Non-Surgical Fat Reduction and Topical Modulation of Adipose Tissue Physiology

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ABSTRACT

Non-surgical fat reduction procedures have gained in popularity over the past few years and remain in great demand. The process results in accumulation of breakdown products, lipid droplets, that are slowly absorbed over a period of months. This paper outlines the physiological process whereby lipid droplets are absorbed through a process of autophagy (lipophagy) involving a repackaging of these droplets to smaller sizes so that macrophages can then cope with digestion of these very large particles. Furthermore, a fat compartment is described within the dermis surrounding the tail of the hair follicle, which is attracting much attention due to its unique phenotype, function, and connection to the deeper subcutaneous fat compartment. This provides an entry route for direct signaling to the subcutaneous fat. Related to these novel concepts, peptides can be designed in liposomal delivery systems to target lipid droplet breakdown via the hair follicle entry route. This concept is elucidated in this paper.

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INTRODUCTION

Body sculpting and non-surgical fat removal procedures have become increasingly popular over the past few years. Non-invasive fat removal technologies include low level laser therapy, infrared light, ultrasound, radiofrequency, and cryolipolysis.[1] Non-invasive body contouring procedures can be classified by the energy source deployed or the effect on the fat tissue, that of short term metabolic size reduction or long term permanent fat cell death.[2] The latter process is more commonly chosen with fat cell destruction occurring in a number of ways: thermal necrosis following high frequency focused ultrasound; pulsed focus ultrasound that cavitates adipose tissue non-thermally; cryolipolysis that causes cold associated cellular apoptosis; and radiofrequency induced electroporation of the fat cell membrane.[2] In addition, injectable lipolytic agents may also be used to decrease smaller fat tissue volumes. In all the above-mentioned processes, the adipose cell is damaged to differing degrees and varying degrees of inflammation ensue, however, in all, the content of the adipocyte, the lipid droplet, is released into the extracellular space to be slowly absorbed by macrophages over ensuing weeks or months.

It is this process that deserves further attention – defining the mechanism of cellular destruction, examining the process of waste product/lipid droplet elimination, and seeking potential access routes for topical products which may optimize resolution of programmed fat tissue destruction.

Pathophysiology of Fat Reduction Processes

Radiofrequency (RF)

Changes in the cellular structure following fat tissue exposure to RF has been termed pyroptosis. This relates to the focal delamination of the cell membrane seen in adipocytes approximately 2 weeks following RF exposure. This is followed by tears in the cell membrane with multiple pores evident, large enough for lipid droplets to pass through resulting in a reduction in adipocytes. This irreversible electroporation of the adipocyte cell membrane results in significant volume loss and cell death over a number of weeks.[2]

Cryolipolysis

Programmed, silent (non-inflammatory) cell death is the purported mechanism of action accompanying cryolipolysis. The theory is based on greater susceptibility of lipid filled adipocytes to cold as opposed to other water filled cells.[3] However, there are current disputes around the non-inflammatory nature and cell wall disruption claimed with cryolipolysis.[2,4] In addition, crystallization and cold ischemic injury of adipocytes inducing subsequent apoptosis (inflammatory, not silent) is also a suggested mechanism of action.[5,6]

Ultrasound induces multiple pores and even rupture of the cell membrane and plasma membrane surrounding the lipid vacuole by cavitation. This allows leakage of triglyceride droplets from the droplet into the extracellular space.[7]

It would appear that the lipolytic effect of these technologies all precipitate a change or disruption in the cell structure, which release mediators, particularly TNFα, inducing lipolysis through intracellular signaling cascades, metabolites, and lipid droplet-associated proteins.[8]

In general, irrespective of the cause, the lipolytic process induces an innate immune response with activation of resident macrophages within the adipose tissue. The cellular changes occurring in all adipocytolytic processes described above involve damage to the cell membrane in one form or another, releasing saturated fatty acids from the lipid contents, which triggers local inflammation in contrast to the classic description of cellular silent apoptosis.[4] The released saturated fatty acids induce macrophage activation with release of cytokines such as tumor necrosis factor alpha (TNF-α) and other inflammatory mediators from the macrophages and the adipocytes themselves.[4] This inflammatory process and TNF-α induction can improve the efficacy of the procedures by stimulating the lipolytic process,[8] however the great challenge to these macrophages is the ultimate digestion of these large particles that may delay the process of dissolution seen over many months following these procedures.

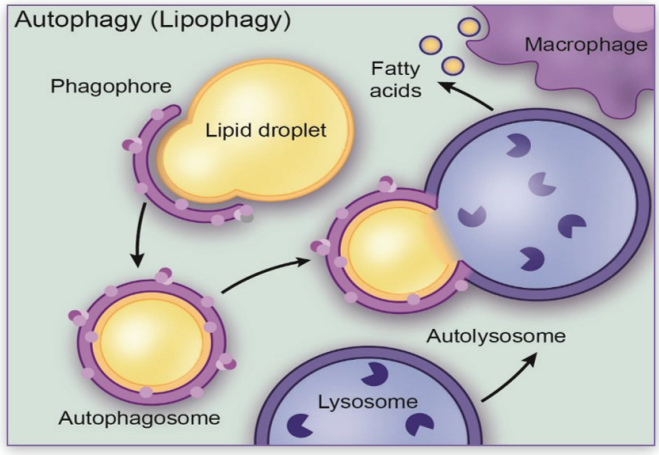
Autophagy/Lipophagy

Macrophages play important roles in the clearance of dead and dying cells, and in particular, of apoptotic cells. In fact, persistence of apoptotic cells following defective clearance has been found to be associated with the development of certain autoimmune diseases.[9] Lipid droplets are extremely large in size and challenging for smaller macrophages to phagocytose, often necessitating a clustering of macrophages surrounding one droplet (crown-like effect) secreting lysozymes in an effort to engulf these large bodies.

Fortunately, the process of autophagy is a great help in dealing with large intracellular components. Simply put, autophagy is the cells' way of repackaging very large organelles and intracellular bodies (such as lipid droplets) so that macrophages can cope with their digestion.[10] The process involves sequestering a portion of the cytoplasmic organelle or droplet, surrounding it with a portion of cell membrane and delivering it to a lysozyme for digestion (Figure 1). This creates smaller components that can then be digested by macrophages. In recent years, studies have demonstrated that lipid droplets are taken up by autophagy to cope with lipid mobilization and droplet digestion – this process, termed lipophagy, specifically targets lipid droplets and adipose cellular debris for digestion.[10,11]

Autophagic activity decreases with age in most tissues resulting in waste product accumulation with aging – the decrease in lipophagy, in particular, and the resultant increase in intracellular lipid droplets, may contribute to atherosclerosis,

FIGURE 1. Autophagy facilitates very large sized fat cells and lipid droplets (much bigger than macrophages) to be digested. The process takes place by surrounding part of the fat droplet with a cell membrane part (phagophore), this then breaks down the droplet into a smaller more digestible size, it fuses with a lysosome that pours enzymes into the droplet further breaking it down into a still smaller size. This particle can now be digested by macrophages, which are drawn into the area by the process.



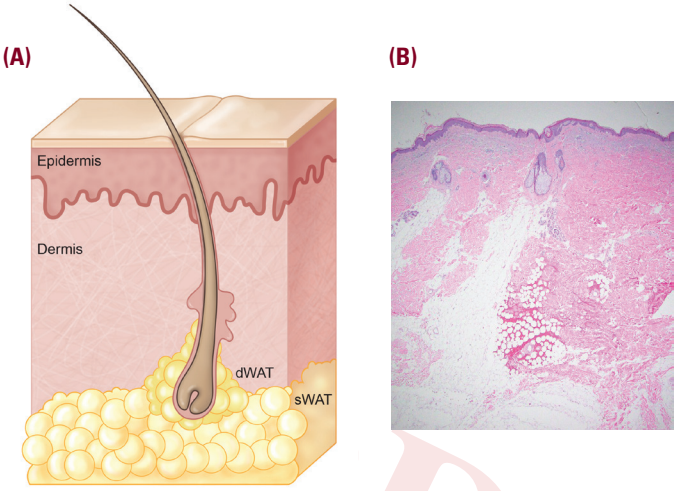
hypercholesterolemia, and other elements of metabolic syndrome of aging.[12]

Dermal White Adipose Tissue (dWAT): Redefining Fat Compartments

White adipose tissue (WAT) is no longer considered a simple energy depot and is now recognized as an organ with major endocrine and metabolic effects. Traditionally, WAT was considered as two distinct anatomical depots, namely subcutaneous (sWAT) and visceral white adipose tissue (vWAT), with differences in cellular and metabolic effects.[13] More recently, a new type of WAT, dermal WAT (dWAT), has been identified and is recognized as playing a major role in skin processes such as hair follicle growth, thermoregulation, wound healing, and signal transmission.[13] This layer lies in the reticular dermis encasing mature hair follicles[14] (Figure 2a and b). In addition, this fat compartment is associated with specific adipose stem cell phenotypes (CD24+), distinctly different to sWAT, suggesting unique functions of this fat depot.[13] dWAT is organized in cone-like structures (often identified in deeper skin graft donor areas) and is particularly associated with scarring when exposed at that depth.[15,16]

Alterations of dWAT content have been identified in aged skin with chronic photo damage (particularly UVA) causing replacement of adipocytes by fibrotic structures, likely an adipocyte-myofibroblast transition.[16] These dermal adipocytes are extremely important due to their plasticity and their ability to change their phenotype in a very short time. In addition, they communicate with sWAT forming spatial 'fat bridges' between

FIGURE 2. Schematic and histological representation of dWAT demonstrating direct bridging between dWAT and sWAT compartments in cartoon form **(A)** and histological view **(B)**.



these two compartments, thus connecting regions which can be directly affected by UV radiation with much deeper layers of fat and paracrine signaling between these compartments has been demonstrated.[16] This provides a mechanism for direct communication to sWAT by topical application targeting dWAT directly or facilitating even more focused transmission by hair follicle mode of entry. Furthermore dWAT can modulate its structure and turnover of adipocytes at far higher rates than sWAT – this makes dWAT a first responder to UV radiation and topical applications.[16] To expand on this concept, it is now theorized that soluble factors (IL-6, IL-8, MCP-3 etc.) produced in the upper dermis during UV exposure diffuse into sWAT via dWAT and trigger modification of sWAT metabolism and differentiation.[16] This discovery is extremely important, not only in explaining the ability of topical preparations to affect sWAT but also in creating a new protection target for anti-aging indications.

Another interesting aspect of the dWAT compartment is its unique relationship with the hair follicle and the dynamic reciprocity that is enjoyed between these two anatomic regions through Wnt beta catenin signaling pathway.[17] Research of this signaling has revealed that dWAT is directly stimulated in unison with anagen and regresses through telogen.[17] This is a logical relationship when one considers the evolutionary function both regions serve in relation to thermoregulation. A practical corollary to this research is a note to be cautious when injecting substances such as deoxycholate for fat dissolution – if injected too superficially with direct effect on dWAT, telogen may be induced and alopecia may result, thus injecting in the correct region is paramount to avoid this possibility. It will be interesting to monitor the occurrence of alopecic episodes with this technology following definition of this novel anatomic region.

Adipose Tissue Signaling from the Top

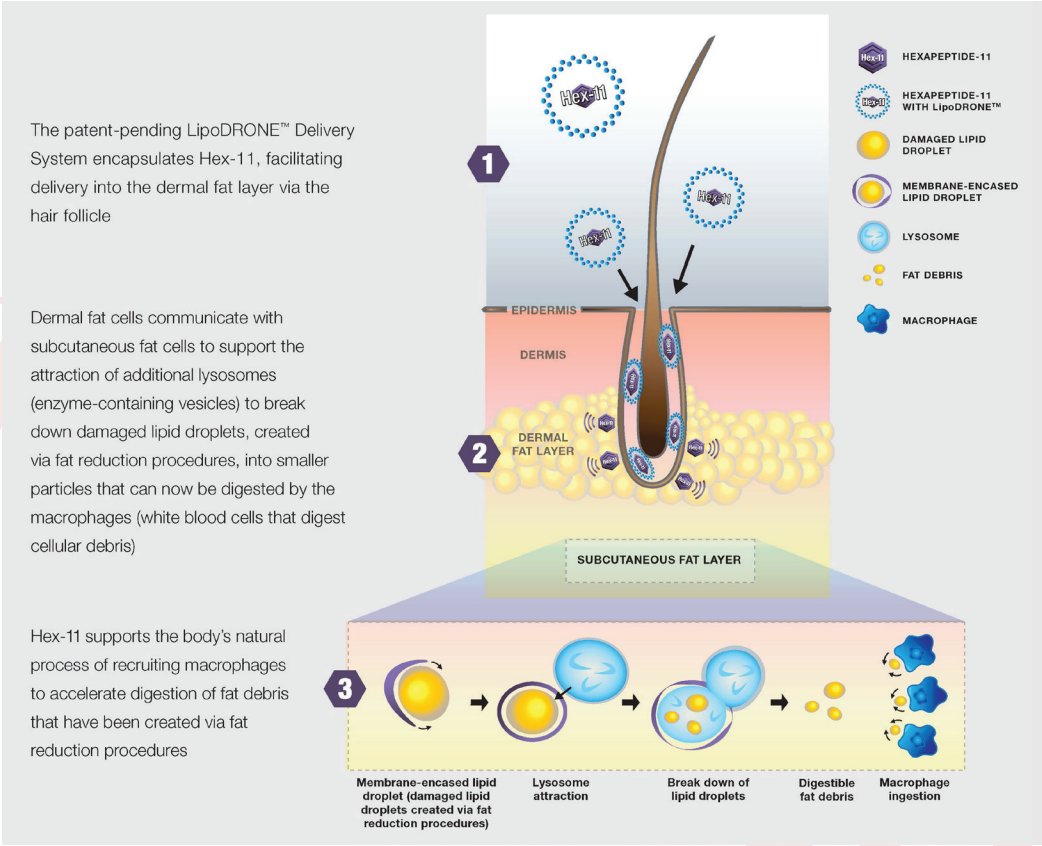
Having defined the anatomy, it is apparent that various mechanisms exist for topical preparations to communicate with subcutaneous fat. These may involve complex signaling mechanisms or direct communication in some cases. In effect, three possible channels exist for topical application to achieve subcutaneous signal transmission:

1. Keratinocyte signaling. This is the most indirect route which relies on complex signaling occurring from the skin surface to tissues below, as far down as sWAT compartments. This signaling mechanism was identified principally when investigating the hair follicle fat tissue relationship. It was found that the activation of epidermal Wnt/β-catenin signaling promoted adipocyte differentiation and hair growth. This signaling pathway is initiated by epidermal keratinocytes synchronizing patterns of hair follicle growth and expansion of the dermal adipocyte layer.[17] Recent studies have demonstrated that activation of epidermal Wnt/β-catenin mediates effects by secreted factors, including insulin-like growth factor 2 and bone morphogenetic proteins 2 and 6.[17,18] The relationship was discovered related to evolutionary thermoregulatory mechanisms where increased anagen hair follicle activation was accompanied by adipogenic differentiation and increased adipose tissue, that is hair growth and adipose tissue thickness worked together to achieve heat insulation. Given that the epidermis and dermis are separated by a basement membrane, this change in adipose tissue was demonstrated to occur by release of secreted factors noted above.[17]

2. Interfollicular absorption through intact stratum corneum. This is limited to molecules with molecular weights less than 1000D, preferably closer to 500D. Small peptide molecules (500-800D) are better suited to this mode of absorption. In addition, lipid moieties (palmitoyl, myristoyl) or liposomal carriers may be added to further enhance absorption of these small molecules. Fortunately, as demonstrated above, the absorption may not need to be down to subcutaneous depths but dWAT may facilitate communication with sWAT from higher levels within the dermis. Growth factors may vary from 15000D to 150000D in MW so almost no absorption is possible through this route.

3. Hair follicle dWAT transmission. As elucidated above, this fat compartment provides an exciting new area for exploration from anti-aging perspectives and from topical application routes providing the bridge between hair follicle surrounded dWAT to underlying sWAT. As opposed to rodents where a distinct panniculus carnosus layer separates dWAT from sWAT, in humans the two merge together providing direct access to the sWAT via the hair follicle channel.[14] Below the skin surface, the hair follicles provide

FIGURE 3. Full process of peptide activity starting with liposomal encapsulation, entry to base of hair follicle where it acts as a reservoir for delivery to dermal white adipose tissue (dWAT). Direct communication with subcutaneous white adipose tissue (sWAT) then allows the process of autophagic 'repackaging' of the lipid droplets to begin.



a large surface area for potential absorption of compounds, because of an enfolding of the epidermis extending deeply into the dermis.[19] Characteristics for entry into the hair follicle unit include low molecular weight, generally not more than 600 Da. In addition entry to sWAT via the hair follicle can be further facilitated by the use of specifically designed liposomes which provide an ideal carrier for the topical agent.[19] It has been suggested that the size of a particle determines the depth of follicular penetration.[20] Topically applied liposomal encircled peptides tend to diffuse and accumulate in the hair follicle, this deposit acting as a reservoir for ongoing functional activities. Particles of ~300 nm in size penetrate more deeply into the pilosebaceous unit than non-particle contents.[19] Additionally, particles less than 200nm have demonstrated superiority over non-particle formulations in terms of storage behavior.[21] Thus, a targeted approach of liposome (lipoDRONE™) surrounded Hex-11 has been designed to deliver quick and efficient loads of peptide to the base of the hair follicle where it provides an ongoing reservoir for delivery to surrounding dWAT which then directly communicates with sWAT producing the desired autophagic responses (Figure 3).

Unifying Hypothesis of Topical Modulation of Adipose Tissue

Non-surgical fat reduction and body sculpting are currently extremely popular procedures. The common end result of the technologies described above is slow dissolution of the products of adipose cell breakdown. Macrophage digestion and excretion of these end products is dependent on adequate break down of these droplets into sized packages suited to macrophage efficiency. As described above, an important process in achieving this end is autophagy (or lipophagy), which involves repackaging of the large droplets into manageable sizes. Although not commonly recognized, peptides exist with specific amino acid sequences that have a profoundly positive effect on the autophagic process.

One such peptide, a hexapeptide, was selected to investigate its potential in dealing with lipid droplet byproducts. The peptide was first subjected to gene expression tests (Predictive Bio, Carlsbad, CA) to assess responses appropriate to autophagic functions. Primary dermal fibroblasts were plated in cell culture media and allowed to grow to 90% confluency. Cells were then harvested and plated at 10,000 cells per cm^2 in 6-well dishes and cultured for 3 days. On day 3, the peptide was added to the

TABLE 1.

Genes Induced by Hexapeptide-11 by 2-fold or More		
Gene	**Fold Change**	**Function**
AMBRA1	3.59	The activating molecule in Beclin-1-regulated autophagy (Ambra1), also known as autophagy/Beclin-1 regulator 1, is part of the autophagy signaling network. Ambra1 has emerged as an important platform for autophagy and an early autophagy regulator[22]
ATG4A	3.29	This gene encodes a member of the autophagic protein family[23]
PSMB5	2.56	Proteasome subunit beta type-5 as known as 20S proteasome subunit beta-5 is a protein that in humans is encoded by the *PSMB5 gene*
CASP3	2.24	Caspase-3 is a predominant player in the execution of apoptotic cell death. However, recent studies indicated that capase-3 plays a role in autophagic processes mediating cross-talk between the autophagic and apoptotic programs[24]
ATG5	2.00	Atg5 is characterized as a protein specifically required for autophagy. In addition it has pro-apoptotic properties[25]

mix and treated and untreated cells were analyzed by real time PCR analysis at 72 hours.

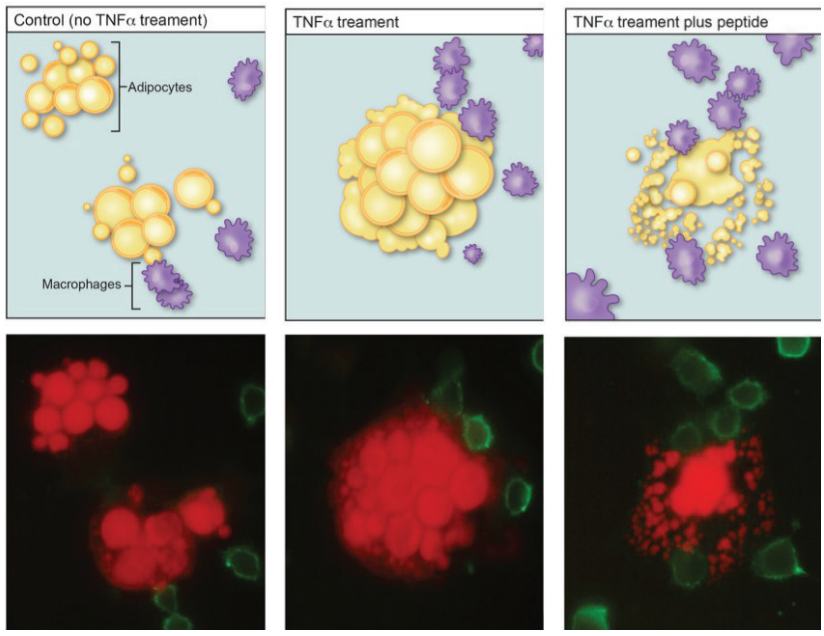
Results: 72-hour treatment with hexpeptide 11 increased expression of 5 major autophagic genes (AMBRA1, ATG4A, PSMB5, CASP3, and ATG5) by 2-fold or greater (Table 1).

Next, an in vitro model was designed to assess efficacy of the peptide with lipid droplet breakdown and macrophage clustering. 3T3-L1 adipocytes were cultured and developed; macrophages were then added to the adipocytes. They were then exposed to TNF-alpha (~25nM) for 24 hours, which caused apoptosis in approximately 70% of adipocyte cells. This provides a perfect model for adipose apoptosis as seen in non-surgical fat reduction. One set of macrophage adipocyte co-culture was also exposed to the hexapeptide and adipose droplet breakdown and macrophage clustering was observed in each group. Macrophages and adipocytes were stained to facilitate identification. The experiment was completed in triplicate.

Results (Figure 4): Co-culturing of macrophage and adipocytes revealed free floating cells with little macrophage clustering. The addition of TNF-α increased droplet breakdown and macrophage clustering. The most significant adipose breakdown and macrophage clustering was observed following the addition of the peptide.

The gene expression studies and co-culture model thus provide encouraging in vitro validation of peptide efficacy. Targeting of the hair follicle point of entry for the peptide is achieved by combining the peptide with a liposome designed specifically for follicular penetration (particle size of liposome vesicles ~185nm) as described above, ensuring entry to the dWAT compartment.

FIGURE 4. (Left) Free floating co-cultured adipocytes and macrophages; (Middle) Addition of TNF-α shows early adipocyte breakdown and macrophage clustering; (Right) Peptide addition causes further adipocyte droplet breakdown and significant macrophage clustering.



In summary, a final formulation has been designed including an active peptide targeting lipid droplet breakdown and macrophage clustering, encased in a liposome delivery system ensuring absorption through the hair follicle route, delivery to dWAT and ensuing signaling to sWAT compartments. Clinical tests comparing the formulation with a sham product following hot and cold non-invasive fat reduction procedures have been completed (in submission) and others are underway. Results thus far are extremely exciting and publications will follow.

Unresolved issues particularly with cryolipolysis, include 'Paradoxical adipose hyperplasia' where unexpected overgrowth of adipose tissue occurs in the area of treatment in a small number of patients.[26,27] Pathogenesis is unknown and may well be related to droplet persistence or exosome stimulation of adipogenesis in these patients although no direct evidence exists for this at this time.

CONCLUSION

Non-surgical fat reduction remains an extremely popular procedure with varied technologies being used for this indication. When utilizing a technology that accesses a non-invasive closed route to the target tissue, it presents a challenge for removal of waste products created by the process. This paper presents some novel approaches for entry to the target region via topical applications and for subsequent breakdown and repackaging of lipid droplets through the autophagic/lipophagic mechanism. Thus, by designing liposome encased peptides, these may be preferentially delivered via hair follicles to dWAT, a newly recognized fat compartment that communicates directly with sWAT in humans. The peptides, primed to stimulate autophagic processes, result in further lipid droplet breakdown and macrophage clustering. This combination shows promise for facilitating and hastening recovery following non-surgical fat reduction procedures.

DISCLOSURE

Alan D. Widgerow is Chief Medical Officer of Alastin Skincare Inc (Carlsbad CA). Products and funding for trials provided by company. Dr. Suzanne Kilmer is a consultant for Alastin Skincare, Inc. Dr. W. Grant Stevens is a consultant for Alastin Skincare, Inc.

REFERENCES

1. Garibyan L, Sipprell WH, 3rd, Jalian HR, Sakamoto FH, Avram M, Anderson RR. Three-dimensional volumetric quantification of fat loss following cryolipolysis. *Lasers Surg Med* 2014;46(2):75-80.
2. Duncan DI, Kim TH, Temaat R. A prospective study analyzing the application of radiofrequency energy and high-voltage, ultrashort pulse duration electrical fields on the quantitative reduction of adipose tissue. *J Cosmet Laser Ther* 2016;18(5):257-67.
3. Manstein D, Laubach H, Watanabe K, Farinelli W, Zurakowski D, Anderson RR. Selective cryolysis: A novel method of non-invasive fat removal. *Lasers Surg Med* 2008;40(9):595-604.
4. Pereira J, Cavalcante Y, Wanzeler R. The role of inflammation in adipocytolytic nonsurgical esthetic procedures for body contouring. *Clin Cosmet Investig Dermatol* 2017;10:57-66.
5. Kilmer SL, Burns AJ, Zelickson BD. Safety and efficacy of cryolipolysis for non-invasive reduction of submental fat. *Lasers Surg Med* 2016;48(1): 3-13.
6. Ingargiola MJ, Motakef S, Chung MT, Vasconez HC, Sasaki GH. Cryolipolysis for fat reduction and body contouring: safety and efficacy of current treatment paradigms. *Plast Reconstr Surg* 2015;135(6):1581-90.
7. Bani D, Quattrini Li A, Freschi G, Russo GL. Histological and ultrastructural effects of ultrasound-induced cavitation on human skin adipose tissue. *Plast Reconstr Surg Glob Open* 2013;1(6):e41.
8. Grant RW, Stephens JM. Fat in flames: influence of cytokines and pattern recognition receptors on adipocyte lipolysis. *Am J Physiol Endocrinol Metab* 2015;309(3):E205-13.
9. Gregory CD, Devitt A. The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? *Immunology* 2004;113(1):1-14.
10. Maixner N, Kovsan J, Harman-Boehm I, Bluher M, Bashan N, Rudich A. Autophagy in adipose tissue. *Obes Facts* 2012;5(5):710-21.
11. Wang CW. Lipid droplets, lipophagy, and beyond. *Biochim Biophys Acta* 2016;1861(8 Pt B):793-805.
12. Singh R, Cuervo AM. Lipophagy: connecting autophagy and lipid metabolism. *Int J Cell Biol* 2012;2012:282041.
13. Cappellano G, Ploner C. Dermal white adipose tissue renewal is regulated by the PDGFA/AKT axis. *Stem Cell Investig* 2017;4:23.
14. Driskell RR, Jahoda CA, Chuong CM, Watt FM, Horsley V. Defining dermal adipose tissue. *Exp Dermatol* 2014;23(9):629-31.
15. Chiang RS, Borovikova AA, King K, et al. Current concepts related to hypertrophic scarring in burn injuries. *Wound Repair Regen* 2016;24(3):466-77.
16. Kruglikov I, Scherer PE. Skin aging: are adipocytes the next target? *Aging* 2016; 8(7):1457-68.
17. Donati G, Proserpio V, Lichtenberger BM, et al. Epidermal Wnt/beta-catenin signaling regulates adipocyte differentiation via secretion of adipogenic factors. *Proc Natl Acad Sci USA* 2014;111(15):E1501-9.
18. Plikus MV, Guerrero-Juarez CF, Ito M, et al. Regeneration of fat cells from myofibroblasts during wound healing. *Science* 2017;355(6326):748-52.
19. Jain SK, Verma A, Jain A, Hurkat P. Transfollicular drug delivery: current perspectives. *Research and Reports in Transdermal Drug Delivery* 2016.
20. Verma D. Particle size of liposomes influences dermal delivery of substances into skin. *Int J Pharm* 2003;258(1-2):141-51.
21. Vogt A, Combadiere B, Hadam S, et al. 40 nm, but not 750 or 1,500 nm, nanoparticles enter epidermal CD1a+ cells after transcutaneous application on human skin. *J Invest Dermatol* 2006;126(6):1316-22.
22. Cianfanelli V, De Zio D, Di Bartolomeo S, Nazio F, Strappazzon F, Cecconi F. Ambra1 at a glance. *J Cell Sci* 2015;128(11):2003-8.
23. Marino G, Uria JA, Puente XS, Quesada V, Bordallo J, Lopez-Otin C. Human autophagins, a family of cysteine proteinases potentially implicated in cell degradation by autophagy. *J Biol Chem* 2003;278(6):3671-8.
24. Wu H, Che X, Zheng Q, et al. Caspases: a molecular switch node in the crosstalk between autophagy and apoptosis. *Int J Biol Sci* 2014;10(9):1072-83.
25. Codogno P, Meijer A. Atg5: more than an autophagy factor. *Nature Cell Biology* 2006;8(10):1045-8.
26. Jalian HR, Avram MM, Garibyan L, Mihm MC, Anderson RR. Paradoxical adipose hyperplasia after cryolipolysis. *JAMA Dermatol* 2014;150(3):317-9.
27. Seaman SA, Tannan SC, Cao Y, Peirce SM, Gampper TJ. Paradoxical adipose hyperplasia and cellular effects after cryolipolysis: a case report. *Aesthetic Surg J* 2016;36(1):NP6-13.

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