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# DSC ANALYSIS OF HUMAN FAT TISSUE IN ALCOHOL-INDUCED AVASCULAR NECROSIS OF THE FEMORAL HEAD
## A preliminary study

*Cs. Pintér*[1], *G. Bognár*[1], *B. Horváth*[1], *T. Sydo*[2], *E. Ligeti*[3], *J. Pulai*[4] *and D. Lőrinczy*[5*]

[1]Department of Traumatology and Orthopaedics, Veszprém County Hospital, 8200, Kórház str. 1, Hungary
[2]Department of Internal Medicine, Veszprém County Hospital, 8200, Kórház str. 1, Hungary
[3]Department of Pathology, Veszprém County Hospital, 8200, Kórház str. 1, Hungary
[4]Department of Rheumatology, Veszprém County Hospital, 8200, Kórház str. 1, Hungary
[5]Institute of Biophysics, University of Pécs, Faculty of Medicine, 7624 Pécs, Szigeti str. 12, Hungary

The osteonecrosis or avascular necrosis of the antero-superior part of the human femoral head (ANFH) often causes incongruity in the hip joint and leads to severe pain and disability in middle aged patients. This abnormality often requires surgical intervention, mainly total hip arthroplasty (THA). The possible pathomechanisms of ANFH are fat embolism, arterial occlusion, fatty necrosis of osteocytes and intraosseous hypertension. Factors have been proposed in the literature that can lead to this condition are alcohol abuse, steroid therapy, metabolic changes, dyslipidaemia. In some case we can not verify any factor; these are the so called idiopathic ANFH cases.

We observed macroscopic variation in colour and consistency of the subcutaneous fat tissue in patients with ANFH compared to osteoarthritis or hip fracture during THA procedures. Subcutaneous fat tissues during THA from a patient with ANFH due to alcohol abuse were compared with an otherwise healthy patient who underwent surgery due to traumatic hip fracture. No histological changes were notified in the size; shape of adipocytes and in the cells of the septae of the connective tissue. Gas chromatography showed that the patient with alcoholic ANFH had less long chain fatty acids. DSC revealed, that in case of non-necrotic sample as a reference, during heating between 0–100°C two separable transitions are with $T_m$=5.7 and 9.9°C, total $\Delta H$= –20.8 J g$^{-1}$. In –20–100°C range endotherms with $T_m$= –10.9 and 4.95°C, total $\Delta H$= –75.8 J g$^{-1}$ could be detected. In case of alcohol-induced avascular necrosis we have found endotherms between 0–100°C with: $T_m$=7.3°C, total $\Delta H$= –26.9 J g$^{-1}$, and heating between –20–100°C: $T_m$= –0.25°C, total $\Delta H$= –103.3 J g$^{-1}$ thermal parameters. The alteration in the fatty acid profile did not cause histological changes, but we were able to detect it with analytical methods e.g. DSC and gas chromatography.

***Keywords:*** *DSC, fatty acids, gas chromatography, osteonecrosis*

## Introduction

The most common localization of the avascular osteonecrosis is the loading zone of the femoral head [1, 2]. The process starts with oedema of bone marrow, abnormal circulation, and it leads necrosis of the subchondral region of antero-superior part of the human femoral head of different extent [3–6]. The incongruity of the femoral head yields to secondary degenerative changes and end stage osteoarthritis [7]. The common clinical presentation encountered by orthopedic surgeons is that of a middle aged actively working male patient population in 80% is affected [1, 7].

In advanced stage of ANFH it is world wide accepted to use cementless total hip arthroplasty (THA) aiming for minimal bone loss with respect to the patients relatively young age [8].

The ANFH is responsible for up to 12% of total hip arthroplasties [9]. We observed macroscopic variation in colour and consistency of the subcutaneous fat tissue in patients with ANFH compared to osteoarthritis or hip fracture during THA procedures. The fatty tissue of ANFH patients crumbled slightly, its colour seems to be brighter yellow than usual.

Our observation is also supported by pathologist's macroscopic finding during autopsy.

According to them similar difference can be observed in the retroperitoneal fatty tissue of patients with history of alcohol abuse. We proposed that the changes due to the altered fat metabolism that contributed to the ANFH can be observed in the subcutaneous fat tissue in the molecular level [10, 11].

The goal of the present study was to investigate the subcutaneous fat tissue structure by histology, to analyse its fatty acid composition by gas chromatography (GC), and its quality through differential scanning calorimetry (DSC).

* Author for correspondence: denes.lorinczy@aok.pte.hu

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*Akadémiai Kiadó, Budapest, Hungary*
*Springer, Dordrecht, The Netherlands*

## Experimental

*Materials and methods*

In this study we compared the results of two groups, one of diagnosed alcoholic patients suffering from ANFH and the other of otherwise healthy patients with hip region fracture. The groups were formed by 5–5 patients. All of them were male, with normal hematologic values. The mean age of the ANFH group was 51 years, (from 44 to 56), mean age of control group was 53 years (from 48 to 56). ANFH patient was operated by THA, anterolateral surgical exposure was performed. The control group underwent different kinds of osteosynthesis.

Tissue samples

Fat tissue samples were taken from the subcutis by anterolateral incision of the surgical exploration for THA procedure (ANFH group) and by other surgical exposures during different kinds of osteosynthesis at the same region (control group). Our activities were done under the proper law paragraphs and valid permission.

Histology

For the histological examination the specimens were fixed in 10% neutralized formalin for a week, embedded in paraffin, and cut to a thickness of 5 μm. All sections were stained with oil red and hematoxylin-eosin. The histological morphometry was performed with Nikon Eclipse E400 light microscope (usual magnification 400×) to examine the changes of the fat tissue structure. The remaining tissue samples were frozen and kept in a freezer (–21°C) until the further analysis.

Fatty acid analysis

Boron-trifluoride methanol extraction was used for the fatty acid-profile definition in 10 samples, and then the fatty acids were transformed to fatty acid-methylesters, (FAME) and then were stored in *n*-hexane medium until the FAME profile was analysed by a Chrompack CP 9000 type gas chromatograph. FAMEs were separated on a 100 m×0.25 mm quartz capillary column (CS-Sil 88). Both the injector (splitter) and detector (FID) were maintained at temperature 270°C. Helium gas was used as the carrier gas using pressure 235 kPa, with flow rate at the detector 1 mL $min^{-1}$. The temperature-programme of column: maintain the temperature of 140°C for 10 min; then rise to 10°C $min^{-1}$ until 235°C, and maintain isotherm until 26 min [12, 13].

Differential scanning calorimetry (DSC)

The thermal unfolding of the fat tissue samples were monitored by Setaram Micro DSC-II calorimeter. The experiments were conducted between 0 and 100 as well as –20 and 100°C. The heating rate was 0.3 K $min^{-1}$ in all cases. Conventional Hastelloy batch vessels were used during the denaturation experiments. Typical sample wet masses for calorimetric experiments were between 200–250 mg. Pure alcohol was used as a reference sample. The sample and reference vessels were equilibrated with a precision of ±0.1 mg. There was no need to do any correction from the point of view of heat capacity between sample and reference vessels. Calorimetric enthalpy was calculated from the area under the heat absorption curve by using two-point setting Setaram peak integration.

## Results and discussion

*Histological findings*

Regularly the cellular content of adipose tissue is approximately 50% adipocytes, with the remaining 50% being preadipocytes, macrophages, and the stromal vasculature fraction of fibroblasts, endothel cells. Approximately 80% of adipose tissue mass is lipid, and over 90% of the lipids are stored as triglycerides.

In our investigations no histological changes was notified in the size and shape of adipocytes and in the cells of septae of connective tissue (Fig. 1).

*Fatty acid analysis*

The percentage of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) fractions of fatty acid methyl esters (FAME) of alcoholic and control patients were determined by GC (Table 1). The samples were compared quantitatively and qualitatively. In SFA fraction there were no significant difference between the two groups (alcoholic:control=26.03:27.01). In MUFA fraction

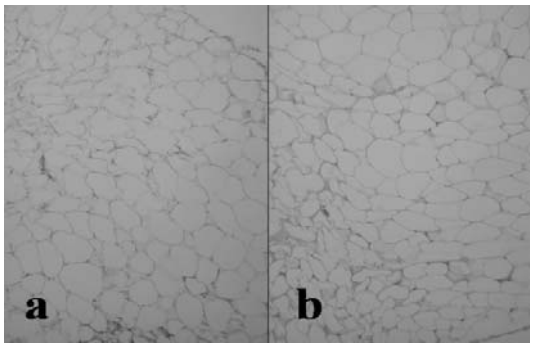


**Fig. 1** Characteristic histological picture of subcutaneous fatty tissue (a – control group, b – ANFH group)

we found a higher value in alcoholic group, than in the control group (alcoholic:control=67.72:51.99). In PUFA fraction there was a significant difference, alcoholic patients had much lower PUFA content than controls (alcoholic: control=6.24:20.57). In control group the most important part (85%) of storaged fat consists of palmitic acid (20%), oleic acid (46%) and linoleic acid (19%). In alcoholic patients on the whole same amount of palmitic acid (21%), significantly increased amount of oleic acid (53%) and decreased to a third part of linoleic acid (6%) could be detected. There were no significant difference between the two groups in myristic acid, pentadecanoic acid, margaric acid, eicosenic acid amounts. Of note, it was interesting the absence of long-chain PUFA-s (eicosatrienoic acid, arachidic acid, docosapentanoic acid), and the relatively decreased amounts of α-linoleic acid, heneicosanoic acid and eicosadienoic acid in alcoholic group (Table 2).

**Table 1** The mean values of saturated, monounsaturated and polyunsaturated fatty acids in alcoholic and control patients

| | alcoholic | control |
|---|---|---|
| SFA | 26.03 | 27.01 |
| MUFA | 67.72 | 51.99 |
| PUFA | 6.24 | 20.57 |

**Table 2** GC shows the distribution of fatty acid methyl esters (FAMEs) between C 12–C 22 atomic chains (data in average %±sd.)

| | | Alcoholic | Control |
|---|---|---|---|
| Type of fatty acid | | Fatty acid methyl ester/% | |
| lauric acid | 12:0 | 0.24±0.02 | 0.63±0.05 |
| myristic acid | 14:0 | 1.85±0.11 | 1.98±0.11 |
| myristoleic acid | 14:1 | 0.42±0.04 | 0.23±0.02 |
| pentadecanoic acid | 15:0 | 0.14±0.01 | 0.16±0.01 |
| palmitic acid | 16:0 | 21.42±2.02 | 20.19±2.06 |
| palmitoleic acid | 16:1 | 12.94±1.22 | 4.95±0.47 |
| margaric acid | 17:0 | 0.22±0.02 | 0.22±0.02 |
| stearic acid | 18:0 | 2.02±0.17 | 3.63±0.33 |
| oleic acid | 18:1 | 53.47±4.89 | 45.93±4.31 |
| linoleic acid | 18:2n6 | 5.97±0.61 | 18.75±1.78 |
| eicosenic acid | 20:1 | 0.89±0.09 | 0.88±0.08 |
| alpha-linolenic acid | 18:3n3 | 0.10±0.01 | 0.47±0.05 |
| heneicosanoic acid | 21:0 | 0.14±0.01 | 0.20±0.02 |
| eicosadienoic acid | 20:2 | 0.17±0.01 | 0.41±0.04 |
| eicosatrienoic acid | 20:3n6 | <0.1 | 0.36±0.03 |
| arachidic acid | 20:4n6 | <0.1 | 0.33±0.02 |
| docosapentanoic acid | 22:5n3 | <0.1 | 0.66±0.06 |

*DSC results*

Non-necrotic fat samples were used as a control during the denaturation experiments. As it can be seen from Fig. 2a the melting in 0–100°C temperature range exhibited a big endotherm thermal domain with two components. The proper thermodynamic parameters were 5.7 and 9.9°C as melting temperatures ($T_m$), and –20.8 J g$^{-1}$ as total calorimetric enthalpy change ($\Delta H$). The second heating of the sample showed a mild, but non significant change in the thermal parameters as well as in the shape of DSC scan. Figure 2b reports about the melting in –20+100°C range. The observed parameters were $T_m$s= –10.9, 4.95 and 10.2°C with a $\Delta H$= –75.8 J g$^{-1}$, as total calorimetric enthalpy change. The cooling to –20°C caused a structural rearrangement in the fat tissues, that resulted in the different $T_m$ and $\Delta H$ compared to Fig. 2a. The second heating revealed a significant structural rearrangement (Fig. 2b) caused by the first

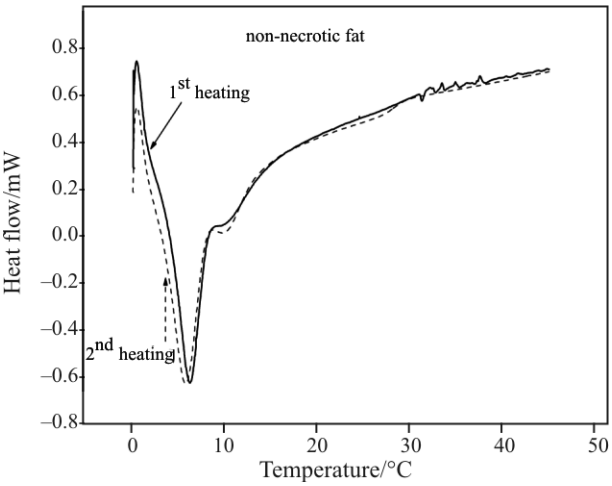


**Fig. 2a** Thermal denaturation of control samples in 0–100°C range

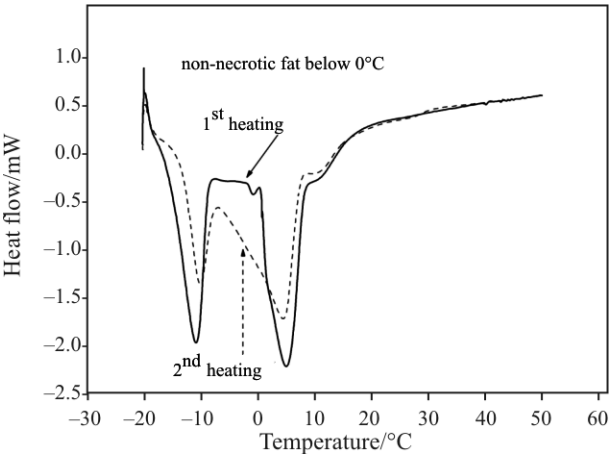


**Fig. 2b** Thermal denaturation of control samples in –20+100°C range

heating and recrystallisation, where the second endotherm's thermal domains were mainly affected. In case of patients with alcohol induced avascular necrosis the thermal denaturation significantly differs in 0–100°C temperature range from the control one (Fig. 3a). The DSC scan shows only one thermal domain with $T_m$=7.3°C, total $\Delta H$= –26.9 J g$^{-1}$. The second heating exhibits about 5°C temperature shift and 70% enthalpy decrease. This change is the sign of altered fatty acid composition (Tables 1 and 2). In case of denaturation started at –20°C (Fig. 3b) the proper parameters were $T_m$s= –0.25 and 7.5°C, total $\Delta H$= –103.3 J g$^{-1}$. In case of some patients with bilateral ANFH we performed a second THA operation within two years. Advanced alcoholic and metabolic changes were evident. The second fatty tissue samples were investigated only by DSC. The results supported, that alcohol abuse caused fatty liver makes significant alteration in fat metabolism, wich process can be followed by measuring the fatty acid composition of subcutaneous fatty tissue. A tipical result can be seen in Figs 4a and b, that demonstrate further change in the fatty acid composition.

The diagnosis of ANFH is based on radiographic methods, MRI is the diagnostic tool of choice for identifying early stage of disease [14, 15]. The ANFH is often bilateral, but the disease of contralateral side can occur several years later too [15]. The most commonly used classification by Ficat describes five stages of the disease, from subclinical state to end stage osteoarthritis [3]. The bone death is followed by occurrence of microfractures then bone resorption can lead to bone surface collapse and incongruity [16]. In this stage the abnormalities can be visualised by CT or X-rays [6]. Due to more severe pain, synovitis, disability, loss of range of motion, contractures and atrophic muscles occurring in patients with ANFH compared with osteoarthritis, the treatment modality

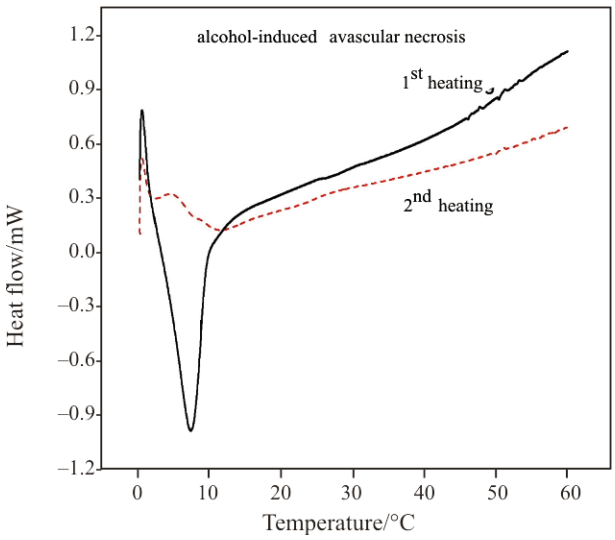


**Fig. 3a** Thermal denaturation of alcoholic affected samples in 0–100°C range

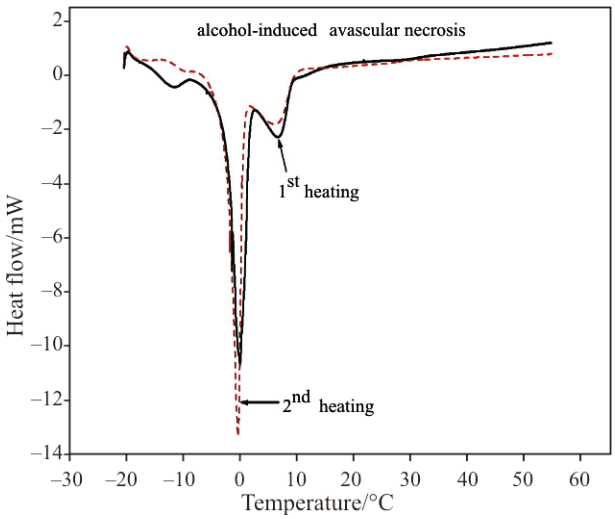


**Fig. 3b** Thermal denaturation of alcoholic affected samples in –20+100°C range

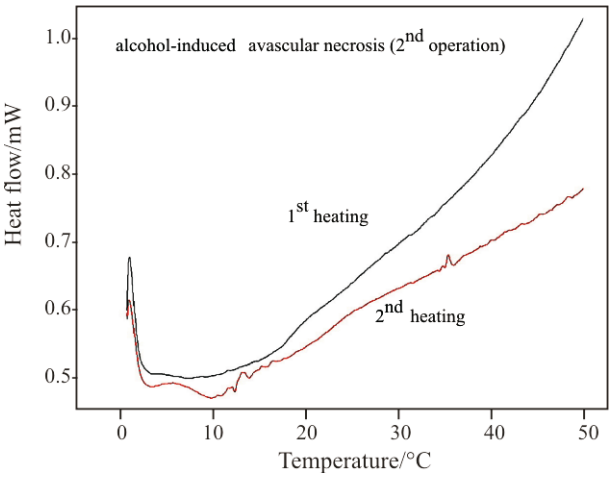


**Fig. 4a** Thermal denaturation of double operated, alcoholic affected samples in 0–100°C range

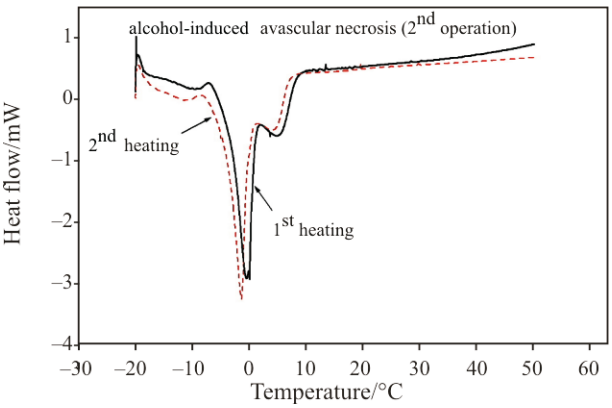


**Fig. 4b** Thermal denaturation of double operated, alcoholic affected samples in –20+100°C range

**Table 3** Suspected ethiological factors in the groups

| Possible ethiologic factors associated with osteonecrosis | |
|---|---|
| **Metabolic diseases** | **Traumatic** |
| Hyperlipidemia | Femoral neck fracture |
| Hyperuricemia/gout | Dislocation or fracture |
| Hepatopathy | Minor trauma |
| Diabetes mellitus | **Drug induced or toxic** |
| Pancreatitis | **Corticosteroid th.** |
| Cushing syndrome | Alcohol use |
| Gaucher's disease | Cigarette smoking |
| **Hemorheologic** | **Inflammatory diseases** |
| Sickle cell anaemia | Systemic lupus erythematosus |
| Intravascular coagulation | Scleroderma |
| Thrombophlebitis | Rheumatoid arthritis |
| Coagulopathies-Thrombophilia | Psoriatic arthritis |
| Caisson (dysbarism) disease | **Other** |
| Chronic renal failure | Pregnancy |
| Hemodialysis | Radiation |
| **Angiopathic** | Organ transplantation |
| Raynaud syndrome | HIV infection |
| Diabetic angiopathy | Idiopathic |

is mostly surgical [6, 7, 15]. In the early stages femoral head conserving techniques, core decompression or osteotomy is preferred [16–18]. When seeking medical attention with painful hip, the involved femoral head usually can not be saved, but this technique can be utilized in the contralateral hip, that was diagnosed with MRI in an early, asymptomatic stage [6, 15, 16].

During the etiological research of ANFH a number of different pathogenetic factors have been implicated, but the causation of atraumatic osteonecrosis of the femoral head is believed to be multifactorial, remains unclear [19–22]. In some cases anatomical factors are associated with a genetic predisposition and exposure to certain risk factors [19], Table 3.

In our experience the alcohol abuse plays the leading role, but since the question is a delicate subject. The answers are most of the time inaccurate, accurate data are not known. In case of chronic alcoholism changes in lipid metabolism and liver steathosis are frequent. Using liver biopsy a strikingly high number of cases with liver steathosis in ANFH cases can be found.

Jones *et al.* [1] demonstrated that fat embolism can appear in the lungs, kidneys and in subchondral vessels of femoral head originating from a fatty liver. They proposed three possible origins for the fat emboli: (1) fatty liver, (2) destabilization and coalescence of plasma lipoproteins and (3) disruption of fatty bone [23]. Alcohol can cause ANFH through multiple pathways: (1) alcoholic hepatopathy and fat embolism from liver, (2) direct toxic effect, (3) alcohol abuse decreases osteogenesis and enhances adipogenesis from marrow stem cells, and (4) alcohol induced neuropathic femoral head changes may be causative agent as well [24–26].

In this study we investigated the altered alcoholic fat metabolism through fatty tissue histology, and found no difference. The GC can show the alterations of stored fatty acids in detail [27, 28]. The DSC is helpful method to reveal the thermal properties of pathologic human tissues [12, 13] and supported the GC findings well.

## Conclusions

We have investigated with different methods the subcutaneous fatty tissue in alcohol induced ANFH versus control group, having macroscopically seen alterations in its colour and consistence. Although we have found no histological changes by microscopic analysis, the analysis by GC revealed significant alterations in the fatty acid content, and similar results were obtained by DSC as well. These alterations in fat metabolism may play a pivotal role not only in pathophysiology of ANFH, but it might cause cardiovascular complications due to different blood lipid content [29]. Further investigations are needed regarding the fatty tissue and plasma lipid level alterations, whether they could have an effect in the outcomes of the femoral head preserving techniques done in early stages of ANFH.

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