CryoLetters **34** (1), 100-105 (2013)
© CryoLetters, businessoffice@cryoletters.org

EVALUATION OF ADIPOCYTIC CHANGES AFTER A SIMIL-LIPOCRYOLYSIS STIMULUS

Hernán Pinto [1 *], Estefan Arredondo [2] and David Ricart-Jané [3]

[1]Instituto de Investigaciones para las Especialidades Estéticas y del Envejecimiento, Barcelona;
[2]Donation and Transplantation Insitute, Barcelona;
[3]Centre de Recerca del Metabolisme, University of Barcelona, Barcelona, Spain.
*Corresponding author email: portatil@ageback.es

Abstract

Lipocryolysis is considered as an effective, well-tolerated non-invasive procedure to reduce local adiposities. However there is little information about its mechanism of action by the procedure. It is proposed that lipid phase transition or crystallization may be an unleashed apoptotic stimulus. Yet, the post-lipocryolysis apoptosis is not easily confirmed, least of all is its correlation with crystallization. In this study adipocytes from rat fat tissue were exposed to a lipocryolysis-session-like stimulus. Lipid changes were observed in all test sample.

Keywords: Lipocryolysis; adipocytes; crystallization; fat tissue; temperature.

INTRODUCTION

Lipocryolysis is a treatment that is spreading quickly around the globe. Several studies have already shown its safety (3, 8) and efficacy (10, 11), making this non-invasive procedure appealing. The theoretic basis of this procedure was proposed a few years ago (9), which claimed that fat reduction could result from the local apoptotic adipocyte destruction as a consequence of a heat extraction triggering stimulus (1, 9). Since then, little evidence has been published with regard to any physiological changes that may lead to fat reduction. Important links of the mode of action are still missing. The correlation between lipocryolysis, lipid crystallization, apoptosis and inflammation remains to be established.

When a lipocryolysis procedure is started, the machines generate vacuum to position the adipose tissue inside the treatment unit and to reduce the local blood flow (9). The combination of vacuum with heat extraction (5) lowers the intra-adipocitary temperature to an extent where the physical thermal stimulus generates cellular changes that will accomplish the therapeutic results (16) without damaging any other structures (2). In general, these thermal changes were named “crystallization”, but the term and the concept are now under debate. An unleashed apoptotic stimulus as a consequence of such changes was believed to be an logical action mechanism. Still, we lack scientific evidence between the empirically-proven efficacy of this procedure and dipocitary necrosis. Oxidative stress (13, 14) and cold stress lipolysis

(15) are among some other theorized mechanisms of the lipocryolytic action. Up to date, only one publication offered some iconographic proof of crystallization after this therapy (9). In that study, the authors used heated pig lard obtained from previously frozen and heated tissue (9). They observed *needle like* crystals at room temperature (21.8ºC) after storage over night at room temperature and *cloudy crystallization* at 10.4ºC after a cooling rate of approximately 10.8ºC/min. But the temperatures-in-time stimuli to which adipocytes were exposed in their study did not resemble to the conditions for performing a lipocryolysis session (11). Other authors cooled human adipose down to 1ºC and never saw crystallization, only liquid-to-gel transition of a fraction of human fat. More evidence is needed to back up the lipocryolysis-induced apoptosis hypothesis. The aim of this study is to evaluate adipocytic changes under a lipocryolisis session-similar-stimulus. Crystallization was considered to be the necessary step for apoptotic stimulus unleashing.

MATERIALS AND METHODS

Samples of adipose tissue

Four male Wistar rats (Harlan Interfauna) were anesthetised with isoflurane, and 1g of retroperitoneal white adipose tissue was extracted from each animal. Sacrifice was performed with cervical dislocation technique in accordance with the National Policy and was approved by the Ethics Committee.

Isolation of fat cells

Petroperitoneal fat was chopped into small pieces and digested in 10 mL of Krebs Buffer Solution supplemented with: 0.6 mg DNAse (Sigma) and 10 mg Collagenase (Type 4, Worthington). Digestion was performed at 37ºC for 30 minutes (Figure 1). EDTA solution (0.1 M, 1 ml) was added to finish digestion process. Tissue remnant was separated from isolated adipocytes by filtration. After washing with Krebs buffer, cellular count was performed. Samples had cellular integrity between 91% and 94%.

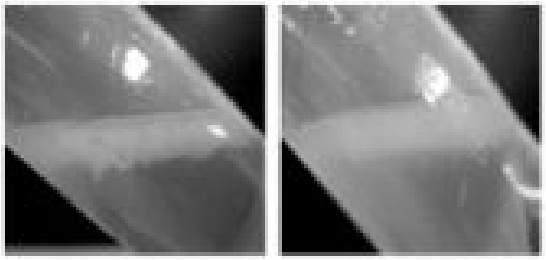

Figure 1. Before (left) and after (right) collagenase action. Small fragments of fat tissue are broken up after 30 minutes of enzymatic digestion.

Cold exposure

50 μL of the isolated adipocyte suspension was placed in slides and exposed to 8ºC for 0, 10 or 25 minutes. The combination of 8ºC and 25 min resembled to lipocryolysis actual session conditions. To prevent sample dry-up, slides were placed inside a small closed glass box. The slides and the glass box where previously cooled down to 8ºC to avoid an abrupt reheat at the moment of microscopy. After 25 min of cold exposure and photography, samples where left at room temperature for 2 h to check on crystal evolution.

Cellular changes

The presence of crystals was evaluated by bright field microscopy (Olympus CH-2) at 40X, 100X and 400X, with an adapted polarizer filter. Light intensity used was the maximum available: 10. To make sure that tissue samples will not been heated up above 10.38ºC, the only temperature at which post-lipocryolysis crystallization has been proved, the temperature

enhancement due to exposition to the microscope light was calculated. Temperature evolution of the samples when placed at the microscope platen centre at room temperature (23.8ºC) and at 12 cm from the light bulge (focus distance x40) was determined by Y= 0,104x + 21.4. This implied an increase in sample temperature of approximately 0.3ºC at the moment of being photographed.

RESULTS

Cellular integrity after collagenase digestion oscillated between 91% and 94%. Before exposure to 8ºC, damaged adipocytes were few and no crystals were seen (Figure 2A and B). Changes were clearly observed after 10 min exposure to 8ºC (Figure 2C and D). When the duration was increased, more crystallization was observed (Figure 2E and F). Changes inside unaltered-resembling-adipocytes were seen, as well as in diverse sized extra-cellular vacuoles formed by the fusion of destroyed adipocytes (Figure 2F). Lipid crystalls presented different levels of structural complexity, from the simple needle-like ones (Figure 3G) to the most intricate star-like ones ~50 μm in diameter (Figure 3H). Crystals did not disappear when the samples were warmed at room temperature (22ºC) for two hours (Figures 4K and 4L)

DISCUSSION

Necrosis occurs.

The apoptotic hypothesis (1, 9) is accepted in the lack of evidence, partly because of the observation of delayed fat reduction response even 60 days after procedure and no severe inflammatory response after treatment (2, 16). In this study, some immediate cellular damaging was seen in all samples after cold exposure. It is not clear whether the observed crystallization would result in later apoptosis or necrosis. New studies addressing the apoptosis process should be performed.

Fatty acid composition.

Not only saturated fatty acids inside adipocytes undergo physical changes when exposed to cold, but also mono and poly unsaturated fatty acids whose lipid-to-gel transition and crystallization temperatures are lower (4, 6). This points to the question whether crystallization induction temperature is different among species, and most important, if this temperature may vary among different subjects of the same species. Since there is a variation in unsaturated fatty acid composition and ratio between individuals (according to pathologies and mainly to nutritional habits), future studies should clarify whether these differences may alter therapeutic outcome.

Crystal analysis. It was proposed that thermal changes could be lipid crystallization or lipid-to-gel phase transition. Some evidence presented in this study may empower this idea, but others certainly not. Temperature affected crystal properties and these may affect therapeutic outcome. Crystal size difference was evidenced between samples exposed to 8ºC for 10 min and samples exposed to 8ºC for 25 min, in which they seemed larger. Figures 2C, 2D, 2E and 2F could be very consistent with a crystallization process, but also with the observation of a lipid to gel transition phenomenon. A different situation arose when a careful analysis of single crystals was performed and polymeric patterns were evidenced: first, adopting lineal conformations resembling needles that added to each other to form bi-dimensional V-like patterns; then, with several “V” like shapes that stoke to one another to form spherical star-like patterns. This is very consistent with a crystallization process. Still, further investigations should provide correlation a) between lipid-to-gel transition and

crystallization overlapping, b) with other possible crystal formation processes; and c) clarify their clinical implications so that the application protocol may be optimized. It is noted that after exposure to 8ºC for 25 min, samples could be left at 22ºC for up to 2h with crystals intact. This irreversibility is consistent with a crystallization process. Further investigations must provide the evidence to evaluate and perhaps regulate irreversible crystal formation process correlating it to: a) apoptosis vs. necrosis ratio, b) immediate vs. delayed clinical results and c) final therapeutic outcome.

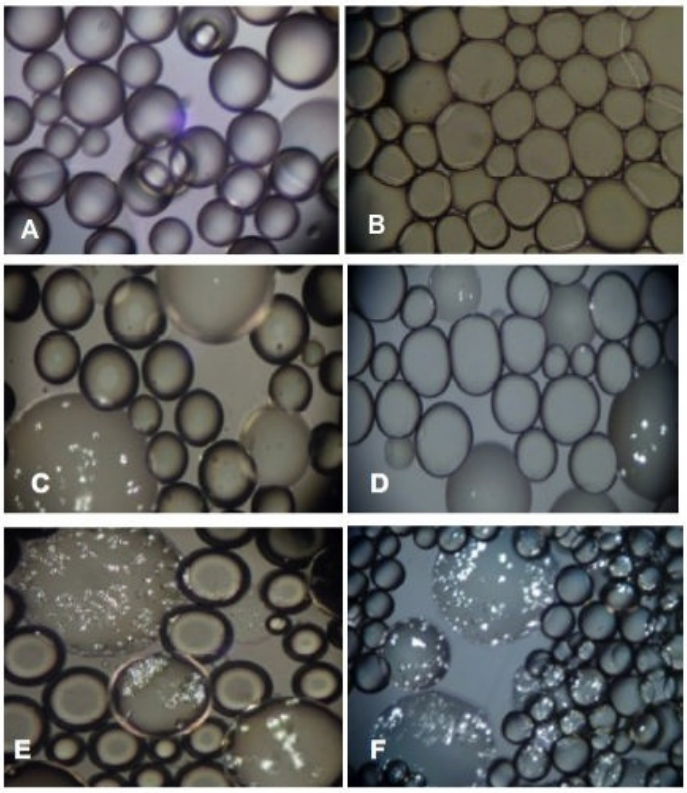

Figure 2. Impact on adipocytes.

A,**B**: control adipocytes: no cold exposure and no crystallization.

C,**D**: exposed to 8ºC for 10 min.

E,**F**: exposed to 8ºC for 25 min

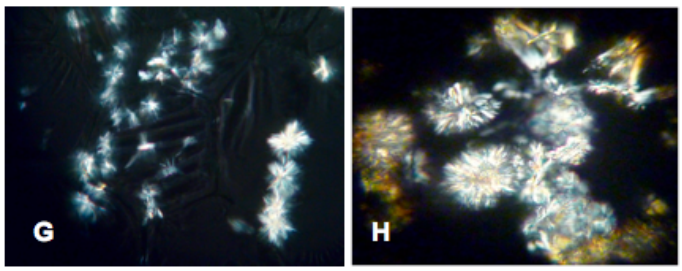

Figure 3. Lipid crystals.

G: exposed to 8ºC for 10 min.

H: exposed to 8ºC for 25 min.

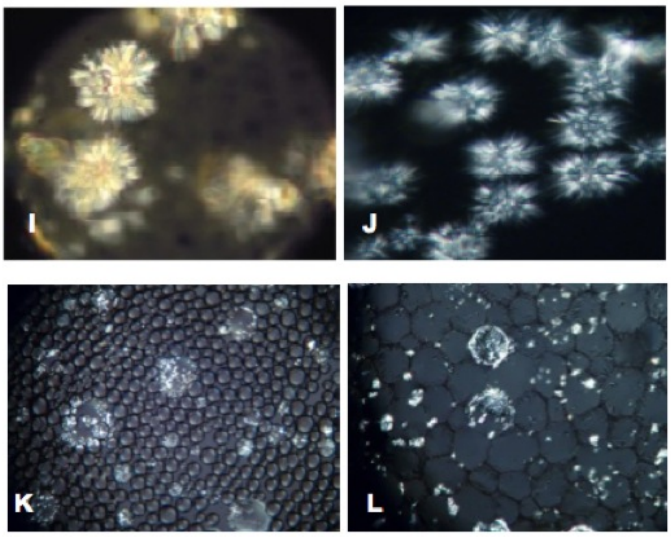

Figure 4. Irreversibility.

I,J: adipocytes reheated at room temperature (22ºC) for 2 h/

K,L: magnification 40X and 100X., reheated at room temperature (22ºC) for 45 min

Acknowledgements: Hernán Pinto is an external medical advisor to Clinipro, S.L., who partialy funded this study. The authors thank Miss Andrea Sallent Font, Dr. Eduardo García-Cruz, Dr. Graciela Melamed, Miss Eva Pardina and Dr. Mercè Durfort for their contribution.

REFERENCES

1. Avram MM, Harry RS (2009). Cryolipolysis™ for Subcutaneous Fat Layer Reduction. *Las Surg Med*. 41:703–8
2. Coleman S, Sachdeva K, Egbert B, Preciado JA, Allison J (2009). Clinical Efficacy of Non-Invasive Cryolipolysis™ and its Eeffects on Peripheral Nerves. *Aesth Plast Surg*. 33:482–8
3. Dover J, Burns J, Coleman S, Fitzpatrick R, Garden J, Goldberg D, Geronemus R, Kilmer S, Mayoral F, Tanzi E, Weiss R, Zelickson B (2009). A prospective clinical study of non-invasive cryolipolysis for subcutaneous fat layer reduction—Interim report of available subject data. *Lasers Surg Med* 41(S21):43.
4. Fay H, Meeker S, Cayer-Barrioz J, Mazuyer D, Ly I, Nallet F et al. (2012) Polymorphism of natural fatty acid liquid crystalline phases. Langmuir. 28(1):272-82.
5. Filardo Bassalo JM (2003). Crónica de los efectos físicos. *ContactoS.* 47:64-70
6. Guendouzi A, Mekelleche SM (2012). Prediction of the melting points of fatty acids from computed molecular descriptors: a quantitative structure-property relationship study. Chem Phys Lipids. 165(1):1-6
7. Hulbert AJ, Rana T, Couture P (2002). The acyl composition of mammalian phospholipids: an allometric analysis. Comp Biochem Physiol B Biochem Mol Biol. 132(3):545-27.
8. lein KB, Zelickson B, Riopelle JG, Okamoto E, Bachelor EP, Harry RS, et al. (2009) Non-Invasive Cryolipolysis™ for Subcutaneous Fat Reduction Does Not Affect Serum Lipid Levels or Liver Function Tests. *Las Surg Med*. 41:785–90
9. Manstein D, Laubach H, Watannabe K, Farinelli W, ZurakowskiD, Anderson RR (2008). Selective Cryolysis: A Novel Method of Non-Invasive Fat Reduction. *Las in Surg and Med*. 40:595-604
10. Nelson AA, Wasserman D, Avram MM (2009). Cryolipolysis™ for Reduction of Excess Adipose Tissue. *Semin Cutan Med Surg*. 28:244-9
11. Pinto H, García-Cruz E, Melamed G (2012). Study to Evaluate the Action of Lipocyolysis. *Cryoletters*. 33: IN PRESS

12. Preciado JA, Allison J (2008). The Effect of Cold Exposure on Adipocytes: Examining a Novel Method for the Noninvasive Removal of Fat. *Cryobiology*. 57:327
13. Rauen U, Polzar B, Stephan H, Mannherz HG, De Groot H (1999). Cold-induced apoptosis in cultured hepatocytes and liver endothelial cells: mediation by reactive oxygen Species. *The FASEB Journal.* (13):155-68
14. Rauen U, Petrat F, Li T, De Groot H (2000). Hypothermia injury/cold-induced apoptosis—evidence of an increase in chelatable iron causing oxidative injury in spite of low O2/H2O2 formation. *The FASEB Journal.* (14):1953-64
15. Velrand A (1999). Cold stress increases lipolysis in humans. *Aviat Space* Environ Med. 70:42-50
16. Zelickson B, Egbert B, Preciado JA, Allison J, Springer K, Manstein D (2009). Cryolipolysis™ for Noninvasive Fat Cell Destruction: Initial Results from a Pig Model. *Dermatol Surg*. 35(10):1462–70

Accepted for publication 10/12/12