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ORIGINAL ARTICLE

Induction of fat apoptosis by a combination of synchronized radiofrequency and HIFEM technology: Human histology study

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Abstract

Purpose: With the growing demand for more effective fat reduction techniques, a combination of synchronized radiofrequency (RF) and HIFEM has been introduced. Preceding studies evidenced the ability of RF+HIFEM to maintain the fat tissue temperature at the levels necessary for adipocyte apoptosis while documenting the induced changes to the fat tissue during the several weeks after the treatment. This study aims to demonstrate the induction of apoptosis by RF+HIFEM technology in the early stages through the assessment of caspase-3 protein, one of the apoptosis-executing proteases.

Design: In this two-arm, single-center, randomized trial, nine human subjects were enrolled and assigned into two groups, either the active group ($N=6$) treated with both RF+HIFEM set at the highest tolerated levels or the sham group ($N=3$) treated with 5% of the maximum RF+HIFEM power, serving as a control. All patients were scheduled to undergo one treatment visit of the abdominal area, two follow-up visits at 8 and 24 h, and one safety visit 7 days after the treatment. A punch biopsy (5 mm in diameter, approximately 10 mm in depth) was obtained from the abdominal area at the baseline and consecutive follow-up visits. Samples were fixed, and cut into 5 μm thick slices, and immunohistochemical staining was used to visualize the Caspase-3, revealing the adipocyte nuclei where apoptosis processes are in progress.

Findings: Documented findings suggest that the temperature threshold of 43–45°C is required to initiate fat apoptosis and consequent reduction in adipocyte number was achieved during the combined treatment with RF+HIFEM. The active group showed an elevated ratio of positively stained nuclei versus all adipocyte nuclei found on the evaluated slices—referred to as the apoptotic index (AI). The AI significantly ($p<0.001$) increased at both 8 h (47.01 ± 10.56%) and 24 h (43.58 ± 6.35%) posttreatment. The Sham group showed no significant change in the AI ($p>0.05$). No adverse events or side effects related to the treatments were observed.

Summary: This study supports previously published evidence on fat reduction after RF+HIFEM treatment, documenting the safe initiation of adipocyte programmed cell death posttreatment.

KEYWORDS
apoptosis, body contouring, fat

1 | INTRODUCTION

Higher body mass index (BMI) not only increases the risk of health complications, but can also affect our mental state. In previous studies, both men and women reported greater dissatisfaction with their body image with increasing BMI. These individuals also experience greater prejudice and discrimination,[1,2] which may result in them striving for a leaner physique. However, long-term maintenance of weight loss via lifestyle changes is challenging when more than 50% of lost weight is gained back within 2 years and nearly 80% is gained back within 5 years after the initial weight loss.[3] Many people then, as a way to improve their body image, opt for cosmetic intervention. While liposuction continues to be one of the most popular choices in cosmetic surgery,[4] it is an invasive procedure that may come with downtime, risks, and potential adverse events.[5] Noninvasive approaches such as ultrasound-assisted liposuction, power-assisted lipoplasty, and laser-assisted lipolysis offer fat reduction via increasing or decreasing the temperature of fat tissue, leading to the induction of various metabolic pathways. Among these pathways, apoptosis emerges as the safest long-term fat reduction solution, without stimulating potentially harmful inflammatory reactions.[6]

Apoptosis is a programmed cell death process crucial to the normal function of various body processes such as cell turnover, embryonic development, proper development, and functioning of the immune system.[7] One of the apoptotic pathways is the intrinsic pathway also known as mitochondrial apoptosis which is initiated by negative or positive stimuli. Negative signals involve the absence of certain growth factors, hormones, and cytokines, while positive stimuli include hyperthermia induced by radiofrequency (RF).[7–9] The stimuli cause the opening of the mitochondrial permeability transition pore, the release of cytochrome c, expression of the Bcl-family members, and activation of initiator caspases-9 leading to activation of effector caspase-3 and 7.[9] Caspases (cysteine-aspartic proteases) are proteolytic enzymes that regulate apoptosis.[10] The ones involved in apoptosis have been classified by their mechanism of action as initiator caspases (caspase-8 and -9) or effector caspases (caspase-3, -6, and -7). Caspase-3 is the main effector caspase, which executes the final stages of apoptotic cell death, catalyzing the cleavage of many key cellular proteins resulting in DNA fragmentation,[9] and degradation of cytoskeletal and nuclear proteins.[11]

The noninvasive procedure combining synchronized radiofrequency (RF) and HIFEM is a safe and effective method[12–14] not only for fat reduction but also simultaneous muscle growth, reaching a desired aesthetic look without downtime. HIFEM is an electromagnetic muscle stimulation technology, that induces brain-independent supramaximal contractions in the muscle tissue via electromagnetic energy, similar to resistance training but with higher intensity,[14] leading to the stimulation of muscle hypertrophy and hyperplasia.[15] Radiofrequency has been widely used successfully for fat reduction by selectively heating the adipose tissue, without damage to the epidermal or dermal layers.[16] Based on the previous histological findings, the suggested mechanism leading to fat reduction by RF is adipocyte apoptosis.[16–19] By heating the adipose tissue to 43–45°C, fat cells lose their integrity[16] and compared to healthy cells, which are round-shaped and of uniform size, cells after treatment with RF were observed to be shrunken and of decreased size,[17,20] with pyknotic nuclei and condensed nuclear chromatin.[17]

This study aims to investigate the induction of fat apoptosis after HIFEM+RF treatment at the molecular level by determining the changes related to caspase-3 activity levels and detecting any side effects and adverse events associated with the BTL-899 treatment of the abdominal area.

2 | METHODS

Nine subjects ($n=9$, eight females and one male) were enrolled and randomly allocated into two groups in the 6:3 ratio, the active group ($n=6$, 23–59 years, BMI = 24.6–34.7 kg/m^2, skin type I–V) and the sham group ($n=3$, 28–61 years, BMI = 25.6–31.9 kg/m^2, skin type II–III). Eligible subjects were those willing to undergo punch biopsies of abdominal fat and did not meet any of the exclusion criteria, such as pregnancy, electronic implants, and other conditions that contraindicate the use of electromagnetic field or radiofrequency or obtaining the biopsy. All study subjects received detailed information about the procedure and sample collection and signed a written informed consent. This prospective single-center single-blinded two-arm study was approved by the Institutional Review Board (IRB) and was registered at the clinicaltrial.gov site (NCT05139745).

The study participants received one 30-min abdominal treatment with the device simultaneously emitting both the HIFEM and RF energies (Emsculpt NEO, BTL Industries, Boston, MA, USA). The treatment parameters were set according to the allocated group. In the active group, the intensity of the RF and HIFEM field was set to a maximally tolerated level, while the sham group received treatment with both energies set to 5% of their intensity, serving as a control.

The histologic analysis was the primary evaluation method of the changes in the adipose tissue. Every subject underwent a punch biopsy (5 mm in diameter, approximately 10 mm in depth) of the abdominal treatment area at baseline (approximately 1 week prior to the treatment), 8 h and 24 h after the treatment. The chosen biopsy timing reflected the assay results of Sundquist et al, where caspase-3 activity continued to increase over the measured 7 h and the estimated apoptotic cell death duration of 6–24 h.[21,22] To minimize the pain and discomfort during sample collection, 1% lidocaine anesthesia was applied to the sample area prior to the sampling. The

wound was immediately closed and disinfected, and later examined at the 7-day safety follow-up visit.

Altogether, three samples were collected from each subject. Each sample was sliced into three 5 μm thick slices and stained with an immunohistochemical staining kit detecting the large fragment of activated caspase-3 (SignalStain® Apoptosis (Cleaved Caspase-3) IHC Detection Kit #12692). Staining resulted in dyeing the pyknotic nuclei in brown-black. Additionally, the slides were stained by the Calleja method (Calleja's Staining Kit FH) to visualize the collagen structures, such as septae, in blue. The laboratory assistant followed the staging protocols provided by the manufacturers. All slides were observed under a microscope (Hitachi Axio Scan.Z1, Carl Zeiss AG, Germany; 20×/0.8NA Plan-Apochromat objective) and photographed by free imaging microscopy software (ZEISS ZEN lite 3.6 blue edition).

ImageJ (64-bit Java 1.8.0_172) was used to evaluate the slices manually, so artifacts such as red blood cells or cells of the immune system were recognized and not included in the evaluation (Figure 1). The region of interest (ROI) was undetermined and ranged from 500×500 to 1000×1000 μm to ensure that all of the adipose tissue in the slide was analyzed. The obtained data were quantified using the apoptotic index (AI). The AI was determined by dividing the number of positively stained nuclei by the total number of nuclei and multiplied by 100.[23]

The Friedman–Nemenyi test was performed to determine the statistical significance of the changes occurring within the group, while the Mann–Whitney test for two independent samples were used to compare the groups. The significance level of $\alpha = 0.05$ was set for all statistical analyses.

3 | RESULTS

All nine patients completed the treatment with RF and HIFEM energies set to 100% of its intensities and the sample collection. However, two participants were withdrawn after biopsy due to their samples not being suitable for histologic evaluation; therefore seven subjects were included in the evaluation, two in the sham group and five in the active group. No adverse events or side effects were reported. Signs of necrosis were not observed while evaluating histological slides.

At baseline, there was no difference between the groups ($p = 0.363$), as the average AI in the active group was 7.34 ± 2.34%, with 7.63 ± 3.06% in the control group. The changes throughout the study were significant ($p < 0.001$) in the active group, while no significant ($p = 0.311$) changes were observed in the control group.

Sample collection 8 h after the treatment showed an average AI of 47.01 ± 10.53% in the active group ($p < 0.001$), while in the control group, the average AI was 7.27 ± 1.88% ($p = 0.319$). At the 24-h follow-up biopsy, the average AI in the active group was 43.72 ± 6.10%. The change was significant compared to the baseline ($p = 0.002$); however, it was not statistically significant compared to the 8-h data ($p = 0.745$), as it plateaued due to the apoptotic time frame. In the control group, 24-h biopsy showed an average AI of 7.36 ± 3.34%, with statistical insignificance compared to both the baseline ($p = 0.955$) and the 8-h data ($p = 0.480$).

The histological evaluation revealed morphological changes within the adipose tissue of the treated group. In the active group (Figure 2), the histological slides show disturbed septae and an increased number of pyknotic nuclei peaking 8 h after the treatment. No changes in the morphology nor the number of apoptotic nuclei were observed in the control group (Figure 3).

4 | DISCUSSION

This study observed signs of adipocyte apoptosis through morphological changes and staining of the apoptotic signaling molecule, activated caspase-3. Caspase-3 cleaves structural and nuclear proteins, including endonuclease inhibitor ICAD upon activation. Cleavage of ICAD releases the endonuclease CAD, degrading chromosomal DNA[24] within the nuclei and causes chromatin

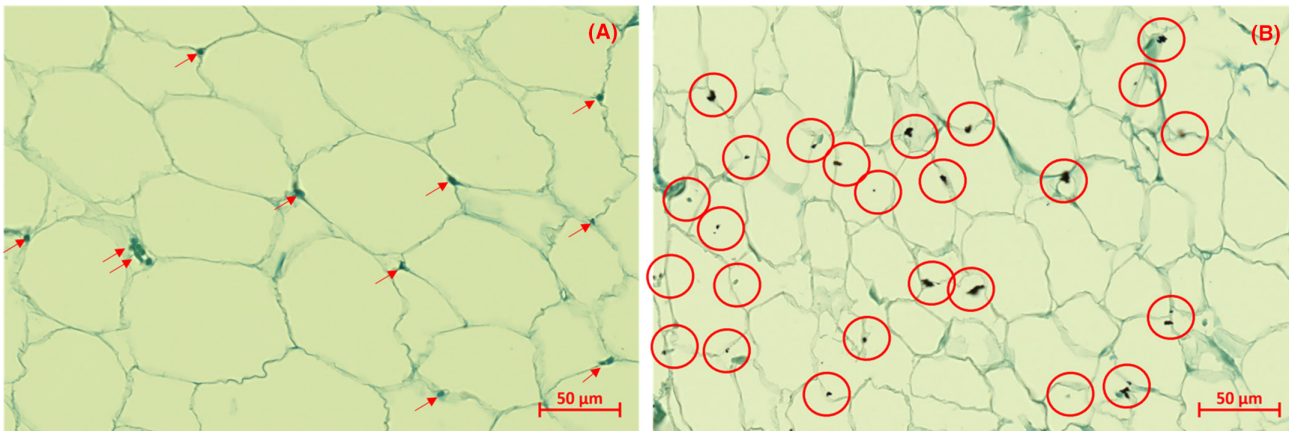

FIGURE 1 A visual representation of the adipose tissue. The left side (A) represents untreated adipose tissue with a healthy nucleus (red arrows), while the right side (B) represents adipose tissue after the treatment with visibly smaller and disturbed adipose cells with pyknotic nuclei (red circles).

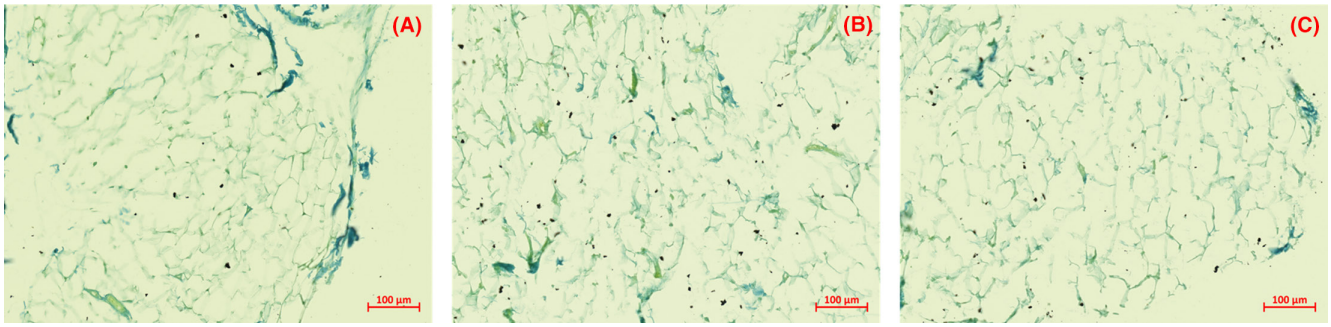

FIGURE 2 Visualization of the changes in the adipose tissue morphology in the active group after the treatment. The number of pyknotic nuclei 8 h after the treatment visibly increased with breached septae, which plateaued 24 h after the treatment. A = baseline, B = 8 h after the treatment, and C = 24 h after the treatment.

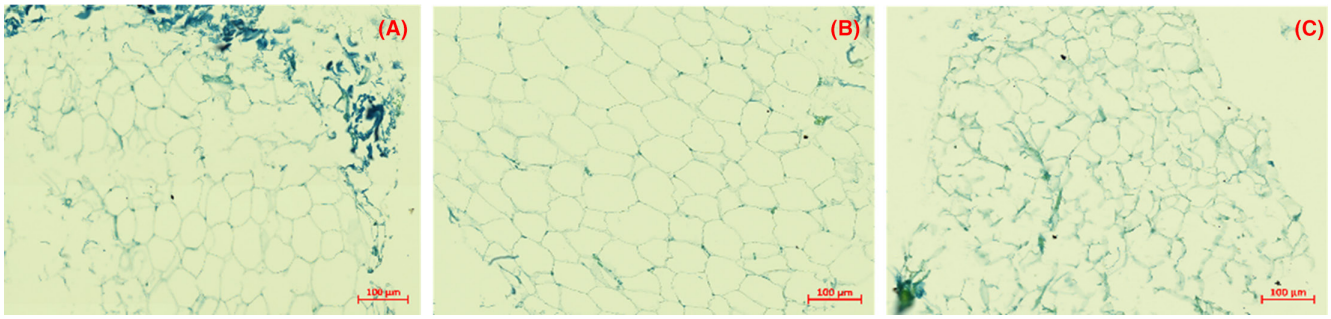

FIGURE 3 The control group showed no changes in morphology or number of pyknotic nuclei compared to baseline. A = baseline, B = 8 h after the treatment, and C = 24 h after the treatment.

condensation. Among the caspase-3 cleaved protein also belongs gelsolin, an actin-binding protein necessary for actin organization and signal transduction. With gelsolin cleavage, the cell can no longer maintain intracellular transportation and cytoskeletal integrity.[7] Caspase-3 activity results in characteristic morphological features of apoptosis, such as nuclear condensation, cell shrinkage, and fragmentation.[21,25]

At the molecular level, the study results support the existing histological findings suggesting that posttreatment fat reduction occurs through apoptosis. The observed increase of AI was 540% (from 7% to 47%, $p < 0.001$) 8 h after treatment, and 496% (from 7% to 43.72%, p-value 0.002) 24 h after treatment. The morphological evaluation showed cell shrinkage and disrupted septae, while the control group had no significant changes ($p = 0.311$) both in AI and morphology. These findings are consistent with the earlier study by McDaniel et al,[18,19] where AI increased by 487% 1 h after treatment.

While there are alternative noninvasive fat reduction methods on the market with apoptosis as the proposed mechanism of fat reduction, this study has proven this theory at both histological and molecular levels for the noninvasive procedure combining synchronized RF and HIFEM. The observed increase in pyknotic nuclei supports the histological evidence,[13,17] and staining for activated caspase-3 confirms the ongoing apoptosis on the molecular level. By staining caspase-3, which executes the final stages of mitochondrial apoptosis, it was ensured that only irreversible adipose cell death was recognized. Signs of necrosis were not observed while evaluating histological slides. While the mechanism of radiofrequency-induced apoptosis is apprehended through hyperthermia, the mechanism of the HIFEM energy is not completely understood yet. Previous study results from Weiss et Bernardy[26] indicate that HIFEM energy standalone induces adipocyte apoptosis, evidenced by the increased AI posttreatment, and increased blood levels of free fatty acids (FFA) and triglycerol, the fat metabolism biomarkers. The suggested mechanism of apoptosis induction is through elevated FFA levels, which have been shown to trigger endo-reticular stress leading to apoptosis.[27–29]

Because additional fat reduction was observed even 1 month after the last treatment,[14,20] further research is needed to observe the changes over a longer period for a more comprehensive understanding of the apoptotic timeline, which is beyond the scope of this study. The outcomes of HIFEM+RF treatment were demonstrated to be visible up to 3 months posttreatment in several treated parts of the body such as the abdomen, upper arms, lateral and inner thighs, flanks, buttocks, and saddlebags.[13,14,30–33]

Due to this method's invasive nature, the sample size was expected to be limited. Although two subjects were withdrawn due to their samples not being suitable for histological evaluation, slicing the collected specimen from every studied subject into three separate evaluated samples provided sufficient data to demonstrate the trend in the tissue. However, the generalizability is limited due to the small sample size. The subgroup was enrolled in this study, and the targeted group for this procedure was included as the type of

treatment-seeking people are predominantly female. Even though this study did not include a control group, the study included a sham group with RF and HIFEM energies set to 5% to replicate the treatment experience and maintain the single-blinded study design. Future research would benefit from including more subjects, photo documentation to visualize the changes in appearance, and a higher rate of follow-ups.

5 | CONCLUSION

This presented study offers molecular evidence that the simultaneous use of RF + HIFEM is causing fat reduction through apoptosis, the safe programmed cell death.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

The Allendale Institutional Review Board (IRB) approved the study. All subjects voluntarily signed a written consent form to have their faces photographed and published.

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