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Improved methods for selective cryolipolysis results in subcutaneous fat layer reduction in a porcine model

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Background/aims: Cryolipolysis is a noninvasive method for the selective reduction of localized fat tissues. It has demonstrated efficacy in both clinical and preclinical trials; however, despite its popularity, its mechanisms of action and evaluation methods are not yet fully defined. The purpose of this study was to improved methods for cryolipolysis using a porcine model.

Methods: The abdomens of female PWG micro-pigs were treated with a cooling device (CRYOLIPO II™), and we examined the treatment effects using photography, three-dimensional photography, ultrasound, gross, and microscopic pathology, and serum lipid level analyses in order to determine the mechanism of action, efficacy, and safety of CRYOLIPO II™.

Results: CRYOLIPO II™ successfully reduced abdominal fat in our porcine model. Gross and microscopic histological results confirmed the noninvasive cold-induced selective subcutaneous fat destruction, and showed increases in pre-adipocyte differentiation and in the activation of lipid catabolism. In particular, we found that CRYOLIPO II™ may increase PPARδ (delta) levels in adipose tissue at 30–60 days post-treatment.

Conclusion: Fat reduction by cryolipolysis was successfully achieved in our porcine model. Thus, our findings indicate that CRYOLIPO II™ may be a promising fat reduction device for body contouring and fat reduction in humans, and that cryolipolysis exerts its effects, at least partly, by targeting the PPARδ signaling pathway. These results show that both investigative and diagnostic potentials capacity.

Key words: cryolipolysis – fat reduction – noninvasive cooling – PPARdelta

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THE WIDE variety of human body contours, as determined by varying fat accumulation patterns, depends on a complex combination of factors such as lifestyle choices, age, sex, and genetic and hormonal influences (1). Excessive fat accumulation in the human body has increased dramatically in the last few decades, and is associated with a number of problems (2). Research efforts aimed at developing new, safe, and effective regimens for fat reduction have increased in recent years (3), with noninvasive alternatives to liposuction that can be performed annually becoming increasingly popular, as patients look for more effective methods of body sculpting (4). Successful selective fat reduction has been reported using a variety of novel methods, including cryolipolysis (5), high intensity focused thermal ultrasound (HIFU) (6, 7), radiofrequency ablation (8), and low-level external laser therapy (9, 10).

Cryolipolysis, which is the controlled application of cold to subcutaneous tissues to reduce the adipose tissues (11), has received FDA approval as a nonsurgical device for selective fat reduction. Previous reports have demonstrated that cryolipolysis results in an inflammatory process culminating in necrotic cell death (12), but the exact mechanism by which this inflammatory process is initiated and reduces the number of fat cells remains unclear.

In a clinical study, cryolipolysis for isolated fat deposits in the flank or on the back was demonstrated to result in a reduction in fat content (5). However, this treatment is associated with a number of side effects, including

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transient local redness, bruising and numbness of the skin, nonuniform fat reduction, sepsis, and even death (13). Therefore, there is an urgent need to develop safer and more effective fat removal techniques, including improved cryolipolysis devices. In this study, the cooling device CRYOLIPO II™ (Classys Inc., Seoul, Korea) was utilized to reduce abdominal fat in a porcine model, and the mechanisms of action were investigated.

Peroxisome proliferator-activated receptors (PPARs) are lipid-activated transcription factors that belong to the nuclear hormone receptor family. The PPAR family members are transcriptional factors that play key roles in lipid homeostasis. The PPAR family includes three isoforms, namely α, β/δ, and γ (14). PPARα has been implicated in the regulation of fatty-acid catabolism by controlling β-oxidation in the liver (15), whereas PPARγ, which is mainly expressed in the adipocytes and placenta is activated by specific compounds, such as prostaglandins and thiazolidinediones, and plays a central role in adipogenesis (16). On the other hand, PPARδ is commonly found in tissues with active lipid metabolism (17), such as adipose tissue, and heart and skeletal muscle. In particular, PPARδ acts as a very-low-density lipoprotein sensor in macrophages (18). Based on these findings, we investigated the protein expression of these nuclear receptors in adipose tissue after cryolipolysis.

The main aims of this study were to evaluate the mechanisms of action, efficacy, and safety and evaluated methods of the CRYOLIPO II™ cooling device in a porcine model, and we found that it effectively resulted in fat reduction of the treated areas.

Materials and Methods

Animals and experimental design

Female PWG micro-pigs ($n = 2$) 6 months of age or older were used in this study. All animals were obtained from the closed barrier unit at Medikinetics (MK, Pyeongtaek, 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). The pigs were administered general anesthesia with medetomidine (Domitor, Pfizer Animal Health Korea, Seoul, Korea) and tiletamine/zolazepam (Zoletil, Virbac, Carros, France) via intramuscular (IM) injection before the abdominal areas were treated using the CRYOLIPO II™ cooling device for 1 h (Classys Inc.). The cooling intensity factor (CIF), which is a measure of the rate of heat extraction from the treatment area, was 24.9 (-44.68 mW/cm^2). During the 1 h procedure, the massage was performed for 30 min, followed by a massage that was performed automatically by the device itself for 10 min. The pigs were followed up for 90 days post-treatment before being humanely sacrificed.

Assessments

Skin surface measurements of the temperature distribution following treatments were performed on the porcine abdominal skin by an FLIR T-250 infrared camera (FLIR system Inc., Danderyd, Sweden). The treatment areas were photographed on day 0 and 90, and the changes in surface area were measured using a three-dimensional (3D) LifeViz Camera (Quantificare, Cedex, France). The fat layer thickness measurements were performed using a Folliscope (LeedM, Seoul, Korea), and the treated and adjacent areas were moreover evaluated using standardized flash photography and diagnostic ultrasound (Bionet, Seoul, Korea) 90 days post-treatment.

Serum lipid analysis

The pigs were restrained manually for collection of blood from the caudal portion of the external jugular vein. Blood samples were centrifuged at 1500 g for 25 min, and the serum or plasma was removed and stored at −20°C until the time of sample analysis. Serum was collected into heparinized tubes (Vacutainer, Becton-Dickinson, CA, USA) containing sodium heparin (143 USP units per tube) and immediately placed on ice. All samples were collected after an overnight fast of at least 12 h. Fasting triglyceride (TG) and cholesterol levels were determined enzymatically (19). Very-low and low-density lipoproteins (VLDL and LDL, respectively) were precipitated by 4% sodium phosphotungstate from the plasma (20), and high-density lipoprotein (HDL) cholesterol was

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measured in the supernatant after centrifugation at 1500 g for 30 min at 4°C.

Histological examination

The porcine skin tissues were fixed with 4% paraformaldehyde (PFA) and embedded in paraffin. Subsequently, 5-μm-thick sections, cut using a microtome, 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 with optimum cutting temperature (OCT) (Tissue-Tek®, Sakura Finetek Inc., Torrrance, CA, USA). Next, 10-μm-thick sections were cut using a microtome, placed on slides, and stained with Oil Red O, followed by counterstaining of the nuclei by hematoxylin.

Immunohistochemical analyses

The sections were stained using mouse monoclonal antibodies against PPARδ (1 : 200, 48-755, Prosci, Poway, CA, USA). Immunohistochemical analyses were performed with a high temperature antigen unmasking technique. In brief, the sections were heated in an unmasking solution (citrate buffer, pH 6.0), washed, and subsequently incubated with the primary monoclonal antibodies 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 the sections were rinsed and counterstained with hematoxylin to visualize the nuclei. Lastly, the sections were dehydrated and covered with permount (Fisher Scientific, Fair Lawn, NJ, USA) and cover slips. Light microscopy was used to assess the histological changes.

Statistical analyses

Statistical comparisons between the treated and untreated groups were performed using one-way ANOVA analyses followed by post hoc Tukey for direct comparison. The results are expressed as means ± standard deviations of at least three independent experiments, and P values of $P < 0.05$: *, $P < 0.01$: **, and $P < 0.001$: *** were considered statistically significant.

Results

Effects of fat layer reduction on visible skin contour changes in a porcine model

To evaluate the effects of the cooling device on fat reduction *in vivo*, we used a porcine model. The pigs were shaved with an electric clipper and the device was pressed against the shaved surface of the skin (Fig. 1a). The cooling conditions were as follows: temperature, −7°C; suction, four steps; time, 1 h. The animals were sacrificed 90 days post-treatment. The test sites and surrounding areas were clinically assessed and photographed at days 0, 30, 60, and 90 post-treatment. For analysis of temperature distribution, the test sites were separated from other regions by contour lines. The average temperature in the contoured areas was 32.7°C (range, 16.4–31.6°C) immediately after the treatment (Fig. 1b).

Porcine skin tissues 90 days post-treatment were examined for changes in body contour and depth by a 3D camera. On day 90, the test sites showed changes in the abdominal skin (Fig. 2a). The reduction in thickness of the upper layer of the subcutaneous fat was confirmed by examination of the gross pathology photographs. As shown in Fig. 3a, fat reduction, as determined by decreased thickness of the porcine skin, was observed immediately after cooling device application (skin thickness of the cold-induced site, 1.57 mm; thickness in the non-cold-induced site, 8.47 mm). Reductions in fat layer thickness were assessed using ultrasound (Fig. 3b) and measurements of the pathologic specimens. Mean reductions in the superficial fat layer of 47% (from 1.27 to 0.68 cm), 44% (0.71 cm), and 57% (0.54 cm) were observed after 30, 60, and 90 days, respectively. Overall, the morphological and histological findings showed that cooling device application resulted in a marked fat reduction and decrease in the depth of porcine skin at 30, 60, and 90 days post-treatment, as compared to day 0. Therefore, our results suggest that the CRYOLIPO II™ cooling device effectively results in fat layer reduction, and consequently in visible skin contour changes in a porcine model.

Inflammatory response and lipid reduction in porcine skin

Porcine cutaneous tissues obtained on days 0, 15, 30, 60, and 90 post-treatment were analyzed

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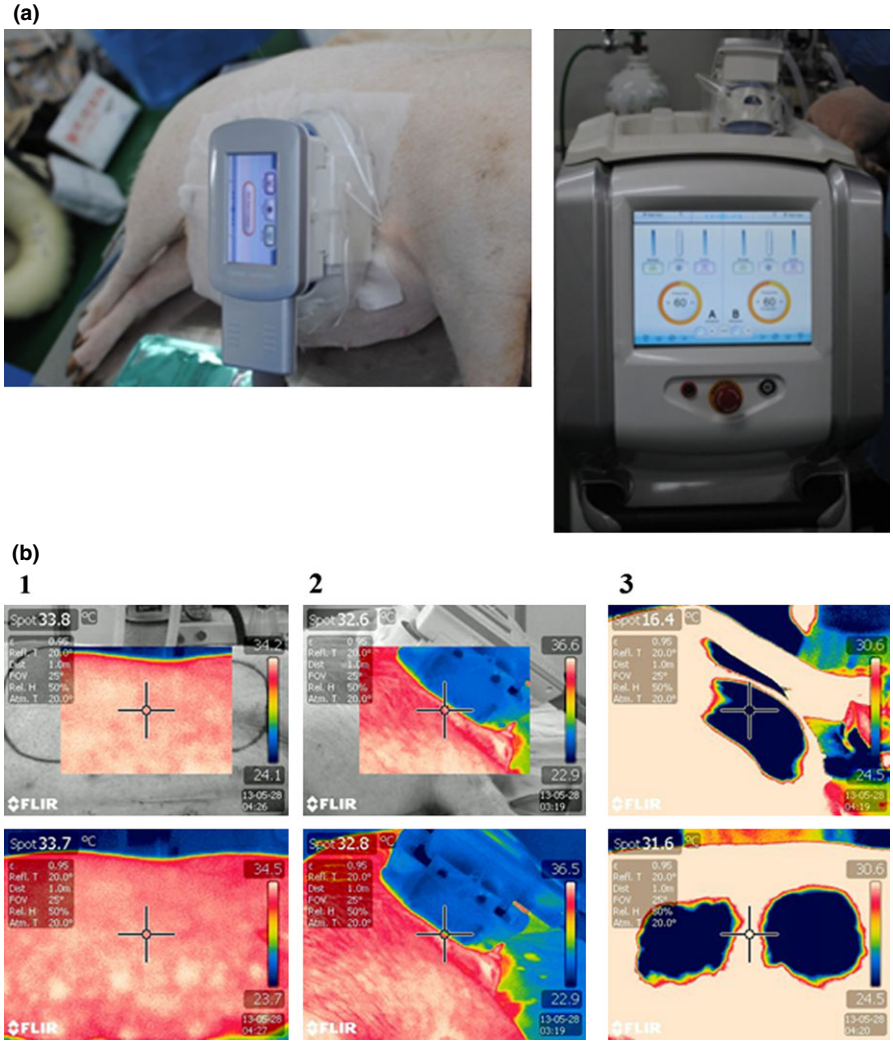


Fig. 1. CRYOLIPO II™ cooling device. (a) The CRYOLIPO II™ cooling device (From Classys Inc.; with permission). (b) Thermal profiles before cooling device application in porcine skin and immediately after the end of the treatment.

for the status of inflammatory response and lipid reduction, using optical microscopy. We detected no injury to the epidermis or the dermis outside the focal region.

Figure 4 shows the results of the cooling device treatment on the adipose tissues. As shown in Fig. 4a, H&E stained sections were used to measure the inflammatory cell response in the deep adipose tissue, and the results of the histological evaluations showed dramatic activation of macrophages (Fig. 4a, c). Interestingly, the inflammatory process was highly apparent at 15 days post-treatment, but had begun to decline 30 days post-treatment. Moreover, Oil Red O staining showed adipocytes with decreased lipid droplets in the fat layer (Fig. 4b), and based on these findings, we concluded that the cooling device effectively induced apoptosis and decreased the mature lipid cells in this porcine model.

Effect of serum lipid levels

Lipoproteins consist of esterified and un-esterified cholesterol, triglycerides, phospholipids, and proteins. There are five major lipoproteins in the blood: chylomicrons, very-low-density lipoprotein, low-density lipoprotein, intermediate-density lipoprotein (IDL), and high-density lipoprotein. We here examined the effects of the CRYOLIPO II™ cooling device on a porcine model using average phenotypic values for total cholesterol, HDL, LDL, and TG. The treated pigs were found to have higher serum cholesterol, HDL, LDL, and TG concentrations during days 0–30 compared to the other time points,

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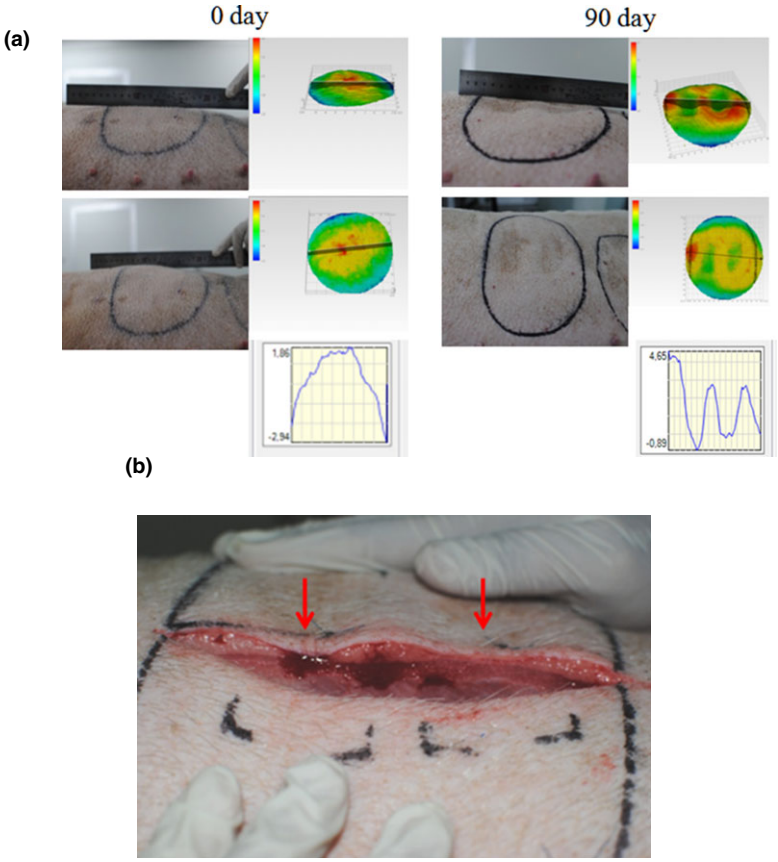


Fig. 2. Skin contour changes and gross pathology. (a) Photographs of the treated areas 90 days after cooling device application showed clear surface contour changes. (b) Gross pathology photograph showing an example of fat layer reduction that contributed to the visible skin contour changes.

with the serum cholesterol, HDL, LDL, and TG concentrations on day 60 post-treatment being significantly lower than on day 0, and the serum LDL-cholesterol concentrations being significantly lower on day 90 compared to day 0. Our results showed that the serum total cholesterol, HDL, LDL, and TG concentrations significantly decreased at 60 days after treatment. These data suggest that the reduced levels of lipoproteins observed in the fat layer 60 days postcryolipolysis may be the result of decreased serum lipid levels, and may lead to activation of lipid catabolism. However, further studies are needed to examine the mechanisms of serum lipid level regulation by cooling devices.

Fat reduction through lipid catabolism activation

To evaluate the mechanism underlying the induction of lipid catabolism in this porcine model, we performed immunohistochemistry on porcine skin specimens using an antibody specific to PPARδ. Previous reports have suggested that PPARδ may play roles in the pathology of diseases associated with lipid-filled macrophages, such as dyslipidemia and atherosclerosis (21, 22). However, the effects on lipid metabolism by direct activation of PPARδ remain controversial. In the porcine model used herein, a noticeable increase of PPARδ protein expression was observed in the adipocytes of the fat layer from day 15 post-treatment, whereas a marked decrease in PPARδ protein expression levels was observed at day 30 post-treatment, as compared with day 0. In particular, the PPARδ staining revealed greatly increased lipid droplet accumulation in the adipocytes. Overall, our findings suggest that cooling devices used for fat reduction may affect the lipid catabolism by activating endogenous lipid metabolites through the PPAR pathway.

Discussion

Cryolipolysis has previously been demonstrated to show efficacy for fat reduction and body sculpting in both clinical and preclinical studies, but despite its popularity, its mechanisms of

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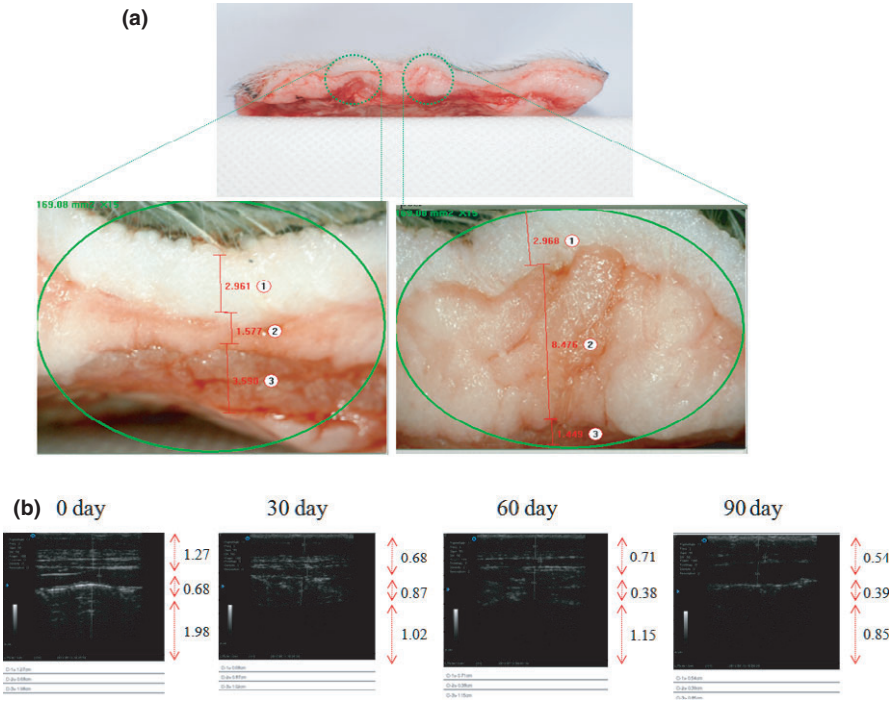


Fig. 3. Changes in fat layer thickness. (a) Fat layer thickness measurement was performed using a Folliscope (computerized hand-held USB camera) at 90 days post-treatment. (b) Photometric comparison of the treatment areas was performed using standardized flash photography and diagnostic ultrasound assessments at days 0 and 90 post-treatment.

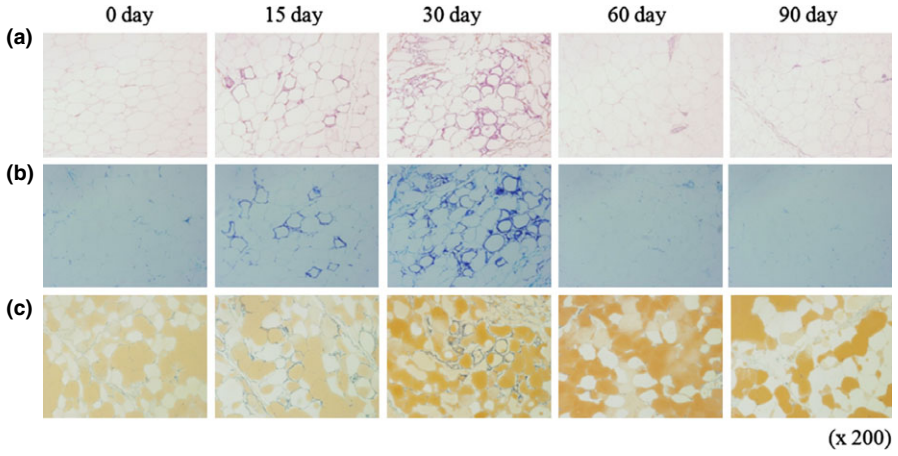


Fig. 4. Effects of cooling device application on inflammatory responses and lipid reduction in porcine skin. Typical photographs of histology from each panel: (a) the effect of the cooling device on porcine abdominal skin was analyzed using hematoxylin-eosin (H&E) staining and (b) toluidine blue staining. Mast cells stained purple. (c) Oil Red O staining. Neutral fat and fat cells stained red. Original magnification, ×100.

action are not yet fully understood (23). Cryolipolysis has been shown to be able to significantly reduce fat layer thickness in a pig model (12), and on the basis of these studies, we aimed to evaluate the effects of cryolipolysis (using the CRYOLIPO II™ cooling device) on abdominal fat reduction *in vivo*. The effects of the cooling device application on the test sites and surrounding areas were examined in a porcine model using photography, 3D photography, ultrasound, and gross and microscopic pathology examinations. Specifically, morphological and histological studies showed that cooling device application resulted in marked fat reduction and decreases of the skin depth. Hence, our results suggest that the CRYOLIPO II™ cooling device effectively resulted in fat layer reduction, which was responsible for the

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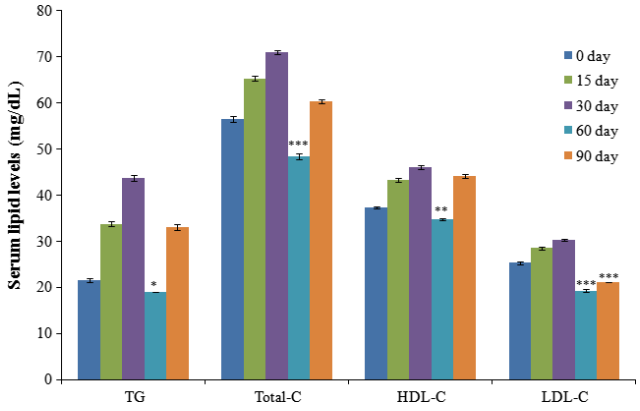


*Fig. 5. Post-treatment changes in serum lipid levels. Levels of serum lipids, including total cholesterol, low-density lipoprotein cholesterol (LDL), high-density lipoprotein (HDL), and triglycerides (TG) were examined. On days 0, 15, 30, 60, and 90 post-treatment, serum was collected from jugular vein blood. Serum lipid levels were determined enzymatically. Data are expressed as means ± standard deviations of duplicate samples. *P < 0.05, **P < 0.01, ***P < 0.001 as compared to day 0.*

visible skin contour changes observed in this porcine model. Moreover, the CRYOLIPO II™ cooling device proved to be safe for subcutaneous fat reduction, with no side effects or adverse events observed by the morphological, histological, or gross pathological analyses.

Furthermore, as shown in Fig. 4a, H&E stained sections of the adipose tissues from the deep fat layer were analyzed for inflammatory cell responses. The results of the histological evaluations showed a dramatic activation of macrophages in the fat layer 15 days post-treatment, which was reduced by day 30.

Interestingly, 30 days after application of the cooling device, the adipose tissue was greatly reduced. Selective cooling of adipocytes has been previously demonstrated to result in enhanced apoptosis (24), suggesting that cryolipolysis may exert its effects by producing injuries to the adipose tissue, thereby resulting in the activation of apoptotic pathways. Furthermore, the subsequent inflammatory response may cause additional damage to the adipocytes that are not immediately affected by the cold exposure (25). Similar to our results, Goldman et al. reported in their study that the inflammatory process became increasingly apparent at days 7 and 14 post-treatment in porcine skin, and that between 14 and 30 days post-treatment, phagocytosis of lipids became apparent, whereas by 30 days post-treatment, the inflammatory process had begun to decline (12).

In this study, Oil Red O staining showed a reduction in the size of the lipid droplets in the adipocytes in the fat layer (Fig. 4b). Therefore, we conclude that the cooling device used herein effectively induced apoptosis and decreased the number of mature lipid cells in our porcine model. These results also confirm previous findings suggesting that fat cells may be more sensitive to cold than other tissues.

Lastly, we examined the effects of the CRYOLIPO II™ cooling device in our porcine model using average phenotypic values for total cholesterol, HDL, LDL, and TG (Fig. 5). In a previous report, preliminary animal and human data suggested that cryolipolysis had no effect on serum lipid profiles (26). However, results from our study, which used a different pig species compared to previous studies, suggest that, 60 days after lipolysis treatment, the lipoproteins in the fat layer are decreased, and that this may be the result of decreased serum lipid levels, and may lead to activation of lipid catabolism. In particular, PPARδ activation is known to result in increased lipid catabolism in adipose tissues and in cardiac and skeletal muscles, and in improved serum lipid levels and insulin sensitivity in animal models. Additionally, PPARδ ligands have been found to prevent weight gain and suppress macrophage-derived inflammation *in vivo* (27). To evaluate the mechanisms underlying the induction of lipid catabolism in our porcine model, we

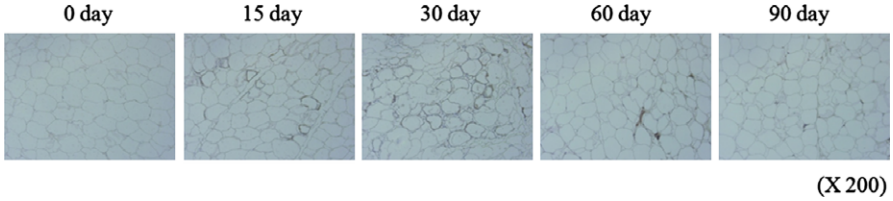


Fig 6. Immunohistochemical identification of PPARδ-positive cells in adipose tissue sections. Skin biopsies were taken 0, 15, 30, 60, and 90 days after treatment, and were analyzed by immunohistochemistry. The PPARδ+ areas are stained by 3,3′-diaminobenzidine (red arrow), with a hematoxylin counterstain to visualize the nuclei. Original magnification, ×100.

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performed immunohistochemistry (Fig. 6). Our results indicate that PPARδ expression levels were markedly increased 15 days post-treatment, as compared with day 0, and these findings suggest that cryolipolysis may result in enhanced lipid catabolism, and that cooling devices may activate endogenous lipid metabolites through the PPAR pathway. However, additional studies are required to validate these results.

Our findings indicate that CRYOLIPO II™ represents a promising fat reduction device for body contouring. These results shows that both investigative and diagnostic potentials capacity, and could serve as a useful methods and device for fat reduction and body sculpting in humans.

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Conflicts of interest

None.

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