3D BIOPRINTED HUMAN ENDOTHELIALIZED ADIPOSE TISSUE AS A NEW PREDICTIVE MODEL FOR IN VITRO EVALUATION

Sandrine HERAUD, Amelie THEPOT, **Morgan DOS SANTOS***

LABSKIN CREATIONS, Edouard Herriot Hospital, Lyon, France

* Corresponding author: Morgan DOS SANTOS, PhD, LabSkin Creations, Edouard Herriot Hospital, 69003m Lyon France, +334 72 11 06 18, morgan.dossantos@labskincreations.fr

Abstract

Background: Human skin is a complex organ harbouring multiple layers, different systems and several distinct cell populations. Current available tissue-engineered skin models often only incorporate the epidermal and dermal layers, and lack of the hypodermis contributing to the mechanical and endocrine properties of the normal human skin. The hypodermis homeostasis is maintained thanks to a pool of precursor cells precommitted to differentiate into adipocytes: the adipose stem cells (ASCs). These cells are essential because mature adipocytes do not proliferate.

Although conventional tissue-engineering strategies led to the development in the past few decades of first generation of engineered hypodermis, such processes are usually time consuming, limited in the shelf-life and confined to the production of constructs having mostly flat and predetermined geometry. To overcome these limitation of conventional hypodermis tissue-engineering schemes, 3D printing combined with advanced tissue engineering represent a promising approach to reconstruct layer by layer a functional 3D human adipose tissue.

Using our previous findings, the aim of this work was to perfect an in vitro 3D bioprinted model of human adipose tissue including a pre-vascularisation by co-bioprinting endothelial cells and ASC.

Experimental Results: To conduct such research, adipose-derived stem cells combined with primary human hypodermal microvascular endothelial cells were mixed in a patented bioink composed of fibrinogen, alginate and gelatin and printed with optimal printing conditions

and printer functions. To allow the development of the microvascularisation of the bioprinted adipose tissue, the bioink was functionalized with a cocktail of crucial growth factors to maintain endothelial cells functionality. When applying the favorable printing technique and conditions, viable tissues were obtained, demonstrating high adipogenic differentiation and capillary-like network formation after 21 days of culture. Histological analysis showed that the printed adipose tissue was morphogically consistent with mature adipocyte cells forming droplets. More importantly, immunohistological analysis revealed the formation of an endothelial microvascular network expressing CD31 homogeneously distributed within the bioprinted adipose tissue. Confocal microscopy analysis further showed the formation of capillary-like structures with a clear lumen. Interestingly, the functionalization of the bioink with growth factors demonstrated similar results compared with their supplementation directly in the cell culture medium. Adipogenic modulators such as caffeine and oleic acid promoted lipolysis and lipogenesis, respectively, of adipocytes by modulating lipid content and adipocyte size, demonstrating the functionality of the 3D bioprinted model in vitro.

Conclusion: For the first time, we have developed a bioprinting method for the generation of endothelialized human adipose tissue with cellular and molecular characteristics closely resembling native human tissue. This unique in vitro model may be a relevant tool to explore molecular mechanisms underlying adipogenesis and to identify dermo-cosmetic active compounds.

Keywords: hypodermis; Adipose Stem Cells (ASCs); bioprinting; vascularization; adipogenesis; lipolysis

1. Introduction.

Human skin is a complex organ harbouring multiple layers, different systems and several distinct cell populations. Current available tissue-engineered skin models often only incorporate the epidermal and dermal layers, and lack of the hypodermis contributing to the mechanical and endocrine properties of the normal human skin[1,2]. The hypodermis homeostasis is maintained thanks to a pool of precursor cells precommitted to differentiate into adipocytes: the adipose stem cells (ASCs). These cells are essential because mature adipocytes do not proliferate[3].

Although conventional tissue-engineering strategies led to the development in the past few decades of first generation of engineered hypodermis, such processes are usually time consuming, limited in the shelf-life and confined to the production of constructs having mostly flat and predetermined geometry. To overcome these limitations, novel regenerative medicine technology-based 3D bioprinting approach, which combines 3D printing platform with advanced tissue engineering, provides a potential solution for the reconstruction of a functional 3D human adipose tissue.

In this view, our laboratory developed a 3D bioprinting approach to accurately deposit cells and biomaterials into precise geometries with the goal of creating physiological and functional biological adipose tissue. To improve efficiency of revascularization of the tissue, this work aimed to engineer an adipose tissue construct with microvascularisation by adding endothelial cells. To conduct such research, adipose derived stem cells combined with human dermal microvascular endothelial cells were mixed in a patented bioink composed of 3 biopolymers and printed with optimal printing conditions and printer functions[4]. To allow the development of the microvascularisation of the bioprinted adipose tissue, the bioink was functionalized with endothelial growth factors to maintain endothelial cells functionality.

To assess adipocytes functionality, adipogenic modulators promoting lipogenesis and lipolysis were tested on our bioprinted adipose tissue.

2. Materials and Methods.

ASC and endothelial cells isolation from human adipose tissue and amplification

Human ASCs were isolated from abdominal lipoaspiration of 4 healthy female donors (mean age 36 yo ± 12 years and body mass index 28.1 ± 2.1) undergoing aesthetic liposuctions following ethical and safety guidelines and in adherence with Helsinki Principles. ASCs were extracted and isolated according to the procedure described by Lequeux *et al.*[3]. Briefly, ASCs were then amplificated in 525 cm² triple cell stack flask at a density of 10,000 cells/cm² in proliferation medium composed of DMEM/Ham's F12 (Life Technologies) in proportion 1:1 supplemented with foetal bovine serum, basic fibroblast growth factor and antibiotics. When confluence was reached ASCs were detached using trypsin containing 0.01% EDTA (Invitrogen) for 5 minutes, amplified until passage 3 and frozen for cell banking.

Human hypodermal microvascular endothelial cells (HHMVEC) were also isolated from lipoaspiration abdominal adipose tissue. HHMVEC were extracted and isolated with the M131 Endothelial Medium (Life Technologies). When confluence was reached cells were detached using Trypsin EDTA for 5 minutes, amplified until passage 2 and frozen for cell banking.

3D Hypodermis Bioprinting

ASCs and HHMVEC were mixed within the developed bio-ink composed of alginate, gelatine, and fibrinogen, and printed in the optimum printing conditions and 3D printer functions. Each condition was printed in triplicate and skin print dimensions were 1,5 cm x 1,5 cm x 0,4 cm (width x length x height). These samples were cultured in optimized differentiation medium at 37°C in a 5% CO2 atmosphere. Induction of ASCs differentiation into adipocytes was initiated using specific differentiation-inductive medium containing optimized cocktail of growth factors and cytokines.

Functionality test with oleic acid treatment and Oil Red O assay

The oleic acid solution was prepared with 5% bovine serum albumin (BSA). Oleic acid was added to the culture medium and samples were then treated with this medium. Oleic-acid-treated samples were compared to the untreated samples.

For Oil red O assay, the hypodermis tissues were washed twice with PBS and fixed with 4% formaldehyde. They were then immersed in isopropanol and stained with Oil Red O solution thereafter at room temperature. After staining, the tissues were washed to remove unbound dye. For the measurement of total dye incorporated into cellular lipid droplets, isopropanol was added and the plates were gyrated at room temperature. Subsequently, 300µl of the extracted dye was transferred in triplicate in a 96-well plate, and the absorbance was measured with a spectrophotometer. Data were normalized by the cell viability measured with an Alamar Blue assay.

Histological and Immunohistological analysis

After 21 days of total cell culture, samples were immediately fixed in neutral buffered formalin 4% for 24h and embedded in paraffin or in OCT compound and frozen at -80°C.

Paraffin-embedded formalin-fixed samples were then cut into 5 µm sections. After dewaxing and rehydration, sections were stained with haematoxylin, phloxin and saffron (HPS) for routine histological analysis.

OCT-embedded samples were cut into 5µm thick sections and stained with Bodipy and counterstained with Hoechst.

For immunohistochemistry, after heat-mediated antigen retrieval treatment, tissue sections were incubated in 5% H_2O_2/3% BSA to inactivate endogenous peroxidases. Non-specific binding was blocked in PBS containing 5% of BSA. Sections were then incubated with the primary mouse monoclonal antibody FABP4 diluted in PBS/BSA 5% overnight at room temperature. After incubation for 1 h with the peroxidase-conjugated secondary antibody (EnVision, Dakocytomation, Glostrup, Denmark), FABP4 antigen was detected with 3,3-diaminobenzidine tetrahydro-chloride (Dakocytomation) as the substrate. Tissue sections were subsequently counterstained using Harris' hematoxylin (25%, Sigma Aldrich).

For immunofluorescence, non-specific binding was blocked in PBS containing 5% of BSA. Sections were then incubated with the primary monoclonal antibodies against perilipin and

CD31diluted in PBS/BSA 5% overnight at room temperature. For, secondary Alexa-conjugated antibodies (Molecular Probes, Invitrogen) were incubated 1 h at room temperature. Nuclear counterstaining using Hoechst was carried out routinely. As a negative control, primary antibody was replaced by the corresponding IgG class.

Image acquisition and analysis

Immunostainings and histology were observed using an Axioskop 2 Plus optical microscope (Zeiss, Le Pecq, France), and images were captured using Axiocam HRc camera (Zeiss) and Zen2 software (Zeiss). Sixteen-bit images were saved in an uncompressed tagged image file format (tiff). Nine representative images were captured for each condition.

Immunofluorescence specimens were visualized using an Observer Z1 optical microscope (Zeiss, Le Pecq, France), and images were captured using LSM700 laser scanning confocal system (Zeiss). Eight-bit images were saved in an uncompressed Zeiss image file format (lsm). Six representative z-stack images were captured for each condition in the same manner.

Image processing and analysis were performed using the software MBF_ImageJ for microscopy. Quantification was realized using an automated ImageJ macro.

Statistical analysis

All data are presented as mean values ± standard deviations. Statistical significance in the data was assessed running Student's t-test. Each set of data relates to a comparison versus untreated control. Statistical significant differences are indicated by asterisks as follows: *P < 0.05, **P < 0.01 and ***P < 0.001.

3. Experimental results.

a. Vascularized adipose tissue model

To reconstruct vascularized adipose tissue, adipose-derived stem cells combined with primary human hypodermal microvascular endothelial cells were mixed in our patented bioink composed of fibrinogen, alginate and gelatin and printed with optimal printing conditions and printer functions.

To allow the development of the microvascularisation of the bioprinted adipose tissue, we evaluated the effect of a cocktail of crucial growth factors to maintain endothelial cells functionality. We compared the effect of this cocktail supplemented directly into the culture medium or added into the bioink (functionalized bioink) with a not supplemented control. We first determined the favorable printing technique and conditions and obtained then viable tissues for these 3 different conditions as demonstrated by HPS histological analysis (Fig. 1, first line).

To evaluate the adipogenesis into the samples, we performed an immunostaining for fatty acid binding protein 4 (FABP4), demonstrating a high adipogenic differentiation for all conditions, with numerous cells containing large vacuoles expressing FABP4 on the edges (Fig. 1, second line). The lipids content was assessed through a bodipy staining, showing the printed adipose tissue contained many large lipids vacuoles (Fig. 1, third line).

Combined together, these data demonstrated that the adipose tissue were morphogically consistent with mature adipocytes forming lipids droplets.

More importantly, immunofluorescence analysis of endothelial marker CD31 expression revealed CD31-expressing endothelial cells, homogeneously distributed within the bioprinted adipose tissue (Fig. 1, fourth line). Interestingly, many endothelial cells were distributed into adipose tissue supplemented with the endothelial growth factor cocktail whereas only few were observed in the not supplemented adipose tissue. We performed an image analysis on CD31 expression to quantify the level of CD31 expression per cells into the tissues (Fig. 1, graph at the bottom). This quantification confirmed a significant higher CD31 expression in tissue supplemented with growth factors cocktail compared to the not

supplemented control. Furthermore, the functionalization of the bioink demonstrated similar results compared with the supplementation directly in the cell culture medium.

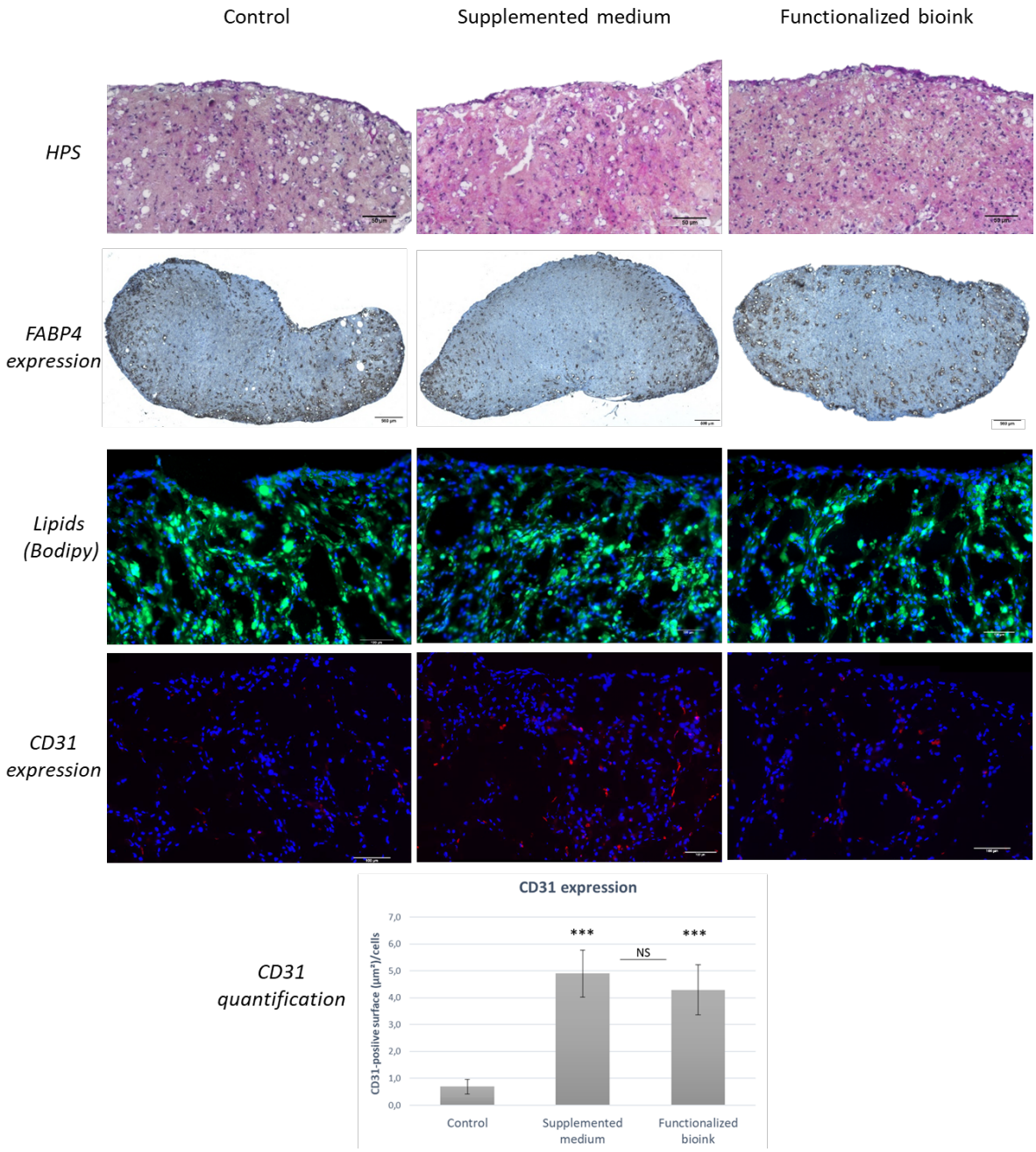


Figure 1: HPS staining, FABP4, CD31 immunostaining and bodipy staining of 3D vascularized adipose tissue reconstructed in a bioink +/- functionalized and cultured +/- with endothelial growth factors.

b. Observation of capillary-like structures into the vascularized adipose tissue

To further explore the vascular structure into the adipose tissue stimulated with the endothelial growth factors supplementation, we performed a co-staining of perilipin and CD31 by immunofluorescence revealed in yellow and red, respectively. Confocal microscopy analysis showed the formation of an endothelial microvascular network expressing CD31, surrounded by numerous mature adipocytes (Fig. 2). Furthermore, after 21 days of culture, the tissues demonstrated the formation of a capillary-like network and these capillary-like structures showed a clear lumen (Fig 2.).

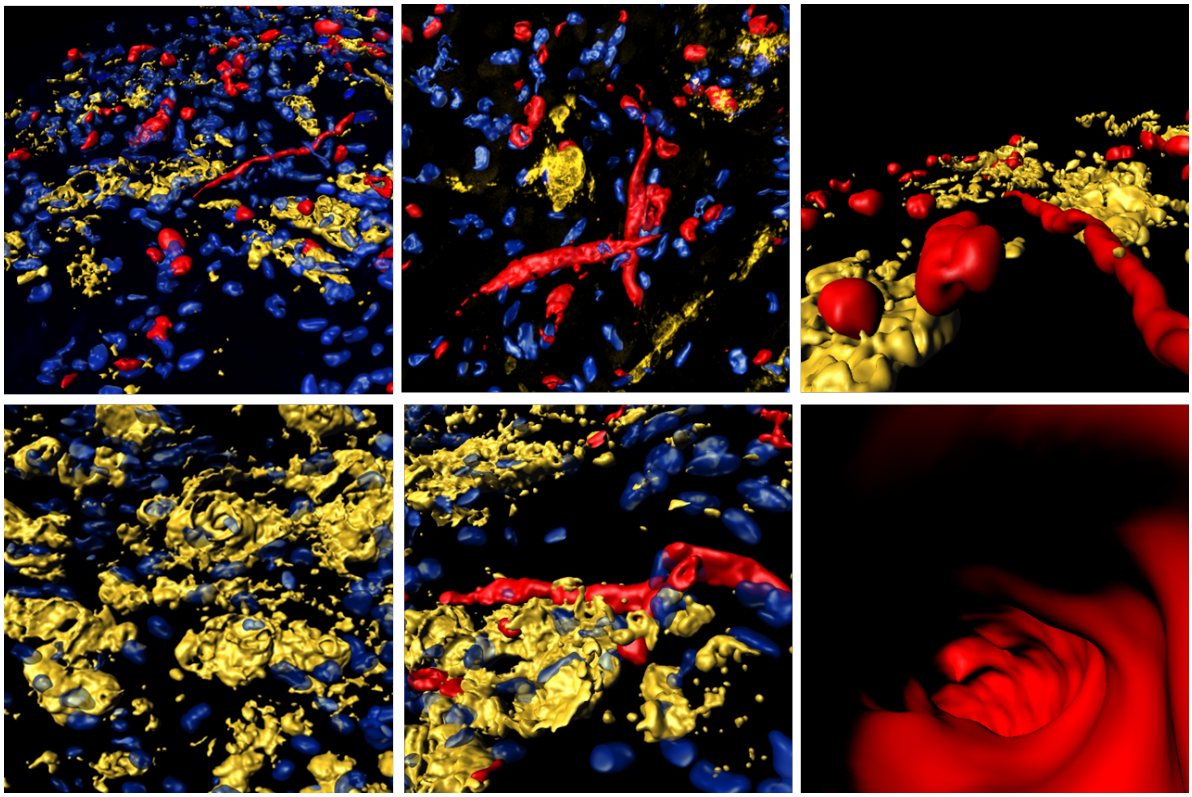


Figure 2: Immunofluorescence analysis of adipose tissue by perilipin (yellow) and CD31 (red) markers, observation under a confocal microscope. Dapi nuclear staining in blue. 3D visual reconstruction with Imaris software.

c. Functionality of the adipose tissue model

After demonstrating the consistent morphology of the adipose tissue model with the physiology, the following step was to assess its functionality through two of the essential functions of adipose tissue: lipogenesis and lipolysis. For this, we selected adipogenic modulators well described in the literature. For the lipogenesis study we added oleic acid to the culture medium.

As a control, we evaluated the effect of oleic acid on adipocytes cultured in 2D. The addition of oleic acid demonstrated a proper lipogenesis function with a significant increase of 96% of lipid accumulation, evaluated with Oil Red O staining and clearly visible on microscopic photos (Fig. 3). Adipocytes in the 3D bioprinted tissue also showed their ability to intake oleic acid with a significant increase of the lipid vacuoles size observed on HPS analysis and with a 28% significant increase of lipid accumulation assed with Oil Red O (Fig. 3).

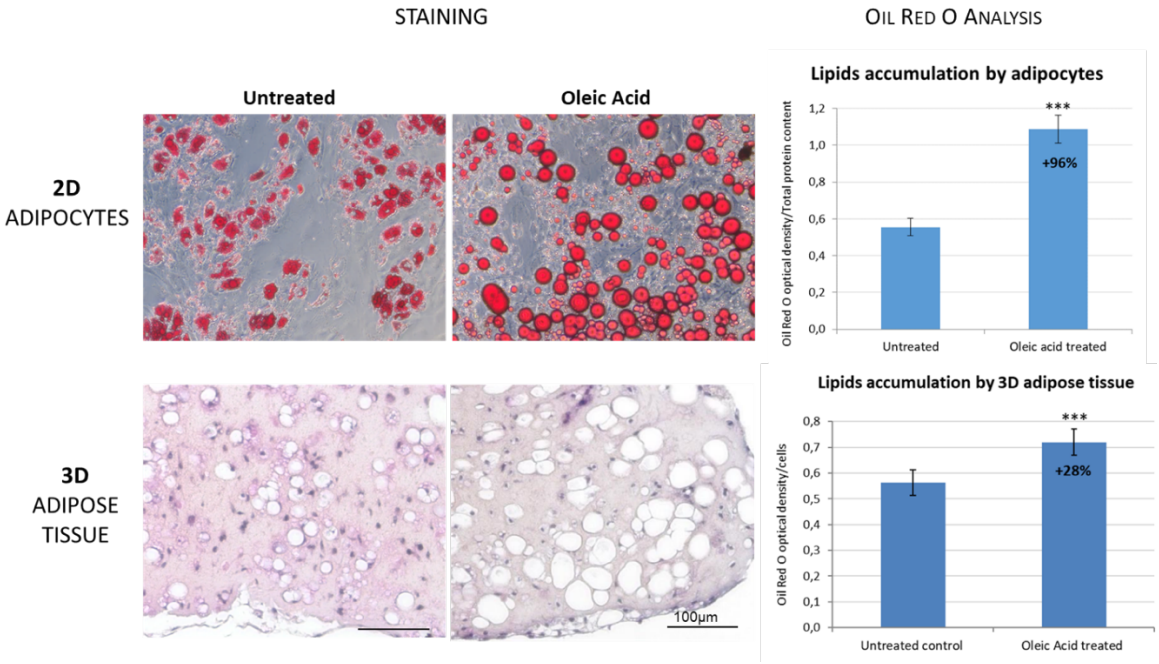


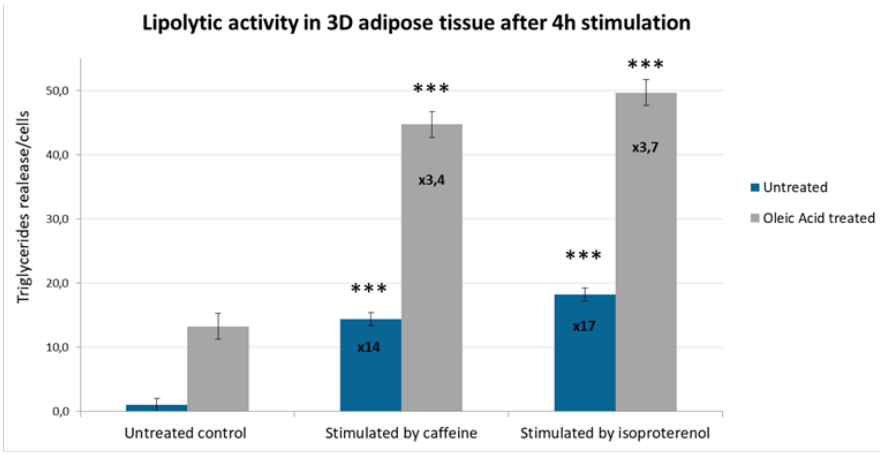
Figure 3: Study of oleic acid supplementation effect on adipocytes and reconstructed adipose tissue by microscopic and Oil Red O analysis.

For lipolysis induction, we chose two highly described adrenergic stimulants: caffeine and isoproterenol. With both of these adipogenic modulators, adipose tissue demonstrated their ability to release lipids after a short-term or a long-term stimulus.

After a 4-hours short-term stimulus with caffeine and isoproterenol, a strong significant increase of triglycerides released into the supernatant was observed (Fig. 4). In basal condition, the release was 14 and 17-fold higher with caffeine and isoproterenol compared with the untreated. In a condition with lipogenesis previously induced by oleic acid, we also observe a lipolytic response to stimuli with a release 3.4 and 3.7-fold higher with caffeine and isoproterenol compared with the control.

After a 1-week long-term stimulus with caffeine and isoproterenol, a significant decrease of total lipids into the adipose tissue was observed through the Oil Red O assay (Fig. 4). In basal condition, the lipids accumulation decreased by 15% and 36% with caffeine and isoproterenol treatment compared with the untreated control. In a condition with lipogenesis previously induced by oleic acid, we also observe a lipolytic response to stimuli with a decrease of 36% and 43% with caffeine and isoproterenol compared to control.

SHORT-TERM
TRIGLYCERIDES RELEASE



LONG-TERM
SLIMMING EFFECT

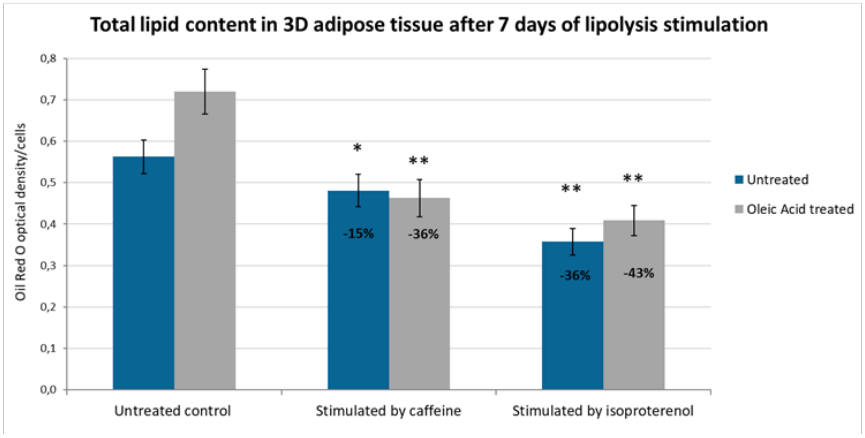


Figure 4: Study of the short and long term lipolytic effect of caffeine and isoproterenol on the reconstructed adipose tissue.

4. Conclusion

For the first time, we have developed a bioprinting method for the generation of endothelialized human adipose tissue with cellular and molecular characteristics closely resembling native human tissue with capillary-like structures showing a clear lumen. To promote the development of the microvascularisation of the bioprinted adipose tissue, the bioink was functionalized with endothelial growth factors.

Furthermore, adipogenic modulators such as oleic acid and caffeine/isoproterenol promoted lipogenesis and lipolysis, respectively, of adipocytes by modulating lipid content and adipocyte size, demonstrating the functionality of the 3D bioprinted model *in vitro*.

This unique *in vitro* model may be a relevant tool to explore molecular mechanisms underlying adipogenesis and to identify dermo-cosmetic active compounds.

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Conflict of Interest Statement.

NONE

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