In Vitro UV-Visible Spectroscopy Study of Yellow Laser Irradiation on Human Blood

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Abstract. This experimental study was performed to investigate the effect of low level yellow laser of 589nm wavelength with various laser irradiation time. Human blood samples with random diseases are irradiated with yellow laser of power density of 450mW/cm² from 10 minutes to 60 minutes at 10 minutes intervals. The morphology of the red blood cell were also observed for different irradiation time. The result shows that there is a significant different in the absorption of light with varying laser irradiation time (p<0.01). The maximum absorption recorded at 40 minutes of irradiation at 340nm peak. Blood smear of the samples reveals that there are observable changes in the morphology of the red blood cell at 40 minutes and 60 minutes of irradiation.

Introduction

Human blood contributed to homeostasis by transporting oxygen, carbon dioxide, hormones, and nutrients to and from the body cells. There are two components in the blood which are blood plasma and formed elements. Blood is about 45% formed elements and 55% blood plasma. The human blood includes three principal components: erythrocytes or red blood cells (RBCs), leucocytes or white blood cells (WBCs), and thrombocytes or platelets. The percentage of total blood volume is occupied by RBCs. RBCs are specialized in transporting oxygen. The nature of this cells that generate ATP anaerobically allow the oxygen to be transported effectively as these cells does not uses the oxygen they transport. The plasma membrane of RBCs is strong and flexible, allowing them to deform and passes through narrow blood vessels and capillaries without rupturing. [1] Laser irradiation induces conformational transitions of the red blood cell membrane. This resulted in changes of the structural states of both erythrocyte membrane proteins and lipid bi-layer. Consequently, the activity of the membrane ion pumps also changed. [2] Irradiation of red blood cells may result in deformability which subsequently will have an effect on the function of the cells if it is given beyond therapeutic level.

Low level laser therapy (LLLT), also known as photobiomodulation involves exposing cells or tissues to low level light. This type of therapy is also known as cold laser therapy as the power densities used produces no heating effect on the tissues. LLLT has a photochemical effect which means the light is absorbed and cause a chemical change. [3] In medicine, light absorption by non-specialized photoacceptor molecules is used as the absorbing molecules. This process can transfer the
energy to another molecule. As a result, chemical reactions occurred in the surrounding tissues. Biological tissues are mostly translucent. As light passes through, most of the light will be scattered rather than absorbed. This is due to the refractive index of the tissues. Absorption of light is via chromophores, including water, various proteins, hemoglobin, and various tissue pigments. [6] Laser therapy has been used to treat numerous conditions that require stimulation of healing and restoration of function. The use of low level laser is widely applied to reduce pain, inflammation and oedema, to promote wound and nerves healing, and to prevent tissue damage. [4][5]

Although LLLT is now used widely in medical field, the underlying biochemical mechanisms is still not well understood. Different laser parameters must be optimal to maximize the effectiveness of the treatment. The effectiveness of laser therapy is characterized by a biphasic dose response curve. [7] The primary reaction after light absorption are based on five different hypotheses, namely redox properties alteration hypothesis, superoxide anion hypothesis, nitric oxide hypothesis, singlet-oxygen hypothesis, and transient local heating hypothesis. The secondary reactions are cellular signaling pathways, including mitochondrial retrograde signaling. Mitochondria are thought to be a likely site for the initial effects of light. This leads to an increase of Adenosine 5'-triphosphate production, inflection of reactive oxygen species, and ratiocination of transcription factors. Later, these effects leads to increased cell migration and cell proliferation. [8] But since mitochondria is absent in red blood cell, further research needs to be done in order to understand the response and the mechanisms of laser radiation interaction with biological tissues.

In therapeutic laser therapy, application of correct dose will stimulate a positive effect, but a higher dose will inhibit the response. [9] Delivering optimal dose is important to maintain the physiological function of the red blood cells. Ergo, this paper investigate the effect of 589nm yellow laser with different exposure time using UV-Vis spectrophotometer.

**Material and method**

I. Blood sample preparation

2.5 to 3 mL human blood sample of a patient of age 20 to 35 is collected into Ethylenediaminetetraacetic acid (EDTA) tube to prevent clotting. The blood sample collected from Pusat Sejahtera, USM Penang is used within 48 hours. The blood sample is mixed thoroughly before it is divided into four plain tube of 0.5 mL each to be irradiated with different exposure time.

II. Laser irradiation

589 nm wavelength yellow laser with output power of is 90mW is used to irradiate the blood sample. The beam diameter is 0.5 cm and the beam area is approximately 0.2 cm². The laser is set 15 cm vertically aligned from the blood sample. 0.5 mL blood sample is irradiated with different exposure times of 10, 20, 30, 40, 50, and 60 minutes.

III. Morphological analysis

A blood smear is performed on the control and irradiated samples. A sample portion of blood is dropped near the frosted end of a clean glass slide and a second glass slide is used as a spreader. The blood is streaked in a thin film over the slide. The blood streak is allowed to dry. The slide is labelled accordingly before it is observed under biological microscope with 40x magnifications. The blood is then placed in a centrifuge for separation of plasma and the RBCs. Packed RBCs were diluted and placed in Kartell
The absorbance result of power density of 450 mW/cm² with laser irradiation time obtained from the UV-Vis Spectrophotometer is plotted in a graph as shown in Figure 1. From the graph, it can be perceived that there are four observable peak at specific wavelength. The first strong absorption peak is observed at 340 nm where the carbohydrate metabolism of blood is at its maximum because of
structural changes of Nicotinamide Adenine Dinucleotide (NAD) into NADH and Nicotinamide Adenine Dinucleotide Phosphate (NADP) into NADPH through reduction process. This reduced form of the coenzyme causes an increased in light absorption. Other peaks can be observed at 414, 542 and 576 nm where is represent the d-f transition of CO-oxyhemoglobin. The absorption of light is at their maximum at 40 minutes of exposure to yellow laser. The light absorption increases from 10 minutes to 40 minutes, but it decreases as the exposure time is extended to 50 and it continues to decrease at 60 minutes. This fluctuations of light absorption is known as biphasic response. The mechanism of LLLT at cellular level has been associated with the absorption of monochromatic visible and near infrared radiation. Effective tissue penetration is maximized at specific optical window. \[15\]

Table 1 reveals that there are significant different (p<0.01) on the blood sample irradiated with different exposure time. This suggest that the irradiation time present an imperative role in the effectiveness of a laser therapy. Determining an optimal irradiation time is necessary. Low level laser is proven to provide significant effect on red blood cells. The irradiation to human blood changes the ability to absorb light. This statement is proved by the changes of light absorption as the irradiation time is manipulated.

Tissue penetration of light and specific wavelength of light absorbed by photoacceptor are of the major parameters to be considered in phototherapy. The biphasic response of LLLT suggest that there are two types of reactive oxygen species (ROS) which are good ROS and bad ROS. \[16\] The reason for the production of good ROS is to be connected with stimulation of mitochondrial electron transport as shown by increases in ATP production. Good ROS can initiate beneficial cell signaling pathways leading to activation of redox sensitive transcription factors. But as the dose is increased, beneficial ROS production in the mitochondria decreases as a result of decreased of ATP production. Bad ROS can damage the mitochondria leading to apoptosis. \[17\]

Mof the light that strikes the biological tissues will be absorbed. This phenomenon is the key for the desired effect on the tissues. The highest mean and also the maximum absorption of light occurred 340 nm after irradiation of 40 minutes to yellow laser. Figure 2 compared the light absorption at 340nm for different irradiation time. The optimum light absorption occurred at 40 minutes of irradiation with the highest absorbance recorded.

Laser has biostimulation effect on biological tissues. Fluctuation of light absorption illustrates the biphasic dose response curve. When the blood sample is irradiated, the enzymatic activity of the membrane sodium (Na\(^+\)) and potassium (K\(^+\)) ion pump changes in dose and fluence-dependent manner. Consequently, the biological function of the cells is stimulated and increases the light absorption. But further increase of irradiation time inhibit the enzymatic activities due to the suppression of the Na\(^+\) and K\(^+\). \[18\] Due to the disruption at the membrane, the MCV reduces due to ion fluxes. Movement of ions causes the cell to loss its shape and become crenated. Hence, light absorption reduces.
Figure 1. Absorption of light at different wavelength and irradiation time.

Table 1. Effect of yellow laser irradiated on blood samples.

| Time (minutes) | Mean value (%) | p-value   |
|----------------|----------------|-----------|
| 10             | 78.346         |           |
| 20             | 95.818         |           |
| 30             | 123.428        |           |
| 40             | 133.312        | .000**    |
| 50             | 128.086        |           |
| 60             | 111.994        |           |

**Correlation is significant at the 0.01 level
Blood smear was done to ensure that the RBCs are in good shape. The RBCs have deformable structures that allow them to recover to their original shape which is an important blood characteristic, especially in capillaries. It has been studied that LLL irradiation causes a change of size of RBCs aggregate. Insufficient RBCs deformability increased the aggregability and morphology. The flow properties of lifespan depends on an adequate oxidative stress response. An alteration and deformability of the RBCs may affect the function of the cells.

Figure 2. Absorption of light at 340nm with different irradiation time.

Figure 3 shows the blood smear of the control sample. The RBCs are in good shape. After 10 minutes irradiation, the blood smear in Figure 4 shows a few abnormal cell shape but it can be neglected as less than 5 observable cells are observed. Figure 5 shows the blood smear for blood sample irradiated at 40 minutes. Abnormal cells observed are keratocytes, dacrocytes, and echinocytes. At 60 minutes of irradiation, keratocytes and echinocytes were observed, as shown in Figure 6.

Referring to the blood smear prepared, most of the abnormal cells observed were echinocytes. RBCs are made up of proteins. As the irradiation increased, the local heat increased. Tremendous local heat can result in denaturation and precipitate stress to the membrane. Therefore, the shape of the cell also changes. Echinocytes formed from the loss of water and potassium due to decreased in ATP generation. The echinocytes can become spherocytes as they loses membrane vesicle. Further loses of the surface area and volume leads to hemolysis.

LLL irradiation changed the morphological properties of the RBCs. Blood smear showed deformed RBCs after irradiation. The amount of deformability of the RBCs increased as the irradiation time increase above threshold. Different output power has different threshold irradiation
time. Although the maximum absorption recorded at 40 minutes of irradiation, the irradiation time cannot be considered optimum as there is observable changes in the RBC's morphological properties.

**Figure 3.** Blood smear of a control sample.

**Figure 4.** Blood smear of a sample irradiated at 10 minutes.

**Figure 5.** Blood smear of a sample irradiated at 40 minutes.

**Figure 6.** Blood smear of a sample irradiated at 60 minutes.
Conclusion

This experiment was performed to study the absorption of light at different exposure time. Analysis of the spectrum from UV-Vis spectrophotometer showed different absorption level when irradiated at different exposure time. The highest irradiation time is 60 minutes but the maximum absorption is at 40 minutes of irradiation to laser. Blood smear showed deformed cells after irradiation. The amount of deformability of the blood cells increased as the irradiation time increase.

Acknowledgement

The authors would like to extend their appreciation to Universiti Sains Malaysia (USM), Penang for the funding of this research (304/PFIZIK/6315023) and Medical Physics and Biophysics Laboratory staff for their assistance and support.

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