Effects of obesity and physical exercise induced weight loss on murine skin connective tissue monitored by in vivo nonlinear microscopy

Doctoral thesis

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Introduction

Due to the increasing incidence of obesity, metabolic syndrome and diabetes, dermatological complications are also becoming more frequent. The most severe consequences include susceptibility to infections, wound healing disorders and ulcerative skin lesions of the lower extremities. Emergence of these pathologies could be the result of connective tissue remodeling. In case of diabetes, the latter is a consequence of proinflammatory mechanisms provoked by subcutaneous adipose tissue enlargement and tissue glycation. Epidermal and dermal tissue could be noninvasively examined by multiple next generation imaging techniques, one of which is nonlinear microscopy. Nonlinear microscopic techniques including second harmonic generation (SHG) is capable of visualization of molecules with high structural regularity, such as the collagen content of the dermis, while coherent anti-Stokes Raman scattering (CARS) can be utilized for selective imaging of lipid molecules.

Our research team participated in a consortium where an innovative device was developed, which is capable of *in vivo* nonlinear imaging. This thesis will discuss murine skin connective tissue alterations due to obesity and sport induced weight loss monitored by nonlinear microscopy.

Aims

In the first long term experiment (I.), leptin deficient ob/ob mice were fed either ad libitum or in a calorie restricted manner. Ad libitum fed C57/BL6 mice served as controls. Collagen structure and adipocytes were examined by *in vivo* SHG and *ex vivo* CARS imaging techniques. Our aim was the identification and description of structural changes in the skin of obese mice. The secondary endpoint was to improve and fine-tune the imaging system for optimal performance.

In the second experiment (II.) C57/BL6 mice were kept on a high fat-, high fat and fructose- or high fructose diet. Mice on normal chow served as controls. Collagen structure and adipocyte sizes were followed by *in vivo* SHG and *ex vivo* CARS techniques. Our aim was to detect, describe and monitor the collagen structure and adipocyte size alterations. We also aimed to demonstrate the benefits and imaging capabilities of our nonlinear microscope system, which can potentially be capable of *in vivo* clinical diagnostics in dermatology.

In our third experiment (III.) wild type obese mice on high fat diet were compared to lean controls on normal chow. Daily voluntary exercise was introduced to the groups for 8 weeks. Our aim was to analyze and follow the collagen structure changes before and after the 8-week long exercise period. We hypothesized that weight loss and sport lead to improvement of the connective tissue in the dermis. We set out to demonstrate this with *in vivo* SHG measurements and *ex vivo* histology.

Methods

Mice and diets

I. experiment:

Three groups were examined during the 30 weeks of the experiment: 1. ob/ob-ND no diet group: ad libitum access to chow and water for ob/ob type mice (n=2). 2. ob/ob- CRD calorie restricted diet group: calorie restriction for ob/ob type mice (n=2). 3. control: ad libitum chow and water for wild type C57/BL6 mice (n=2). Weight changes were registered weekly.

II. experiment:

20 wild type C57BL/6 mice were investigated during the 32 weeks of experiment. Mice were divided into 4 groups: 1. Hfat - high fat group: ad libitum access to high fat (30%) containing chow (n=5). 2. HFru - high fructose group: ad libitum access to normal chow and 20% fructose containing drinking water (n=5). 3. HFHF - high fat – high fructose group: ad libitum access to high fat (30%) chow and 20% fructose containing drinking water (n=5). 4. control group: ad libitum access to normal chow and water (n=5).

III. experiment:

Wild type C57BL/6 mice, divided into two groups, were investigated during the experimental period: 1. HF - high fat group: ad libitum access to high fat (30%) containing chow (n=5). 2. CO –control group: ad libitum access to normal chow and water (n=4).

Voluntary exercise

III. experiment:

High-fat diet was introduced when mice became 8-week-old, then voluntary exercise was initiated when the mice reached the age of 40 weeks. Mice in both groups spent 40 min daily in a hamster wheel for 8 weeks. Weight measurements were taken weekly. After each exercise period, distances covered by the mice have been recorded by a calibrated bicycle computer.

Nonlinear microscopy, SHG/CARS imaging

A nonlinear microscope system capable of combined *in vivo* SHG and *ex vivo* CARS measurements was utilized during the project. This device was developed under the supervision of Robert Szipőcs, PhD at the Hungarian Academy of Science's Wigner Research Centre for Physics.

The quantification of collagen damages was carried out by measuring the intensity of SHG signal converted to integrated optical density by Image J software. The amount of unimpaired collagen in the skin correlates with the proportion of integrated optical density. Adipocytes were pictured through visualizing the symmetric stretching vibration resonance of CH_2 groups in saturated fatty acids by CARS technique.

A broadly tunable, femtosecond pulse Ti:S laser (FemtoRose) operating at 796 nm was utilized for *in vivo*

SHG imaging and images were captured by an Carl Zeiss LSM 7-MP laser-scanning microscope. Special feature of the combined system was, that the FemtoRose was also used as a pump beam for the CARS measurements. An inherently synchronized, two-stage Yb-fiber amplifier unit generated the Stokes pulses at 1028 nm for *ex vivo* CARS imaging of adipocytes. 3D images of the skin samples were obtained by computer controlled accurate positioning of the objective along the z-axis (Z-stack), which enabled us imaging the samples in different tissue depths. The laser beams were focused by a 20x, water immersion objective, 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.

In vivo SHG images were taken in the same sessions under i.p. anesthesia with the use of 1.2% Avertin solution. Before measurements, hair was removed from the dorsal areas with an electric clipper. During evaluation, mice were placed into a restrainer to prevent movement. 6 × 6 mm large regions of interest (ROI) were marked on the back of the mice. In the first two experiments, the same dorsal location on each mouse was used during the four sessions. At the third experiment ROIs were marked on both sides on the back of the mice, 5 mm away from the spine. Images captured at Week 0 from one ROI were used as baseline; then at Week 8, images with the same microscopic setup were taken from the opposite side. Punch biopsy samples were removed form identical locations after the final measurements in the

first two experiments. *Ex vivo* CARS measurements were performed on the skin samples before formalin fixation. Currently this technology allows *ex vivo* imaging only, since reaching a sufficiently deep penetration requires a laser energy that would be harmful for the animals if performed *in vivo.* At the third experiment samples were obtained at week 0 and 8 from the above-mentioned ROIs for before- after comparisons, but we did not perform CARS measurements.

At the first experiment, we took very high resolution images, counting 1024 x 1024 pixeles, during optical sectioning. Acquiring 5 Z-stacks almost needed one hour with this setup. For decreasing possible side effects of general anesthesia picture resolution was decreased to 512 x 512 pixels. For this reason, imaging area was also somewhat reduced but this did not influence the diagnostic accuracy.

Histopathological analysis

After SHG imaging samples were obtained by punch biopsy under general anesthesia. 4-μm sections of formalin-fixed and paraffin embedded samples were stained with hematoxylin-eosin and collagen-specific Weigert van Gieson's staining. The thickness of dermis, subcutaneous adipose tissue, the size of adipocytes and the collagen density in the III. experiment, were examined by digital image analyses.

Statistics

During the first experiment we were not able to perform reliable statistical analysis due to the low number of mice. In the II. and III. experiments normal distribution was confirmed by Shapiro-Wilks test. Groups were compared with one or two-way repeated measures ANOVA, as applicable. Tukey post hoc test was used to validate the data. $P < 0.05$ was considered as significant.

Compliance with ethical standards

All procedures were approved by national authorities (PEI/001/800-6/2015).

Results

Weight changes

I. experiment:

The ob/ob –ND knockout group kept on an ad libitum diet for 30 weeks had a notably higher body weight (84.56g) than the control (30.10g) and the calorie restricted groups (28.43g) at the end of the study. The weight gain in the ob/ob group was almost three times higher than in the other groups.

II. experiment:

Mice in HFat group had significantly higher body weight from week 16 on (35.85±5.3g), compared to

controls (26.4±2.61g). At the end of the study their weight (43±3.7g) was almost fifty percent higher than the weight of controls (27.35±1.87g) and mice in HFru group (28.33±2.2g). 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 (31.4±4.4g) 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.

III. experiment:

The weight of obese mice (44,92±5.57g) was significantly higher than controls (28.17±1.36g) at the beginning of the exercise period. A significant weight loss during the PE from week 5 on in the HF group (37.58±5.3g) has been noted. This significant weight difference between the two groups disappeared by Week 8, HF (31.1±1.66g), CO (27.57±1.65g). The shorter distance covered by the obese mice was probably due to their lower speed, since we did not observe any obvious rest during the 40 min exercise. In addition, there was no increase in the activity of the HF group over the 8-week experiment. On the contrary, the activity of control mice increased gradually. HF (1. week: 0.0756±0.08km, 8. week: 0.0766±0.078km), CO (1. week: 0.166±0.41km, 8. week: 0.092±0.026km).

SHG and CARS evaluation

I. experiment:

During the first three measuring sessions, at weeks 8, 16, and 24, we could not observe significant differences in the SHG intensities among the groups using the same laser and microscope parameters. It was noted, however, that the ob/ob—ND and ob/ob—CRD groups had lower SHG intensity in each measuring time compared to the control group. At the final evaluation time, at week 30, when the ob/ob—ND group had almost tripled its body weight, the SHG intensity decreased remarkably when compared to the controls. The collagen morphology in the ob/ob—CRD group was normal and fiber-rich, as was in the control animals. The ob/ob—ND group displayed reduced dermal collagen content and the SHG intensity anti-correlated with the degree of obesity. Along with these notably enlarged adipocytes could be visualized with the *ex vivo* CARS technology

II. experiment:

At week 8, we observed significant differences in SHG intensity between the groups compared to controls. At week 16, 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. At week 32, 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 session compared to control mice. 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 severe until the last measuring time point.
On *ex vivo* CARS images we could see that adipocytes of HFat and HFHF groups were much larger than in the other groups.

III. experiment:

Before exercise, a significant difference in SHG intensities was seen between the two groups. SHG images revealed a decreased collagen density in the HF group. After exercise, this difference disappeared and the SHG intensity increased significantly in HF mice compared to baseline. The structure of the dermis and SHG intensities did not change in CO mice.

Histopathological analysis

I. experiment:

The adipocytes in the ob/ob—ND group were considerably larger (3678.06 μm2) compared to the control group (1429.12 μm2). Furthermore, along with the enlarged adipocytes in the ob/ob—ND group, the dermal thickness decreased greatly (115.2 μm) compared to

control group (210.42μm). There was no relevant difference between the control and ob/ob-CRD groups (dermis: 176.22 μm, adipocytes: 1712.35 μm2).

II. experiment:

The size of adipocytes was significantly larger in mice of Hfat (229.84±35.78 μm) and HFHF groups (213.58±42.19 μm) compared to control (155.38±20.34 μm) and HFru-diet-fed animals (155.17±37.8 μm). 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 the groups.

III. experiment:

We found a significantly lower collagen fiber density in the HF group (42.57±13.71) compared to CO (62.01±8.55), which showed a remarkable improvement after the PE period (HF: 63.47±8.45, CO: 72.24±11.66). In line with that finding, the dermis became significantly thicker in the HF group (HF BE: 131.94±35.8 μm, HF AE: 210.96±25.82 μm). Furthermore, SAT became thinner (HF BE: 371.3±121.5 μm, HF AE: 215.32±90.09 μm) and adipocytes shrunk significantly in the HF group as a result of exercise (HF BE: 80.45±11.68 μm, HF AE: 46.67±4.12 μm). In the HF group, all parameters examined changed to be similar to the control group after the 8-week exercise.

Conclusions

I. experiment:

- Collagen structure deterioration was successfully visualized *in vivo* with our SHG technique. We concluded that it was satisfactorily sensitive for detection of early changes in collagen structure. It is equally suitable for analysis and follow-up.
- The SHG technique was reliable, which may indicate that this technique could be safely used in clinical diagnostics in the future.
- The CARS system was not suitable for *in vivo* measurements due to technical limitations. Existing limitations on the imaging depth of the CARS system can be overcome by future development of the laser sources.
- We demonstrated that one can identify all major features and changes of skin connective tissue in obesity. We concluded, based on our experimental setup, that lifestyle is more important than genetic predisposition in obesity-related skin deterioration.

II. experiment:

- Early changes in collagen structure could be successfully monitored by *in vivo* SHG technique, however CARS measurements could be only done *ex vivo*, due to the aforementioned limitations. We must note that SHG signal could be influenced by intensive hair growth. For this reason, a proper preparation of the examined site is required.

- Obesity induced by HFat and HFHF diets underlined the significance of lifestyle behind weight gain.
- Behind the smaller weight change of HFHF and especially HFru groups, the appetite decreasing effect of fructose was suspected.
- The significant SHG intensity decrement in the HFru group was not accompanied by adipocyte enlargement nor weight gain. However connective tissue alteration was also demonstrated by histology. Behind these finding glycation processes were hypothesized.
- We concluded, that our nonlinear microscope system is suitable for the early detection of collagen structure alterations. Its sensitivity and specificity makes it appropriate for *in vivo* clinical diagnostics.

III. experiment:

- In line with our former experiments, significantly enlarged adipocytes, subcutaneous fat tissue and significantly decreased collagen density, *in vivo* SHG intensities were detected in the obese animals. We were able to visualize and follow the collagen structure changes during the experiment by *in vivo* SHG technique.
- We concluded, based on our experimental setup, that physical exercise plays a pivotal role in bodyweight control.
- By introducing PE, we were able to show a significant improvement in the quality of connective tissue along with the decrease in the amount of fat tissue and size of adipocytes. The novelty in this study was that favorable

effects of daily physical exercise on collagen fibers of the dermis could be demonstrated *in vivo* by SHG imaging.

- In conclusion, it appears that skin bears with great potential to return to its original state once positive changes take place. It also shows that from the skin's point of view it is never too late to start to exercise.

Publications

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