BIOLOGICAL EFFECTS OF LOW FREQUENCY HIGH INTENSITY ULTRASOUND APPLICATION ON *EX VIVO* HUMAN ADIPOSE TISSUE

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In the present work the effects of a new low frequency, high intensity ultrasound technology on human adipose tissue *ex vivo* were studied. In particular, we investigated the effects of both external and surgical ultrasound-irradiation (10 min) by evaluating, other than sample weight loss and fat release, also histological architecture alteration as well apoptosis induction. The influence of saline buffer tissue-infiltration on the effects of ultrasound irradiation was also examined. The results suggest that, in our experimental conditions, both transcutaneous and surgical ultrasound exposure caused a significant weight loss and fat release. This effect was more relevant when the ultrasound intensity was set at 100% (~ 2.5 W/cm², for external device; ~19-21 W/cm², for surgical device) compared to 70% (~ 1.8 W/cm² for external device; ~13-14 W/cm² for surgical device). Of note, the effectiveness of ultrasound was much higher when the tissue samples were previously infiltrated with saline buffer, in accordance with the knowledge that ultrasonic waves in aqueous solution better propagate with a consequently more efficient cavitation process. Moreover, the overall effects of ultrasound irradiation did not appear immediately after treatment but persisted over time, being significantly more relevant at 18 h from the end of ultrasound irradiation. Evaluation of histological characteristics of ultrasound-irradiated samples showed a clear alteration of adipose tissue architecture as well a prominent destruction of collagen fibers which were dependent on ultrasound intensity and most relevant in saline buffer-infiltrated samples. The structural changes of collagen bundles present between the lobules of fat cells were confirmed through scanning electron microscopy (SEM) which clearly demonstrated how ultrasound exposure induced a drastic reduction in the compactness of the adipose connective tissue and an irregular arrangement of the fibers with a consequent alteration in the spatial architecture. The analysis of the composition of lipids in the fat released from adipose tissue after ultrasound treatment with surgical device showed, in agreement with the level of adipocyte damage, a significant increase mainly of triglycerides and cholesterol. Finally, ultrasound exposure had been shown to induce apoptosis as shown by the appearance DNA fragmentation. Accordingly, ultrasound treatment led to down-modulation of procaspase-9 expression and an increased level of caspase-3 active form.

Ultrasound (US) wave is a sound energy with a frequency higher than 20 KHz that, in the presence

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of a medium (solid, liquid, gas) is transmitted from one molecule to the next. Energy contained within a soundbeam attenuates as it propagates along a tissue due to the reflection and absorption phenomena (1). Considering the same number of cycle wavelengths, low frequency ultrasound penetrates deeper than the high-frequency ultrasound. The amount of energy that reaches a specific site depends on both the ultrasound characteristics (frequency, intensity, amplitude, focus and beam uniformity) and the tissues through which it travels. Therefore, at higher frequencies, the soundbeam will have less energy available to propagate through a tissue since more energy is absorbed. Ultrasound is widely used in medicine for several diagnostic and therapeutic applications (2-5). Most clinically-used ultrasound has a frequency range of 2 and 10 MHz (high frequency and low power). Ultrasound waves can easily transmit through soft parenchymal tissues (i.e. adipose tissue) and is often used for adipose layer reduction and body contouring. The use of ultrasound technology as an emulsifying approach for adipose tissue was introduced in 1987 (6), after which the technique of ultrasound liposculpturing was outlined (7). Thus, ultrasound is often used to improve liposuction for fat reduction (8-13). This technique, known as ultrasound-assisted lipoplasty (UAL), uses an ultrasonic concentrate beam that hits adipose tissue cells in a defined subcutaneous focal area resulting in a series of expansion and compression cycles that exert negative and positive pressure. These differences in pressure cause cell membrane destruction and hence cell death without damage to other tissues. Ultrasonic energy at the settings used for UAL is indeed relatively specific to destroy low density tissue such as adipose tissue (8). Ultrasound alters adipose tissue through micromechanical disruption and cavitation with minimal thermal effect (13-15). Cavitational effects, by causing microcavities in the adipose tissue with consequent cell destruction and fat liquefaction, are considered the most important mechanism through which ultrasound causes tissue disruption, even though the micromechanical effect produced directly by the action of the ultrasonic waves on organic molecules should also be considered (8, 13). Although since 1978 biological effects of ultrasound have been studied (16) and the clinical use of ultrasounds is well documented, the knowledge of the true potential, as well the exact cellular and molecular effects of ultrasound, is still relatively little and mainly based on clinical observation alone. Since the frequencies of most ultrasound generators used in aesthetic medicine range between 1-3 MHz, in the present work, we focused our attention on the biological effects of a new low frequency high intensity ultrasound technology characterized by multifrequency ultrasound wave beams able to allow a higher tissue penetration of ultrasound energy and a lower phenomenon of attenuation when compared to high frequency ultrasound (1-3 MHz). In particular, we investigated the effects of both external and surgical ultrasound-irradiation on *ex vivo* human adipose tissue specimens by evaluating, other than sample weight change kinetics and fat release, also histological architecture as well adipocytolysis and apoptotic cell death induced by treatment. The influence of saline buffer tissue-infiltration on the effects of ultrasound irradiation was also examined.

MATERIALS AND METHODS

External and surgical device

The ultrasound device (Microlipocavitation, Lain Electronic S.r.L. Milan, Italy; Technosinergye Medical Technology, Pomezia, Rome, Italy) is composed of a high-frequency generator, a radiofrequency transmission cable, and a probe with piezoelectric crystal. The device uses modern microprocessors, capable of monitoring the session peak cavitation, to manage the enormous amount of energy produced by low frequency ultrasound (37.2-42.2 kHz). The device is equipped with two probes that have different diameters for different uses: 60 mm and 2 mm for external and surgical treatment, respectively. The surgical handpiece is innovative because it concentrates the low frequency ultrasound only on the tip of the cannula, thus reducing the risks or effects related to the thermal damage.

Reagents

Formaldehyde was purchased from J.T. Baker (Phillipsburg NJ, USA). Triacylglycerol (TAG), Cholesterol (CO), Cholesterol Esters (CE), Ethidium Bromide and Haematoxylin/Eosin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solvents and reagent for DNA extraction and Western Blot analysis were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 10% neutral buffered formalin ready to use,

dehyol absolute, dehyole 95, and paraffin were purchased from Bio-Optica (Milan, Italy). Advanced smart processor (ASP300), automatic stainer (5010 Autostainer XL), and rotative microtome (RM2135) were purchased from Leica Microsystems (Nussloch, Germany). Optical microscope (Axioskop) was purchased from Zeiss (Jena, Germany). Masson's Trichrome Kit were purchased by BioOptica Milano S.p.A., Italia. Primary antibodies for Western Blot and horseradish peroxidase-conjugated goat secondary antibody were purchased from Santa Cruz Biotechnology, Inc.(CA, USA).

Sample collection

Subcutaneous tissue specimens of white human fat were obtained from 8 female patients (age range: 41-55 years) undergoing abdominoplasty, flankoplasty, thigh lift and breast reduction for obesity, weight loss and aging, at the Operative Unit of Plastic Reconstructive and Aesthetic Surgery (Casa di Cura "Di Lorenzo", Avezzano (AQ), Italy) directed by Prof. M Giuliani. Before the study, all patients, who gave written informed consent, had an accurate clinical examination to evaluate the general health condition. All tests were normal.

Fresh *ex-vivo* human tissue specimens (weight range: ~500-800 g), were obtained with intact skin and fat. The range thickness of surgical specimen was between 3-7 cm. After surgery, adipose tissue samples were kept in saline solution (NaCl 0.9%) and immediately transferred to the laboratory for ultrasound-treatment with external or surgical device.

Protocols for ultrasound-irradiation

Tissue samples were rinsed in saline buffer to remove adhering blood and visible fibrous material. Multiple tissue specimens (weight range: 203-275 g) were placed on the holder and untreated or treated with ultrasound as described below. The experiments were performed at room temperature (20°C). Each sample was weighed before and after ultrasound treatment (irradiation time: 10 min). Ultrasound-treatment with external device was carried out on the cutaneous surface of the specimen, applying an ecographic gel to create a sound head-gel-skin interface able to reduce reflection to only 0.1% and then to favor the ultrasound conduction. External device was used with a slow and continuous rotation as in clinical practice. The parameters of transcutaneous treatment were: power 70% (~ 1.8 W/cm²) - 100% (~ 2.5 W/cm²); frequency 37.2-42.2 kHz; exposure time: 10 min; effect detection time: 20 min and 18 h by the end of treatment. Treatment with a surgical handpiece was performed by introducing the cannula in the adipose tissue parallelly to the skin according to the manufacturer's instructions. The parameters of surgical treatment were: power 70% (~13-14 W/cm²) or 100% (~19-21 W/cm²); frequency 37.2-42.2 kHz; exposure time: 10 min; effect detection time: 20 min and 18 h by the end of treatment. In a set of experiments, to improve conduction of the ultrasound through adipose tissue and to promote its cavitational effects, adipose tissue specimens were infiltrated by syringe with a volume of saline solution equal to sample weight. The amount of saline solution spontaneously released by the specimens was evaluated; saline-infiltrated samples were weighed again and then ultrasound-treated with transcutaneous or surgical device for 10 min.

Thin layer chromatography (TLC)

Fat released after treatment was collected, measured and subjected to lipid extraction by the Bligh & Dyer method (17). The extracted lipids were applied on aluminium silica plates (Merck, Darmstadt, Germany). The plates were developed with an eluent mixture (hexane/diethyleter/acetic acid, 70:25:3 v/v) to separate neutral lipids. Triacylglycerol (TAG) and cholesterol esters (CE) were used as standards. After developing the samples, the plates were sprayed with 8% phosphomolybdic acid in ethanol until they were wet and then heated for 10 min at 80°C. TLC stained shows green/dark and green/dark blue spots on a yellow/green background. Silica plates were acquired by densitometer (UVItec Limited BTS-20M, Cambridge UK).

Ultrasound-irradiation and histology of adipose tissue

Multiple small specimens of human adipose tissue were fixed for 48 h in 10% neutral buffered formalin at room temperature over a 48-h period. The samples were washed under running water for 2 h, dehydrated in the ethanol ascendant series with an automatic processor (Leica ASP 300) and then manually embedded in paraffin. 4 μm thick sections were obtained with a rotative microtome and sections on slides were stained either with hematoxylin or eosin with an automatic stainer (Leica 5010 Autostainer XL). Sections were also stained with Masson's Trichrome, according to standard protocols, to visualize collagen fibers. Slides were analysed independently by two pathologists with an Axioskop optical microscope purchased from Zeiss, Germany.

NaOH maceration method and scanning electron microscopy (SEM)

Samples of adipose tissue (10x10 cm blocks) were submitted to the following technique of cellular matrix-controlled digestion to maintain the architecture of the collagen fibers: digestion in water solution of 10% NaOH in ambient temperature for 12 days (18-19); maceration in distilled water for 7 days in ambient temperature; washing with PBS 0.1 M; impregnation with tannic acid to 1% in 0.1 M of PBS; washing with PBS 0.1 M; after-setting with

1% osmium tetroxide in PBS 0.1 M; washing with PBS 0.1 M; freezing in distilled water; cracking with a razor blade; dehydration in increasing series of ethanol; critical point drying; mounting onto aluminum stubs; metalizing with gold. All the observations were made by scanning electron microscopy operating at 10 kV.

DNA extraction and ladder assay

DNA extraction from untreated or ultrasound-treated adipose tissue specimens was performed by incubating samples with digestion buffer containing proteinase K (20 mg/ml) overnight at 37°C. Then phenol, chloroform and ethanol were added to the samples. Sodium acetate 3 M and absolute ethanol were added to the solution. After incubation, the tubes were centrifuged for 10 min at 14,000 rpm, after which the precipitated DNA was washed with 75 and 100% ethanol, resuspended in 30 µl Tris-EDTA buffer and stored at -20°C. DNA was analyzed by 1.5% (w/v) agarose gel containing ethidium bromide (0.5 mg/ml), electrophoresed for 1 h at 100V, photographed under ultraviolet illumination and acquired by densitometer.

Caspase assay

For Western blotting, tissue specimens were minced and homogenized in ice-cold RIPA buffer (phosphate buffer saline pH 7.4 containing 0.5% sodium deoxycolate, 1% Nonidet P-40, 0.1% SDS, 5 mM EDTA, 100 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM PMSF, 2 mM ortovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin). Homogenates were centrifuged and the supernatants were assayed for protein content. 50 µg of proteins were electrophoresed through a 10% SDS-polyacrilamide (PAGE) gel and transferred onto polyvinylidene fluoride (PVDF) membrane sheets. Membranes were blocked with 5% not-fat dry milk for 1 h at room temperature and then incubated overnight at 4°C with rabbit polyclonal anti-caspase 3 (1:100) or rabbit polyclonal anti-procaspase-9 (1:100). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody were used at 1:2000. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL), according to the manufacturer's instructions. Membranes were incubated in stripping buffer (62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 100 mM 2-ME) for 30 min at 50°C, washed three times with PBST for 10 min each, reblocked for 30 min with blocking buffer, and then reprobed with anti-β-actin Ab (1:1000). Band relative densities were determined using TotalLab software (ABEL Science-Ware srl, Italy) and values were given as relative units. Immunoblot data were normalized to β-actin protein levels.

Statistical analysis

Statistical analysis of data was performed by using one-way analysis of variance ANOVA followed by the Student's *t* test (Prism 3.0 GraphPad Software, San Diego, Ca). P value less than 0.05 was used as the significance criterion.

RESULTS

Effect of ultrasound exposure by external device on tissue weight and fat release

To evaluate the effects of trancutaneous ultrasound-irradiation on adipose tissue weight, samples treated for 10 min with the external device at both 70% (~ 1.8 W/cm²) and 100% (~ 2.5 W/cm²) power, were weighed after gentle squeezing of the tissue at 20 min and 18 h from the end of the treatment. Data shown in Fig. 1A and B represent the mean of three independent experiments with ultrasound at 70% or 100% power, respectively. A single ultrasound-treatment of 10 min led to a slight, even if statistically significant, decrease in weight of samples either at 20 min or 18 h from the end of the treatment, suggesting that the overall effect of ultrasound irradiation did not appear immediately after treatment but propagated over time. No significant differences were observed, in these conditions, between ultrasound irradiation at 70% and 100% power. On the other hand, a more relevant weight loss was observed after a single ultrasound treatment with external device in the presence of SB infiltration, in accordance with the knowledge that ultrasonic waves in aqueous solution better propagate with a consequent more efficient cavitation process (11). Sample weight loss, which was evident as early as 20 min from the end of treatment (~8% and ~15% at 70% and 100% power, respectively), was more relevant and particularly high after 18 hours (~16% and ~30% at 70% and 100% power, respectively), thus confirming that the effect of ultrasound is propagated over time. In all experiments, the percentage of weight loss after ultrasound-irradiation of samples previously infiltrated with SB was significantly higher than that observed in the absence of infiltration, either after 20 min or 18 h after treatment ($P<0.01$). In agreement with data on weight loss, a simultaneous and proportional release of fat volume from the tissue could be measured after ultrasound exposure at 70% or 100% power.

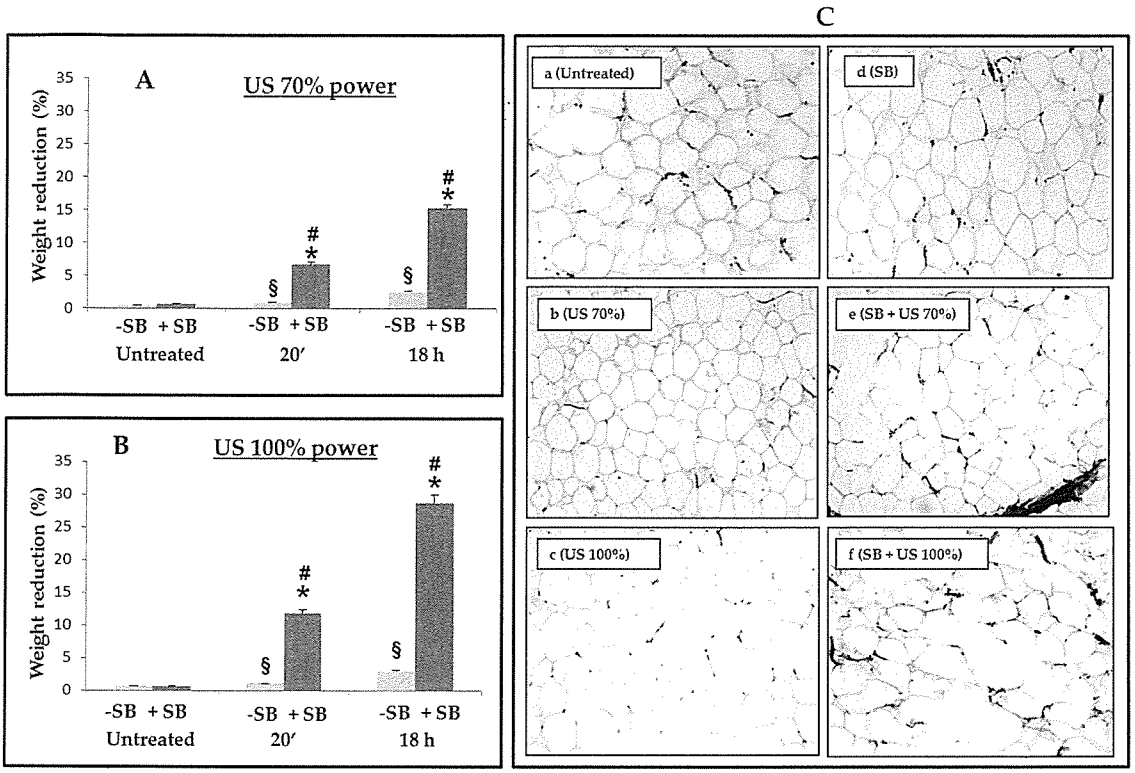

Fig. 1. *Weight reduction and histology of adipose tissue samples after ultrasound irradiation with external device. Weight reduction (%) of adipose tissue samples following ultrasound treatment for 10 min with external device at 70% power (~1.8 W/cm²) (**A**) or 100% power (~2.5 W/cm²), (**B**) with or without saline buffer infiltration (±SB); samples were analyzed and weighed after 20 min and 18 h after treatment. The data shown represent the mean of three independent experiments ±SD (*p value<0.01 compared to control samples; #p value<0.01 when compared to mean weights of non-SB-infiltrated samples; §p value<0.01 when compared to control and non-SB- infiltrated samples). **C**) Sections of untreated or ultrasound-treated adipose tissue samples were stained with haematoxylin and eosin (H&E). a: Section of untreated adipose tissue. b-c: Adipose tissue sections ultrasound-treated with external device for 10 min at 70% and at 100% power, respectively; d: adipose tissue section infiltrated with saline buffer (SB); e-f: adipose tissue sections infiltrated with SB and then ultrasound-treated with external device for 10 min at 70% and at 100% power, respectively. The results from a representative experiment out of three are shown. Magnification of all images is 10X.*

Effects of ultrasound exposure by external device on histology and collagen bundles in adipose and skin tissue samples

Fig. 1C shows a representative experiment aimed to analyze the morphologic characteristics of adipose tissue after external ultrasound-irradiation in the same experimental conditions above-described. Sections of untreated or ultrasound-treated adipose tissue samples were stained with haematoxylin and eosin (H&E). A normal lobular architecture could be seen either in untreated sample (a), or in SB-infiltrated sample (d). In accordance with the experiments on ultrasound-treatment induced sample weight loss and fat release above-described, a clear alteration of adipose tissue architecture could be detected in SB-infiltrated samples after ultrasound-irradiation either at 70% (e) or 100% power (f), which was more relevant than that observed in non-SB infiltrated samples (b, 70% power; c, 100% power). The specimens for histological examination were collected from multiple adipose tissue sample by skin excision. The amount and morphology of the collagen fibers were evaluated under microscope by hematoxylin-eosin and Masson-trichrome staining. We observed that the chemical composition of the collagenous fibrous tissue was modified proportionately to the ultrasound intensity. Fig. 2A shows collagen bundles between adipocyte clusters,

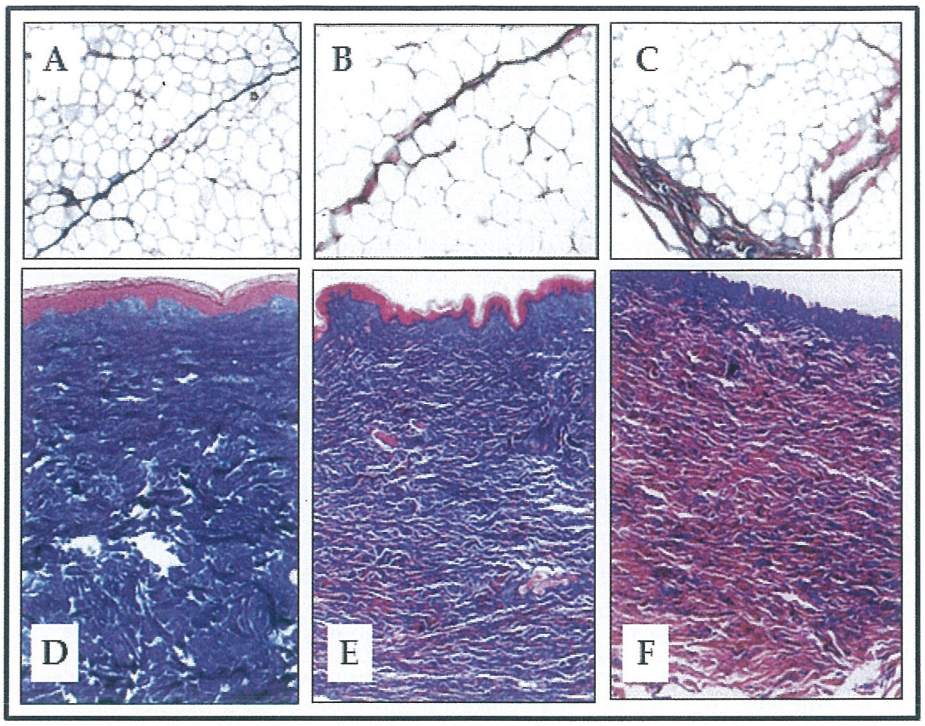

Fig. 2. *Histology of collagen bundles in adipose and skin tissue samples untreated or ultrasound-treated with external device. Tissue sections from untreated or ultrasound-treated samples were stained with the Masson trichrome technique for the detection of collagen fibers (10X magnification).* ***A)*** *Untreated adipose tissue sample.* ***B)*** *Adipose tissue after ultrasound-irradiation for 10 min with external device at 70% power.* ***C)*** *Adipose tissue after ultrasound-irradiation for 10 min with external device at 100% power.* ***D)*** *Untreated skin sample.* ***E)*** *Skin sample ultrasound-treated for 10 min with external device at 70% power.* ***F)*** *Skin sample ultrasound-treated for 10 min with external device at 100% power. The results are representative of one of three independent experiments.*

stained bright blue, a sign of unmodified collagen. Fig. 2, B and C show collagen fiber histology after ultrasound treatments at 70% power and at 100 % power, respectively. The prominent destruction of the collagen bundles is easily observed with the Masson trichrome because of the inability of the collagen fibers to bind the aniline blue. To further verify the ability of ultrasound-treatment to alter collagen fibers, skin collagen with the same histochemical staining was also analysed before and after irradiation. A gradual change of color in treated skin at 70% and 100% power respectively (Fig. 2, E and F) when compared to untreated skin control (Fig. 2D) was observed. The structural changes of collagen bundles present between the lobules of fat cells were further analysed through scanning electron microscopy (SEM). Adipose tissue specimens were treated for 10 minutes with the low frequency external device at 100% power and prepared for microscopy as described in the Materials and Method section. The results obtained by SEM were overlapping with those from light microscopy. In fact, SEM micrographs at low magnifications clearly highlighted how the treatment used induced a drastic reduction in the compactness of the adipose connective tissue (Fig. 3A), with formation of wide and empty lacunae dispersed among the bundles of collagen fibers (Fig. 3B). Specifically, in treated samples an irregular arrangement of the fibers induced an alteration in the spatial architecture and this can easily be seen at higher magnification, when collagen bundles resulted less densely packed (Fig.

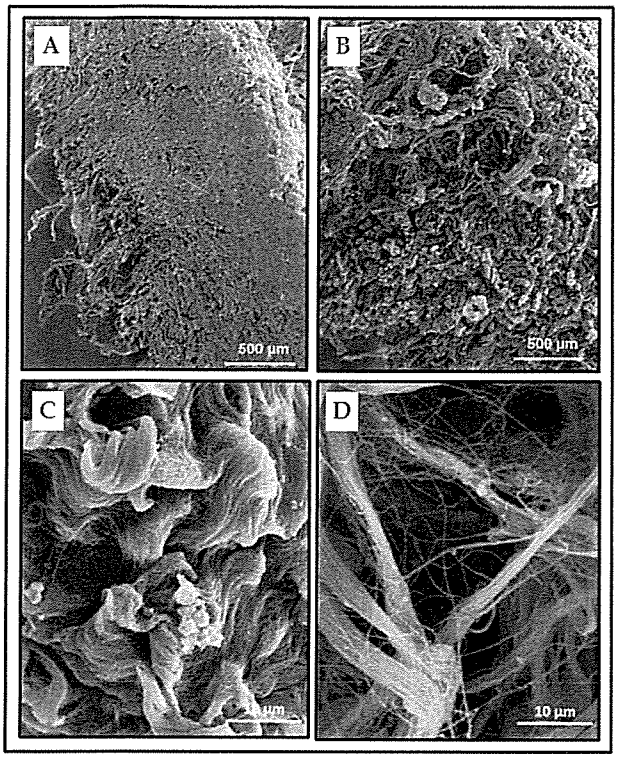

Fig. 3. *Surface ultrastructure by SEM analysis. Adipose tissue samples were ultrasound-treated for 10 min with external device at 100% power and then observed with a scanning electron microscope. SEM micrographs of the collagen bundles between the adipocyte lobules after tissue NaOH maceration from control and treated samples following freeze-fracturing at low magnification (25X) (**A** and **B**, respectively) or high magnification (1000X) (**C** and **D**, respectively). The results are representative of one of three independent experiments.*

3D) compared to controls (Fig 3C).

Effect of ultrasound exposure by surgical device on tissue weight

To investigate the effect of low frequency high intensity ultrasound irradiation at 70% (~13-14 W/cm^2) or 100% (~19-21 W/cm^2) on *ex vivo* human adipose tissue, we also used the surgical handpiece by moving the cannula back and forth in the sample for 10 min. After each ultrasound exposure at both 70% and 100% power, samples were weighed at 20 min and 18 h after treatment. In all conditions, ultrasound exposure induced a weight reduction, this effect being, as expected, much more significant after 18 h from the end of treatment than that observed after 20 min (Fig. 4). To investigate whether these results could be due to mechanical movement of the cannula, samples were treated under the same experimental conditions except that the surgical handpiece was switched off. Results (not shown) indicated that just a slight weight reduction (< 3%) was detectable at 20 min and 18 h when compared to the experiments with the surgical handpiece switched on. Indeed, a significant weight reduction was observed when samples were treated with ultrasound at 70% power (Fig. 4A) and, even more, with ultrasound at 100% power (Fig. 4B). In Fig. 4, A-B are also reported the results of experiments aimed to analyse the effects of ultrasound surgical handpiece treatment (10 min) on SB-infiltrated adipose tissue weight reduction. Similar to the above-reported results with external device, the infiltration of the tissue significantly enhanced the effects of ultrasound exposure, both at 70% and 100% power, with the latter more efficacious, as detected either at 20 min or 18 h after the treatment. In agreement with data on weight loss, a simultaneous and proportional release of fat from the tissue could be measured after surgical ultrasound exposure at 70% or 100% power.

Effect of ultrasound-surgical treatment on adipose tissue histology

Histologic changes of *ex vivo* adipose tissue after ultrasound irradiation with the surgical handpiece for 10 min were also analyzed (Fig. 4C). A standard lobular architecture could be seen in untreated (a) as well in SB-infiltrated samples (d), while a clear injury of ultrasound-treated samples was evident, the cell membranes being disrupted and folded on themselves, with clear and large areas of adipocytolysis, particularly after treatment with ultrasound at 100% power (c and f) compared to samples irradiated with ultrasound at 70% power (b and e).

Effect of ultrasound exposure by surgical device on fat release

Extracellular neutral lipids released after ultrasound-irradiation with the surgical device were analyzed by TLC after loading plate with volumes proportional to those overall collected (highest volume: 50 µl). Adipose tissue samples

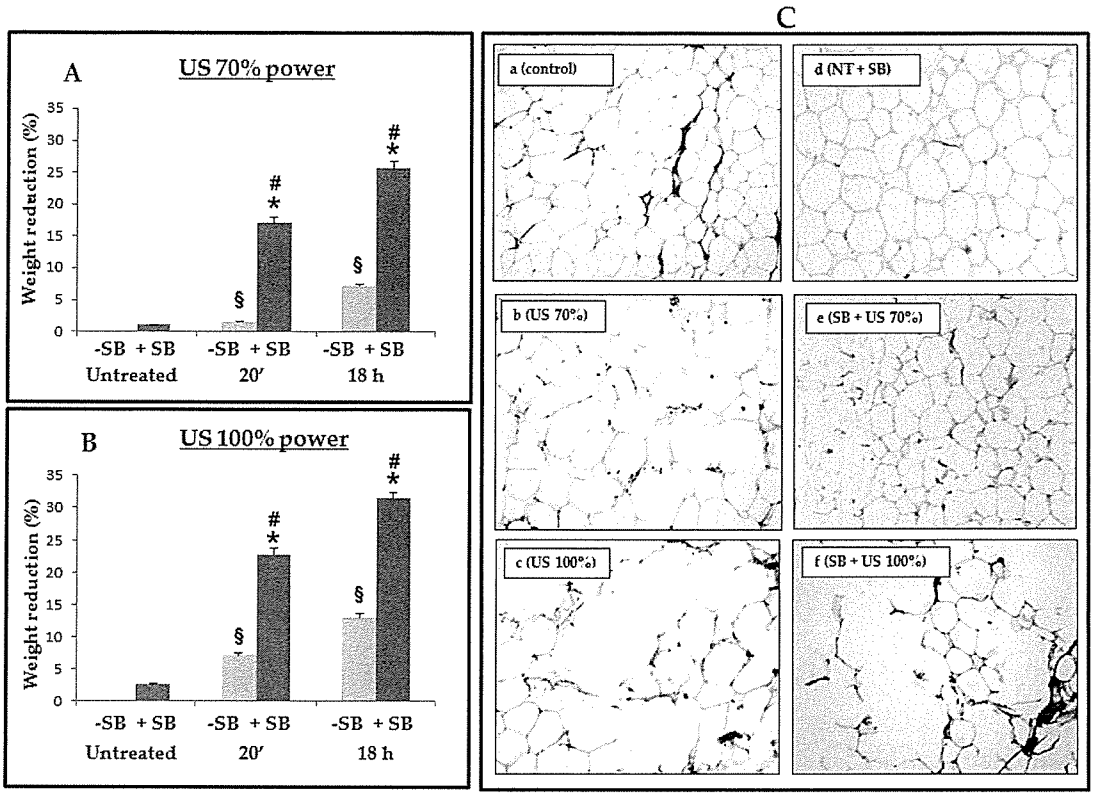

Fig. 4. *Effect of ultrasound exposure with surgical device on adipose tissue weight and histology. Mean weight of untreated or ultrasound-treated adipose tissue samples with surgical device at 70% (A) or 100% power (B) for 10 min with or without saline buffer infiltration (±SB). Samples were analyzed and weighed after 20 min and 18 h after treatment. Shown data represent the mean of three independent experiments ±SD (*p value < 0.01 when compared to SB control samples; #p value<0.01 compared to homologous not SB-infiltrated samples; §p value<0.01 when compared to control non-SB-infiltrated samples). C) Sections of ex vivo adipose tissue samples were stained with haematoxylin and eosin. a: Section of untreated adipose tissue. b-c: Section of adipose tissue treated with surgical device (US) for 10 min at 70% and at 100% power, respectively; d: Section of untreated adipose tissue infiltrated with saline buffer (SB); e-f: Section of adipose tissue infiltrated with SB and treated with surgical device (SB+US) for 10 min at 70% and 100% power, respectively. The images are representative of three separate experiments. All magnifications were at 10X.*

treated for 10 min with the surgical device with the handpiece switched off were used as controls. In all samples, the most intense bands could be referred to triacylglicerols (TAG) (Rf = 0.75) and cholesterol (CO) (Rf = 0.35). Ultrasound irradiation induced an increased release of the total amount of triglycerides and cholesterol when compared to that observed by using the surgical handpiece switched off ($p<0.01$) (Fig. 5, A-B). The intensity of the spots resulted significantly higher after ultrasound-treatment for both TAG and CO, the increase being more relevant at 18 h compared to 20 min. The observed results, confirming the ability of low-frequency ultrasound to induce adipocytolysis and, consequently, the extracellular release of fat, mainly TAG and CO, also suggest, in the meantime, that low frequency ultrasound-irradiation treatment did not affect the integrity and molecular structure of these neutral lipids.

DNA laddering and caspase activities

To investigate whether ultrasound irradiation, in addition to causing cell lysis, was also able to induce apoptotic cell death, DNA fragmentation detected by agarose gel electrophoresis was evaluated. Fig. 6 shows a DNA electrophoresis of multiple samples of adipose tissue treated for 10 min with the external or surgical ultrasound device and is representative of three independent experiments. While the DNA extracted from both control (untreated) (lane 1)

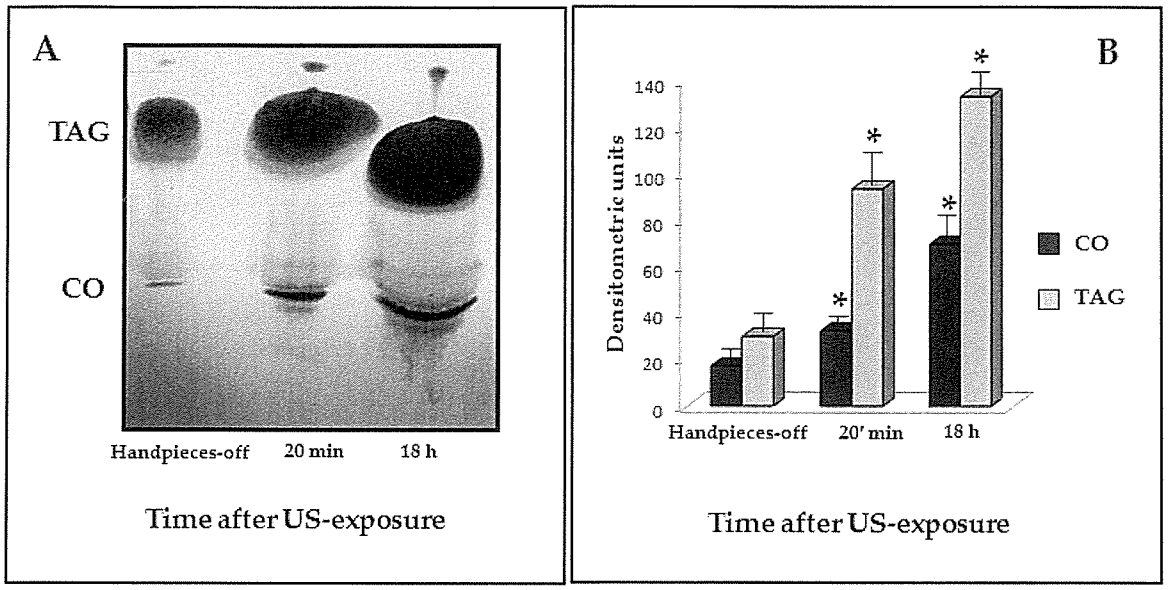

Fig. 5. *Released fat volumes and detection of extracellular lipids by TLC.* ***A)*** *Released fat volumes collected at 20 min or 18 h after the treatment (10 min) with ultrasound surgical device at 70% or 100% power. Shown data represent the mean of three independent experiments (±SD).* ***B)*** *Thin layer chromatography (TLC) of neutral lipids released from adipose tissue sample following treatment for 10 min with handpiece switched off or ultrasound exposure for 10 min at 100% power. Eluent mixture used was hexane/diethyleter/acetic acid (70:25:3 v/v). A representative TLC analysis of three experiments is shown. TAG triacylglycerols (Rf = 0.75), CO cholesterol (Rf = 0.35). (B) Spot intensities of TLC are reported as densitometric units. Data represent the mean of three independent experiments ±SD (*p value < 0.01 when compared to results obtained with handpiece-off treatment).*

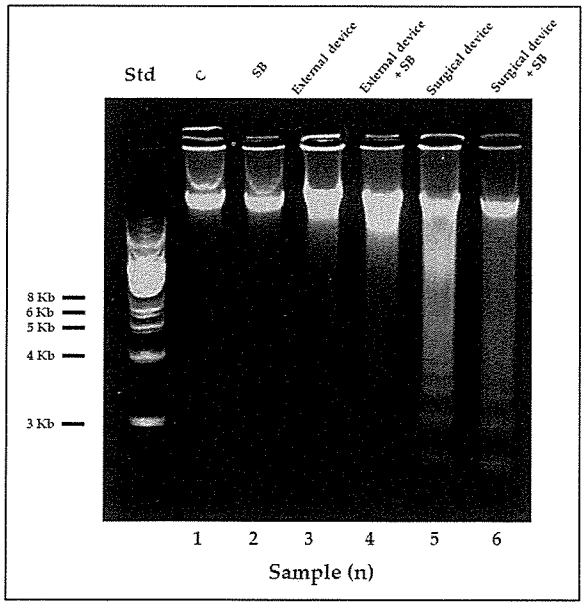

Fig. 6. *DNA ladder. Agarose gel electrophoresis, stained with ethidium bromide, showing the effect of both external and surgical ultrasonic device on DNA of adipose tissue samples The data shown are relative to samples processed 18 h after the end of treatment. Std lane is 1 kb DNA molecular weight standard. Lane 1: not treated adipose tissue sample (C=control). Lane 2: SB-infiltrated sample. Lane 3-4: samples exposed to external device for 10 min (100% power) in the absence or presence of SB-infiltration, respectively; Lane 5-6: samples exposed to surgical device for 10 min (100% power), in the absence or presence of SB-infiltration, respectively. A representative gel is shown out of 3 independent experiments with similar results.*

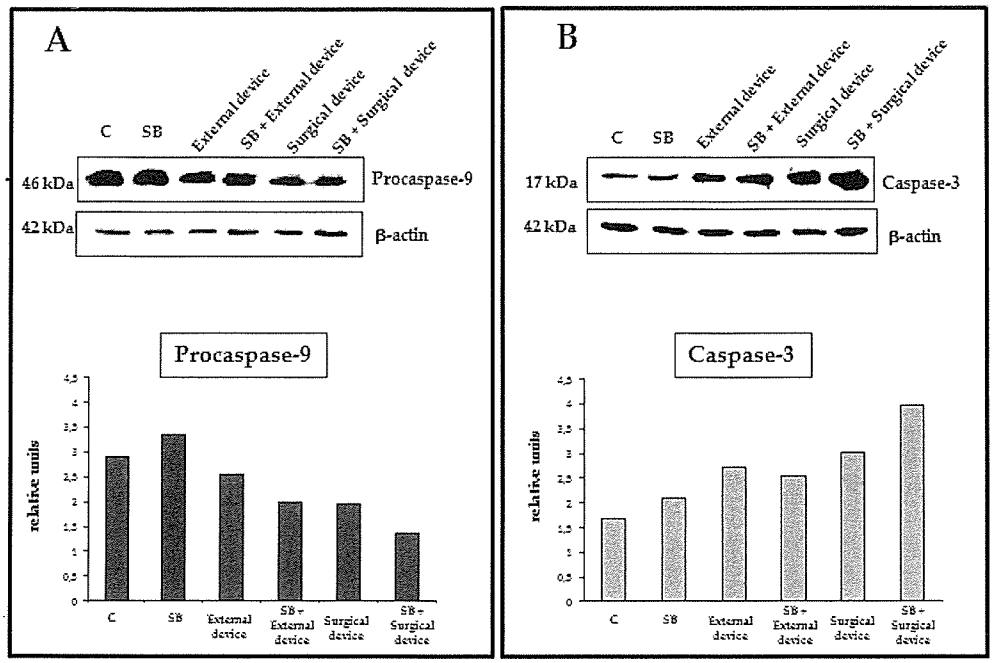

Fig. 7. *Western blot analysis of procaspase-9 and caspase-3. A) Immunoblotting assay for the inactive precursor of caspase-9 (procaspase-9) and (B) executive caspase-3 enzyme in adipose tissue protein extracts. C = control (not treated); SB = Saline Buffer-infiltration; External device: ultrasound-irradiation (10 min,± SB-infiltration) with external device (100% power; 18 h after the end of treatment); Surgical device: ultrasound-irradiation (10 min, ± SB-infiltration) with surgical handpiece (100% power; 18 h after the end of treatment). Histograms showing the densitometric analysis of procaspase-9 and caspase-3, respectively, as normalized versus β-actin levels. Values are given as relative units. The data shown are from one representative of three independent experiments.*

and SB-infiltrated sample (lane 2) appeared intact, DNA extracted from samples treated with external ultrasound showed a slight fragmentation recognized by smears, either in the absence or presence of SB-infiltration (lanes 3 and 4, respectively), However, treatment with the surgical handpiece led to a clearly detectable DNA fragmentation with a striking, typical "ladder pattern", either in the absence or presence of SB infiltration (lanes 5 and 6, respectively). These results prompted us to analyze the caspases involved in the ability of low frequency-ultrasound to trigger an apoptotic pathway. Thus, the caspase-9 dependent apoptotic pathway was investigated by analyzing the levels of its inactive precursor, i.e. procaspase-9. Fig. 7A shows that control tissue exhibited basic high levels of procaspase-9 which were clearly downregulated in the samples treated with both the external and surgical device, either in the absence or presence of SB infiltration. Also in this case, the effect was more evident in the presence of SB when compared to SB-uninfiltrated samples. In addition, downmodulation of procaspase-9 levels appeared more consistent in surgically-treated samples. As expected, the processing of procaspase-9 occurring in ultrasound-irradiated samples was associated to an increased level of active form caspase-3, which plays a key role in initiation of cellular events during apoptosis. As shown in Fig. 7B, in samples treated with both the external and surgical device, a significant increase of the 17 kDa active caspase-3, either in the absence or presence di SB-infiltration compared to control samples, could be detected. In accordance with results on procaspase-9, surgical treatment appeared to be more significant compared to the external treatment, and the SB infiltration was associated to a more consistent increase of active caspase-3 levels compared to homologous SB uninfiltrated tissue.

DISCUSSION

In the present study, the effects of high intensity

low frequency ultrasound exposure on human adipose tissue samples *ex vivo* were investigated by using either the transcutaneous approach or surgical treatment. Even if the frequency level was the same (37.2-42.2 kHz), the physical parameters of external ultrasound treatment were not comparable with those of surgical treatment. Indeed, the intensity (power) values were significantly different being, when set at 100% power, about 2.5 W/cm^2 and 19-21 W/cm^2, for external and surgical irradiation, respectively. Thus, the aim of our work, rather than comparing the two systems, was to evaluate their biological effects on adipose tissue *ex vivo* by simulating *in vitro* conditions used for clinical applications. The results show that a single ultrasound exposure for 10 min with the external device led to a slight but significant weight reduction of tissue after 18 hours from the end of treatment. Interestingly, after sample infiltration with saline buffer, the extent of weight loss and the extracellular fat volume were much greater than those observed in the absence of infiltration, in accordance with the knowledge on the increased effectiveness of cavitational process induced by ultrasound in the presence of water (13). Of interest, the effect of exposure to ultrasound on the tissue weight continued after the end of treatment. In fact, the weight values, as measured at 20 min or 18 h from the end of exposure to ultrasound showed a gradual increase in weight loss that reached the highest level after 18 h. The experimental conditions (*ex vivo* tissue samples) did not allow measurements at longer times. As expected, both in the presence and absence of infiltration with SB, the weight loss, as well the released fat levels associated with the ultrasound treatment appeared greater when the ultrasound generator was set at 100% power compared to 70% power. In agreement with these findings, the observations of histological sections of adipose tissue samples after ultrasound exposure showed damage in the membranes of fat cells and a disruption of the tissue that could be well associated with the above-described results on weight loss. The experiments designed to demonstrate possible alterations in inter-adipocyte collagen fibers clearly showed significant damage also at this level. These results could be reached even on sections of the skin overlying adipose tissue. The histological observations in optical microscopy were also confirmed with scansion electron microscopy (SEM). Further studies are needed to better understand the molecular alterations of the different types of collagen induced by high intensity low frequency ultrasound as well as to understand whether the observed effects represent a stimulus to induce a tissue repair process which includes stimulation of collagen neosynthesis.

Treatment with a surgical handpiece led to a weight reduction and a fat release more relevant than those detected with the external device. Even in these conditions, SB-infiltrated samples suffered a greater weight reduction after surgical ultrasound exposure when compared to controls, confirming that an aqueous solution increased ultrasound-induced cavitational phenomenon. The analysis of the composition of lipids in the fat released from adipose tissue after ultrasound treatment with surgical device showed, in agreement with the level of adipocytolysis, a significant increase in the prevailing lipids (i.e. triglycerides and cholesterol), suggesting, in the meantime, that the irradiation probably did not lead to molecular structural alterations. The potential of ultrasound exposure to induce apoptosis appears particularly interesting. A single treatment with external ultrasound exposure led to a slight degree of DNA fragmentation as shown by the appearance of the typical smear of DNA subjected to agarose gel electrophoresis. The exposure to ultrasound with the surgical handpiece caused a very advanced level of DNA fragmentation, as evidenced by the classic pattern of DNA laddering. These data were supported by down-modulation of procaspase-9 expression which implies an increase of the active form (caspase-9) that cleaves the procaspase-3 to active caspase-3, finally inducing apoptosis (20-22).

In view of the increasing use of high-intensity and low frequency ultrasonic technology, in medicine and in surgery and the scientific rationale behind it, our data provide additional tools for a better understanding of the beneficial or side effects of ultrasound irradiation and also to design appropriate clinical studies.

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