Characteristics and Young's Modulus of Collagen Fibrils from Expanded Skin Using Anisotropic Controlled Rate Self-Inflating Tissue Expander

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Key Words

Mechanical properties · Collagen fibril · Skin · Anisotropic controlled rate self-inflating tissue expander

Abstract

Mechanical properties of expanded skin tissue are different from normal skin, which is dependent mainly on the structural and functional integrity of dermal collagen fibrils. In the present study, mechanical properties and surface topography of both expanded and nonexpanded skin collagen fibrils were evaluated. Anisotropic controlled rate self-inflating tissue expanders were placed beneath the skin of sheep's forelimbs. The tissue expanders gradually increased in height and reached equilibrium in 2 weeks. They were left in situ for another 2 weeks before explantation. Expanded and normal skin samples were surgically harvested from the sheep (n = 5). Young's modulus and surface topography of collagen fibrils were measured using an atomic force microscope. A surface topographic scan showed organized hierarchical structural levels: collagen molecules, fibrils and fibers. No significant difference was detected for the D-banding pattern: 63.5 ± 2.6 nm (normal skin) and 63.7 ± 2.7 nm (expanded skin). Fibrils from expanded tissues consisted of

loosely packed collagen fibrils and the width of the fibrils was significantly narrower compared to those from normal skin: 153.9 ± 25.3 and 106.7 ± 28.5 nm, respectively. Young's modulus of the collagen fibrils in the expanded and normal skin was not statistically significant: 46.5 ± 19.4 and 35.2 ± 27.0 MPa, respectively. In conclusion, the anisotropic controlled rate self-inflating tissue expander produced a loosely packed collagen network and the fibrils exhibited similar D-banding characteristics as the control group in a sheep model. However, the fibrils from the expanded skin were significantly narrower. The stiffness of the fibrils from the expanded skin was higher but it was not statistically different.

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Introduction

Skin tissue expansion is a procedure that enables additional tissue to be formed for use in surgical reconstruction. Self-inflating tissue expanders overcome the limitations associated with the conventional silicone balloon expander, which requires a periodical injection of saline solution into the balloon to slowly stretch the overlying skin and stimulate growth [1]. This hydrogel expander

absorbs body fluid, which leads to a gradual swelling of the device to a definite volume and size, thus stretching the overlaying skin and stimulating growth [2, 3]. Similar devices have also been used in surgical reconstruction for burns, scars [4, 5] and cleft palate [6]. More recently, anisotropic self-inflating hydrogel expanders have been introduced, where the direction of expansion could be controlled [2, 3].

Mechanical properties of skin depend on the structural elements of the three main layers: epidermis, dermis and hypodermis. Each layer exhibits different properties in order for the skin to perform its physiological functions. However, studies have shown that the central role of dermal collagen is maintaining the elastic property of skin tissue [7]. The elastic property of the dermis is directly associated with the arrangement of its constituents. Collagen types I and III are primarily formed in the dermal layer of the skin. The collagen is made up of specific sequences of amino acid to form three woven polypeptide chains. The three polypeptide chains are twisted together to form collagen helixes, known as collagen molecules. These molecule bundles form strands of collagen fibrils, which assemble to become collagen fibers [8]. The fibrils are cylindrical in shape: diameter in the range of 10–500 nm with a periodically banded pattern of ~67 nm known as D-banding [9]. Hodge and Petruska [10] first explained about distinct patterns of fibrils via the appearance of dark and bright bands in their collagen molecule model, termed the gap and overlap regions, respectively. D-banding is measured from the length of overlap to the gap region.

Due to the distinct hierarchical level of collagen structure, many experiments have been conducted to investigate the relationship between these structures and the mechanical properties of tissue. Previous studies have shown that the mechanical behavior of tissue is dependent on the collagen fibrils [11–15]. Information about the mechanical properties of collagen is not only essential to explain the macroscopic biophysics of different tissues but can also contribute to the understanding of the microscopic structure of collagen fibrils themselves.

The structural properties of collagen fibrils have been widely studied using X-ray diffraction [11, 12, 16–18]. In recent years, numerous studies on the structural and mechanical properties of biological samples at an ultrastructural level have been successfully conducted using second-harmonic generation multiphoton imaging [19, 20] and atomic force microscopy (AFM) [15, 21–30]. Second-harmonic generation multiphoton microscopy is an imaging technique that is capable of studying living tissue *in vivo*, such as skin tissue analysis, without the need of

staining and fixation. When examining dermal collagen, this technique utilizes the second-harmonic signal intensity generated by laser to produce images of collagen fibers, thus enabling qualitative and quantitative assessment of their orientation, type and distribution [19, 20].

The advent of AFM enables researchers to view surface topography of collagen samples at high resolution by exploring the interaction forces between a sharp tip and the sample surface. The topography is imaged by tapping parallel lines on the surface of a sample with a sharp tip fixed at the end of a cantilever. Force spectroscopy is one of the modes available in AFM to conduct mechanical testing and this mode is performed once the imaging has been stopped by approaching and retracting the cantilever tip from the sample while the interaction between the tip and sample (cantilever deflection) is measured [31].

A tissue expander is widely used to obtain additional tissue in clinical practice. However, the quality of the expanded tissue produced remains a focal area of research [32–34]. Previous studies have reported the mechanical properties of bulk-expanded tissue [35–39]. However, since the mechanical behavior of skin is dependent on the collagen fibrils, this study aimed to evaluate the surface topography of expanded skin and Young's modulus of its collagen fibrils when the anisotropic controlled rate self-inflating tissue expander is used.

Materials and Methods

Animal Protocol

Animal ethical approval was obtained from the Institutional Animal Care and Use Committee (IACUC) of the University Putra Malaysia, Malaysia (R031/2013). Adult male Dorper sheep (2 years old) with an average weight of 40 kg were used in this study; 10 sheep were assigned equally to the control and expanded skin groups. All surgical procedures were performed under general anesthesia. The sheep were injected with ketamine 10% (Pharmaniga, Malaysia) and deep anesthesia was maintained with 5% isoflurane (Piramal, India).

Anisotropic controlled rate self-inflating tissue expanders (Ox-tex Ltd., Oxford, UK) 20 mm in diameter and 3 mm thick were used in this study. The expanders were manufactured specifically for this study and designed to expand at a controlled rate in unidirection over a period of 2 weeks. The surgical field was thoroughly cleansed with 0.015% chlorhexidine gluconate and 0.15% cetrimide solutions (Baxter Healthcare Ltd., UK). A subcutaneous pocket was created by blunt dissection in a tension-free manner 5 mm away from the incision site. The expander was then slid through and implanted within the preformed pocket in the dorsolateral region of the hind limb (fig. 1a). The surgical wound was sutured with interrupted 5-0 vicryl (Ethicon Inc., Johnson & Johnson, UK). In addition, three other sutures were tied posterior to the expander to prevent it from displacement and to close up the dead space.

Fig. 1. The normal skin dorsolateral region of the forelimb (a) and the expanded skin (b).

Baseline photographs (fig. 1b) and radiographs (fig. 2) of the expanded skin group were taken. The sheep were monitored and the height of the implanted tissue expander was measured and recorded on a daily basis for 4 weeks prior to euthanasia.

Euthanasia was carried out using pentobarbital. The expanded skin was surgically removed (fig. 3a, b), snap frozen and stored at -20°C . Wet skin thickness was measured using a digital caliper (Mitutoyo, Japan) and samples were prepared for AFM.

AFM Imaging

The sample was sectioned to a thickness of $50\text{ }\mu\text{m}$ using Cryostat (Leica, Germany), embedded on a glass slide and rinsed with deionized water at room temperature prior to imaging. Imaging and force spectroscopy were performed using NanoWizard[®] 3 AFM (JPK Instruments, Germany) on the reticular layer of the dermis as this layer is comprised of densely packed collagen fibers. Besides, testing on the reticular layer instead of the papillary layer reduces the chances of mistakenly tapping on the epidermis region. The specimen was initially observed under the microscope (TopViewOptics; JPK Instruments) attached to the AFM and adjusted accordingly to define the area of interest and the cantilever was placed directly above this area. For consistent imaging across samples, a contact mode cantilever with a resonant frequency of 13 kHz and a spring constant of 0.2 N/m with a tip radius of 0.01 nm was employed in the imaging with a constant force of 10 nN in air. Two channel windows were observed during imaging: the height channel corresponds to the topography of the sample and the error signal channel is for direct observation of the collagen fibrils. The maximum scanning area in x and y directions was $10 \times 10\text{ }\mu\text{m}$ and the maximum height (z-range) was $7.5\text{ }\mu\text{m}$.

Atomic Force Spectroscopy

The cantilever was initially calibrated by performing force spectroscopy on a clean glass slide using the calibration manager mode (NanoWizard[®]; JPK SPM Data Processing software (JPK Instruments)). The linear slope of the contact region from the force curve was then fitted to determine the sensitivity. The actual spring constant of the cantilever was determined using the thermal noise

Fig. 2. X-ray image of expanded skin tissue with the yellow circle (color in online version only) illustrating the position of the tissue expander beneath the skin of the limb.

Fig. 3. a Deflected expanded skin. **b** Expanded skin at explantation.

Fig. 4. Tissue expander swelling ratio against number of days.

method. Force spectroscopy was performed on the collagen fibrils identified from the topographic scan by generating 10 points on the overlap regions of the fibrils. Force curves were generated for each point to measure Young's modulus. The maximum loading was adjusted according to the roughness of the fibril so that the indentation depth was small enough to avoid the influence of the underlying substrate.

Data Processing

All images and force spectroscopy data were processed using JPK SPM Data Processing software (JPK Instruments). The images were filtered to generate better images, while the force spectroscopy data were processed to calculate Young's modulus (E).

Measurement of D-Banding and Width of Collagen Fibrils

The characteristic of D-banding was determined by measuring the length of the gap and overlap regions of collagen fibrils [15]. The surfaces were first imaged at a $10 \times 10 \mu\text{m}$ scan area to identify the collagen fibril pattern followed by rescanning at a higher resolution with a scan area of $5 \times 5 \mu\text{m}$. The D-banding and the width of the collagen fibrils were measured as described by Janko et al. [28]. The D-banding was measured along the longitudinal axis of the collagen fibrils and the distance between the valleys of three

full-width waves from the cross-section profile was measured and divided by three to obtain the D-banding. The width of single collagen fibrils was measured perpendicular to the longitudinal axis of the fibrils. From the cross-section profile, the width of the fibrils was measured in terms of the length for one wave.

Determination of Young's Modulus

Young's modulus was derived from fitting the contact region of the force curve using the Hertzian model. The model approximates the sample as an anisotropic and linear elastic solid. Besides, it was assumed that the indenter was not deformable and there were no additional interactions between the sample and the tip. The cantilever obeys Hooke's Law, $F = kx$, where F is the force exerted on the sample, k is the spring constant of the cantilever and x is its deflection. Considering the use of a 0.01-nm paraboloid tip in this experiment, Young's modulus for each curve was calculated using the following equation:

$$z = \left| \frac{3k(d - d_0) \times (1 - \nu^2)^{2/3}}{1E\sqrt{R}} \right| + (d - d_0) + z_0, \quad (1)$$

where d_0 and z_0 are the corresponding values of the cantilever deflection and the z-piezo extension at the contact point, E is the Young modulus, ν is the Poisson ratio and R is the tip radius.

All images and force spectroscopy data were processed using JPK SPM Data Processing software (JPK Instruments).

Statistical Analysis

The data collected were analyzed using Student's t test (SPSS version 22.0) at 95% confidence interval. Normal distribution of data sets was confirmed using the Shapiro-Wilk test.

Results

Tissue Expander Swelling Ratio

Figure 4 illustrates the swelling ratio of the tissue expander recorded on a daily basis for 4 weeks. The graph shows that the tissue expander gradually increased in height and reached equilibrium in 2 weeks. Figure 1b and these results confirmed that the expander behaves anisotropically with the controlled rate as designed by the manufacturer. Upon reaching equilibrium, the tissue expanders were left in situ for another 2 weeks before explantation.

Surface Topography

The collagen fibrils appeared to be stacked in a basket weave-like structure for both groups, as shown in figure 5. In addition, it was observed that networks of collagen overlapped each other. The normal skin is composed of densely packed collagen fibrils that overlap each other. In contrast, the expanded skin consists of a loosely packed network of collagen fibrils.

Fig. 5. Typical AFM images of normal (a) and expanded (b) skin.

Table 1. D-banding, width and Young's modulus of collagen fibrils taken from normal and expanded skin

	Normal skin	Expanded skin	p value
D-banding, nm	63.5±2.6	63.7±2.7	0.781
Width, nm	153.9±25.3	106.7±28.5	0.001
Young's modulus, MPa	35.2±27.0	46.5±19.4	0.345

D-Banding of Collagen Fibrils

Table 1 summarizes the mean and standard deviation of the D-banding pattern measured from more than 50 collagen fibrils for both groups. For the expanded skin samples (fig. 6), the topographic analysis along the longitudinal axis (line AB) of collagen fibrils yielded an average D-banding value of 63.7 ± 2.7 nm, while the D-banding pattern of normal skin was 63.5 ± 2.6 nm. No significant difference was evident between the groups.

Width of Collagen Fibrils

For the expanded skin sample, the width measured along line CD (fig. 6) yielded a mean width of 106.7 ± 28.5 nm. In comparison, the mean width of collagen fibrils in normal skin was 153.9 ± 25.3 nm. The width of collagen fibrils in both groups was statistically different ($p < 0.05$; table 1).

Atomic Force Spectroscopy

More than 60 curves were generated for the force spectroscopy of normal and expanded skin. Figure 7 repre-

Fig. 6. a Distinct D-banding pattern in expanded skin at higher magnification (scan area of $3 \times 3 \mu\text{m}$). b Typical fibril profile measured along the lines AB and CD.

Fig. 7. The typical force-distance curve recorded from one point each for normal and expanded skin. Young's modulus determination was conducted in the 'approach' mode.

sents one point indentation for each of the normal and expanded skin samples for the determination of Young's modulus in the 'approach' mode. Young's modulus of the collagen fibrils for normal skin was 35.2 ± 27.0 MPa, whereas that for the expanded skin was 46.5 ± 19.4 MPa (table 1). However, there was no significant difference between the groups.

Discussion

From the qualitative and quantitative data, it is evident that the implanted tissue expander stretched the skin and consequently stretched the collagen fibrils. It is believed that the stretching of fibrils is due to the viscoelastic characteristic of collagen fibrils [16, 27, 29]. When stress is applied, the rearrangement of structural collagen fibrils can happen in three different ways: the straightening of collagen molecules themselves, the sliding of collagen molecules from each other or the expulsion of water molecules from the fibrils, resulting in the reorganization of the water network. Fratzl et al. [17] reported that the increase of D-banding occurred at a higher strain due to the stretching of collagen triple helices.

Initially, in biological equilibrium, the skin is in a state of resting tension [40]. Further, the collagen fibrils are arranged in a dense and crimp pattern. As the tissue expander gradually inflates and stretches the skin, the fibrils start to rearrange themselves parallel with each other in

order to resist deformation. As the collagen fibrils attempt to align themselves and become straighter, the resting tension increases. As a result, it becomes stiffer and produces a higher Young's modulus [40]. Young's modulus of the collagen fibrils was found to be in the range of 0.1–11.5 GPa [14, 27, 30], which is in agreement with the present study, although towards the lower end. The main differences can be accredited to the fact that the present study utilized the nanoindentation technique, which represents Young's modulus of the surface rather than the bulk one-dimensional tensile stiffness along the collagen fibril axis as reported by Graham et al. [27] and Eppell et al. [14]. Further, the difference between the present values and those of previous findings could also be attributed to the different origin of collagen tested.

One of the limiting factors in this study is the large standard deviation of Young's modulus for both groups. This is to be expected for biological tissue samples, firstly, because variation could occur between the sheep themselves. During the expansion period, some sheep might be more active than others. Therefore, there is a slight chance that activities may influence the skin's characteristics. The activities probably cause the skin to exhibit a different sensitivity to the test condition, such as some tissue becoming stiffer than other. Increasing the number of sheep could probably decrease the standard deviation. However when using mammals of a higher species, the number used is always a limiting factor.

Secondly, the variability between individual collagen fibrils could be a contributory factor, as suggested by Wenger et al. [30]. Moreover, they also reported that the mechanical properties of collagen fibrils are dependent on the direction of force used during testing: either perpendicular or axial to the fibrils. However, no value for Young's modulus was reported. In this present work, the force was applied perpendicular to the collagen fibrils at 10 different indentation points twice. The large standard deviation could probably be addressed by increasing the number and frequency of indentation points. Thirdly, difficulty arises during the selection of similar size fibrils due to their complicated network arrangement, thus obtaining a clear profile is sometimes challenging. Therefore, fibrils of varying diameter were selected, hence producing large variations in Young's modulus. Svensson et al. [41] have suggested that larger fibrils produce a higher modulus.

Tissue expansion is dependent on the viscoelasticity of skin to increase the surface area in response to intrinsic and extrinsic forces [42]. Previous research has suggested that the increase in tissue surface area following expansion resulted from new tissue being generated by a phe-

nomenon termed mechanical creep [43–45]. This phenomenon is further substantiated by Wilhelmi et al. [46], who suggested that mechanical creep is due to the elongation of skin beyond its inherent extension, such as in the presuturing technique. In addition, they also suggested that tissue expansion is the consequence of biological creep, a generation of new tissue due to a persistent chronic stretching force. Based on the swelling ratio of the tissue expander over time (fig. 4), we hypothesized that mechanical creep occurred during the initial 2 weeks after implantation as the skin gradually stretched at a constant force over time. At this phase, mechanical creep increases the skin surface area due to the viscoelastic nature of the skin without new tissue formation. In contrast, upon reaching equilibrium (maintenance period), the skin is likely to regain its biomechanical properties. This suggestion is also supported by Zeng et al. [38]. The formation of new tissue during this period could restore the biomechanical properties in the skin. However, histological studies are required to reinforce this hypothesis.

Surgical operations are reported to induce an inflammatory reaction and generate an enormous amount of free radicals, which could damage collagen fibers, as reported by several authors [47, 48]. In the present study, an incision wound was made 5 cm away from the implantation area. This procedure was carried out to reduce inflammation at the implantation area. It was also done to reduce any effect on ongoing collagen synthesis and breakdown, remodeling the extracellular matrix around the incision wound during the healing process, which can

be a confounding factor in the results of a study [49]. This technique also minimizes wound tension caused by tissue expansion, which may lead to suture failure and wound dehiscence.

Conclusion

An anisotropic controlled rate self-inflating tissue expander successfully expanded skin tissues in a sheep model. This novel device expanded orthotropically and its effect on the collagen fibrils revealed that the D-banding did not change significantly from the control group. The collagen fibrils were observed to be aligned towards the direction of the principal strain and the fibrils were significantly narrower. The increase in the stiffness of the fibrils following expansion was not statistically significant.

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Disclosure Statement

All authors declare that they have no conflicts of interest in the conduct of the study.

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