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ABSTRACT

Elastic fibers, a major component of the extracellular matrix of the skin, are often exposed to ultraviolet (UV) radiation throughout mammalian life. We report on an *in vitro* study of the alterations in bovine nuchal ligament elastic fibers resulting from continuous UV-A exposure by the use of transmission electron microscopy (TEM), histology, mass spectrometry, and solid state ¹³C NMR methodologies. TEM images reveal distinct cracks in elastic fibers as a result of UV-A irradiation and histological measurements show a disruption in the regular array of elastic fibers present in unirradiated samples; elastic fibers appear shorter, highly fragmented, and thinner after UV-A treatment. Magic angle spinning ¹³C NMR was applied to investigate possible secondary structural changes or dynamics in the irradiated samples; our spectra reveal no differences between UV-A irradiated and non-irradiated samples. Lastly, MALDI mass spectrometry indicates that the concentration of desmosine, which forms cross-links in elastin, is observed to decrease by 11 % following 9 days of continuous UV-A irradiation, in comparison to unirradiated samples. These alterations presumably play a significant role in the loss of elasticity observed in UV exposed skin.

Introduction

Elastin, the principal protein component of the elastic fiber, is an extracellular matrix protein of human tissues that require elasticity such as the arteries, lungs, and skin. Elastin plays an important role in providing these tissues, and others, the ability to stretch while maintaining healthy cells [1–5]. In the skin, the majority of elastin is located in the reticular dermis. Mature elastin is a system of interconnected fibers that are encompassed by other proteins such as elaunin fibers and oxytalan fibers [6]. Tropoelastin, the monomer of elastin, is a large 72 kDa protein which is cross-linked by desmosine or isodesmosine to form elastin. The vast majority of elastin is produced during fetal development, and the first few years of life, after which the expression of tropoelastin sharply decreases [7]. Consequently, connective tissues rely on elastin that is formed early in life [8]. When skin is damaged through UV exposure and/or oxidation it may lose elasticity, and low levels of tropoelastin production may result in irreparable damage. Recent research into repairing damaged elastic fibers has focused on integrating tropoelastin into the skin; one challenge in this approach

relates to transferring the protein across the epidermis. Another approach has been to increase tropoelastin expression through the use of small molecules such as all-*trans* retinoic acid [9,10].

The effects of photoaging are closely connected to elastin damage through UV exposure. It has been found that the major histopathological alteration to photoaged skin is an accumulation of poorly organized elastin, termed solarelastosis. UV irradiation has been shown to induce the expression of matrix metalloproteinases (MMPs), which cause degradation of extracellular matrix such as elastin and collagen. Irradiation of skin was found to increase the expression of human macrophage elastase (MMP-12) mRNA 11.9 fold within 16 h of UV exposure [11]. Other studies have found that elastin production is increased after exposure to UV radiation, which may result in large amounts of abnormal elastic material in the skin. In one study, irradiation of mice with ultraviolet B (wavelengths = 290–320 nm) yielded an 8.5 fold increase in promoter activity, while ultraviolet A (wavelengths = 320–400 nm) only resulted in an 1.8 fold increase in promoter activity. These results indicate that ultraviolet B has a significant impact on the accumulation of elastosis, while ultraviolet

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A contributes as well, but to a lesser extent [12]. In another study, by tagging elastic material with antibodies and conducting immunofluorescence microscopy, it was found that solar elastosis is primarily derived from elastic fibers and not from preexisting or newly synthesized collagen [13].

While the relationship between UV radiation and photoaging of skin has been established, the exact change that elastin undergoes when exposed to UV radiation is still unclear. One common belief is that elastic fibers are denatured or cleaved by UV radiation, and that most of the elastotic material is produced after exposure to UV radiation or its disorganized structure gives aged skin its characteristic wrinkled appearance. In previously reported studies, electron microscopy, histology, TEM (transmission electron microscopy), and SEM (scanning electron microscopy) have been applied to probe macroscopic changes to the elastic fiber in aged and photodamaged skin [14–17]. For all biological damage associated to sun exposure, UV-B contributes 80% whereas UV-A contributes only 20% even though approximately 95% of terrestrial UV-radiation is UV-A [18]; more work is needed to better understand the microscopic changes that UV-A irradiation causes to the structure of elastin. We conducted this *in vitro* study to uncover changes on elastic fibers that undergo when exposed to high intensity UV-A irradiation. Elastic fibers were then examined histologically and by TEM to observe macroscopic changes that resulted from irradiation. ^{13}C solid state NMR spectroscopy was performed to measure possible structural alterations of elastin. Mass spectrometry was also implemented to quantify changes in the relative amount of desmosine cross-links. These combined methods provide new information relating to the detrimental effects of UV-A irradiation to the structure of the elastic fiber and of elastin.

Materials and methods

Sample preparation

Bovine nuchal ligament elastic fibers purchased from Elastin Products Company, LLC (Owensville, MO) were used for this study. These samples were purified by Elastin Products Company using a known protocol [19] and were free of fat, collagen, smooth muscle cells, and other connective tissue. In a prior study, we showed that the protocol used for isolating elastin did not alter the structure of the protein, or the concentration of cross-links [20]. Elastic fibers were completely immersed in distilled water while being irradiated with a 3U40W UV-A lamp (Cnlight Co, China) with a center wavelength of 365 nm which was placed 10 cm above the sample. The wavelength distribution of the UV-A lamp is shown in Figure S1 (supplementary). During irradiation, the system was covered with a shield to ensure that the sample was isolated from other light and were submerged in water during irradiation. The irradiation intensity was 12 mW/cm^2 and samples were continuously irradiated for 9 days. The intensity of the UV-A lamp is therefore approximately 3 times higher than that of the sun, when directly overhead (located at the zenith).

Histology and microscopy

Unirradiated elastic fibers and fibers following 9 days of UV-A irradiation were used for the histological study. For histology, a small amount of sample was placed overnight in 100 ml phosphate buffered saline. The samples were then placed in Lieca Cryo-Gel (SPI supplies Product Ref-02694-AB), sectioned on a Leica CM1850 cryostat at $10\ \mu\text{m}$, and stained using the Sigma-Aldrich elastic stain kit (REF HT25A-1KT) following a modified version of a previously reported protocol [21]. The slides were gently rinsed with 95% ethanol, and then placed in xylene for a few seconds. Cover slips were then mounted using an Eukitt quick-hardening mounting medium (Sigma-Aldrich REF 03989) and left to dry overnight. All sections were photographed using a National Optical DC4-156-S digital microscope at a magnification of $10\times$.

Transmission Electron Microscopy (TEM)

Bovine nuchal ligament elastic fibers were immersed in 0.1 M phosphate buffered solution (pH-7.4) for 1 h. All the samples were stained with osmium tetroxide and embedded in epoxy resin. Samples were sliced along the plane that was perpendicular to the fiber axis, with a thickness of 60 nm and examined in a JEM-2000EX transmission electron microscope. The accelerating voltage used was 120 kV.

^{13}C NMR experimental parameters

Prior to the NMR experiments, unirradiated and 9 days UV-A irradiated samples were immersed in distilled water and solid-state NMR experiments were carried out on hydrated samples. ^{13}C NMR experiments were performed using a Bruker Avance (Billerica, MA) spectrometer at a magnetic field strength of 21.10 T. All the experiments were carried out using a 4 mm center packing rotor with an insert to keep the samples hydrated, as well as to center the samples with respect to the RF coil. ^{13}C MAS (magic angle spinning) spectra were measured using a DEPTH sequence (to suppress background carbon signals arising from rotor inserts and the probe head) [22,23] with 80 kHz TPPM decoupling [24] at $(300 \pm 1)\text{ K}$ and direct polarization. The spinning speed was set to 14.5 kHz for all the samples and spectra were acquired by accumulating 18,800 scans. The $^{13}\text{C}\ \pi/2$ pulse was 54 μs and the recycle delay was 6 s. Analysis of data was performed using MATLAB and matNMR with a Gaussian multiplication broadening factor of 100 Hz. ^{13}C NMR spectra were referenced to adamantane (TMS = 0 ppm).

Sample hydrolysis and quantification with labeled desmosine

Elastic fiber samples for this study were lyophilized for 24 h prior to hydrolysis. Approximately, 2.1–2.2 mg of each sample was placed into a solution containing 300 μl of 6 M HCl and 1 μl of 0.5% w/w phenol solution. The sample and solution mixture was placed into a vacuum hydrolysis tube, flushed with nitrogen gas, and then evacuated. The samples were kept at $110\ ^\circ\text{C}$ for 96 h, afterward the solvent was frozen in liquid nitrogen and lyophilized for 8–10 h. After lyophilization, each sample was suspended in a 50 μl solution of 94.5% 0.14 M sodium acetate, 0.5% triethylamine, and 5% acetonitrile (v/v/v) at a pH of 7.5. Resuspended samples were diluted to 100 fold. Labeled d_4 -desmosine standard (Toronto Research Chemicals, Toronto, Canada) at final concentration of 10 pmol/ μl was mixed with the diluted samples in three different ratios 1:1, 3:1, and 1:3. The relative amount of desmosine in each sample was quantified with respect to mass spectrometric peak intensity of standard desmosine in MS^2 mode. For statistical analysis of the data, a *t*-test was used assuming our data followed a normal distribution, and using the standard deviations in each samples studied. Null hypothesis probability was measured indicating the level of significance of our data.

MALDI-MS quantitative analysis

MALDI- MS^2 experiments were performed using a Thermo LTQ XL ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with a vacuum MALDI source. α -Cyano-4-hydroxycinnamic acid (CHCA) purchased from Sigma-Aldrich (St. Louis, Missouri, USA) was recrystallized and used to prepare the matrix solution. Solid CHCA was added in a solution of 0.1% trifluoroacetic acid, 70% acetonitrile, and 29.9% HPLC grade water until saturation. The solution was centrifuged and supernatant liquid was used as the matrix solution. 1 μl of sample mixture containing different ratio of standard and sample were placed in 9 μl of CHCA matrix solution. The final solution was vortexed, and 1 μl of mixture was spotted on the MALDI plate. Spotted points were air dried prior to inserting the plate into the MALDI mass spectrometer for analysis.

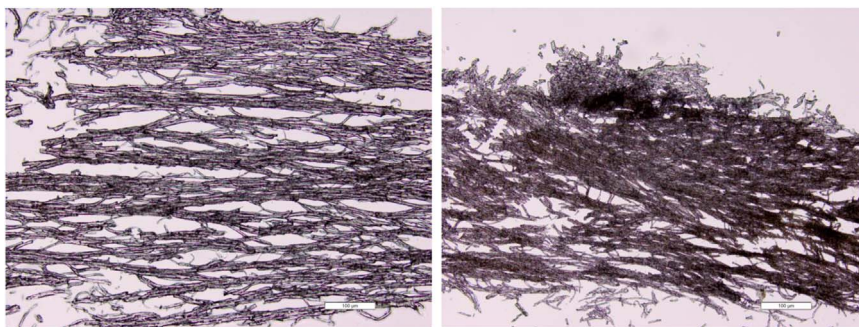


Fig. 1. Histological images of unirradiated (left) and 9 days irradiated (right) bovine nuchal ligament elastic fibers. Images were photographed at 10× magnification and elastic fibers are stained black. The onset of fragmentation and disruption in ordering of elastic fibers is evident following UV-A exposure. The scale bar in the figures shown is 100 μm.

MALDI-MS² analysis was performed using a laser energy of 3.1 μJ, for 150 scans with the AGC set to 10,000 ions. Precursor ions of unlabeled desmosine ($m/z=526.3$) obtained from hydrolysis of the elastic fibers studied and d₄ labeled desmosine standard ($m/z=530.3$) (Toronto Research Chemicals, Toronto, Canada) were selected in a single isolation window ($m/z=529 \pm 6$), and fragmented with a normalized collision energy = 35, activation Q = 0.25, and activation time = 30 ms. The most intense product ions, at $m/z=397$ for unlabeled desmosine and $m/z=401$ for d₄-desmosine, were used for relative quantification.

Results and discussion

UV-A induced alterations of the elastic fiber

To follow the effect of UV-A exposure on elastic fibers, bovine nuchal ligament fibers with and without UV-A irradiation were investigated. Representative histological images of 9 days UV-A irradiated and unirradiated elastic fibers are shown in Fig. 1. From the figure, it is clear that in an unirradiated sample, multiple dense layers of elastic fibers are arranged in a regular fashion; the fibers appear relatively straight and are not fragmented. This regular pattern is dramatically disrupted after UV-A irradiation by 9 days (Fig. 1, right panel). UV-A exposure results in fragmentation of elastic fibers and alters the regularly ordered arrangement of the fibers as well. Moreover, elastic fibers appear thinner compared to that of unirradiated samples; the observed alterations in the elastic fibers due to UV-A irradiation follows the work previously reported in the literature [16,15]. It is known that the level of elastase increases due to UV-irradiation *in vivo* which initiates elastin degradation [15,25,26]. Imayama et al. applied SEM and TEM to study the elastic fibers of dermal connective tissue and showed that UV-irradiation alters the elastic fiber by formation of new elastic fibers which later form an irregular network [15]. In their study, they irradiated the soles of Sprague-Dawley rats with UV-B (130 mJ/cm² for 3 min/day up to 12 weeks) and therefore it was possible to follow the formation of new elastin. In the present *in vitro* study, cells have been removed by the purification step, consequently, elastic fibers are not repaired. Thus, this *in vitro* measurement allows for characterization of alterations that occur as a result of break down in the cross-links of elastin, discussed below, which in turn degrade their structure.

TEM was used to follow the macroscopic alterations of UV-A exposure on elastic fibers. Fig. 2 shows TEM images of the sample without UV-A exposure and with 9 days of UV-A exposure. In the images, the elastic fibers are in white denoted ‘a’, the interstitial spaced between fibers is denoted ‘c’ and cracks within or on an edge of fibers denoted ‘b’. Due to UV-A irradiation, pronounced cracks are observed in the 9 days irradiated sample, both in the interior of the elastic fiber and on the edges. Similar to the histological measurements, elastic fibers appear sparser and thinner in the 9 days irradiated sample, compared to the unirradiated sample.

UV-A exposure may initiate microscopic changes of the structure or dynamics of the principal protein, elastin, in the elastic fiber. To follow these changes, ¹³C MAS NMR experiments were carried out. Chemical shift assignments of all major amino acids (e.g. glycine, proline, valine) that comprise the spectra shown in Fig. 3 were made following previously reported measurements of elastin and its related peptides [20,27–31]. A detailed discussion of the peak assignments and structures observable in elastin has been reported elsewhere [20,32]. Experimental studies of the structure of elastin by modern NMR methodology is difficult due to the large, highly cross-linked nature of the protein. Even with isotopic labeling, degeneracies present in the spectra make structural elucidation challenging—in the present work we apply magnetic resonance spectroscopy to reveal secondary structural changes or dynamics due to UV-A exposure. All ¹³C peaks in these samples (unirradiated and 9 days irradiated) appear in the same position and no significant chemical shift differences were observed. These measurements reveal that any microstructural changes of elastin following 12 mW/cm² of UV-A irradiation for 9 days do not appear to alter the ¹³C NMR chemical shifts of the most abundant amino acids that comprise the protein.

Biochemical changes of elastin due to UV-A exposure

The detrimental effects of UV rays on the cell and extracellular components (e.g., elastin, collagen, etc) of skin are already known to some extent [25,33,16,34,14,18]. Sun exposed skin showed abnormalities which include fiber disintegration, thickening, and proliferation of the elastic fiber [14]. A marked decrease in the linearity of the elastic fiber is also observed due to UV exposure [16]. Chatterjee et al. observed skin wrinkling when hairless mice were exposed to UV-B irradiation and followed different biochemical parameters. Biochemical parameters such as water, elastin, and glycosaminoglycan content was observed to increase where as the collagen content remained the same after UV-B irradiation [25]. However, a different study showed that both UV-A and UV-B irradiation increase the concentration of elastin while the collagen content remain unaffected [34]. To obtain qualitative assessment of the macroscopic damage in elastic fiber that occurs in 9 days of UV-A exposure and to relate these changes to the actinic damage, we compare the UV-A irradiation from the lamp used in this study with that of the sun over the same period of time. Taking the total intensity of light on earth surface (1400 W/m²) one finds that the energy deposition from the UV-A lamp was 2.85 times higher compared to that of continuous exposure to the sun for a duration of 9 days. For this calculation we assumed that only 3 % of the incident solar energy is UV radiation, and that the sun is at the zenith [35]. This computation does not account for any attenuation of the UV radiation, e.g. through the dermis, which may act as a layer of protection to the elastic fibers and reduce the intensity of the incident radiation.

Desmosine and isodesmosine form the cross-linking domains of elastin and their relative concentrations may be used as a measure of biochemical changes. The effects of UV radiation on isolated desmosine

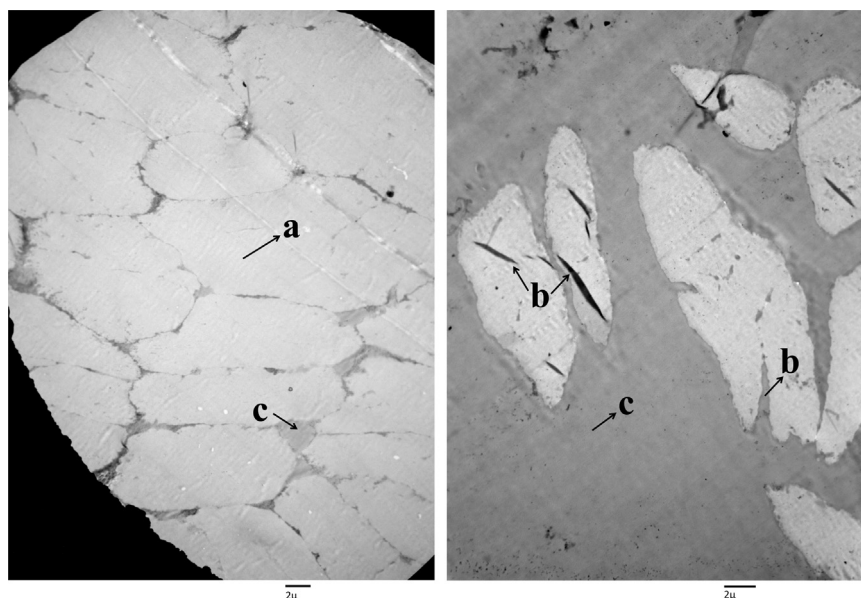


Fig. 2. TEM images of (left) unirradiated and (right) 9 days irradiated elastin samples taken cross-sectionally (fiber axis points into the page). In the images, the light gray color (a) denotes an elastic fiber, (b) cracks on the edge or within the elastic fiber as a result of UV-A exposure, and (c) the interstitial space between fibers.

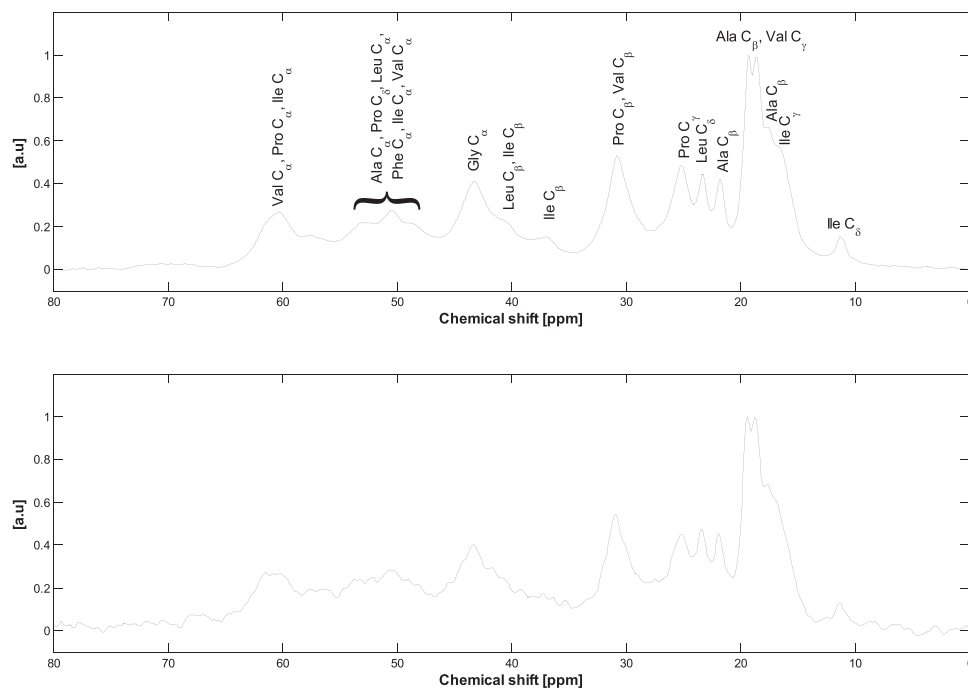


Fig. 3. ^{13}C MAS NMR spectra of aliphatic region of unirradiated (top) and 9 days UV-A irradiated (bottom) elastic fibers recorded at 300 K. Peak assignments were made using values published in the literature, as discussed in the text. The spectra were acquired by accumulating 18,800 scans at 14.5 kHz magic angle spinning speed at a magnetic field strength of 21.10 T.

Table 1

Relative desmosine content (per mg lyophilized mass) in unirradiated and 9 days UV-A irradiated bovine nuchal ligament elastic fibers studied. The relative amount of desmosine is reported by taking the ratio of unlabeled desmosine to the d_4 desmosine normalized by the amount of desmosine in the unirradiated sample. Average values were calculated considering all the data points in each dilution for three different sets of hydrolysis for each sample. Error bars denote the variance propagated across all measurements (9 measurements: 3 different dilution ratios). The results of a *t*-test are presented in the table indicating the probability that the measurements are statistically different comparing 9 days irradiated and unirradiated samples.

Sample	Amount of Desmosine	<i>t</i> -test (%)
no irradiation	1.00 ± 0.01	–
9 days irradiation	0.89 ± 0.04	99.9

and isodesmosine have been reported previously by Baurain and coworkers [36]. Their study showed photolytic reaction results free lysine due to breaking down of pyridinium rings of (iso)desmosine. However, the production of lysine depends on several factors such as irradiating wavelength, pH, and irradiation time. The formation of lysine was 35% when they irradiated desmosine at 274 nm for 25 min, whereas, for isodesmosine the irradiation dose was 285 nm for 50 min and yield was 78%. At wavelengths of 320–400 nm (UV-A), the absorbance in desmosine is ≤ 0.05 [36]. In the present study, we quantify the reduction of desmosine due to UV-A in bovine nuchal ligament elastic fibers by MS/MS experiments. In these measurements, the unirradiated sample was used as a control. The relative amount of desmosine in the 9 days UV-A irradiated sample was normalized with

the amount of desmosine in the unirradiated sample and the results are presented in Table 1. From the table, the relative amount of desmosine in the 9-days irradiated sample was found to decrease by approximately 11%. The decrease in the relative content of desmosine arises from the breakdown of cross-links as a result of the irradiation, as each measurement was made per unit mass of the lyophilized sample prior to hydrolysis.

A previous *in vivo* study on hairless mice skin showed that the desmosine content increases when treated with UV-A or UV-B [34,25]. Chatterjee et al. observed an increase in elastin content per unit area of mice skin with UV-B exposure (40 mJ/cm²) of 18 weeks irradiating three times a week [25]. However, Johnston et al. did not observe any statistically significant difference in the relative desmosine content when purified elastin was treated using 1 and 10 J of UV-A [34]. In the current *in vitro* study, an 11% decrease in desmosine content is observed in 9 consecutive days of UV-A irradiation using approximately 42 kJ, whereas in the previous work by Johnson et al., 1 J for 6.25 min was employed [34]. Though it is known that UV-B is more detrimental to the aesthetic and mechanical functionality of skin, studies till now have demonstrated that UV-A causes minimal damage to skin. However, the results obtained in the present study show that UV-A may play a greater role in photoaging of skin than previously believed. Thus, the findings of this work indicate that cross-linking of tropoelastin is measurably reduced following UV-A irradiation and this breakdown may alter its biomechanical characteristics during prolonged exposure if not repaired. Chung and coworkers [11] have observed a marked increase in MMP-12 activity following UV exposure, which causes degradation of the extracellular matrix, including elastin and collagen. The reduction of desmosine following UV-A exposure revealed in this *in vitro* study of elastic fibers indicates that additional alterations are present before MMP-12 is expressed and other biological processes are initiated.

Similar to photoaged skin, cutis laxa is characterized by loose, redundant, inelastic, and permanently wrinkled skin. Cutis laxa is a clinically and genetically heterogeneous connective tissue disease [37]. It is known that in cutis laxa, abnormal mutant tropoelastin (120 kDa) deposits result in disruption of the elastin-microfibrillar interaction and interferes with elastic fiber function [38]. A common feature of cutis laxa is a dramatic reduction in elastin content without significant alterations to the collagen component of connective tissue; elastic fibers appear scarce, thin, fragmented, and with less microfibrillar components [39,37,40]. The morphological changes to the elastic fiber as a result of cutis laxa are quite similar to the changes observed in UV exposed elastin. In addition to these morphological changes, a biomechanical study of cutis laxa shows reduction in the elastic modulus by 32% and viscoelastic modulus by 53%, whereas retraction time increased by 85% compared to the control, indicating mechanical changes occur that are similar to the changes observed in aged skin [41,42]. In the present *in-vitro* study the biochemical analysis also showed that UV-A irradiation breaks the cross-links in elastin which may play a significant role in the already known mechanical degradation of photoaged skin [43].

Conclusions

This work highlights changes in elastic fibers as a result of UV-A exposure. TEM measurements show that UV-A exposure results in cracks of the elastic fiber pointing to macroscopic alterations. Mass spectrometric measurements show a decrease in desmosine content by 11% in a 9-days UV-A irradiated sample; this change arises from a breakdown of cross-links. Histological images show a disruption in the regular array of elastic fibers as well as fibers appearing shorter and thinner following UV-A exposure. ¹³C NMR magic angle spinning methods were applied to study possible secondary structural changes or dynamics of the major amino acids of elastin, the principle protein component of the elastic fiber. Our NMR measurements indicated that

while the amount of desmosine in the UV-A irradiated sample was reduced in comparison to the unirradiated sample, the NMR chemical shifts of the most abundance amino acids of the protein and the observable line-widths appear the same. While previous *in vivo* studies pointed to an increase in desmosine in the skin when treated with UV-A or UV-B, this *in vitro* study shows a reduction of desmosine of the elastic fiber. These microscopic changes, coupled with the fragmentation of the elastic fiber, are likely to play a significant role in the already known loss of elasticity in aged, UV exposed skin.

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Appendix A. Supplementary data

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Transparency document. Supplementary material

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