X-ray diffraction analysis confirms intra-adipocitary lipid crystallization after a lipocryolysis-like stimulus

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Abstract. Lipocryolysis always claimed localized-fat-reduction to be a consequence of local apoptotic adipocyte destruction triggered by intracellular triglyceride crystallization. The whole idea is now under debate, for it has been suggested that the physical changes occurring in adipocytes after lipocryolysis could be better explained by a lipid gel-like transition process rather than by lipid crystallization. Since a) lipocryolysis claims apoptosis to be the key to adipocyte destruction and clinical-result achievement and b) it considers crystallization to be a necessary step for the apoptotic stimulus unleashing, any effort to untangle, prove or discard this process is very important.

Key words. Crystallization, gel-like transition, lipocryolisis, x-ray diffraction.

Introduction

Lipocryolysis is a treatment that combines heat extraction with vacuum in a specific way It is a safe technology [1, 2] that is effective for localized fat reduction [3, 4] for aesthetic purposes (e.g.: trunk fat) and for functional improvement (e.g.: lipomas), among others. When first heard-of [5], the theoretic knowledge that backed-up the empiric localized-fat-reduction-results was limited and claimed local apoptotic adipocyte destruction as a consequence of a heat extraction triggering stimulus [5, 6]. By combining vacuum and heat extraction by Peltier Effect [7], intra-adipocitary temperature can be lowered to an extent where the physical changes that will finally lead to the reported therapeutic results [8] occur. This happens without damaging any other tissue [9]. Apoptotic adipocytolysis as a consequence of intracellular changes was the first and most logical action mechanism proposed for lipocryolysis. It was assumed that adipocytolysis was a biological consequence of intracellular lipid crystallization, but the concept is now under debate. Some authors are reluctant to it and suggested that changes could have been better explained by a gel-like transition process rather than by lipid crystallization. Solid evidence that may explain the low-degree adipocytary necrosis observed is still needed, because the exact correlation between lipocryolysis, crystallization, gel-like bahaviour, apoptosis, necrosis and inflammation remains hidden. Our last study contributed partially to solve this issue [10], arriving to several conclusions that ultimately led to this communication. Some of the presented evidence was very consistent with both, crystallization and lipid gel-like behaviour. But there were some specific facts that doubtlessly leaned the equilibrium towards crystallization:

i) different crystal patterns were reported: needle-like, v-like, star-like and sphere-like [10]. Under certain cooling rates and conditions, needle-like pattern [11, 12] and spherulitic growth [13] are very well known for triglycerides β crystallization polymorphism.

ii) after an 8ºC exposure during 25´, samples were left at room temperature (22ºC) and photographed while slowly reheated-up. 120´ after cold exposure, some crystals disappeared but others remained intact. This behaviour was previously observed and related to crystallization [14].

iii) temperature and exposure time affected the observed structures in a similar way to what was previously described for lipid crystal thermodynamics and kinetics [13, 15].

iv) birefringent structures were 50 μm approximately, which was consistent with crystals [13].

Serious iconographic evidence already backed-up natural fat crystallization after lipocryolysis [10], though some authors have cooled human adipocytes down to 1ºC and never saw crystallization but gel-like behavior. Studies that approach the crystallization process of natural (multi-component) fat are rare. Since the aim of this observational report was to elucidate the nature of the intra-adipocitary changes observed, X-ray crystallography (XRD) seemed the most suitable option. XRD is based on x-ray diffraction when breaking through solids. Incident x-rays interact with the electrons of the sample and bring information about sample atomic composition and spatial disposition. In crystals, atomic arrangement is periodically repeated and thus x-ray diffract in specific patterns [16].

Material and methods

Adipose tissue.

Four male Wistar rats (Harlan Interfauna) were anesthetized with isoflurane. Sacrifice was performed with cervical dislocation technique. White adipose tissue was obtained via laparotomy. 2 g of retroperitoneal fat were chopped into small pieces and digested in 20 mL of Krebs Buffer Solution (Hepes 1.25 mM, NaCl 12 mM, KCl 0.6 mM, MgSO4·7H2O 0.12 mM, CaCl2 0.1 mM, albumine 2 g and glucose 0.045 g) supplemented with 10 mg Collagenase (Type 4, Worthington).

Isolation of fat cells.

Digestion was performed at 37ºC for 40 minutes under constant gentle shaking. EDTA solution (0.1 M, 2 ml) was added to finish digestion process and was incubated for 5 more minutes. Tissue remnant was filtered and separated from isolated adipocytes by buoyancy for five minutes. Adipocytes were recovered without buffer, to reduce possible analytical interferences. Adipocytes were then separated into two vials.

Cold exposure

In order to generate the same birefringent structures obtained before [10], one vial was exposed to 8ºC for one hour (figure 1). The other vial (basal) was used as a control immediately after its obtaining, with no birefringent structures expected.

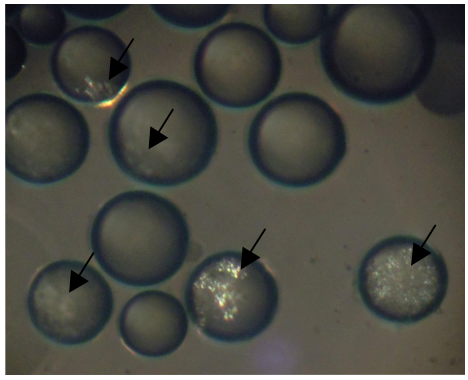

Figure 1. Crystals formed inside adipocytes (arrows).

Crystal Obtaining

The presence of crystals was assured 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 were not heated-up above 10.38ºC (the only temperature at which post-lipocryolysis crystallization has been reported [5], temperature enhancement due to microscope light exposition was calculated [10].

X-Ray Diffraction

Samples were sandwiched between low absorbing 3.6 microns polyester films. A *PANalytical X'Pert PRO MPD* θ/θ powder diffractometer of 240 millimetres of radius, in a configuration of convergent beam with a focalizing mirror and transmission geometry was used. Wave length Cu Kα radiation was λ = 1.5418 Å. Work power: 45 kV – 40 mA. Incident beam slits defining a beam height of 0.4 millimetres. Incident and diffracted beam 0.02 radians *Soller* slits. *PIXcel* detector: Active length = 3.347 º. 2θ/θ scans from 1 to 40 º2θ with a step size of 0.026 º2θ and a measuring time of 200 seconds per step. DRX processed samples: *background* (polyester with no adipocytes), *basal* (sample with no crystals, processed immediately after

adipocyte isolation) and *cooled* (isolated adipocytes after temperature treatment).

Results

X-ray diffraction analysis

Results are shown in figure 2. The green line represents the polyester signal (background), with a spacing value of 0.54 nm. The blue line represents the basal signal, with no evident peak. The sample *per se* provides a considerable background, because most of the sample is not crystallized and has an amorphous structure. No birefringent structures could be seen in basal samples. Finally, the red line corresponds to the cooled sample. Polyester signal is covered by the background noise. Cooled sample XRD analysis showed peaks A and B over the background. There is a third peak (C) not easily seen. D-space and peak positions are summarized in table 1.

Peak	Position	d-space (nm)
A	2.11	4.19
B	19.29	0.46
C	19.54	0.45

Table1. XRD: position and d-spacing of peaks.

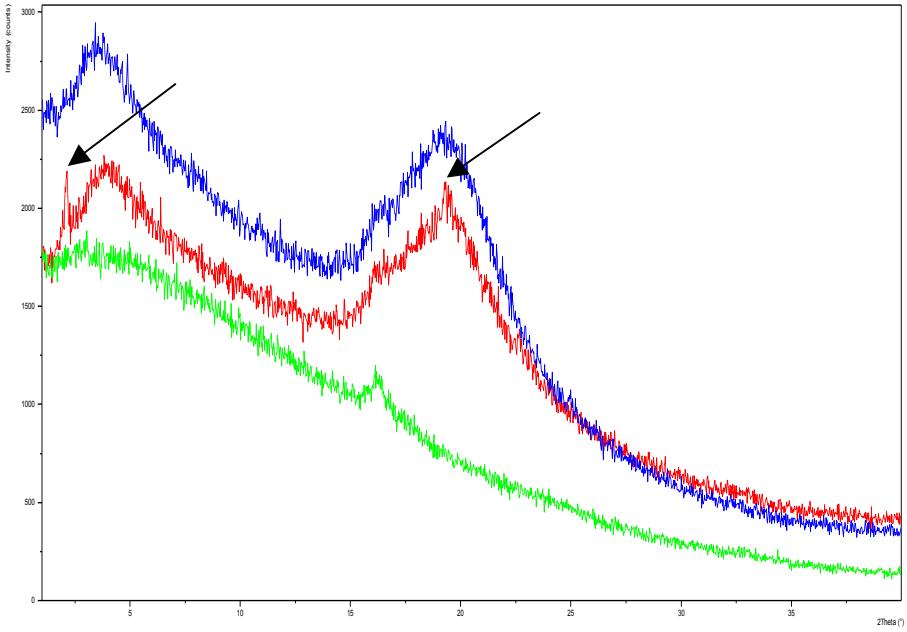

Figure 2. XRD: sample analysis (position vs. counts) showing peaks A and B (arrows)

Discussion

A) Some important information could be gathered from previous studies:

i) Birefringence is consistent with crystals. The intra-adipocitary structures analyzed by XRD in this report were already described by our team as birefringent under polarized microscopy [10]. These structures have also been thoroughly analyzed by many other authors during the last 50 years [16].

ii) Size and shape are consistent with crystal structures. Needle-like pattern [12] as well as spehrulitic growth [13] are characteristic of specific triglyceride crystallization polymorphisms.

iii) Intra-adipocitary structures behave like crystals. Cooler temperatures and longer exposure times affect at least structure size.

iv) Theoretic predictions for crystalline structures are met. Needle-like crystal aggregation may result in regular or irregular spherulitic growth according partially to the applied cooling rate. Evidence of irregular sphere-like growth after a lipocryolisis-like stimulus was already provided by our team [10].

B) The observed XRD pattern for the analyzed sample was similar to the previously reported by Higaki et al, when analyzing crystalline structures in pure lipid mixtures [11, 14]. Recorded XRD peak position and spacing were the same that have characterized specific triglyceride crystallization polymorphisms for the last 50 years [16]. Sample peak shape also resembled natural fat crystal XRD peak shape [11, 14], with broad bases due to the low amount of crystalline structures in relation to the total liquid content of the sample (amorph).

Because our observations have been confirmed by XRD analysis and because they have been strictly coherent with a huge body of evidence, we concluded that the intra-adipocitary structures observed after lipocryolisis-like stimuli are crystals and that probably, lipid crystallization is involved in lipocryolysis therapeutic outcome.

Further investigations are urgently needed for: a) understand more precisely the driving forces of crystallization in natural fats and b) correlate lipocryolysis, crystallization, gel-like bahaviour, apoptosis, necrosis and inflammation.

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