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Evaluation of the Wound Healing Response After Deep Dermal Heating by Fractional Micro-needle Radiofrequency Device

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ABSTRACT

Background: Fractional Radiofrequency Microneedles (FRM) are minimally invasive devices that use inserting bipolar radiofrequency for deep dermal heating, has been introduced. We investigated the tissue response after FRM according to different energy levels in porcine skin.

Methods: Porcine back skin was used in the study. A FRM device was composed of 49 insulated needles. Needles were vertically inserted with 1.5mm depth and four different energy levels were used to examine wound healing response chronologically. Histologic evaluation was done by hematoxylin & eosin (H&E) and heat shock proteins (HSP) 47 staining for immediately after, 2 days after, 14 days after, 28 days after and 10 weeks after the procedure. RT-PCR was done for various cytokines including HSP47, HSP72, metalloproteinase (MMP), and extracellular matrix (ECM) proteins.

Results: FRM treatment generated a thermally coagulated zone localized in the reticular dermis, without damaging the epidermis. The coagulation necrosis zone in H&E staining was replaced by new collagen tissue over 10 weeks. RT-PCR studies revealed an increase in HSP, MMPs, and ECM proteins. In the high energy level procedure, an increased number of fibroblasts were found.

Conclusion: FRM treatment induced a dermal remodeling process including neocollagenesis in the deep dermis. From this result, FRM is expected to provide a good and positive efficacy for skin rejuvenation.

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INTRODUCTION

Various methods have been utilized to rejuvenate aged skin, skin laxity, and facial rhytides. Ablative laser resurfacing had been the gold standard dermatologic approach for facial rejuvenation.[1-3] Although the ablative laser resurfacing is highly effective, the risk of unwanted side effects was high and the postoperative recovery period was too long.[4,5] Non-ablative skin resurfacing was introduced in an effort to minimize healing time and patient discomfort,[6,7] but the post-treatment effects were unsatisfactory. To overcome the limitations of both ablative and non-ablative laser resurfacing, fractional resurfacing was developed.[8-10] However, the conventional fractional laser system used the epidermis as energy channels but the risk for epidermal burn and hyperpigmentation could not be avoided, and it was impossible to deliver high energy exclusively deep in the dermis.[11,12]

Recently, fractional radiofrequency microneedles (FRM) were introduced for skin rejuvenation.[12-14] By delivering electric energy by using minimally a invasive bipolar radiofrequency microneedle device, it can create thermal damage in deep dermis. In this study, we studied the wound healing response in porcine subjects after the FRM procedure using histologic, immunohistochemical, and molecular techniques.

METHODS

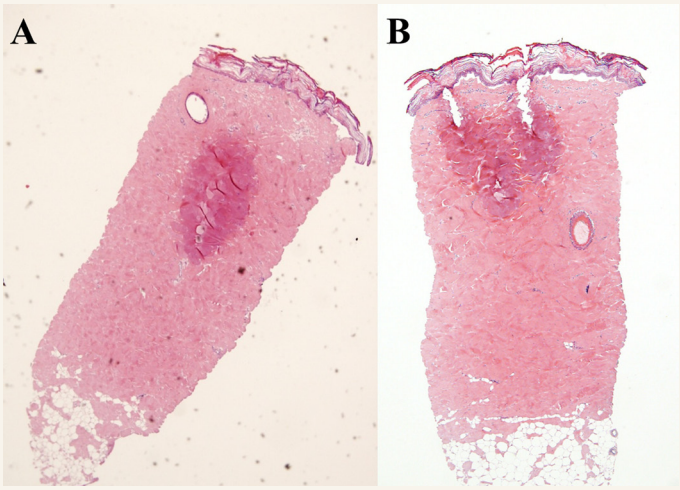
Animal Study Using FRM System

The FRM system (INTRAcel; Jeisys, Seoul, Korea) delivered bipolar RF energy directly within the dermis via 49 micro-needle electrodes in 1cm^2 area. The micro-needles electrodes were nonconductive except for the tip to protect the epidermis. Needle length can be chosen (0.5mm, 0.8mm, 1.5mm, 2.0mm) for

TABLE 1.

Different Energy Levels Used in FRM Treatment			
	Watts/cm²	msec	mJ/cm²
EL1	12.5	100	1250
EL2	32	50	1600
EL3	50	50	2500
EL4	12.5	500	6250

FIGURE 1. H&E staining images after FRM treatment. Immediately after the procedure (EL2) **a)** x40), coagulation necrosis zone was observed in deep dermis. Two nearby necrotic columns were connected to each other making the form of bridges in high energy level treatment (EL3) **b)** x40).



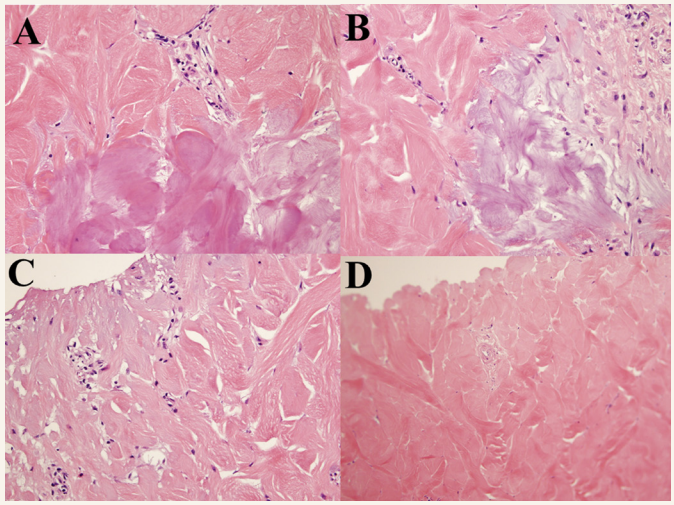
various clinical situations. Needles automatically penetrate into the skin vertically, and RF is emitted 0.2 seconds after needle inserting for safety. The amount of RF emission differs according to the energy level.

One micropig, aged 4 months, was enrolled. General anesthesia was given using enflurane and oxygen. The hair of the minipig was cut with electrical cutters. The micropig underwent treatment on their back using a FRM system with different energy levels (Table 1). To evaluate the wound healing response following FRM treatment, biopsies were taken immediately after FRM treatment, at 2 days, 14 days, and 28 days, and at 10 weeks after FRM treatment from the micropig.

Histologic Studies and RT-PCR Studies

To determine the deep dermal heating effects of FRM treatment, the wound healing responses were assessed histologically. Immediately after FRM treatment, 2 days, 14 days, and 28 days, and at 10 weeks after FRM treatment, two 2 mm biopsies were performed from each of the energy level sections. The biopsies from each area of different FRM treatment were fixed in 10% formalin overnight and paraffin-embedded. The paraffin sections were sliced and stained with hematoxylin & eosin (H&E) and heat shock protein (HSP) 47.

FIGURE 2. H&E staining images after FRM treatment showed the dermal wound healing process (EL3). Inflammatory cell infiltrations surrounding the coagulation necrosis were found at day 2 **a)** x200). At day 14, damaged collagens were started be absorbed **b)** x200). At day 28, the proliferation of young fibroblasts was obvious **c)** x200). At day 70, the lesion is almost replaced by new collagen **d)** x200).



To assess the changes in gene expression in dermal remodeling after FRM treatment, a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used. Two 1.5 mm biopsies were taken from each of the energy levels immediately after FRM treatment, 2 days, 14 days, and 28 days, and 10 weeks after FRM treatment. ß-Actin, ß-globin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a housekeeping gene. The ratios of amplification density were calculated by dividing the intensity value for the gene of interest by the intensity value for housekeeping gene.

RESULTS

Deep Dermal Fractional Heating was Repaired With Time

In order to assess the tissue responses immediately after FRM treatment, histologic evaluation was done by H&E staining. Immediately after FRM treatment, the thermally damaged area was apparent in the deep reticular dermis separated by an almost-intact epidermis (Figure 1A). In certain instances, two nearby necrotic columns were connected to each other making the form of bridges (Figure 1B).

To assess the inflammatory responses and the collagen synthesis after FRM treatment, histologic evaluation was done using H&E staining. The deep dermal fractional heating initiated the wound healing process. Scant infiltration of inflammatory cells

FIGURE 3. HSP47 staining images after FRM treatment (EL4). Minimal HSP47 expression was observed immediately **a)** x200). The intensity of HSP47 expression progressively increased through day 14 **b)** x200), and remained prominent at day 28 **c)** x200) and 10 weeks **d)** x200), demonstrating the neocollagenesis after FRM treatment.

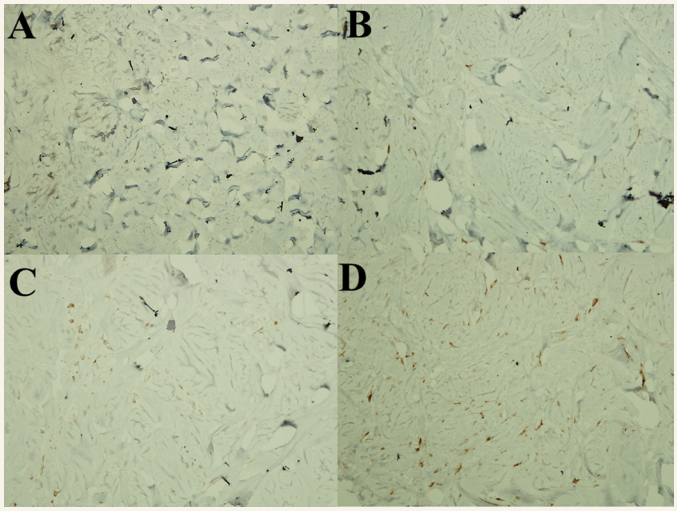
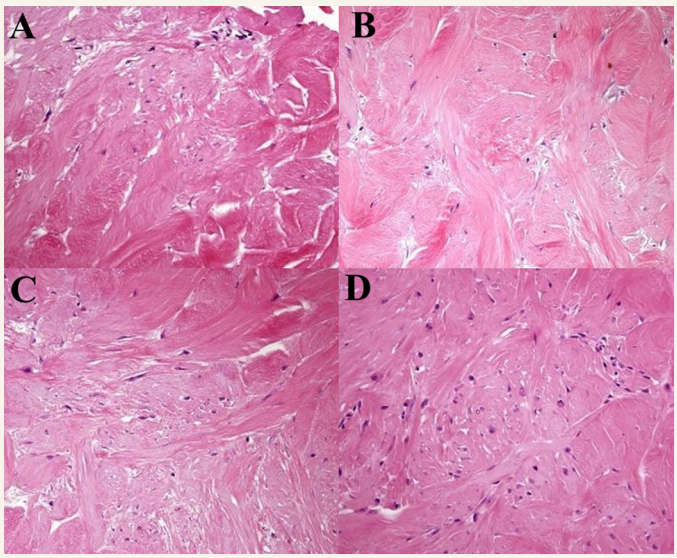


FIGURE 4. H&E staining images after 70 days following FRM treatment of each energy level were compared **a)** EL1; **b)** EL2; **c)** EL3; and **d)** EL4; x200). Higher energy level treatment **c)** and **d)** correlates with more collagen fiber production and increased cellularity.



around the treated areas was observed by 2 days after FRM treatment (Figure 2A). The number of inflammatory infiltrates had increased to 14 days after FRM treatment (Figure 2B). After 28 days of FRM treatment, the inflammatory infiltration was reduced, however, proliferation of the young fibroblasts was found (Figure 2C). In addition, the thermally coagulated collagen after FRM treatment was replaced by new collagen. Fourteen days after FRM treatment, the damaged collagen was partially absorbed

FIGURE 5. HSP47 staining images for 70 days **a)** EL 3 and **b)** EL4; x100). HSP47 expression is increased in high energy level treatment **b)**. It means higher energy level is related with more collagen production.

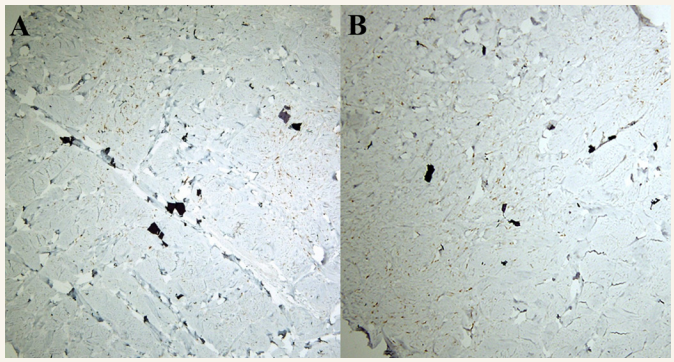
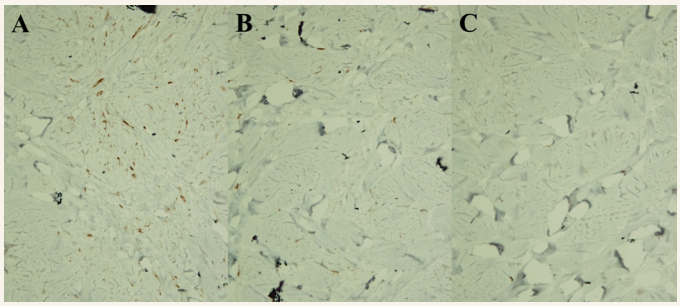


FIGURE 6. HSP staining images for 70 days following EL 3 FRM treatment. HSP47 expression was increased both in the target areas **a)** x200) and between two coagulation columns **b)** x200). However, no expression of HSP47 was observed beyond the treatment depth **c)** x200).



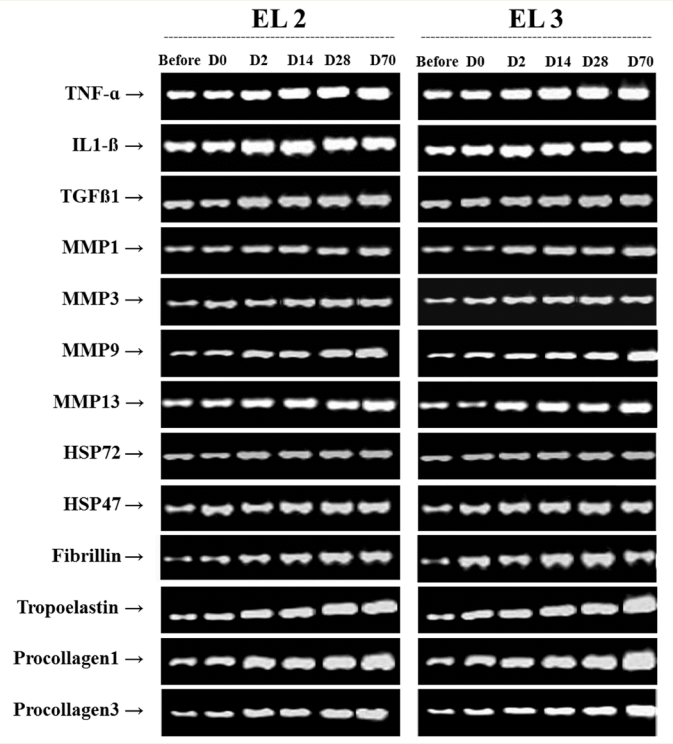
and the irregularly arranged new collagen tissue replaced some of the degenerated lesion (Figure 2B). The damaged collagen was fully replaced by 70 days after FRM treatment (Figure 2D).

Immunohistochemical staining for HSP 47, the chaperone of procollagen, was done to assess the neocollagenesis after FRM treatment. HSP 47 also showed a wound healing response after the FRM treatment. After 28 days of FRM treatment (Figure 3A), the HSP47 staining started to be observed and increased HSP staining was found up until 70 days after FRM treatment (Figure 3B).

Neocollagenesis After FRM Treatment is Correlated With Degree of Damage

In order to determine whether neocollagenesis had correlated with the degree of damage after FRM treatment, various energy levels (level 1 to 4) were used to assess the collagen synthesis, proliferation of fibroblasts and HSP47 expression. After 70 days of FRM treatment, the damaged collagen was fully replaced after FRM treatment (Figure 4). Compared to the new collagen of low level FRM treatment (level 1 and 2; Figure 4A and 4B), that of high level FRM treatment (level 3 and 4; Figure 4C and 4D)

FIGURE 7. RT-PCR results of FRM treatment. Among the MMPs, the MMP-9 and MMP-13 showed increased gene expression after 2 days of FRM treatment. The gene expression of HSP 47 also increased after 2 days of FRM treatment. In addition, the gene expressions of extracellular matrix proteins were increased.



was thicker and denser. In the high level FRM treatment (level 3 and 4; Figure 4C and 4D), the proliferation of fibroblasts were evident, on the other hand, in the low level FRM treatment the proliferation of fibroblasts were scant (level 1 and 2; Figure 4A and 4B). In addition, the immunohistochemical staining for HSP 47 also showed correlation with the degree of damage. The HSP 47 staining of level 4 FRM treatment (Figure 5B) showed a greater amount of positive staining as compared to that of level 3 FRM treatment (Figure 5A).

Changes After FRM Treatment are Confined to Treatment Depth

To examine the depth of the collagen remodeling induced by FRM treatment, we observed HSP47 staining after FRM treatment. HSP 47 was markedly increased in the area of FRM treatment (Figure 6A). The dermal area between the FRM treatment areas also demonstrated a moderate increase in the HSP 47 staining (Figure 6B). However, regardless of the energy level, no expression of HSP47 was observed beyond the treatment depth (Figure 6C).

Molecular Changes of the Wound Healing Process Were Found After FRM Treatment

To further examine the changes after FRM treatment, RT-PCR was performed to compare the mRNA expression immediately after FRM treatment, 2 days, 14 days, and 28 days, and 10 weeks after FRM treatment (Figure 7). The genes invested were selected based on the previous studies.[12,15] They were cytokines (TNF-α, IL-1ß, and TGF-ß1), heat shock proteins (HSP72 and HSP47), metalloproteinases (MMP-1, MMP-3, MMP-9, and MMP-13), and extracellular matrix proteins (Fibrillin, Tropoelastin, Procollagen1, and Procollagen 3). Compared to the control, the gene expression of cytokine remained stable. Among the MMPs, the MMP-9 and MMP-13 showed increased gene expression after 2 days of FRM treatment. The gene expression of HSP 47 also increased after 2 days of FRM treatment. In addition, the gene expressions of the extracellular matrix proteins had increased. Among the extracellular matrix proteins, the gene expression of tropoelastin and procollagen 1 were more than 3 fold greater than that of control by day 70 after FRM treatment.

DISCUSSION

To rejuvenate the aged skin, many treatment modalities including ablative and non-ablative lasers were introduced. Ablative lasers such as the CO2 laser or Er:Yag laser showed satisfactory results.[16,17] However, these ablative lasers had caused significant down-time, prolonged erythema, pigmentation change, and even scarring.[11,18] On the other hand, non-ablative lasers, which can be used with fewer side effects, showed less favorable results.[6,7] To overcome the limitation of previous laser treatment, fractional photothermolysis was introduced to treat photoaged skins, periorbital wrinkles, and acne scars and showed effective results.[8-10] After the treatment of fractional photothermolysis, multiple small thermal injuries surrounded by viable tissue were left and the healing process was accelerated by surrounding viable tissue.[19] However, the conventional fractional photothermolysis had limitations: epidermal damage over the thermal injury zone and inaccurate control of treatment depth.[13,20] Recently, FRM were introduced to overcome the limitations of conventional fractional photothermolysis. The FRM device delivered thermal energies induced by bipolar radiofrequency using microneedles.[12,14] In this study, we investigated the wound healing response in porcine subjects after the FRM treatment using histologic, immunohistochemical, and molecular techniques.

We found that the thermally damaged area was located in the reticular dermis and that the coagulated collagen was separated by an intact epidermis and dermis. In addition, we found that the size of thermally damaged dermis can be controlled by changing the delivered energy level. These results suggest that a FRM device can produce dermal fractional damage without damaging the overlying epidermis. In addition, we also found that FRM treatment initiated the wound healing process. Fourteen days after FRM treatment, inflammatory cell infiltration was found and proliferation of fibroblasts was found after 28 days of FRM treatment. The absorption of damaged collagen and new colla-

gen tissue can be observed after FRM treatment. These results are consistent with the previous report that the FRM treatment started the wound healing response of inflammation, proliferation, and remodeling.[12]

It was reported that HSP47 was increased when the collagen synthesis increased and that HSP47 acts as molecular chaperone in collagen synthesis.[21] To assess the collagen synthesis after FRM treatment, the immunohistochemical staining for HSP47 was done. We found that the HSP47 staining was evident after 28 days of treatment and increased up until the 70 days after treatment. These results supported the histologic study that the FRM treatment induced neocollagenesis. Moreover, the increased HSP47 staining after 70 days of treatment suggests that the effect of FRM treatment in skin rejuvenation can persist as long as 10 weeks after treatment.

"To assess the collagen synthesis after FRM treatment, the immunohistochemical staining for HSP47 was done."

In this study, the results of RT-PCR of inflammatory cytokines were stable, while gene expression of heat shock proteins, metalloproteinases, and extracellular matrix proteins had increased. Among the increased matrix proteins, fibrillins and tropoelastins, which consist of elastic fiber, had increased as well as procollagens. Metalloproteinases, which degrade degenerated dermis, are important in the wound healing response. In a previous study using a CO2 laser,[15] ablative laser resurfacing induced an increased expression of MMP-1, 3, 9, and 13. However, the temporal pattern of increased expression was different: the increased expression of MMP-1 and MMP-3 had) decreased, while the increased expression of MMP-9 and MMP-13 had been sustained. In this study, the expression of MMP-9 and MMP-13 had increased until after 10 weeks of treatment, while MMP-1 and MMP-3 did not show significant change. Based on these results, we supposed that the FRM treatment did not initiate the prominent inflammatory process, which is induced by inflammatory cytokines and collagen degradation by MMP-1 and MMP-3. These result are in accordance with a previous report, which used another FRM system.[12] In addition, the sustained high expression of MMP-9 and MMP-13 after FRM treatment could act important role in the skin rejuvenation. Orringer et al[15] suggested that the sustained increased expression of MMP-9 could facilitate the formation of new collagen by degrading the residual degenerated collagen, while MMP-13 was associated in the remodeling of newly synthesized collagen. In another study,[22] the MMP-9 and MMP-13 during the late phase of wound healing process was necessary in the scarless healing in nude mice. Based on the RT-PCR results and previous studies, we can suggest that the FRM treatment can induce neocollagenesis and the remodeling of collagen in dermis without significant inflammation and destruction of the dermis.

The conventional fractional photothermolysis treatment can induce diverse complications.[23-25] Among the complications, the postinflammatory hyperpigmentation is troublesome in darkly pigmented patients.[23,24] The postinflammatory hyperpigmentation can be associated with the epidermal damage after fractional photothermolysis treatment. In the conventional fractional photothermolysis, increasing the depth and the size of coagulated dermis allowed for increased damage in epidermis and the risk of complications. However, the FRM treatment has clear advantages: the epidermis is intact after FRM treatment. In addition, we found that by the adjustment of the energy level, control the thermal zone size for FRM treatment can occur. In addition, the FRM treatment system used in this study also can control the depth of thermally coagulated zone by adjusting the insertion depth of the electrode needle. The FRM system can provide the safe treatment while overcoming the limitations of conventional fractional photothermolysis.

In conclusion, the 10 week observation of the FRM treatment has shown dermal remodeling process with the increased production of HSP47 and procollagen, and a full replacement of thermally denatured collagen into a new collagen. In addition, the gene expression of metalloproteinases and elastin was also increased in RT-PCR. Thermally denatured collagens were introduced in the reticular dermis without damaging the epidermis and could be controlled by adjusting the energy level and electrode needle depth. The Fractional Radiofrequency Microneedle system can be expected to provide a reliable treatment option for a skin tightening, wrinkle reduction, scars, and pore treatment.

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DISCLOSURES

The authors have not declared any conflicts of interest.

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