS, Yaar M, Gilchrest BA. Thymidine dinucleotide mimics the effect of solar simulated irradiation on p53 and p53-regulated proteins. J Invest Dermatol 1999;112:25–31.
[15] Breuckmann F, Gambichler T, Altmeyer P, Kreuter A. UVA/UVA1 phototherapy and PUVA photochemotherapy in connective tissue diseases and related disorders: a research based review. BMC Dermatol 2004;4:11.
[16] Morita A, Werfel T, Stege H, Ahrens C, Karmann K, Grewe M, et al. Evidence that singlet oxygen-induced human T helper cell apoptosis is the basic mechanism of ultraviolet-A radiation phototherapy. J Exp Med 1997;186:1763–8.
[17] Xiang Y, Liu G, Yang L, Zhong JL. UVA-induced protection of skin through the induction of heme oxygenase-1. Biosci Trends 2011;5:239–44.
[18] Nisar MF, Parsons KS, Bian CX, Zhong JL. UVA irradiation induced heme oxygenase-1: a novel phototherapy for morphea. Photochem Photobiol 2015;91:210–20.
[19] Ju M, Chen K, Chang B, Gu H. UVA1 irradiation inhibits fibroblast proliferation and alleviates pathological changes of scleroderma in a mouse model. J Biomed Res 2012;26:135–42.
[20] Inada SA, Kamiyama S, Akasaki I, Torii K, Furuhashi T, Amano H, et al. Development of an ultraviolet A1 light emitting diode-based device for phototherapy. Open Dermatol J 2012;6:13–24.
[21] Inada SA, Amano H, Akasaki I, Morita A, Kobayashi K. Effect of UV irradiation on the apoptosis and necrosis of Jurkat cells using UV LEDs. Proc SPIE 2009;7231:1–6.
[22] Matthews YJ, Halliday GM, Phan TA, Damian DL. Wavelength dependency for UVA-induced suppression of recall immunity in humans. J Dermatol Sci 2010;59:192–7.
[23] Nistico SP, Saraceno R, Chiricozzi A, Giunta A, Di Stefani A, Zerbinati N. UVA-1 laser in the treatment of palmoplantar pustular psoriasis. Photomed Laser Surg 2013;31:434–8.
[24] Yamamoto T, Kuroda M, Nishioka K. Animal model of sclerotic skin: III: Histopathological comparison of bleomycin-induced scleroderma in various mice strains. Arch Dermatol Res 2000;292:535–41.
[25] Akhmetshina A, Venalis P, Dees C, Busch N, Zwerina J, Schett G, et al. Treatment with imatinib prevents fibrosis in different preclinical models of systemic sclerosis and induces regression of established fibrosis. Arthritis Rheum 2009;60:219–24.
[26] Avouac J. Mouse model of experimental dermal fibrosis: the bleomycin-induced dermal fibrosis. Methods Mol Biol 2014;1142:91–8.
[27] Yamamoto T, Takagawa S, Katayama I, Yamazaki K, Hamazaki Y, Shinkai H, et al. Animal model of sclerotic skin. I: Local injections of bleomycin induce sclerotic skin mimicking scleroderma. J Invest Dermatol 1999;112:456–62.
[28] Herrmann G, Wlaschek M, Lange TS, Prenzel K, Goerz G, Scharffetter-Kochanek K. UVA irradiation stimulates the synthesis of various matrix-metalloproteinases (MMPs) in cultured human fibroblasts. Exp Dermatol 1993;2:92–7.
[29] Mempel M, Schmidt T, Boeck K, Brockow K, Stachowitz S, Fesq H, et al. Changes in collagen I and collagen III metabolism in patients with generalized atopic eczema undergoing medium-dose ultraviolet A1 phototherapy. Br J Dermatol 2000;142:473–80.
[30] Ju M, Chen K, Chang B, Gu H. UVA1 irradiation inhibits fibroblast proliferation and alleviates pathological changes of scleroderma in a mouse model. J Biomed Res 2012;26:135–42.
[31] Grabbe J, Welker P, Humke S, Grewe M, Schopf E, Henz BM, et al. High-dose ultraviolet A1 (UVA1), but not UVA/UVB therapy, decreases IgE-binding cells in lesional skin of patients with atopic eczema. J Invest Dermatol 1996;107:419–22.
[32] Gobello T, Mazzanti C, Sordi D, Annessi G, Abeni D, Chinni LM, et al. Medium- versus high-dose ultraviolet A1 therapy for urticaria pigmentosa: a pilot study. J Am Acad Dermatol 2003;49:679–84.
[33] Kronauer C, Eberlein-Konig B, Ring J, Behrendt H. Influence of UVB, UVA and UVA1 irradiation on histamine release from human basophils and mast cells in vitro in the presence and absence of antioxidants. Photochem Photobiol 2003;77:531–4.
[34] Yoshizaki A, Iwata Y, Komura K, Ogawa F, Hara T, Muroi E, et al. CD19 regulates skin and lung fibrosis via Toll-like receptor signaling in a model of bleomycin-induced scleroderma. Am J Pathol 2008;172:1650–63.
[35] Godar DE. UVA1 radiation triggers two different final apoptotic pathways. J Invest Dermatol 1999;112:3–12.
[36] Mikita N, Kanazawa N, Yoshimasu T, Ikeda T, Li HJ, Yamamoto Y, et al. The protective effects of ultraviolet A1 irradiation on spontaneous lupus erythematosus-like skin lesions in MRL/lpr mice. Clin Dev Immunol 2009;2009:673952.
[37] Guhl S, Hartmann K, Tapkenhinrichs S, Smorodchenko A, Grutzkau A, Henz BM, et al. Ultraviolet irradiation induces apoptosis in human immature, but not in skin mast cells. J Invest Dermatol 2003;121:837–44.
[38] Maeda T, Yamamoto T, Imamura T, Tsuboi R. Impaired wound healing in bleomycin-induced murine scleroderma: a new model of wound retardation. Arch Dermatol Res 2016;308:87–94.
[39] Gambichler T, Rotterdam S, Tigges C, Altmeyer P, Bechara FG. Impact of ultraviolet radiation on the expression of marker proteins of gap and adhesion junctions in human epidermis. Photodermatol Photoimmunol Photomed 2008;24:318–21.
[40] Edstrom DW, Porwit A, Ros AM. Effects on human skin of repetitive ultraviolet-A1 (UVA1) irradiation and visible light. Photodermatol Photoimmunol Photomed 2001;17:66–70.
[41] Khodaeiani E, Fakhrjou A, Amirnia M, Babaei-Nezhad S, Taghvamanesh F, Razzagh-Karimi E, et al. Immunohistochemical evaluation of p53 and Ki67 expression in skin epithelial tumors. Indian J Dermatol 2013;58:181–7.
[42] Ansarin H, Daliri M, Soltani-Arabshahi R. Expression of p53 in aggressive and non-aggressive histologic variants of basal cell carcinoma. Eur J Dermatol 2006;16:543–7.
[43] Burren R, Scaletta C, Frenk E, Panizzon RG, Applegate LA. Sunlight and carcinogenesis: expression of p53 and pyrimidine dimers in human skin following UVA I, UVA I + II and solar simulating radiations. Int J Cancer 1998;76:201–6.
[44] Seite S, Moyal D, Verdier MP, Hourseau C, Fourtanier A. Accumulated p53 protein and UVA protection level of sunscreens. Photodermatol Photoimmunol Photomed 2000;16:3–9.
[45] Yamamoto T, Nishioka K. Increased expression of p53 and p21 (Waf1/Cip1) in the lesional skin of bleomycin-induced scleroderma. Arch Dermatol Res 2005;296:509–13.
[46] Gudkov AV, Gurova KV, Komarova EA. Inflammation and p53: a tale of two stresses. Genes Cancer 2011;2:503–16.
[47] Rombold S, Lobisch K, Katzer K, Grazziotin TC, Ring J, Eberlein B. Efficacy of UVA1 phototherapy in 230 patients with various skin diseases. Photodermatol Photoimmunol Photomed 2008;24:19–23.
[48] Tuchinda C, Kerr HA, Taylor CR, Jacobe H, Bergamo BM, Elmets C, et al. UVA1 phototherapy for cutaneous diseases: an experience of 92 cases in the United States. Photodermatol Photoimmunol Photomed 2006;22:247–53.
[49] Jacobe HT, Cayce R, Nguyen J. UVA1 phototherapy is effective in darker skin: a review of 101 patients of Fitzpatrick skin types I-V. Br J Dermatol 2008;159:691–6.
[50] Lisby S, Gniadecki R, Wulf HC. UV-induced DNA damage in human keratinocytes: quantitation and correlation with long-term survival. Exp Dermatol 2005;14:349–55.
[51] Macve JC, Norval M. The effects of UV waveband and cis-urocanic acid on tumour outgrowth in mice. Photochem Photobiol Sci 2002;1:1006–11.
[52] De Fabo EC, Noonan FP, Fears T, Merlino G. Ultraviolet B but not ultraviolet A radiation initiates melanoma. Cancer Res 2004;64:6372–6.
[53] Dooley A, Low SY, Holmes A, Kidane AG, Abraham DJ, Black CM, et al. Nitric oxide synthase expression and activity in the tight-skin mouse model of fibrosis. Rheumatology (Oxford) 2008;47:272–80.