ORIGINAL ARTICLE

IWJ WILEY

Fractal analysis of rat dermal tissue in the different injury states

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Funding information
National Natural Science Foundation of China, Grant/Award Numbers: 30872685, 81272110

Abstract

Scar formation and chronic ulcers can develop following a skin injury. They are the result of the over- or underproduction of collagen. It is very important to evaluate the quality and quantity of the collagen that is produced during wound healing, especially with respect to its structure, as these factors are very important to a complicated outcome. However, there is no standard way to quantitatively analyse dermal collagen. As prior work characterised some potentially fractal properties of collagen, it was hypothesised that collagen structure could be evaluated with fractal dimension analysis. Small-angle X-ray scattering technology (SAXS) was used to evaluate the dermis of rats exposed to graft harvest, burn, and diabetic pathologic states. It was found that almost all collagen structures could be quantitatively measured with fractal dimension analysis. Further, there were significant differences in the three-dimensional (3-D) structure of normal collagen versus that measured in pathologic tissues. There was a significant difference in the 3-D structure of collagen at different stages of healing. The findings of this work suggest that fractal analysis is a good tool for wound healing analysis, and that quantitative collagen analysis is very useful for assessing the structure of dermal collagen.

KEYWORDS
burns, collagen structure, diabetes, fractal dimension, quantitative analysis, wound model

Key Messages

- fractal analysis can be applied for studying the three-dimensional structural change quantitively
- the study showed that the structure of dermal tissue in diabetes skin was altered before wound and could not be back to normal after wound, which

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Haisong Xu and Jingde Zhang contributed equally to this work.

- suggested the recessive impairment in the diabetic skin and bad healing quality of diabetic skin
- the study also showed the structural differences among the species, which suggested the importance of the ideal scar modelling

1 | INTRODUCTION

Common complications after skin injury are scar formation and chronic ulceration, which greatly affect physical and psychological health and increase social and economic burdens. There were no ideal ways to treat these complications, and the mechanisms behind their occurrence are still not fully understood.[1]

Wound healing is a highly complex, programmable, and dynamic process. If normal wound repair goes awry, two major outcomes can occur: scar formation and chronic ulceration. Scar formation is the result of collagen overproduction, while chronic ulceration is the result of insufficient collagen production. Collagen forms a scaffold for dermal tissues[2] and is known to play an important role in wound healing.[3,4] Changes in collagen components, quantities, and structure are closely related to the molecular events that occur during wound healing.[5] However, it is not well understood how collagen structure changes during wound repair, and how these changes are related to molecular events that occur during injury and wound healing. As there are no widely accepted quantitative methods for collagen analysis, most prior research has been qualitative in nature.[2] This means that changes in collagen structure cannot be monitored or dynamically predicted.

Khorasani et al[5] and Frisch et al[6] published works describing quantitative measurements of collagen via fractal dimension analysis using microscopic and transmission electron microscopy (TEM) images. However, their works had two limitations: (a) their research was performed using two-dimensional images of sections of dermal tissue, which does not reflect the full thickness of the dermis; (b) they did not provide a way to verify if the collagen was fractal.

Ueda et al[7] also performed a three-dimensional quantitative analysis of collagen tissue using multiphoton microscopy. However, they also could not completely analyse the full thickness of the dermal tissue because of the limitations of the laser beam that they used and the nature of the structure of collagen. The strength of the laser beam was significantly reduced between the papillary dermis and the reticular dermis, which resulted in incomplete visualisation of the reticular dermis. Collagen fibres have a complex entangled and tightly packed structure, which fundamentally precluded the identification of fibre continuity using this technique.

In this study, a fractal dimension analysis was performed using small-angle X-ray scattering (SAXS) to validate clinical observations about changes in the collagen architecture of animal models. SAXS records the elastic scattering of X-rays at very low angles on the nanometre scale when X-rays penetrate the inhomogeneous structure of a sample.[8] X-rays can penetrate the full thickness of the skin with a high resolution. Fractal dimension (D) analysis measures the percentage of certain particles that occupy a given space. In this work, the particles were the scattering signals of the X-rays from the skin. This approach is generally used to measure objects with loose or dense internal structures. In the former, SAXS measures the density of the internal structure. In the latter, SAXS measures the smoothness of the surface because the internal structure is too compact to be explored efficiently.[9] SAXS is used to analyse objects that exhibit self-similarity and is currently used to analyse almost all irregular and complex biologic tissues, such as neurons,[10] alveoli,[11] and capillary beds.[9] As collagen tissue has similar features to these structures, it was hypothesised that collagen structure could be analysed with fractal dimension.

2 | MATERIALS AND METHODS

2.1 | Sprague-Dawley (SD) rats

SD rats of either gender were provided by the Experimental Animal Center of the Chinese Academy of Science. Animal procedures were approved by the Animal Use and Care Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. All methods were performed in adherence to internationally relevant guidelines and regulations. Mean rat mass was 250 ± 25 g. An intraperitoneal injection of 50 mg/kg pentobarbital (Jiangsu Hengrui Medicine Co. Ltd., Jiangsu, China) was used for anaesthesia. The dorsum of each rat was shaved, and a depilatory agent was applied to remove the remaining hair while avoiding damage to the skin. The surgical area was sterilised with benzalkonium bromide. Following the completion of the experimental protocol, rats were euthanised via CO_2 asphyxiation and cervical dislocation.

2.2 | Different skin thickness of skin grafts

After anaesthesia induction, the full thickness of the dorsal skin of 10 rats was removed. Then, the epidermis faced up, different thicknesses of skin were cut with a dermatome at a controlled scale to produce different thicknesses of skin grafts.[12] Dermal thicknesses of 0.1, 0.2, 0.3, 0.4, 0.6 mm, and full-thickness were used in this study.

2.3 | Different burn depths

Thirty rats were equally distributed into groups that received superficial partial-thickness burns, deep partial-thickness burns, and full-thickness burns (n = 10 each). A metal column 1 cm in diameter and 2 cm in height was heated in boiling water (100°C) for 15 minutes, then applied to the dorsum of each rat without pressure after its surface was wiped off with cotton gauze. Different depths of burns were created with different contact durations: 3 seconds for a superficial partial-thickness burn, 5 seconds for a deep partial-thickness burn, and 8 seconds for a full-thickness burn.[13,14] All of the wounded areas were removed for testing the day after creation.

2.4 | Different time points after wound creation

Twenty SD rats were used in this experiment. A 2×2 cm^2 full-thickness skin wound was created by a scalpel blade. Wounds were allowed to heal naturally with sterile Vaseline gauze dressings, which could be changed carefully every 3 days. Normal tissues were harvested when the wound was created, and wound tissues were harvested 2 weeks (10 rats) and 4 weeks (10 rats) after creation.

2.5 | Diabetic skin

Twenty SD rats were used in this experiment. A single dose of 62 mg/kg streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO) was injected intravenously to induce diabetes.[15] Blood glucose levels were tested with a blood glucose monitoring system (Glucotrend 2, Roche Diagnostics, Mannheim, Germany). A diabetic state was diagnosed when polydipsia, polyphagia, polyuria, weight loss, and hyperglycaemia (16.7 mmol/L) were noted. Rats were hyperglycaemic for 8 weeks before surgery. Full-thickness wounds (1.0 cm diameter) were made on the back of each rat with a sterilised punch equidistant from the midline. Tissues that were cut with the punch were used as the diabetic control group. Wound tissues and the surrounding areas were harvested 2 and 4 weeks after injury.

2.6 | Small-angle X-ray scattering (SAXS)

Skin samples were immersed in 0.5% dispase (Roche, Mannheim, Germany) at 4°C for 12 hours after the excess subcutaneous tissues were trimmed and de-epithelialised samples were lyophilised for 48 hours.[16]

SAXS measurements were performed with a BL16B1 beamline at the Shanghai Synchrotron Radiation Facility (SSRF) in Shanghai, China. A 10 keV X-ray was used as the incident beam. Scatter intensity was recorded with a Rayonix SX-165 CCD detector (Rayonix, Evanston, IL, USA). Data were averaged to create a one-dimensional SAXS profile via circular averaging with the Fit2D software (ESRF, France). Signals were treated as a function of the modulus of the scattering vector: $q = (4\pi/\lambda) \sin\theta$, where λ was the wavelength and 2θ was the scattering angle. Different objects had different θ. q was defined as the modulus of the scattering vector at the peak maximum.

2.7 | Fractal dimension

When the scattering vector q was within the range $1/R < <q < <1/r_0$, it was called a fractal range. R was the statistical fluctuation of electron density and correlated with mean coherence length, which was formed by the interaction between the wavelength of the incident wave and the electron density. $R = r(r) = \frac{<\eta k\eta j>r}{<\eta^2>}$, where $<\eta^2>$ represents the mean square of the fluctuation of the electron density and r_0 was the radius of the scattering element $r_0 = \sqrt{-3\alpha}$ and represents the gyration radius of the system.

Graphically, α was the slope of the curve InI (q) ∼ Inq, which indicates that two scattering units k and j were possibly in the same phase or had the same possible electron density in a random direction and a given distance r. The slope was like a smooth line (Figure 1). If the slope was not a line, then the sample did not have fractal geometry.

There were two kinds of fractals according to different values of α: mass fractal and surface fractal. In a mass fractal ($0 < \alpha < 3$), the fractal dimension (Dm) is $D = \alpha$. The larger the D, the denser the particle distribution.

Simply speaking, if the ideal object, such as a ball, has a uniform density, its D is 3 (three power of its radius, which was the equation of the volume). As the density of the collagen structure is non-uniform, its fractal dimension is lower than 3. In a surface fractal ($3 < \alpha < 4$), the fractal dimension is Ds = $6-\alpha$, which falls between 2 and 3. The smaller the D, the smoother the particle surface is (Figure 1).
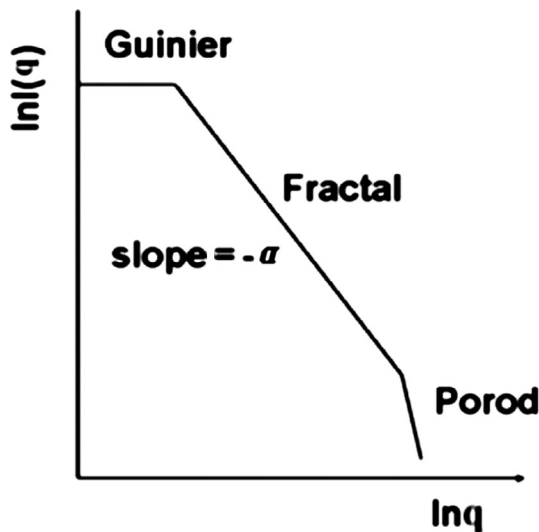
FIGURE 1 Illustration of fractal dimension as determined using SAXA. The median line shows fractal dimension, and the slope was the number of fractal dimensions of an object[9]

Simply speaking, the surface area of an object was proportional to the second power of its radius if the surface was very smooth, yielding a fractal dimension of 2. If the surface was not smooth and full of creases, the surface area would be higher than the second power of its radius, yielding a fractal dimension larger than 2. If D was larger than 4, then the object may not possess any fractal geometry.[8,17,18]

2.8 | Statistical analysis

All data were expressed as the Mean $\pm$ SD of at least three independent experiments. One-way analysis of variance (ANOVA) and Student's t test were used to measure statistical differences. Statistical significance was $P < .05$.

3 | RESULTS

3.1 | Different skin thickness

Normal skin and the split-thickness skin grafts (STSG) had different fractal geometry. The collagen structure of the normal skin was highly compact ($\alpha > 3$), while all of the α values of the STSGs were lower than 3, suggesting that the structure of the dermal tissue was damaged during cutting. The α values increased with skin graft thickness, suggesting the density of the structure increased. However, there was a reversion between the 0.3 and 0.4 mm groups. The D of the 0.4 mm group was significantly less than that of the 0.3 mm group, indicating more destruction (Table 1).

TABLE 1 D values of different depths of skin tissues

Skin graft (mm)	α	Dm/Ds
0.1	2.56 + 0.03*	2.56 + 0.03 Dm
0.2	2.73 + 0.03^	2.73 + 0.03 Dm
0.3	2.87 + 0.02&	2.87 + 0.02 Dm
0.4	2.69 + 0.04#	2.69 + 0.04 Dm
0.6	2.95 + 0.05	2.95 + 0.05 Dm
Full thickness	3.53 + 0.06	2.47 + 0.06 Ds

*$P < .05$ between 0.1 mm grafts and 0.2 mm grafts.
^$P < .05$ between 0.2 mm grafts and 0.3 mm grafts.
&$P < .05$ between 0.3 mm grafts and 0.4 mm grafts.
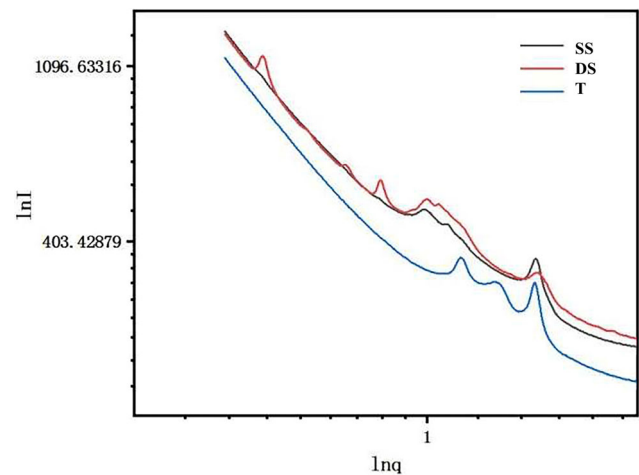#$P < .05$ between 0.4 mm grafts and 0.6 mm grafts.

FIGURE 2 Fractal analysis of different burn depths. There were various peaks at different depths as calculated with InI (q) $\sim$ Inq, which suggests there were no fractals, while others in this study showed smooth lines like Figure 1, which suggests some fractal quality. DS, deep second-degree burn; SS, superficial second-degree burn; T, third-degree burn

3.2 | Different burn depth

After the q value in the abscissa and the I value in the ordinate were logged, there was no smooth fractal line such as that shown in Figure 1. There were several peaks on the lines. The locations of the peaks differed by burn thickness, which demonstrated that there were no fractal dimensions (Figure 2).

3.3 | Different time points after wound creation

The wound healed 2 weeks after wound creation. The α values were larger than 3 in both the normal and injured tissues 4 weeks after wound creation, suggesting that both groups belonged to the surface fractal. However,

TABLE 2 _D_ values 2 and 4 weeks after wound creation

Skin samples	α	Dm/Ds
Normal	3.53 + 0.06	2.47 + 0.06 [Dm]
2 wk after wound	2.70 + 0.16	2.70 + 0.16 [Dm]
4 wk after wound	3.38 + 0.10*	2.63 + 0.10 [Ds]

*P < .05 between Normal and 4 weeks after wound.

TABLE 3 _D_ values in normal versus diabetic rats

Skin samples	α	Dm/Ds
Normal	3.53 + 0.06	2.47 + 0.06 [Dm]
Diabetes	2.64 + 0.14	2.64 + 0.14 [Ds]

TABLE 4 _D_ values 2 and 4 weeks after wound creation in diabetic rats

Skin samples	α	Dm
Diabetes	2.64 + 0.14*	2.64 + 0.14
2 wk after wound	2.05 + 0.13**	2.05 + 0.13
4 wk after wound	2.35 + 0.13***	2.35 + 0.13

*P < .05 between pre-injury and 2 weeks post-injury.
**P < .05 between 2 weeks and 4 weeks post-injury.
***P < .06 between pre-injury and 4 weeks post-injury.

α was smaller than 3 2 weeks after wound creation, which meant it belonged to the mass fractal. _D_ was significantly higher 4 weeks after wound creation compared with normal tissue (Table 2), suggesting that tissue structure 4 weeks after wound creation was not as delicate as normal tissue and that the texture of the wounded tissue was rougher than normal tissue.

3.4 | Diabetes skins

The α of all diabetic skin samples was less than 3, which suggests the structure of the diabetic skin was damaged before the injury (Table 3). There were significant differences between diabetic skin 2 and 4 weeks after wound creation. The _D_ increased with time during wound healing but did not exceed that of the diabetic control groups. Interestingly, the wound healing time of diabetic skin was the same as that of normal wounds, although the size of the wound area was smaller (Table 4).

4 | DISCUSSION

Skin injury often results in scar or chronic ulcer formation, which affects physical and psychological health and increases social and economic burden. There are no ideal ways to treat these complications, and their underlying mechanisms are unclear.[1]

Dermal tissue is mostly composed of collagen fibres, which self-assemble into a net-like structure and form the backbone of the extracellular matrix. The structure of collagen is currently thought to play an important role in scar formation and wound healing,[2-4] although our knowledge to this end is still incomplete.

Research about the structure of collagen should include both qualitative and quantitative studies. Qualitative research helps differentiate among normal, scar tissue,[19-23] and chronic ulcers, while quantitative research illustrates the dynamic, continuous changes in the structure of collagen structure from normal to pathologic state or vice versa, allowing us to real-time monitor collagen structure and to use these data to predict the progression and outcome of a disease. Qualitative research on collagen is currently widely accepted,[19] while quantitative research is not.

Quantitative collagen analysis was previously proposed. However, there were shortcomings to these early studies. Some were based on 2D images from sections,[5,6] which did not reflect the full thickness of the dermal tissues. Other works were based on images that were not of full-thickness dermal tissues.[7]

To develop a truly quantitative three-dimensional methodology for analysing the whole skin, fractal dimension analysis with SAXS was used in this study. SAXS has been previously shown to penetrate the full thickness of the dermis with a high degree of resolution.[16] Fractals are geometric concepts formulated by the mathematician Benoit Mandelbrot in 1973.[24] Fractals are a quantitative description of an object, with characterisation of irregularity and self-similarity under any defined scale. The shape of a self-similar object does not change when the measurement scale is changed because any part of it may be similar to the original object. The size and geometric parameters of an irregular object differ when it is inspected at increasing resolutions, which show more details and imperfections within it. This is also believed to be the main attribute of the morphologic complexity of cells and tissues.[25,26] Fractals have been widely used in almost all irregular and complex biologic tissues, such as neurons,[10] alveoli,[11] and capillary beds.[9]

The collagen structure of the dermis was hypothesised to have both irregular and self-similar features.[16,21,22] Fetal and adult collagen also looks histologically similar.[27] Our previous clinical research confirmed that the collagen structure has fractal attributes (data not shown here). To further examine this hypothesis, several animal models related to wound formation were created, samples were collected and tested with SAXS, fractal features were detected, and those features then underwent fractal dimension analysis.

The present work showed that collagen density fluctuated around that of normal collagen during wound healing. The D was changed from Dm to Ds, which meant that the density was sparser early after injury, but became denser over time. The D was higher than that of the controls 4 weeks after wound creation, which suggests that the structure of the healed tissues was rougher and not as delicate as that of normal tissues. This study was different from our clinical research, in which all α values were lower than 3 despite an increasing trend in density from 2 to 4 weeks (data not shown). This may be because rats are not more likely to create scar than human beings.

The α values of diabetic rats were less than 3, which represents sparser collagen geometry than that of the normal skin. This suggests that the collagen ultra-structure was significantly altered in diabetic animals. Wound healing time in diabetic animals was also longer than that of the controls. Compared with the wound tissues of normal rats, all of the scar tissue of diabetic wounds still had a lower D ($\alpha < 3$) after the wound healed, despite the increase in D during remodelling. This suggests that the healing quality of diabetic skin is lower than that of non-diabetics. This may also indicate that the skin of diabetics is more susceptible to recurrent injury and ulceration than that of non-diabetics.[28]

For patients with large skin defects, skin grafts are the best choice. There are many types of skin grafts: split-thickness skin grafts (STSG) and full-thickness skin grafts (FTSGs). The STSG was further divided into the thin split-thickness skin graft (TSTSG), the intermediate split-thickness skin graft (ISTSG), and the thick split-thickness skin graft (ThSTSG). All of which is based on the thickness of the dermis. The dermis can be divided into the papillary dermis in the superficial layer, and the reticular dermis in the deepest layer.[2] The mean thickness of dorsal rat skin was 1.18 mm, while that of the papillary dermis was 300 μm.[29-31] Therefore, in our experiment, graft thicknesses of 0.1, 0.2, and 0.3 mm can be defined as TSTSG, 0.4 mm grafts were ISTSG, and 0.6 mm were ThSTSG.[30] TSTSG contains the papillary dermis, ISTSG contains the full thickness of the papillary layer and the superficial reticular layer, and ThSTSG contains the full thickness of the papillary dermis and a deeper extent of the reticular layer. The results of the present work showed that the α of any of the STSGs was less than 3 compared with normal skin, suggesting that the collagen structure of the skin is destroyed during STSG graft harvest. There was a trend towards increased D with increased graft thickness except for the ISTSG, whose D was strikingly lower than 0.3 mm grafts and equivalent statistically to that of 0.2 mm grafts. The reason for this might be that it was difficult to cut near the interface between the papillary layer and the reticular layer because the reticular layer has a higher density than the papillary layer, making the collagen more easily damaged during ISTSG harvest than during deeper graft harvest.

The present work supports our hypothesis that collagen tissue is fractal, except in the case of simulated burns. Fractal dimensions did not exist in burned skin regardless of depth, which was consistent with our prior clinical (data not shown) and benchtop research.[16] This may be the result of thermal denaturation, which could dramatically change collagen structure by making it lose its self-similarity. Why did the heating power change the periodicity of the collagen tissue at nanoscale, making them disappear at different locations? This needs further research.

In conclusion, our study provides a standard for performing fractal analysis on skin, confirmed the results of our prior clinical work, and identified several quantitative differences in collagen structure between normal and pathologic skin. This may make it possible for clinicians to dynamically monitor for and intervene on changes in collagen structure, thereby improving the quality of wound healing and providing clarity on the biologic mechanisms behind wound healing and regeneration.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT
Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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