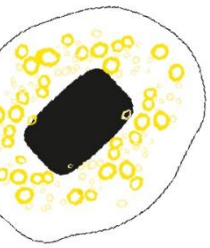
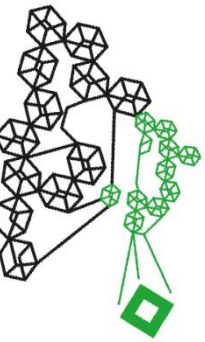


Effect of phototherapy on fat-fed hepatocyte



Bachelor thesis

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Effect of phototherapy on fat-fed hepatocyte

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Preface

This thesis is the final work for my Bachelor degree of Biomedical Engineering at the University of Twente titled *"Effect of phototherapy on fat-fed hepatocyte"* and was conducted at the Translation Liver Research (TLR) group at the Department of Medical Cell Biophysics (MCBP).

I want to thank my supervisor Ruchi Bansal and my daily supervisor Amit Khurana for their support, advice and motivation during my thesis. I also want to thank everybody at the TLR that was open to my asking all kinds of questions, not all relevant. And I would like to thank my external committee member, Liliana Moreira Teixeira Leijten.

Although I am sad that my thesis is coming to an end, I looking forward to the future with new knowledge, experiences and skills of which I am very great full. I hope you will enjoy reading my bachelor thesis.

Summary

Non-alcoholic steatohepatitis (NASH) is an excessive built up of fed in the liver that inhibits the liver to perform all it functions and can lead to liver cirrhose. It is also the most common liver disorder in western countries. Besides a big change in living style and diet, there is no know working treatment to heal the liver. In recent years, research has been done to see if photobiomodulation (PBM) could be used for treatment of the liver. PBM is a non-invasive, non-thermal light therapy that uses visible and near-infra red (NIR) light to stimulate the cells so that they can reduce inflammation, accelerate wound repair, restore the body's homeostatic balance and help with neurological pain and pathologies. In this research, two iCAP devices were used, one with LED's with a wavelength of 660 nm (red) and one with a wavelength of 810 nm (near infra-red or NIR). The HepaRG, primary human hepatocytes, cells were treated with a combination of oleic and palmitic acid, isopropanol control and control treatment which was left on the cells for 24 hours. When the treatments were added, the iCAP device was placed on the 12 well plates for 30 minutes. To analyse the working of the iCAP device multiple methods were used, namely: oil red o, Hoechst, TMRE and DCFDA staining and an alamar blue assay. When analysing the results, it shows no effect of the 660 nm wavelength LED's but some positive effect of the 810 nm wavelength device. Further research to use 660 nm for treatment of NASH is not advised, but for 810 nm further research is necessary to see the extent of the effect on the cells.

Samenvatting

Niet-alcoholische steatohepatitis (NASH) is een aandoening waarbij er een ophoping van vet in de lever is. Hierdoor kan de lever niet of minder goed functioneren en kan, wanneer onbehandeld, lijden tot levercirrose. Het is de meest voorkomende lever ziekte in de westerse wereld. Momenteel is de enige behandeling voor NASH een drastische verandering in levensstijl en dieet, er is nu geen werkende medicijn behandeling om de lever weer te kunnen genezen. Recentelijk wordt er onderzoek gedaan naar de toepassing van photobiomodulation (PBM) bij behandelingen van NASH. PBM is een non-invasieve, niet thermische licht therapie dat gebruik maakt van zichtbaar licht en near infra-red (NIR) om de cellen van het lichaam te stimuleren. Deze stimulatie helpt het lichaam met het bestrijden van ontstekingen, het balanceren van de hormoonhuishouding, het verminderen van neologische pijn en pathologisch en het versnellen van de wondgenezing. In dit onderzoek wordt gebruik gemaakt van twee iCAP devices, elk met 12 LED's waarvan een device met LED's met een golflengte van 660 nm (rood) en het tweede device met LED's met een golflengte van 810 nm (NIR). HepaRG cellen worden gebruikt als primaire menselijke hepatocytes waarop een behandeling van 24 uur wordt uitgevoerd van oliezuur en palmitinezuur, een isopropanol controle en een controle behandeling. Wanneer de verschillende behandelingen zijn toegevoegd aan de cellen, worden ze voor 30 minuten behandeld met het licht. Om de behandeling te analyseren zijn er verschillende methodes gebruikt, oil red o, Hoechst, TMRE en DCFDA staining en een alamar blue assay. Bij het analyseren van de resultaten is gebleken dat er in dit geval geen positief resultaat is gekomen uit de cellen behandeld met een golflengte van 660 nm, maar dat er wel een positief effect is bij de cellen die behandeld zijn met 810 nm. Er is meer onderzoek nodig naar 810 nm om te kijken of dit ook toegepast kan worden om NASH te kunnen behandelen.

Abbreviations

ATP	Adenosine triphosphate
CCO	Cytochrome c oxidase
DCF	2’,7’-dichlorofluorescein
DCFDA	Dichlorodihydrofluorescein diacetate
EMT	Electromagnetic radiation
HCC	Hepatocellular carcinoma
HepaRG	Liver hepatocytes cell line
HepG2	Liver hepatocellular carcinoma cell line
IPA	Isopropanol
IR	Insulin resistance
LED	Light-emitting diodes
LLLT	Low Level Laser Therapy
MMP	Mitochondrial membrane potential
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NIR	Near-infrared light
NO	Nitric oxide
OA	Oleic acid
PA	Palmitic acid
PBM	Photobiomodulation
PHH	Primary human hepatocytes
ROS	Reactive Oxygen Series
TMRE	Tetramethylrhofdamine ethyl ester

1. Introduction & Theoretical Background

1.1 NAFLD/NASH

Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatic fat accumulation. The complete mechanisms of NAFLD is not completely understand at the moment, but it is mostly associated with insulin resistance (IR) and it affects individuals that have 3 out of 5 of metabolic syndromes (obesity, hypertension, diabetes, hypertriglyceridemia, hyperlipidaemia). There is also steatosis present in more than 5% of hepatocytes. It has a large spectrum of symptoms, following advances in the progression from liver lesions to fatty liver, and may lead to cirrhosis upon disease worsening (scarring of the liver) [1] [2].
As the name suggests, NAFLD is a liver disease where the cause of the liver disease is not excessive alcohol or secondary causes, such as hepatitis C, or Coeliac disease, rather the use of high fatty diet classically called, "western diet" and engaging in a sedentary life-style and lack-of physical activity. It is possible for a patient to have both a chronic liver disease and also NAFLD, which may aggravate the disease progress [1].

The different phases of NAFLD are shown in Figure 1 below. The first phase of NAFLD is non-alcoholic fatty liver (NAFL) characterized by steatosis, here the fat in the hepatocytes is more than 5% and the fat starts to push the nucleus to the edge of the cell. With IR, cells become less responsive to insulin, so the fat storage in the cells goes up and the fatty acid oxidation goes down. This decreases the secretion of fatty acids into the bloodstream and increases the synthesis and uptake of free fatty acids in the liver. This causes fat droplets to form and grow in the hepatocytes. The results in loss of function of the hepatocytes, which become vulnerable to degradation of unsaturated fatty acids. Here the unsaturated fatty acids, acids with one or more double carbon bond, react with a hydroxyl radical to form water and a fatty radical, this causes oxidative stress. These radicals are unstable and will react with non-radicals. This can result in damages to the lipid membrane which results in mitochondrial dysfunction and at the end also cell death, which results in inflammation of the liver and makes the liver go to the second phase of NAFLD. [3]

The second phase is non-alcoholic steatohepatitis (NASH), a more aggressive stage with inflammation of the liver and a higher change of permanent liver damage due to liver fibrosis, where fibrotic tissue is formed that changes the architecture of the liver. Here the extracellular matrix is damaged and is aggravated by oxidative stress due to lipotoxicity. The fibrosis causes the hepatic stellate cells (HSC) to become active and produce more extracellular matrix, which result in inflammatory triggers and oxidative stress resulting in the HSC to produce an excess of collage deposition, characterized by liver scarring. Both the first and second phase are reversible with a change in lifestyle and treatment. [3] [4]

However, uncontrolled progression of phase two may lead to phase three: cirrhosis of the liver. Wherein the scar tissue has overtaken healthy liver parenchymal tissue and leading to severe dysfunction of the liver resulting in permanent liver damage. The last stage of NAFLD is hepatocellular carcinoma (HCC), a form of liver cancer. The only treatment at the moment for cirrhosis and HCC is a liver transplant. [1] [4]

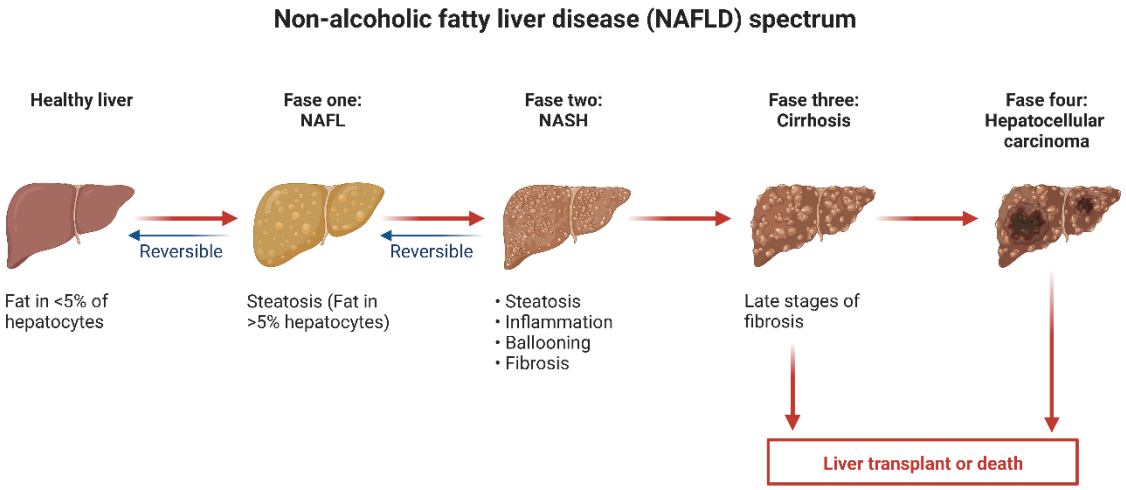

Figure 1: Spectrum of NAFLD

Depending on the diagnostic method, about 17-46% of the adults in Western countries are diagnosed with a type of NAFLD, which makes it the leading chronic liver disease in the Western world and the second most common reason for a liver transplant [1] [5] [6].

As a result of the liver damage that NASH causes, multiple processes in the liver are distorted. It causes oxidative stress by the overproduction of reactive oxygen species (ROS) which disrupt the metabolic activity of the cells. Under healthy circumstances, ROS are product by the mitochondrial electron transport chain by the metabolism of oxygen. They play a role in cell signalling and homeostasis. When the hepatocytes get damage because of the extra fat in the cells, ROS become in overproduction and this results in oxidative damage to the cells. It can result in lipid peroxidation of the membrane, intracellular oxidative modification of proteins and oxidative damage to DNA. The fatty acids become radical and unstable. Because of the instability of the radical fatty acid, it is highly reactive and if the reaction is not terminated fast, it will lead to damage of the cell membrane, which consists of lipids with which the radical fatty acid can react [7] [8].

At the moment there are no clinically approved therapeutics for the management of NASH. The different managements options used for NAFLD are to reverse the factors that contribute to the IR. This include change in lifestyle, dietary alterations and physical activity, and symptomatic drug treatments, mainly to help with blood glucose levels. However, the drug treatments that are currently available and in development are not sufficient for all the patients with NASH [1] [4] [9].

Nakano et al. [10] showed that artificial sunlight might have a positive effect on the progression of NAFLD in rats, so might light be a possible non-invasive treatment for these types of liver diseases?

1.2 Photobiomodulation

Photobiomodulation (PBM) [11] [12], also known as Low Level Laser Therapy (LLLT) [11], is a technique that uses red and near-infra red light to induce photobiological processes in cells [12] [13] [14]. These days LED's (light-emitted diodes) are used instead of a low level laser, because of the low energy density, availability and the low costs of LED's in comparison to lasers. Another benefit of LEDs is that they do not heat up the cells, which can cause damage to the cells.
PBM is part of the research area of photomedicine, which is on the one hand the study and treatment of diseases caused by exposure to light, and on the other hand the diagnostic and therapeutic applications of light for detecting and curing diseases [12]. Here, the focus is on the therapeutic applications of light. LLLT was discovered 55 years ago by accident, when experiments

were performed to see whether or not low-energy defocused red laser light could be save to use on the skin, the experiment had the unexpected site effect of acceleration of hair regrowth. After this discovery, the same group of researchers also discovered that the defocused red laser enhanced wound healing [15]. The complete mechanics of PBM are still unknown, but it is though to enhances the mitochondrial ATP production, cell signalling growth factor synthesis, attenuates oxidative stress. Now it is used in multiple places, for pain relief, inflammation, wound healing, sport injuries and neurodegenerative diseases, such as dementia [14] [16]. It is also cosmetically used to combat skin aging and acne [15].

PBM is a non-pharmacologic therapy using the near-infrared spectrum of light, see [14]. For this experiment LED's with a wavelength of 660 nm and with a wavelength of 810 nm will be used. Previously experiments with the iCAP device at the University of Twente have shown a positive effect of emitting light with a wavelength of 660 nm on (diabetic) wound-healing and macrophage-driven inflammation [17] [18]. This research wants to investigate of the 660 nm wavelength can also be used for fat-fed hepatocytes. The 810 nm wavelength device has not been used in research by the university before, but Staelens et. all. shows that it has potential to be used in the medical field. [13]

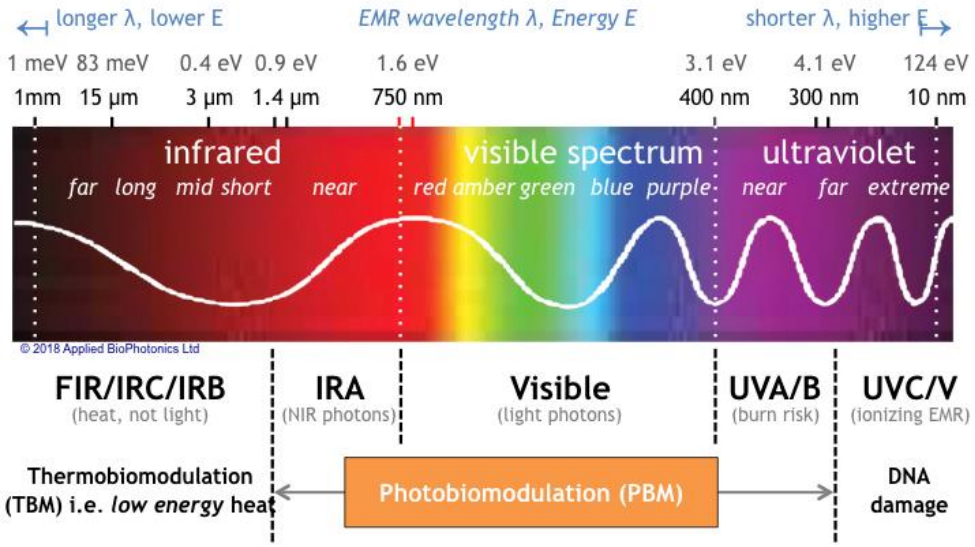

Figure 2: Electromagnetic spectrum of light [19]

Figure 2 shows the electromagnetic radiation (EMR) spectrum of light. Light is a form of energy and is both a particle (photon) and a wave. The LED's emit the photons, which when hit the cells, some of the photons will be absorbed.

When the cell absorbs a photon, the mitochondria of the cell absorbs the energy of the photon and can utilize that energy in the production of adenosine triphosphate (ATP). ATP is a compound that provides energy for the cells. The process of the photon absorption also leads to the creation of ROS, which leads to gene transcription, where nuclear factor κB (NF-κB) is the most important one. The transcription of the nuclear factor results in cellular repair and healing. Further, it leads to unclogging of nitric oxide (NO), which results in the NO being released back into the system. NO is a secondary messenger and participates in cellular communication. It also dilates the blood vessels and has a positive effect on the blood circulation [11]. **Fout! Verwijzingsbron niet gevonden.** shows a schematic depiction of this process.

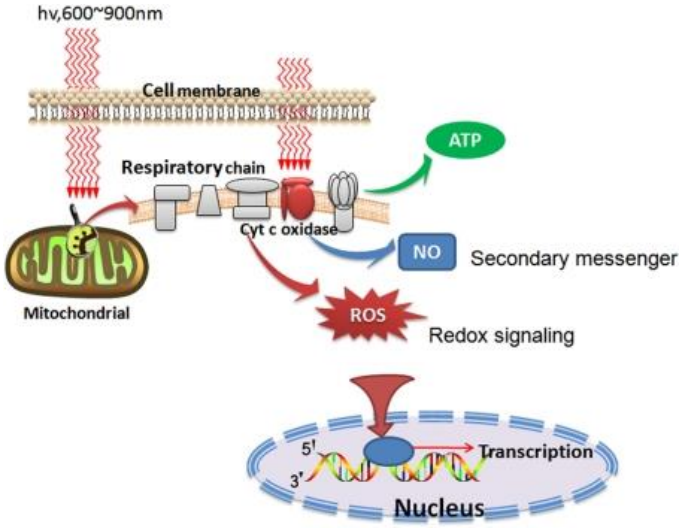

Figure 3: Schematic depiction of the cellular signalling pathways triggered by PBM [11]

The mechanism of pharmacological activity of PBM is via the production of ATP by the mitochondria, by modulation of the mitochondrial enzyme cytochrome c oxidase (CCO), a chromophore. CCO accepts the photon energy of NIR wavelengths. This results in an increased proton electrochemical potential, ATP synthesis, increased RNA and protein synthesis. It also results in an increase in oxygen consumption, mitochondrial membrane potential and enhanced synthesis of NADH and ATP.

Visible (380-780 nm [20]) and near-infrared light (NIR, 780-2500 nm [20]) results in the same photo response of the cell, the pathway is however different, as show in Kim et. all. The visible light ensures a photochemical reaction, mainly on the CCO and by this the visible light is mostly responsible for the ATP synthesis. Near-infrared induces a photophysical reaction in the cell membrane. At the end, it also results in more ATP synthesis, but NI through the cellular membrane transport mechanisms [14].

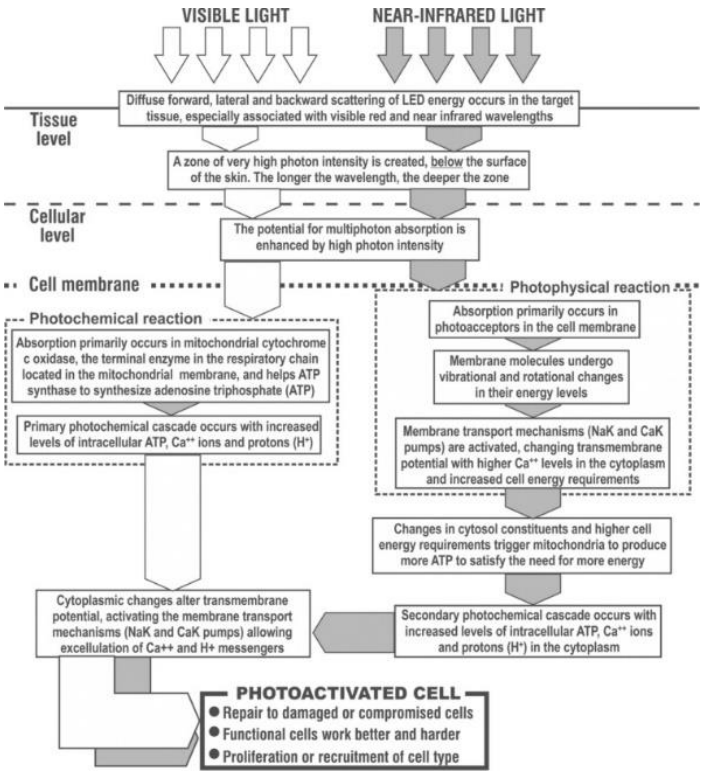

Figure 4: The process of cellular photoactivation by low level light therapy (LLLT) [14]

2. Research design

2.1 Aim of this research

To examine the pharmacological effects of photobiomodulation (with a wavelength of 660 nm and 810 nm) on fat-fed hepatocytes

2.2 Hypothesis

Photons might have a positive effect on the mitochondrial activity of the cells, which can result in faster breakdown of the fatty acids (fat) thereby reducing fatty-acid (fat)-induced toxicity, oxidative stress. This results in reducing inflammatory and fibrotic disease responses.

2.3 Novelty

At the moment, there are no reports studying the effect of PBM on fat-fed hepatocytes. This research therefore is novel and very innovative studying the effect of PBM (with a wavelength of 660 nm and 810 nm) on fat-fed hepatocytes to evaluate its clinical potential.

2.4 Objective

1) To examine the effect of 660 nm LED (red light) treatment on fat-fed hepatocytes
2) To examine the effect of 810 nm LED (near infra-red light) treatment on fat-fed hepatocytes
   Sub-objectives are:
   i) To examine the effect of PBM (660 nm and 810 nm) on cell proliferation or rescuing fat-induced lipotoxicity in fat-fed hepatocytes.
   ii) To examine the effect of PBM (660 nm and 810 nm) on lipid accumulation and metabolism in fat-fed hepatocytes.
   iii) To examine the effect of PBM (660 nm and 810 nm) on oxidative stress induced in fat-fed hepatocytes.
   iv) To examine the effect of PBM (660 nm and 810 nm) on mitochondrial activity in fat-fed hepatocytes.

2.5 Research design

For this research I worked with HepaRG cells. These are “healthy” primary human hepatocytes, in comparison with the more used HepG2, which are cancerous hepatocytes [21]. In recent studies [22], HepaRG cells have been found the be a good alternative to primary human hepatocytes (PHH), which gives a better representation of the real world NASH situation.

For resembling NASH-phenotype, HepaRG cells were treated with a combination of oleic acid and palmitic acid, most commonly fatty acids present in our diet. These are also most commonly used fatty acids to induce NASH phenotype in cellular models.

For my research I used the iCAP LED device, which contains 12 LEDs that fit a 12 wells cell culture plate and have a fixed wavelength of 660 nm and 810 nm. Previous research at the University of Twente has shown that the use of the iCAP device with LEDs with a wavelength of 660 nm has a positive effect on the (diabetic) wound-healing [18] and the reduction of macrophage-driven inflammation and cytokine storms [17].

To analyse the effect of the light treatment I used Alamar blue assay for the assessment of metabolic activity of the hepatocytes, Oil Red O staining to see the remaining oil after the treatment and fluorescence staining. Tetramethylrhodamine, ethyl ester (TMRE) staining was used for measuring the mitochondrial activity and DCFDA staining was used to observe the ROS.

2.5.1 iCAP module

Two light modules were used, both with a fixed wavelength, one with a wavelength of 660 nm and the second one with 810 nm. The module is designed to fit on a 12 well plate and is built with 12 Light Emitting Diodes (LEDs), see Figure 5. The LED's are located in the middle of the well, so that the well is irradiated as homogeneously as possible on all the wells.

Because of the properties of LED's, it is assumed that the emitted power is equivalent in all the wells in the plate and the iCAP device, with an output power of 5 mW per LED.

Figure 5: iCAP device in use

2.5.2 Treatment

2.5.2.1 Palmitic Acid (PA)

Fatty acid, most common saturated fatty acid in nature. Palmitic acid has toxic properties, this makes the concentration of PA leading in the experiment. The concentration of PA used is 0,4 mM, as higher concentrations may result in too much cell death.

2.5.2.2 Oleic Acid (OA)

Oleic acid is the most common monounsaturated fatty acid in nature. For inducing lipotoxicity, the ratio between Oleic Acid and Palmitic Acid is 2:1. So with a concentration of 0,4 mM of PA, gives a concentration of 0,8 mM of OA.

2.5.2.3 Isopropanol (IPA)

Both oleic acid and palmitic acid were dissolved in isopropanol for this research. So, because of this a control with just isopropanol was also used, the concentration of IPA will be the combined one of OA and PA, which makes it a concentration of 1,2 mM IPA.

2.5.2.4 Control

A negative control will also be performed, here the cells will not be treated with either oleic acid, palmitic acid, the combination of the acids or isopropanol, but will receive the same light treatment and incubation times as the treated cells.

2.6 Principles of the methods used

2.6.1 TMRE

Tetramethylrhofdamine ethyl ester (TMRE) can be used to label active mitochondria with a red fluorescent marker. TMRE is a positively charged dye that accumulates in active mitochondria that are negative in charge, due to the mitochondrial membrane potential (MMP), which is negatively charged. We hypothesized that there will be more mitochondrial activity in the cells treated with light than cells not treated with light. A higher mitochondrial activity means a higher ATP production and a stronger fluorescence presence. This correlates with the working of PBM [23] [24]. To analyse the TMRE staining, an user-defined image scoring will be performed.

2.6.2 DCFDA

Dichlorodihydrofluorescein diacetate (DCFDA) is a blue fluorescent staining and is used to measure the activity of ROS molecules, such as hydroxyl and peroxyl. When DCFDA enters the cell, the cellular esterases deacetylated the DCFDA, making a non-fluorescent compound. When this compounds react with a ROS, the highly fluorescent 2',7'-dichlorofluorescein (DCF) is oxidized. DCF can be detected with a fluorescence microscope at a wavelength of 485/535 nm. This means that the more ROS are precent in the cells, the more DCFDA is converted into DCF with a higher fluorescent signal [7] [25] [26]. To analyse the DCFDA staining, an user-defined image scoring will be performed.

2.6.3 Hoechst staining

Hoechst dye is used for staining of the minor groove of double-stranded DNA in the cell nucleus [27]. This acts like a control staining for the fluorescent staining, to see whether or not cells are present and are correctly stained for the TMRE and DCFDA staining. To analyse the Hoechst staining, an user-defined image scoring will be performed.

2.6.4 Oil Red O

Oil Red O it is a fat-soluble dye that stains neutral triglycerides and lipids red [28]. ORO is an accurate method for detecting and quantifying hepatic steatosis in human liver biopsies [29]. It makes it possible to observe under the microscope how much lipid droplets are still present. To analyse the oil red o staining's, an user-defined image scoring will be performed.

2.6.5 Alamar Blue

Alamar blue is a reagent that is used as a cell health indicator. The active ingredient in alamar blue is resazurin, a non-fluorescent blue, cell permeable indicator, which when entered in a living cell, will be metabolised to resorufin. Resorufin is a red compound that is highly fluorescent and can be quantified with a spectrometer. Alamar blue is used to measure the viability of the cells by measuring the metabolic activity. [30] [31]

3. Methods

3.1 Culture

The type of cells that were used for this research were the HepaRG. The cells were cultured in Williams medium supplemented with L-glutamine, 10% FBS, 5 µg/ml insulin and 0.5 µM of hydrocortisone. The experiment was started with the HepaRG cells at passage 8 and the cells were kept in the incubator with 5% CO_2 and at a temperature of 37°C. In Table 1 the description of each experiment and the passage number is noted.

For the alamar blue assay, for experiments 2, 5 and 6, 100.000 cells were seeded per well and for experiment 150.000 cells were seeded per well.

For the oil red o staining, for experiment 1, 3 and 4, 100.000 cells were seeded per well and for experiment 7 till 9, 75.000 cells were seeded per well.

For the fluorescent staining, experiment 10 till 12, 75.000 cells were seeded per well

The cells were plated in 12 wells plate, for every experiment two plates were used, one which would be treated with light and the other as the no light control. After the cells were plated in the 12 well plates, the plates were put in the incubator for 24 hours, after which the treatment was started. For the complete protocol, see in Appendix: Protocol 1 and Protocol 2. Cell splitting was done according to Appendix: Protocol 9

Table 1: Passage number for the different experiments

Experiment			Passage number	Cells in well
1	660 nm	ORO	P8	100.000
2	660 nm	AMA	P8	100.000
3	660 nm	ORO	P10	100.000
4	810 nm	ORO	P10	100.000
5	660 nm	AMA	P12	100.000
6	810 nm	AMA	P12	100.000
7	810 nm	ORO	P8 (new line)	75.000
8	810 nm	AMA	P8	150.000
9	810 nm	ORO	P10	75.000
10	810 nm	Fluorescence	P10 (Fail)	75.000
11	810 nm	Fluorescence	P12	75.000
12	810 nm	Fluorescence	P12	75.000

3.2 Treatment

The HepaRG cells were stimulated with two fatty acids, oleic acid (OA) and palmitic acid (PA) in a combination of 0,8 mM and 0,4 mM, respectively. For experiment 1 till 6, a treatment with used oleic acid with a concentration of 0,8 mM and palmitic acid with a concentration of 0,4 mM was used. The isopropanol control with a concentration of 1,2 mM and a control without an oil treatment and only medium were also used in the study. The different treatments were added to 1% bovine serum albumin (BVA) in the Williams medium. For experiment 1 till 6, the well plate design in Figure 6 was used, for experiment 7 till 10 the well plate design in figure 7 was used an for experiment 11 and 12 well plate design as shown in Figure 8 was used.

After the oil treatments were added to the well plates, the plates were put in the incubator at 5% CO_2 and a temperature of 37°C and one plate was treated with light for 30 minutes in the incubator. To use the iCAP device, the cover of the well plate was removed in the incubator and the iCAP device,

after being disinfected, was inserted on top of the well plate. After 30 minutes, the iCAP device was removed and the cover was placed back on the well plate. The oil treated well plates were kept in the incubator for 24 hours. For the complete protocol, see in Appendix: Protocol 3 and Protocol 4

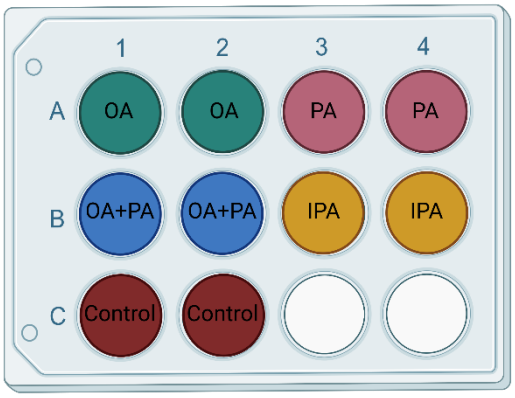

Figure 6: 12 well plate design, experiment 1-6

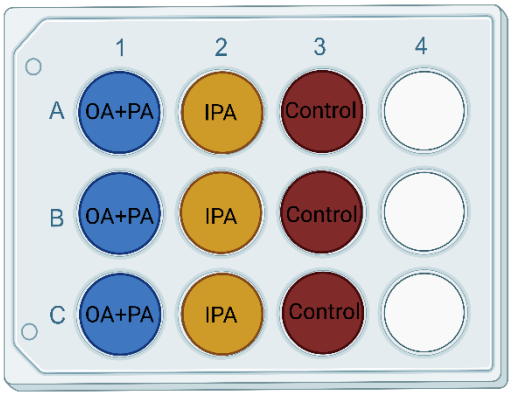

Figure 7: 12 well plate design, experiment 7-10

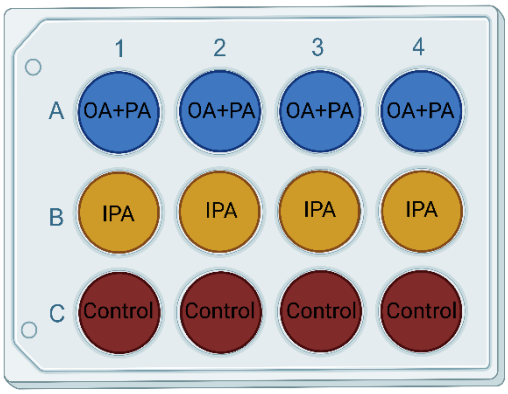

Figure 8: 12 well plate design, fluorescent staining, experiment 11 and 12

3.3 Alamar blue

After the 24 hours oil treatment, the wells were washed three times with PBS and a 10% alamar blue assay was added to the wells in the dark, including in the empty wells as a negative control. After which, the well plates were put in the incubator for 6 hours. After 6 hours, in the dark, two times 100 µl of the medium of each well was added in a 96 black wells plate and covered with aluminium foil. The black wells plate was analysed with a spectrometer and the data was processed with Excel and GraphPad Prism 5. For the complete protocol, see in Appendix: Protocol 5.

3.4 Oil red o staining

After the 24 hours oil treatment, the wells were washed ones with PBS and in PBS transported to the fume hood for the oil red o staining. 4% formaldehyde was made with ice-cold PBS in the fume hood and the cells were fixated with the 4% formaldehyde for 20 minutes. After the fixation, the cells were washed with 60% isopropanol and stained with 60% Oil red O in MilliQ solution for 15 minutes. After a wash with 60% isopropanol, the cells were counterstained with hematoxylin for three minutes. After the three minutes, the well plates were washed with running tap water for 5 minutes and the cells were kept in some tap water. The well plates were sealed with Parafilm M and kept in the refrigerator till looked under the Nikon TI-E Inverted Microscope. 30 minutes before looking under the microscope, the wells plate were left out the refrigerator. To analyse the pictures, ImageJ was used. For the complete protocol, see in Appendix: Protocol 6 and Protocol 7.

3.5 Fluorescent staining

After the 24 hours oil treatment, the medium was removed from the wells and 40 µM of DFCDA in Williams E medium solution was added to the wells and incubated at 5% CO_2 and a temperature of 37°C. After 15 minutes, TMRE was added to get a 200 nM solution and 0,5 µl of Hoechst 33342. The wells were incubated for 30 minutes after which they were washed three times with PBS and the last wash was left. Images were taken with the Nikon TI-E Inverted Microscope, for the Hoechst staining, the DAPI filter was used, for TMRE staining, the PE staining was used and for DCFDA staining, FITC filter was used. The images were analysed with ImageJ. For the complete protocol, see in Appendix: Protocol 8.

Figure 9 shows a schematic overview of the different treatments and analysing methods that were used.

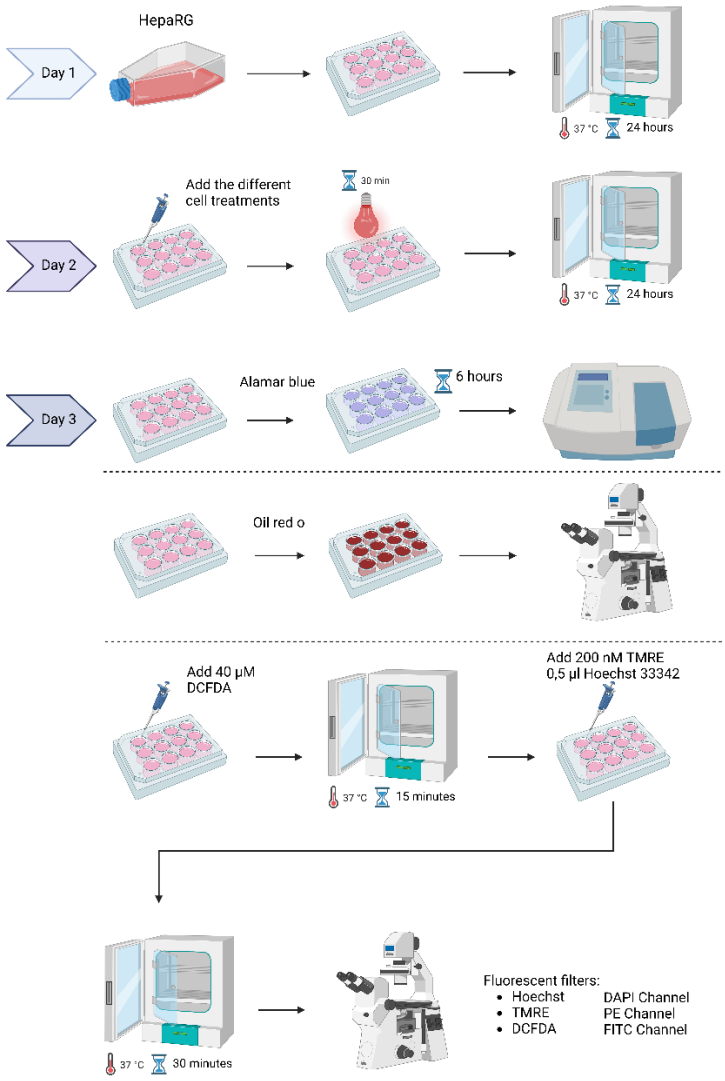

Figure 9: Schematic of the different experiments

4. Results

4.1 Oil Red O

4.1.1 660 nm

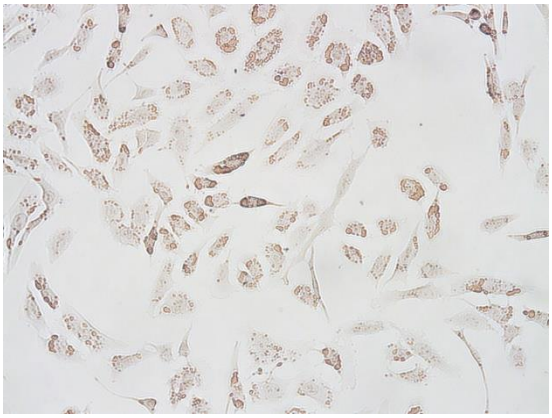

Figure 10: Oleic & palmitic acid treatment, no light control

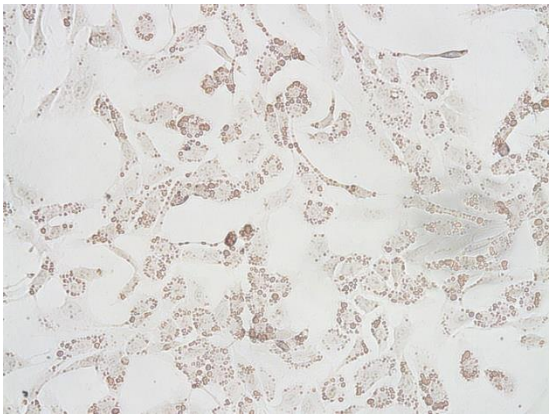

Figure 11: Oleic & palmitic acid treatment,660 nm light

Figure 12: Isopropanol treatment, no light control

Figure 13: Isopropanol treatment, 660 nm light

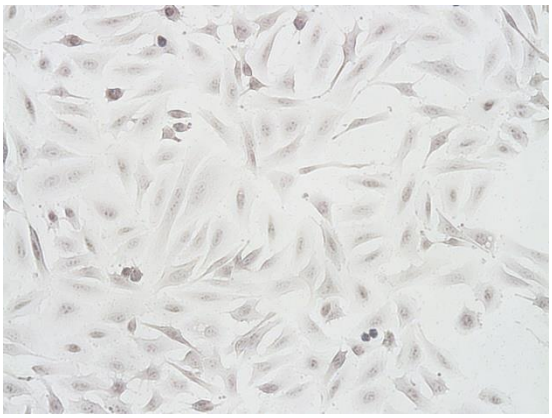

Figure 14: Control treatment, no light control

Figure 15: Control treatment, 660 nm light

Figures 10, 12 and 14 show the results of the ORO staining on the cells that were not treated with light and figures 11, 13 and 15 are the ORO results of the cells treated with 660 nm light for 30 minutes. The results of these staining were inconclusive for the cells treated with 660 nm light. When comparing the pictures of the wells that were not treated with light and the pictures of the wells that were treated with light, not a substantial different was observed.

4.1.2 810 nm

4.1.2.1 No light

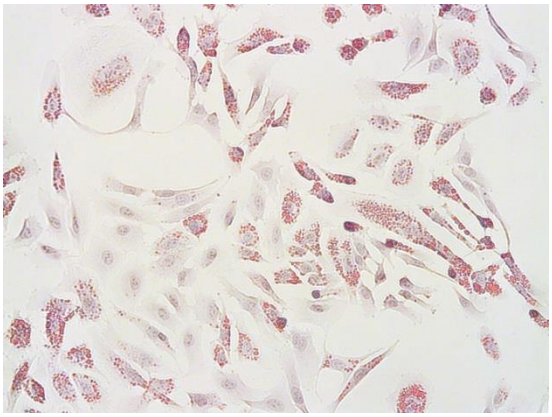

Figure 16: Oleic & palmitic acid treatment, no light control

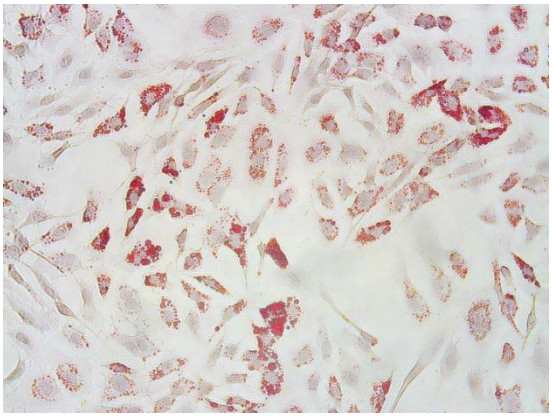

Figure 17: Oleic & palmitic acid treatment, 810 nm light

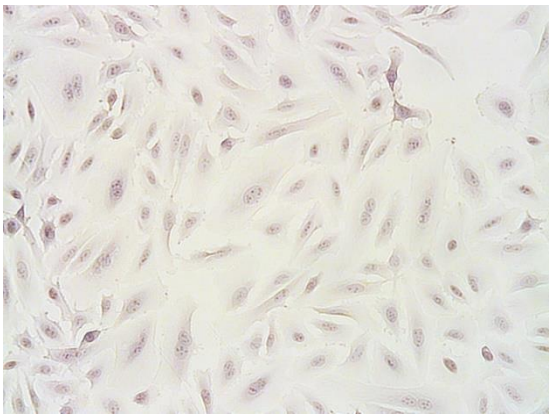

Figure 18: Isopropanol treatment, no light control

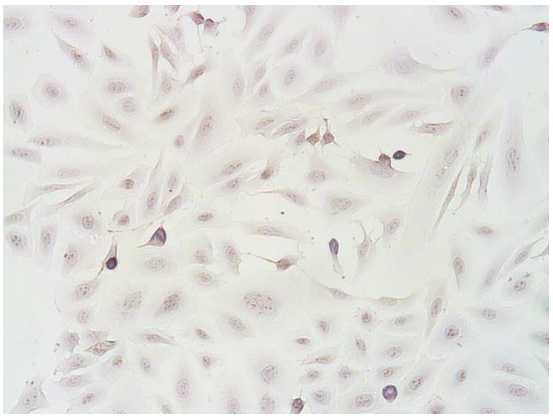

Figure 19: Isopropanol treatment, 810 nm light

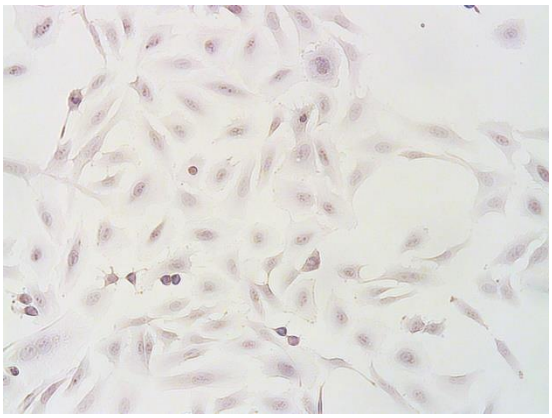

Figure 20: Control treatment, no light control

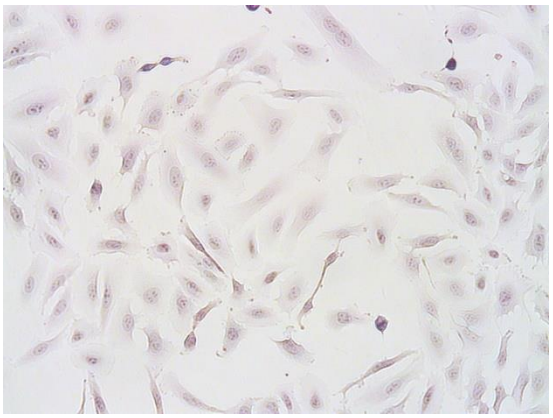

Figure 21: Control treatment, 810 nm light

Figures 16, 18 and 20 show the results of the ORO staining on the cells that were not treated with light and figures 17, 19 and 21 are the ORO results of the cells treated with 810 nm light for 30 minutes. The pictures shown a small difference in the ORO staining between the cells that weren't treated with light and the cells that were.

4.1.3 ORO analysis: user-defined image scoring

To analyse the ORO staining further, each well was scanned and rated by eye on a scale of 1 to 5 with 1 score for no to little oil red o staining and 5 score for a lot of oil red o staining. The average of the ratings are shown in figure 22-24 with figure 22 for the cells treated with 660 nm light and figure 23 for the cells treated with 810 nm light. Figure 24 shows a combined graph of the ratings of the two different light treatments. When comparing the different graphs, not a high different in rating can be observed, the difference between the staining of the different treatments was very small. It did show a small positive effect on the cells that were treated with light of 810 nm wavelength.

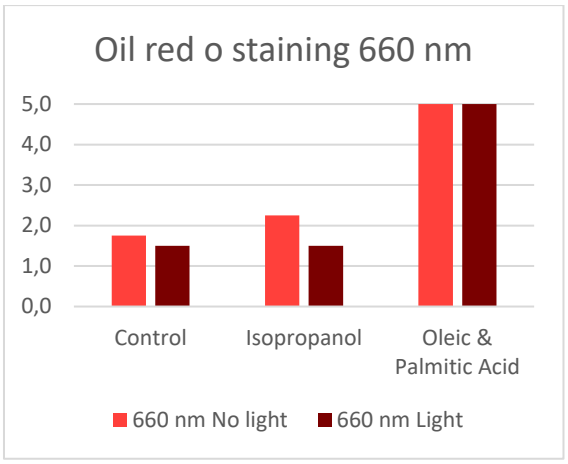

Figure 22: oil red o staining of 660 nm experiments

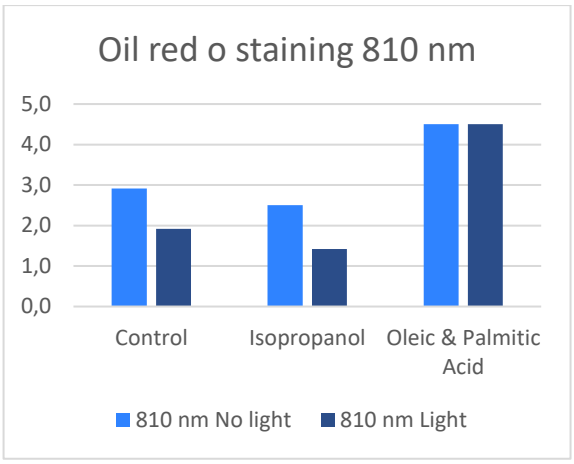

Figure 23: oil red o staining of 810 nm experiments

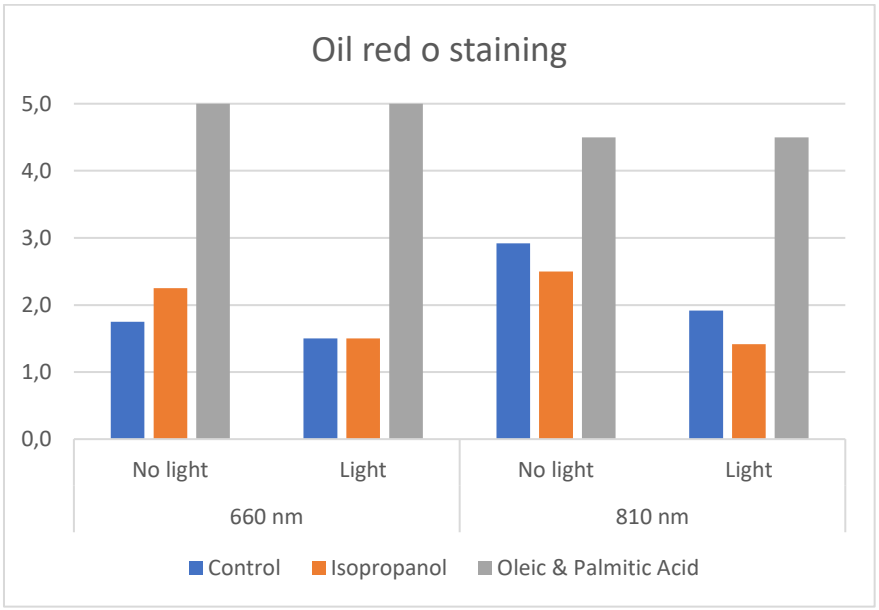

Figure 24: Average rating of the oil red o staining

4.2 Alamar Blue

4.2.1 660 nm

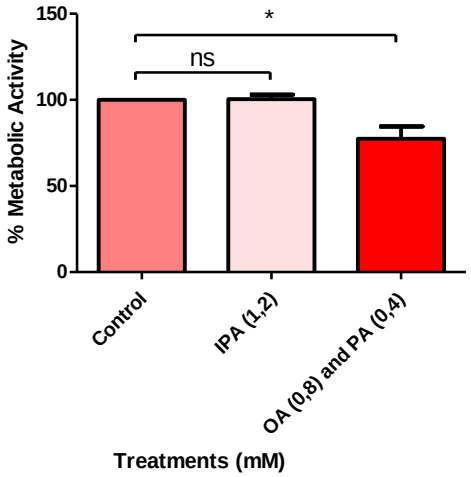

*Figure 25: no light treatment, * =p<0.5, ns=not significant*

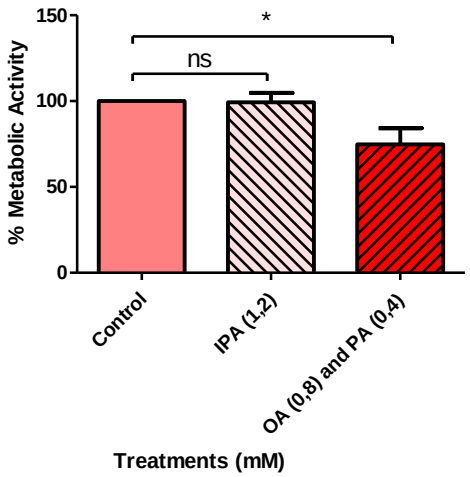

*Figure 26: 660 nm, light treatment, * =p<0.5, ns=not significant*

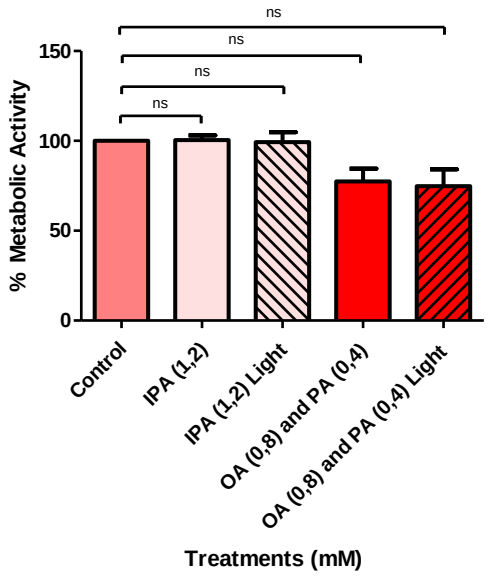

*Figure 27: 660 nm, both no light and light treatment, * =p<0.5, ns=not significant*

Figures 25-27 show the alamar blue results of the cells treated with 660 nm and the controls, with figure 25 and 26 the cells treated without and with light separately and figure 27 shows the combined results of the alamar blue assay. The results are displayed as % metabolic activity normalized to the untreated control. The measurements were carried out in duplicate in two independent experiments. The lipid treated cells shown no statistical differences.

The results show no statistical positive effect of the light treatment, this corresponds with the results of the oil red o staining.

4.2.2 810 nm

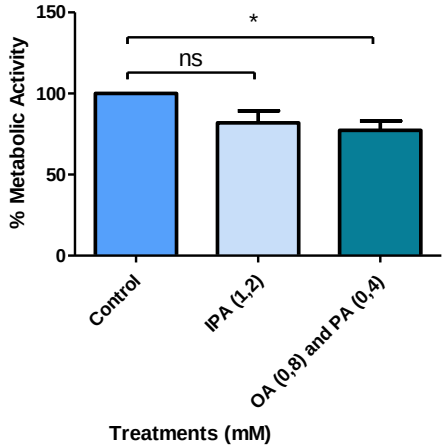

*Figure 28: 810 nm, no light treatment, * =p<0.5, ns=not significant*

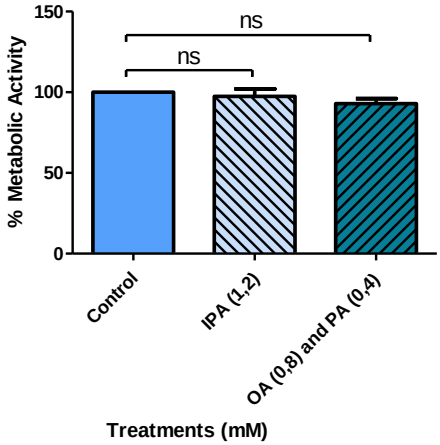

*Figure 29: 810 nm, light treatment, * =p<0.5, ns=not significant*

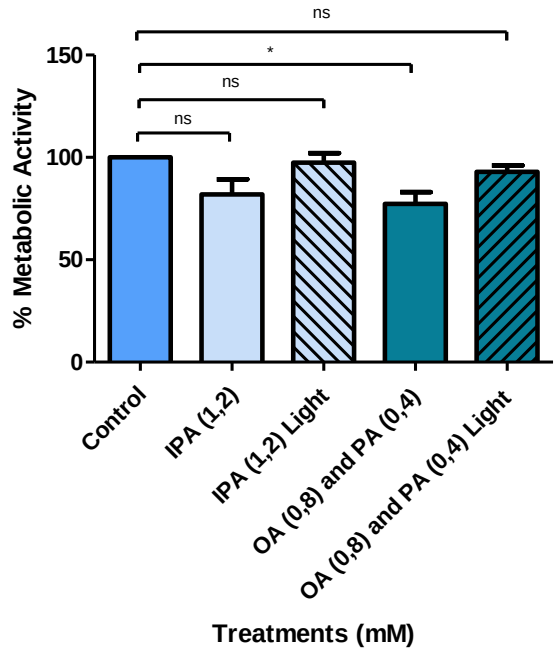

*Figure 30: 810 nm, both no light and light treatment, * =p<0.5, ns=not significant*

Figures 28-30 show the alamar blue results of the cells treated with 810 nm and the controls, with figure 28 and 29 the cells treated without and with light separately and figure 30 shows the combined results of the alamar blue assay. The lipid treated cells showed a small difference in the metabolic activity. The results are displayed as % metabolic activity normalized to the untreated control. The measurements were carried out in two independent experiments, one with the measurement in duplicate, the second the measurements were conducted in triplicate. The lipid treated cells shown no statistical differences, but they do show a small positive effect of the light treatment. Because of this effect, although small and not significant, the discussion was made to continue with only treating the HepaRG cells with the light of 810 nm wavelength.

4.3 Fluorescent staining

Figure 31 shows the rating given to the wells about the amount of fluorescence that was present after the staining treatment. The ratings were given by evaluating the wells through a Nikon TI-E Inverted Microscope and by observing the amount of fluorescence, where 1 score is almost no fluorescence and 5 score is max fluorescence.

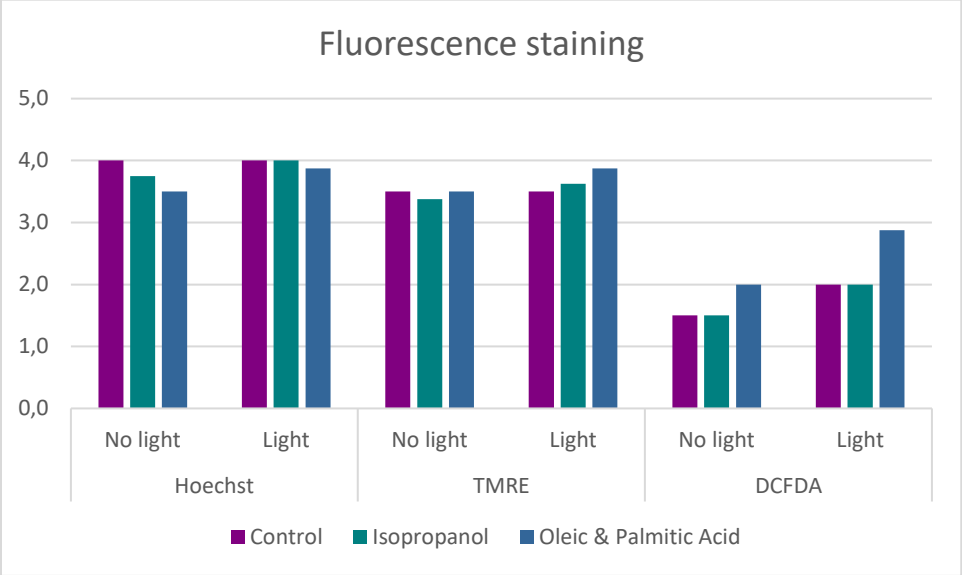

Figure 31: Fluorescent staining rating

The experiment was carried out in quadruple in two independent experiments. Figure 31 shows a small increase in fluorescent of Hoechst staining between no light and light treatment of isopropanol and oleic & palmitic acid, this can be explained by there being more cells still alive after the two different treatments. It also shows a small increase with the TMRE staining.

Figure 32 and Figure 33 shows the results of the fluorescent staining. It shows separately the three different staining's, with Hoechst coloured blue, TMRE coloured red and DCFDA coloured green, and the combination of Hoechst with TMRE and Hoechst with DCFDA. This so it is possible to see if the TMRE and DCFDA staining's did stain a living cell.

Figure 32 shows the results of the fluorescent staining with the cells not treated with light and Figure 33 shows the results of the fluorescent staining with the cells treated with 810 nm light for 30 minutes. When comparing the pictures of the wells not treated and treated with light, it is difficult to see a huge difference between the wells. It shows a small difference in fluorescent of TMRE.

For DCFDA the fluorescent was weaker in all the wells and the difference between the well plates that were analysed first and the second experiment were greater than of the Hoechst and TMRE staining. The well plates that were analysed first had a higher DCFDA fluorescent staining than the second analysed plate. This different was less present with the Hoechst and TMRE staining. The DCFDA staining was also lower than expected, which can mean that the levels of ROS was low in all the HepaRG cells or that the staining protocol is not correct for this specific experiment.

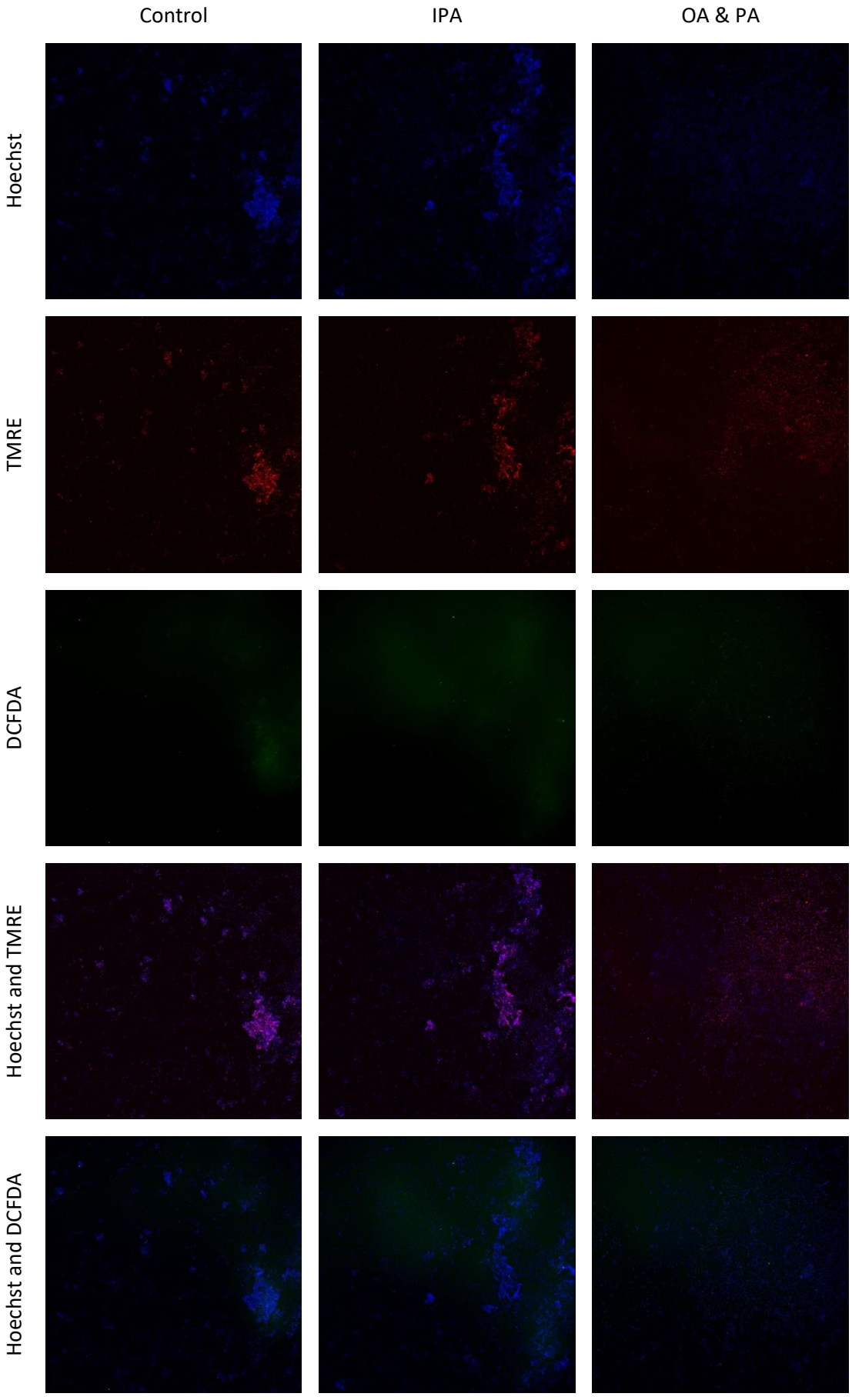

Figure 32: Hoechst, TMRE and DCFDA staining of the no light control wells at 4x magnification

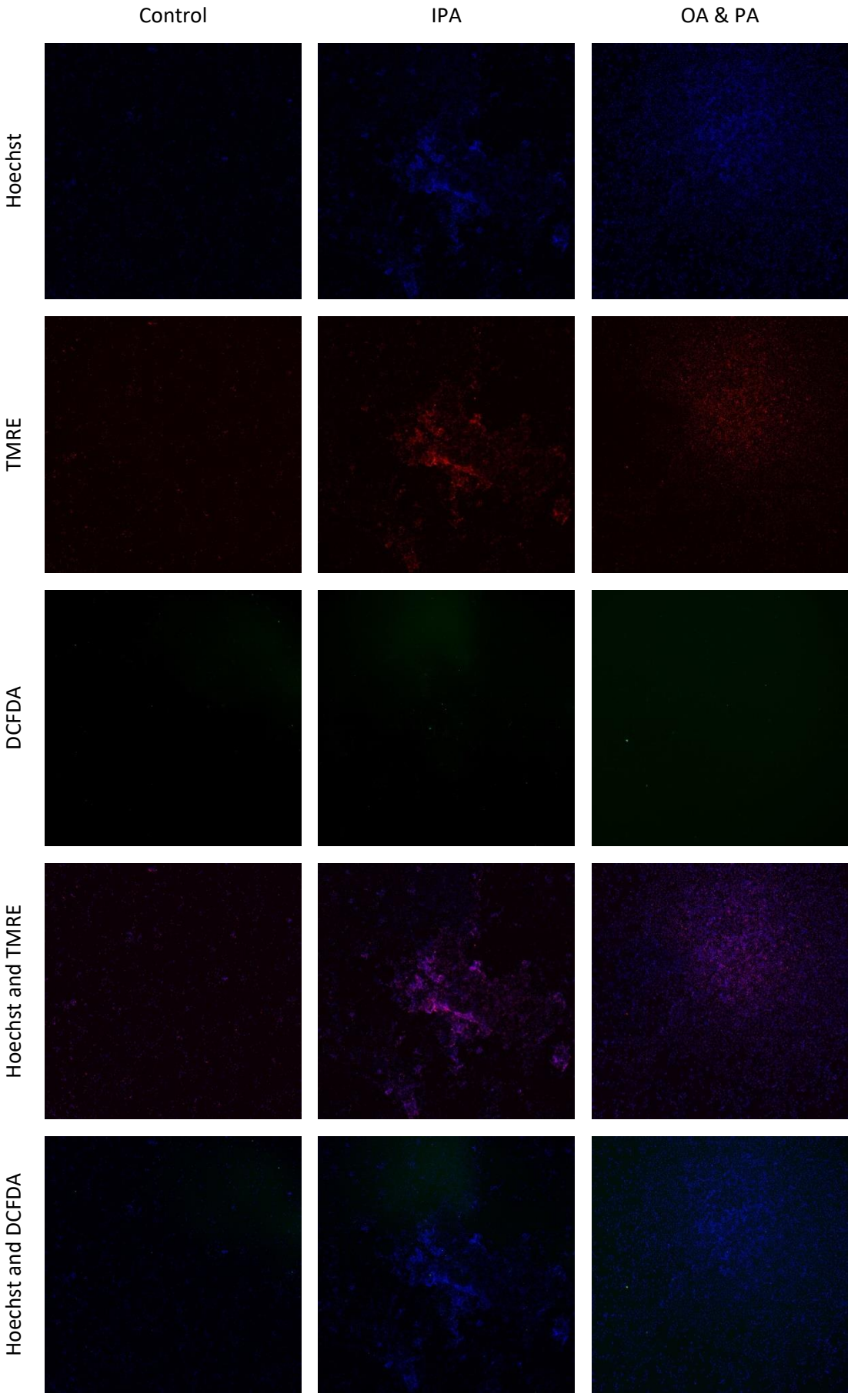

Figure 33: Hoechst, TMRE and DCFDA staining of the 810 nm treated wells at 4x magnification

5. Discussion

The aim of this study was to investigate the effect of PBM with wavelengths of 660 nm (red) and 810 (near-infra red) on fat-fed hepatocytes and to see if it is possible to use PBM as an effective treatment for NASH. To investigate this aim, an *in vitro* study was preformed, with different analysis methods, like the alamar blue and different types of staining. At the moment, the treatment options for NASH are limited and not all the options have a good result [1]. The *in vitro* study was done with the HepaRG, a primary human hepatocyte cell line.

Oil red o staining was performed to determine whether the cells have metabolised the lipid treatment, the treatment was carried out on either two or three wells and in duplicate. This was investigated with a Nikon TI-E Inverted Microscope and the staining was rated on a scale from 1-5, with 1 being no to a little staining and 5 a lot of staining. For the 660 nm wavelength, the results were inconclusive. The observation of the amount of ORO staining on the cells were about the same for the wells treated with and without light. For the 810 nm wavelength, a small difference was observed. It was also observed that a cell density of 100.000 cells per well was at the high side, so for the second 810 nm ORO staining, a lower density of 75.000 cells per well was used. This resulted in a clearer look at the amount of oil red o staining present in the cells.

The alamar blue assay was used to determent the health of the cells after the lipid treatment. Healthy cells reduce the blue non-fluorescent resazurin in resorufin, which is red in colour and highly fluorescent. When the different treatments are normalized in comparison to the non-light treated control wells, it shows the viability of the cells. This experiment was carried out on either duplicate or triplicate in two independent experiments. The HepaRG have a slow metabolic activity, which resulted in a longer incubation period of 6 hours and for the cell density to be higher for the final experiment. For the 660 nm wavelength, the alamar blue assays were also inconclusive. With these and the ORO results, the conclusion was drawn that for this study 660 nm did not have an effect on the fat-fed hepatocytes and the decision was made to not continue with the 660 nm wavelength iCAP device for this study. The alamar blue assays from the cells treated with 810 nm wavelength did show some improvement in comparison with the cells that weren't treated with the iCAP device. The difference in metabolic activity of the cells treated with the two different wavelength can be explained by the different mechanics of the PBM effect of visible or near-infrared light (see Figure 4). The NIR results in a higher energy requirement from the cell, which triggers the mitochondria to produce more ATP and thus more metabolism of resazurin to resorufin. [14]

To determent if PBM has an effect on the oxidative stress and the mitochondrial activity of the cells, staining's with fluorescent markers were carried out and examined under a Nikon TI-E Inverted Microscope. The well plates of the second experiment were observed under the microscope at a later point in the day than the first experiment. During this time, the well plates were kept at room temperature. It looked like this had an effect on especially the DCFDA stained cells, here the different between the first plate and second plate was the greatest. TMRE was used to look at the active mitochondria of the cells and DCFDA to look at the oxidated stress of the cells. The staining of the cells was rated on a scale from 1-5, with 1 being none to a little fluorescent and 5 a lot of fluorescent. The results of these staining showed that the 810 nm iCAP device has a positive effect on the fat-fed hepatocytes. The TMRE staining was higher, this means that there are more active mitochondria than in the cells that were not treated with light. A higher mitochondrial activity is one of the workings of PBM, especially with NIR [11] [14]. The DCFDA staining was also more present in the cells which were treated with light, which means that there is ROS present in those cells. It is expected that the DCFDA staining will be less fluorescence with the cells treated with light. PBM has as an effect that there is less ROS in the cells, which would result in less DCFDA being oxidized to DCF. Which these DCFDA

results, it is necessary to look further into the application of DCFDA staining with HepaRG. It might be necessary to change the protocol of the DCFDA staining.

6. Conclusion

The initial aim of this study was to examine the effect of LEDs with the wavelength of 660 nm and 810 nm on fat-fed hepatocytes and to see whether or not it is a positive addition to the treatment of NASH. This research has shown that, although useful in different diseases, 660 nm wavelength does not have a positive effect on the lipid treated cells, when applied for 30 minutes. For 810 nm this research shows that there is a possible positive effect in *in vitro* study, and it would justify to investigate further.

7. Future recommendation & perspective

The results of this research do indicate a positive effect of PBM with near-infra red light on the fat-fed HepaRG and this makes it a candidate for possible treatment of patients with NASH. The possible advantaged can be big, it is a relative low cost treatment with no known side effects.

To implement PBM in clinical use can be relatively simple, to put a light source with a wavelength of 810 nm on the body of the patient, at the location of the liver for 30 minutes. It is a simple and non-invasive procedure.

More research is needed to see if these effects are indeed because of PBM and what other effects it might have. qPCR and ELISA analyses might also be performed in the future to get more data. qPCR can show information about the expression of specific genes and ELISA to see measure the enzyme activity if the cells.

The analysing of the ORO and fluorescent staining could also be explored more and looked at to see if a quantifiable analyse method can be performed.

The 660 nm wavelength however, has shown no effect on the fat-fed HepaRG's and more research on this wavelength on hepatocytes is not recommended. Other research has shown that 660 nm can be positive for example for wound healing, but not to cure NASH.

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Appendix

Protocol 1

Day 1: Protocol cell plating 5 treatments

Preheat the media, PBS and trypsin

1. Extract the media
2. Wash 3x with 3 mL PBS
3. Add 2 mL of trypsin to the flask (divide well, don't remove)
4. Incubate for 5 minutes
    a. Use this time to get the necessary supplies (well plates, flask)
5. Tab the bottom with force
6. Add to 6 mL media and resuspend with force
7. Count the cells using the cell counter
    a. Add 10 µl of cell suspension to one side of the chip and both sides
    b. Focus on the cells
    c. Press cell count
        i. Make sure the two different sides have similar cell/mL number
    d. Calculate average
8. Dilute the cells with media to appropriate concentrations of cells needed
    a. Make 2 tubes with the right concentration of cells, one for the OilRedO plates and one for the AMA plates.
    b. Concentration needed for first half of experiments
    c. Concentration needed for second half of experiments
        i. ORO and fluorescent staining: 75.000 cells per well
        ii. AMA: 150.000 cells per well
9. Add 1 ml of cell suspension per well (shake well in 8-form)
10. Control using the microscope if the cells are present.
11. Put the plate in the incubator for 24 hours and continue with treatment.
12. Use the remaining cells in the flask and 14 ml of medium to make a new T75 flask for next passage.

Protocol 2

Day 1: Protocol cell plating 3 treatments

Preheat the media, PBS and trypsin

1. Extract the media of the T175 flask
2. Wash 3x with 3 mL PBS
3. Add 2mL of trypsin to the flask (divide well, don't remove)
4. Incubate for 5 minutes
    a. Use this time to get the necessary supplies (well plates, flask)
5. Tap the bottom with force
6. Add to 6 mL media and resuspend with force
7. Count the cells using the cell counter
    a. Add 10 µl of cell suspension to one side of the chip, to both sides
    b. Focus on the cells
    c. Press cell count
        i. Make sure the two different sides have similar cell/ml number
    d. Calculate average
8. Dilute the cells with media to appropriate concentrations of cells needed
    a. Make 2 tubes with the right concentration of cells, one for the OilRedO plates and one for the AMA &qPCR plates.
    b. Concentration needed
        i. Total wells
            1. ORO and fluorescent staining: 75.000 cells per well
            2. AMA: 150.000 cells per well
9. Add 1 ml of cell suspension per well (shake well in 8-form)
10. Control using the microscope if the cells are present.
11. Put the plate in the incubator for 24 hours and continue with the treatment.
12. Use the remaining cells in the flask and 14 ml of medium to make a new T75 flask for next passage.

Protocol 3

Day 2: Protocol lipid 5 treatment

1. Get a filter with 0,2 µm filter, 10 mL syringe (both sterile) and a 50 mL flask
2. Preparing 1% BSA medium. Weigh the BSA in a tube in the big lab, add the medium in the fume hood in the ML-II lab. Put it in the water bath to preheat and dissolve.
3. Prepare oil treatments (mix the OA and PA)
4. Take out the Alamar blue plates, remove the medium
5. Add 1 mL of the treatments according to the 12 well plate design.
6. Put the plates in the incubator. When in the incubator, remove lid of the well plate and place the light module on the designated plate. Leave the light module on the plate for 30 minutes.
    a. **Check if the light is on!!**
7. After 30 minutes, take the light module of the plate and place the lid back on the plate. Take the OilRedO plates out for oil treatment.
8. Remove the medium and add the 1 mL of the treatments according to the 12 well plate design.
9. Put the plates in the incubator. When in the incubator, remove lid of the well plate and place the light module one the designated plate. Leave the light module on the plate for 30 minutes.
    a. **Check if the light is on!!**
10. After 30 minutes, take the light module of the plate and place the lid back on the plate.

Protocol 4

Day 2: Protocol lipid 3 treatment

1. Get a filter with 0,2 µm filter, 10 mL syringe (both sterile) and a 50 ml flask
2. Preparing 1% BSA medium. Weight the BSA (in fridge) in a tube in the big lab, add the medium in the fume hood in the ML-II lab. Put it in the water bath to preheat and dissolve.
3. Prepare oil treatments (mix the OA and PA).
4. Take out the plates, remove the medium
5. Ad 1 mL of the treatments according to the 12 well plate design.
6. Put the plates in the incubator. When in the incubator, remove lid of the well plate and place the light module one the designated plate. Leave the light module on the plate for 30 minutes.
    a. **Check if the light is on!!**
7. After 30 minutes, take the light module of the plate and place the lid back on the plate. Take the OilRedO plates out for oil treatment.
8. Remove the medium and add 1 mL of the treatments according to the 12 well plate design.
9. Put the plates in the incubator. When in the incubator, remove lid of the well plate and place the light module one the designated plate. Leave the light module on the plate for 30 minutes.
    a. **Check if the light is on!!**
10. After 30 minutes, take the light module of the plate and place the lid back on the plate.

Protocol 5

Day 3: Protocol Alamar Blue (ABA)

Start with this assay 6 hours before the end of the treatment OR make the alamar blue solution after the end of the treatment.

Alamar blue is photosensitive, turn off the light!

1. Preheat Alamar blue solution, PBS and medium
2. Remove medium and was 3X with 1 mL PBS
3. Add 1 mL of medium and ad 110 µl per well of Alamar blue
    a. 10% Alamar blue solution to each well (110 µl per well)
4. Incubate the well plate for 6 hours
5. After incubation take a black base 96-wells plate. Transfer 100 µl of each well in two different wells in the 96-well plate.
6. Cover the plate with aluminium foil.
7. Take the plate to the plate reader
    a. Insert the plate without the lid on
    b. Lab MCBP: Select AMB
    c. Choose the selected wells
    d. Save the file on a USB.

Protocol 6

Day 3: Protocol OilRedO, 5 treatments

1. Preheat 1 flask of PBS (need 20 ml)
2. Prepare a 60% isopropanol solution in MilliQ
    - Stock is 100%
3. Take 9 mL of PBS in a separate flask and put in the freezer
4. Extract the media and wash the cells 1x with 1 ml of PBS.
5. Prepare 4% formaldehyde in ice-cold PBS in the fume hood in the big lab
    - Formaldehyde stock is 37%. Dilute 1:10 to achieve a concentration of ~4%
6. Fix the cells with formaldehyde (400 µl per well) for 20 minutes at RT
7. Prepare OilRedO working solution fresh from stock solution:
    - Cover 2 x15ml flasks with aluminium foil, one unfiltered, one filtered
    - According to ratio 3 ml OilRedO stock : 2 ml MilliQ
    - Strain with a 0.45 µm filter
    - Keep covered until use
8. Wash cells with 60% isopropanol (500 µl per well) for 5 minutes
9. Incubate cells with OilRedO solution for 15 minutes (500 µl per well) keep the plate covered
10. Wash cells with 60% isopropanol (500 µl per well) for 5 minutes
11. Counterstain cells with hematoxylin for 3 min – 500 µl per well
12. Wash in running tap water for at least 5 min
    - Aim on an empty well preferably
13. Leave some water in the wells
14. Take photos using the microscope

Protocol 7

Day 3: Protocol OilRedO, 3 treatments

1. Preheat 1 tibe of PBS
2. Prepare a 60% isopropanol solution in MilliQ
    - Stock is 100%
3. Take 9 mL of PBS in a separate tube and put in the freezer
4. Extract the media and wash the cells 1x with 1 ml of PBS.
5. Prepare 4% formaldehyde in ice-cold PBS in the fume hood in the big lab
    - 9 ml of ice-cold PBS and 1 ml of 37% Formaldehyde
    - (Formaldehyde stock is 37%. Dilute 1:10 to achieve a concentration of ~4%)
6. Fix the cells with formaldehyde (400 µl per well) for 20 minutes at RT
7. Prepare OilRedO working solution fresh from stock solution:
    - Cover two 15 ml flasks with aluminium foil, one unfiltered, one filtered
    - According to ratio 3 ml Oil Red O stock : 2 ml MilliQ
    - Strain with a 0.45 µm filter
    - Keep covered until use
8. Wash cells with 60% isopropanol (500 µl per well) for 5 minutes
9. Incubate cells with OilRedO solution for 15 minutes (500 µl per well) keep the plate covered
10. Wash cells with 60% isopropanol (500 µl per well) for 5 minutes
11. Counterstain cells with hematoxylin for 3 min – 500 µl per well
12. Wash in running tap water for at least 5 min
    - Aim on an empty well preferably
13. Leave some water in the wells
14. Take photos using the microscope

Protocol 8

Day 3: Protocol DCFDA, TMRE and Hoechst v2

1. Make 40 µM DCFDA solution
2. Remove the medium from the wells
3. Add 1 ml of the 40 µM DCFDA solution
4. Incubated for 15 minutes at 37°C
5. TMRE is in the refrigerator (in box “Assays”) or in the freezer at -30°C
6. Take the plates from the incubator and add 20 µl of TMRE 5x working solution to get 200 nM of TMRE and add 0.5 µl of Hoechst 33342 to each well, shake wells well
7. Incubated for 30 minutes at 37°C
8. Wash the wells three times with PBS, leave last wash
9. The cells were then viewed under the Nikon TI-E Inverted Microscope
    a. For the Hoechst staining, DAPI channel (blue)
    b. For the TMRE staining, PE channel (red)
    c. For the DCFDA staining, FITC channel (green)

Protocol 9

Cell splitting

HepaRG cell culture (T-25 tissue treated)

Media: William E + 10% FBS + 1% L-Glutamine + 1% pen/strep + 5 µg/ml insulin + 0.5 µM hydrocortisone

Protocol:

From T-75 to T-175

Preheat the media (30 ml), PBS (6 ml) and trypsin (half an hour before passage) and check the flask

1. Extract the media using the aspiration system
2. Wash 3x with 2 ml PBS
3. Add 0.75 ml trypsin (divide well)
4. Incubate for 5 min at room temperature, tap against the sidings (control using the microscope if the cells came lose)
5. During incubation add 25 ml in a new T-175 culture flask, write on the flask the cell type, passage number, date and your name (write this also down in your lab journal)
6. Add 5 ml medium to the trypsinized cells and resuspend
7. Add all the cell suspension to the new flask
8. Control using the microscope if the cells are present in the flask