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Lipocryolysis: Cooling speed affects adipocyte survival

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Abstract: New knowledge about crystallization has surfaced recently, since some of the last lipocryolysis publications have focused on how it works [4, 5]. However, although its action is better understood day after day, there is a sense of feeling that its clinical outcome could be improved. Kinetic particularities of crystal formation process allowed us to think that the cooling speed might be determinant in the results achieved in a lipocryolysis session. Adipocytes from six male rats where isolated and then exposed to 1ºC/min, 2ºC/min and 6ºC/min cooling speeds until they reached 8ºC and were kept at that temperature for 30 minutes. Cell damage was significantly higher in 1ºC/min cooling speed than in other cooling speeds.

Keywords: Lipocryolysis, Cooling Speed, Adipocyte

1. Introduction

New knowledge about crystallization has surfaced recently, since some of the last lipocryolysis publications have focused on how it works [1, 2]. However, although its action is better understood day after day, there is a sense of feeling that its clinical outcome could be improved. A number of alternative crystal structures are a characteristic property of all lipids [3]. This is due to the fact that there are different possibilities of packing the long hydrocarbon chain into a crystal lattice. This phenomenon is called polymorphism and each different crystal structure is called a polymorphic form of the lipid [4]. Crystallization is a reversible consequence of lipocryolysis. In previous studies, we presented evidence of various post-session intra-adipocitary changes [1, 2]: crystal size and patterns, crystal irreversibility, crystal formation thermodynamics and kinetics, and crystal diffraction properties. However, no study has been performed to date addressing the issue of cooling speed.

The physics underneath lipid crystal formation are complicated. Kinetic particularities of this process allowed us to think that the cooling speed might be determinant in the variety and amount of the crystals being formed after a lipocryolysis session.

The aim of this study has been to roughly assess the possibility of enhancing lipocryolysis results by subjecting rat adipocytes to a lipocryolysis-like stimulus with different cooling speeds: 1, 2 and 6ºC per minute.

2. Material and Methods

Six male Sprague Dawley rats, 60-day-old, weighing between 250 and 310g, were included in this study. Animals were housed in a controlled environment (lights on from 8:00 AM to 8:00 PM; temperature at 23+/- 2ºC and humidity 40-50%) and fed *ad libitum* with a standard chow diet (Harlan Interfauna Ibérica) and water. Experimental groups, summarized in Table 1, were exposed to different cooling speeds, and inducted for lipolysis or not. Samples were separated into six experimental groups. For each cell isolation, the isolated adipocytes were distributed in 16 vials: 8 vials for the adipocytes in the control group and 8 vials for the adipocytes in the treated group. In each group, 4 vials were used to stimulate lipolysis with isoproterenol (ISO+) and 4 vials were not stimulated with isoproterenol (ISO-). Each vial contained 150 µL of the suspension of adipocytes.

Retroperitoneal white adipose tissue (WAT) was obtained by laparotomy. 2g of WAT were digested with 20ml of Krebs Buffer (Hepes 1.25 mM, NaCl 12 mM, KCl 0.6 mM, MgSO 4 ·7H 2 O 0.12 mM, CaCl 2 0.1 mM, 2 g bovine albumin -fraction V-, Sigma and 0.045 g glucose) and 10 mg of collagenase Type 4, Worthington. Tubes were incubated at 37ºC in a bath with mild agitation for 40 minutes. No controlled atmosphere was used during the experiments, but Krebs buffer used for incubations and washes was gassed

previously half an hour with carbogen. To stop digestion, 2 mL of 1 mM EDTA were added and incubated for 5 additional minutes. Samples were then filtered with a piece of 100% nylon fabric to remove undigested tissue debris and the isolated adipocytes were collected in a syringe (without piston) connected to a stopcock. The syringe was kept in vertical position for 5 more minutes to enhance the flotation of the adipocytes in the buffer. The infranatant buffer was discarded and the isolated adipocytes were washed twice with 10 mL of Krebs buffer. Lastly, adipocytes were re-suspended in 2.5 mL of Krebs buffer.

Before starting the assay, 25 µL from each vial were diluted 1/60 in Krebs buffer to perform the cell counts. At the same time, the number of cells with crystals (figure 1) was observed and counted by bright field microscopy (Olympus CH-2) at 40X and 100X, with a polarizer filter. After removing the volume to perform the cell counts, 100 µL of every vial were diluted 1/4 with Krebs buffer. Subsequently, 600 µL of Krebs ADA buffer (Krebs buffer supplemented with 0.2 mg/mL of adenosine deaminase, Roche) were added to the ISO- samples, and 590 µL of Krebs ADA buffer plus 10 µL of isoproterenol (Sigma) 1 mM were added to ISO+ samples, in order to activate lipolysis. All samples were incubated for 30 minutes in a thermostatic bath at 37° C, with gentle agitation. After that, samples were immediately placed on ice to stop the reaction. Adipocytes were discarded by flotation and the infranatant was frozen at -20°C forl further analysis.

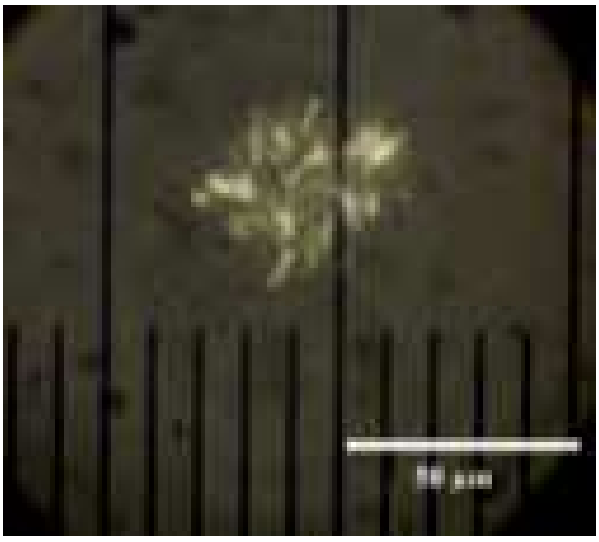

Figure 1. *Intra-adipocyte crystal observed at cooling speeds of 1ºC/min. (Olympus CH-2, 100X, polarizer filter).*

Adipocytes were then exposed to 1ºC/min, 2ºC/min and 6ºC/min cooling speeds according to their experimental group until they reached 8ºC and were kept at that temperature for 30 minutes (figure 2). Because cooling speed affected the total cooling time, samples were subjected to different total cooling times: 35 minutes for cooling rate 6ºC/min, 45 minutes for cooling rate 2ºC/min and 60 minutes for cooling rate 1ºC/min. 6ºC/min cooling rate resembled most actual lipocyrolisis protocols.

Glycerol was measured in the infranatants obtained. The "Free Glycerol Reagent" (Sigma) enzyme kit was used. It is based on the transformation of the glycerol present in samples to glycerol-1-phosphate by the glycerol kinase. Glycerol-1-phosphate generates hydrogen peroxide by the action of glycerol phosphate oxidase, which, in turn, oxidizes a chromogen that produces a colored compound with absorbance at 540 nm.

In all samples, cells and crystals were observed and counted by bright field microscopy (Olympus CH-2) at 40X and 100X, with a polarizer filter. Adipocytes that looked intact where considered and counted as "cells" (5ul, 1/40 dilution). Adipocytes that presented any particularity (membrane disruption with content extrusion or crystal inclusion) were considered and counted as "damaged cells".

Statistical analysis was performed with SPSS version 17 for Windows (IBM Corp., Armonk, NY, USA). Normal distribution assumption was assessed with a Shapiro-Wilk test and homoscedasticity assumption was assessed with a Levene test. According to their results, groups´ differences were assessed with ANOVA and Bonferroni test or with Kruskal-Wallis and Mann-Whitney tests.

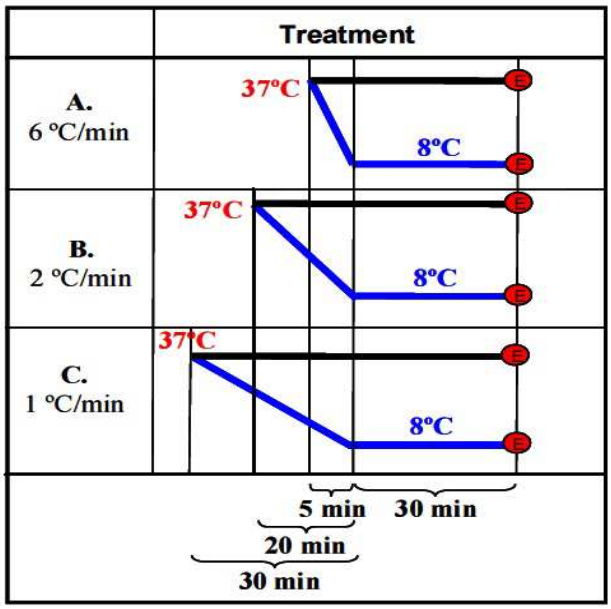

Figure 2. *Three tempering conditions: from 37ºC to 8ºC, at cooling speeds of 6ºC/min, 2ºC/min and 1ºC/min, and 30 min at 8ºC (blue line). Control groups for each condition: 37ºC (black line).*

3. Results

Cell count. The percentage of cells that resulted damaged due to the elapsed time throughout the assays (basal damage) was determined from cell counts performed before and after the treatment in control samples (kept at 37°C throughout assays). The average basal damage observed in the control samples was 1.2% ± 2.1, and it did not change regardless of the control group. Basal damage was subtracted from the one obtained in the samples subjected to the different cooling rates to establish the net damage.

a) Before cold exposure. Normal distribution and

homoscedasticity assumptions were accepted (Shapiro-Wilk and Levene tests respectively). Groups were founded to be comparable.

b) After cold exposure. Normal distribution and homoscedasticity assumptions were accepted (Shapiro-Wilk and Levene tests respectively).

A one-way-ANOVA (figure 3) test detected statistically significant differences between experimental groups exposed to different cooling rates (p=0.0168). Bonferroni test (alpha risk =0.017 for each comparison) located these differences between the 1ºC/min cooling speed and the other two cooling speeds, but not between the 2ºC/min and the 6ºC/min cooling speeds. Cell damage was significantly higher in 1ºC/min cooling speed than in other cooling speeds.

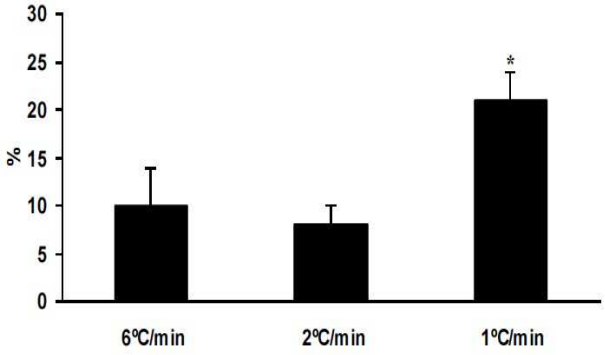

Figure 3. *Percentage of damaged cells after exposure to different cooling speeds. In samples subjected to a cooling rate of 6°C/min there was a 10±4% of cell damage. When the cooling rate was 2°C/min the cell damage was similar, 8±2% and when a cooling rate of 1ºC/min wa s used, the percentage of cell damage raised to 21±3%. ANOVA p=0.0168**

4. Discussion

We have been using the "cell count" variable in our last studies as a direct and simple way of evaluating cell damage (since flow cytometry has limitations when it comes to handle adipocytes). As expected, the Bonferroni test showed significant differences between the group exposed to 8ºC and those left at room temperature (25ºC). This is not a new concept: now-a-days we have plenty of evidence of lipocryolysis adipocyte destruction [5, 6, 7]. But in this study we saw that slower cooling rates (1 ºC/minute) produced higher "immediate" cellular damage than faster cooling rates (2 and 6 ºC/minute). 1ºC/ minute rate seemed to be the most effective treatment to reduce adipose depots. Further researches should confirm this finds and should also evaluate its applicability, since the fact of lowering the cooling speed necessarily implies an increment in the duration of the session.

We have also observed that cold exposure might have stimulated lipolysis in some samples. When 6ºC/min and 2ºC/min cooling rates were applied, cell crystallization and lipolysis impairment were correlated. When 1ºC/min cooling rate was applied, they were not. Higher cell destruction and crystal formation are not coherent with reduced lipolytic rates. This lowered net lipolytic response could have been due to an increased basal lipolytic rate instead of an impaired response to isoproterenol. This find could be very interesting, because if the remaining live cells may have an enhanced lipolytic rate, and therefore increasing their lipid consumption, this would account for a sensitizing effect of lipocryolysis. Again, further researches should confirm this claim, since it is the first time this effect is being reported.

Another interesting find was related to crystal formation. No crystals were detected in the control samples, though different patterns of crystallization were detected in the samples exposed to cold. When a cooling rate of 6°C/min was applied, crystallization was achieved in 15±1% of the cells, and a matrix crystallization pattern that filled the cytoplasm of the adipocytes was observed. When a cooling rate of 2°C/min was applied, crystallization was observed in 19±1% of cells. A Student´s T test showed significant differences with the mean crystallization observed at 6ºC/min: p=0.02. The crystallization pattern was mostly a matrix inside the cell but also a 2%±0.3 of cells presented the needle-like form of crystals. Further crystal morphological analysis should reveal useful information.

Finally, when driving conclusions, it has to be taken into account that there is a certain degree of variability in the response to cooling treatments (cell mortality and crystal formation) since humans have with different WAT composition than rats.

References

[1] Pinto H, Arredondo E, Ricart-Jané D. Study for the Evaluation of Adipocytic Changes after a Simil-Lipocryolysis Stimulus. *Cryoletters* 34(1) (2013)100-5

[2] Pinto H, Pardina E, Ricart-Jané D. X-ray diffraction analysis confirms intra-adipocitary lipid crystallization after a lipocryolysis-like stimulus. *Cryoletters* 34(6) (2013) 619-23

[3] Pinto H, Pardina E, Ricart-Jané D. X-ray diffraction analysis confirms intra-adipocitary lipid crystallization after a lipocryolysis-like stimulus. *Cryoletters* 34(6) (2013) 619-23

[4] Coleman S, Sachdeva K, Egbert B, Preciado JA, Allison J. Clinical efficacy of non-invasive Cryolipolysis™ and its effects on peripheral nerves *AesthPlast Surg* 33(2009) 482–8

[5] Pinto H, García-Cruz E, Melamed G. Study to Evaluate the Action of Lipocryolysis. *Cryoletters* 33(3) (2013):176-80

[6] Manstein D, Laubach H, Watannabe K, Farinelli W, ZurakowskiD, Anderson RR. Selective cryolysis: a novel method of non-invasive fat reduction. *Las in Surg and Med* 40(2008)595-604

[7] Zelickson B, Egbert B, Preciado JA, Allison J, Springer K, Manstein D. Cryolipolysis™ for noninvasive fat cell destruction: initial results from a pig model. *Dermatol Surg* 35(10) (2009) 1462–70