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Mid-Long Term Effect of Non-Ablative High Radiofrequency Therapy on the Rabbit Dermal Extracellular Matrix*

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Abstract— **This study quantitatively investigated the postoperative effects of radiofrequency (RF) application on the normal dermal extracellular matrix (ECM) of *in vivo* rabbits. Postoperative effects were evaluated by histology and atomic force microscopy analysis of dermal tissues treated using three RF energy levels (10~30 W) and either a single- or multiple-pass procedure. Progressive changes in the morphology of rabbit dermal ECMs were investigated over a 30-day postoperartive period. All RF-treated groups, except for the low energy group (10 W), displayed more prominent inflammatory responses compared to the control. This inflammatory reaction was more prominent a day after application. Dermal tissues 30 days after RF application exhibited prominent myofibroblast activity associated with ECM contractile activity during wound healing in addition to chronic inflammation. A decrease in the morphology of dermal ECMs after RF application continued until seven days postoperatively. The ECM diameter increased to near baseline at 30 days postoperatively. Low energy and multi-pass applications resulted in greater collagen fibril contraction and recovery at the ultra-structural level at 30 days postoperatively than did a single high energy application.**

I. Introduction

A non-surgical, non-ablative radiofrequency (RF) application based on the generation of a powerful electro-thermal effect throughout the dermis has been developed as an effective method to improve skin laxity. The mechanism underlying the improvement of facial wrinkles via RF therapy is generally accepted to be the initial induction of skin collagen contraction as a result of the heat generated by RF and subsequent regeneration throughout the following weeks [1]. The Radiowave Technology urgitron Dual Frequency™ RF (Elman International) is one of most commonly used RF devices that employs a proprietary capacitive coupling method to facilitate more efficient transmission of heat into the dermis without damaging the epidermis [2]. This technology is expected to attain a better therapeutic effect with a lower risk of complications compared to other therapies.

From clinical study, it is difficult to qualitatively evaluate the effect of improvement after RF and to define appropriate treatment durations and intensities. Therefore, in the present study we examined the effect of RF on the changes in the collagen fibril diameter of normal rabbit skin with atomic force microscopy (AFM) that was previously used for the short-term investigation of the therapeutic effect of RF application on animal skin [3]. The collagen fibril diameter was assessed on the nano-scale one month after treatment to determine the appropriate duration and intensity of treatment using AFM.

II. Materials and Methods

A. Animal

A 25 male New Zealand white rabbits supplied by a single breeder were used. Rabbits were six months old with a mean weight of 2.5±0.4 kg. Each animal was housed individually in a standard rabbit cage maintained at 22~24°C with alternating 12-hour light/dark periods. Adequate anesthesia was administered prior to all procedures to minimize pain. All animal use procedures were approved by the Ethical Committee of Kyung Hee University College of Medicine (KHMC-IACUC11-024) and were in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

B. RF Application

Animals were anesthetized by an intramuscular injection of 15 mg/kg ketamine and 6 mg/kg xylazine. Additional ketamine and xylazine were properly administered as needed to maintain the level of anesthesia during the experiment. Coupling mono-polar RF energy was administered to the four limb regions of each rabbit through a non-ablative skin tightening handpiece using a Surgitron Dual FrequencyTM RF Device (Ellman International Inc., Oceanside, NY). The neutral plate of the device was placed on the abdomen of each rabbit. RF Cool Gel DHPG8 (Ellman International Inc.) was used to cool the outer surface of the rabbit skin while RF energy heated and modified the soft tissue beneath the skin. Each rabbit was treated with a power of 10, 20, and 30 W and a pulse duration of 1200 msec, receiving single (1-) and multiple (3-) passes. A total of 100 skin punch biopsies (Ø 4 mm, biopsy punch, Stiefel Laboratorium, Offenbach/M., Germany) were harvested from all four limbs of a single

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control rabbit (n=4), and 24 RF-treated rabbits (n=96) at postoperative days 0, 2, 7 and 30. Biopsy specimens were taken adjacent to the treated site from the clipped back of each rabbit. Each biopsy specimen was immediately fixed in 4% buffered paraformaldehyde and stored until subsequent histological and AFM assessments.

C. Histology

Fixed tissues were embedded in paraffin and sectioned at 2~3 μm on the plane perpendicular to the surface of the specimen. Standard hematoxylin-eosin (H&E) and Masson's trichrome (MT) staining was performed and the results were evaluated by two pathologists blinded to the experimental conditions. The expression of substrate was calculated for each tissue using a computerized software analyzer.

D. Histology

AFM topographic images were obtained at postoperative days 0, 2, 7, and 30 using a NANOS N8 NEOS (Bruker, Herzogenrath, Germany) equipped with a 42.5×42.5×4 μm^3 XYZ scanner and two Zeiss optical microscopes (Epiplan 200×/500×). Each dermal surface was scanned in air with a scanning area of 5×5 μm^2 and a scan speed of 0.8 line/sec. AFM tapping mode imaging was performed under 35% relative humidity using the silicon cantilever with an integral pyramidal shaped tip (SICONG, Santa Clara, CA). The nominal tip radius and height were <10 nm and 12~16 μm, respectively. Six representative microscopic topographical images per time point for each group were used for quantitative analysis of the degree of irreversible collagen fibril injury. The diameter of rabbit dermal collagen fibrils was measured by two observers blinded to the experimental conditions using a Scanning Probe Image Processor (SPIP version 4.8, Image Metrology, Lyngby, Denmark) and Gwyddion software.

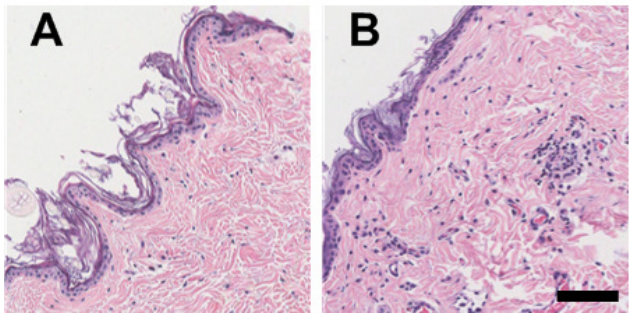

Figure 1. Representative histological (H&E, 200×) images of dermal tissue. (A) Normal dermal tissues showing normal epithelium and epidermis, (B) RF-treated dermal tissues 30 days after application (20 W, multiple-pass) showing thickened epidermis and increase in cells. Scale bar = 100 μm.

D. Statistics

The density of MT staining is expressed as the mean ± standard deviation. The dimensions of dermal collagen fibrils are expressed as means ± standard errors. Statistical analysis was performed to compare the density differences in collagen fibrils between groups using a multiple range of one-way ANOVA. A Friedman test was performed to compare the morphological differences in collagen fibrils at each time point. P-values < 0.05 were considered significant.

III. Results and Analysis

A. Inflammatory Response

On postoperative day 0 (immediately after RF application), the dermal tissues treated with more than 20 W showed significantly more prominent acute inflammatory responses compared to the control. Inflammation increased with a greater number of passes and as time passed. On postoperative day 30, RF-treated animals showed chronic inflammation. There was an increase in the thickness of the epidermis or granular cell layer, associated with the development of rete ridges, but this difference was not significant.

B. Myofibroblast Activation

On postoperative day 30, the dermal tissues treated with RF energy showed more prominent myofibroblasts, associated with reflecting contractile activity of the ECM during wound healing compared to the untreated dermal tissues. Interestingly, groups that underwent multiple passes of treatment at 20 W, about 110-fold increase, showed greater myofibroblast/collagen density (>60%, $p < 0.001$, ANOVA).

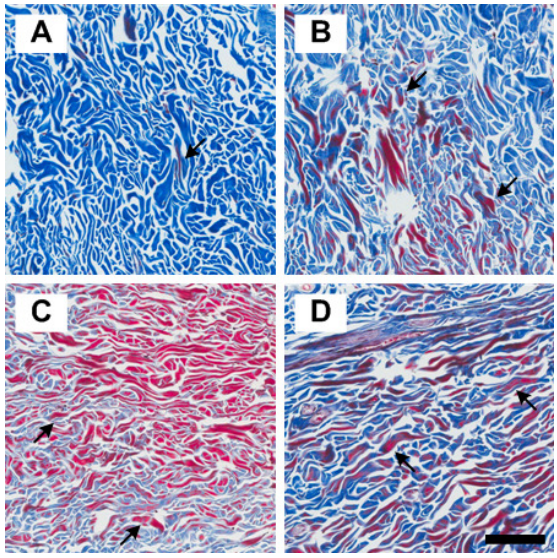

Figure 2. Representative histological (MT, 200×) images of (A) normal dermal tissues and RF-treated dermal tissues 30 days after application with multiple passes of (B) 10 W, (C) 20 W, and (D) 30 W. Myofibroblasts are indicated by red staining (arrows). Scale bar = 100 μm.

C. Nanostructural Change

The control rabbit showed an irregular parallel arrangement of collagen fibrils with clear axial periodicity. The mean diameter was 116.38±3.18 nm (n=50) and the mean fibrillary D-periodicity was 67.86±0.40 nm (n=50), ranging from 62.60~72.90 nm. All RF-treated rabbits showed irreversible collagen fibril injury and either increased or decreased diameter compared to the control. The collagen contraction and recovery was more prominent in the multiple pass procedure than in the single pass procedure. The lower energy

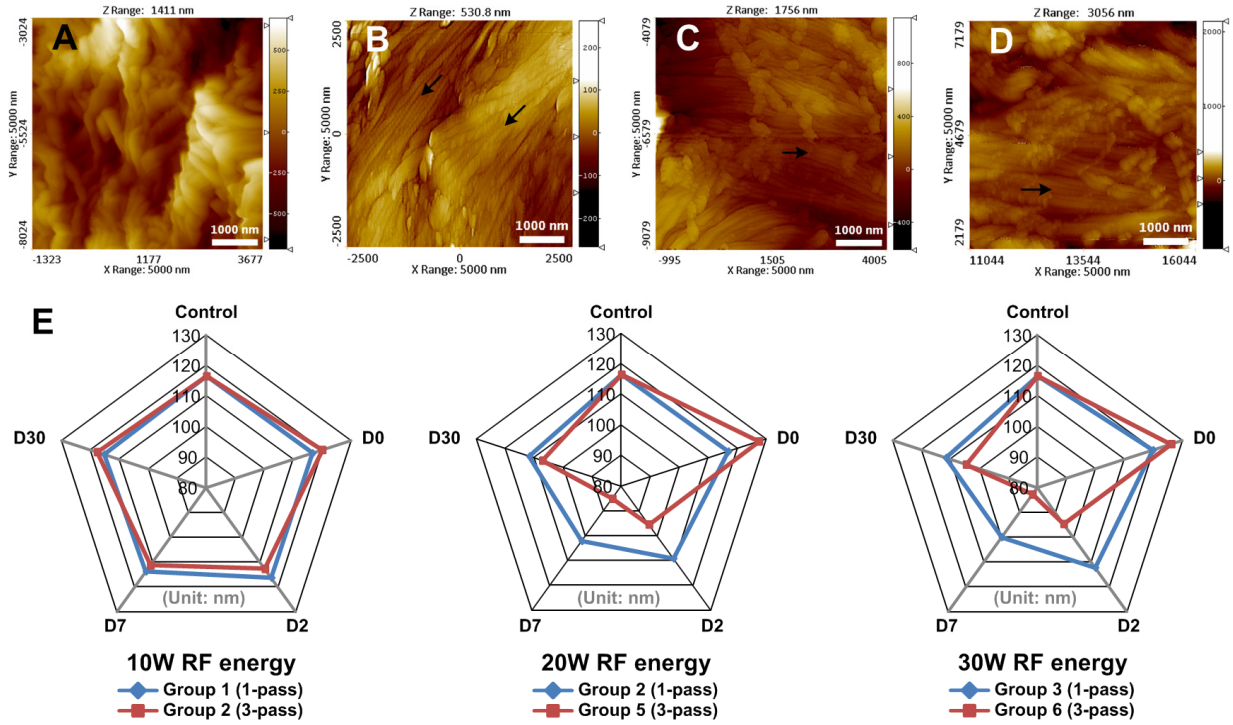

Figure 3. Representative AFM tapping mode topographical images (5×5 μm^2) of (A) normal dermal tissues and RF-treated dermal tissues (B) 2 day, (C) 7 day, and (D) 30 day post-treatment with multiple passes of 20 W. All RF-treated dermal tissues showed irreversible collagen fibril injury and either increased or decreased diameter as well as a well-aligned collagen structure (arrows) compared to the control. Scale bar = 1000 nm. (E) Effects of 10, 20, and 30 W energies applied with the same RF pass procedure on the diameter of rabbit dermal collagen fibrils over the 30-day post-treatment period.

(10 W) treatment resulted in less fibril contraction compared to the higher energy (over 20 W) application.

D. Analysis

This study quantitatively evaluated the nanostructural effects of RF application on the morphology of dermal ECMs in live rabbits using a representative RF machine over a 30-day post-treatment period. Collagen contraction was defined as a decrease in both the diameter of dermal collagen fibrils and the ratio of myofibroblast density to collagen density. Progressive collagen remodeling and production was defined as a change in the dimensions of the dermal collagen fibrils. This study used AFM combined with histological assessments as a novel method to examine the short and mid-long term effects of RF applications on dermal tissues through morphological changes in collagen fibrils.

This study has three main findings; (i) RF application led to a decrease in the diameter of collagen fibrils after two days postoperatively. The decreased fibril diameter recovered to near baseline during the 30 days following the seventh postoperative day; (ii) RF application caused the irregular parallel arrangement of collagen fibrils to become regular with clear axial periodicity; (iii) A greater number of RF passes and a higher energy level promoted a change in the dimensions of the collagen fibrils.

The finding of an immediate decrease in collagen fibril diameter after RF application is consistent with previous reports [4]. RF application caused thermal injury, which completely or partially denatured the fibril helix, possibly injuring dermal collagen fibrils and causing swelling [5]. This injury was likely the catalyst for the wound healing response. This decrease is also thought to be caused by the release of various chemotactic factors [6]. In a study that used transmission electron microscopy (TEM) to study tendons, Alaseirlis et al. [7] found that the diameter of injured collagen was smaller than that of normal collagen. They suggested TNF-α, an inflammatory cytokine, influenced wound healing by regulating macrophage differentiation and down-regulat ing ECM production. Our histological and AFM findings are consistent with those findings. On postoperative days 2 and 7, RF-treated skin showed more inflammatory cell ingrowth than untreated skin. Images from AFM showed smaller diameter and regular parallel arrangement of dermal collagen fibrils in RF-treated skin than in untreated skin. The next healing phase, proliferation, was characterized by an increase in fibril diameter as a result of DNA synthesis for Type-III collagen, and an influx of water and cellular components. The remodeling stage was distinguished by a decrease in matrix synthesis and an increase in Type-I collagen synthesis, leading to tightening of the skin matrix. These findings agreed with the present histopathologic and AFM results.

RF application has been recognized as the most impressive method for skin rejuvenation. Negative side effects of the treatment include tissue overheating due to improper protocols. The original protocol called for a single pass of high energy, which caused significant side effects such as scars and skin indentations [8] as well as patient discomfort. Using TEM to analyze collagen fibril architecture, Kist et al. [4] found similar changes after multiple passes of low energy compared to a single pass of high energy. These findings agree

with ours, in that irreversible collagen fibril injury occurred not only with increasing energy level, but also the number of passes.

IV. Summary

This study found RF application directly led to collagen contraction and recovery. It may have caused tissue tightening due to the breakage of intramolecular hydrogen bonds. Similar to previous reports, our results suggest that collagen fibril contraction after RF tissue-tightening stimulates a collagen repair and synthesis process that likely gives rise to the tightening of the dermal tissue that is observed clinically. The results suggest that low energy and multi-pass application results in greater collagen fibril contraction and recovery at the nanostructural level than a single high energy application. It appears that low energy application leads to more consistent results as well as greater patient comfort and safety. Limitations of this study include the small sample size and use of normal tissues. Further studies are required to evaluate the long-term effects of various protocols for RF tissue-tightening application using animal models with damaged skin and biological factors.

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