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DSC ANALYSIS OF HUMAN FAT TISSUE IN STEROID INDUCED OSTEONECROSIS
A preliminary study

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Osteonecrosis (ON) of the femoral frequently occurs after steroid medication. One of the final pathways leading to steroid induced ON is thought to be pathologic fat metabolism. The pathobiological mechanism underlying the induction of fat metabolism outslides by steroids leading to ON has not been fully elucidated. The purpose of this study was to examine the intraoperative obtained gluteal fat tissue from ON patients with histology, gas chromatography (GC) and differential scanning calorimetry (DSC) and to compare them with otherwise healthy patient's samples. The histological sections showed no significant differences compared with the control group. GC revealed that fraction of saturated fatty acids decreased in ON samples from mean values of controls of 24% to 21, the polyunsaturated fraction from 20 to 14%. The monounsaturated acids showed an increase from mean rate of 52% of the controls to 65% of steroid treated samples. DSC curves correlate with chromatographic analysis of the tissue fatty acids (Steroid treated, heating between 0–100°C: T_m=5.7°C, ΔH= –15.8J/g^{-1}; heating between –20–100°C: T_m= –9.96 and 5.85°C, ΔH= –59.17 and –16.2 J g^{-1}. Non-necrotic, heating between 0–100°C: two separable transition with T_m=5.7 and 9.9°C, total ΔH= –20.8 J g^{-1}; heating between –20–100°C: T_m= –10.9 and 4.95°C, total ΔH= –75.8 J g^{-1}.)

Our preliminary findings are rather tendentious. Further investigations are needed with higher sample rate and under other anamnestic circumstances too.

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

Introduction

Osteonecrosis (ON) of the femoral head is a rather common disorder causing disability of the hip joint. It develops in the high load-bearing region of the femoral head. The necrotic subchondral region of the bone looses of its load-bearing capacity, the chondral surface collapse which results the incongruity of the joint.

Femoral capital ON, formerly a rather rare disease in clinical practice, is currently met with increasing frequency. It is most common in the second to fifth decades of life. The typical patient is a male, in his mid 30s [1]. As such patients have a longer life span, and are economically active, this can be costly to society; while joint replacement is the definitive treatment, it has a much higher failure rate in this group of patients, possibly reflecting base disease and younger age [1]. In our age osteonecrosis is responsible for up to 12% of total hip arthroplasties [2].

A number of different factors have been implicated in the development of ON, but although it has been recognised for over 100 years, the biological mechanisms involved remain unclear [3, 4]. Causation of atraumatic ON of the femoral head is believed to be multifactorial, in some cases associated with both a genetic predisposition and exposure to certain risk factors. The list of risk factors of ON is on the rise, say, corticosteroid therapy, alcoholism, infections, and storage disorders, physical or thermal damage, autoimmune diseases, marrow infiltrating disorders, and coagulation defects [5–9]. Conditions such as autoimmune disease like systemic lupus erythematosus, organ transplant, etc. are regularly treated with significant doses of steroids, often for prolonged periods, and there is a clear association with ON [1, 10–17].

One of the pathways thought to play a rule in development of ON is steroid induced outslide of fat metabolism [18, 19].

The intraoperative observation of the authors is that definitive differences are present in macroscopic appearance and consistent of fat tissue by ON treated patients compared with non-osteonecrotic patients.

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The aim of this study is to establish the differences between fatty acid profiles and thermal characteristics of peripheral fat tissue in patients with steroid associated ON and to compare them with control samples from traumatic patients. Histological analysis was performed with light microscope, the lipid composition was determined by gas chromatography (GC), and the thermal phase transitions were studied by DSC.

Experimental

Materials and methods

Tissue samples

5 fat tissue samples from steroid treated ON patients were taken by anterolateral incision of the surgical exploration for total hip arthroplasties from the subcutis. Steroid treated samples are taken from patients permanently treated with prednisolon for autoimmune diseases. The daily intakes varied between 10–30 mg. 5 control samples were taken from the same anatomical region, from patients treated with traumatic anamnesis. By control patients no permanent medication, alcohol consumption or system disease occurred. In case of one patient a second surgical intervention occurred on contralateral side (this sample was analysed with DSC alone). 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 haematoxylin-eosin. The histological analysis was performed with Nikon Eclipse E400 light microscope (usual magnification 400×) to examine the changes of the fat tissue structure.

Fatty acid analysis

The fatty acid profile of the 2×5 samples determined. Methyl esters were extracted from fat tissue and methylated by the boron trifluoride-methanol method. Analysis of the fatty acids was carried out with a Chrompack CP 9000 gas chromatograph, using a capillary column (100 m, 0.25 mm) with CS-Sil 88 (FAME). The injector port and detector temperature were maintained at 270°C. An isothermal program was utilized with column temperature, 140°C; helium carrier (235 kPa) flow rate at the detector 1 mL min^{-1}. The oven temperature was programmed to maintain a temperature of 140°C for 10 min, then rise to 240°C at 10°C min^{-1} and maintain for 26 min.

DSC measurements

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

Histology

Typically, the cellular content of fat tissue samples was found approximately 50% adipocytes, with the remaining 50% being the stromal vasculature fraction of fibroblasts, endothelial cells, macrophages and preadipocytes. No definitive differences could be observed in microscopic staining of steroid treated patient's sections in relation to control sections (Fig. 1).

Fatty acid analysis

Table 1 shows the chemical composition of the steroid treated and the control samples and their blends expressed in percentage of fatty acids. Values reported are the mean values and standard deviations. Control sample had a significant amount of oleic acid (mean: 46%), followed by palmitic (mean: 20%), linoleic (mean: 19%). On the other hand, the main fatty acid found in steroid treated samples was oleic acid, but with a significant higher rate (mean: 56%) followed by the decreased amount of palmitic

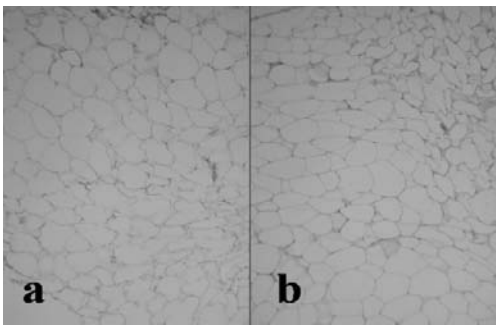

Fig. 1 Histological sections of the fat tissue samples. (a – control sample, b – steroid treated patient sample. No significant difference can be observed.)

Table 1 Fatty acid profiles of human fat samples

Fatty acid profile	Control	Steroid tr.
Lauric 12:0	0.63±0.05	0.16±0.01
Myristic 14:0	1.98±0.11	1.40±0.13
Myristoleic 14:1	0.23±0.02	0.28±0.02
Pentadecanoic 15:0	0.16±0.01	0.10±0.01
Palmitic 16:0	20.19±2.06	16.77±1.48
Palmitoleic 16:1	4.95±0.47	7.74±0.69
Margaric 17:0	0.22±0.02	0.12±0.11
Stearic 18:0	3.63±0.33	2.03±0.19
Oleic 18:1	5.93±4.31	55.79±0.51
Linoleic 18:2n6	18.75±1.78	12.97±0.11
Eicosenic 20:1	0.88±0.08	0.73±0.08
α-linolenic 18:3n3	0.47±0.05	0.21±0.02
Heneicosanoic 21:0	0.20±0.02	0.14±0.01
Eicosadienoic 20:2	0.41±0.04	0.26±0.02
Eicosatrienoic 20:3n6	0.36±0.03	0.24±0.02
Arachidic 20:4n6	0.33±0.02	0.76±0.06
Docosapentanoic 22:5n3	0.66±0.06	0.30±0.03

(mean: 17%) and linoleic acid (mean: 11%), respectively. By steroid treated patients the fatty acid analysis revealed a decrease of the main saturated fatty acids such as palmitic (16:0) and stearic (18:0), as well as the decrease of the main polyunsaturated fatty acid linoleic acid (18:2n6). The summary fraction of saturated fatty acids decreased from mean values of 24 to 21%, the polyunsaturated fraction from 20 to 14%, respectively. In case of the main monounsaturated fatty acids such as oleic (18:1) and palmitoleic acids (16:1) an increase can be observed. The total percentage of monounsaturated acids showed an increase from mean rate of 52% of the controls to 65% of steroid treated samples.

DSC analysis

Healthy (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 (ΔH). 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_ms= –10.9, 4.95 and 10.2°C with a ΔH= –75.8 J g^{-1}, as calorimetric enthalpy change. The cooling to –20°C caused a structural rearrangement in the fat tissues, that resulted in the different T_m and ΔH compared to Fig. 2a. The second heating revealed a significant structural rearrangement (Fig. 2b) caused by the first heating and recrystallisation, where the second endotherm's thermal domains were mainly affected. In case of patients with steroid treatment 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=5.7°C, total ΔH= –15.8 J g^{-1}. The second heating exhibits two smaller endotherms with about 5°C temperature shifts in both direction, and 80% 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_ms= –9.9 and 5.8°C, with ΔH= –59.2 and –16.2 J g^{-1}, respectively (Table 2). These results supported that steroid treatment made significant alteration in fat metabolism, which can be followed by measuring the fatty acid content of subcutaneous fatty

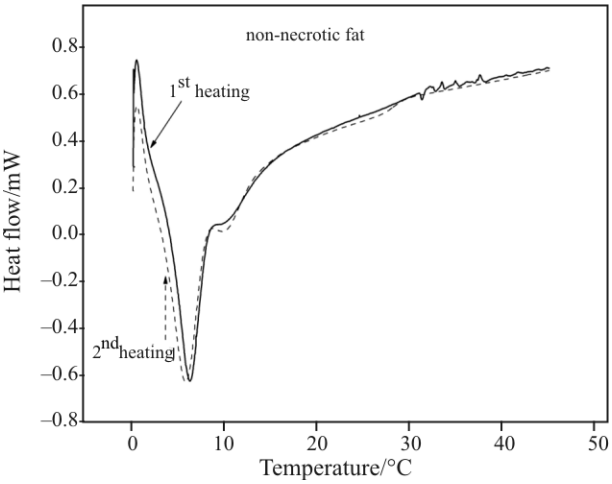

Fig. 2a Characteristic DSC heating curve of control sample (heating: 0–100°C)

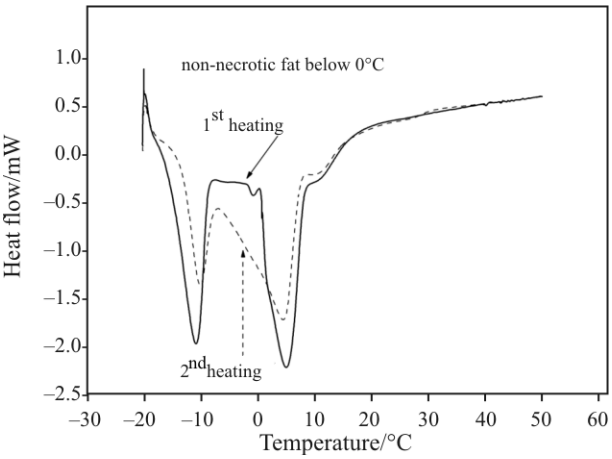

Fig. 2b Characteristic DSC heating curve of control sample (heating: –20–100°C)

tissues (Table 1). In case of (some) a patient(s) unfortunately we have to perform a second surgical intervention too within two years. A typical result can be seen in Figs 4a and 4b that demonstrate further change in the fatty acid composition, mainly in those components that can melt above 0°C.

Our understanding of the pathogenesis of the non-traumatic variants of ON is incomplete [20]. More than ten hypotheses have been proposed. Fat embolism, microfracture, intraosse-ous hypertension, vasculitis, and intravascular coagulation are some well accepted theories [5–9].

Glucocorticoids have been the focus of studies on the pathogenesis of osteonecrosis. Although statistical data show that steroids may be implicated in one-third of all cases of ON, the precise mechanism of action of the steroids have not been determined [21–28]. The increased use of steroids for immunosuppression after organ transplantation, as treatment for autoimmun diseases, and for chemotherapy has resulted in an increased risk of osteonecrosis [10–17]. It is difficult to determine whether ON is related to the disease process or its treatment. Multivariate analysis

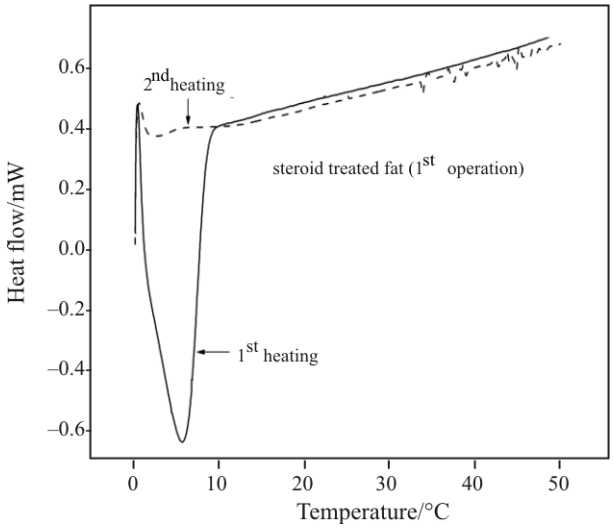

Fig. 3a Typical DSC heating curve of sample from steroid treated patient (heating 0–100°C)

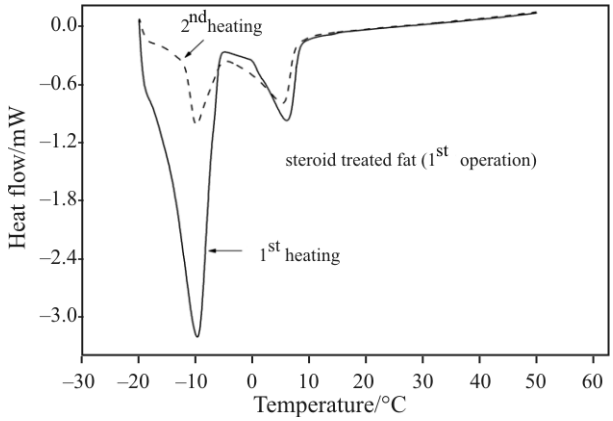

Fig. 3b Typical DSC heating curve of sample from steroid treated patient (heating –20–+100°C)

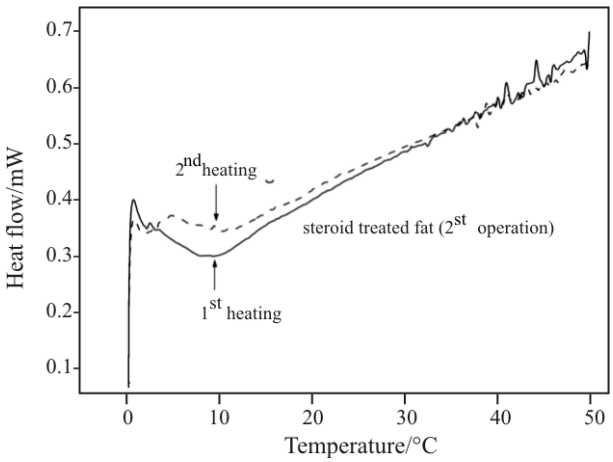

Fig. 4a DSC scan of steroid treated patient after reoperation within 2 years (0–100°C)

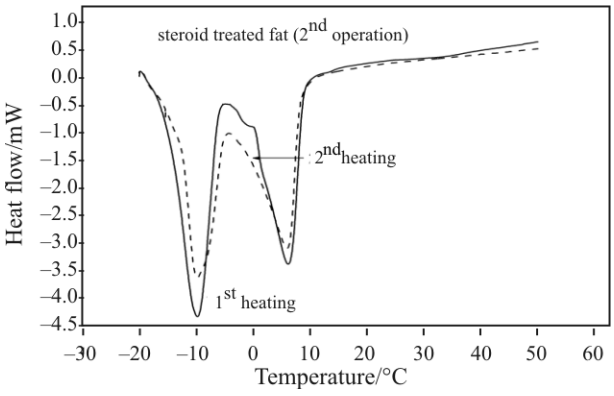

Fig. 4b DSC scan of steroid treated patient after reoperation within 2 years (–20–+100°C)

Table 2 Thermal parameters: T_ms are melting temperatures, ΔH stands for calorimetric enthalpy normalised for mass (data are average±s.d.)

	No. of samples	0–100°C	–20–100°C
Steroid tr.	5	T_m=5.7±0.45°C, ΔH=–15.8±1.2 J g^{-1}	T_{m_1}=–9.96±0.9°C, ΔH_1=–59.17±3.7 J g^{-1}, T_{m_2}=5.85±0.49°C, ΔH_2=–16.2±1.51 J g^{-1}
Control	5	T_{m_1}=5.7±0.4°C, T_{m_2}=9.9±0.9°C, ΔH=–20.8±0.17 J g^{-1}	T_{m_1}=–10.9±1.1°C, T_{m_2}=4.95±0.39°C, ΔH=–75.8±6.9 J g^{-1}

has suggested that high dose steroid use is an independent variable despite such confounding factors [1].

Steroid treatment produces a hyperlipidaemic state that often leads to osteoporosis but not usually osteonecrosis and it is not possible to predict which patients of the many treated with steroids will develop ON. While a minimal dose and duration of steroid is necessary to cause osteonecrosis, the amount has not been determined and there is marked patient variability. Between 5 and 10% of those receiving high dose steroids go on to develop osteonecrosis of the hip [1]. What distinguishes those patients that do develop osteonecrosis from those that do not, has yet to be determined.

However, the regulation of adipogenesis may involve complex mechanisms and interactions between multiple regulatory elements [29–31]. Steroid induced adipogenesis and the systemic changes in fat metabolism are major contributors to steroid induced ON [32, 33]. Fat cell hypertrophy and abnormal metabolism of fat have been demonstrated both in patients who have ON and in animals that have been treated with steroids [5, 6]. Lipotoxicity of pathologic fat tissue in metabolic disease is a known phenomenon characterized by ectopic fat deposition in muscle and liver and deposition in the pancreas [34–39].

The authors suppose is, that the structural and biochemical changes in peripheral fat tissue observed in steroid treated patients can stand together with the pathogenesis of ON too. DSC is a validly efficient method for the demonstration of structural changes in pathology of human motive apparatus [40, 41]. To the authors knowledge no previous study analysed the chemical and thermal properties changes of peripheral fat tissue in steroid induced ON.

Present authors' results suggest that fat profile and thermal behaviour of the samples from steroid treated osteonecrotic patients are different from the control samples. Despite this, no definitive differences could be observed in microscopic picture of steroid treated sections in relation to control sections (Fig. 1). Based on this establishment, the differences are supposed to be on molecular level.

GC revealed by steroid treated patients a decreased amount of the main saturated fatty acids such as palmitic (16:0) and stearic (18:0) as well as the decrease of the main polyunsaturated fatty acid linoleic acid (18:2n6). The summary fraction of saturated fatty acids decreased from 24 to 21%, the polyunsaturated fraction from 20 to 14%, respectively. In case of the main monounsaturated fatty acids such as oleic (18:1) and palmitoleic acids (16:1) an increase can be observed. The total percentage of monounsaturated acids showed an increase from rate 52% of the controls to 65% of steroid treated samples.

We can not answer directly by our DSC investigation which fatty acid compounds in what kind of measure could explain the observed alterations in the thermal parameters, but it is clear that there is a significant relation between the GC and DSC results. In case of a twice operated patient, the thermal properties changes proceeded by tissue sample taken by second surgical intervention, which can probably indicate a progression in fat metabolism disorder. Further measurements are wanted to clarify this connection, e.g. determination of melting temperatures of pure fatty acid compounds and of their mixture in the same rate as they can be found in the control and steroid treated patients.

Conclusions

The results of the authors are rather tendentious. The DSC analysis associated with DC supposed to be a feasible method in the investigation of thermal and chemical consequences of the fat tissue samples in ON induced by different risk factors. Further investigations are needed, with higher sample rate and under other anamnestic circumstances, to better identify the ethiology and the possible pathological pathways leading to ON.

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