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Estrogen Depletion Results in Nanoscale Morphology Changes in Dermal Collagen

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Abstract

Tissue cryo-sectioning combined with Atomic Force Microscopy (AFM) imaging reveals that the nanoscale morphology of dermis collagen fibrils, quantified using the metric of D-periodic spacing, changes under the condition of estrogen depletion. Specifically, a new subpopulation of fibrils with D-spacings in the region between 56 and 59 nm is present two years following ovariectomy in ovine dermal samples. In addition, the overall width of the distribution, both values above and below the mean, has increased. The change in width due to an increase in lower values of D-spacings was previously reported for ovine bone; however, this report demonstrates that the effect is also present in non-mineralized collagen fibrils. A non-parametric Kolmogorov-Smirnov test of the cumulative density function indicates a statistical difference in the sham and OVX D-spacing distributions ($p < 0.01$).

Introduction

The dermis layer of skin is primarily composed of Type I collagen fibers (85–90%), elastic fibers and glycosaminoproteoglycans(GAGs).(Castelo-Branco *et al.*, 1993) Collagen fibrils account for skin's tensile strength and resilience whereas the elastic fibers contribute to the elasticity and extensibility of skin.(Goldsmith, 1991) Unlike collagen in bone, which is frequently remodeled to maintain its mechanical strength, skin collagen has a remarkably long half life under normal conditions,(Verzijl *et al.*, 2000) and thus suffers long term

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degradation due to skin ageing. The severity of skin ageing differs by the anatomical locations: sun protected skin suffers mainly intrinsic ageing effects associated with time such as fine wrinkles and reduced elasticity whereas sun exposed skin suffers both intrinsic and extrinsic ageing (exposure to external influences such as UV radiation), where the severity and rate of the pathological changes including deep wrinkles, pigmentation and melanoma formation are exacerbated. (Bologna, 1995; El-Domyati *et al.*, 2002; Gilchrist, 1996; Naylor *et al.*, 2011) The process of skin aging leads to decreased skin collagen content, moisture and elasticity; (Brinca *et al.*, 1987; Uitto, 1986) A recent study has shown that collagen fibrils are fragmented in aged human skin. These changes in the extracellular environment affect fibroblast attachment and production of matrix metalloproteinases (MMPs), which in turn accelerates extracellular matrix (ECM) degradation. (Fisher *et al.*, 2009) This work underscores the importance of characterizing collagen in order to better understand mechanisms of effects of aging upon skin.

Estrogen has many beneficial and protective effects on skin physiology and functions including maintenance of hydration and skin thickness, wound healing, and reduction of skin cancer risk. (Brinca, 2000) On the molecular level, estrogen exerts its effect by interacting with surface or intracellular estrogen receptors. Intracellular estrogen receptors ER- α and ER- β have been identified in dermal fibroblasts. (Haczynski *et al.*, 2002) The cellular responses triggered by the level of estrogen involve gene transcription/expression, as well as cytoplasmic signaling pathways. On the macroscopic level, aging and especially the onset of menopause causes a series of deteriorations in skin tissue physiology as a consequence of compositional and structural alterations in the ECM proteins. Postmenopausal women suffer from loss of dermal collagen content at an average rate of 2 % per postmenopausal year over a period of 15 years. (Brinca *et al.*, 1987; Brinca *et al.*, 1983; Brinca *et al.*, 1985; Brinca *et al.*, 2005) Decreased amounts of elastic fibers and GAGs in postmenopausal years lead to compromised skin elasticity and less binding with water respectively. (Sherratt, 2009; Uitto, 1986; Waller and Maibach, 2006) The thinning of the dermal layer and loss of water gradually results in wrinkle formation. On the microscopic level, little is known about the ultrastructural changes of dermal proteins that accompany aging and menopause. In this study, we examine the effect of estrogen depletion on the nanoscale morphology of collagen fibrils in ovine dermis. We employ the metric of fibril D-spacing which captures a number of structural features including the molecular structure of the collagen, the three dimensional fibril formation, and the associated post-translational modifications.

Studies indicating the D-spacing in collagen exists as a distribution of values ranging from about 64 to 73 nm were recently reported for murine bone, dentin, and tendon tissue. (Wallace *et al.*, 2010a) The importance of using a technique that measures the fibril D-spacing on a fibril by fibril basis, as opposed to X-ray or optical methods which average over micron to millimeter scales when obtaining D-spacing data, was highlighted by studies examining the effect of genetic changes, *Osteogenesis Imperfecta*, or estrogen depletion upon the D-spacing distribution in bone tissue. (Wallace *et al.*, 2010b; Wallace *et al.*, 2011) In both cases, the average D-spacing values did not change significantly but the distributions of D-spacings were significantly different.

Long term ovariectomy in ovine leads to compromised compact bone viscoelastic properties, which are similar with the conditions in postmenopausal women.(Les *et al.*, 2005) Mineralization, architecture and remodeling parameters of OVX ovine bones have been characterized, and intriguingly only poor correlation between viscoelastic mechanical properties and these parameters were found.(Les *et al.*, 2004) Additional quality factors that come from non-mineral components of bone are speculated to play a crucial role in decreasing bone viscoelastic properties. Similar biochemical and biomechanical effects have been noted between estrogen deprived skin and bone.(Ozyazgan *et al.*, 2002; Pierard *et al.*, 1995; Pierard *et al.*, 2001) We are interested in characterizing the fibrillar collagen D-spacing in hope of better understanding the mechanisms of mechanical failure in ovariectomized tissue. D-spacing has been demonstrated as an effective evaluation of fibril strain in bone and tendon previously. Mechanical stretching on the tissue level can lead to increased fibril level strain, therefore increased D-spacings.(Gupta *et al.*, 2010; Gupta and Zioupos, 2008) Atomic Force Microscopy (AFM) imaging of the ultrastructure of type I collagen provides a means to probe the integrity of the matrix protein and its association with macroscopic pathologies. Previously we have found a dramatic difference in the D-spacing population distribution between sham control and OVX ovine cortical bone, suggesting long term estrogen deprivation leads to a decrease in fibril D-spacing. (Wallace *et al.*, 2010b)

In this study, we quantify the D-spacing distribution present in ovine skin and examine the effect of estrogen depletion upon the distribution. It is shown that collagen in skin also exhibits a distribution of D-spacing values, as opposed to the singular value of ~67 nm for tendon and bone or 65 nm for skin, typically discussed in textbooks and reviews, and that this distribution changes upon estrogen depletion. This study demonstrates that the distribution of D-spacings is independent of degree of tissue mineralization. It is particularly interesting to note that estrogen depletion causes similar changes in the nanoscale morphology of fibrils in both skin and bone.

Results

The combination of cryostat sectioning and AFM imaging has been recently highlighted by Graham *et al.* as an advantageous tool for morphological studies of collagen matrix protein structures in soft tissues.(Graham *et al.*, 2010) Although histological data reveal the orientation and organization of collagen fibril bundles in dermis, the resolution is limited in resolving fibril organizations within a bundle. AFM imaging can overcome this issue and representative images of fibril bundles from ovine dermis are illustrated in Figure 1. Qualitatively, on the 50 micron scale and above, the fibril bundles were randomly oriented in a wavy pattern; within a fibril bundle, on the order of 10 micron scale, collagen fibrils were bundled in a parallel longitudinal direction and individual fibrils crossing the bundle domains were frequently observed (see the arrow heads in figure 1b and 1e). The function of these crossing fibrils is unclear.

Quantitatively, the characteristic collagen fibril D-spacing was measured and employed as the main morphological metric. For each biopsy, at least 60 fibrils from a minimum of four and an average of five randomly selected 50 μm locations were analyzed. The difference in

the number of fibrils obtained for each biopsy is due to variation in collagen abundance at the location of AFM tip engagement. Measurements from each skin biopsy were pooled together to yield the average D-spacing (Figure 2). The mean values for five sham ovine were 62.0, 61.6, 62.7, 63.1, and 62.6 nm. The mean values for the OVX ovine were 61.8, 61.3, 60.7 and 62.5 nm. The means from sham and OVX are not significantly different ($p=0.249$) when compared with the two tailed student T-test.

Examining the population histogram (Figure 3A) revealed that the sham D-spacing distribution spans between 59 and 66 nm whereas the OVX population spans between 56 and 67 nm. The major difference between these populations arises from the percentage of fibrils with D-spacings from 56 to 59 nm – 14.6% in OVX group and 1.6% in Sham group. Note that these changes in distribution do not have a significant impact on the mean D-spacing values which are 61.9 nm for the OVX and 62.3 nm for the sham specimens. The distributions are not strictly Gaussian and the OVX distribution in particular appears bimodal, making the use of the mean value statistically incorrect. We provide it here so that a rough comparison to previous literature can be made; however, to correctly analyze the data a non-parametric method must be employed.

In order to determine the statistical significance of these distributions, a cumulative density function (CDF) was plotted and evaluated using the nonparametric Kolmogorov-Smirnov test (Figure 3B). The CDF highlights the cumulative difference in the 56–62 nm region and the distributions were found to be significantly different ($p<0.001$).

Depending on species and tissue type, mature collagen fibril diameter varies dramatically. In developed ovine dermis, collagen fibril diameter is about 100 nm.(Flint *et al.*, 1984) To evaluate the effect of estrogen depletion, fibril diameters were measured by averaging from fibril bundle width. The results indicated that OVX ovine dermis have similar fibril diameters with Sham ovine. In the case of Sham ovine, fibril diameter ranges from 80 nm to 180 nm, with an average of 130 ± 30 nm. OVX ranges from 80 nm to 160 nm and has an average of 120 ± 20 nm. In collective tissues, fibril diameters are typically assessed in the cross-section plane, diameter measurements in the axial plane are limited in accuracy because fibril overlapping is inevitable in tissue sections. Averaging from parallel bundles remedies this problem to a certain degree and ensures ± 10 nm accuracy (for more details see the supporting information).

In order to explore the effect of estrogen on collagen content in ovine dermal skin, we performed Sirius red staining followed by polarized light microscopic imaging. Because the birefringence is highly specific to fibrillar collagen due to its uniaxial anisotropy,(Cuttle *et al.*, 2005; Junqueira *et al.*, 1978) the staining serves as a good indication of collagen fibril abundance. Figure 4 indicates higher abundance of fibrillar collagen in Sham dermis ($p<0.05$) and a qualitatively thicker fibril bundle width than in OVX dermis.

Discussion

AFM is a non-destructive alternative for imaging biological tissues under aqueous conditions; however, imaging bulk skin tissue using AFM can be challenging because

collagen fibril bundles are surrounded by a sol-gel of hydrophilic GAGs and subcutaneous adipose fat. Recently Graham and coworkers reported a combined tissue cryo-sectioning and AFM imaging method that provides excellent resolution of the ECM components in skin, cartilage, aorta, and lung.(Graham *et al.*, 2010) The sample preparation greatly facilitates AFM imaging and characterization of biological tissues while in the meantime avoids fixation, chemical staining, and high vacuum.

In order to evaluate the nanomorphology of collagen fibrils present in dermis, we selected the D-spacing as a reliable quantitative marker. We have previously demonstrated that the application of 2-dimensional Fast Fourier Transforms (2D-FFT) allows an accurate evaluation of this prominent fibril feature. The D-spacing arises from a parallel staggered packing of collagen monomers which lead to alternating gap and overlap zones along the longitudinal axis of a fibril, as illustrated by the two-dimensional Hodge-Petruska model. (Hodge and Petruska, 1963) Recent X-ray crystallographic work by Orgel *et al.* provides additional three-dimensional insight which supports a supertwist microfibril model.(Orgel *et al.*, 2001) These structural models indicate that quantitative analysis of the D-spacing should be sensitive to changes in the collagen molecule triple helix, the molecular packing, and intermolecular cross-linking effects. For example, the single amino acid substitution of a cysteine residue for glycine-349 results in nanoscale morphology changes observed in the collagen fibril D-spacing distribution. Moreover, the free energy changes induced by amino substitution correlate with clinical severity of *Osteogenesis Imperfecta*.(Lee *et al.*, 2011)

Quantitative analysis of ovine dermis collagen D-spacings indicates a distribution of values is present ranging from 56 to 67 nm with a mean value of 62 nm. Although AFM has an excellent ability to differentiate differences in the D-spacing within tissue, the absolute value is limited by the calibration process. The average value of the distribution of 62 nm is close to previous literature values obtained by X-ray scattering. Purslow reported 67 nm D-spacing in rat skin;(Purslow *et al.*, 1998) others reported lower values of about 65 nm for skin.(Brodsky *et al.*, 1980; Gathercole *et al.*, 1987; Stinson and Sweeny, 1980) These techniques have spot sizes of microns and thus average over too large an area of the skin structure for observation of a D-spacing distribution. The observation of this distribution in dermal collagen provides further evidence that a distribution of values is an intrinsic aspect of collagen fibrillar structure. A similar distribution has previously been observed for another non-mineralized Type 1 collagen tissue, murine tail tendon, as well as for the mineralized collagen tissues murine dentin and bone and ovine bone.(Wallace *et al.*, 2010a; Wallace *et al.*, 2010b; Wallace *et al.*, 2011) The observation of the distribution is possible because of the fibril by fibril analysis using the AFM data.

The influence of bulk tissue stress on collagen fibril D-spacings has been subject of numerous studies. Gupta *et al.* (2008) demonstrated a connection between fibril strain and D-spacing. They noted a 0.3 nm increase in D-spacing in bone as measured by small angle X-ray scattering (SAXS) under mechanical stretching. For bone, fibril strain accounts for only a fraction of the total tissue strain, suggesting that interfibrillar sliding and shear of the proteoglycan-rich matrix takes up the remainder of the tissue strain. With regards to tendon, Puxkandl *et al.* (2002) demonstrated up to a 1 nm change when a 3% macroscopic strain was employed and a 0.2 nm change at a 1% strain. D-spacing changes varied between 0.2 and 2

nm at tendon fracture. The most general conclusion from the comparison of this data to the distribution of D-spacings that we report, which has a width of 12 nm, is that materials strain effects on D-spacing are not large enough to explain the D-spacing distribution observed in either mineralized or non-mineralized biological tissues. The strain effects tend to be about an order of magnitude too small.

One limitation of the current study is that we used dorsal skin exposed to ultraviolet (UV) radiation as opposed to skin protected from extrinsic UV radiation. Ovine dermis is considerably thicker than human dermis;(Dellmann and Eurell, 1998) in addition a layer of wool equivalent of SPF 30 protection also makes it difficult to assess how much photoageing is induced in these dermal tissue samples as compared with human samples. (Fleet, 2006; Forrest and Fleet, 1986) However given that the Sham and OVX ovine were provided with the same sheltering condition, the effects observed in this study signify change in the hormonal level rather than differential UV radiation exposure.

Estrogen is known to play important roles in mediating connective tissue physiology and function. Estrogen depletion associated with menopause causes detrimental effects on connective tissues. In skin, estrogen depletion is associated with declining dermal collagen content, skin thickness, water-holding capacity, and skin elasticity. In terms of mechanical properties, a steep increase in skin extensibility was noted in women during perimenopause (Pierard *et al.*, 1995) and ovariectomized rats exhibit an increased Young's Modulus in the skin.(Ozyazgan *et al.*, 2002) Reduced estrogen level also impairs the rate and quality of wound healing: in postmenopausal women and in ovariectomized female rodents, a marked delay in wound healing was reported.(Ashcroft *et al.*, 1997; Calvin *et al.*, 1998) Hormone replacement therapy was found to partially reverse these effects and topical application of estrogen on wounded skin accelerated wound healing.(Ashcroft *et al.*, 1999) In addition, Pierard and coworkers noted a positive correlation between bone mineral density and skin viscoelasticity in women.(Pierard *et al.*, 2001)

Collagen ultrastructure in ovine bone demonstrated significant change with estrogen depletion, 28 % of fibrils in OVX ovine have D-spacings lower than 64 nm, while sham-operated ovine contained 7% of such fibrils with low D-spacings.(Wallace *et al.*, 2010b) The results presented here show that similar changes occur in dermal collagen nanomorphology of upon estrogen depletion. Although the percentage of low D-spacing fibrils (less than 59 nm) is lower in dermis, 14.6% in OVX group and 1.6% in Sham group, the result is persistent in all five OVX animals we examined. Bone is a mineralized connective tissue while dermis is only constituted of macromolecular proteins. Thus, the results indicate the changes in collagen nanomorphology results from changes in the protein structure, most likely post-translational modifications, and/or the structural interactions with other tissue proteins such as decorin,(Danielson *et al.*, 1997) and is not a mineralization related structural change.

Fibril diameter has been employed previously as a key measure of ultrastructural change. A number of diseases and tissue malfunctions are associated with changes in collagen fibril diameter. Decorin and lumican knockout rats and type V collagen deficient mice showed one-fold increase in fibril diameters.(Wenstrup *et al.*, 2004; Yeh *et al.*, 2010) Ovariectomy

has been shown to decrease expression level of proteoglycans including decorin (Danielson *et al.*, 1997) and lumican (Markiewicz *et al.*, 2007). In this study, average collagen fibril diameter in sham is about 130 ± 30 nm, and 120 ± 20 nm in OVX, the difference is less than 10 % and considered negligible given the limited accuracy in the analysis. Thus, estrogen depletion exerts an anisotropic effect on skin collagen's ultrastructure. It is unclear whether decorin and lumican deficiency are associated to collagen fibril D-spacing changes, this will be the subject of future studies.

In conclusion, estrogen depletion causes a change in the nanoscale morphology of dermal collagen, quantitatively demonstrated by change in the D-spacing metric. The morphology changes are similar to those previously observed for the changes in bone collagen suggesting that estrogen depletion acts upon a structural aspect of the collagen molecule and/or associated proteins and is intrinsic to the fibril formation process.

Material and methodology

Animals

Six-year-old Columbia-Rambouillet cross ovine were anesthetized and ovariectomized (OVX, $n = 5$), the control group was subjected to a sham surgery (Sham, $n = 5$) [Colorado State University, ACUC #03-010A-02] as part of a larger study. Two years after the surgery, the animals were sacrificed with an intravenous overdose of a barbiturate, and skin specimens were procured on the dorsal thoracolumbar region centered at the midline, a region that is subject to both intrinsic and extrinsic ageing. Specimens were wrapped in saline-soaked towels, placed in a plastic zip lock bag, and frozen at -20 °C.

Cryostat sectioning

First, $1 \text{ cm} \times 1 \text{ cm}$ skin specimens were cut with subcutaneous fat layer removed using a scalpel blade. Samples were then embedded in Tissue-Tek optimal cutting temperature (OCT) solution (Sakura Finetek Inc., Torrance, CA, USA) and frozen at -20 °C. $10 \mu\text{m}$ thick thin-sections of skin were obtained using Microm HM550 Cryostat (Thermo Scientific Inc., Walldorf, Germany) and transferred onto glass slides. The dermal sections were rinsed with ultrapure water for 5 minutes and kept at -20 °C prior to the AFM study on the next day. The combined cryo-section and AFM imaging was described in a recent report by Graham and others (Graham *et al.*, 2010).

Atomic Force Microscopy (AFM) imaging and analysis

The AFM imaging on OVX and Sham dermal sections was carried out in air using a PicoPlus 5500 AFM (Agilent), in contact mode with SNL-10 AFM probes (Bruker AFM probes, nominal tip radius 2 nm, force constant 0.25 N/m). The setpoint and gains were optimized in each scan to maintain a minimum level of tip-sample contact and no lateral dragging were observed in the images. Line scan rates were set at 2 Hz or lower at 512 lines per frame. Image analysis and measurements were performed using SPIP software (V5.0.8, Image Metrology; Horsholm, Denmark). Collagen fibril D-spacings were measured using 2D fast Fourier Transform (FFT) toolkit of SPIP software, detailed description and validation can be found in previous studies (and the supporting information) (Wallace *et al.*,

2010b) In short, a straight fibril with at least nine D-bands was selected and marked by a rectangular box as the region of interest (ROI), the 2D FFT transformation is carried out on the ROI, the periodic information (i.e. D-spacing) was obtained from the FFT image. This method provides measurements with an uncertainty of 0.8 nm, based on that the bin size in the population histogram was set to 1 nm.

Statistical analysis of AFM data

Statistical analyses utilized PASW (Version 18, SPSS Inc.). A p value of less than 0.05 was considered significant for all analyses. The mean D-spacing values for all Sham ovine (n = 5) and OVX ovine (n = 5) were compared using two-tailed student T-test. In order to examine differences in the population distribution of fibril nanomorphology between sham and OVX groups, the Cumulative Distribution Function (CDF) of each group was calculated and Kolmogorov-Smirnov (KS) test was used to test for statistical significance between distributions. This test is sensitive to changes in the mean and standard deviation of a distribution.

Picrosirius red staining and image capture

7 μm thick tissue sections were thawed for 15 minutes and fixed in 2% paraformaldehyde for 20 minutes. The slides were then incubated in 0.1% Sirius Red in saturated picric acid for 65 minutes at room temperature. After washing in water, they were placed in 1% Acetic Acid for 30 minutes. The sections were then dehydrated through ethanol and xylene and mounted in Permount medium (Fisher Scientific, USA). To visualize the birefringent collagen, a polarized light microscopy (Zeiss Axioskop 2) with a SPOT 2e CCD camera was used to capture the images. Exposure time for red color was 0.1 second. All slides were photographed on the same day. The relative collagen content was calculated based on the staining intensity (normalized by the tissue area), and student t-test was used to compare them statistically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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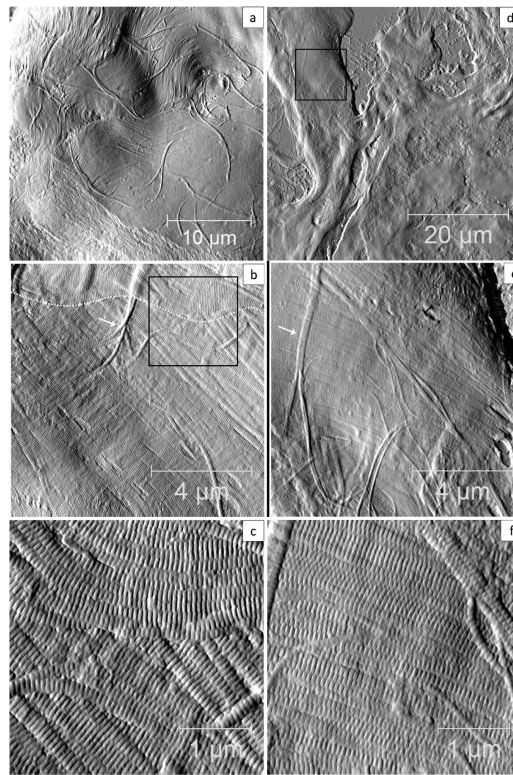


Figure 1.

AFM deflection images of dermis contain domains of collagen fibril bundles. Panel a and b are representative images from a sham dermis sample; panel c and d are images from an OVX dermis sample. Panel a captures potentially two fibril bundles (the rough area at the bottom of the scan is caused by microtome sectioning). Panel b shows one fibril bundle on top of another (The boundary is marked by the white dashed line), notice that a few fibrils that are underneath one bundle are on the surface of another bundle. Panel c is the region marked by the black box in panel b. Panel e and f capture the only region with collagen fibrils found in the 50 micron area of OVX dermis (panel d).

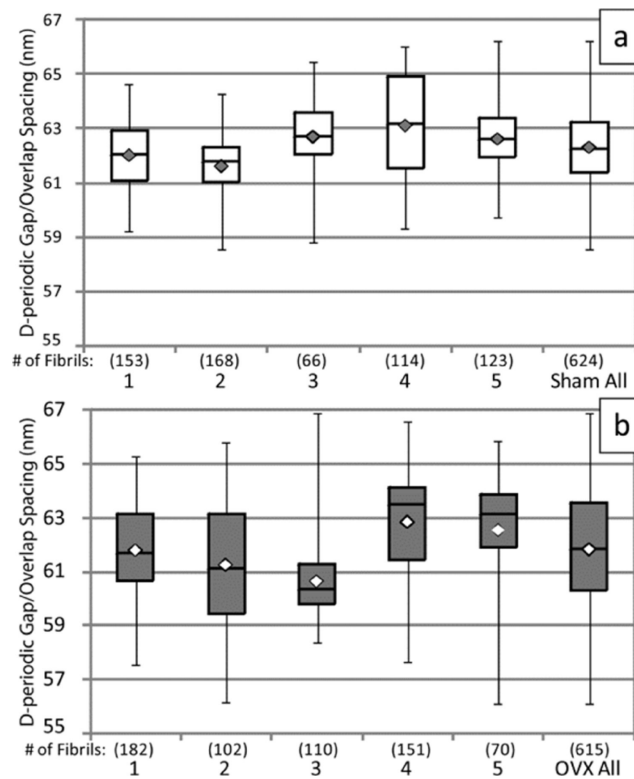


Figure 2. D-Periodic Gap/Overlap spacings from individual sham ($n = 5$, panel a) and OVX ($n = 5$, panel b) ovine dermis. The box-plot shows the interquartile (middle 50% of data), the horizontal line inside the box is the mean, the diamond is the median, and the whiskers provided the minimum and maximum observation. The numbers in parentheses are numbers of fibrils measured in each specimen. Two tailed student T-tests between Sham and OVX group suggests no differences between the means ($p = 0.249$).

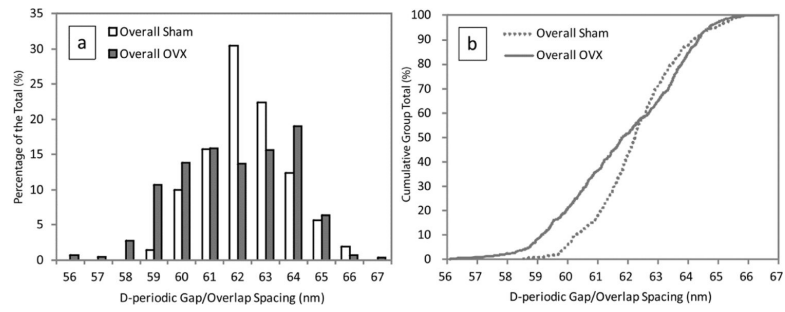


Figure 3.

Histogram and cumulative distribution function of D-Periodic spacings from Sham and OVX ovine skin (each contains five animals). Panel a shows the histogram representation of D-periodic gap/overlap spacings from sham and OVX ovine skin (1 nm bin size). Panel b is the Cumulative Distribution Function (CDF) calculated from each group. Kolmogorov-Smirnov test performed the data distributions indicates significant difference ($p < 0.001$).

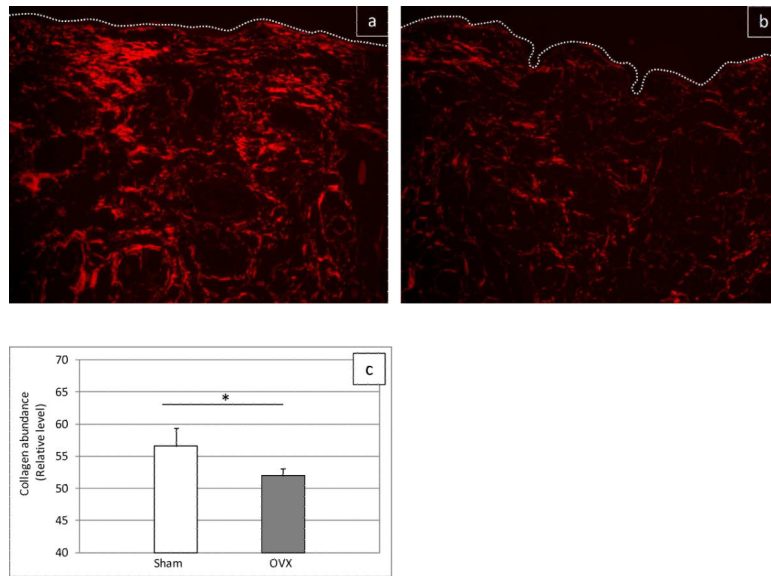


Figure 4. Sirius red staining reveals the abundance of fibrillar collagen content in Sham and OVX dermis. Panel a and b are polarized light microscopic images of Sham and OVX dermis, respectively. The magnification is 10 \times , dash lines represent epidermis. Panel c shows the collagen abundance measured from the staining intensity (* $p < 0.05$).