# Can light be used to treat obesity and diabetes?

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ABSTRACT

This document is highly theoretical lab method paper. It may be used to perform thermogenesis from several cells in a living organism instead of only brown adipocytes. We perform thermogenesis from white adipocytes. The purpose is to uptake the blood glucose and lipids to produce heat and subsequent weight loss and to diminish blood glucose levels for people who suffering from diabetes and obesity. In fact thermogenesis consumes energy (i.e blood glucose and blood lipids). So we now describe a novel and original lab method based on thermogenin (called thermogenin-like system, UCP1-like system or TLS) that is involved in heat production and glucose/lipids capture by white adipocytes.

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## Introduction

1 This document is highly theoretical lab method paper. It may be used to perform thermogenesis from several cells in a living organism instead of only brown adipocytes. We perform thermogenesis from white adipocytes. The purpose is to uptake the blood glucose and lipids to produce heat and subsequent weight loss and to diminish blood glucose levels for people who suffering from diabetes and obesity. In fact thermogenesis consumes energy (i.e blood glucose and blood lipids). So we now describe a novel and original lab method based on thermogenin (called thermogenin-like system, UCP1-like system or TLS) that is involved in heat production and glucose/lipids capture by white adipocytes.

## *In vitro* assays

2 **Culture experiment.**The cell culture experiments were carried out in a category 2 culture room, under a type II biological safety hood, BH 2004 D, with vertical laminar flow.

**Cell culture medium**.A 10% FCS (Fetal Calf Serum) solution was used as culture medium. A -SVF solution (without SVF) was prepared on the basis of a volume of 500 mL and was composed of: 385.4 mL of sterile water, 50 mL of EMEM 10X (Eagle's Minimum Essential Medium), 14.6 mL of bicarbonates 7.5%, 10mL HEPES 1M(4-(2-HydroxyEthyl)-1-PiperazineEthaneSulfonic acid),5mL of non-essential amino acids (MEM 100X (Minimum Essential Medium)), 5mL of gentamicin, 5mL of L-alanyl-L-glutamine 200mM (GlutaMax™, a more stable analog of L-glutamine providing L-alanine and L-glutamine). In order to obtain a 10% FCS solution, 45mL of -SVF solution was added to a tube and then topped up with 5mL of pure FCS.
**Cell preparation.**White adipocytes cell line was used.
The ampoules containing the cells were maintained in liquid nitrogen (-80˚C) then thawed in a water bath at 37˚C. The cells were then suspended in 9 mL of culture medium and then centrifuged (1000 revolutions/min for 10 minutes).

After centrifugation and to eliminate the freezing medium, the supernatant obtained was eliminated and the The suspension was placed in a 25cm flask contain
cells were resuspended in 1 mL of culture medium.ing 5mL of culture medium. This flask was then kept in an incubator containing 5% of$CO_2$(Carbon dioxide), an atmosphere saturated with humidity and at 37˚C. After 24 hours of incubation, the cells adhere to the bottom of the flask and proliferate, forming a cell carpet. The medium was then renewed every 48 hours to optimize cell proliferation.
After 6 to 7 days of culture, the cells occupy 80% of the surface of the flask, justifying a detachment of the cells by means of a treatment with trypsin to avoid inhibition of proliferation by contact and thus avoid distorting the results of the subsequent experiences.

**Detachment of cells**.The culture medium was removed using an aspiration syringe then 10 mL of PBS (Phosphate Buffered Saline) were added in order to eliminate the trypsin inhibitors present in the culture medium previously removed. The PBS was then aspirated.
400µLof trypsin were then applied to the cells, together with a chelator of divalent cations, EDTA (Ethylene Diamine Tetra-Acetic acid). This step made it possible to detach the cells from the culture dish. After a few minutes of waiting, the trypsin previously applied was neutralized by adding 10 mL of culture medium, reflux was carried out to homogenize the cell suspension.
**Malassez cell** count.Cell counts were performed on Malassez cell.
**Transient transfections.**A detachment of the cells was initially carried out according to the protocol described above.Afterwards 2 eppendorfs of 1.5mL each containing 1 000 000 cells were prepared.
The eppendorfs were then centrifuged (1000 rpm for 7 minutes, 4˚C). The supernatant was aspirated and the pellet containing the cells was saved.
100 µL ofnucleovector®Kit V were added in the eppendorfs to resuspend the pellet then 2µg of DNA constructs (see below), were each added in a different eppendorf. All of the medium contained in the eppendorfs was taken and then placed in a transfection tank.
Transfection was then carried out using the Nucleofector II transfection apparatus (transfection by
electroporation) set up with the appropriate transfection program. (program MDA-MB-231, X013). 500µL of culture medium were added to each of the two tanks to restore the cells to favorable conditions, the medium present in the two tanks was then removed and then transferred back to two separate eppendorfs. The two eppendorfs were kept at 37°C for 5 minutes before transferring the transfected cells to the boxes provided for the various tests of the study, these boxes were placed in an incubator until they were used. All the transfections were validated using the RT-PCR (Reverse Transcription Polymerase Chain Reaction) method described below.
**Real-time quantitative RT-PCR.**The cell medium was aspirated then 1mL of Trizol® was added to lyse the cells.
The lysate was then stored at -80°C until extraction of the nucleic acids.On the day of extraction, the cell lysate was thawed then the total RNAs were extracted.
After drying, the pellet was rehydrated with22µLof water. The amount of genetic material was estimated by measuring absorbance with the Nanodrop spectrophotometer. For that,1µLsamples were introduced at the level of the spectrophotometer and a concentration of total RNAs in the solution as well as the purity of the
total RNAs were obtained. A reverse transcription from2µgof total RNAs was then carried out. The purpose of this step is to synthesize cDNA (complementary DNA) of RNA in order to allow the polymerase
used in qPCR (Quantitative Polymerase Chain Reaction) to bind to its substrate. To solutions containing2µgof
RNA, or0,2µg/µL of RNA, were added10µLof reaction mix. For10µL, the mix contained1µLRT (Reverse
Transcriptase),1µLRNase inhibitors,0,8µLdNTP (DeoxyriboNucleotides TriPhosphates) (100nM),2µLprimers (Reverse and Forward),2µLof RT buffer and3,2µLsterile water (experimental data provided with the reverse transcription kit). The reverse transcription cycle started with 10

minutes at 25˚C in order to prepare the samples. Then, the samples were brought to 35˚C for 120 minutes, which allowed the hybridization of the primers and the synthesis of the cDNAs. Finally the samples were brought to 85˚C for 5 minutes in order to stop the synthesis and separate the primers.
Quantitative PCR reactions were then performed on the LightCycler® from Roche in 420-well microplates and using the ABsolut kitTMqPCR SYBR® Green Mixes (ABgene) in accordance with the supplier's instructions. To do this, a DNA intercalator, SYBR green, was used, which emits fluorescence in contact with double-stranded
DNA, with a maximum intensity at 550 nm. This fluorescence was detected and measured by the LightCycler microspectrofluorimeter®, which allowed quantification of the number of copies of the cDNA sequence of interest. At each end of the cycle, the fluorescence is therefore measured and it is possible to visualize the exponential increase in real time of the quantity of amplicons generated. Finally, the number of cycles necessary for the fluorescence of the target gene to be detectable is inversely proportional to the initial quantity of the mRNA of interest. This number of cycles was calculated and compared for a given gene between different conditions. The target sequences were amplified from specific primers and using polymerase activating at high temperature. For each sample, a reaction medium was prepared containing4,6µLof reaction mix (SYBR®Green, Taq (Thermus AQuaticus) polymerase, dNTP, enzyme buffer and MgCl2),2,2µLof cDNA diluted to the 20th,0,5µLto prime forward,0,5µLprimer reverse (both at 102µM) and 1,6µLsterile water.
The reference gene used was actin, in order to verify that the variations in the number of cycles obtained for a gene of interest between different conditions were indeed due to a difference in the initial quantity of mRNAs of this gene and not to the presence of Taq polymerase inhibitors between two conditions or the fact
that less genetic material was collected between two conditions.
**DNA constructs for transfections *in vivo.*** The first step of this model is the in vitro transfection of white adipocytes with adeno-associated viral (AAV) vectors. The following transgenic DNA constructs are used in vivo: the light-driven inward H+ pump PoXeR targeted to the inner mitochondrial membrane, firefly luciferase, luciferin-regenerating enzyme (LRE), cysteine racemase and thioesterase.
**Measure of blood glucose and lipids levels.** Use specific kits adapted to white adipocytes.
**Estimate oxygen consumption and ATP levels.** Use specific kits adapted to white adipocytes.

3 Measure blood glucose and lipids levels, oxygen consumption and ATP levels with specific kits.

## *In vivo* assays

4 The TLS may be used in normal cells to promote uncoupling-induced weight loss without significant adverse effects. It may be possible to express PoXeR (and other transgenes of the TLS) under the control of a weak promoter to reduce the functionality of the system and subsequent weight loss. It may be also possible to express PoXeR (and other transgenes) under

the control of a
strong promoter to enhance weight loss without significant side effects. In addition, it is interesting to obtain a spatial control of the system in order to restrict transgenes expression to the adipose tissue. For this reason, we propose to use the following
transgenic DNA constructs in vivo:
[Adiponectin promoter]-PoXeR targeted to the inner mitochondrial membrane
[Adiponectin promoter]-firefly luciferase
[Adiponectin promoter]-LRE
[Adiponectin promoter]-cysteine racemase
[Adiponectin promoter]-thioesterase

5 PoXeR is a natural light-driven inward proton pump found in Parvularcula oceani, a deep-ocean marine bacterium. This pump controls the unusual directionality opposite to normal proton pumps and may lower the proton motive force when expressed in the inner mitochondrial membrane, producing heat similarly to thermogenin. A study shows that PoXeR can be expressed in mouse neural cells and it is functional in these cells [1]. Another study shows that it is possible to construct photoenergetic mitochondria in cultured mammalian cells expressing another light-driven proton pump derived from Haloterrigena turkmenica [2]. Taken together, these results suggest that PoXeR may be a good candidate for the TLS in vivo in mammals.

6 **Injections.** Because the bioluminescence of firefly luciferase decreases over time, it is necessary either to continually bring the substrate (i.e firefly luciferin) by continuous infusion and/or to use a system that regenerates the firefly luciferin from oxyluciferin, the product of the reaction. A study shows that oxyluciferin is enzymatically regenerated into firefly luciferin by LRE in vitro. This system can be improvable by using cysteine racemase and thioesterase. Finally, it is possible to concentrate the substrate firefly luciferin in adipose tissue in vivo by using a suitable linker (firefly luciferin prodrug). Then, mice are injected with a firefly luciferin or a firefly luciferin prodrug so that the active firefly luciferin is mainly released in adipose tissue.

Now, the TLS needs to be sensitized to the light. In fact, all trans retinal is required for light-induced PoXeR activation in vivo.
All trans retinal may be not naturally present in adipose tissue, thus it is necessary to administer it by continuous infusion, it is not enzymatically regenerated in contrast to firefly luciferin. Because all trans retinal is hydrophobic it can be encapsulated in liposomes to reach the adipose tissue in vivo. Hydrophobic compounds have affinity to the phospholipid bilayer of liposomes. Finally, few hours after all trans retinal injection, mice are injected with a firefly luciferin or a firefly luciferin prodrug.

Estimate in vivo the weight loss, blood glucose levels or measure body temperature of mice (see below in summary). We expect that glucose and lipids blood are decreased and oxygen consumption are increased.

## Summary

7 1) Firstly, use the previous transgenic DNA constructs for the in vitro transfection of white adipocytes by electroporation. Estimate blood glucose and lipids levels. Estimate ATP levels and oxygen consumption (because thermogenesis consumes oxygen and glucose). If these experiments are conclusive, perform in vivo experiments.

2) Use the previous transgenic DNA constructs for the in vivo transduction of adipose tissue with AAV vectors. In fact, a study indicates that AAV-mediated genetic engineering of white and brown adipose tissue is possible in adult mice.

3) Then, use a continuous infusion of all trans retinal in mice. Few hours after all trans retinal injection, use a continuous infusion of firefly luciferin or firefly luciferin prodrug.

4) Finally, estimate in vivo the weight loss, blood glucose levels or measure body temperature of mice using infrared imaging. Indeed, a study shows that it is possible to measure brown adipose tissue thermogenesis using infrared imaging in mice.