

New System Delivering Microwaves Energy for Inducing Subcutaneous Fat Reduction: In - Vivo Histological and Ultrastructural Evidence

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Abstract

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BACKGROUND: Recently, it has been developed a new technology for the reduction of subcutaneous adipose tissue through a non-invasive treatment by microwaves. The main objective of the present study is to demonstrate the feasibility of utilising a non-invasive, localised microwaves (MW) device to induce thermal modifications into subcutaneous adipose tissue only by a controlled electromagnetic field that heats up fat preferentially. This device is provided with a special handpiece appropriately cooled, directly contacting the cutaneous surface of the body, which provides a calibrated energy transfer by microwaves.

AIM: In this paper, microscopic and ultrastructural modifications of subcutaneous adipose tissue induced by microwaves irradiation are evaluated.

METHODS: Our experimental plan was designed for collecting biopsy samples, for each skin region treated with a single irradiation session, 1) before treatment (control), 2) immediately after treatment, 3) after 6 hrs, 4) after 1 month, 5) after 2 months. Bioptic samples from each step were processed for light microscopy and transmission electron microscopy. At the same time, each region where biopsies were collected was subjected to ultrasound examination. Recorded images permitted to evaluate the thickness of different layers as epidermis, dermis, hypodermis, connective fasciae, until to muscle layer, and related modifications induced by treatment.

RESULTS: In every biopsy collected at different time-steps, epidermis and superficial dermis appeared not modified compared to control. Differently, already in the short-term biopsies, in the deep dermis and superficial hypodermis, fibrillar connective tissue appeared modified, showing reduction and fragmentation of interlobular collagen septa. The most important adipose tissue modifications were detectable following 1 month from treatment, with a significant reduction of subcutaneous fat, participating both the lysis of many adipocytes and the related phagocytic action of monocytes/macrophages on residuals of compromised structures of adipocytes. In the samples collected two months following treatment, the remnants of adipose tissue appeared normal, and macrophages were completely absent.

CONCLUSIONS: Ultrasound, microscopic and ultrastructural evidence are supporting significant effectiveness of the new device treatment in the reduction of subcutaneous fat. In this paper, the possible mechanisms involved in the activation of the monocytes/macrophages system responsible for the removal of adipocytes residuals have also been discussed.

Introduction

The trafficking of fatty acids into and out of adipocytes is a physiological mechanism regulated by

a complex series of proteins and enzymes and is under control by a variety of hormonal and metabolic factors (Thompson 2011) [1]. Many Authors have presented theoretical and experimental papers using methods and devices with as objective the reduction of adipose tissue. Particular interesting a recent paper

(Asan 2017) [2] in which it has been considered the properties of some tissues related to the absorption of microwaves energy in different models using equivalent phantom and ex-vivo measurements. The most of experimental evaluations available in the literature, related to fat reduction, have been performed *in vitro* conditions, which only in few cases could represent what and how happens *in vivo*, in the complex dynamics inside tissues and organs in the individuals. In this paper, we present morphological observations on the subcutaneous adipose tissue following microwaves irradiation of the skin.

A controlled electromagnetic field @2,45 GHz, perpendicularly applied to the skin, is selectively absorbed by the subdermal fat layer thanks to the dielectric properties of the different tissues (epidermis, dermis, fat) crossed by the applied EMF. The high-controlled emission of the EMF can establish a perfect coupling only in the presence of a fat layer, due to the absorption characteristics of this tissue at the established frequency.

In the present *in-vivo* study on an animal model, 7 minutes thermal exposure to 50°C, histological and ultrastructural evidences show conservation without damage to the epidermis and dermis layers, while the subdermal fat is modified through a series of molecular processes stimulating a massive delivery of fat droplets and the beginning of “auto-adipolysis”.

Material and Methods

A mathematical model was used to correlate the frequency of the electromagnetic field and the dielectric properties of the tissues to induce localised hyperthermia only in the subcutaneous fat layer. The non-invasive pseudo-transcutaneous electromagnetic field (Coolwaves™ by Onda, DEKA, Florence, Italy) was applied to thermally induce adipocytes' damage. During the treatment, applying a transmitting handpiece on the surface of the skin, the electromagnetic field energy was delivered into the subcutaneous adipose tissue (SAT) of a Vietnamese pig (this study on the animal model was carried out in full compliance with international guidelines for safety and compliance with their use) with a 50.000 J total dose delivered in 7 minutes over a cutaneous area 15 x 15 cm². The handpiece used was maintained at a temperature of 5°C through a specific cooling system.

The experimental plan was designed on the basis of a single irradiation session, and for each skin region treated, biopsy samples were collected and processed for light and electron microscopy, owing to the following steps: T0 (before treatment, as control), T1 (immediately after treatment), T2 (6h following treatment), T3 (1 month following treatment) and T4 (2

months following treatment).

Temperature monitoring was performed superficially by the thermometric infrared camera vision system and internally, in the subdermal fat layer (at 7mm depth), by sterile thermometric glass fibre. The epidermal temperature in the treated area was always observed in a safety-range of 15-25°C, while at 4 to 7 mm in depth was of 50°C (Figure 1A and 1B).

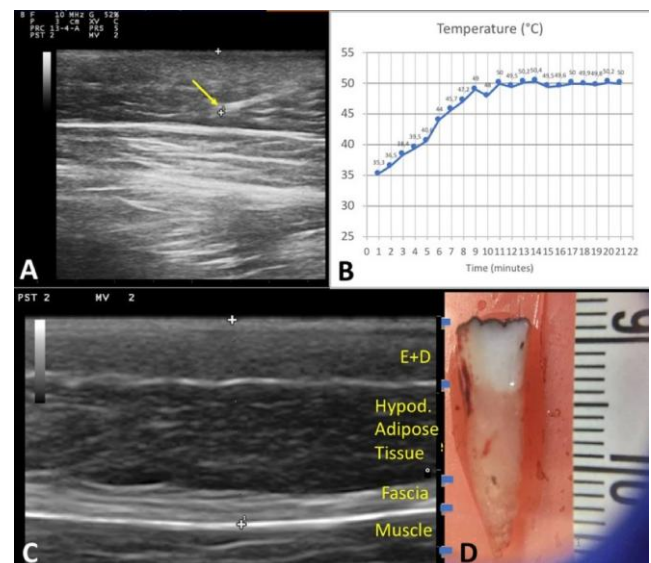


Figure 1: A and B are showing the tip of the temperature probe (arrow) by ultrasound A) and the temperature measured inside the hypodermal layer B) respectively; C) and D) correlation of ultrasound image C) and biopsy sample D) tissue layers; The different layers are clearly identifiable: Epidermis and Dermis (E + D), Subcutis (Hypodermal Adipose Tissue), Fascia, Muscle

In each time step, biopsy samples were collected, immediately immersed in the fixative solutions and processed both for light microscopy and transmission electron microscopy.

Light microscopy

Biopsy samples were immediately immersed in a 4% paraformaldehyde/sodium phosphate buffer solution for 24 hrs and then processed (dehydration, paraffin embedding and sectioning) for light microscopy. Sections were stained with Haematoxylin and Eosin, and other sections were stained with Picrosirius Red for collagen staining.

Electron microscopy

Fixation was performed by immersion of biopsy samples in a 2.5% glutaraldehyde (EM grade)-2% paraformaldehyde in 0.1M sodium cacodylate buffer solution (pH 7.3) for 6 hours at 4°C. After washing in the same buffer, samples were post-fixed for 2 h in osmium tetroxide 1.33% in 0.1 M s-collidine buffer, dehydrated in a graded series of ethanol (30%, 50%, 70%, 80%, 95%, 100%), propylene oxide and finally embedded in epoxy resin Epon 812. Semithin

(0.2 μm) and ultrathin (40-60 nm) sections were obtained at the ultramicrotome Reichert Ultracut S provided with a diamond knife. Semithin sections were stained with Toluidine blue and ultrathin sections, previously collected on 200 μm mesh copper grids, were counterstained with lead citrate and uranyl acetate. A Zeiss EM 902 transmission electron microscope, operating at 80 kV with an objective aperture of 30 / 60 μm , was used for direct observation. Electron micrographs were recorded on Kodak 4489 Electron Image film and finally digitised with an Epson Perfection V750 Pro scanner at 1200 dpi.

Results

The description of our observations will follow the time sequence of samples collection, using the most suitable presentation of our findings to better understand the functional dynamics.

Before sample collection, it has been used ultrasound imaging to define the thickness of the different structural layers, particularly the depth and extension of the subcutaneous tissue as the target of the microwave energy transfer (Figure 1C) and compare it with biopsy sample (Figure 1D).

At time T0, defining the control samples collected before the treatment, microscopic morphology of adipocytes appeared normal, spherical in shape, with the whole volume of cells appearing filled with a homogeneous content, surrounded by a very thin layer of peripheral cytoplasm, where an elongated and flattened nucleus was well identifiable (Figure 2A). All around adipocytes and between them, a loose connective tissue was appreciable, with some cells (fibroblasts) and an extracellular matrix constituted by collagen fibrils relatively dispersed inside a faint ground substance (Figure 2A).

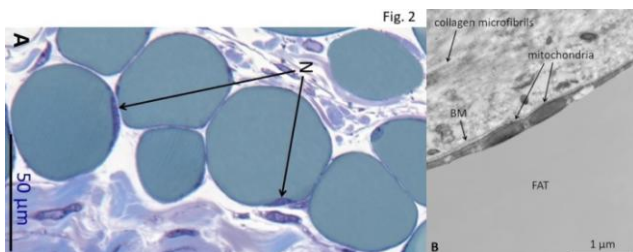


Figure 2: T0 (Control); A) Semithin section at the light microscope from epoxy resin embedding, stained with toluidine blue. Adipocytes show peripheral nucleus (N) with dispersed chromatin, a very thin peripheral layer of cytoplasm, and a homogeneous lipidic content. Around them, elongated fibroblasts and bundles of collagen fibres are also observable; B) Ultrastructure of the very thin peripheral cytoplasmic of an adipocyte and inside it two elongated mitochondria were observable. A continuous basal lamina close to the plasma membrane and collagen microfibrils in the surrounding connective tissue is also visible. The homogeneous fat is constituting the inner content of the adipocyte

Small blood vessels were also identifiable. At the electron microscope (Figure 2B), the very thin peripheral cytoplasm appeared to contain dispersed organelles, as mitochondria, rare profiles of the endoplasmic reticulum and small vesicles involved realistically in the physiological transport of materials (lipids included), responsible of the correct homeostatic processes across the cytoplasm to and from the interstitial connective tissue.

At time T1 (immediately the following treatment), small single or multiple vesicles were visible at the light microscope in the peripheral cytoplasm of adipocytes (Figure 3A).

At time T2 (6 hrs after treatment), many vesicles containing material very similar to the big lipidic content of the adipocyte were detectable inside adipocyte peripheral cytoplasm. In ultrathin sections observed at the electron microscope, the whole peripheral cytoplasm was completely occupied by vesicles containing a relatively electron transparent material. This feature appears particularly evident in the oblique sections of the inner surface of the peripheral cytoplasm as a holey structure (Figure 3B). In a relatively thick section at the electron microscope, we observed blebbing structures at the surface of adipocytes (Figure 3C) projecting part of their lipidic content towards the interstitial connective tissue.

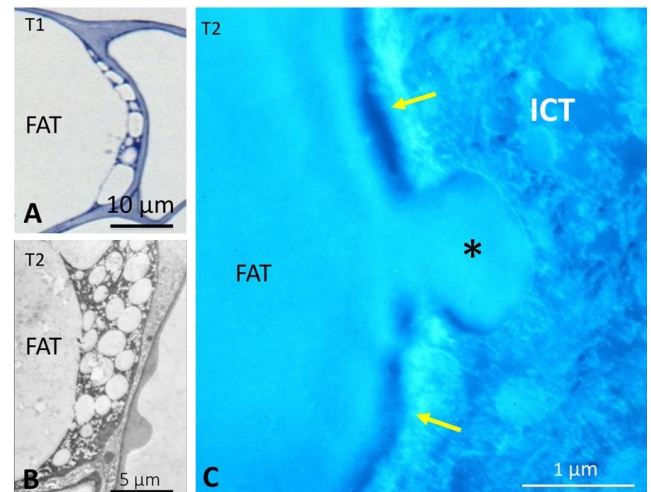


Figure 3: T1, T2, T3; A) T1 (immediately the following treatment): Semithin section at the light microscope from epoxy resin embedding, stained with toluidine blue. Inside the thin peripheral cytoplasm of adipocytes, some vesicles with the content similar to the big lipid content of cells are visible; B) T2 (6 hrs following treatment): at the electron microscope the peripheral cytoplasm of adipocytes shows numerous vesicles occupying the whole thickness of the cytoplasm. In the oblique sections, the internal cytoplasmic surface appears fully of vesicles; C) T3 (6 hrs following treatment): the high magnification at the electron microscope represents the superficial blebbing as a direct extrusion of a lipid droplet (asterisk) through peripheral cytoplasm from the inside of an adipocyte (left) to the outside, in the interstitial connective tissue (ICT). Arrows mark the thin peripheral cytoplasm of the adipocyte

Further, some cytological details of adipocytes involved in these mechanisms, have been observed at the electron microscope, as mitochondrial

swelling with few disorganised cristae and interruptions of the inner membrane, interruptions of the plasma membrane, dilations of the endoplasmic reticulum (data not shown). The whole of these features suggests adipolysis via necrotic processes.

At time T3 (1 month after treatment), observing transverse sections of the wall of adipocytes, numerous vesicles were evident inside an electron-dense cytoplasm. Some of them appeared as invaginations of the inner side of the peripheral cytoplasm with a content continuous with the big lipidic sphere of the adipocyte; others appeared as openings towards the interstitial connective tissue. These features are suggesting a mechanism of endo-exo-cytosis of lipids from the inside of the adipocyte to the outside towards the interstitial connective tissue. At time T3, in some areas of the biopsies an inflammatory infiltrate was detectable. This infiltrate appeared constituted by numerous cells penetrating the interstitial tissue, singularly or grouped encircling single adipocytes (Figure 4A).

The infiltrate is constituted mainly by monocytes distributing free in the interstitial connective tissue or close contact with adipocytes, covering their external surface. Single monocytes appeared provided with cytoplasmic processes projecting free in the interstitial connective tissue or directly contacting free vesicles released by adipocytes. The first contact of monocytes with lipids released by necrotic adipocytes is realistically suggested by a direct contact representing the result of the "find me" action of the extracellular lipid structures towards monocytes and the starting of the mechanism "eat me" stimulating the phagocytic action of macrophages. The following step in the mechanism of adipolysis observed at the same time T3 (after 1 month from treatment) is represented by the formation of Crown-Like-Structures as the most typical feature. CLS are constituted by single monocytes closely related one with the other forming a pluricellular structure directly encircling adipocytes (Figure 4A). Inside these cells, small droplets appear very similar to the adipocyte content, suggesting the starting of a phagocytosis mechanism. In a following step of the mechanism suggested by images, single monocytes forming the "crown" around adipocyte, fuse their cytoplasm forming a unique big cytoplasm with many nuclei inside (syncytium) all around adipocytes (Figure 4B), realistically for a more effective action of phagocytosis of the content of these cells and the cytoplasmic residuals of the same necrotic adipocytes. Remark in Figure 4B the small dimensions of the represented adipocyte, due to the action of fat removal by macrophages. At the electron microscope, a single monocyte of the crown appears closely contacting the surface of the adipocyte (Figure 4C).

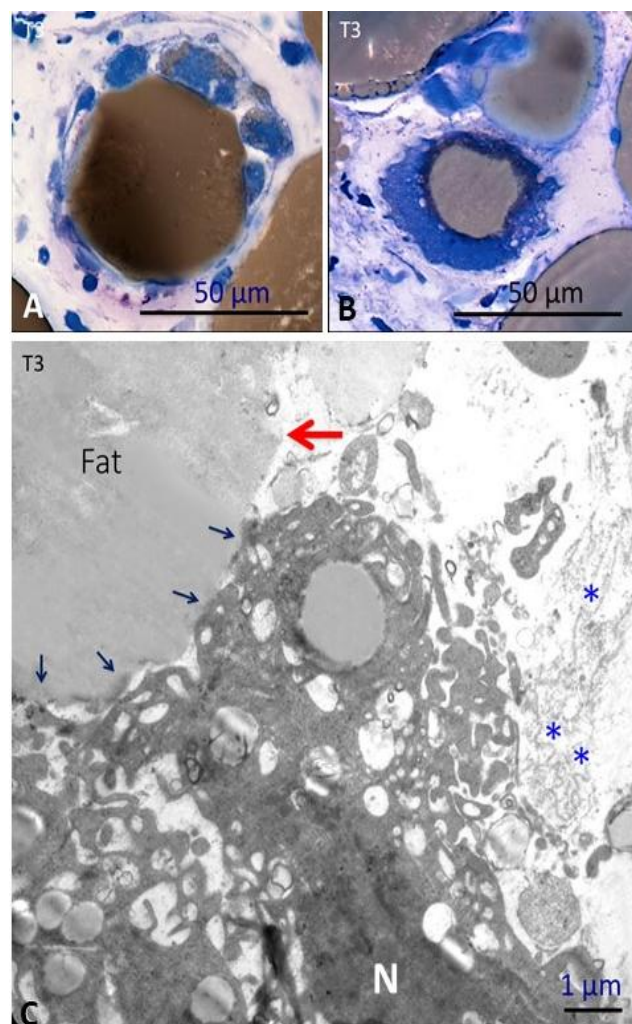


Figure 4: T3 (1 month following treatment); A) Light microscopy of a "Crown-Like-Structure", constituted by a crown of single monocytes, joined between them by thin cytoplasmic processes, distributed all around an adipocyte. These monocytes appear to contain in the cytoplasm small droplets similar to the content of the adipocyte. Realistically these droplets are the result of phagocytosis due to macrophage activity; B) The smaller adipocyte appears completely surrounded by a multinucleated structure originated by the fusion of single monocytes forming a single syncytial macrophage. The small dimensions of the adipocyte are realistically due to stimulated phagocytosis of the fat of necrotic adipocyte. Semithin sections from epoxy resin embedding, toluidine blue-stained; C) In the high left part of the electron micrographs (red arrow), a portion of the big lipidic content of the adipocyte is directly contacting the interstitial tissue. The remnant part of this content is directly contacting the plasma membrane of a macrophage (small black arrows), which has already internalised in his cytoplasm numerous lipid droplets. The lack of the peripheral cytoplasm of the adipocyte demonstrates that this adipocyte has completed the process of cellular death. Furtherly, the basal membrane, far from the adipocyte, appears highly disorganised inside interstitial tissue (asterisks); N: macrophage nucleus

The surface of this cell appears expanded in numerous and complex superficial projections towards both the interstitium and the surface of adipocyte, constituting a sort of labyrinth of spaces realistically ready to endocytosis. Inside this cell, some small droplets of phagocytosis are detectable (Figure 7C). Adipocyte peripheral cytoplasm appears highly fragmented or completely absent, permitting a direct

exposure of the adipocyte content to the interstitium and direct contact between the plasma membrane of macrophage and the big lipid droplet of the same adipocyte. Furtherly, remark the absence of the basal membrane at the surface of the necrotic adipocyte. Disorganised residuals of this structure are recognisable in the interstitium externally to the macrophage (Figure 4C).

The interstitium of the adipose tissue is constituted by a loose connective tissue containing finely distributed collagen fibres and a highly permeable ground substance formed by glycosaminoglycans, proteoglycans and multi adhesive glycoproteins. The interstitial connective tissue is also provided with cells, like fibroblasts, mesenchymal cells capable of differentiation, migrating cells, endothelial cells forming the inner lining of blood and lymphatic vessels. Highly extended lymphatic vessels have been detected even at the light microscope, as a delicate structure with a very fine endothelial wall expanded between adipocytes. Inside lymphatic vessels, free macrophages containing lipid droplets. Realistically, following phagocytosis, macrophages migrate inside connective tissue, then pass through the thin endothelial wall of lymphatics and finally they are transported with the lymph to lymph nodes where the terminal lysis of the phagocytised material is performed (data not shown).

At time T4 (two months after treatment) the normal structure of the adipose tissue appear restored, as shown in semithin sections from epoxy resin embedding (Figure 5A). Normal features of adipocytes morphology two months after treatment are also confirmed at the electron microscope (Figure 5B).

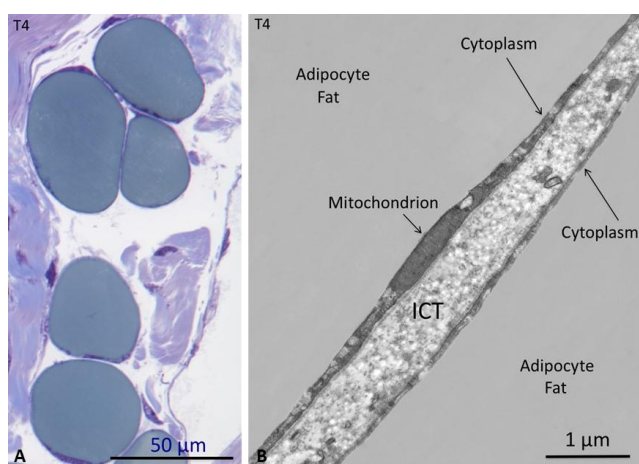


Figure 5: T4 (2 months following treatment); A) Semithin section at the light microscope from epoxy resin embedding, toluidine blue-stained. Adipocytes appear as TO (control), without any vesicle in the thin peripheral cytoplasm and a very thin interstitial tissue between them. No cell infiltrate detectable; B) At the electron microscope, the peripheral cytoplasm of two adjacent adipocytes, with the interstitial connective tissue (ICT) between them, shows normal features, as in the control samples. Abnormal modifications of the plasma membrane or cytoplasmic structures are never detectable. The very small vesicles observable in the peripheral cytoplasm, as in control, are related to physiological trafficking of molecules across peripheral cytoplasm of adipocytes

The effectiveness of the treatment in terms of fat reduction has been demonstrated by ultrasound measurements of thickness from the surface of epidermis to the muscle layer, significantly reduced due to the fat reduction (Table 1).

Table 1: Comparative Table of Thickness from the epidermal surface to the muscle (excluded)

Time Table	Thickness Abdomen A (right)	Thickness Abdomen B (left)
T0 Control	2.7 cm	3.1 cm
T1 Immediately post treatment	2.6 cm	3.0 cm
T2 6 hrs after treatment	2.38 cm	2.59 cm
T3 1 Month after treatment	1.38 cm	1.3 cm
T4 2 Months after treatment	1.31 cm	1.4 cm

Discussion

Controlled hyperthermia-and maybe other effects not directly evaluable due to the treatment (among them the resonance of some molecular species-both structural and enzymatic-with the frequency of irradiation) determines in the adipose tissue (with particular effect on hypertrophic adipocytes), severe adipolysis with necrotic-like features which in turn stimulate an immune response with the active participation of monocytes and macrophages. Realistically, it could result in adipocytes a response of functional surcharge in the transporting mechanisms through membranes of the peripheral cytoplasm. In adipocytes, some key-structures for the life of cells appear involved, as the plasma membrane, the outer and inner membranes of mitochondria, and the membranes of the endoplasmic reticulum. The plasma membrane is essential for maintaining the intracellular microenvironment and for the whole of the finely controlled transmembrane transport processes which guarantee the correct ionic and molecular gradients and the related homeostatic equilibrium with the extracellular environment. Mitochondria are the cytoplasmic organelles responsible for oxidative phosphorylation, providing adenosine triphosphate (ATP) for most energetic cellular processes. Endoplasmic reticulum (particularly the rough e. r.) is the site of protein synthesis in the polyribosomes attached to the membranes of the organelle, controlling at the same time the 3D conformation of proteins form which specific biological functions depend. Concerning mitochondria, it is possible to consider that the increased catabolism induced by irradiation, in the mitochondria of hypertrophic adipocytes would start a condition of oxidative stress with production of Reactive Species of Oxygen and free radicals (Giordano 2013) [3], promoting an excess of delivery of fatty acids. The excess of transport of different molecular species through lipid droplets well identifiable morphologically at the electron microscope (Kranedonk 2014) [4]-fatty acids, adipokines and pro-inflammatory molecules –

to the outside of the cell towards the interstitial connective tissue (Giordano 2013) [3], overtake the normal physiologic homeostatic flow through the very thin peripheral cytoplasm of adipocytes (Gao 2017) [5].

As a consequence of these structural and functional events, “necrosis-like” modifications are initiated, similarly to what happens in experimental obesity demonstrated in mouse and humans (Cinti 2005) [6]. Through a chemotactic mechanism mediated by receptors specifically called “find me”, microparticles delivered by adipocytes stimulate the recruitment of cells, both resident and coming from the blood, specifically monocytes and macrophages (Eguchi 2015) [7]. To all that, it follows the starting of phagocytosis through molecular triggering signals (“eat me” signals) of phosphatidylserine (a glycerophospholipid) delivered by adipocytes (Krahling 1999, Engin 2017) [8], [9]. They are also released pro-inflammatory mediators, as (TNF) α , nitric oxide synthase, interleukin (IL)-6 and (IL)-1 β . The most of macrophages arrange in close relationship with adipocytes which have started the necrotic process, constituting characteristic “Crown Like Structures” (Cinti 2005) [6]. In these structures, activated monocytes and macrophages, starting with single contacts, arrange constituting extended multinuclear syncytia, as demonstrated in our observations. The function of these structures is the removal of the excess of free fatty acids and lipid droplets free in the interstitial tissue, together with the residuals of necrotic adipocytes. The big amount of small insoluble lipid droplets released from adipocytes constitute an important cytotoxic source of cholesterol and free fatty acids, which can damage other adipocytes (Unger 2002) [10]. Because of that, the removal of these molecular species from the interstitial tissue by macrophages represents a defence mechanism to restore the physiological homeostatic equilibrium. To all that it is added the release of further other molecules chemoattractant for macrophages, also stimulating the recruitment of blood monocytes through diapedesis from vessels with synergistic action of resident macrophages (Curat 2004) [11].

The excess of free fatty acids, via “Toll-like receptors” (Nguyen 2007) [12], stimulate the immune-system of the interstitial tissue, represented by resident cells (subpopulation of macrophages also identified as “dendritic cells”). These cells, similarly to dendritic cells and Langerhans cells in the epidermis, perform patrolling functions in the interstitium and favour the recall of other monocytes from the blood. In these cells, phagocytic functions are stimulated and optimised through the aggregation of single monocytes forming multinucleated phagocytosing macrophages which arrange all around the most damaged adipocytes. In this process are also involved the lipases in the adipocytes (Fujimoto 2011) [13], as enzymes acting on constitutive triglycerides inside

adipocytes, with the delivery of glycerol and fatty acids. In this way, these molecules are delivered by adipocytes which already started a necrotic process, to which significantly contribute Reactive Oxygen Species (Ventura 2004, Green 2014) [14], [15]. These molecular species aggravate the cytotoxic stress, with rupture of cells membranes (plasma membrane, mitochondria and endoplasmic reticulum membranes), with free delivery of both cell fragments (or molecular components no more contained inside membranous compartments) and enzymes into the interstitial tissue. Here, through phagocytosis, macrophages remove these materials from interstitium and after that, migrate inside lymphatic vessels in order to complete terminal lysis of phagocytized material from involved adipocytes

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