

Histological examination of skin tissue in the porcine animal model after simultaneous and consecutive application of monopolar radiofrequency and targeted pressure energy

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Abstract

Background: The cosmetic appearance of skin is substantially influenced by the organization of connective fibers and underlying subcutaneous tissue. It has been previously documented that radiofrequency and pressure energies alone are able to improve skin appearance; however, detailed histological evaluation should be done to determine their synergistic effect.

Aims: This histological study investigates the difference between simultaneous and consecutive application of monopolar radiofrequency with targeted pressure energy on porcine skin.

Methods: In a total of four weekly abdominal treatments, simultaneous emission of the energies was applied to two pigs (12 minutes per session); additionally, two pigs were treated consecutively (12 + 12 minutes per session). The 5th pig served as a control subject. Biopsies were obtained at baseline, after the 4th treatment, and at 1-month follow-up. Primary outcomes were to document changes of dermal and hypodermal tissues.

Results: In the treated subjects, the amount of collagen and elastin fibers increased significantly ($P < .001$). At follow-up, simultaneous application showed a significantly higher increase in collagen and elastin fibers (by 59% and 64%, respectively), when compared to consecutive. Thickness of the dermis increased more in the pigs treated simultaneously (+848.8 μm /50.17%; $P < .001$). Treated tissue also showed the upper part of dermis to be rich in blood vessels and better organized interlobular septa in hypodermis. No significant change was observed in the control subject.

Conclusion: Simultaneous application produces significantly more profound changes, when compared to consecutive treatment. Further research is needed but our findings represent a new potential treatment of various skin conditions like cellulite or laxity.

KEYWORDS

collagen, dermis, elastin, radiofrequency, targeted pressure energy

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1 | INTRODUCTION

Public health awareness coupled with body image perception is increasingly changing how people look at both objective and subjective cosmetic problems such as scars, cellulite, breast size, or rhytides. Following patients' demand, more than 4.8 million cosmetic procedures were performed in the United States in 2018 according to the American Society for Aesthetic Plastic Surgery.¹

Cellulite is a common cosmetic concern that is present in at least 80% of postpubertal women.² It seems that the dimpled or orange-peel appearance of the skin's surface usually observed on thighs, abdomen, and/or buttocks is due to structural changes of the skin, accumulation of subdermal fat, and decreased lymphatic drainage in the area, as a result of insufficient vascularization and metabolism.³ The cosmetic appearance of the skin is also substantially influenced by the quality of connective tissue. Collagen and elastin fibers are especially responsible for the elasticity and firmness of the skin as they contribute to healthy and smooth skin texture.⁴ Moreover, the connective tissue in subcutaneous fat forms fibrous bands which envelop clusters of adipocytes and thus maintain the integrity of the fat tissue.^{5,6} It has been evidenced that the severity of cellulite corresponds with the relative high ratio of adipose vs connective tissue volume, while a strong network of connective tissue in the fat layer may prevent adipocytes from protruding into the overlying dermis.⁷

Treatments of cellulite aim to reduce skin laxity, improve the blood/lymphatic circulation and the metabolic rate, diminish the subdermal fat, and increase the density of connective tissue.^{2,3,8-11} In general, these changes are induced either by heating or mechanical stimulation of dermis and the underlying fat layer. Various non-invasive technologies such as ultrasound, laser, pressure waves, and radiofrequency (RF) are used to improve the appearance of the skin by inducing effects such as fat lipolysis/reduction, neocollagenesis, neoelastinogenesis, and neoangiogenesis.^{2,3,8,9,12-19}

Despite different mechanisms of action, RF-induced heating of dermal and subdermal tissues and pressure energy have similar effects. It was recently reported that monopolar radiofrequency simultaneously applied with targeted pressure energy (TPE) is a viable new method for noninvasive treatment of cellulite and skin laxity in postpubertal women.^{10,20} While the RF field heats the dermal and subdermal tissues to primarily achieve remodeling of loose connective fibers and fat reduction, the TPE applies pressure to the treated area and, besides the connective tissue remodeling, it increases local metabolism and lymphatic drainage.

In this study, porcine animal model of the human skin²¹ was used to investigate the hypothesized superior effect of these two energies applied simultaneously in comparison with a consecutive therapy. We sought to document positive changes in dermal and subdermal tissues with increasing numbers of collagen and elastin fibers, as well as signs of angiogenesis and reduction of adipocytes size by means of lipolysis. Documenting fat cells lysis is beyond the scope of this study since pure weight loss rarely if ever diminishes cellulite.²

2 | MATERIALS AND METHODS

2.1 | Ethical considerations

All experiments in this study were performed in concordance with relevant laws and regulations on the protection of animals used for scientific purposes. An ethical committee approval was received prior to commencement of this study. A porcine model was chosen for this experiment because the pigskin is remarkably similar to the human skin.²¹ This study design was therefore considered suitable to provide safety and efficacy evidence which can be applicable for use on humans.

2.2 | Subjects and experimental groups

For ethical reasons, the minimum possible number of animals (N = 5) was used. The sample size of the study was chosen to align as close as possible to the 3 Rs principle of animal experimentation.

The pigs were approximately 140 days old (average weight of 70 kg). The animals were kept in normal environmental conditions under an 11 hours day/13 hours night light cycle and were given access to food and water ad libitum.

One animal served as a control. Two animals were consecutively treated; first with monopolar RF and then with TPE. The other two animals were treated simultaneously with RF and TPE energies.



FIGURE 1 Investigated device. EMTONE combines radiofrequency and targeted pressure energy in one single applicator

2.3 | Experimental device

The device used in this study (EMTONE, BTL Industries Inc; see Figure 1) consists of two units with combined output in one single applicator. The first unit generates radiofrequency field, which provides heating to the dermal and subdermal layers. The second unit provides targeted pressure energy with frequency of 10 Hz and pressure strength of 1.5-4 bar propagating into the dermal and subdermal layers.

2.4 | Treatment protocol

Prior to treatment, the animals were first anesthetized with ketamine (10 mg/kg, intramuscular) and then kept under general anesthesia by dosage up to 20 mg/kg, iv. The animals were treated on the abdominal area (*Regio abdominis lateralis*; approximately 25 square inches) in four sessions during a 4-week period (one procedure per week). Conductive cream was applied prior to the procedure.

With respect to the temperature increase caused by the RF component, the skin temperature was monitored and maintained in the range of 40-45°C with the help of the built-in IR thermometer in the device's applicator and a Fluke Ti200 thermal camera (Fluke Corp.).

2.4.1 | Consecutive treatment (CT) group

In each of the four sessions, first, a monopolar RF was applied for 12 minutes by means of the EMTONE device. The power was set to 75%-85% of the device output (max. 150 W). Immediately after the RF therapy, TPE was applied to the same abdominal area for another 12 minutes. The power of the TPE was set to 4 units on the device pressure scale (1.5-4 bar). One consecutive session thus comprised 24 minutes of net application time.

2.4.2 | Simultaneous treatment (ST) group

The parameters of the simultaneous use of RF and TPE were identical to those used in the consecutive treatment group, only delivered at the same time. The procedure lasted 12 minutes.

2.5 | Biopsy acquisition

Biopsies were taken under general anesthesia before the first treatment, after the 4th treatment, and 1-month after the last treatment (follow-up). At each stage of the experiment, samples were taken from each of the five pigs (total of 15 tissue biopsies) with a Kruuse Biopsy Punch 8 mm (KRUUSE). The biopsies were obtained perpendicularly to the skin surface, so the tissue samples contained

epidermis, dermis, and hypodermis. The wounds were treated with chlorhexidine and tetracycline afterward.

The samples for histology examination were fixed in a 10% aqueous solution of neutral buffered formalin (Merck KGaA) at room temperature for 48 hours. After the fixation, the pieces were washed in running water, dehydrated in increasing concentrations of ethanol, cleared by incubation in xylene, and embedded in paraffin. The serial sections (3-5 μm thick) were performed with a microtome (Leica RM2235).

2.6 | Histology

Ten slices were prepared from each biopsy specimen. Five of them were stained for collagen and five for elastin. Quantitative analysis of collagen and elastin was performed with the ImageJ software²² by semi-automatic segmentation in HSB (Hue-Saturation-Brightness) color system. The collagen and elastin fibers were selected and their densities were expressed as a portion of area (range from 0 to 100), which they encompassed in the studied images of approximate size 0.4 mm², covering reticular and papillary dermis. In addition, on each slice the dermis thickness, collagen bundles thickness or elastin fiber thickness and the cross-sectional area of adipocytes were measured. The presence of blood vessels in studied samples was qualitatively examined and commented by experienced histologist.

For collagen examination, de-waxed and hydrated paraffin sections were stained with Weigert's hematoxylin, washed in acidified water, dehydrated in ethanol, cleared in xylene, and mounted in Entellan[®] (Merck KGaA).

Elastin fibers were visualized with Orcein. De-paraffin and hydrated slides were stained for 30 minutes in 1% Orcein solution, passed twice in 70% ethanol, stained in Harris Hematoxylin for 1 minute, dipped in acid alcohol, dehydrated in ethanol, cleared in xylene, and mounted in Entellan[®] (Merck KGaA).

Microscopic observations and estimations of adipose tissue were performed with a Leica DM1000 LED microscope, equipped with the Leica Application Suite Core Software. The software automatically determines the area of adipocyte cells per slice and expresses the mean area of cells.

2.7 | Statistical analysis

The results are expressed as the mean \pm standard deviation (SD). All data points were analyzed with the JASP software (version 0.9.2.0; University of Amsterdam) using a two-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison tests to reveal the significant difference in means of the studied groups (simultaneous/consecutive/control). Multiple paired t tests were performed to assess any significant differences between data taken after the final treatments and at the 1-month follow-up. The level of significance was set at $\alpha = 0.05$, and it was adjusted using Bonferroni correction in the case of multiple comparisons.

3 | RESULTS

During the treatments, no side effects were registered apart from mild transient erythemas caused by the heating and the physical interaction between the applicator and the skin. The erythema subsided in approximately 1 hour after the treatment. During the histology examinations, no lasting or permanent injuries to the dermal or the subdermal tissues were observed.

Table 1 lists the average collagen/elastin amounts, dermis thickness, collagen bundles thickness, elastin fiber thickness, and the cross-sectional area of the adipocytes measured in this study in the treated pigs. Results of the control group showed a stability of measured parameters in time (changes in studied quantities were shown to be negligible and therefore statistically insignificant).

3.1 | RF/TPE induces neocollagenesis and neoeLASTINogenesis

It should be noted that both consecutive and simultaneous treatments (hereafter, CT and ST, respectively) resulted in increased collagen and elastin fiber amount (Table 1, Figure 2 and also Figure 6). While both CT and ST showed a significant increase in the two proteins after the fourth treatment, as well as at 1-month follow-up ($P < .001$), the RF/TPE applied simultaneously showed a significantly higher increase. In conclusion, there was observed by 59% and 64% greater improvement of collagen and elastin fibers in ST subjects, respectively, compared with CT 1 month post-treatment ($P < .001$). These differences strongly suggest a synergistic effect between RF and TPE when used simultaneously.

Overall, 1 month after the last treatment the area encompassed by collagen fibers in measured slices increased by 10.34 (CT) and by 16.47 (ST) when compared to baseline. The increase in collagen fiber area registered immediately after the fourth treatment was 9.80 (CT) and 13.84 (ST), respectively. At follow-up, collagen fibers were covering on average up to 79% (ST) of studied area. Interestingly, the difference in the amount of collagen between the fourth treatment and the 1-month follow-up (hereafter, AF and FU, respectively) was statistically significant only in the simultaneously treated animals (Bonferroni $\alpha = 0.004$, $P < .001$), indicating the synergistic effects of RF and TPE.

The area encompassed by elastin fibers increased on average by 4.12 and 5.27 (CT, AF, and FU, respectively; $P < .001$), and 6.84 and 8.63 (ST, AF, and FU, respectively; $P < .001$). At the follow-up, elastin fibers were covering on average up to 15% (ST) of studied area. None of the differences observed between 4th treatment and 1-month follow-up were statistically significant ($P \geq .10$).

Figure 3 shows changes in the collagen bundles thickness and the elastin fiber thickness (see also Table 1). Again, greater and significant improvement was measured in the ST group in comparison with the CT. Especially, the increased thickness of elastin fibers was mild to modest in case of CT, showing no change after the treatments and only 23.46% change at the follow-up (yet significant against control

at $P < .001$). The enlargement of collagen fibers at the FU examination compared with the AF results was statistically significant only in ST subjects ($P < .001$).

3.2 | RF/TPE increases dermal thickness

Both consecutive and simultaneous treatments resulted in an increased dermal thickness with the latter showing greater effect (Table 1 and Figure 4). Compared with the baseline, dermal thickness in the CT group showed a 26.56% and 38.06% increase (AF and FU, respectively; $P < .001$). Simultaneous treatments showed an even greater increase compared with the CT group (AF = 41.51% and FU = 50.17%; $P < .001$).

The dermal thickness showed a higher increase in ST pigs compared with the animals treated consecutively (by 70% AF and by 44% FU, $P < .001$), suggesting once again that a greater synergistic effect is possible when RF and TPE are used simultaneously.

3.3 | RF/TPE affects hypodermal tissue

The average area of fat cells decreased in both CT and ST groups with the difference between the two groups being negligible (Figure 5; AF $P = .98$ and FU $P = .91$). Compared with the control, in both treated groups the area of adipocytes decreased significantly by approximately 25% (see Table 1). one-month follow-up samples from both CT and ST animals showed that the fat cells had not grown in size compared to when measured immediately after the 4th treatment. This observation may indicate that metabolism in the treated sites was still accelerated, as the signs of enhanced vascularization were seen at 1-month follow-up (Figure 6 and Figure 1). As expected, the occurrence of blood vessels was increased in both CT and ST groups compared with the control.

Apart from the recognized increase in collagen and elastin thickness observed in the dermis, the histological examination revealed that collagenous and elastic fibers constituting the hypoderm septa were also influenced by the RF/TPE energies. At the 1-month follow-up, the interlobular septa were visibly better organized in the pigs treated simultaneously, as shown on Figure 5.

4 | DISCUSSION

This study shows that the simultaneous application of monopolar RF and targeted pressure energy positively influences both the dermal and subdermal tissues. Importantly, the simultaneous use of the two energies exhibits clearly measurable synergistic effect compared with consecutive treatment. This was primarily demonstrated by more profound elevation in the connective tissue amounts. Specifically, both collagen and elastin fibers showed enhanced thickness that resulted in a denser and more compact papillary and reticular dermis. In comparison with consecutive treatment, there was

TABLE 1 Collagen and elastin fiber amount and thickness, dermis thickness, and adipocytes' area. Data expressed as the mean \pm standard deviation (SD). The differences against baseline are given as well in percent where appropriate. *P*-values generated by ANOVA are presented in separate columns

Tx Group/Units	Baseline (\pm SD)	AF (\pm SD)	FU (\pm SD)	Baseline vs AF	Baseline vs FU	<i>P</i> -value (vs Baseline)
Area of collagen fibers [-] ^a						
CT	64.00 (2.41)	73.80 (1.50)	74.34 (3.23)	9.80	10.34	<i>P</i> < .001
ST	62.60 (2.12)	76.45 (1.26)	79.08 (0.94)	13.84	16.47	<i>P</i> < .001
<i>P</i> -value (ST vs CT)		<i>P</i> < .01	<i>P</i> < .001			
Area of elastin fibers [-] ^a						
CT	6.50 (2.44)	10.62 (1.50)	11.77 (2.01)	4.12	5.27	<i>P</i> < .001
ST	6.15 (0.96)	12.98 (1.82)	14.78 (2.68)	6.84	8.63	<i>P</i> < .001
<i>P</i> -value (ST vs CT)		<i>P</i> < .01	<i>P</i> < .001			
Collagen fiber thickness [μ m]						
CT	15.44 (4.45)	25.60 (4.90)	27.13 (5.38)	10.16 (65.77%)	11.69 (75.69%)	<i>P</i> < .001
ST	15.59 (5.22)	28.30 (6.00)	33.35 (8.80)	12.71 (81.54%)	17.76 (113.96%)	<i>P</i> < .001
<i>P</i> -value (ST vs CT)		<i>P</i> < .01	<i>P</i> < .001			
Elastin fiber thickness [μ m]						
CT	1.15 (0.23)	1.13 (0.46)	1.41 (0.40)	-0.01 (-0.94%)	0.27 (23.46%)	<i>P</i> > .05; <i>P</i> < .001
ST	1.15 (0.40)	1.46 (0.34)	1.89 (0.42)	0.30 (26.33%)	0.74 (64.05%)	<i>P</i> < .001
<i>P</i> -value (ST vs CT)		<i>P</i> < .001	<i>P</i> < .001			
Dermis thickness [μ m]						
CT	1553.84 (74.21)	1966.56 (166.95)	2145.28 (145.45)	412.73 (26.56%)	591.45 (38.06%)	<i>P</i> < .001
ST	1691.90 (110.14)	2394.26 (93.13)	2540.72 (117.16)	702.36 (41.51%)	848.82 (50.17%)	<i>P</i> < .001
<i>P</i> -value (ST vs CT)		<i>P</i> < .001	<i>P</i> < .001			
Adipocytes' area [μ m ²]						
CT	2109.03 (533.24)	1643.16 (578.42)	1664.57 (446.52)	-465.88 (22.09%)	-444.47 (21.07%)	<i>P</i> < .05; <i>P</i> < .01
ST	2175.49 (632.85)	1667.84 (630.21)	1591.95 (547.04)	-507.64 (23.33%)	-583.54 (26.82%)	<i>P</i> < .05; <i>P</i> < .001
<i>P</i> -value (ST vs CT)		<i>P</i> > .05	<i>P</i> > .05			

Abbreviations: AF, after 4th treatment; CT, consecutive treatment; FU, 1-mo follow-up; ST, simultaneous treatment; Tx, Treatment.

^aArea of collagen and elastin fibers in examined slices of approximate size 0.4 mm²; range 0-100.

a more profound collagen fiber increase of 59% and also a more profound elastin fiber increase of 64% in pigs treated simultaneously. This increment of connective tissue led to a substantially thicker dermis at the 1-month follow-up while we observed on average an additional 591.45 μ m (CT) and 848.82 μ m (ST) of dermal tissue. The interlobular septa in adipose tissue also showed to be better organized at 1-month follow-up in pigs treated simultaneously (Figure 5).

As a marker of lipolysis, we measured the area of adipocytes. Any decrease in the fat cell size is an indicator of intracellular lipid content's reduction.²³ Our observations indicate that both CT and ST were similarly effective regarding the subdermal lipolysis, although the maximal relative (26.82%) and absolute (583.54 μ m²) levels of improvement were achieved in ST pigs. Trelles et al have found that RF-based treatment of cellulite produced a decrease in the lipid content and changes in their membranes. The authors believe that this would lead to cell ruptures and their death, as well

as, extrusion of the lipid content out of cells.³ Our findings corroborate these results, as we observed a reduction in adipocytes' size due to decreased content of intracellular lipid reservoirs after four treatment sessions. The fat cells shrinkage is believed to be due to accelerated cellular metabolism and hence the release of free fatty acids by the radiofrequency field,¹⁵ and partially due to increased vascularization (discussed below) induced by both RF/TPE energies.^{12,13,19,24}

Besides lipolysis, RF and targeted pressure energy are also known to induce neocollagenesis and neoangiogenesis. Meyer and co-authors reported that the application of RF leads to the increased dermal thickness and type I collagen in rats. More importantly, the study also showed an increase in the expression of fibroblast growth factor 2 (FGF2), an important signaling protein involved in angiogenesis, and consecutively in the microvessel density.¹³

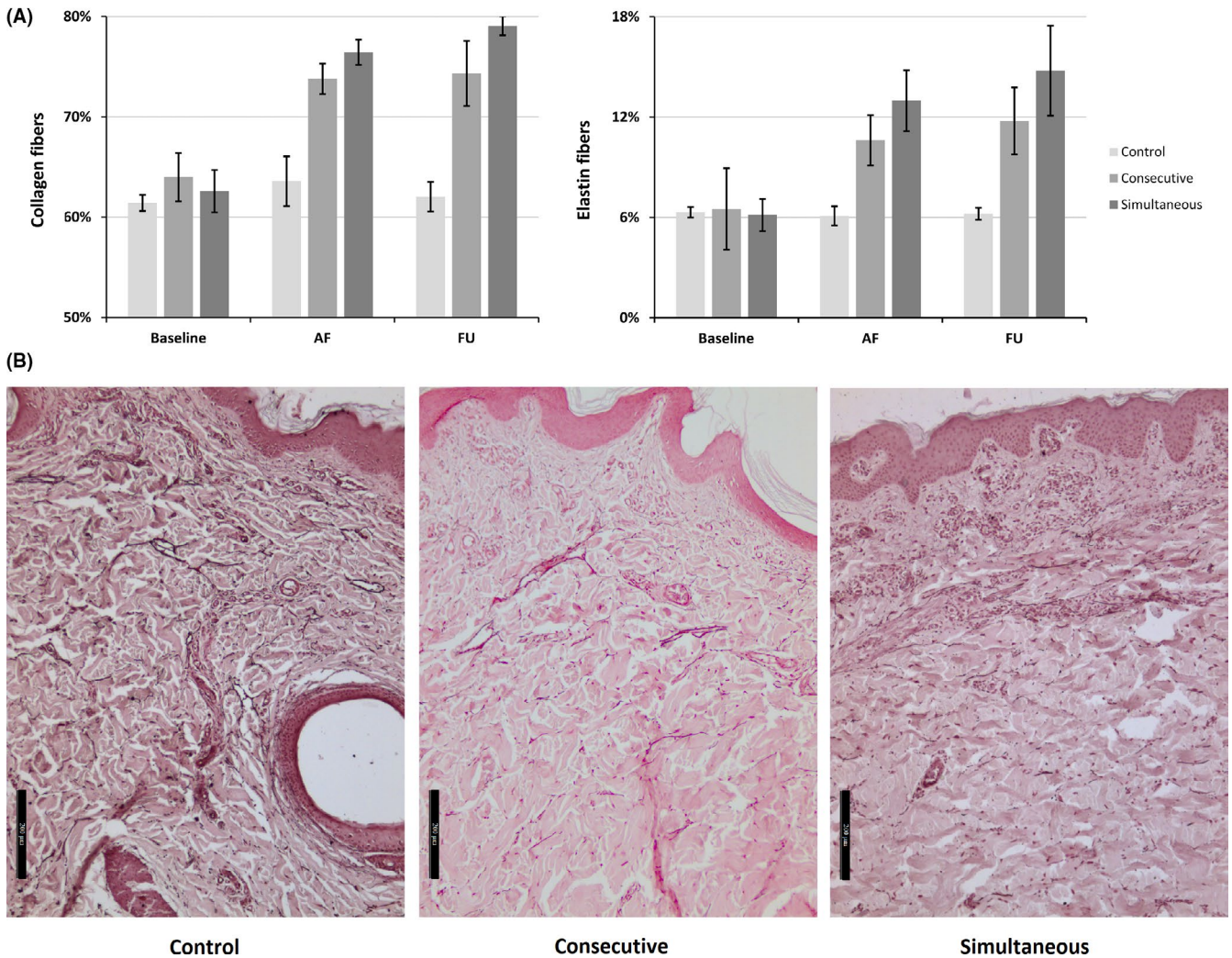


FIGURE 2 Increase of collagen and elastin amount—area encompassed on examined slices (A, mean ± SD). B, shows illustrative histological comparison of control/consecutive/simultaneous samples at 1-mo follow-up. The collagen and elastin fibers are denser and thicker in the consecutive/simultaneous samples. Samples of treated tissue are also showing greater vascularization, especially in the upper part of reticular dermis. Detail of the dermis, bar 200 μm

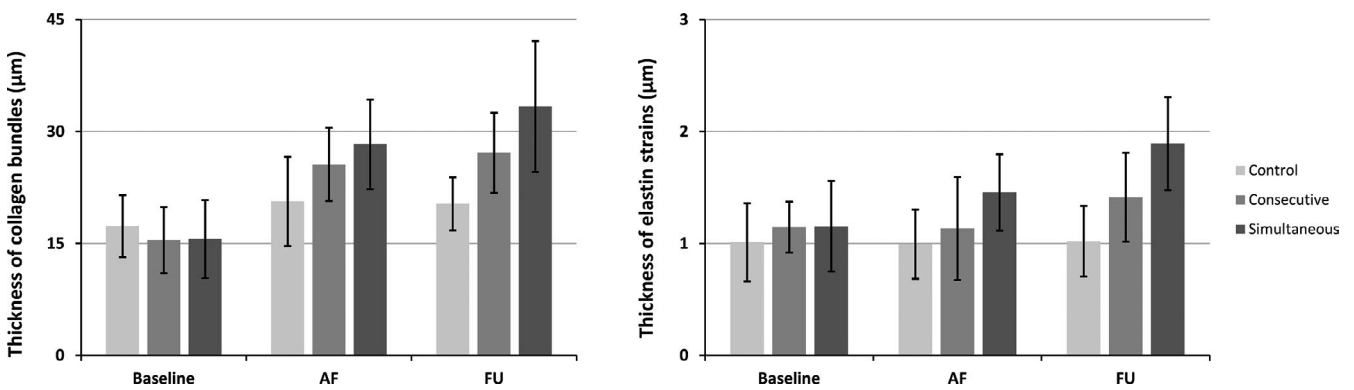


FIGURE 3 Collagen bundles and the elastin fiber strain thickness in μm (mean ± SD)

Fibroblasts, the cells maintaining the skin's structural integrity, are very similar to osteoblasts, the bone-forming cells. It is well documented that the osteoblasts are responding to

mechanic stimuli which upregulate numerous genes.²⁵ Targeted pressure energy administered externally to the tissue is a form of mechanical stimulation which leads to both osteogenesis and

FIGURE 4 Dermis thickness in μm (A, mean \pm SD). B shows illustrative assessment of dermis thickness (D). Subcutaneous tissue (H) is seen below the reticular dermis; bar 500 μm

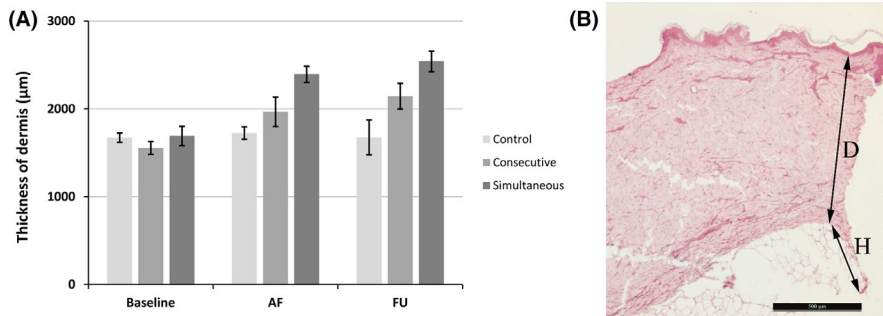


FIGURE 5 A, Visualize adipocytes and interlobular septa in hypodermis before and 1 mo after simultaneous treatment; bar 500 μm . Adipocytes are visibly smaller after RF/TPE treatment, while septa are visibly thicker and better organized. 5b shows results of measurement of adipocytes' area in μm^2 (mean \pm SD)

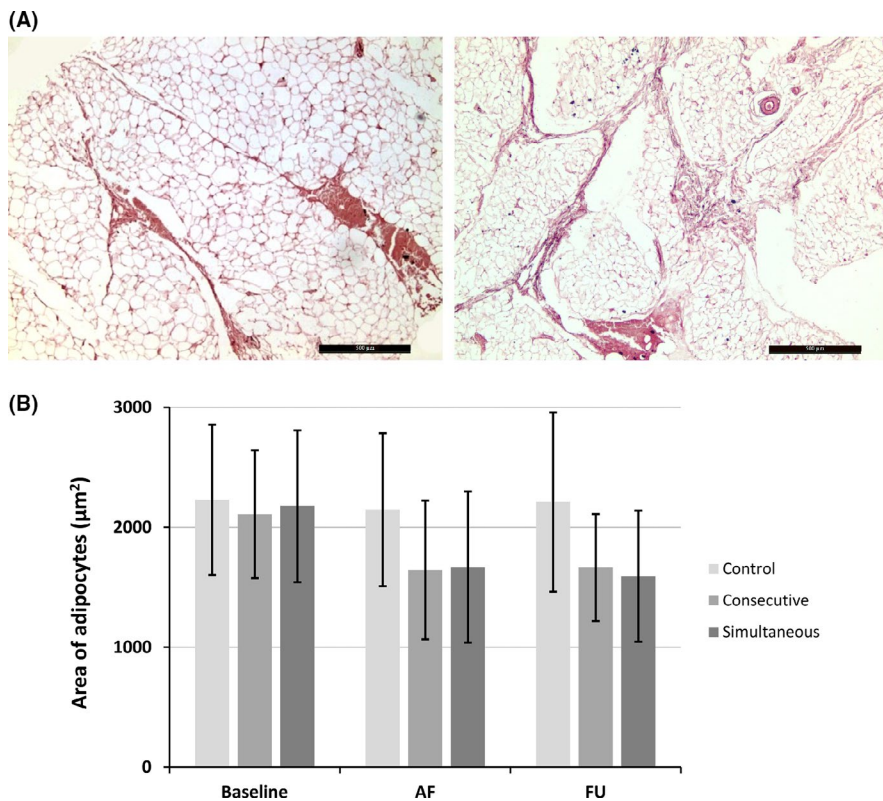
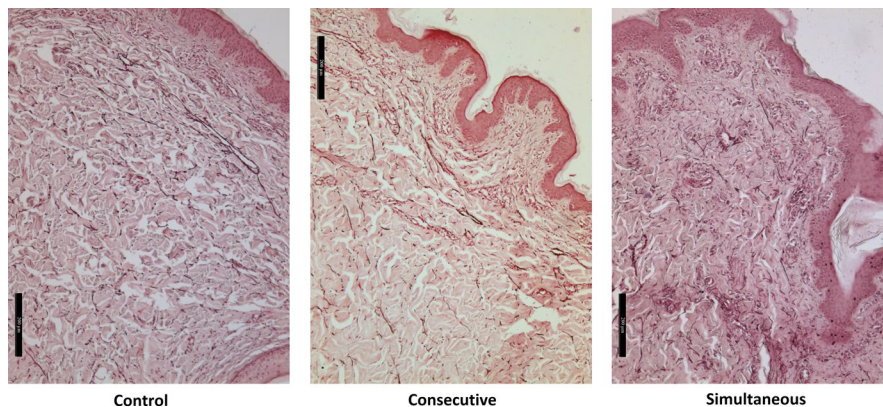


FIGURE 6 Illustration of increased vascularization. Samples were taken at 1-mo follow-up. Consecutive/simultaneous samples are showing greater vascularization, denser organization, and thickness of connective fibers in comparison with the control. Detail of the dermis; bar 200 μm



angiogenesis, stimulating both the osteoblasts and the fibroblasts, respectively.^{12,19,24,26} De Lima Morais et al further observed that pressure energy leads to an overexpression of FGF2 and its

receptor, fibroblast growth factor receptor 1 (FGFR1), leading to angiogenesis. Moreover, they also reported that mechanical stimulation led to increased collagen synthesis and dermal thickness¹²

also observed in our study, particularly in synergy with the RF energy. It has been documented that the application of mechanical stress affects thermal stability of collagen molecules, resulting in a more efficient thermal stimulation and consequent remodeling,^{27,28} which may explain the more pronounced results of simultaneous application documented in our study.

Angiogenesis and an increased metabolic rate are important factors contributing to the treatment of cellulite, due to the fact that the condition is at least in part caused by insufficient lymphatic drainage.^{2,3} The combined application of RF and TPE, which produced an increased vascularization in this animal study, may be therefore a suitable combination for noninvasive treatment of cellulite.¹⁰ Despite the fact that microvessel density was not measured in our study, the evaluation of histological samples (see Figure 6) revealed that RF/TPE combination used simultaneously might be a more powerful tool for the induction of angiogenesis than any consecutive or single application treatment.

Elastinogenesis and collagenesis induced by the RF/TPE application may possibly further improve the visual appearance of the skin. A thicker dermis with decreased laxity (due to increased elastin content¹⁰) would be more resistant to bulging caused by the underlying fat cells. In addition, the reduction in adipocytes' size may decrease the bulging effect as well. Moreover, the better-organized septa in adipose tissue should also favorably support the outcomes of therapy as the strengthened network of fibrous bands maintains the integrity of the subcutaneous layer and prevents the fat lobules from protruding into the dermis.^{5,6}

This histological study might benefit from longer follow-up observational period. However, the length of the follow-up (1 month after the last treatment) is comparable to other existing histological studies examining the effect of RF treatment on human skin.^{9,14,15,29-31} These experiments report collagen (and elastin) increase in the skin after RF irradiation with follow-ups ranging from 1 week to 3 months, showing great diversity among the experimental designs. On the other hand, one of the strengths of our study is the quantitative objective evaluation of the composition of dermal/subdermal tissue and verification of the observed changes by the examination of control subject.

5 | CONCLUSION

This porcine model study indicates that the simultaneous application of RF and targeted pressure energy may be a suitable treatment for various skin conditions such as cellulite and skin laxity. The combined application of these energies showed enhanced synergistic effects in several instances. Compared with the consecutive application of the modalities, the simultaneous use of these two fields exhibits a more beneficial effect on animal dermal and subdermal tissues, with regard to connective fiber density and dermal thickness. Additionally, a decrease in lipid content was achieved, reducing the size of adipocytes. Further research is needed to confirm the enhanced vascularization observed in the dermal tissue.

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CONFLICT OF INTEREST

Brian M. Kinney MD is a medical advisor to BTL. Dian Kanakov MD and Penka Yonkova PhD have no conflicts to declare.

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