Selective Cryolysis: A Novel Method of Non-Invasive Fat Removal

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Background and Objectives: Excess fat poses a host of local and systemic problems. Various energy sources, for example, laser, ultrasound, and radiofrequency electric current have been studied as potential non-invasive treatments aimed at local destruction of subcutaneous fat. Cryosurgery at very low temperatures is routinely used for non-specific tissue destruction, however the potential for tissue-specific cold injury has not been investigated. This study describes non-invasive cold-induced selective destruction of subcutaneous fat.
Materials and Methods: Black Yucatan pigs under general anesthesia were exposed within test sites to preset temperatures of 20, -1, -3, -5, and $-7°C$ for 10 minutes. Gross and histological assessments were performed immediately, 1 day, 2, 7, 14 and 28 days post-cold exposure for four pigs, and up to 3.5 months for one pig. Additionally, six pigs were exposed between $-5°C$ and $-8°C$ for 10 minutes, at sites covering approximately 15% body surface area, followed by serum lipid level determinations at various time points up to 3 months.
Results: A lobular panniculitis was induced by cooling, followed for some test sites by grossly obvious loss of several mm of subcutaneous fat occurring gradually during the 3.5 months study period. Loss of adipocytes, the appearance of lipid-laden mononuclear inflammatory cells, and local thickening of fibrous septae were noted. Typically there was no clinical or histological evidence of injury to skin, and no scarring. Serum lipids were not significantly increased.
Conclusions: Prolonged, controlled local skin cooling can induce selective damage and subsequent loss of subcutaneous fat, without damaging the overlying skin. Selective cryolysis warrants further study as a local treatment for removal of adipose tissue. Lasers Surg. Med. 40:595–604, 2008. © 2008 Wiley-Liss, Inc.

Key words: cryolipolysis; cold; panniculitis; fat removal; pig; selective cryolysis; cryo; body contouring

INTRODUCTION

There is increasing public, medical, and scientific awareness regarding the many hazards of excess adipose tissue. Western culture and media often promote a slim body image. Excess body fat poses systemic health problems, and is frequently associated with a dissatisfaction of the body shape. The systemic medical problems include risks for cardiovascular disease, type II diabetes and cancer, associated particularly with excess visceral fat [1,2]. Secondary problems due to overweight include musculoskeletal problems, arthritis and difficulty exercising. Body shape and image are negatively impacted by unwanted local subcutaneous fat. Diets, exercise, medications and/or gastric surgeries may effectively control obesity [3]. Various cosmetic procedures and devices have been developed to remove or reduce unwanted local subcutaneous fat. However, the majority of these are procedures pose potential complications due to their invasive nature, or may lead to poor or minimal results.

Conventional fat removal is achieved surgically by abdominoplasty [4] or by liposuction, which has evolved as a minimally invasive alternative, to remove fat through a cannula inserted through several small skin incisions [5]. Liposuction is typically safe, when performed by highly skilled physicians and can be performed as an office procedure with tumescent anesthesia. However, it remains a risk of infection, local or widespread scarring, non-uniform fat reduction, perforation, hemorrhage, sepsis and death [6,7]. Due to these potential side effects, downtime and requirements for sterile procedures, there is a need for a non-invasive procedure that also allows for effective fat removal.

Various non-invasive devices including laser [8], radio frequency [9] and ultrasound [10] are geared to overcome these limitations, by trying to selectively damage subcutaneous fatty tissue using various forms of energy delivered into the subcutaneous fat. Focusing (ultrasound), greater attenuation in fat (laser, radiofrequency) and/or concurrent application of surface cooling are used to generate maximum effects of the emitted energy below the surface at the level of the fatty tissue. The selectivity of such modalities is achieved by the physical interaction between the tissue and emitted energy.

Cooling is commonly employed to protect the epidermis and skin during laser or intense pulsed light treatments [11]. These applications are not aimed to have any therapeutic effect due to cooling per se. In contrast, cryosurgery with liquid nitrogen or other cryogen is widely used for non-selective tissue destruction of actinic keratosis, warts, pigmented lesions, superficial tumors and other lesions [12,13]. Cells are irreversibly damaged by freeze/thaw cycle(s) when the tissue temperature plummets far below the freezing point of tissue water [14]. Typically, liquid nitrogen at $-196°C$ is used for exposure times in the range of seconds. Apparently all types of human cells are susceptible to damage from conventional cryosurgery. In clinical cryosurgery the factors that define the extent of cold injury are the freezing rate, coldest temperature produced, the duration of freezing and the thawing rate [15]. In an in vivo dog model it was shown that cryosurgery for 3 minutes resulted in 100% viable dermis and epidermis when the intradermal temperature range was between 0 and $-10°C$ [15].

There are hints that adipose tissue is preferentially sensitive to cold injury. A rare clinical entity of cold-induced fat necrosis in infants, has been well described and sometimes called "popsicle panniculitis" [16]. Inflammation of the buccal fatty tissue occurs after infants suck for a prolonged time on frozen treats. Another uncommon clinical entity, equestrian panniculitis [17], was described in females after horse riding with tight pants in cold climates. These unusual clinical observations suggest that human adipose tissue may be preferentially damaged by exposure to cold.

The purpose of this study was to test in an animal model whether selective damage to subcutaneous fat can be achieved by controlled cold application to the skin surface, within a temperature range well above conventional cryosurgery, and using application times long enough to extract heat from subcutaneous fat. The results of three complementary animal pilot studies are reported here [18]. The term "selective cryolysis" was chosen to describe the intentional, selective damage of adipose tissue by controlled cooling.

METHODS

The following studies were performed at different time points with distinct emphasis. Therefore different cooling devices were used. For the initial exploratory study a simple cold applicator was employed, the dosimetry study used an electronically regulated thermoelectric chiller in order to maintain a well-controlled, preset surface temperature, and the lipid level study was performed with an applicator that had an increased size to facilitate the cold exposure of relative large areas.

Exploratory Cold Exposures ($N = 1$)

A black Yucatan pig (female, 6 months of age, approximately 25 kg) was used to explore the feasibility of removing subcutaneous fatty tissue by local, non-invasive cold exposure.

This pig was exposed to a laboratory cooling prototype device that had as the cold applicator a slightly convex, circular copper plate (curvature radius approximately 10 cm, and diameter 5.5 cm), designed to be pressed firmly against the skin surface. The copper plate was cooled with a laboratory chiller (Neslab RTE 100, Thermo Electron Corp. Newington, NH) by circulating a cold antifreeze solution at $-7°C$ through a heat exchanger chamber attached to the copper plate. The chiller was operating in constant temperature mode. The cold applicator was mounted to a spring-loaded handpiece that required a pressure of approximately 150 N to fully compress the spring, providing a pressure of approximately 6 N/cm^2 (450 mmHg) upon the skin.

Cold exposures were performed under general anesthesia using Telazol/Xylazine 4.4 mg/kg i.m.+2.2 mg/kg i.m. and inhalant anesthetics (Isoflurane (1.5–3.0%) with oxygen (3.0 L/min)) delivered by mask. Several test sites were mapped with microtattoos on the flank, buttock and abdomen. All test sites were shaved before exposure. A glycerol/water lotion was applied between the skin and the cooling device to increase thermal contact. The device was applied with such force during the exposure that the spring was fully compressed in an attempt to inhibit blood flow within the test sites. In 3 out of 11 test sites (see Table 1) the test sites were located within the flank area. At these sites the application pressure was reduced (spring only partially compressed) in order to avoid suppression of the respiration by prolonged mechanical compression of the rib cage. Exposure time varied arbitrarily between 5 and 21 minutes. This pig was observed for 3.5 months for the appearance and persistence of local fat loss, and then euthanized to obtain gross and histological sections stained with hematoxylin and eosin. The amount of fat loss in each test site was estimated relative to adjacent unexposed fat layer thickness at the margins of each cold exposure site.

Dosimetry Study ($N = 4$)

The study was performed on four female black Yucatan pigs (retired breeders) of 2–3 years of age, body weight approximately ca. 100 kg.

Cryolysis Device for Fatty Tissue

The Zeltiq prototype device (Zeltiq, Pleasanton, CA) contained a thermoelectric TE cooling element assembled with an active surface cooling area of 3×4 cm^2, and a maximum heat flux of approximately -10 W/cm^2 at $\delta T = 0°C$ and -6 W/cm^2 at $\delta T = 30°C$. The excess heat from the TE element was removed by a PolyScience 5306 (PolyScience, Niles, IL) liquid chiller operating as a close loop system. A variable, preset plate temperature was maintained constant during each cold exposure, by electronic regulation according to temperature sensors embedded within the cooling plate. Most test sites were performed with a single flat cold applicator simply held against the skin surface (Fig. 1a). In this configuration, subcutaneous fat is cooled through the overlying dermis while its deep fascia margin is still in contact with underlying muscle. The deep fascia between fat and muscle

TABLE 1. Gross Observations—Pilot Exposure Study

Site	Temperature [°C]	Time [minutes]	Location	Indentation	Relative fat loss of superficial fat layer
1	Control		Abdomen	−	9%
2	−7	5	Flank	+	NA
3	−7	5	Flank	+	66%
4	−7	10	Abdomen	+	NA
5	−7	10	Buttock	++	79%
6	−7	10	Buttock	+	57%
7	−7	10	Buttock	+	NA
8	−7	21	Buttock	+	NA
9	−7	11	Buttock	+	NA
10	−7	10	Buttock	++	NA
11	−7	15	Flank	+	66%

Exposure parameters, test site location, clinical observations and relative superficial fat layer reduction. Indentation was graded at the time point of euthanasia (−, not noticeable; +, minor; ++, marked). The percentage of relative fat loss within the superficial fat layer as compared to the adjacent unexposed area was determined. Some samples were not evaluated ex vivo (NA).

is a sliding layer (allowing muscular motion); due to this mobility, a "fold" of skin and subcutaneous fat can be easily created by pulling. Test sites were also exposed in "fold configuration" (Fig. 1b), using two cooling plates of the same size applied on the sides of the skin fold with pressure.

Cold Exposures

All procedures were performed under general anesthesia as described above. All test sites were shaved before exposure. Approximately 60 cold exposure sites and 10 control sites were mapped and individually marked at the corners with India Ink microtattoos at the fist visit within the flanks, abdomen and buttocks. Each Yucatan pig was exposed within mapped test sites at selected time points (28 days, 14, 7, 2, 1 and immediately ~1 hour) before euthanasia with preset plate temperature settings (20, −1, −3, −5, −7°C) for 10 minutes. Each exposure temperature was performed in duplicate at each time point. At each procedure day the assignment of the test sites to the different exposure temperature and follow up intervals was randomly assigned to avoid potential anatomic or other systematic bias. To ensure good thermal contact from skin to applicator, an ultrasound gel (Ultrasound Gel, Medline,
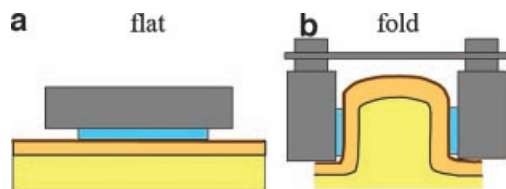

Fig. 1. Schematic of different exposure configurations. Colors represent: device housing (gray), cold element with optional built-in temperature sensor (blue), epidermis (dark brown), dermis (light brown), subcutaneous fatty tissue (yellow). **a**: "Flat" exposure of a single cold element. **b**: "Fold" exposure with two cooling elements simultaneously cooling an elevated and compressed treatment fold volume.

Mundelein, IL) was applied to the skin before each cold exposure. Flat exposures were performed holding the device with a contact pressure of approximately 1 N/cm^2 (~75 mmHg) to the skin. Because the "fold" application required stretching the skin into a fold between two cooling plates, these test sites were limited to the abdomen which has sufficient skin laxity in the pig. Substantial pressure was applied to the skin on each side of the "fold," sufficient to reduce or eliminate blood flow within the skin fold.

Test sites and surrounding areas were clinically assessed and standardized photography of individual test sites was performed. A diagnostic ultrasound imager (SonoSite 180 with a 7.5 MHz linear transducer, SonoSite, Inc, Bothell, WA) was also used for some test sites. After euthanasia, tissue was processed for gross pathologic and histologic evaluation. Deep (skin, fat, underlying muscle) tissue vertical sections were stained with hematoxylin and eosin and evaluated microscopically to assess the level of fat damage as well as potential damage to the dermis or epidermis. In addition to blinded qualitative assessment, the extent of inflammation within the adipose tissue close to the dermal−fat junction was graded by a blinded investigator on a scale from 0 to 3. Slides exhibited in Figure 5 were used as reference for the grading. Intermediate half grades were allowed in case the clear assignment to a specific level was ambiguous.

Besides these test sites, some additional test sites were used in order to measure the tissue temperatures in vivo during cold exposure. Thin thermocouples designed for tissue insertion (Hyp-1 type, 30 gauge, Omega, Stamford, CT) were put at the surface of the skin and inserted laterally just below the dermal−fat junction. Depth and proper location of the tip of the inserted couple was confirmed by the ultrasound device. Superficial and deep thermocouples were sutured into place to minimize displacement and movement artifacts during cold exposure. These test sites were not used for histological evaluation as

the inserted thermocouples could have induced tissue alterations.

Statistical Analysis

Repeated-measures mixed model analysis of variance (ANOVA) was used to evaluate differences in fat damage (0–3 ordinal scale) according temperature and time with damage examined at multiple test sites from the same animal. F-tests were used to determine effects of temperature and time on fat damage and Akaike's information criterion (AIC) was used to assess goodness-of-fit of the statistical model.

Lipid Level Study ($N = 6$)

Six adult female black Yucatan pigs (retired breeders) 2–3 years old, weighing approximately 100 kg were used. The animals were fasted for 12 hours prior to cold exposure under general anesthesia to minimize the risk of aspiration, and were otherwise maintained on a standard porcine diet with feedings ad lib. Test sites were exposed to a large-area laboratory prototype cooling device consisting of a flat copper plate of 2.5 cm thickness and 11.5×11.5 cm^2 area, mounted to a thermoelectric cooling element. The thermoelectric TE cooler was a LHP-800CP (TECA Corp., Chicago, IL) with a specified maximum heat flux of approximately -1.5 W/cm^2 at $\delta T = 0°$C and -0.5 W/cm^2 at $\delta T = 30°$C. The entire cooling element had a weight of 6 kg. An on-off thermostatic temperature controller (TC3300, TECA Corp.) was used in this system. Embedded within the surface of the copper plate was a temperature sensor connected to a control unit that triggered a relay to provide power to the thermo electric plate, when the sensor temperature was above a preset temperature. Similar to a common house thermostat with on-off rather than proportional power control, there was fluctuation of the copper plate temperature by approximately 1°C around the set point temperature. The copper plate was pre-cooled to the set temperature before it was applied to the test sites, therefore causing an enhanced heat flux during the initial phase of the exposure. The excess heat from the TE cooler was removed by a MLA 270 DS-115 Melcor (Melcor Corp., Trenton, NJ) liquid chiller operating as a close loop system.

Each Yucatan pig was exposed in approximately 15 skin areas of 11.5×11.5 cm^2 for 10 minutes, equivalent to about 15% body surface area, with the cold plate set at $-5°$C to $-8°$C. The weight of the applicator resulted in a contact pressure of approximately 0.5 N/cm^2 or 38 mmHg. No additional manual force was applied to the device. To ensure good thermal contact from skin to applicator, an ultrasound gel (Ultrasound Gel, Medline) was used and the hairs of the pigs were shaved prior to each exposure. Immediately after each of these exposures, a 1 minute massage in the test sites was performed using a commercially available vibrating massager (Brookstone, Inc., Merrimack, NH).

For each animal, all cold exposure procedures were performed on a single day under general anesthesia as described above. The subsequent blood draws were performed after i.m. sedation on animals fasted for 12 hours. Pigs were observed clinically and blood samples were obtained after a 12-hour fast prior to treatment, within 1 hour and 1 day, 1 week, and 1, 2, and 3 months post-treatment. In addition, lipids were evaluated throughout the time course (pre-exposure, post-exposure, 1, 3, 14 days, 1, 2, and 3 months) using repeated-measures ANOVA. Two-tailed values of $P < 0.05$ were considered statistically significant using the post hoc Fisher least significant difference procedure. Data are presented in terms of the mean and standard error of the mean (SEM). Statistical analysis was performed using the SPSS software package (version 16, SPSS, Inc., Chicago, IL).

RESULTS

Exploratory Study

Cold exposures were apparently well tolerated; the pig recovered quickly from the analgesia and there were no signs of distress or pain evident at any time after the procedure. There was no apparent skin injury in any of the exploratory animal test areas. A slight increase in pigmentation was noted after one week for some sites. There was no hypopigmentation, scarring, or textural change evident in any test area. Exposure conditions and outcome for the test sites are summarized (Table 1).

On gross observation, the highest degree of selective fat loss was obviously seen as a smooth indentation matching the size and shape of the cooling device, in a test site (#5) on the buttock for which firm pressure was easily maintained due to lack of respiratory motion. The gross appearance of tissue from this site after 3.5 months is shown in Figure 2. In a circular area corresponding to the size of the laboratory prototype cooling device, subcutaneous fat thickness was reduced. Subcutaneous fat in pigs generally shows two layers on gross examination; two distinct fat layers can be seen. In this sample the reduction of fat was confined to the superficial fat layer while the deeper fat layer appeared unchanged. Approximately 80% of the upper fat layer thickness or 40% of total fat layer thickness was removed in the cold exposure site located at the buttock. Pigs also exhibit intradermal adipocytes. The corresponding histology of this site showed marked reduction of intradermal

Fig. 2. Gross vertically cut tissue appearance from exploratory study, showing substantial fat loss at 3.5 months. A single cold exposure at a device temperature of about $-7°$C, 10 minutes exposure time was applied. White arrow indicates exposure site. The dermis–fat junction is marked with the upper dashed line, and the interface between the upper and lower fat layers is marked with the lower dashed line. It can be seen that the upper fat layer was reduced. The total fat loss was approximately 40%. Bar represents 1 cm.

fat as compared with the adjacent unexposed control. The distance between the septae within the exposure site appeared reduced. Only very minor inflammation with few scattered lymphocytes was seen.

Dosimetry Study

None of the test sites exhibited, at the tested temperatures or follow up intervals, any clinically or histologically evident damage of the epidermis or dermis. Various degrees of inflammation confined to the superficial fat was observed (Fig. 3).

A time line of the histological grading is exhibited in Figure 4. Figure 4a exhibits normal appearing adipocytes
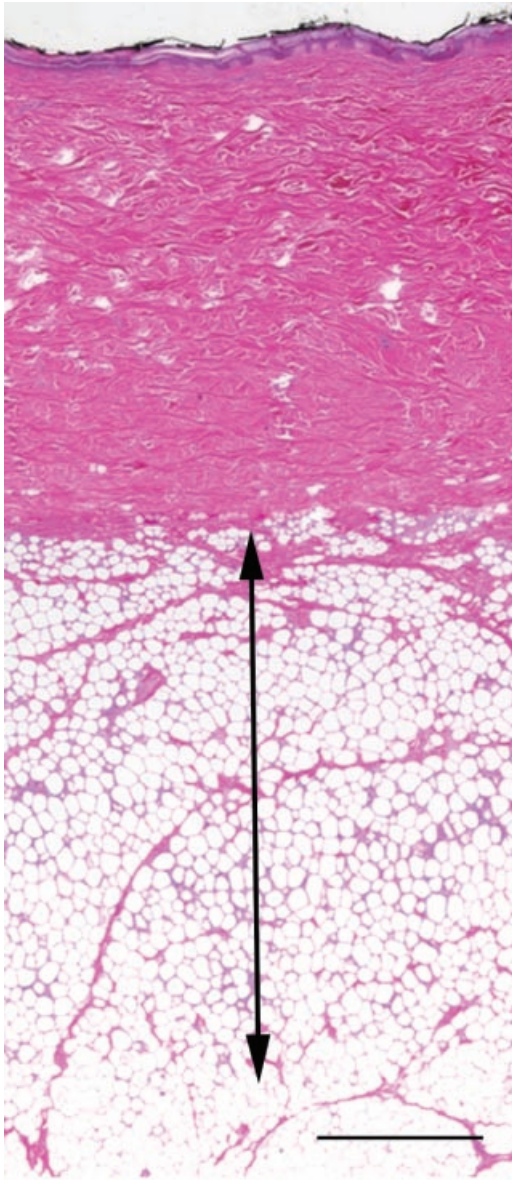
Fig. 3. Representative histology (composite for better image quality) exhibits selective panniculitis (black arrow) within the superficial 3–4 mm fat layer. No evidence of epidermal, vascular or dermal damage is present. Bar represents 1 mm.

immediately and 1 day after exposure for all temperatures tested. Two days after cold exposures of −1°C and below inflammation limited to the subcutaneous fat and within several mm of the fat–dermal interface became evident as localized clusters of mixed neutrophil and mononuclear cell inflammatory infiltrate, in a predominately lobular pattern of panniculitis. Adipocytes were histologically apparently still intact at 2 days after cold exposure. At 7 days (Fig. 4b) after cold exposure no changes were apparent in the skin, lobular panniculitis was more intense compared with 2 days after exposure, and the inflammatory infiltrate was more mononuclear. At 14 and 30 days after cold exposure, there were no apparent skin changes and lobular panniculitis was still more intense. The average size of the adipocytes appeared reduced and a wider range of adipocyte size was present. Lipid-laden mononuclear inflammatory cells were abundant, consistent with a phagocytosis process (Fig. 4c,d).

The degree of cold-induced lobular panniculitis was also dependent on the cold temperature used. Histological grading of the extent of inflammation at the dermis fat junction was performed without knowledge of the exposure conditions, by a single blinded investigator. Table 2 and Figure 6 summarize the data related to cold temperature, and time after cold exposure.

Fat damage was significantly greater at lower temperatures ($F = 16.11$, $P < 0.001$) and increased significantly over time ($F = 32.77$, $P < 0.001$). A control (unexposed sites) was evaluated at 37°C in 39 sites with no damage observed in 34 sites, scores of 0.5 in two, and a score of 1.0 in one site.
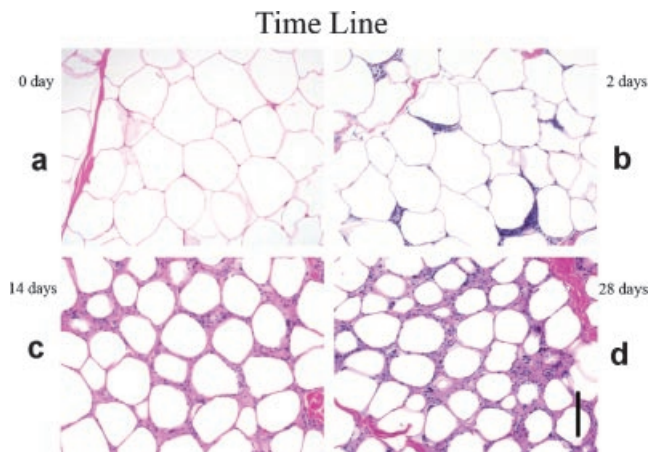

Fig. 4. Representative histological time line for the adipose tissue at the dermal–fat interface. A single cold exposure was applied (−7°C, 10 minutes, flat cold plate). Bar represents 100 μm. **a**: Within the first hour: Normally shaped and sized adipocytes, no inflammatory cells. **b**: 2 days: Clusters of inflammation (mainly neutrophils) embracing individual adipocytes. **c**: 14 days: dense lymphocytic infiltrate, occasional macrophages, some reduction of adipocyte cell size. **d**: 30 days: similar to 14 days, however in addition multinucleated giant cells (macrophages) and further reduction of adipocyte cell size.
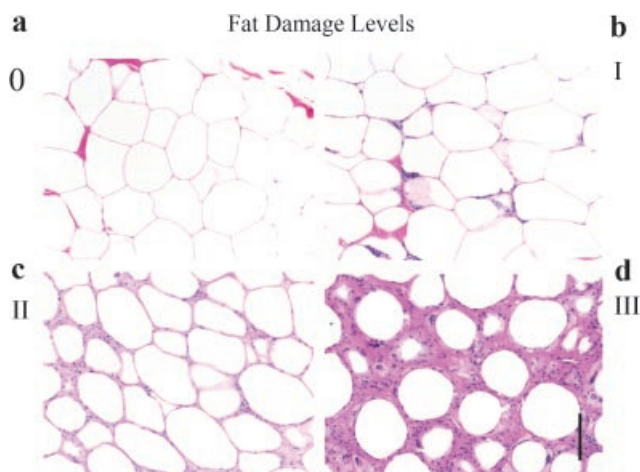
Fig. 5. Calibration slides to exhibit different levels of panniculitis at the superficial fat layer as used for grading of fat damage related to exposure parameters and follow up interval. Results of evaluation shown in Figure 6. For these representative slides the lipid area fraction, the percentage of the image attributable to fat, was determined by image analysis. Bar represents 100 μm. **a**: Level 0, no evidence of inflammatory cells, normal shaped and sized adipocytes. Fat area fraction 97%. **b**: Level 1, minor and scattered inflammation embracing individual adipocytes, fat area fraction 94%. **c**: Level 2, more than approximately 50% of adipocytes exposed to inflammatory cells, some fat size reduction, fat area fraction 80%. **d**: Level 3, most adipocytes engulfed by multilayer of inflammatory cells, marked reduction of average adipocyte cell size, fat area fraction 52%.

No differences were detected in fat damage at 1 day between any of the temperature conditions ($P > 0.25$). As shown in Table 2, compared to 20°C there was significantly more fat damage at 2 days for temperatures of −7°C ($P < 0.001$), −5°C ($P < 0.001$), −3°C ($P = 0.015$), and −1°C

($P = 0.021$). Similarly, greater fat damage was observed at 7 days for −7°C, −5°C, and −3°C (all $P < 0.001$) and −1°C ($P = 0.008$). At 14 days, fat damage was greater at −7, −5, −3°C (all $P < 0.001$) and −1°C ($P = 0.002$). At 28 days, fat damage was significantly greater for −7°C ($P < 0.001$), −5°C ($P = 0.004$) with a borderline difference demonstrated at −3°C ($P = 0.049$) and no difference at −1°C ($P = 0.26$). Figure 6 illustrates the fat damage over the time course from post-exposure to 28 days at each temperature with the greatest fat damage occurring at 14 days for all temperature levels.

A representative temperature plot obtained during a cold exposure is exhibited in Figure 7. The plot shown in Figure 7a was obtained with blood flow and the plot shown in Figure 7b was produced after the pig was euthanized (no blood flow).

None of the flat application test sites within the dosimetry study exhibited obvious indentation. However, most of the skin fold applications demonstrated did show marked indentation. Figure 8 exhibits a representative outcome of a fold application of −7°C for 10 minutes after 1 month. The comparison of ultrasound pre (Fig. 8a) and post 1 month exposure (Fig. 8b) reveals about 3 mm of fat loss that matches the extent of indentation. This amount for fat loss matches clinical indentation (Fig. 8c). The histology revealed fat necrosis down to the muscle layer with clear sparing of the muscle from damage (Fig. 8d).

Lipid Level Study

The animals tolerated the cold exposure well and recovered quickly from the procedure without any apparent distress or pain. Approximately 30% of all test sites had freezing of the skin during the procedure, evident by whitening and hardening of the skin after the cold plate was removed. Typically, a brisk erythematous reaction was observed with a delay of few minutes in such sites. In general, the skin on the abdomen was more prone to freezing than the skin on the flank. No evidence of marked

TABLE 2. Comparison of Fat Damage for Different Temperatures Relative to 20°C

Temperature	2 Days	7 Days	14 Days	28 Days
−7°C	0.80 ± 0.11	1.90 ± 0.22	2.05 ± 0.15	2.20 ± 0.28
−5°C	0.79 ± 0.25	1.46 ± 0.14	1.80 ± 0.24	1.82 ± 0.19
−3°C	0.50 ± 0.18	1.05 ± 0.20	1.56 ± 0.22	1.20 ± 0.22
−1°C	0.44 ± 0.15	1.73 ± 0.13	1.10 ± 0.20	0.68 ± 0.23
ANOVA	*P*-value	*P*-value	*P*-value	*P*-value
−7°C	<0.001*	<0.001*	<0.001*	<0.001*
−5°C	<0.001*	<0.001*	<0.001*	0.004*
−3°C	0.015*	<0.001*	<0.001*	0.049*
−1°C	0.021*	0.008*	0.002*	0.264

Data are mean ± SEM. Fat damage (0−3 scale). No significant differences were observed at 1 day compared to 20°C for any temperature. At 2, 7, 14, and 28 days, fat damage was significantly greater for each temperature compared to 20°C, except for −1°C at 28 days.
ANOVA = analysis of variance.
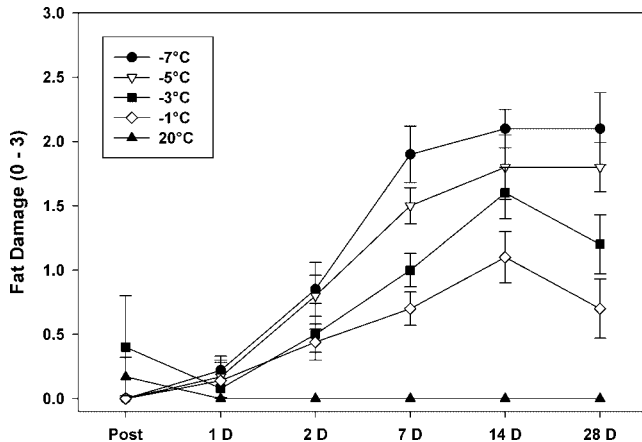*Statistically significant.

Fig. 6. Histological grading of panniculitis at dermal–fat interface according to reference slides shown in Figure 5. The extent of inflammation is related to exposure temperature and time. Error bars are standard error of the mean.
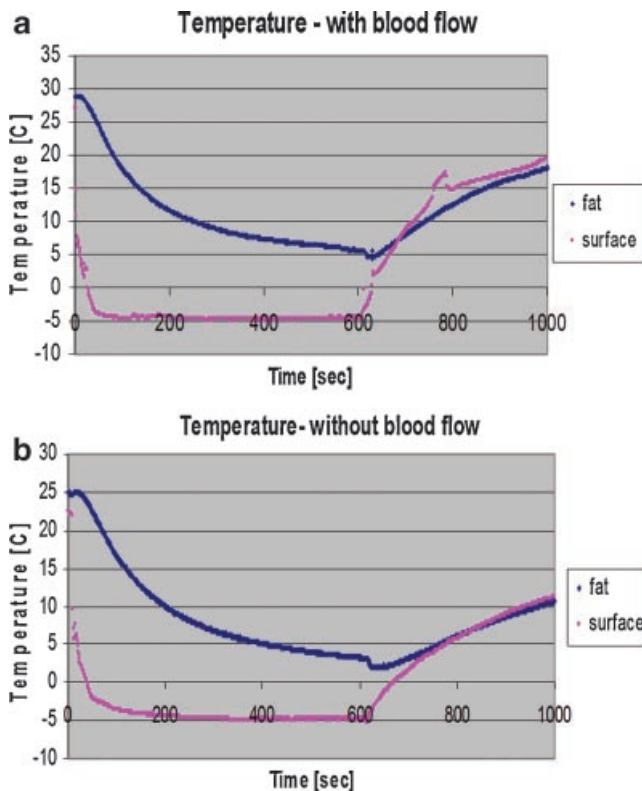

Fig. 7. Temperature profile during cold exposure with Zeltiq prototype (preset device temperature −7°C, 10 minutes, flat application) one thermocouple was put at the interface between cooling device and skin (red), and one thermocouple was inserted into the superficial fat close to the dermal–fat interface (blue). Two measurements were (**a**) with blood flow; (**b**) without blood flow (pig was sacrificed with couples maintained in place) prior to exposure.
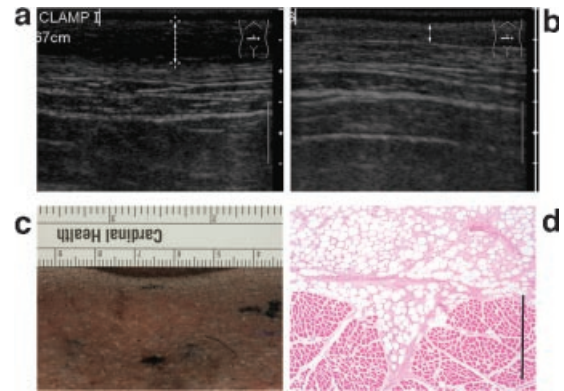

Fig. 8. Marked fat loss of pig from dosimetry study after fold application with approximately 50% fat loss. The indentation matches with the extent of fat loss as measured by ultrasound. **a**: Ultrasound image of test site before exposure and fat layer (white arrow). Bar represents 1 cm. **b**: Ultrasound 1 month post-exposure reveals significantly reduced fat layer (white arrow) and increased echo within fat layer. **c**: Tangential photograph reveals indentation of approximately 4 mm depth without evidence of any pigmentary changes. Scale exhibited at ruler. **d**: Corresponding histology of the interface between deep fat layer and muscle layer exhibits marked panniculitis. Muscle layer without appears completely normal without any signs of inflammation. Bar represents 1 mm.

depression was noted at the test sites of the pigs. For some test sites that showed skin freezing, superficial damage evident as epidermal necrosis occurred thereafter. Several of these sites became transiently hypopigmented, which had mostly resolved at 3 months. No scarring or ulceration was observed at any of the test sites.

In the animals with ~15% body surface area, cold exposure for 10 minutes between −5° and −8°C, blood triglyceride and cholesterol levels were measured immediately, 1, 3, 7, 14 days and 1, 2, and 3 months post-exposure. The results of all six pigs are exhibited in Figure 10. There was a temporary decrease in serum triglycerides immediately following the cold exposures, attributed to fasting prior to and during general anesthesia. Otherwise, no significant change in serum lipids was noted at any time after cold exposure as determined using repeated-measures ANOVA.

DISCUSSION

Our results indicate that non-invasive cooling at the skin surface can cause selective damage and loss of a substantial volume of subcutaneous fat in a pig model. We name this process "selective cryolysis" of fatty tissue. Many important details about selective cryolysis remain to be studied.

It is clear that a delayed, cold-induced lobular panniculitis is involved, presumably in response to direct cold-induced injury of adipocytes. In this study, inflammation and adipose tissue loss were well correlated. Both proceeded for many weeks following a single, local exposure to cold, reaching an apparent maximum around 4 weeks after
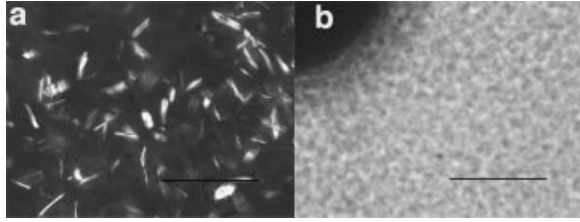
Fig. 9. Crystallization of pig fat at various temperatures and cooling rates. The images were obtained by crossed-polarized microscopy of a 1 mm quartz cuvette filled with heated pig lard obtained from previously frozen and heated samples procured from the test sites of the dosimetry study. Bar represents 1 mm. **a**: exhibits needle like crystals at room temperature (21°C) after storage over night at room temperature. **b**: exhibits cloudy crystallization at 10.3°C after a cooling rate of approximately 10°C/minute.

cold exposure and having resolved about 3 months after cold exposure. In its early inflammatory phase, panniculitis may further damage adipocytes. In its later phase, we observed that phagocytosis appears to account for removal of adipocytes and loss of fat tissue. Temperature and time of application are both important to induce selective cryolysis of fatty tissue. In this study, we used skin surface temperatures near the freezing point of tissue water, applied for 10 minutes to allow time for sufficient transfer of heat from subcutaneous fat though the overlying dermis. A skin surface temperature as high as −1°C induced within the various tested anatomical locations in average a mild superficial panniculitis, and the anatomic depth of panniculitis and of fat loss was increased when lower temperatures were applied.
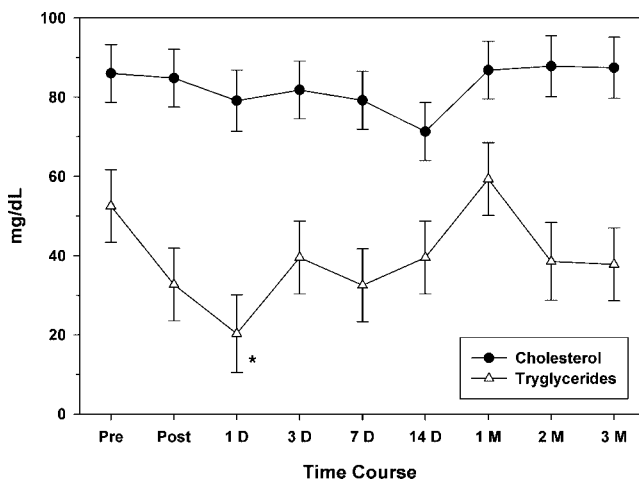

Fig. 10. Lipid levels over time after a single cold exposure (−5°C, 10 minutes) of approximately 15% body surface area. Triglycerides and cholesterol were not significantly elevated vs. a pre-procedural control at any of the following time points: Immediately, 1, 3, 7, and 14 days and 1, 2, and 3 months. The significant drop of triglycerides at 1 day post-exposure (*) can be explained by fasting before and during general anesthesia.

The extent of inflammation was typically greatest near the fat–dermis interface. For the flat application test sites, this band-like distribution of panniculitis extended only about 2–4 mm into the fatty tissue. This probably explains why, in spite of relatively marked inflammation within the superficial fatty tissue, there was no noticeable indentation due to fat loss for the test sites of the flat application. Samples which exhibited much deeper inflammation, also exhibited marked indentations as shown in Figure 8. This test site involved the "fold" exposure geometry, which we tested as a means of optimizing the amount and depth of fat cooling. Such test sites exhibited inflammation down to the muscle layer, and the extent of skin indentation in these sites matched nicely the observed loss of fat layer thickness. Taken together, these findings show that the amount of fat loss is related to both severity and thickness of cold-induced panniculitis.

Fat cooling takes time, as shown in Figure 7 during a −7°C flat application. More than 3 minutes were needed for the superficial fat to reach 10°C at the dermal–fat interface. There are many reasons for the slow cooling of fat. Heat must be extracted from adipose tissue through the overlying dermis, which is several millimeter thick. Dermal blood flow can effectively limit fat cooling by convectional heating of the affected volume. Cold-induced vasoconstriction may reduce dermal blood flow. Applied pressure analogous to a blood pressure cuff can stop local blood flow entirely, eliminating convective heat exchange. For the flat applicators in our dosimetry study, these pressures were not reached. In contrast, the "fold" applicator and the exploratory convex applicator used for the sample shown in Figure 2, were each applied with sufficient pressure to stop or nearly eliminate any cutaneous blood flow. With these high-pressure applicators deep panniculitis and a large volume of fat loss occurred, when compared with the flat applicator used at the same surface temperature. This may also explain the absence of marked indentations for the lipid level study. The pressure used for this applicator was likely not sufficient to suppress blood flow effectively. The exact roles of blood flow in selective cryolysis remain to be determined. Potentially, reperfusion injury may also play a role. Dermal and adipose tissue blood flow were not measured in this study.

Skin and fat can be formed into a fold over most of the body surface because the deep fascia is mobile. Cooling a "fold" of dermis and subcutaneous fat clamped between two cooling elements, further optimizes fat cooling via changing geometry. For flat applicators, there is always a temperature gradient with depth as illustrated in Figure 7. In contrast, when heat is extracted from both sides of a fold in the absence of blood flow, temperature of tissue inside the fold between the cold applicators can become nearly as cold as the skin surface device.

We found that selective cryolysis is remarkably selective at both the gross and microscopic levels to the subcutaneous fatty tissue—neither epidermis, dermis nor underlying muscle tissue were affected in any of the test sites. For example, Figure 8 shows adipose tissue inflammation down to the deep muscle layer, with sharply demarcated sparing

of the muscle itself. In every test site, skin was colder than the underlying fat. Therefore, some mechanism exists by which adipose tissue is intrinsically more sensitive to cold, at temperatures that are easily survived by skin. The most likely mechanism, hypothesized when "popsicle panniculitis" was first described, is that crystallization of the cytoplasmic lipids in adipocytes occurs at temperatures well above the freezing point of tissue water. In essence, "lipid ice" forms at much higher temperatures than water ice. Indeed, we have observed at temperatures around $10°C$ that pig fat solidifies, and when observed under cross polarized light, needlelike crystals can be seen within the tissue (Fig. 9). Triglycerides can crystallize around $10°C$ depending on chain length, cooling rate and degree of saturation [19]. Crystallization requires energy, expressed as the latent heat of freezing. It can be seen in Figure 7 that the rate of temperature decrease in superficial fat during treatment with a flat applicator, changes at around $10°C$. This deflection is consistent with a latent heat exchange due to crystallization of adipocyte lipids. This mechanism is also consistent with the observed need in this study for the time of cold application to be apparently longer than simply related to heat transfer and heat capacity of the tissues involved. It appears that heat must be extracted from the fat, not only to cool it, but to crystallize it. Taken together, these observations suggest but do not prove that lipid crystallization is responsible for selective injury to adipose tissue. This mechanism may pose challenges for developing selective cryolysis of fatty tissue clinically, as it is known that pigs have a higher percentage of saturated fatty acids compared to humans [20]. Efficient cooling under optimized conditions may be required. On the other hand, the clinical entity of "equestrian panniculitis" [17] suggests that this is possible in at least some adults, and it also indicates that the concurrent application of cold with motion (e.g., massage) may have some synergistic effects. However, the results of the "lipid level study" have shown that cold exposure followed by massage did not result in significant fat loss (for the conditions used). As also a different cooling applicator was used, further studies are necessary to determine the effects of massage or motion during and after cold exposure. We would like to comment on the observation that the dosimetry study and the exploratory study did not result in any epidermal damage, while the lipid level study resulted in occasional epidermal damage. The reason for this is not known at this point. It can be speculated that the increased thickness of the copper plate may resulted in an initially enhanced heat flux causing a rapid temperature drop and surface freezing at some test sites. This would suggest that future studies should also carefully monitor the heat flux during the procedure. Also the post-exposure massage may have contributed to some epidermal trauma.

This study has other implications. For example, the practice of freezing fat grafts at $-20°C$ for extended periods prior to reimplantation, is likely to result in graft failure over several months. On the other hand, stem cells and preadipocytes may not be as susceptible to freeze injury.

"Popsicle panniculitis" has not been reported to result in noticeable, permanent facial fat loss. However, the number of infants who experienced this rare entity and were specifically examined later in life, may be zero. If cytoplasmic lipid crystallization is the dominant cellular damage mechanism, one would expect stem and preadipocyte cells to survive, because they contain much less lipid than mature adipocytes. On the other hand, these cells may succumb during the subsequent panniculitis, and/or fail to differentiate due to changes in the adipose tissue micro-environment. In our study, there was no evidence that the fat lost after cold exposure could regenerate, although the longest duration of our observations was 3.5 months. At this point, we know little to nothing about the longevity of fat loss induced by selective cryolysis. Alternative mechanisms may explain or potentiate selective cryolysis. These include death of adipocytes mediated by oxidative stress, either as a result of hypothermia as suggested by Rauen and de Groot [21] or as part of reperfusion injury [22]. Nishikawa et al. [23] demonstrated that at normothermic conditions, the fatty tissue is particularly susceptible to reperfusion injury. Localized vasoconstriction which can be triggered by cold exposure may potentially enhance such reperfusion injury. Such mechanism would also be consistent with our observation that firm pressure during application cold exposure appears to enhance the efficacy and depth of fat loss. In adipose tissue, it remains unclear whether the inflammation causes death of adipocytes, or vice versa, whether death of adipocytes causes inflammation, or both. The time course of inflammation which is delayed for a few days, then seen initially as a neutrophilic infiltrate around individual clusters of adipocytes, suggests that piecemeal adipocyte necrosis may initiate the process.

Cryolysis of fatty tissue is possible due to biological selectivity. Hereby we refer to biological selectivity as a specific response (e.g., inflammation) that is confined to a certain tissue (e.g., fat), although other tissues were exposed to a similar or more intense physical stimulus (e.g., colder temperature of the epidermis and dermis as compared to the fat).

Finally, there is the question of where does the fat go, which is lost gradually over weeks to months after a single exposure to cold? In this study, there was no significant rise in serum lipids at any time point after treating ~15% of the animals' body surface. Instead, a significant transient decrease in serum triglycerides was observed, attributable to fasting prior to and during general anesthesia on the day of cold treatment. Possibly, fat loss was so gradual that an increase in circulating lipid levels was not measurable. More likely, the appearance of numerous lipid-laden mononuclear cells at 2 weeks and beyond strongly suggests that the adipocytes are undergoing apoptosis and being removed by phagocytosis, presumably via activated macrophages. This cellular transport process would not be expected to cause any increase in serum lipids. This process is likely to follow usual pathways for adipose tissue turnover. Each year, about 10% of body fat is recycled through adipocyte apoptosis [24].

CONCLUSIONS

In conclusion, we describe and define "selective cryolysis," the intentional destruction of adipose tissue by cooling at temperatures that do not substantially affect adjacent tissues. Potentially, this may be developed into a clinical alternative treatment for fat removal.

Prolonged, controlled local skin cooling can induce selective damage and subsequent loss of subcutaneous fat, without damaging the overlying skin. Selective cryolysis warrants further study as a local treatment for removing adipose tissue.

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