Research Article

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Spectroscopy Study of Packed Erythrocytes Irradiated by 532 nm Low Level Laser

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Abstract

Background: *Low-level laser wavelengths have been used in a variety of medical applications due to their ability to modulate blood rheology and enhance microcirculation. The reaction of human blood to low-level laser irradiation (LLLI) offers valuable information regarding the interaction of laser light with tissues.*

Objective: *The aim of the study was to observe whether in vitro irradiation affects packed red blood cells and whole blood.*

Methods: *Blood samples were each split into two equal aliquots labelled as control (non- irradiated) and irradiated groups. Irradiated sample was exposed to LLLI output powers of 60, 80, and 100 mW at 532 nm wavelength with various irradiation time ranged from 30, 60, 90, 120, and 150 s.*

Results: *Irradiated samples for packed red blood cells (RBCs) before mixing with plasma have a lower absorption range compared to irradiated samples after mixing with plasma.When compared to whole blood, RBCs have a higher absorption range. Blood samples smeared showed variations in the packed RBCs morphology between the control samples. Blood samples smeared showed no changes in the red blood cell morphology between the control and irradiated samples.*

Conclusion: *The results revealed that different irradiation exposure durations resulted in significant differences in light absorption of RBCs and whole blood. The optimum laser dose obtained from the result for RBC packed cell is 100 mW at 150 s while for whole blood is 80 mW at 60s exposure time.*

Keywords: Low Level Laser, Blood, UV-Vis Spectroscopy, Irradiation

1. Introduction

Laser is an acronym for light amplification by stimulated emission of is a powerful light beam that produced intense heat when focused at a short distance [1,2]. Lasers are instruments producing or amplifying coherent radiation at frequencies in the electromagnetic spectrum's infrared, visible, or ultraviolet regions [3]. Many lasers are used in medicine for surgery, such for cutting and debulking soft tissues [4]. While, low level laser intensity (LLLI) contributions more towards therapeutic measures of different pathological situations like healing of wounds and inflammations as well as pain treatment initiated by Mester and colleagues efforts, who applied a low-energy (1 J/cm2) ruby laser in wound healing enhancement therapy [5]. Several researchers have reported that laser biostimulation effects depend on several factors and parameters like wavelength, beam range, dosage, laser intensity, irradiance, specific absorption rate (SAR), polarization and most importantly considering the irradiated cells [6,7]. Some of the researches indicated that lasers emissions within powers output of 1 – 500 mW and energy densities (0.04 – 50 J/cm2) at a monolayer of cells or target tissue are considered to be LLLI. This irradiation prevents apoptosis, stimulates cell proliferation improvement, control cell migration, and adhesion at these low levels of visible light exposure. It's also been discovered that laser irradiation causes red blood cell membrane conformation transformations, which are caused by changes in the structural states of both erythrocyte membrane proteins and lipid bi-layers, resulting in changes in membrane ion pump activity [8]. LLLI is not thermal or an ablative mechanism although considered as a photochemical effect similar to the photosynthesis process in plants where the absorbed light causes a chemical change [9]. In the literature, experimental analysis of laser effects between visible and near-infrared wavelengths displayed significant variations with better results achieved with a visible wavelength [10]. Further work is required to elucidate the effects of low-level laser irradiation on human blood cells therefore the study is very pertinent in understanding the inter-

action mechanisms of laser irradiation of the biological tissues. The purpose of this research is to investigate in vitro effects of different low-level 532 nm wavelength laser doses on normal red blood cells (RBCs) in human blood. Light's biological effects differ according to wavelength, time of light exposure, light intensity (dose or fluence) given. For light-induced biological processes to occur, the photoacceptor molecules in the cells will absorb light. While high output power lasers ablate tissues, low-power lasers are proposed to activate tissue and promote the processing of cells. This low irradiation is incorporated with conventional medicine with ongoing work to determine whether a demonstrable impact occurs. For wound healing, phototherapy with various light sources (i.e., illumination, ultra-violet irradiation, lasers, and light-emitting diodes) was used. Sunlight was used by Ancient Greeks to cure numerous skin disorders [11]. Irradiation therapy with low power output lasers or light-emitting diodes within the red to the near-infrared region (630-1000 nm) has been used in soft tissue injuries treatment over the past 40 years and shown to facilitate tissue rejuvenation in both in vivo and in vitro [12,13].

2. Materials and Method

2.1 Blood Sample Preparation

The research was carried out by using 40 fresh human blood samples obtained from 17 males and 23 females within the age 21 to 60 years, without previous history of any major diseases or treatment from Hematology Laboratory, Wellness Center, Universiti Sains Malaysia. 3 mL of blood samples each were collected in test tubes with EDTA (1.3 mg/mL blood anti- coagulation substance). The collected samples were immediately analyzed after collection by dividing each sample into two aliquots for use as control (non-radiated) and irradiated samples.

2.2 Irradiation of Sample

The 532 nm wavelength green laser (beam aperture of 5 mm) is calibrated and warmed up before irradiation to achieve stable beam output. The sample is placed vertically under the laser with an upright position at 6 cm apart between the sample and laser is 6 cm. The blood samples then irradiated at varies output power (60, 80, and 100 mW) with different exposure times of 30, 60, 90, 120, and 150 s.

2.3 Blood Smear Preparation

The morphology of blood components was examined using blood slides prepared before and after laser irradiation under light microscopy. A droplet of well-stirred blood was pipetted at the end of a microscopic slide at 1 cm from the edge. With the aid of a spreader slide carrying a chipped edge placed in front of the blood droplet inclined at an angle about 30^{o} - 45^{o} to the blood. Spreader slide was pulled back into the blood drop, so that the blood spreads along its edge, and then pushed in other direction to the end of the smear slide resulting in a thin layer smear for microscopic examination. The smeared blood on the slide was then left to air dry for about 3 min.

2.4 UV-Vis Spectrophotometer

Shimadzu UV-Vis spectrophotometer used was calibrated for each experiment to ensure the accuracy and precision of the instrument. The spectral bandwidth of the instrument is 1 nm with a scanning range of 200 to 1000 nm. The instrument is used to determine the diluted blood light absorption capability for the control and irradiated samples. The reference cell contained 4 mL normal saline solvent in polystyrene cuvette of volume 4.5 mL with 10 mm length. The 4 µL of packed RBCs was diluted and mixed thoroughly with 4 mL of normal saline. Both samples are placed together in the UV-Vis Spectrophotometer to get the absorbance reading of the diluted packed RBCs. The spectrum obtained is analyzed using UV Probe version 2.3 software.

3. Result and Discussion

3.1 Absorption Spectrum of Packed RBC Irradiation

The presented results in Figures 1 (a)-(f) show difference between non-irradiated and irradiated samples before and after mixing the plasma. The first peak of the absorption spectrum of the packed RBC irradiated was observed to occur at 340 nm wavelength at different time durations. The 340 nm is where the metabolism of the blood carbohydrate is considered to be at its highest peak due to structural variations in Nicotinamide Adenine Dinucleotide (NAD) to NADH and Nicotinamide Adenine Dinucleotide Phosphate (NADP) to NADPH by reduction mechanism. This reduced coenzyme form increases high absorption. Further peaks were observed at 414, 542 and 576 nm where co-oxyhemoglobin d-f is represented [14-16]. The highest absorption at this 340 nm wavelength for irradiated samples before mixed plasma is at 60 s (Figure 1 (a)) and after mixed with plasma again at 150 s (Figure 1 (b)) irradiation time for output power of 100 mW. When output power increased to 80mW, the absorption light has increased at exposure time of 30 – 60 s. Then, as the time increased from 60 to 90 s the absorption is decreasing. (Figure 1 (c) and 1 (d)). But when output power is reduced to 60mW, the absorption light is decreasing for exposure time of 120 and 150 s (Figure 1 (e) and (f)). The light absorption fluctuations are recognized and known as biphasic responses.

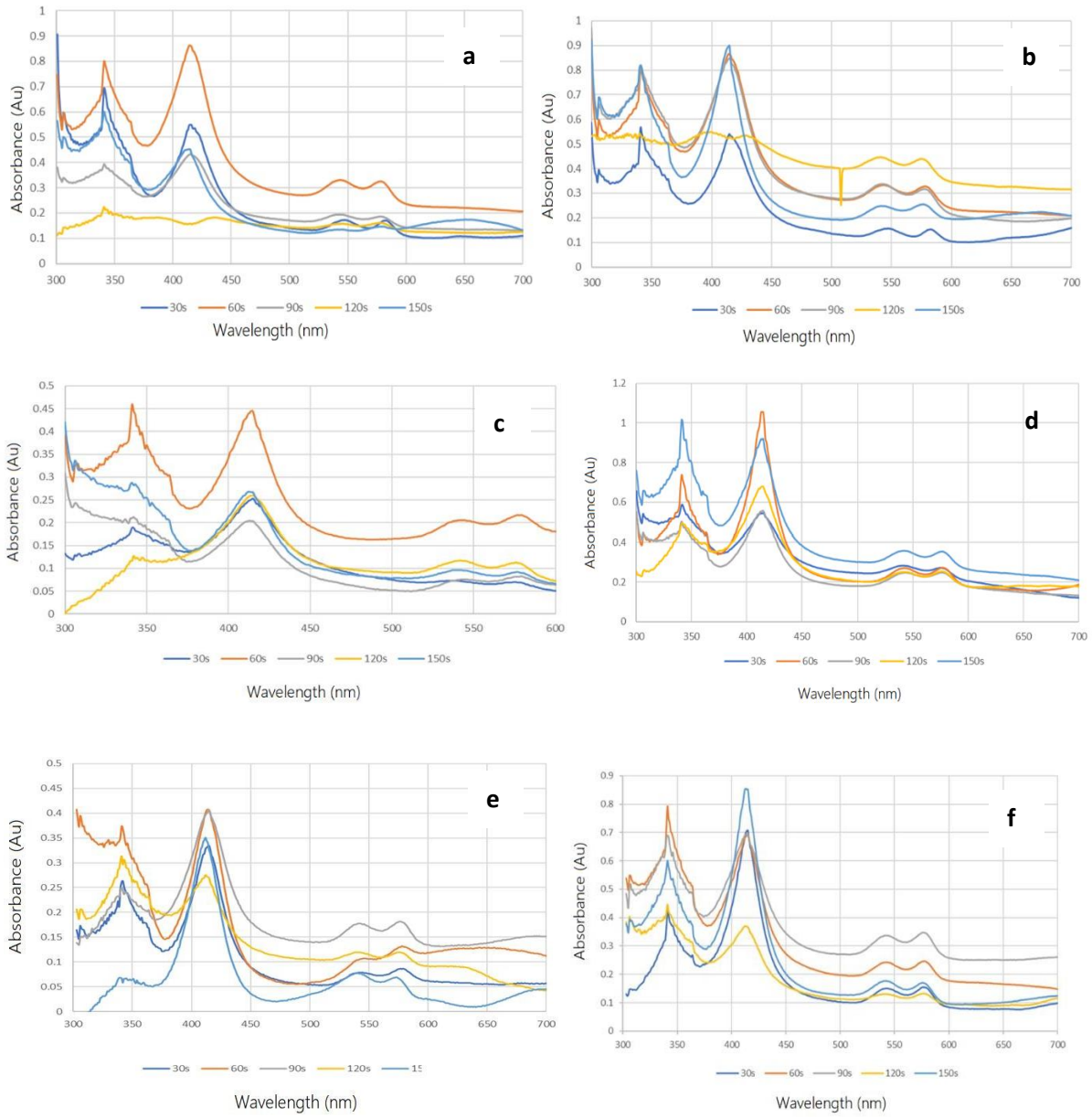


Figure 1: (a) Difference of Absorption Spectrum before Mixed Plasma at 100 mW; (b) Difference of Absorption Spectrum after Mixed Plasma at 100 mW; (c) Difference of Absorption Spectrum before Mixed Plasma at 80 mW; (d) Difference of Absorption Spectrum after Mixed Plasma at 80 mW; (e) Difference of Absorption Spectrum before Mixed Plasma at 60 mW; and (f) Difference of Absorption Spectrum after Mixed Plasma at 60 mW.

3.2 Absorption Spectrum for Whole Blood Irradiation

Similarly, for whole blood irradiation, it can be observed that the first peak is around 340 nm, 419, 544 and 583 nm. Tissues penetration by the ultraviolet light (UV) and the absorbed wavelength by the photoacceptor are the essential factors in laser therapy. The absorption has to do with the biphasic responses of the laser therapy which show the two types of reactive oxygen species (ROS), that is the great ROS and bad ROS [17,18]. The purpose for the good ROS production is to be linked with the enhancement of mitochondrial electron transportation, as shown by the rise in the ATP production. The good ROS can activate beneficial cells signaling pathways that lead to the redox sensitive transcription factors activation. Though, at increased exposure, the beneficial ROS production in mitochondria decreased due to reduced output of ATP [19]. The one considered as bad ROS can harm the mitochondria that lead to apoptosis [20]. Figures 2 (a), (b), and (c) compared the difference absorption between non-irradiated and irradiated samples for different irradiation time. The UV-light that hits the biological tissues is been absorbed. This phenomenon is the gateway for the desired effect on the tissues.

Absorption of solute is linearly dependent on its concentration since absorption is ideal for quantitative. The UV-light absorption parameters of molecules depend on the molecular environment and that of the chromophore's mobility. Multiwavelength UV-visible spectroscopy is a relatively simple technique that can provide a considerable quantity of information. The UV-visible spectrum of blood contains information on the light absorption and scattering parameters of suspended particles in it [21-23].

The method of laser irradiation at the cellular level has been affiliated with the uptake of monochromatic UV-visible and near-infrared radiation. Efficient tissues penetration is maximized in a limited optical range [24].

As the light hits the sample, energy light facilitates energy from bonding or non-bonding orbitals to one of the vacant anti-bonding orbitals. The electron excites from full orbital to zero anti-bonding orbitals. If the energy is the same as the wavelength energy it will absorb the wavelength energy. The high-energy transition absorbs high-wavelength light. In the UV- Visible spectrophotometer, at a wavelength of between 200 and 1000 nm, the energy transition would be between i) π bonding and π anti bonding ii) n-non-bonding to π anti bonding iii) n- non-bonding to antibonding. The molecules must then contain either π bond or anti-bonding orbital atoms [25,26]. Low-level laser radiation alters the ATBase activity of the membrane ion pumps in the dose and fluence rate-dependent manner. At the same time alters have been observed in integral parameters such as cell stability, membrane lipid peroxidation levels, intracellular reduced glutathione levels. The fractionation of the light dose significantly changed the membrane to laser radiation. Change in tryptophan fluorescent parameters of erythrocyte membrane proteins and the raise in lipid bilayer fluidity measured by pyrene monomer/excimer fluorescence proportion were observed [27].

Laser has an impact on biological tissues by bio-stimulation. Light-absorption fluctuation shows the biphasic dosage response curve. When the blood sample is irradiated, the enzymatic vigor of the sodium membrane (Na+) and potassium (K+) ion pumps modify in dose and fluence-dependent way. As a consequence, the biological work of the cells is stimulated and the absorption of light raises. But further increase in irradiation time inhibits enzymatic activity due to suppression of Na+ and K+. Because of the membrane cutout, the MCV decreases due to ion fluxes, motion of ions caused the cell to lose its shape and become peeler. Therefore, light absorption decreases [28].

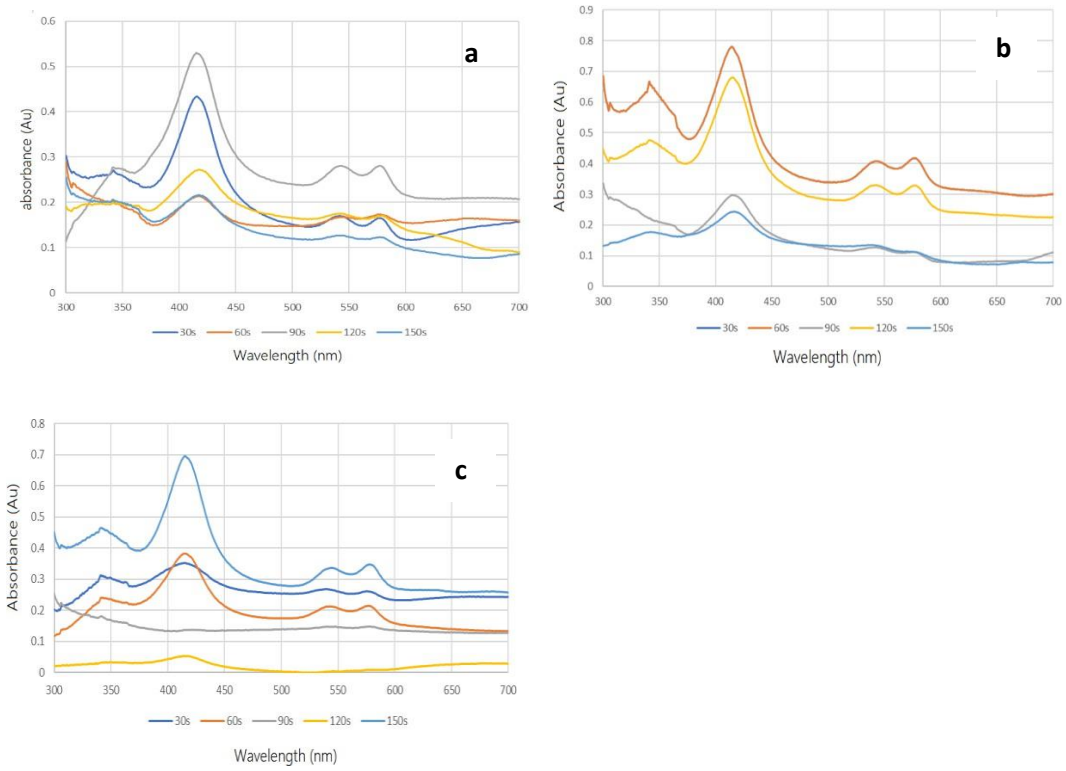


Figure 2: (a) Difference between whole blood at 60 mW; (b) difference between whole blood at 80 mW; and (c) difference between whole blood at 100 mW.

3.3 LLLI-Induced Changes in RBCs Morphology

To observe the effects of laser irradiation on the RBC shape, 40 whole blood smear were prepared after irradiation by green laser 532nm at different output powers. No hemolysis or morphological changes of the erythrocytes were observed. The RBCs were found to maintain their shape after irradiation with effective laser wavelength 532nm at powers 60, 80, and 100 mW, as shown in Figure 3 compared with non-irradiated RBCs. Based on the Figure 3, the morphology for all the irradiated blood does not get affected by all laser parameter.

Previous studies have concentrated on the effects of LLLI on blood behavior as a whole (cells, plasma proteins, microcirculation, and other rheological properties), whereas the current research concentrated for the most part on the effects of LLLI on certain blood parameters separately. This part of the present study attempted to evaluate the in vitro effects of LLLI on RBC. For this experiment, all the cells are normal compared to the previous study that showed abnormal cells commonly known as echinocytes.

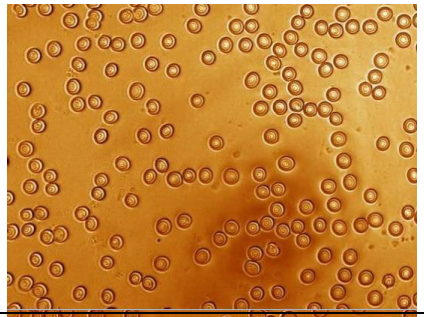
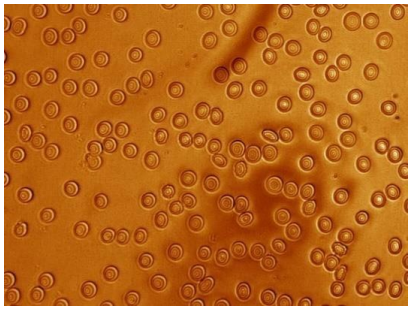
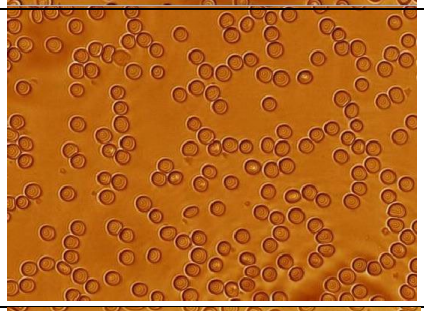
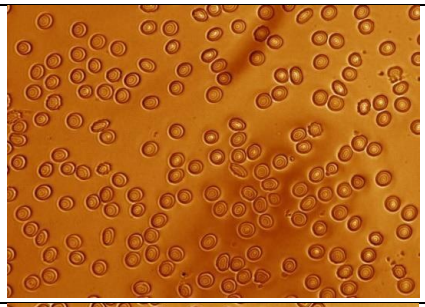
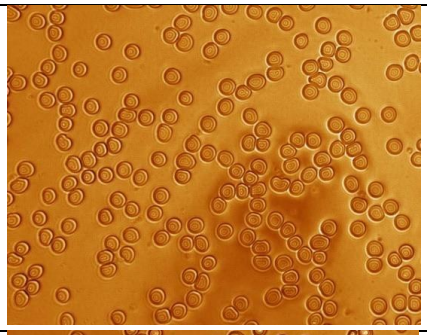
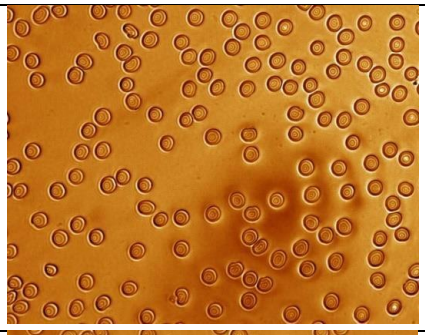
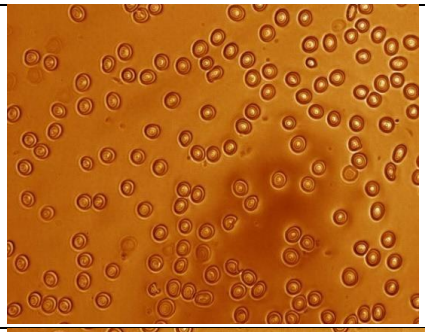
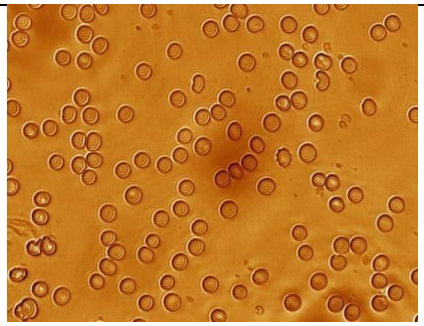
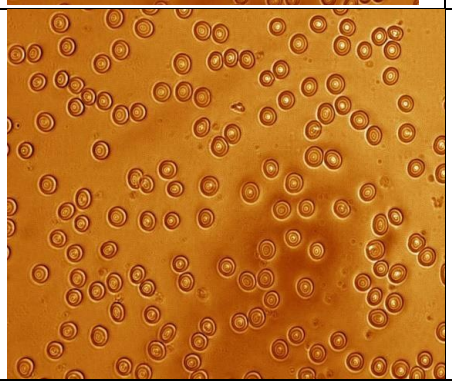
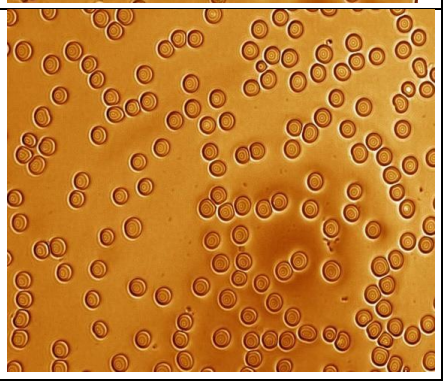
Time irradiation (s)	Non-irradiated	Irradiated
30		
60		
90		
120		
150		

Figure 3: Microscopic appearance of a blood smear at ×40 magnification before and after LLLI of blood samples at 532nm wavelength.

The RBCs consists of proteins, therefore as the irradiation becomes excessive, then increases the local heating. The enormous local heat resulted in denaturation and precipitate stress to the membrane. This triggers the membrane to be under shear stress that changes cells morphology. Echinocytes produced from water and potassium loss as a result of a decline in the production of ATP. Echinocytes can become spherocytes as they lose the vesicle of the membrane. Further loss of surface area and volume leads to hemolysis [29]. However, from the observation for this study, laser parameters used are at low intensity and only caused photochemistry effect instead of photothermal.

4. Conclusion

The research studies of the effects of LLLI on blood are considered to be of great importance to elucidate the mechanisms of action of LLLI in tissues. Different methods of therapy by blood irradiation have been developed and used in clinical practice with beneficial effects. The fact that the response of pathological cells to LLLI differs from the response of healthy cells suggests that LLLI could also be diagnostic method for cellular membrane alterations. Low level laser is proven to have effect on red blood cell. Analysis of the spectrum from UV-Vis spectrophotometer showed different absorption level when irradiated at different exposure time. The highest absorption time is 150 s, but the maximum absorption for irradiated samples before mixed plasma is at 60 s of irradiation to laser and less absorption is at 30 s irradiation time. For irradiated samples after mixed plasma again, the maximum absorption at 150 s irradiation time and less absorption is at 90 s irradiation time. For the whole blood the maximum absorption is at 30 s and less absorption is at 120 s irradiation time. From the experiment, it is established that LLLI of green laser 532 nm wavelength at different laser parameter does not adversely affect human blood cells. Considering the experimental conditions, the highest absorption peak obtained without affecting the blood for RBC packed cell is at 100 mW with 150 s exposure times and 80 mW at 60 s exposure time for whole blood irradiation is therefore considered that this dose value is the optimum for beneficial effects. The conclusion is made as the radiation maintains the shape of cells without leading to negative phenomena such as spherocytosis and hemolysis that are normal in non-irradiated blood.

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