

# Isolated Rat Adipocytes are Still Capable of Inducing Lipolysis after a Lipocryolysis-Like Thermic Stimulus

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## Abstract

Lipocryolysis has already been established as a safe and effective technology. Though there is broad consensus that lipocryolysis is not a harmful technology, very little has been said about the functionality of those adipocytes that remain in the body after the procedure. The aim of this study has been to roughly assess the lipolytic activity of the adipocytes that suffer lipocryolysis. Rat white adipocytes were isolated, tempered and exposed to isoproterenol and cell count and glycerol concentration were measured. We found that the adipocyte count was significantly higher in the samples not exposed to cold and that adipocytes were still capable of lipolysis after the treatment. These findings suggested that cold can damage adipocytes and that it may not impair lipolysis in the adipocytes that remain in the body after the treatment.

**Keywords:** Lipocryolysis; Glycerol; Adipocyte

## Introduction

Lipocryolysis has already been established as a safe [1,2] and effective technology [3]. New knowledge about crystallization vs. lipid-to-gel transition 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, side effects have not been fully studied yet. Though there is broad consensus in that lipocryolysis is not a harmful technology, as well as evidence of results being safely achieved without collateral tissue damage [6], very little has been said regarding the functionality of the adipocytes that remain in the body after the procedure. Crystallization 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 [7]. 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 [8]. In previous studies, we presented evidence of various post-session intra-adipocytary changes [4,5]: 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 adipocyte metabolic function restoration after a lipocryolysis session. The aim of this study has been to roughly assess the lipolytic activity of the adipocytes that suffer lipocryolysis.

## Materials and Methods

Three male Wistar rats, 8-week-old, weighing between 250 and 300g, were included in this study. All procedures involving animals were approved by the Committee on Animal Bioethics and Care at the University of Barcelona and the Government of Catalonia, Spain. Animals were housed in a controlled environment (lights on from 8:00 AM to 8:00 PM; temperature at 23 ± 2°C and humidity 40-50%) and fed *ad libitum* with a standard chow diet (Harlan InterfaunaIbérica) and water. Experimental groups, summarized in Table 1, were exposed to room or cold temperatures, and inducted for lipolysis or not. Lipolysis was inducted with isoproterenol that yields glycerol and fatty acids from triglyceride rupture due to the action of lipases.

Samples were separated into four experimental groups. Each group was n=12. Lipolysis was inducted with isoproterenol only in two specific groups.

Retroperitoneal White Adipose Tissue (WAT) was obtained by laparotomy. 2 g of WAT were digested with 20 ml of Krebs Buffer (Hepes 1.25 mM, NaCl 12 mM, KCl 0.6 mM, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.12 mM, CaCl 20.1 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 37°C 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. 0.3 mL of this solution was separated into two 0.15 mL vials for each group. Adipocytes were exposed to 8°C ± 1°C during 25 minutes [9]. Before tempering, 0.025 mL was extracted from every tube and diluted 1/40 in Krebs buffer to perform cell count. After tempering, a second cell and crystal count was performed. The remaining 0.1 mL in each vial was diluted to 1/4 with Krebs buffer. Subsequently, 0.6 mL of Krebs ADA buffer (Krebs

Group	Temperature (25 minutes)	Lipolysis
CB	Room Temperature	No
CL	Room Temperature	Isoproterenol
FB	8°C	No
FL	8°C	Isoproterenol

**Table 1:** Experimental groups CB: control group; CL: lipolysis control group; FB: cold group; FL: cold and lipolysis group.

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buffer supplemented with 0.2 mg/mL of adenosine deaminase, Roche) was added to the CB and FB group samples, and 0.59 mL of Krebs ADA buffer plus 0.01 mL of isoproterenol (Sigma) 1 mM was added to the CL and FL group samples, in order to enable lipolysis. All samples were incubated for 30 minutes in a thermostatic bath at 37°C, with gentle agitation. Samples were then immediately placed on ice to stop the reaction. Adipocytes were discarded by flotation and the infranatant was frozen at -20°C until further analysis.

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. Thereafter, the 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” (5 ul, 1/40 dilution). Adipocytes that presented any particularity (membrane disruption with content extrusion or crystal inclusion) were considered and counted as “damaged cells”. Intact adipocytes were considered and counted as “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.

## Results

### Cell count

**Before cold exposure:** Normal distribution assumption was accepted and homoscedasticity assumption was rejected ( $p=0.042$ ). Kruskal-Wallis test showed no statistically significant differences between the groups that were found to be comparable.

**After cold exposure:** Normal distribution assumption was accepted and homoscedasticity assumption was accepted. ANOVA test detected statistically significant differences between experimental groups after 25 minutes of cold exposure ( $p<0.001$ ). Bonferroni test (alpha risk=0.017 for each comparison) located these differences between room temperature groups and cold-exposed groups, but not between the cold exposed groups themselves. Cell count was significantly higher in room temperature groups than in cold-exposed groups. Cell count after cold exposure was: CB group 104.42 (16.03), CL group 112.25 (15.96), FB group 80.00 (14.50) and FL group 85.00 (20.86).

### Glycerol concentration

Normal distribution assumption was accepted and homoscedasticity assumption was rejected ( $p<0.001$ ). Kruskal-Wallis showed statistically significant differences in glycerol concentration ( $p<0.001$ ) between groups. Since we were particularly interested in analyzing the adipocyte lipolytic response after the cold stimulus, we additionally compared the two cold-exposed groups with a Mann-Whitney U-test that showed no statistically significant differences between them (CL group: mean 348.15, SD 112.1 and FL group: mean

332.07, SD 130.59).

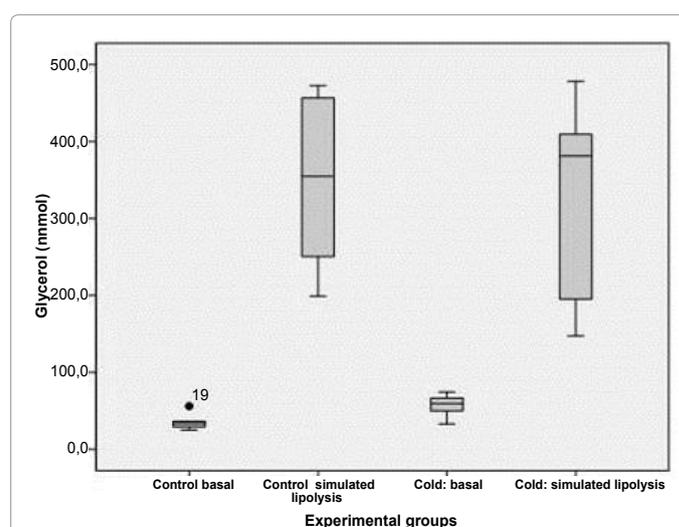
## Discussion

### Cell damage

The results of the present study will be added to the broad body of evidence already supporting the fact that lipocryolysis damages adipocytes [1,5,6,9,10]. Cell count is a direct and simple way of evaluating cell damage. Bonferroni test showed significant differences between the groups exposed to 8°C and those left at room temperature (25°C). Yet, these differences will only account for direct and immediate cell damage-which has never been proposed as part of the lipocryolysis action mechanism-, only partially related to apoptosis (crystal inclusions), which was the original action mechanism proposed for lipocryolysis [9]. Though adipocyte necrosis possibly plays a minor role in lipocryolysis [4], it will be important to determine its correlation with the damages we have witnessed in the adipocytes immediately after the cold stimulus. New researches should also provide correlation between the increasingly observed adipocyte damage immediately after cold stimulus and the lack of inflammation after lipocryolysis treatments. Adipocyte necrosis enhancement looks promising for lipocryolysis optimization and should be a main focus for future lipocryolysis studies.

### Glycerol concentration

Though it has not been possible through the Kruskal-Wallis test to detect which of the four groups exhibited statistically significant differences in glycerol concentration, the test did show that these differences existed between some of them. It can easily be seen in figure 1 that CB and FB groups (with no added isoproterenol) have extremely lower glycerol concentrations than CL and FL groups (with added isoproterenol). The comparison between CL and FL groups showed no differences in glycerol concentration. This fact was very important, as it may account for an undamaged lipolytic capability of the adipocytes that remain in the body after the procedure, or at least for a partially preserved lipolysis path. But the fact of measuring virtually the same amount of glycerol before and after the cold stimulus was paradoxical.



**Figure 1:** Glycerol concentration (nmol/per million cells) before cold exposure: control basal -CB- without isoproterenol and control simulated lipolysis -CL- with isoproterenol, and glycerol concentration after cold exposure: cold basal -FB- without isoproterenol and cold simulated lipolysis -CL- with isoproterenol. Adipocytes respond to isoproterenol induction before and after cold stimulus

Since this particular issue was never attended before, we could only hypothesize. The most promising possibility was that cold could also play a role in resetting adipocyte lipolysis sensibility. Some preliminary data of our studies showed that glycerol production differed according to the thermal stimulus inflicted to the adipocyte: cooling speeds, final temperature and/or tempering cycles. Another possibility was that the glycerol concentration observed was the result of a normal glycerol production in undamaged adipocytes plus the one produced by fully working enzymes in damaged adipocytes. In previous studies of the CeReMet team, it was stated that LPLs will not keep working in dead adipocytes without heparin [11]. The same happens to HSL, because lipolysis requires ATP. This will have to be assessed thoroughly in the future though it seems evidence points elsewhere. Future studies should confirm this claim by assessing full restoration of lipolysis and other adipocytic metabolic processes after this therapy.

We conclude that Lipocryolysis confirms its usefulness for adipocyte damage, and that necrotic cell destruction, previously thought irrelevant, is starting to prove to be important or at least promising for results enhancement. In the future, it may no longer be considered a minor process. It seems lipocryolysis does not cause any damage on the adipocytes that remain in the body after the procedure, which look undamaged. Though lipases are difficult to denature and they may still be present and working in unviable adipocytes, this is improbable and post-lipocryolysis lipolytic capability seems to be preserved. Further researches will be needed to confirm or reject these statements for physicians to be able to a) extend the lipocryolysis cold exposure time and b) investigate the possibility of altering crystal polymorphisms formation in order to achieve higher adipocyte necrosis and apoptosis rates that could represent further improvements of clinical results.

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