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PRE AND POST LIPOCRYOLYSIS THERMIC CONDITIONING ENHANCES RAT ADIPOCYTE DESTRUCTION.

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Abstract

New knowledge about crystallization vs. lipid-to-gel transition has surfaced recently, since some of the latest publications on lipocryolysis have focused on its action mechanism. As a result, new opportunities for technical improvements and clinical outcome optimization have opened up. The food industry has been working with lipid crystal polymorphisms for decades, and tempering seems to be the easiest method of external conditioning, in addition to being harmless. In this study, several temperature treatment patterns (TTP) were applied to isolated rat adipocytes. The survival of the adipocytes exposed to the different TTPs and the formation of crystals in the surviving adipocytes, were assessed and analyzed. Pre and post lipocryolysis thermic conditioning changed lipocryolyisis crystallization process and showed an enhancement in adipocyte destruction that could represent an important step in improving clinical results.

Key words: Lipocryolysis; temperature treatment patterns; preconditioning; post-conditioning.

INTRODUCTION

Crystalization is the chosen term to express the post-lipocryolysis intra-adipocyte changes, for the existence of a number of alternative crystal structures is a characteristic property of all lipids [1]. This is due to the fact that there are a number of 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 or modification of the lipid [2]. New knowledge about crystallization vs. lipid-to-gel transition has surfaced recently [3], since some of the latest publications on lipocryolysis have focused on its action mechanism [4]. Apoptotic adipocytolysis as a consequence of intracellular changes was the first and most logical action mechanism proposed for lipocryolysis [5]. It was assumed that adipocytolysis was a biological consequence of intracellular lipid crystallization [6], although this concept has been thoroughly debated lately. Though direct and immediate necrotic cell death -which has never before been proposed as part of the lipocryolysis action mechanism- apparently

has no correlation to apoptosis -which was the original action mechanism proposed for lipocryolysis [5]- , adipocyte necrosis possibly plays a minor role in lipocryolysis. It is still considered to be a secondary process, but its impact on cellular destruction can be seen, has been confirmed [4], and definitively should not be overlooked.

In previous studies, we presented evidence of several post-lipocryolysis intra-adipocitary changes [3, 4]: crystal sizes and patterns, crystal irreversibility, crystal formation, and crystal diffraction properties. X-ray diffraction (XRD) crystallography established that post-lipocryolysis crystal structures corresponded mainly to the lipid ß polymorphism, though α and α´ polymorphisms could also be seen, but in a very small proportion [3]. Different crystal patterns were reported after lipocryolysis sessions: needle-like, v-like, star-like and sphere-like [3, 7]. Under certain cooling rates and conditions, the needle-like pattern [8, 9] and a spherulitic growth [7] are very well known for triglyceride β crystallization polymorphism. All these studies contributed to a better understanding of the action mechanism of lipocryolysis. As a result, new opportunities for technical improvements and clinical outcome optimization have opened up.

The kinetics and thermodynamics that drive the formation, growth, stabilization, melting and destruction of lipid crystals are extremely complicated [10]. Inter-conversion between these three polymorphisms is an extremely appealing process: the possibility of leaning crystal formation towards the most effective polymorphism for adipocyte destruction could be very important for clinical outcome enhancement.

Food industry has been working with lipid crystal polymorphisms for decades [11, 12], and tempering seemed to be the easiest method of external conditioning, in addition to being harmless. Tempering is a very well known way of altering lipid structure [13], but it has never been done *in vivo* as part of a medical treatment, where physiological temperatures and pain are natural limits. In this study, several temperature treatment patterns (TTP) were applied to isolated rat adipocytes. The aims of this work have been to a) compare the number of surviving adipocytes exposed to the different TTPs and b) observe the formation of crystals in these adipocytes.

MATERIAL AND METHODS

Seven male Sprague-Dawley rats (Harlan Interfauna Ibérica, Barcelona; Spain), 56-day-old, weighing between 250 and 300g, were included in this study. Tissues were obtained taking advantage of animals used in another experiments which procedures were approved by the Committee on Animal Bioethics and Care of the University of Barcelona and the Generalitat of Catalonia (Autonomous Regional Government); Spain. 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. Animals were anesthetized with isoflurane and killed by cervical dislocation. A laparotomy was performed to obtain retroperitoneal WAT.

Approximately 2g of WAT were digested with 10 mg of collagenase Type 4, Worthington, in 20mL of Krebs buffer (Hepes 1.25mM, NaCl 12mM, KCl 0.6mM, MgSO4·7H2O 0.12mM, CaCl2 0.1mM, bovine albumin –fraction V- Sigma 2g and glucose 0.045g). The tissue was 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 the digestion, 2mL of 1mM EDTA were added and incubated for 5 additional minutes. Sample was 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 an additional 5 minutes to enhance the flotation of the adipocytes in the buffer. The infranatant buffer was discarded and the isolated adipocytes were washed twice with 10mL of Krebs buffer. Finally, adipocytes were re-suspended in 2.5mL of Krebs buffer.

Each tempering condition was analyzed as a different treatment pattern (TTP). Every TTP had its isolated adipocytes distributed into 12 vials: 6 vials for the adipocytes included in the control group and the other 6 for the adipocytes included in the treated group (simple randomization). Each vial contained 0.15mL of the adipocytes' suspension. Before starting the assay, 0.025mL from each vial were diluted 1/40 and 1/60 in Krebs buffer to perform the cell counts. 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.

The different temperature treatment patterns (TTPs) analyzed, are summarized in figure 1. Some adipocytes were cooled down until they reached 8°C and were kept at this temperature for 30 minutes. This was called the Modified Basal Treatment (MBT) pattern. Other adipocytes were submitted to the MBT pattern, but immediately afterwards suffered a post-lipocryolisys conditioning by re-heating them until they reached 38°C or 40°C where they were kept for 10 more minutes. These two TTPs were called the POST38 pattern and the POST40 pattern respectively. There was also pre-lipocryolysis conditioning by heating the adyipocytes until they reached a temperature of 40°C at which they were kept for 5 minutes and after which they were submitted to the MBT pattern. This was called the PRE 0 pattern. Finally, there were combined pre and post-lipocryolysis conditioned adypocytes. They were heated until they reached a temperature of 40°C, at which they were kept for 5 minutes, and then they were submitted to the POST 38 pattern or the POST 40 pattern. These two last TTPs were called the PRE38 pattern and the PRE40 pattern respectively. Control samples were kept at 37ºC throughout the whole procedure and they were called, in general, the C pattern.

Statistical analysis was performed with SPSS version 17 for Windows (IBM Corporation, Armonk, NY, USA).

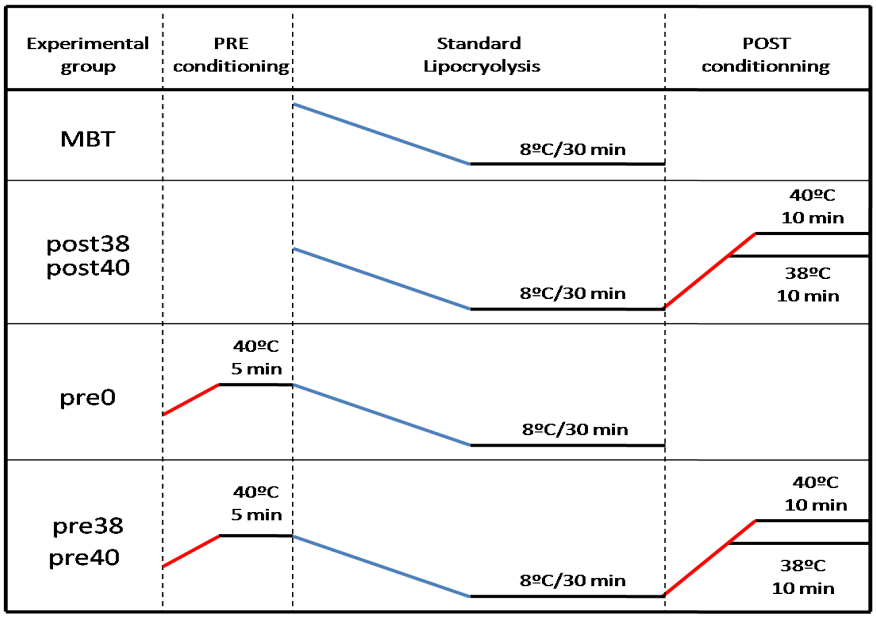

Fig 1. Temperature treatment patterns (TTPs). Heating (red line), cooling (blue line) and stabilized temperature (black line). 40ºC during pre-conditioning, 8ºC during standard lipocryolysis, 38ºC or 40ºC during post-conditioning. Control groups (constant room temperature) are not shown. MBT: modified basal treatment. Min: minutes.

RESULTS

Each temperature treatment pattern had its own control group which remained exposed to 37ºC for the same period of time and was not submitted to any tempering. For the number of cells present in these control samples, assumptions of normal distribution and homogeneity of variances were assessed and accepted with Shapiro-Wilk and Levene (F_0=1.701; p=0.150) tests, respectively. A oneway ANOVA was performed and showed that there were no statistically significant differences in the final cell number of the control groups (p=0.86). No cell on any control group showed any crystallization.

As regards the number of cells in the groups exposed to the different TTPs, the normal distribution assumption was assessed and accepted with a Shapiro-Wilk test, but the Levene test showed significant differences in the homogeneity of variances (F_0=2.853; p=0.023), so a Kruskal-Wallis test was performed which showed that there were significant differences between experimental groups (p=0.001) (figure 2). Since this test cannot locate the differences between two specific TTPs, a Student T was performed. MBT pattern was chosen because is the TTP that represents most accurately the actual lipocryolysis efficacy, and it was compared to the PRE 38 pattern due to the sample distribution observed when plotted (figure 2). Student T test showed that the

mean cell difference between MBT and PRE38 samples was 515.67 (IC 95% 247.66 to 783.67) cells, which was statistically significant (p=0.002).

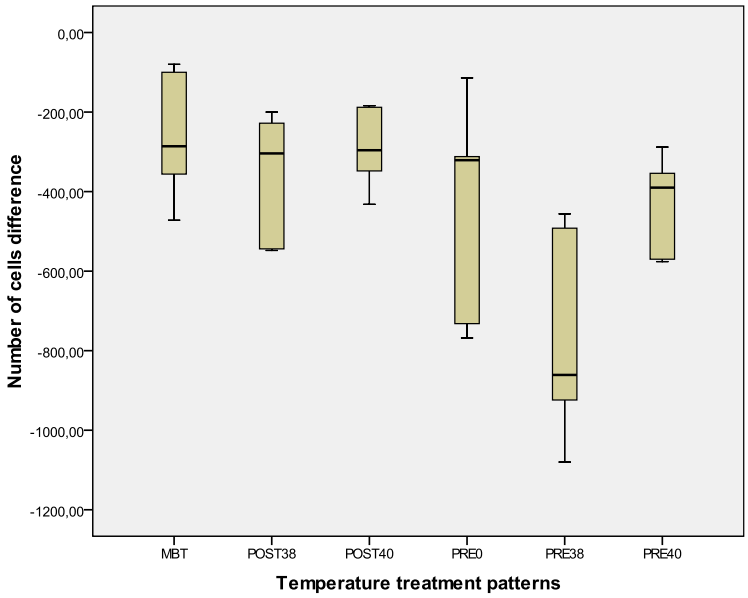

Fig 2. Cell difference. This variable is the difference between the number of cells that remained alive after treatment and the initial number of cells. It stands for immediate cell death.PRE 38 shows the lowest mean, with a significant differences to that of MBT (p=0.002), representing the TTP with the lowest number of cells alive after cold exposure, and thus accounting for the most effective TTP for adipocyte destruction.

The normal distribution assumption for the number of cells with crystals was assessed and accepted with a Shapiro-Wilk test, but again, a Levene test showed significant differences in the homogeneity of variances (F_0=3.739; p=0.006). A Kruskal-Wallis test was then performed and showed that there were significant differences between experimental groups (p<0.001) (figure 3). Like before, the mean number of cells with crystals of the MBT and PRE38 patterns was compared using a Student T test that showed that the there was a difference of 153.887 (IC 95% 83.23 to 224.54) cells, which was statistically significant (p=0.001).

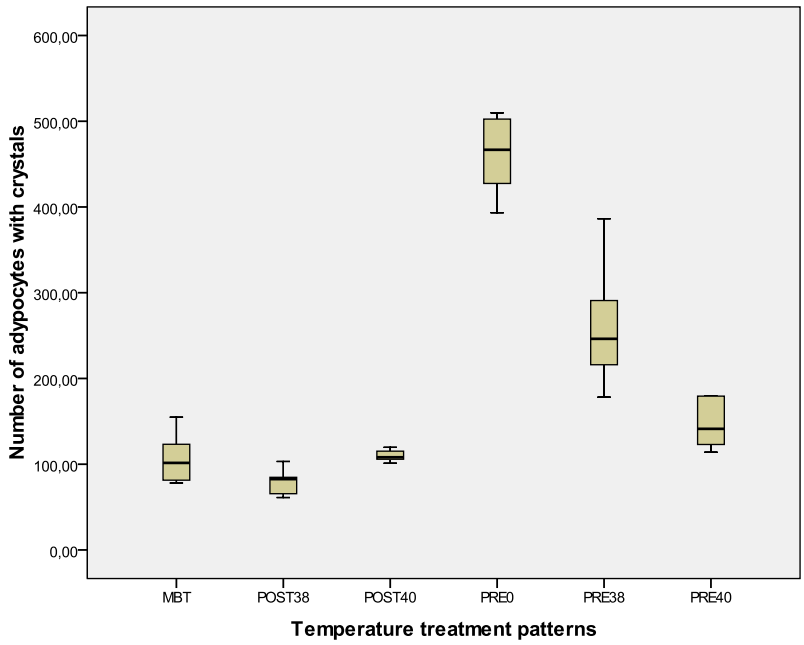

Figure 3. Number of cells with crystals. It is the number of cells that seem intact under the microscope after treatment, and that have crystals inside. It stands for the number of cells that may suffer apoptosis. PRE 0 and PRE 38 patterns show significant differences with MBT pattern (p=0.001).

DISCUSSION

From the very beginning, it has been claimed that lipocryolysis achieved its results by triggering apoptosis. The empiric results associated with the absence of inflammation provided a "keyhole" where the only key that fit in was apoptosis. Still, in every study we performed, we were able to identify non-apoptotic cell death up to some extent, normally in a very small proportion. These findings opened our eyes to the fact that there were at least two processes coexisting beneath the action of lipocryolysis which could be used to optimize its results.

Food industry has been tempering lipids for decades and for different reasons [14], although the scenario was obviously and absolutely different. Lipid mixtures with two or three compounds –phases- cannot be compared to multiphase *in vivo* systems where the lipid variety is almost infinite and where biological limitations when heating or cooling living tissues play a major role. Of course some of these limitations do not exist when working with *in vitro* systems, yet triglyceride polymerization, crystal kinetics and thermodynamic principles remain the same.

Lipid crystallization is a very complicated process that, among others, will be based on: lipid molecular composition, structure, applied temperature, tempering protocol, nucleation process, crystal growth rate, and even crystal impurities. As shown

by other authors [8, 9, 15] a change as slight as 1ºC in the re-heating temperature (or in any of these variables) may result in a crystallization process with other characteristics that may represent a different outcome.

As regards the cell death, there were important differences between some TTPs. In this case, the usefulness of the Kruskal-Wallis test was limited to a confirmatory role. When looking at Figure 2, it was easily seen that PRE38 pattern had the lowest mean, which was much lower than the MBT pattern mean. Since MBT pattern scrupulously resembled a lipocryolysis treatment, even having in mind that the sample was small and that the dispersion was wide, we decided to compared the means of these two TTPs and found that the differences between them were significant. Immediate cell destruction was considered to be a minor or secondary process of the action of lipocryolysis but since cell death of PRE38 pattern tripled that of MBT pattern, this consideration should be reviewed. As mentioned in the introduction, lipocryolysis impact on immediate cellular destruction should never be overlooked again.

Differences were also seen in the microscope analysis when counting the number of cells that looked intact and that had crystals inside, once the tempering stimulus was completed. Crystals are inherent to lipocryolysis, but the exact correlation between them, their properties, and the clinical outcome are yet to be established. However, intuition claims that some characteristics of the crystals could be desirable. Although it might not actually be the case, it seems logical to assume that a higher number of crystals and a bigger crystal size will mean bigger complications for the cell normal functioning, and probably will induce apoptosis in more adipocytes. Figure 3 allows us to see the differences in the number of cells with crystals for every TTP tried out. The Kruskal-Wallis test confirmed it but could not locate between which specific TTP took place. The number of adipocytes with crystals for PRE38 pattern was roughly 2.5 times that of MBT pattern. A Student T test showed that this difference was significant.

PRE 0 pattern showed encouraging data as well, but it was discarded because the cell death was not significantly different from that of standard lipocryolysis or MBT pattern. In the future, when new researches provide a better correlation between crystal formation and apoptosis for lipocryolysis, this TTP may gain and require further attention.

We conclude that the PRE38 pattern substantially improves a) immediate cell destruction and b) crystal formation in the remaining adipocytes.

When all these findings are analyzed together, not only cold can be considered once again as a useful adipocyte killer agent, but also some other facts are slowly being confirmed: a) necrotic cell destruction, previously thought irrelevant, is proving to be important and promising for results enhancement, and in the future, it may no longer be considered a minor process secondary to apoptosis, and b) it seems that Lipocryolysis does not cause any damage on the adipocytes, which look undamaged under the microscope. Further research is needed to confirm this statement for physicians to be able to a) extend the lipocryolysis cold exposure time for their patients and b) investigate the possibility of altering crystal polymorphisms formation with other

tempering patterns in order to achieve higher adipocyte necrosis and apoptosis rates that should provide further improvements in clinical results.

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