Comparative study on high-frequency ultrasonography and histological structure of the skin: Relationship between collagen/elastin content and echogenicity

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Abstract

Background: High-frequency ultrasonography is used to non-invasively visualize the skin. However, few studies have focused on the association between ultrasound images and actual histology. Here we focused on the comparison of high-frequency ultrasound images and their actual histology using Cesarean scar tissue removed during delivery.

Methods: This study included 12 pregnant women who provided a written informed consent. The ultrasound images of scars were captured prior to delivery using a high-frequency ultrasound device. Scars were sampled by an obstetrician, and collagen and elastin staining were performed on the samples. The amount of collagen or elastin was estimated by an intensity analysis using immunohistochemistry images. Results: Only low-echogenicity signals were observed in the scar tissue, although a tight structure was present. Echogenicity was significantly correlated with the elastin-positive area but not with the collagen-positive area. Conclusion: The echogenicity of ultrasound images appeared to be more closely associated with the amount of elastin than with the amount and/or density of collagen fibers.

Key words: ultrasonography, collagen, elastin, dermis, scar

Introduction

Ultrasonography is not limited to diagnostic use; it has recently been used for nursing purposes, such as visualizing skin deterioration, catheter failure, and edema. For example, it has been proven to be useful for predicting and assessing pressure ulcers12) and visualizing peripheral intravenous catheters5)−6). Lymphedema can also be assessed by ultrasonography7)−8).

Another interesting use of ultrasonography is for imaging of the skin structure. As the skin is thin (approximately 2.5 mm at the thickest) and is present on the outermost layer of our body, a high-frequency (>20 MHz) ultrasonography device has been used for skin visualization. Several studies have demonstrated the relationship of the skin structure and its function with related diseases using such a device9)−12). In addition, the echogenic quantification of high-frequency ultrasonography has been used for assessing the skin structure. For example, Horii et al.
and Matsumoto et al. revealed that dermal echogenicity is inversely correlated with the severity of obesity.\(^{13,14}\)

One another possible application of ultrasonography for nursing might be a prediction of pressure ulcer recurrence, especially on the scar. The rate of pressure ulcer recurrence is reported to be as high as 63% in the patients with spinal cord injury, implying the scar tissue is more susceptible to external disturbances.\(^{15}\) In addition, since a scar is characterized by a stiff but not elastic tissue inside the skin, it may cause the deep tissue injury by stresses around the scar.\(^{16}\) It might be beneficial to assess several parameters of the scar, such as size, depth, and echogenicity, for the prevention of the recurrence of pressure ulcer on the scar. Before that, however, we need to know what factors are causing the change of echogenicity in the skin and scar.

Despite the high popularity of ultrasonography for skin analyses, few studies have focused on the relationship between actual histology and echogenicity. The attenuation coefficient and propagation speed of ultrasound are reportedly correlated with the collagen content;\(^{17,18}\) however, these studies did not demonstrate the direct comparison between echogenicity and the skin structure. Recently, Minematsu et al. reported very interesting findings.\(^{19}\) In that study, they used pig skin to demonstrate the relationship between actual histology and ultrasound images and found that dermal echogenicity is not the same even if the skin structure is similar. In addition, the skin structure is characterized not only by collagen fibers but also by elastic fibers,\(^{20}\) which has not yet been considered. Considering such limited findings, it is safe to assume that the relationship between the actual skin structure and echogenicity is still unknown. Therefore, we were motivated to investigate this relationship by comparing the actual skin histology and the high-frequency ultrasonography images, especially the echogenicity images, of human skin.

Seeking for the condition in which the ultrasound images is obtained before the actual human skin is harvested, we proposed the idea of utilizing the Cesarean scar tissue of a pregnant woman. During the second Cesarean delivery, the previous Cesarean scar will be removed and disposed: the disposed skin tissue contains scar with normal portion of skin, which can be used for a standard histological analysis. The ultrasound images can be obtained before the fixed date of Cesarean delivery. In addition, the scar with aberrant but plenty of collagen fibers and few elastic fibers can be compared with the normal portion of skin in one sample.

This study aimed to compare the actual histology, especially the density of collagen and elastin, and the echogenicity in the dermis using Cesarean scar tissue with adjacent normal skin.

**Methods**

1. Ethical considerations

This study was approved by the Medical Ethics Committee of Kanazawa University (approval number 507-3) and that of Asanogawa General Hospital (approval number 30) where the ultrasound images and skin samples were collected. This study was conducted in accordance with the Declaration of Helsinki. A written informed consent was obtained from all participants.

2. Participants

We included pregnant women who had previously undergone a Cesarean delivery, would be undergoing a Cesarean delivery during the forthcoming delivery, and had agreed to participate in this study. Twelve pregnant women [age, 24-40 years; body mass index (BMI) just before delivery, 20.0-28.4] who fulfilled these criteria were recruited.

3. Ultrasonography of scar and the adjacent normal skin

The ultrasound images of Cesarean scar and the adjacent normal skin were first captured using a 20-MHz ultrasound device (DermaScan\(^{\circledR}\); Cortex Technology, Hadsund, Denmark) with a gain profile of 3 and a level of 10. The ultrasound images were captured in the morning of the same day of delivery. The intra- and inter-rater reliabilities of echogenicity measurement were evaluated beforehand by inter-class correlation coefficient (ICC): the intra-rater reliability ICC (1,1) was 0.924 (95% confidence interval: 0.792-0.980) and inter-rater reliability of two different raters ICC (2,1) was 0.975 (95% confidence interval: 0.893-0.994).

4. Sample preparation

A Cesarean scar that was excised by an obstetrician during the delivery was immediately fixed in cold 4% paraformaldehyde/phosphate-buffered saline.
(PBS) solution (Wako Pure Chemical Industries, Osaka, Japan) for 24 h at 4°C. After fixation, the midpoint of the scar was trimmed and cryoprotected with 30% sucrose (Wako Pure Chemical Industries) in PBS for additional 24 h. The trimmed scar tissue was then embedded in optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan) and frozen using liquid nitrogen. Cryosections of 12-μm thickness were prepared and stored at −20°C until further analysis.

5. Hematoxylin-eosin (HE) staining
For HE staining, the cryosections were washed and stained with Mayer’s hematoxylin (Wako Pure Chemical Industries) for 2 min, followed by washing and counter-staining with 1% eosin Y (Wako Pure Chemical Industries) for 2 min. The cleared sections were mounted using Multi Mount 220 (Matsunami Glass Ind., Osaka, Japan) and observed using a bright-field microscope (ECLIPSE E600, Nikon Instech, Tokyo, Japan).

6. Immunohistochemistry
Immunohistochemistry against type I collagen or elastin was performed as follows. Cryosections were first air-dried, and antigen was retrieved with 10 mM citrate buffer (pH: 6.0) for 5 min at 121°C. The sections were washed with 0.1% Triton X-100 in PBS (PBST) and blocked with 2% normal goat serum in PBST for 1 h at 23°C. After blocking, the sections were incubated with mouse monoclonal anti-collagen I antibody (1: 100; ab90395, Abcam, Cambridge, UK) or mouse monoclonal anti-elastin antibody (1: 100; ab77804, Abcam) overnight at 4°C. The next day, the sections were washed and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1 : 200 : A11001, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at 23°C. The sections were finally washed, counter-stained with 2 μg/mL of 4',6-diamidino-2-phenylindole, and mounted with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific). All preparation slides were photographed using a fluorescent microscope (ECLIPSE E600) with a camera (DS-Fi1c, Nikon Instech) at the same exposure time. All the staining procedures were performed on all samples at the same time, and the image acquisition was done at the next day of staining.

7. Image analysis
The echogenicity of the normal and scar portions of the skin were quantified by the embedded program in DermaScan software using a Shape 4 rectangle (4.06 mm × 0.37 mm). The area–normalized echogenicity (range, 0–255) was calculated by multiplying the value of total intensity in percentage by 2.55 (255/100). The immunoreactivity of collagen or elastin was quantified on the fluorescent microscopy images using ImageJ software (National Institute of Health, Bethesda, MD, USA). The regions of interest (3–5 positions) were randomly selected within the normal or scar portion of the image, and the immunoreactive area and mean intensity (area–normalized intensity) were calculated. Using representative images of collagen or elastin immunohistochemistry, the threshold to distinguish collagen- or elastin-positive or negative was determined. In this study, the threshold was set at 25 in 256 (0–255) tones for all images. The average of the immunoreactive areas or mean intensities were used as indices of immunoreactivity for collagen or elastin.

8. Statistics
Data were expressed as means ± standard deviations for normally distributed data or by the boxplot (25th, 50th, and 75th percentile with min–max whisker) for non-normally distributed data. The data were first assessed for their normality using Shapiro-Wilk test with an alpha level of 0.05. The area–normalized echogenicity, collagen- or elastin–positive area, and collagen immunoreactivity were compared using Welch’s *t*-test or Mann–Whitney *U* test. The correlation between echogenicity and the collagen- or elastin–positive area was evaluated using Pearson’s product–moment correlation (coefficient denoted as *r*; for normal distribution of both variables) or Spearman’s rank correlation (coefficient denoted as *ρ*; for non–normal distribution of either variables). All statistical analyses were performed by using R statistical package (ver. 3.3.3 x64).

Results

1. Echogenicity of the scar and normal portions of the skin
The representative ultrasound images of scar and the adjacent normal skin are shown in Figure 1. The area–normalized echogenicity in the scar portion of the skin was significantly less than that in the normal portion of the same sample (Figure 1E, *p* = 0.009).

2. Structure of the scar and adjacent normal skin
Despite the lack of echogenicity in the scar (Figure
1), a compact and tight globular structure without any skin appendages or luminal structures was observed in the scar (Figure 2).

3. Collagen density and immunoreactivity of the scar and normal portions of the skin

Next, we sought to find differences in the skin components between the scar and normal portions of the skin. As shown in Figure 3, no significant difference was observed in collagen density or immunoreactivity between the scar and normal portions of the skin (Figure 3B, \( p = 0.15 \) for collagen density; Figure 3C, \( p = 0.23 \) for collagen immunoreactivity).

4. Elastin density of the scar and normal portions of the skin

As another component of the skin, elastin density was measured (Figure 4). The elastin-positive area was significantly less in the scar portion of the skin than in the normal portion of the skin (Figure 4B, \( p < 0.001 \)).

5. Correlation between echogenicity and the collagen- or elastin-positive area

Finally, the correlation between echogenicity and the collagen- or elastin-positive area was analyzed. Consistent with the immunohistochemistry (Figures 3 and 4), the correlation between echogenicity and the collagen-positive area was not significant (Figure 5A, \( r = 0.34, p = 0.17 \)), whereas a significant correlation was observed between echogenicity and the elastin-positive area (Figure 5B, \( r = 0.53, p = 0.026 \)).
Discussion

In this study, we aimed to demonstrate the relationship between ultrasound images and the actual skin structure characterized by collagen and elastin composition because there has been almost no consensus about the relationship of echogenicity with skin structures in literature.

In some studies, echogenicity was quantified as an index of collagen density or structural changes\(^{12-14,23}\). However, the literature describing the relationship between the actual skin structure and echogenicity is limited. In some studies, the ultrasound attenuation and backscatter coefficients were reportedly correlated with the skin structure\(^{17,18}\), the skin histology and echogenicity was just observed\(^{24}\), and the scar yielded low echogenicity\(^{25}\). Very recently, Minematsu et al. have demonstrated that echogenicity does not always correspond with dermal appearance, although the skin structure with appendages is consistent with the findings on the ultrasound images\(^{19}\). Therefore, the relationship between echogenicity and the skin structure is still ambiguous.

In this study, almost no echogenicity was observed in the scar tissue, although very compact structure comprising collagen alone was observed (Figures 1–3, 5). From these findings, it is plausible that collagen density is not the only determinant of

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Figure 3  Collagen immunohistochemistry of the scar and adjacent normal portion of the skin
(A) Collagen immunohistochemistry image of the scar and adjacent normal portion of the skin. (B) Comparison of the collagen-positive area. (C) Comparison of collagen intensity (immunoreactivity) in the collagen-positive area. Epi, epidermis; a.u., arbitrary unit. Scale bar in (A), 100 \(\mu\)m.

Figure 4  Elastin immunohistochemistry of the scar and adjacent normal portion of the skin
(A) Elastin immunohistochemistry image of the scar and adjacent normal portion of the skin. (B) Comparison of the elastin-positive area. Epi, epidermis. Scale bar in (A), 100 \(\mu\)m.
echogenicity. In contrast to collagen fibers, the amount of elastin was significantly correlated with echogenicity (Figures 4, 5). Elastic fibers comprising elastin and accessory proteins are important for maintaining skin elasticity by supporting collagen fibers. Considering that echogenicity increases as structural complexity increases, skin echogenicity may be related to the elastin-dependent collagen complexity rather than to the collagen content.

Histologically, the more elastic fibers present in the tissue, the tissue becomes more elastic and flexible. In contrast, the hard and inelastic scar tissue is considered to be more vulnerable to external forces. Therefore, the amount of elastic fibers in the scar can be used as a predictor of susceptibility against pressure that leads to recurrence of pressure ulcer. In this study, the amount of elastin was found to be correlated with the echogenicity; thus the echogenicity could be one of the possible parameters that can predict pressure ulcer recurrence. To verify this, our team is now preparing a clinical observational study focusing on the relationship between ultrasound images and pressure ulcer recurrence.

Obesity has been found to affect dermal echogenicity in Japanese males; thus the same might be true for the female participants in this study. However, there were no significant correlations between BMI versus echogenicity, collagen-positive area, or elastin-positive area (echogenicity: \( r = 0.22, p = 0.57 \); collagen: \( r = -0.08, p = 0.84 \); elastin: \( r = -0.38, p = 0.31 \); data not shown). Therefore, BMI can be considered as having no effect on the dermal echogenicity in this study, although this might be just because of the different sex (male or female).

Because scarring is an abnormal pathological condition of the skin, it might not be appropriate to compare it with normal skin. However, collagen immunoreactivity was not significantly different between the scar and normal skin (Figure 3C), implying the normal properties of collagen fibers.

There is a limitation regarding the feasibility in a clinical setting: the ultrasound device. In this study, a high frequency (20 MHz) ultrasound device was used to visualize the shallow layer of the skin; however, the probe of commercially-available portable ultrasound device has a frequency usually less than 10 MHz. Further studies with a conventional probe should be warranted for clinical application, although the scar is distinguishable from normal dermis and it might be relatively easy to be visualized.

**Conclusion**

By comparing skin collagen and elastin distribution with ultrasound images using Cesarean scar tissue, we demonstrated that skin echogenicity was not only determined by collagen fibers but also by the amount of elastic fibers.
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Conflict of interest

The authors have declared that no competing interests exist.

References


原著

高周波超音波診断装置を用いた皮膚イメージングと皮膚内部組織の比較: コラーゲン・エラスチン量とエコー輝度の関係

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要 旨

背景：高周波超音波診断装置（エコー）は皮膚の非侵襲的イメージングに用いられているが、実組織との関係を検討した知見は少ない。本研究の目的は、エコー画像と実際の皮膚組織に着目し、手術の際に除去される帝王切開痕を用いて比較することである。方法：本研究では、書面によるインフォームドコンセントを得られた12名の妊娠を対象とした。出産前に瘢痕部と周囲皮膚をエコーで撮影した。産科医によって瘢痕組織が採取され、コラーゲン・エラスチン染色に供された。染色画像を用いてコラーゲン量とエラスチン量の定量を行った。結果：瘢痕部は密な構造であったが、低エコーであった。エコー輝度とエラスチン陽性面積に有意な相関が認められたが、コラーゲン陽性面積とは有意な相関は認められなかった。結論：エコー輝度はコラーゲンの量や密度よりもむしろ、エラスチンの量より相関することが明らかとなった。

キーワード：超音波診断、コラーゲン、エラスチン、真皮、瘢痕

キーメッセージ
1. 今回の研究は看護・介護のどのような問題をテーマにしているのか？
   研究を行うきっかけとなったことはどのようなことか？
   皮膚や創内部を可視化するために超音波診断装置（エコー）が用いられているが、エコー画像と実組織を比較した研究は少ない。
   エコー画像と、皮膚内部の構造タンパク質（コラーゲン・エラスチン）の関係を明らかにすることをテーマとした。
2. この研究成果が看護・介護にどのように貢献できるのか？あるいは、将来的に貢献できることは何か？
   エコー輝度の高低に加えて、実際に皮膚の内部で起こっている現象を推定できるようになると期待される。
3. 今後どのような技術が必要になるのか？
   エコー輝度と内部組織の対応に加えて、皮膚機能（組織耐久性や弾性など）を評価する必要がある。