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Improved methods for evaluating pre-clinical and histological effects of subcutaneous fat reduction using high-intensity focused ultrasound in a porcine model

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Background: Non-invasive body sculpting procedures are becoming increasingly popular. High-intensity focused ultrasound (HIFU) treatment is a non-surgical fat reduction procedure that permanently destroys unwanted abdominal fat. Despite its increasing popularity, evaluation methods for the procedure have not yet been fully developed.
Aims: The objective of this study was to develop evaluation methods for HIFU for non-surgical, permanent fat reduction in the anterior abdomen using a porcine model.
Methods: The abdomens of female pigs (*Sus scrofa*, $n = 7$) were treated with a HIFU device (SCIZER™, Classys Inc, Seoul, Korea). We examined treatment effects using photography, ultrasound, gross and microscopic pathology, and serum lipid and liver function level analysis, carbon tracer test, and histological examination in order to determine the mechanism of action, efficacy, and safety of the procedure.
Results: HIFU treatment effectively reduced abdominal fat in a porcine model; it accurately treated the target subcutaneous fat layer and the subcutaneous fat was reduced effectively via ultrasonic measurement after HIFU treatment. On histological staining (H&E, toluidine blue, oil red O and immunohistochemistry), we found that subcutaneous fat reduction occurred effectively via accurate treatment of the targeted subcutaneous fat layer. On hematological assay, there were changes within normal range, and values remained stable after 48 h. Via carbon tracer test, the migration of activated macrophages was identified within the axillary lymph node (LN). PPAR-delta, a protein defined by immunohistochemistry staining, was overexpressed in the early stage on days 1 and 7, but a gradual decreasing pattern was confirmed.
Conclusion: We successfully used a HIFU device for body contouring and fat reduction in a pre-clinical study. These results provide that the essential clues toward the effective evaluation, guiding selection of the appropriate diagnostic investigations.

Key words: HIFU – subcutaneous fat reduction – non-invasive body sculpting – improved methods

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HIGH-INTENSITY focused ultrasound (HIFU) has been used for the non-invasive treatment of prostate cancer (1), uterine fibroids (2), neurological disorders (3), and various other cancers (4). It has also been evaluated as a method for the selective destruction of adipose tissue (5).There is an increasing interest in non-invasive fat reduction methods as an alternative to liposuction. Laser energy (6), radio frequency (7), and low-level laser therapy (8) have been investigated. These technologies have not met efficacy expectations and have had reported safety issues (9). Furthermore, with increasing use of HIFU treatment, the mechanisms of action and assessment methods on adipose tissue need to be better understood.

HIFU does not affect skin and organs outside of the focal area. The precisely focused energy is able to destroy fat cells and cause tissue temperature increases great enough to induce necrosis without injury to the surrounding

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tissue (10). As such, HIFU works in principle by maintaining a high temperature over a short time period and leads to coagulative necrosis, killing cells. As tissue temperature rapidly increases over 70°C with HIFU treatment, fat tissue is destroyed through effective coagulative necrosis despite the short exposure time (11). In addition, the fibrosis layer contracts through thermal denaturation. When macrophages are recruited around the injured tissue, chemotactic signaling and tissue damage cause an inflammatory response. Through the lymphatic system, macrophages digest free lipids and cell debris in the liver (12). The temperature difference between the target tissue area and the surrounding area is considerable, and a clear margin between the site of necrosis and the surrounding normal tissue can be found easily via histological examination (13). In this study, we evaluated the efficacy and safety of a HIFU device for the reduction in subcutaneous fat tissue. We used the SCIZER™ focused ultrasound device, which was designed to apply non-invasive therapeutic focused ultrasound to achieve a thermal effect on adipocytes in the subcutaneous fat layer, in order to reduce and improve the contours of localized fat deposits.

The objective of this study was to pre-clinically and histologically evaluate the efficacy, safety, and mechanism of action of a HIFU device for subcutaneous fat reduction.

Materials and methods

Animals and experimental design

Female pigs ($n = 7$, for efficacy test: 6, safety test: 1) were used in this study. All animals were obtained from the closed barrier unit at CRONEX (Hwasung, Korea) and were housed individually under controlled environmental conditions (temperature, 18–22°C; relative air humidity, 30–70%; 15 air changes/h; 12:12-h light-dark cycle). After each pig's general condition and weight (range, 95.5–100 kg) were confirmed, they were placed under preliminary anesthesia by intra-muscular injection using a 3-mL mixture of zoletil 50 (tiletamine hydrochloride plus zolazepam hydrochloride, Virbac S.A, France) and Rompun (xylazine hydrochloride, Bayer) at the ratio of 6 : 4. The subject was then moved to an operating room, laid on the operating table and 2 mL of the same injection mixture was applied via IV injection. Using a laryngoscope, the airway was secured and an intubation tube (8.5 Fr) was inserted. Terel solution (isoflurane, Piramal Critical Care, Inc., Bethlehem, PA, USA) and oxygen was mixed at a ratio of 2 : 1, and the mixture was used as inhalation anesthesia before the abdominal areas were treated using the SCIZER™ HIFU device (Classys Inc., Seoul, Korea). HIFU energy levels from 60 J/cm^2 (three treatments of 180 J/cm^2). The pigs were followed up for 90 days post treatment before being humanely sacrificed. All procedures involving animals were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of CRONEX in Korea (IRB Number: 201506003).

Observation and examination

Thermal changes of the intra-dermis were measured for evaluation using the data logger (ZR-RX25, OMRON, Japan), which was connected with thermocouples. Skin surface measurements of the temperature distribution following treatment were performed on porcine abdominal skin using a FLIR A-Series infrared camera (FLIR system Inc., USA). Subcutaneous fat layer thickness measurements were performed using a folliscope (LeedM, Seoul, Korea), and the treated and adjacent areas were further evaluated using standardized flash photography and diagnostic ultrasound (Acuson P300™ LA523 Transducer, Siemens Medical Solutions, Malvern, PA, USA).

Carbon tracer test

The carbon tracer test was based on the principle that when India ink (BD Diagnostic Systems, Sparks, MD, USA) is injected at the procedure site after HIFU treatment around axillary lymph nodes, activated macrophages combine with carbon particles within the India ink and then move to nearby axillary lymph nodes. After three passes of HIFU treatment, the axillary lymph node was extracted from the sacrificed pigs on day 30. The objective of this test was to identify inflammatory reactions related to migrating macrophages produced by the focal area, which were dyed with India ink (dark blue or black blue).

Histological examination

Tissue biopsy was done before HIFU treatment (0), and at days 1, 7, 15, 30, 60, and 90. Tissue

was fixed with 4% paraformaldehyde and embedded in paraffin. Next, 5-μm-thick sections were transferred to probe-on-plus slides (Fisher Scientific, Pittsburg, PA, USA) and stained with hematoxylin and eosin (H&E) and toluidine blue. The skin biopsy samples were stored at −80°C and placed in a cryomold at the optimum cutting temperature (OCT) (Tissue-Tek®, Sakura Finetek Inc., Torrance, CA, USA). Subsequently, 10-μm-thick sections were stained with oil red O.

Immunohistochemical analyses

Sections were heated in an unmasking solution (citrate buffer, pH 6.0), washed, and subsequently incubated with PPARδ (1 : 200, 48-755, Prosci, Poway, CA, USA) at room temperature for 1 h. This procedure was followed by incubation with secondary antibodies (Envision Detection kit K5007, DAKO, Glostrup, Denmark). The reaction products were developed using 3,3′-diaminobenzidine, and sections were rinsed and counterstained with hematoxylin. Next, the sections were dehydrated and covered with permount (Fisher Scientific, Fair Lawn, NJ, USA) and cover slips.

Results

Focal penetration depth measurement and intra/extra-dermal temperature evaluation after HIFU treatment

HIFU subcutaneous fat reduction effects should have a predictable focal distance as HIFU accurately treats the target. In order to confirm the accuracy of HIFU, the focal distance and thermal injury zone (TIZ) of the HIFU treatment were examined. The focal distance was captured by a digital camera with digital image processing software. The focal distance and the focal area volume of the target point were then measured from the skin surface downward to the dermis. After five independent focal distance measurements using digital measurement, the value was 13.02 ± 0.2128 (Fig. 1b).

Next, we performed HIFU on the 50 × 50 mm square field prepared on the porcine abdominal skin (Fig. 1a). The power was set at 60 J/cm^2 (three treatments of 180 J/cm^2). The HIFU cartridge (frequency: 2 MHz, focal depth: 13 mm) led to coagulation at the target point for penetration. HIFU delivered heat to the subcutaneous layer of the focal area such that it only affected fat cells in the focal area

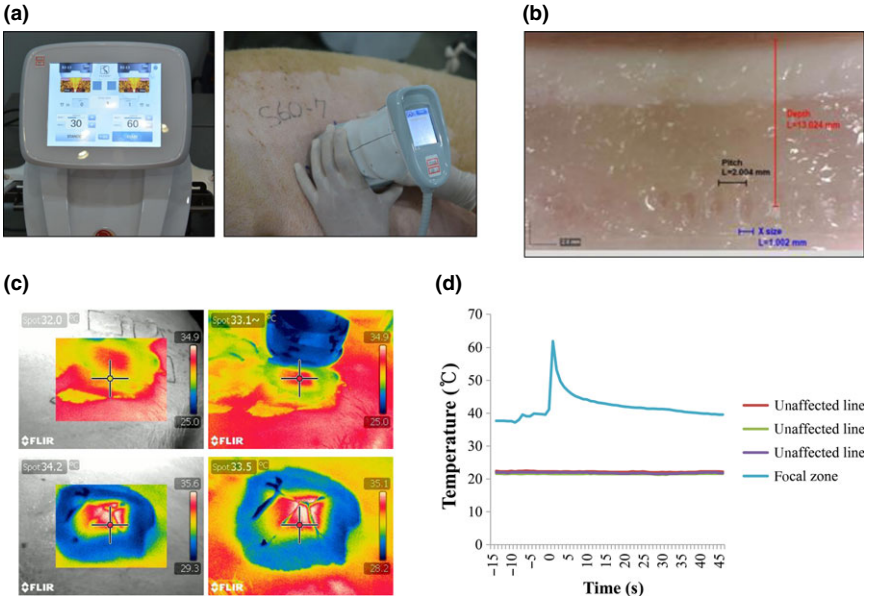


Fig. 1. Performance evaluation of focal distance, volume of focal area, intra-tissue ultrasound measurement for HIFU treatment. (a) The SCIZER™ HIFU device (obtained from Classys Inc., with permission). (b) Focal distances after treatment by cartridge (2 MHz, 13 mm) ex vivo. Image presents the focal distances and focal area volume of the target coagulation point after treatment. (c) Surface temperature changes of the porcine model during application of HIFU and after device removal immediately after treatment. HIFU delivered heat intensively only to the subcutaneous fat layer of the focal area and did not affect surrounding skin tissue. (d) Measurement of the intra-dermal temperature of porcine models during HIFU treatment. Excluding body temperature, a maximum of 30°C (approximately ~70°C) was generated for about 1–2 s. HIFU, High-intensity focused ultrasound.

and reduced subcutaneous fat tissue. To validate whether the test settings appropriately generated temperature, the skin surface temperature of the porcine models was measured with a thermal camera. The skin surface temperature was established at about 33.1~35.6°C, which was higher than that of the non-treated area (Fig. 1c).

The temperature of the focal intra-dermal area was then measured. Thermocouples connected to a data logger were inserted into the skin and measured the heat generated from the focal area after insertion. The temperature was 55~70°C, which was about 30°C higher than body temperature. Temperature transmission of about 70°C to the focal area can instantly induce tissue necrosis, so that the temperature was used to induce targeted subcutaneous fat reduction (Fig. 1c).

Changes in serum lipid levels after HIFU treatment

For safety evaluation, visual inspection by dermatologists was conducted after HIFU treatment in order to evaluate skin injury or changes. After procedures at 60 J/cm^2, a slight rash appeared, but largely disappeared an hour later. There was no difference between treated and normal tissue on day 1. As there was no significant injury to the tissue, HIFU appears to be safe (data not shown).

Blood sampling at the jugular vein was performed before HIFU treatment (0), and 2, 4, 8, 12, 24, 48, and 72 h after the procedure. Lipid profiles (triglyceride, total cholesterol, HDL, LDL, and free fatty acid) and total bilirubin, AST, ALT, and ALP levels were measured from the serum separated from the blood sample (Fig. 2). Data were obtained by methods described in detail in our previous studies (14). Free fatty acid level, which represents the degradation of triglycerides in fat cells lysed into the blood in fatty acid form, was higher than normal. However, it returned to normal 12 h after HIFU treatment. It also returned to normal at 48 h after HIFU treatment. This result is explained as fat tissue was rapidly decreased in just the focal fat area. The lipid profile

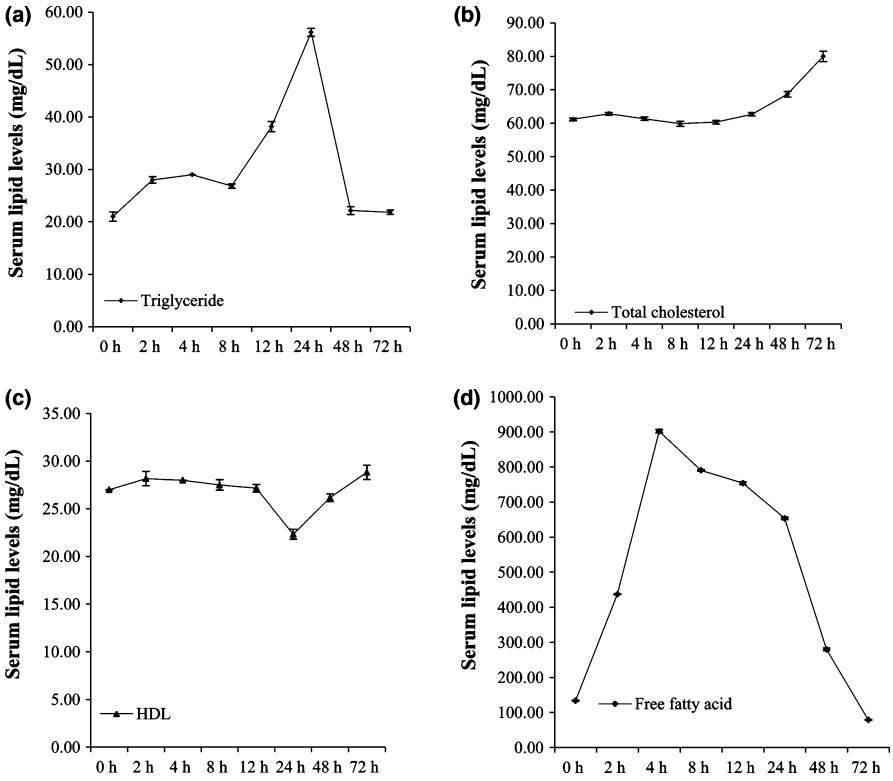


Fig. 2. Post-treatment changes in serum lipid levels and liver dysfunction test. Blood sampling from the jugular vein was conducted before HIFU treatment (0), and 2, 4, 8, 12, 48 and 72 h after treatment. After separating serum from the blood samples, laboratory testing was carried out. Levels of serum lipids, including (a) total cholesterol, (b) low-density lipoprotein cholesterol (LDL), (c) high-density lipoprotein (HDL), and (d) triglycerides (TG) were examined. Data are expressed as the mean ± standard deviation of duplicate samples. HIFU, High-intensity focused ultrasound.

increased temporarily until 24 h, but all values returned to normal after 48 h. Hepatic function values were also temporarily increased, but returned to normal.

Effect of histological change after HIFU treatment

After HIFU treatment was performed, a 6-mm punch biopsy was undertaken for histopathological examination of skin tissue. Fixed in 10% formalin solution, a paraffin block was made from the tissue and then H&E, toluidine blue, and oil red O staining were performed. Tissue biopsy was done before HIFU treatment, and at days 1, 7, 15, 30, 60, and 90. On H&E staining, we found rapid shrinkage of subcutaneous fat tissue after HIFU treatment, and rapid reduction or thickening of collagen within the focal area. We could identify no injury nerve fibers within the tissue. The procedure was deemed to be safe, and did not affect the tissue except for the focal area. In particular, at day 7, after toluidine blue staining, the macrophage activity in tissue was enhanced and fat cell apoptosis was indirectly confirmed as progressed. Fat cell injury by heat and recovery were assessed through oil red O staining. Fat cells or fat droplets easily decreased or disappeared in the tissue at locations closer to the focal area (Fig. 3).

Next, India ink was injected at the procedure site after HIFU treatment around the axillary lymph node (LN). Activated macrophages combine with carbon particles inside India ink and then move to the LN nearby. After three passes of HIFU treatment, the lymph node was extracted from sacrificed pigs in order to evaluate the inflammatory reaction of migrated macrophages dyed with India ink produced from the focal area. We checked India ink drainage at the cortical or medullar regions of the lymph node (Fig. 4).

Subcutaneous fat reduction by HIFU treatment

On ultrasonography, subcutaneous fat thickness reduction was found at the HIFU treatment site. The subcutaneous fat thickness was clearly reduced as the whole skin layer was pulled upwards (Fig. 5a). We performed subcutaneous fat layer thickness measurement after whole skin layer extraction.

At day 90 after HIFU treatment, subcutaneous fat layer thickness was measured by folliscope after whole skin layer extraction of the treated site and its surrounding area. Fat layer thickness reduction was observed at the treatment site (0.40 cm) compared to the non-treatment site (0.58 cm). Collagen thickening and contraction were identified at the subcutaneous fat border around the focal area (Fig. 5b).

Immunohistochemistry staining for PPAR-delta was performed until day 30. As a factor

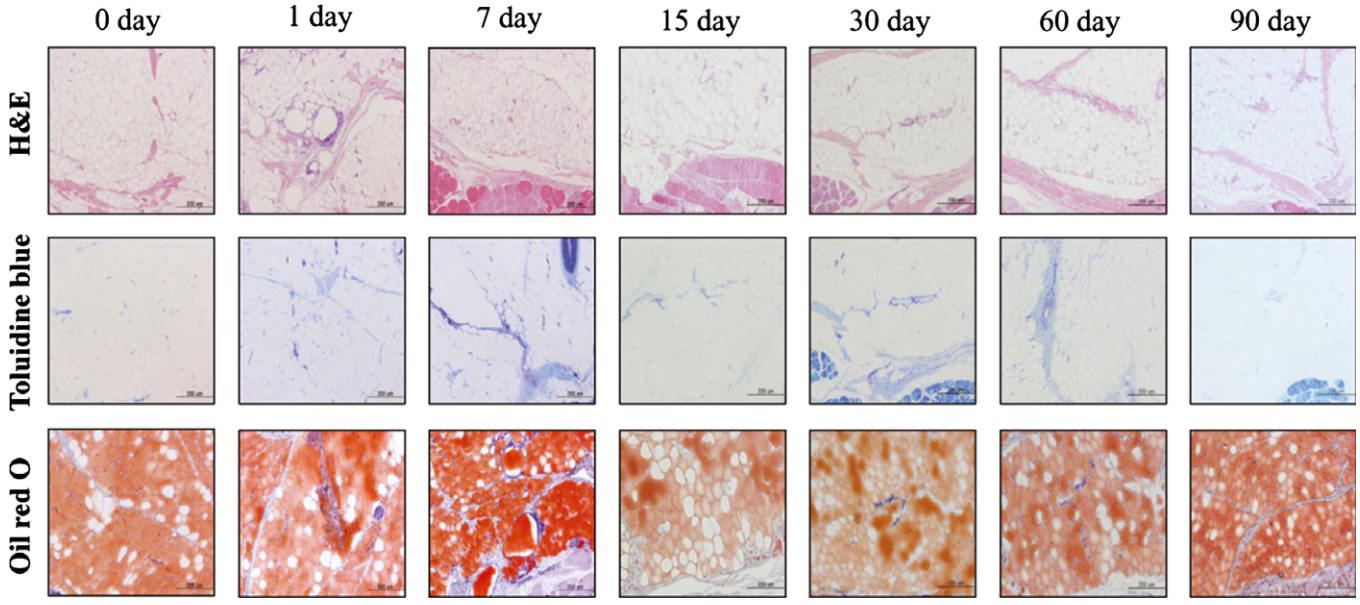


Fig. 3. Histological changes after treatment were observed using various staining methods. Tissue biopsy was done before HIFU treatment (0), and on days 1, 7, 15, 30, 60 and 90. The histological effect of the cooling device on porcine abdominal skin was analyzed using hematoxylin-eosin (H&E) staining and toluidine blue staining: mast cells stained purple. Oil red O staining: neutral fat and fat cells stained red. Bar = 200 μm. HIFU, High-intensity focused ultrasound.

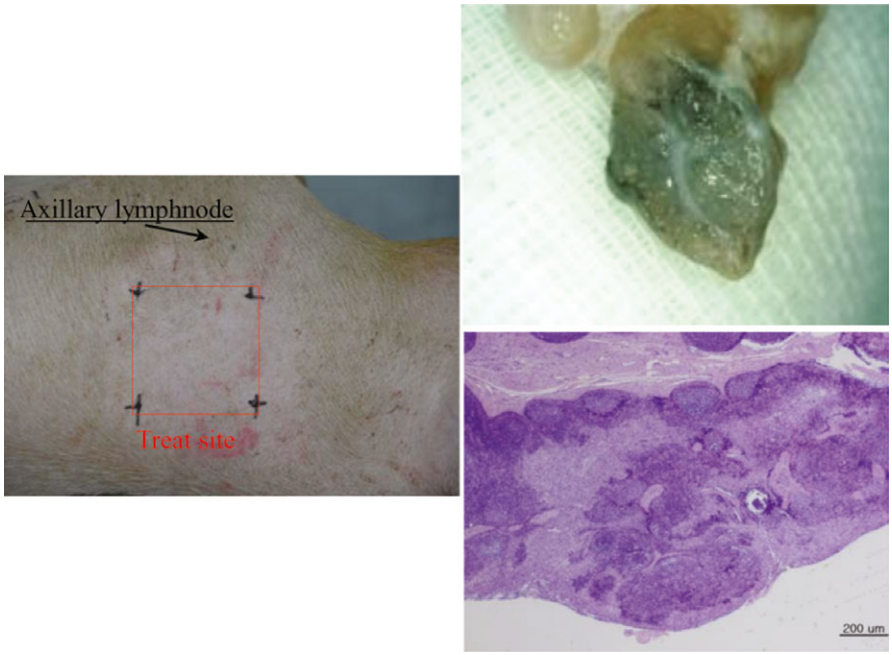


Fig. 4. Effect of HIFU treatment on carbon tracing in a porcine model. Macrophage activity after HIFU treatment was identified by injection of India ink. The axillary lymph node was extracted on day 30 after treatment. Bar = 200 μm. HIFU, High-intensity focused ultrasound.

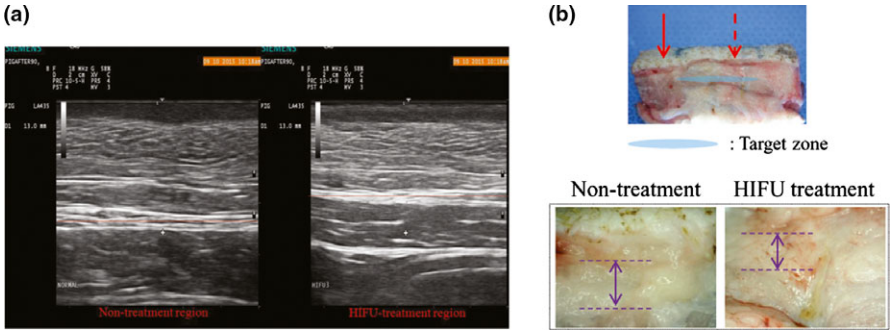


Fig. 5. Measurement of changes in fat layer thickness. (a) Fat thickness reduction at the target site was found from tissue extraction on day 90 after HIFU treatment. Ultrasonography before the procedure and on days 1, 7, 15, 30, 60, and 90 with HIFU treatment. (b) Subcutaneous fat thickness examination was performed on day 90. Skin thickness was clearly reduced. HIFU, High-intensity focused ultrasound.

involved in the lipogenesis mechanism, PPAR-delta (perosixome proliferator-activated receptor delta) functions as a lipid-sensing nuclear receptor (15). We evaluated its role in lipid catabolism as known, although it has not yet been clearly established. In this study, we demonstrate that PPAR-delta expression largely increased 30 days after HIFU treatment, which may reduce subcutaneous fat tissue rapidly and easily (Fig. 6).

Discussion

Non-invasive treatments for subcutaneous fat reduction include laser and suction massage combination therapy (16), pneumatic pressure massage therapy (17), low-frequency ultrasound diathermy (18), HIFU, and several others (19). HIFU is a non-invasive method that causes necrosis of subcutaneous adipose ('fat') tissue by localizing (e.g., focusing) thermal energy. Therefore, HIFU treatment can be concentrated in a precise subcutaneous area to produce fat lysis and can induce marked heating leading to adipocyte necrosis in the treatment area.

Liposonix devices [Food and Drug Administration (FDA) approved for non-invasive waist circumference reduction] focus their pulses of high energy (2 MHz) at the depth of the subcutaneous fat to disrupt discrete areas of adipocytes without damaging surrounding structures (20). Several studies have analyzed the safety

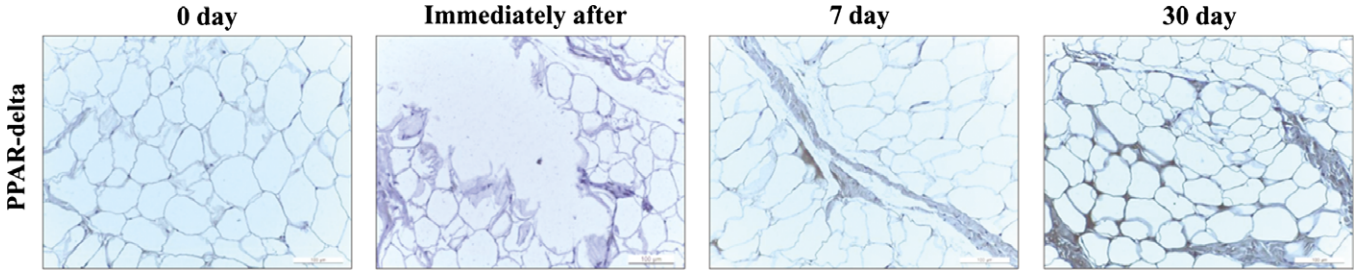


Fig. 6. Immunohistochemical identification of fat destruction and recovery in adipose tissue sections. Adipose tissue biopsies were taken 0, 15, and 30 days after treatment, and were analyzed by immunohistochemistry (n = 3). The PPARδ+ areas were stained with 3,3′-diaminobenzidine, with a hematoxylin counterstain to visualize the nuclei. Bar = 100 μm.

and efficacy of this device's ability to reduce local fat deposits (12, 21). However, a better understanding of the HIFU device and this principle, along with improved methods for the evaluation of fat reduction, are needed.

We performed a visual inspection and evaluated instrument evaluation after SCIZER™ [Cartridge (2 MHz, 13 mm)] treatment of the abdomen in a porcine model. We found no surface injury or heat injury on the skin of the HIFU treatment site. On ultrasonography, we found that HIFU treatments were performed accurately at the target site and there was effective subcutaneous fat reduction. At day 90, we extracted the whole skin layer at the procedure site and the surrounding area, and measured the subcutaneous fat thickness by folliscope. At the procedure site, thickness was reduced and the fat tissue was decreased compared to surrounding non-procedure areas. There was collagen contraction and thickening at the border of the subcutaneous fat layer, close to the focal area. Tissue staining (H&E, toluidine blue, oil red O, immunohistochemistry) revealed that HIFU treatment did not affect surrounding tissue, but accurately targeted the subcutaneous fat layer of the focal area, resulting in a significantly effective reduction in fat cell size and subcutaneous fat. Collagen bundle contraction and thickening were found at the border of the subcutaneous fat layer, close to the focal area.

PPAR-delta proteins defined by immunohistochemistry staining were overexpressed in the early stage on days 1 and 7, but a gradual decrease pattern was confirmed over time. It was hypothesized that the rapid reduction in subcutaneous fat would affect the recovery pathway and lipid catabolism (14). Cell necrosis caused by local energy absorption, which leads to physical disruption or elevates cell temperature to a level or for a period of time that the adipose structure cannot survive (22). Through laboratory tests, we revealed that fat reduction in the focal area did not affect the lipid profile and hepatic function. All laboratory results returned to normal 48 h after the procedure. Through a carbon tracer test, we identified the migration of activated macrophages in axillary LN.

In this study, we examined the efficacy and safety of SCIZER™ for subcutaneous fat reduction. Our findings indicate that SCIZER™ accurately treated the target subcutaneous fat layer, reducing subcutaneous fat effectively via ultrasonic measurement after HIFU treatment. At day 90 of the study, when the thickness of the subcutaneous fat layer was checked by folliscope after the whole skin layer including the procedure site and the surrounding area was extracted, it was confirmed that the thickness of the treated subcutaneous fat layer was thinner than untreated areas. This result supports the efficacy of subcutaneous fat reduction via HIFU. Totally, our results indicate that both investigative and diagnostic potential capacity for noninvasive body contouring method.

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Funding sources

None.

Conflicts of interest

None.

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