

Biological effects of low frequency high intensity ultrasound application on ex vivo human adipose tissue

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Key words: low frequency ultrasounds, human adipose tissue, weight reduction, collagen fibers, apoptosis.

Summary

In the present work the effects of a new low frequency high intensity US technology on human adipose tissue *ex vivo* were studied. In particular, we investigated the effects of both external and surgical US-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 US irradiation was also examined. The results suggest that, in our experimental conditions, both transcutaneous and surgical US exposure caused a significant weight loss and fat release. This effect was more relevant when the US 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). 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 propagates with a consequent more efficient cavitation process. Moreover, the overall effects of US irradiation did not appear immediately after treatment but persisted over time being significantly more relevant at 18 h from the end of US-irradiation. Evaluation of histological characteristics of US-irradiated samples showed a clear alteration of adipose tissue architecture as well a prominent destruction of collagen fibers which were dependent on US 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 evidenced how US 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 US treatment with surgical device showed, in agreement with the level of adipocyte damage, a significant increase mainly of triglycerides and cholesterol. Finally, US exposure had been show to induce apoptosis as shown by the appearance DNA fragmentation.

Accordingly, US treatment led to down-modulation of procaspase-9 expression and an increased level of caspase-3 active form.

ACKNOWLEDGEMENTS

The Lain Electronic is greatly acknowledged for technical support and for the provision of the ultrasound device.

The Authors thank Gasperina De Nuntiis (Department of Health Science, University of L'Aquila, L'Aquila, Italy) for technical assistance.

Introduction

Ultrasound (US) wave is a sound energy with a frequency higher than 20 KHz that, in the presence 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 cycles wavelength, low frequency ultrasound penetrates deeper than the high-frequency ultrasound. Theoretically, using a single wave cycle so that the phenomenon of attenuation is excluded, the penetration depth is equal to the wavelength $\lambda = v/\nu$. The amount of energy that reaches a specific site depends on both the US characteristics (frequency, intensity, amplitude, focus and beam uniformity) and the tissues through it travels. So, at higher frequencies, the soundbeam will have less sound energy available to propagate through a tissue since more energy is absorbed. US are widely used in medicine for several diagnostic and therapeutic applications (2-5). Most clinically used US have a frequency range of 2 and 10 MHz (high frequency and low power). US 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 US technology as an emulsifying approach for adipose tissue has been introduced in 1987 (6), afterwhich the technique of US liposculpturing was outlined (7). Thus, US are often used to improve liposuction for fat reduction (8-13). This technique known as US-assisted lipoplasty (UAL) uses an ultrasonic concentrate beam that hits adipose tissue cells in a defined subcutaneous focal area resulting in a series of expansions and compression cycles that exert negative and positive pressure. These differences in pressure cause cell membranes 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). US alter adipose tissue through micromechanical disruption and cavitation with minimal thermal effect (13-15). Cavitation effects, by causing microcavities in the adipose tissue with consequent cell destruction and fat liquefaction, are considered the most important mechanism

through which US 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). Cavitation refers to the oscillatory activity of vapor-filled bubbles and produces important cell fragmentation and diffusion of the lipid material through the intercellular space (8, 13). Although since 1978 biological effects of US 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 US are still relatively little and understanding of ultrasound-adipose tissue interaction is predominantly subjective and mainly based on clinical observation alone, particularly for low frequency US. Indeed, the frequencies of most US 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 US technology characterized by multifrequency US wave beams able to allow a higher tissue penetration of US energy and a lower phenomenon of attenuation when compared to high frequency US (1-3 MHz). In particular, we investigated the effects of both external and surgical US-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 US irradiation was also examined.

Materials and Methods

External and Surgical Device

The ultrasound device (Microlipocavitation, LAIN ELECTRONIC S.R.L. Milan, 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 US (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 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, U.S.A.). Triacylglycerol (TAG), Cholesterol (CO), Cholesterol Esters (CE), Ethidium Bromide and Haematoxylin/Eosin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phosphate-buffered saline (PBS) was obtained from Euro Clone (West York, UK). All solvents and reagent for DNA extraction 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 - Cod. 21-010802IC. All reagents for Western Blot analyses were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies, rabbit polyclonal anti-caspase 3 and rabbit polyclonal anti-pro-caspase 9, and, horseradish peroxidase-conjugated goat secondary antibody, were purchased from Santa Cruz Biotechnology, Inc.(CA, U.S.A).

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 US-treatment with external or surgical device.

Protocols for US-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 US as below described. The experiments were performed at room temperature (20°C). Each sample was weighted before and after US treatment (irradiation time: 10 min). US-treatment with external device was realized 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 (fig. 1A). 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; effects' detection time: 20 min and 18 h by the end of treatment. Treatment with surgical handpiece was performed by introducing the cannula in the adipose tissue parallelly to the skin (fig. 1B) according to the manufacturer's instructions. The surgical handpiece was gently moved with slow to-and-fro movements, like the clinical US-assisted liposuction. 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; effects' detection time: 20 min and 18 h by the end of treatment. In a set of experiments, to

improve conduction of the US through adipose tissue and to advantage its cavitation 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 weighted again and then US-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 Bligh & Dyer method (17). TLC was performed on 20 x 20 cm aluminum silica plates (Merck, Darmstadt, Germany). Eluent mixture (hexane/diethyleter/acetic acid, 70:25:3 v/v) (100 ml) was introduced in an elution tank to separate neutral lipids. The extracted lipids were applied on silica plates as thin rows at 1.5 cm distance above the bottom of the silica plate, air dried and placed immediately in the elution tank. The solvent was allowed to ascend up to 3 cm from the top of the plate, and then the plate was removed, air-dried and stained. Triacylglycerol (TAG) and cholesterol esters (CE) were used as standards. TLC staining was achieved by dissolving 8% phosphomolybdic acid in ethanol and vaporized it on plates 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).

US-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. 4µm tick sections were obtained with a rotative microtome and

sections on slides were stained either with hematoxylin and 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. Glasses were analysed independently by two pathologists with an optical microscope "Axioskop" purchased by 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 per 12 days (18, 19); maceration in distilled water per 7 days in ambient temperature; washing with PBS 0,1M; impregnation with tannic acid to 1% in 0.1M 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 US-treated adipose tissue specimens was performed by incubating samples with digestion buffer containing proteinase K (20 mg/ml) overnight at 37°C. The samples were then added phenol, chloroform and ethanol. Sodium acetate 3M 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 (UVItec Limited BTS-20M, Cambridge UK).

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 at 600 x g for 30 min at 4°C and the supernatants were assayed for protein content. 50 µg of proteins were electrophoresed through a 10% SDS-polyacrilamide (PAGE) gel under reducing condition. Proteins were transferred onto polyvinylidene fluoride (PVDF) membrane sheets and nonspecific binding sites were blocked for 1 h at room temperature in 20 mM TRIS-HCl buffer, 55 mM NaCl and 0.1% Tween 20 pH 7.4 (TST) containing 5% non-fat dry milk (blocking solution). Membranes were then incubated overnight at 4°C with rabbit polyclonal anti-caspase 3 (1:100) or rabbit polyclonal anti-pro-caspase 9 (1:100). After extensive washings with TST, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:2000). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL), according to the manufacturer's instructions. 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 total protein load by quantification of all samples in a single assay before loading and confirmation of equal loading by image analysis and scanned Coomassie blue-stained gels after blotting (20).

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 US exposure through external device on tissue weight and fat release.

To evaluate the effects of transcutaneous US-irradiation (Fig. 1A) 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 weighted after gentle squeezing of the tissue at 20 min and 18 h from the end of the treatment. Data shown in fig. 2A and B represent the mean of three independent experiments with US at 70% or 100% power, respectively. A single US-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 US irradiation did not appear immediately after treatment but propagated over time. No significant differences were observed, in these conditions, between US irradiation at 70% and 100% power. On the other hand, a more relevant weight loss was observed after a single US treatment with external device in the presence of SB infiltration, in accordance with the knowledge that ultrasonic waves in aqueous solution better propagates 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 US-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 from the tissue could be measured after US exposure at 70% or 100% power (fig. 2C).

Effects of US exposure through external device on histology and collagen bundles in adipose and skin tissue samples.

In fig. 3 is shown a representative experiment aimed to analyse the morphologic characteristics of adipose tissue after external US-irradiation in the same experimental conditions above described. Sections of untreated or US-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 US-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 US-irradiation either at 70% (E) or 100% power (F), which was most relevant than that observed in SB-not 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 was 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 US intensity. Figure 4A show collagen bundles between adipocytes clusters, bright blue stained, a sign of unmodified collagen, figures 4B and 4C show collagen fibers histology after US treatments at 70% power and at 100 % power, respectively. The prominent destruction of the collagen bundles is well observed with the Masson trichrome because of the inability of the collagen fibers to bind the aniline blue. To further verify the ability of US-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 (figs. 4E and 4F) when compared to untreated skin control (fig. 4D) 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 low frequency external device at 100% power and prepared for

microscopy as described in Materials and Method section. Results obtained by SEM were overlapping with those from light microscopy. In fact, SEM micrographs at low magnifications clearly evidenced how the used treatment induced a drastic reduction in the compactness of the adipose connective tissue (fig. 5A), with formation of wide and empty lacunae dispersed among the bundles of collagen fibers (fig. 5B). 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. 5D) if compared to control (fig 5C).

Effect of US exposure through surgical device on tissue weight

To investigate the effect of low frequency high intensity US irradiation at 70% (~13-14 W/cm²) or 100% (~19-21 W/cm²) 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 (fig. 1B). After each US exposure at both 70% and 100% power, samples were weighed 20 min and 18 h after treatment. In all conditions, US exposure induced a weight reduction, being this effect, as expected, much more significant after 18h from the end of treatment than that observed after 20 min (fig. 6). 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 off. Results (not shown) indicated that just a slight weight percent reduction (< 3%) was detectable at 20 min and 18 h when compared to switched-on surgical handpiece experiments. Indeed, a significant weight reduction was observed when samples were treated with US at 70% power (fig 6A) and, even more, with US at 100% power (fig. 6B). In fig 6A-B are also reported the results of experiments aimed to analyse the effects of US surgical handpiece treatment (10 min) on SB-infiltrated adipose tissue weight reduction. Similar to above reported results with external device, the infiltration of the tissue significantly enhanced the

effects of US exposure, both at 70% and 100% power, with the latter more efficacious as detected either at 20 min or 18 h after the treatment.

Effect of US-surgical treatment on adipose tissue histology

Histologic changes of *ex vivo* adipose tissue after US irradiation with surgical handpiece for 10 min were also analyzed (fig. 6). A standard lobular architecture could be seen in untreated (fig. 6A) as well in SB-infiltrated samples, while a clear injury of US-treated samples was evident being cell membranes disrupted and folded on themselves, with clear and large areas of adipocyte lysis, particularly after treatment with US at 100% power (fig. 6C-D) when compared to samples irradiated with US at 70% power (fig. 6B-E).

Effect of US exposure through surgical device on fat release

To associate surgical US-induced adipocytolysis and sample weight reduction with the release of fat, the volumes of released fat were measured either at 20 min or 18 h after US-irradiation (10 min, 70% or 100% power). In agreement with data on weight loss, a simultaneous and proportional release of fat from the tissue could be measured after surgical US exposure at 70% or 100% power (fig. 7A). Extracellular neutral lipids released after US-irradiation with surgical device were also analyzed by TLC after loading plate with volumes proportional to those overall collected (highest volume: 50 μ l). Adipose tissue samples treated for 10 min with the surgical device with the handpiece-off were used as controls. In all samples, the most intense bands could be referred to triacylglycerols (TAG) ($R_f = 0.75$) and cholesterol (CO) ($R_f = 0.35$). US irradiation induced an increased release of the total amount of triglycerides and cholesterol when compared to that observed by using the surgical handpiece-off ($p < 0.01$) (fig. 7B-C). The intensity of the spots resulted significantly higher after US-treatment for both TAG and CO, being the increase more relevant at 18 h when compared to 20 min. The observed results, confirming the ability of low-frequency US to induce adipocytolysis and,

consequently, the extracellular release of fat, mainly TAG and CO, also suggest, in the meantime, that low frequency US-irradiation treatment didn't affect the integrity and molecular structure of these neutral lipids.

DNA laddering and caspase activities

To investigate whether US-irradiation, in addition to causing cell lysis, was also able to induce apoptotic cell death, DNA fragmentation as detected by agarose gel electrophoresis was evaluated. Figure 8 shows a DNA electrophoresis of multiple samples of adipose tissue treated for 10 min with external or surgical US device and is representative of three independent experiments. While the DNA extracted from both control (untreated) (lane 1) and SB-infiltrated sample (lane 2) appeared intact, DNA extracted from samples treated with external US showed a slight fragmentation, recognized by smears and/or multiple bands in the lower (low molecular weight) portion of the gel led to a DNA smear or slight laddering suggesting a DNA damage (lanes 3-5). However, treatment with surgical handpiece led to a clearly detectable DNA fragmentation with a striking, typical "ladder pattern" (lanes 6-8). These results prompted us to analyze the caspases involved in the ability of low frequency US 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. 9 shows that control tissue exhibited constitutive levels of procaspase-9 which are slightly upregulated after SB infiltration. In samples treated with both external and surgical device, procaspase-9 is strongly and significantly downregulated, thus suggesting a procaspase-9 processing and a consequent caspase-9 activation after both treatments. As expected, processing of procaspase-9 occurred in US 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. 9, in samples treated with both external

and surgical device, a significant increase of the 17 kDa active caspase-3, when compared to control samples, could be detected.

Discussion

In the present study, the effects of low frequency high intensity US 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 US 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/cm² and 19-21 W/cm², for external and surgical irradiation, respectively. So, 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 US exposure for 10 min with 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 weight loss extent as well extracellular fat volume were very much larger than those observed in the absence of infiltration, in accordance with the knowledge about the increased effectiveness of cavitation process induced by US in the presence of water (13). Of interest, the effect of exposure to US on the tissue weight will continue 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 increasing 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 presence and absence of infiltration with SB, the weight loss as well the released fat levels associated with the US treatment appeared greater when US 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 US exposure showed damage in the membranes of fat cells and a disruption of the tissue that could be well associated with the results above described 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 played even on sections of the skin overlying adipose tissue. The histological observations in optical microscopy observations were also confirmed with scanning 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 US as well as whether the observed effects may represent a stimulus to induce a tissue repair process which include stimulation of collagen neosynthesis.

Treatment with 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 US exposure when compared to controls, confirming that an aqueous solution increased US-induced cavitation phenomenon thus leading to a more significant loss of adipose tissue. In addition to the expected damage caused by the introduction of the cannula into the tissue, histological changes in areas distant from the lesion mechanically caused by the handpiece were even more evident when compared with those observed following treatment with the external exposure to US, being US irradiation at 100% power more efficient when compared to 70% power. The analysis of the composition of lipids in the fat released from adipose tissue after US 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 US exposure to induce apoptosis appears particularly interesting. A single treatment with external US 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 US 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 (21-23).

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

ACKNOWLEDGEMENTS

The Lain Electronic is greatly acknowledged for technical support and for the provision of the ultrasound device.

The Authors thank Gasperina De Nuntiis (Department of Health Science, University of L'Aquila, L'Aquila, Italy) for technical assistance.

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LEGENDS TO FIGURES

Figure 1.- US-treatment of adipose tissue samples with external (A) or surgical (B) device. (A) Adipose tissue sample treated *ex vivo* with external device. External device treatment was realized on the cutaneous surface applying an ecographic gel to create a head-gel-skin interface. (B) Adipose *ex vivo* tissue sample treated with surgical device. Surgical device treatment was realized applying a cannula moving on in the adipose subcutaneous tissue.

Figure 2.- Weight reduction of adipose tissue samples after US irradiation with external device. Weight reduction (%) of adipose tissue samples following US treatment for 10 min with external device at 70% power ($\sim 1.8 \text{ W/cm}^2$) (A) or 100% power ($\sim 2.5 \text{ W/cm}^2$) (B) with or without saline buffer infiltration ($\pm \text{SB}$); samples were analyzed and weighted after 20 min and 18 h after treatment. Shown data represent the mean of three independent experiments ($\text{SD} < 10\%$) (* p value < 0.01 when compared to control samples; # p value < 0.01 when compared to mean weights of not SB-infiltrated samples; § p value < 0.01 when compared to control and not SB-infiltrated samples). (C) Released fat collected at 20 min or 18 h after the treatment (10 min) with US external device at 70% or 100% power. Shown data represent the mean of three independent experiments ($\text{SD} < 10\%$).

Figure 3.- Histology of adipose tissue samples after US irradiation with external device. Sections of untreated or US-treated adipose tissue samples were stained with haematoxylin and eosin (H&E). A: Section of untreated adipose tissue. B-C: Adipose tissue sections US-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 US-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 was 10X.

Figure 4.- Histology of collagen bundles in adipose and skin tissue samples untreated or US-treated with external device. Tissue sections from untreated or US-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 US-irradiation for 10 min with external device at 70% power. (C) Adipose tissue after US-irradiation for 10 min with external device at 100% power. (D) Untreated skin sample. (E) Skin sample US-treated for 10 min with external device at 70% power. (F) Skin sample US-treated for 10 min with external device at 100% power. The results from a representative experiment out of three are shown.

Figure 5.- Surface ultrastructure by SEM analysis. Adipose tissue samples were US-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 out of three independent experiments.

Figure 6.- Effect of US exposure with surgical device on adipose tissue weight and histology. Mean weight of untreated or US-treated adipose tissue samples with surgical device at 70% (A) or 100%

power (B) for 10 min with or without saline buffer infiltration (\pm SB). Samples were analyzed and weighted after 20 min and 18 h after treatment. Shown data represent the mean of three independent experiments (SD<10%) (* p value < 0.01 when compared to SB control samples; # p value<0.01 when compared to homologous not SB-infiltrated samples; § p value<0.01 when compared to control not 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.

Figure 7.- 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 US surgical device at 70% or 100% power. Shown data represent the mean of three independent experiments (SD<10%). (B) Thin layer chromatography (TLC) of neutral lipids released from adipose tissue sample following treatment for 10 min with handpiece-off or US 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 ($R_f = 0.75$), CO cholesterol ($R_f = 0.35$). (B) Spot intensities of TLC are reported as densitometric units. Data represent the mean of three independent experiments (SD<10%) (* p value < 0.01 when compared to results obtained with handpiece-off treatment).

Figure 8. 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 previously infiltrated with saline buffer. 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 3-4-5: samples exposed to external device for 10 min (100% power); Lane 6-7-8: samples exposed to surgical device for 10 min (100% power). A representative gel is shown out of 3 independent experiments with similar results.

Figure 9. Western blot analysis of procaspase 9 and caspase 3. (A) Immunoblotting assay for the inactive precursor of caspase 9 (pro-caspase 9) and executive caspase-3 enzyme adipose tissue protein extracts. C = control (not treated); SB = Saline Buffer-infiltration; External device: US-irradiation (10 min) with external device (100% power; 18 h after the end of treatment); Surgical device: US-irradiation (10 min) with surgical handpiece (100% power; 18 h after the end of treatment). (B) Immunoreactive bands, normalized against the total extract proteins after Blu Comassie staining, were determined using TotalLab software (ABEL Science-Ware srl Italy) and values were given as relative units. (C-D) Histograms showing the densitometric analysis of procaspase 9 and caspase 3 respectively. Data represent the mean of 3 independent experiments \pm SD; * p value < 0.001).