Diet-induced obesity skin changes monitored by \textit{in vivo} SHG and \textit{ex vivo} CARS microscopy

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Abstract: Obesity related metabolic syndrome and type 2 diabetes have severe consequences on our skin. Latest developments in nonlinear microscopy allow the use of noninvasive, label free imaging methods, such as second harmonic generation (SHG) and coherent anti-Stokes Raman scattering (CARS), for early diagnosis of metabolic syndrome-related skin complications by 3D imaging of the skin and the connective tissue. Our aim was to study effects of various types of diet-induced obesity in mice using these methods. We examined mice on different diets for 32 weeks. The collagen morphology was evaluated four times \textit{in vivo} by SHG microscopy, and adipocytes were examined once at the end of experiment by \textit{ex vivo} CARS method. A strong correlation was found between the body weight and the adipocyte size, while we found that the SHG intensity of dermal collagen reduces considerably with increasing body weight. Obese mice on high-fat diet showed worse results than those on high-fat - high-fructose diet. Animals on high-fructose diet did not gain more weight than those on ordinary diet despite of the increased calorie intake, but their collagen damage was nonetheless significant. Obesity and high sugar intake damages the skin, mainly the dermal connective tissue and subcutaneous adipose tissue, which efficiently can be monitored by \textit{in vivo} SHG and \textit{ex vivo} CARS microscopy.

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1. Introduction

Metabolic syndrome (MS) has become a major public-health issue worldwide with significant clinical importance and a steady increase. It is diagnosed when three of the following metabolic or physical conditions are present: hypertension, central obesity, dyslipidemia and insulin resistance. These factors directly increase the risk of type 2 diabetes and cardiovascular diseases, such as atherosclerosis and ischemic heart disease [1, 2]. The prevalence varies worldwide as obesity depends on social relations, culture, environmental,
demographic and economic conditions. According to the estimation of International Diabetes Federation, one-quarter of the world’s adult population has MS [3]. The “obesity epidemic” is principally driven by an increased consumption of fat and sugar rich, calorie-dense foods, called as „Western-type“ diet [4].

Besides increased risk for cardiovascular diseases and type 2 diabetes, obesity and diabetes-related skin complications must not be ignored. In the case of diabetes almost half of patients suffer from dermatological complications. The most common skin manifestations of DM are impaired wound healing, foot ulcer, also known as “diabetic foot” and premature aging of the skin. In the background of these diseases we find metabolic, immunologic, circulatory and innervation disorders [5].

Adipose tissue, apart from adipocytes, also contains other cell types, such as pre-adipocytes, fibroblasts, macrophages and endothelial cells [6]. In obesity not only the visceral, but also the subcutaneous adipose layer is increased (hyperplasia) and adipocytes become enlarged (hypertrophy). This leads to secretion of free fatty acids (FFA), such as palmitic acid and inflammatory cytokines that counteract the effect of insulin and promote insulin resistance [7, 8]. Furthermore, FFAs and inflammation induce dermal collagen degradation by increasing matrix- metalloproteinase-13 and decreasing collagen-1-alpha and elastin gene expressions. Fibroblast proliferation is also decreased, which suggests that thickened subcutaneous fat layer negatively controls the function of dermal fibroblasts [8]. It was also demonstrated that besides cells in the connective tissue, elevated glucose level and abnormal insulin signaling also inhibit the proliferation and differentiation of keratinocytes [9, 10].

Another important diabetes related biochemical process is tissue glycation. It is a spontaneous non-enzymatic reaction between free amino groups on long-lived proteins and carbonyl groups of reducing sugars. These lead to formation of advanced glycation end products (AGEs) [11]. Accumulation of AGEs is a physiologic process during intrinsic aging, however in case of diabetes it is significantly accelerated. Increased glycation and accumulation of AGEs in various tissues and serum have an important role in the pathogenesis of diabetic complications [12]. Most common AGEs, such as pentosidine, pyrroline, and N-carboxymethyl lysine are endogenous fluorophores that allows their measurement by noninvasive autofluorescence microscopy. Lutgers et al. previously described that skin autofluorescence was significantly higher in type 2 diabetic patients compared to controls in different age groups. Nevertheless, this technique has some limitations, since it is not capable of measuring non-fluorescent AGEs, furthermore there is an overlap between excitation spectrums of other fluorophores, such as amino acids, e.g. tryptophan [13].

The above mentioned restrictions may be overcome by novel nonlinear optical microscopic techniques, which were rapidly evolving over the past two decades. These 3D imaging methods include two-photon excitation fluorescence (TPEF) [14, 15], second harmonic generation (SHG) [16–19] and coherent anti-Stokes Raman scattering (CARS) microscopy [20]. A common feature of these methods is the laser source operating in the near-infrared (NIR) spectral range. This enables a deep penetration into the living tissue and high-resolution in vivo imaging. Investigating the skin is an obvious choice because it contains several endogenous chromophores in its different layers that can be excited in the spectral range of 700-1300 nm [17]. Based on these characteristics, nonlinear microscopy is suitable for label-free in vivo analysis of the skin.

In our present work we aim to identify and follow the effects of different diets to murine skin. Our expectation was that both collagen and the adipocytes would show changes, and we can visualize both by combined in vivo SHG and CARS microscopy. It is well known that three-dimensional in vivo structures of well-ordered protein assemblies, such as collagen, microtubules and muscle myosin are suitable for SHG imaging [16]. By now, in vivo SHG imaging of the dermal collagen has become a widely used and approved technique in
dermatology [15, 17, 18] and cosmetology [19]. According to previous studies [15, 17], we expect that purely dermal collagen and fibroblast cells can generate SHG signal in the skin. In principle, label-free, two-wavelength CARS microscopy is suitable for \textit{in vivo} visualization of adipocytes if the frequency difference of the pump and Stokes wavelengths are properly set to the strong vibration resonance of fatty acids at 2845 cm$^{-1}$ [20, 26]. However, during the experiment it became evident that we could not perform CARS imaging \textit{in vivo} in the depth required, that is why full-thickness punch biopsies were taken from identical dorsal locations and \textit{ex vivo} CARS imaging was performed once at the end of the experiment.

We think that nonlinear optical imaging techniques can provide valuable information about the connective tissue alterations and adipocyte sizes at an early stage, prior to the formation of visible lesions. By this experiment, we would like to demonstrate the benefits of this technology and advocate for its use as a fast and accurate detection of diabetes- and obesity-associated dermatological disorders.

2. Materials and methods

2.1 Animals, diets and imaging methods

Six-week-old female C57BL/6J mice were purchased from Charles Rivers Laboratories (Sulzfeld, Germany). Mice were randomly divided into four groups. Each group consisted of five mice that were kept on different \textit{ad libitum} diet for 32 weeks. For induction of obesity the first group was fed with high fat diet (HFat), using chow that contained 30% fat (SSNIFF, Germany). The second group received drinking water with 20% fructose and a standard chow for the induction of skin glycation processes as a consequence of high fructose intake (HFru). Fructose concentration was defined based on former \textit{in vivo} studies [21, 22]. As a model of unhealthy „Western diet” the third group was fed with high fat chow (30%) and 20% fructose enriched drinking water (HFHF). A fourth group of mice were kept on ordinary diet and served as controls. The body weight was recorded once weekly during the experiment. Our experimental protocol was submitted to and approved by the Semmelweis University Animal Care and Use Committee.

Alterations of dermal collagen were followed by \textit{in vivo} SHG imaging. Measurements were performed four times throughout the experiment. At first, mice were anesthetized with intraperitoneal injection of 1.2% Avertin (Sigma Aldrich, Hungary) solution. The dorsal hair was removed by shaving and plucking, prior to each measurement to avoid leaving residual hair behind that may potentially interfere with the imaging. The use of chemicals for depilation was intentionally not considered to prevent non-specific signals during measurements. After hair removal mice were secured in a custom designed mouse restrainer in order to ensure the precise positioning of the examined skin areas. A drop of distilled water, as a liquid contact medium, was spilled on the hairless dorsal skin and then covered with a glass slide. Glass covered dorsal skin area was placed under the microscope objective and measured multiple times. The location of dorsal skin was chosen in each measuring time approximately at the same region of the base of the tail in order to avoid the breathing movements. For each mouse, five z-stack images were recorded and analyzed. The imaging area was 410 x 410 μm$^2$.

To visualize the adipocytes, we used CARS imaging method once during the experiment at week 32. Currently this technology allows \textit{ex vivo} imaging only, since reaching a sufficiently deep penetration requires a laser energy that would be harmful for the animals if performed \textit{in vivo}. Therefore 4 mm full-thickness punch biopsies were taken from the dorsal skin after mice were euthanized. Images were recorded on samples placed upside-down, in the direction of hypodermis to epidermis, in order to achieve a better image quality. After CARS measurements samples were fixed in 4% buffered formalin, embedded in paraffin and sections were stained with hematoxylin-eosin. Additionally, van Gieson staining was used to demonstrate the changes in collagen morphology. Two microscopic fields of view were
captured from each section per mouse. Dermal thickness was evaluated on van Gieson stained sections by using Digimizer Image Analysis Software (Ostend, Belgium). The thickness was measured in fifteen different points per area in each fields of section regarding one mouse and then mean thickness and standard deviation of each mice were calculated. The perimeter of adipocytes was evaluated similarly with measuring twenty cells per area.

2.2 Imaging setup

The optical setup used for combined SHG/CARS imaging of the skin is shown in Fig. 1. 3D microscopic images were taken by a commercial *Axio Examiner LSM 7 MP* laser scanning 2P microscope (Carl Zeiss, Germany), the detection optics of which was slightly modified for background free SHG imaging of the collagen and CARS imaging of the adipocytes. A broadly tunable, femtosecond pulse Ti-sapphire laser (*FemtoRose 100TUN NoTouch*, R&D Ultrafast Lasers Ltd, Hungary) operating at 796 nm was used for SHG imaging of collagen. The laser delivered nearly transform limited, $\tau_{\text{FWHM}} \approx 190$ fs pulses at a $\approx 76$ MHz repetition rate. It was also used as a pump beam for the CARS measurements. An inherently synchronized, two-stage Yb-fiber amplifier unit (*CARS Stokes Unit*, R&D Ultrafast Lasers Ltd, Hungary) generated the Stokes pulses at 1028 nm with a FWHM bandwidth of $\approx 10$ nm for CARS imaging of adipocytes. Optical pulses of the Yb-amplifier were compressed by a grating pair compressor. Synchronization (i.e., temporal overlapping of the pump and Stokes pulses) and spatial overlapping of the pump and the Stokes beams is assured by a *FemtoCARS Laser Unit* (R&D Ultrafast Lasers Ltd, Hungary). Average powers of the two laser beams measured at the sample surface were $\approx 20$ mW and $\approx 10$ mW for the Ti:sapphire laser and the Yb-amplifier, respectively. The SHG signal of collagen and the CARS signal of adipocytes were efficiently separated between the two NDD detectors by a long-pass dichroic beamsplitter (LP555). The signal to noise ratio was improved by narrowband ($\Delta \lambda \approx 10$ nm) bandpass filters respectively centered at around the SHG signal wavelength for collagen ($\lambda \approx 398$ nm) and the anti-Stokes signal wavelength of adipocytes ($\lambda \approx 649$ nm). This latter wavelength corresponds to the CH$_2$ symmetric stretching vibration resonance of saturated fatty acids in our setup. Further details on the optical setup can be found in Ref [23].

3D images of the skin samples were obtained by computer controlled accurate positioning of the objective along the z-axis (“z-stack images”), which enabled us imaging the samples in different tissue depths. The laser beams were focused by a 20x, water immersion objective.
(W-Plan – APOCHROMAT 20x/1.0 DIC (UV) VIS-IR, (Carl Zeiss, Germany), which resulted in an approximately 0.6 x 0.6 mm² imaging area and a ~0.5 µm spatial resolution in the x-y direction, while 1.5 µm in the z direction.

2.3 Statistical analysis

On graphs bars represent the means and error bars show the standard deviations (SD). We used MedCalc and OriginPro statistical software (Microsoft Office USA, Real Statistics, Charles Zaiontz USA, MedCalc Belgium, OriginLab USA). Groups were compared with one or two-way ANOVA, as applicable.

3. Results

3.1 Body weight of mice

Mice in HFat group had significantly higher body weight from week 12 on (p <0.05), compared to controls (see Fig. 2). At the end of the study their weight was fifty percent higher than the weight of controls and mice in HFru group. Interestingly, the weight increase of mice kept on high fat and high fructose diet was more modest than mice in high fat group, yet the difference reached a significant value by week 16 (p <0.05) when compared to controls. The body weight of animals in the high fructose group was relatively constant during 32 weeks; the increase was similar to control mice: their body weight was significantly lower than HFat and HFHF group from week 24 on.

![Graph A: Increase of body weight in mice groups during 32 weeks.
Graph B: Mean weight of mice in various groups at week 32.](image)

Fig. 2. A: Increase of body weight in mice groups during 32 weeks. B: Mean weight of mice in various groups at week 32. Week 0, 8, 16 and 32 indicate the number of weeks passed after the beginning of different diets. Statistical significance level of differences was performed using two-way ANOVA. *p<0.05 compared with control, #p<0.05 compared with HFat, †p<0.05 compared with HFru group ‡p<0.05 compared with the same group at week 8. The error bars represent the standard deviation (SD).

3.2 Changes in collagen morphology

At the first time point of measurement, at week 8, we observed significant differences in SHG intensity between the groups on various diets compared to controls (see Fig. 3). However, we must note that during this measurement several mice were in anagen phase of their hair growth cycle. For this reason, the quality of most of the SHG images was somewhat unsatisfactory, thus these results should be considered with great care. At week 16, skins of mice in HFat and HFHF groups showed significantly lower SHG intensities than controls. In addition, HFru-diet fed mice displayed a moderate but not significant decrease.
Fig. 3. A: 3D SHG images show the structure of dermal collagen of one representative mouse from each group. B: Results of calculated SHG intensity. Week 8, 16 and 32 indicate the number of weeks passed after the beginning of different diets. The degradation of dermal collagen in HFat and HFHF groups are clearly visible from week 16 as well as in HFr u group at week 32. The images were projected in the z-direction and were captured from skin surface to deeper layer of the dermis (z = 70 µm). Imaging area is ~410 x 410 µm². The results of SHG intensity changes determined by WCIF ImageJ software. Statistical analysis was performed using two-way ANOVA. *p <0.05 compared with control, #p<0.05 compared with HFat group, §p<0.05 compared with the same group at week 8, †p<0.05 compared with the same group at week 16, ‡p<0.05 compared with the same group at week 24. Error bars represent the standard deviation (SD).
At week 32, as the animals in the HFat group almost doubled their body weight, the mean integrated SHG intensity decreased significantly compared to the previous time points. Mice in HFru group were found to have a moderate decrease in SHG intensity in each measuring sessions compared to control mice, however it was only the last two evaluations when they reached the level of statistical significance. In addition, during the last measuring session, their SHG intensity was significantly higher than the HFat group. The collagen structure of control mice was relatively normal and fiber-rich all through the 32 weeks of the experiment. The HFat and HFHF-diet-fed mice had degraded dermal collagen morphology along with the degree of obesity. In HFru group the changes of collagen fibers were clearly visible, but not drastic until the last measuring time point.

3.3 Changes in sizes of adipocytes

On ex vivo CARS images differences in sizes of adipocytes are clearly seen among the various groups of mice (see Fig. 4). Images shown are in orthogonal view, where the sizes of adipocytes may be seen in two dimensions. In addition, the third axis with the thickness of cells is also visible to demonstrate a 3D-like appearance. We can see that fat cells of HFat- and HFHF-induced obese mice were much larger than in the other groups. The thickness of subcutaneous adipose layer of these mice was nearly double than the control mice. HFru-diet-fed mice did not have larger adipocytes than controls, which is not surprising, given that their body weight was nearly the same.

Fig. 4. CARS images show the size of adipocytes of one representative mouse from each group. The differences between size of adipocytes and thickness are clearly visible. The images were projected in the direction of hypodermis to epidermis in order to better image quality. Imaging area is ~212 x 212 µm².

3.4 Histology

The size of adipocytes was significantly larger in mice of HFat and HFHF groups compared to control and HFru-diet-fed animals (Fig. 5(A)). Furthermore, the degradation of dermal collagen in mice of HFru, HFat and HFHF groups was obvious, while the control sections showed normal collagen morphology. The dermal thickness showed no significant difference between various groups. However, the perimeter of adipocytes was significantly higher in HFat and HFHF compared to controls and in HFat compared to HFru mice (Fig. 5(B)).
4. Discussion

Metabolic processes in obesity and diabetes have already been extensively elucidated by invasive methods and molecular biological experiments. In our present study, we set out to investigate and follow these processes \textit{in vivo} by SHG microscopy and \textit{ex vivo} by CARS technique. In this work, we described the major morphological features and changes of murine skin, including dermal collagen alteration and enlargement of subcutaneous adipocytes and the effect of various diets. We observed significant differences in body weight among mice in various diet groups as early as 12 weeks. Along with the degree of obesity, our results indicated a definite decrease of SHG intensity mainly in HFat group, where mice had almost a double body weight compared to that of the control group. The 20% fructose solution showed no effect on body weight, however, dermal collagen glycation and degradation were clearly visible at the end of experiment. Also, the adipocyte diameter in this group remained unchanged. Therefore, high fructose diet affected dermal collagen through a pathway that could be independent from the enlargement of adipose tissue. Interestingly, the combination of high fat and high fructose diet, which represented the “Western diet”, in contrary to our expectation, has not caused significantly higher changes either in body weight or in collagen degradation than the high fat diet per se. Indeed, it seems that the high fat component of “Western diet” might play the major part of its effects on the skin. Although high fructose can induce degradation over time on its own, whereas it seems that it does not have a synergic effect when combined with high fat intake.

These findings were also confirmed by histological evaluations (Fig. 4). The visualization of dermal changes, due to the altered metabolism, was carried out by using \textit{in vivo} SHG and \textit{ex vivo} CARS techniques. Our results demonstrated that the obesity- and glycation-related skin alterations can be visualized \textit{in vivo} with high sensitivity. However, we must note that these imaging methods still have some limitations: during anagen hair cycle \textit{in vivo}, high resolution imaging is difficult due to the strong scattering of focused laser beam on hair pigments. Therefore an efficient hair removal is indispensable. Furthermore, during \textit{in vivo} measurements, fluctuation of laser power may occur, which leads to reduced SHG intensity.
At present, our CARS imaging system is only capable of \textit{ex vivo} measurement, thus this method requires surgery to remove skin samples. For this reason, the size of adipocytes was visualized only once, at the end of our experiments.

Nonlinear optical imaging techniques, such as TPEF, SHG and CARS have been widely used for imaging of various chromophores of the skin [24–26] and employed for the diagnosis of several skin diseases [27, 28]. Lipids do not show fluorescence after excitation and they are difficult to tag with fluorophores, however CARS microscopy is able to provide label free imaging based on vibration of \textit{CH}$_2$ bonds [29]. Recently CARS and SHG imaging methods have been successfully combined and used for imaging the structure of murine mammary gland and tumor stroma. Le et al. showed that collagen content of mammary gland decreased as adipocytes became larger in the glands of obese rats. Furthermore, the stroma of mammary tumors had higher collagen content in obese rats than the tumors of control animals. Their results suggested a direct correlation between obesity and extracellular matrix collagen content in mammary tumor stroma [30]. In another study Urasaki et al. examined the visceral adipose tissue of obese and lean mice. They demonstrated that CARS, TPEF and SHG label-free imaging could be powerful complements to biochemical assays for studying the functions of adipocytes [31]. Currently the number of \textit{in vivo} human studies with CARS technology is limited. Huang et al. investigated the adipose tissue on freshly excised human skin \textit{ex vivo} using SHG and TPEF imaging methods [32]. König et al. combined the \textit{in vivo} CARS and TPEF imaging techniques and investigated the properties of healthy and psoriasis-affected human skin. They found that the psoriasis-affected and healthy skin show different lipid distribution in the layers of epidermis [33]. The study also suggests that \textit{in vivo} CARS imaging technique is suitable for investigation of skin barrier functions, however, for imaging of subcutaneous lipids further technical developments are required.

We conducted our study on mice, but similar experiments can be performed in the future on humans also with our novel, Yb-fiber laser based, handheld TPEF/SHG microscope imaging system [34]. The system works at 1 micron, therefore the laser beam can penetrate deeper into the skin and can be used without the risk of provoking photochemical or thermal damage to the skin.

5. Summary

In our study we described the major features and morphological changes of the obese/diabetic murine skin \textit{in vivo}. It was also demonstrated that nonlinear microscopy is capable of detecting lipid molecules and collagen with high sensitivity. Our results confirmed previous findings that obesity and tissue glycation accelerates the degradation of dermal collagen, which may promote the formation of diabetes-related skin lesions. We demonstrated that these complex metabolic processes can be monitored accurately by the use of \textit{in vivo} SHG and \textit{ex vivo} CARS microscopy that could be useful imaging tools for early diagnosis of diabetes-related skin lesions.

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