In Vitro Effects of Low Level Laser Irradiation on Blood Protein

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Abstract. Blood proteins serve mostly as drug’s transporters and play a crucial role in laser therapeutic purposes. Its low level laser irradiation (LLLI) reveals the mechanisms for the efficacy of antiviral therapy. This research work is interested in elucidating protein (ceruloplasmin, fibrinogen (α-chain), complement C2, albumin, and α-1-antitrypsin) level perturbation by single (single exposure) and multiple exposures (fractionation) LLLI. Protein level after LLLI reveals protein behaviour essential for progression of laser therapy used in protein-related pathologies. Whole blood (mixed with EDTA) irradiation performed with diode laser (λ = 405 nm). Optical microscope and polyacrylamide gel electrophoresis were used for RBC morphology image acquisition and semi-quantification of protein level respectively. Our results show that crenated RBC has minimal effect on protein level perturbation for dose between 32.59 J/cm² and 130.35 J/cm², single exposure has an overall increased protein level for all five proteins at dose 130.35 J/cm², and multiple exposures has an overall decreased protein level for all five proteins at dose 97.77 J/cm². Optical density change was seen most in fibrinogen (α-chain) and least in albumin for single (fibrinogen (α-chain): 423 %; albumin: 59 %) and multiple exposures (fibrinogen (α-chain): -71 %; albumin: -30 %). A large variation in protein level perturbation for high molecular weight proteins, while slight variation for low molecular weight proteins were also observed for single exposure at dose 130.35 J/cm² (except α-1-antitrypsin at 32.59 J/cm²). This data provides evidence of the biostimulation effects of low level laser irradiation on blood proteins and the 2 hours’ adequate incubation time for decreasing protein level.

1. Introduction
The human blood proteins influence a lot of recuperation processes in the body tissues mostly that of patients undergoing different medical treatments [1]. Low level laser irradiation (LLLI) is now widely used in different branches of medicine as a result of its recent experimental research boost and clinical proofs of cogent therapeutic effectiveness and the non-existence of complications and negative effects [2]. It has a significant effect on the blood proteins.

According to Genkin, et. al., (1989), their experimental research had shown that irradiation with laser light induces processes that evidently lead to several changes in the charge of the blood proteins. The
resultant of these effects depends on several factors like the dose, incubation time after irradiation and the cellular properties of the tissues. Also research by Burduli and Kgutnova 2010, concluded that the ceruloplasmin content which is an essential copper-carrier protein in the blood, and actively in the iron metabolism in the blood plasma considerably increased in chronic pancreatitis patients during diseases exacerbation. Therefore, irradiation by method of laser intravenous process of the patients’ blood to complex therapy with chronic pancreatitis controlled the ceruloplasmin content in the blood plasma [1,3,4].

As for protein, ceruloplasmin spectral properties were reported by Artyukhov’s group [5]. Absorption maxima for ceruloplasmin peak at 278 nm, and sharply decrease up to 300 nm. Past 300 nm, absorption of photon becomes constant at 0.2, normalized to maximum absorption. Irradiation of ceruloplasmin with UV light at dose 0.2265 J/cm² was also performed, resulting in no change of absorption peak. Zhang et al. (2015), reported fibrinogen absorption peaks at 218 nm and 275 nm [6]. At 400 nm, absorption is valued at 0.25 and gradually decreases down to 0 at 650 nm. Albumin absorption peak is strongest at 204 nm followed by a weak absorption at 277 nm, agreeing with Lin and Chen, (1997) [7,8]. Above 350 nm, albumin absorption is near-zero. All three proteins (ceruloplasmin, fibrinogen, albumin) have weak absorption at 405 nm, rendering any change in protein level arising from the indirect effect of photon absorption [5,8].

Most authors agree that the irradiation with a helium-neon low level laser affects some changes in the charge of the blood proteins to some degree. This showed that changes in protein binding can affect some antiviral activity and the management of the patient [2,3]. Although they fail to observe or study these causes of the change within these charges on the proteins which will help in elucidating the fundamental mechanism for the efficacy of laser therapeutic treatment. This study reveals the changes in protein level for the serum albumin, fibrinogen (α-chain), ceruloplasmin, complement C2, and α-1-antitrypsin post-LLL. This will improve the efficient and optimal laser irradiation therapy and blood condition during storage.

2. Experimental Methods

2.1. Sample Acquisition
Whole blood samples (~2-4 ml) were taken on the same day of experimental procedures using venepuncture method from Wellness Center, Universiti Sains Malaysia (USM) after ethics approval by the Human Research Ethics Committee USM, under protocol code USM/JEPEM/16060208. Blood sample were collected in Ethylenediamine tetraacidic acid (EDTA) tube which act as anti-coagulant [9,10,11]. Total number of ten blood samples obtained from 6 males and 4 females of range of 22 to 36 years old without any known condition of diagnosed diseases. Four of the blood samples were experimented for single (1 control and 5 irradiated exposures) and six for multiple (1 control and 3 irradiated exposures) observations and exposures. The blood samples collected are used within 48 hours and were stored in 4 °C environment whenever idle.

2.2. Preparation of Samples and Evaluations
A diode laser of wavelength 405 nm at an output power of 19.2 mW with 3 mm beam diameter was used in the low level laser irradiation of the samples. Beam area of irradiation was 0.0707 cm². There are two types of irradiation: single exposure and fractionation. For single exposure, exposure time is between 2 to 10 minutes while the fractionation total irradiation time is maxed at 6 minutes. Different doses were administered within the range of 16.2942 to 146.6478 J/cm² based on varying exposure time durations and energy of irradiations. The sample morphology and physiology conditions were monitored using OLYMPUS XC50 optical microscope.

A Mini-PROTEAN sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE) serves in the work to semi-quantify the protein level. It is being used for all electrophoresis runs and employed the Discontinuous Buffer System that enable proteins to begin forced migration via electric field feedback down resolving gel, separated by size. The 2x concentration SDS-PAGE sample buffer
(Morris Formulation) was used for sample loading. The sample buffer was prepared from 0.125 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 0.004 % Bromophenol Blue, 10 % β-mercaptoethanol, and 20 % glycerol. Running buffer used consist of three-part mixture Tris/glycine/SDS at 1x concentration at pH 8.3 and room temperature. The SDS-PAGE gels were imaged with Epson Image Scanner III (GE Healthcare) in tandem with Epson Scan software.

2.3. Protein Level Analysis
EPSON Scan software was used to acquire high-quality gel images. No alteration was implemented to the acquired gel images and were analysed using ImageJ software. Protein level changes were semi-gauged through computing optical density. Blue-tint gels were converted into 32-bits grayscale image type. Chromatein prestained protein ladder was used as molecular weight marker. Optical density of each gel lane was computed using Gel Analyze Tool. A rectangular area (yellow box) consisted of the desired protein band was selected for each lane. Optical density plot was generated using Plot Lanes feature, where Y-axis and X-axis denotes optical density and vertical gel position, respectively. Area under peak was selected by Straight Tool and numerical optical density computed using Wand Tool. Five proteins were selected for analyses according to molecular weight reported by Schenk et al. [12]. According to Schenk, et. al., (2008), molecular weight for ceruloplasmin, fibrinogen (α-chain), complement C2, albumin, and α-1-antitrypsin are 122,2 kDa, 95 kDa, 83.3 kDa, 69.3 kDa, and 46.7 kDa, respectively [12]. Difference in optical density was computed using the equation 1:

\[
\text{% difference} = \frac{\text{irradiated} - \text{control}}{\text{control}} \times 100\% 
\]

3. Experimental Results
Quantitative changes of the spots on the electrophoresis gels for single exposure in the irradiated group are compared to the control blood sample. These quantitative changes were shown by signal intensity per pixel, i.e., optical density (OD) multiplied by the number of pixels in the selected spot (area) [13] as in Figure 1. The OD_normalized which is for the irradiated is compared with that of control optical density, where the percentage (%) difference for each irradiation times are given in bar plots. The gel electrophoresis of both irradiation modes (single and the fractionation methods) are presented.

**Figure 1.** Gel electrophoresis for a) single exposure with Lane 1: ladder, Lane 2: Control aliquot, Lane 3: 2 minutes, Lane 4: 4 minutes, Lane 5: 6 minutes, and Lane 6: 8 minutes and b) fractionation with Lane 1: ladder, Lane 2: Control aliquot, Lane 3: 1st fraction, Lane 4: 2nd fraction, and Lane 5: 3rd fraction.
3.1. The single exposure study
The sample of RBC morphology for the single exposure were captured for 2, 4, 6, and 8 minutes irradiations from the same blood sample as shown in Figure 2. Changes in protein component are presented in bar plot presented in Figure 3.

![Figure 2](image)

**Figure 2.** Shows the RBC morphology for a) control, b) 2 minutes irradiation, c) 4 minutes irradiation, d) 6 minutes irradiation, and e) 8 minutes irradiation. The crenated RBC are seen marked with blue circles (magnification 100X) and the rest magnification of 40X.

The comparative analysis of the individual proteins samples optical density perturbation is shown in Table 1 with the highest percentage value for fibrinogen (α-chain), and lowest for albumin. The % difference variation seems to increase as protein molecular weight increases. According to Schenk et al., (2008), molecular weight for ceruloplasmin, fibrinogen (α-chain), complement C2, albumin, and α-1-antitrypsin are 122.2 kDa, 95 kDa, 83.3 kDa, 69.3 kDa, and 46.7 kDa, respectively [12]. Besides molecular weight dependent, % difference variation could arise from different whole blood samples used. Its local and global maxima are adequately summarized as well.

3.2. The fractionation study
In fractionation study, the gel used for optical density analysis in multiple exposures is shown in Figure 1(b). The general bar plots of ceruloplasmin, fibrinogen (α-chain), complement C2, albumin, and α-1-antitrypsin normalized optical density perturbation is shown in Figure 4. Each fraction at 2 minutes irradiation, followed by 2 hours incubation time before next fraction. Irradiated aliquots normalized to control aliquot (0-minute irradiation). The laser irradiation induced largest optical density change in fibrinogen (α-chain) Figure 4(b) and lowest in albumin Figure 4(d). The fractionation study shows more crenated RBCs compared to single exposure study. No crenation was observed for control just as in the single exposure study in Figure 2.
Table 1: Local and global maxima from one whole blood sample of single exposure study and the variations for 3 different whole blood samples also presented.

|                  | Ceruloplasmin | Fibrinogen (α-chain) | Complement C2 | Albumin | α-1-antitrypsin |
|------------------|---------------|----------------------|---------------|---------|----------------|
| Local Maxima     | 2.17          | 3.49                 | 2.13          | 1.50    | 1.44           |
| Global Maxima    | 2.71          | 5.22                 | 2.44          | 1.59    | 1.79           |
| Local Maxima     | 7%-203%       | 44%-251%             | 20%-113%      | 27%-50% | 44%-48%        |
| Variation for 3 samples | 7%-203%       | 44%-251%             | 20%-113%      | 27%-50% | 44%-48%        |
| Global Maxima    | 22%-315%      | 56%-423%             | 36%-144%      | 30%-59% | 52%-81%        |
| Variation for 3 samples | 22%-315%      | 56%-423%             | 36%-144%      | 30%-59% | 52%-81%        |

Figure 3: Bar plot of a) ceruloplasmin, b) fibrinogen (α-chain), e) complement C2, d) albumin, and e) α-1-antitrypsin normalized optical density perturbation.
4. Discussion

In the research work, the influence on the human protein in vitro after low laser irradiation was reflected by the characteristic optical density (OD) analysis of these protein. For the single exposure study, the OD of all protein (ceruloplasmin, fibrinogen (α-chain), complement C2, albumin and α-1-antitrypsin) in the irradiated blood were observed significantly higher to that of the control aliquot for the all doses. This demonstrated changes in protein binding after laser irradiation which can be antiviral and contributes to patient management. Perhaps, the protein absorbance of 405 nm is minima therefore rendering any variation in protein level as the indirect effect of photon absorption but the stimulation mechanism [5,6,8]. According to Mikhaylov (2015), it has been reported to achieve an increase in biosynthetic activity. This change in protein binding causes a clinically focal change in the relationship.
amid laser exposure and the occurred mechanism in blood. Since blood proteins have significant effects on individual laser irradiation and the efficacy of disease or antiviral therapy for HIV-infected patients, diabetic etc. [15].

The ceruloplasmin (CP) is known as a copper-containing glycoprotein that has a molecular weight of 132 kDa [16]. It is synthesized mainly in the liver and has many functions; such as an antioxidant, a ferroxidase, for copper transport, anti-inflammation, and proinflammatory activities. Irradiation of blood protein for 2 minutes (dose = 32.59 J/cm²) increases ceruloplasmin optical density to local maxima (OD_{normalized} = 2.17), decreases to local minimum (OD_{normalized} = 1.46) at 6 minutes (dose = 97.77 J/cm²), and increases again to global maximum (OD_{normalized} = 2.71) at 8 minutes (dose = 130.35 J/cm²) as in Figure 3(a). All the variations show a significant ceruloplasmin level increases over that of control optical density. This may be that CP is an acute phase reactant, and its serum concentration is increased during irradiation inflammation, infection, and trauma largely as a result of increased gene transcription in hepatocytes mediated by the inflammatory cytokines [17,18].

It has been ascertained, that during laser irradiation of ceruloplasmin, the functioning of intramolecular electron transport chain is shaken. It is supposed that these changes are caused by the interdomain interactions in a protein molecule [16,19]. In the fractionation study, significant decreasing trend for optical density was noticed. The first fractionation (dose of 32.59 J/cm²) decreases ceruloplasmin optical density of the control (OD_{normalized} of 1) to local minima (OD_{normalized} = 0.787), the second was observed below zero maxima (OD_{normalized} = 0.905) at dose of 65.18 J/cm², the third was global minima (OD_{normalized} = 0.735) at dose of 97.77 J/cm² as shown in Figure 4(a). The multiple exposure demonstrated a contrary result to that of single exposure study, optical density for ceruloplasmin decreased comparably to the control. The incubation time might play a role in the decreased optical density. During incubation, anti-inflammatory effects of LLLI could have kicked in, rendering a reduced optical density [20,21].

Fibrinogen role is that of a coagulation factor to promote endothelial repair and also has a C3 complement function. It correlates with erythrocyte sedimentation rate(ESR). Irradiation of 2 minutes (dose of 32.59 J/cm²) increases fibrinogen (α-chain) optical density to local maxima (OD_{normalized} = 3.49), decreases to local minimum (OD_{normalized} = 2.94) at 6 minutes (97.77 J/cm²), and increases again to global maximum (OD_{normalized} = 5.23) at 8 minutes (130.35 J/cm²) as in Figure 3(b). Formation of reactive oxygen species (ROS) via mitochondria oxygen metabolism pathway post-LLLI has been reported (Kim, 2002). Elevated ROS triggers local inflammation. It is also known that fibrinogen binds to other protein such as albumin and platelets [4,22]. Energy from laser irradiation could potentially break protein-/platelets-bonded fibrinogen [22]. The implication of the broken-free unbonded fibrinogen (α-chain) is an increased optical density. Such a conjecture aligns with fibrinogen as anti-inflammatory constituents.

There is an observed decreased trend for optical density in the fractionation exposure, the first exposure at dose of 32.59 J/cm² decreases fibrinogen (α-chain) optical density from that of the control to a local minima (OD_{normalized} = 0.375). For the second exposure dose of 65.18 J/cm² the optical density was below zero maxima (OD_{normalized} = 0.831). The third was global minima (OD_{normalized} = 0.290) at fraction dose of 97.77 J/cm² as in Figure 4(b).

Optical density for fibrinogen (α-chain) tends to decrease after incubation time. The observed decreased of fibrinogen optical density after incubation time maybe as a result of the anti-inflammatory effect of LLLI that could decreased the optical density. LLLI could had induced production of ROS and dissociation of bonded fibrinogen complex right after irradiation [23]. Dissociation of fibrinogen increases its level and act as intrinsic anti-inflammatory agent against ROS-induced local inflammation. However, majority of fibrinogen are used to mediate anti-inflammatory responses during incubation, rendering a much lower level before next fraction. Perhaps due to the decreased level of fibrinogen, the dissociation of fibrinogen increases at the 2nd fractionation. With fibrinogen level almost back to its original value, dissociation of fibrinogen could be low at the 3rd fractionation. A higher fractionation could confirm the downward trend of fibrinogen, due to mentioned postulation. Quantification of fibrinogen dissociation is warranted to elucidate fibrinogen behaviour under laser irradiation.
Albunin is a hepatic protein synthesis known as a small molecule but important diagnostic and prognostic determinant, as well as a useful therapeutic agent [24]. Irradiation of 2 mins (dose of 32.59 J/cm²) increases albumin optical density to local maxima (DO normalized = 1.50), decreases to local minimum (DO normalized = 1.31) at 4 mins (65.18 J/cm²), and increases again to global maximum (DO normalized = 1.59) at 8 minutes (130.35 J/cm²) as in Figure 3(d). Abundant Na⁺ ions in extracellular space is also known to be adsorbed by albumin via electric field [25]. Albumin’s ability to bind to other components is due to its intrinsically strong negative charge. When albumin adsorption occurs, structural change will ensue [26]. It could be that LLLI disrupts albumin’s electric field, freeing charged compounds and render stand-alone albumin. This explains the increased optical density of albumin in single exposure study.

There is an observed decreased trend for optical density with the first fractionation at dose of 32.59 J/cm² which decreases albumin optical density to local minima (DO normalized = 0.750). The second occurred below zero maxima (DO normalized = 0.968) at a dose of 65.18 J/cm² and third which global minima (DO normalized = 0.698) at a dose of 97.77 J/cm² as in Figure 4(d). Daily synthesis and degradation of albumin is highly regulated in vivo (Rothschild et al., 1973). The daily average of albumin degradation is a staggering 15 g for a healthy individual [27]. It is likely that degradation of albumin occurs in vitro, compounded by unregulated milieu. Although single exposure data showed an increased optical density for albumin, the added incubation time facet could annul LLLI effect given the significant degradation rate. This explains an overall decreased in optical density for fractionation study. Hao and Lawrence (2004), reported CO₂ laser decreases albumin adsorption [28]. A decrease in albumin adsorption implies a higher level of stand-alone albumin. This explains the increased optical density at the 2nd fractionation. Also albumin’s decreased optical density could result from albumin-fibrinogen complex [4,29].

Complement serves as important tool for protection against microorganisms and it contribute to the pathophysiology of a number of autoimmune diseases [30]. The complement C2 is the coded protein by the C2 gene and is part of the classical pathway of the complement system that serves as a multi domain serine protease [30]. The complement components deficiencies of the classical activation pathway C2 leads to increased susceptibility to bacterial infection [30&31]. Irradiation of 2 minutes (dose of 32.59 J/cm²) increases complement C2 optical density to local maxima (DO normalized = 2.13), decreases to local minimum (DO normalized = 1.53) at 6 minutes (97.77 J/cm²), and increases again to global maximum (DO normalized = 2.44) at 8 minutes (130.35 J/cm²) as shown in Figure 3(c). The significant increase may have resulted from the laser activation of the complement C2 that results to several molecules mediating chemotaxis production, opsonisation and degranulation of mast cell that contributes pathogenic organisms’ elimination and inflammation [31&32].

The fraction exposure again demonstrated a decreased trend of optical density for the complement C2 compare with the control. The first dose of 32.59 J/cm² decreases complement C2 optical density to local minima (DO normalized = 0.822), then a sudden increased to maxima (DO normalized = 1.007) at second dose of 65.18 J/cm². There was an observed decrease again to local minima (DO normalized = 0.727) at the third exposure dose of 97.77 J/cm² as all shown in Figure 4(c). The Complement C2 is a protein that aid in antigen lysing via classical pathway. In the classical pathway, complement C2 will be cleaved into two parts; C2a and C2b by C1q [33]. One of absorption peak for complement C2 is at 386 nm wavelength, with substantial absorption at 405 nm [34]. It is possible that complement C2 absorbs 405 nm and subsequently energy-cleaved into its constituents. This may explain the decreased optical density of complement C2 after fractionations. A study on complement C2 constituents can illuminate LLLI cleaving capability. Alpha(α) -1 antitrypsin (AAT): AAT is a 52 kDa, single-chain glycoprotein with a 394 amino acid sequence and is known as a serine protease inhibitor (serpin) responsible for the breaks down of the neutrophil elastase [18,35]. It protects the blood cells against the neutrophil elastase activity. AAT deficiency can result to hepatitis, liver cirrhosis, and panacinar emphysema etc. It is noticed to significantly increased after irradiation of 2 minutes (dose 32.59 J/cm²) optical density increases to global maxima (DO normalized = 1.79), decreases to local minimum (DO normalized = 1.07) at 6 minutes (97.77 J/cm²), and increases again to local maximum (DO normalized = 1.44) at 8 minutes (130.35
J/cm²) as in Figure 3(e). The increase after irradiation maybe connected to AAT binds to free fatty acids [36]. A decreasing trend for the optical density of α-1-antitrypsin in a fractionation study was observed. The first exposure dose of 32.59 J/cm² decreases α-1-antitrypsin optical density to local minima (ODnormalized = 0.848). The second was below zero maxima (ODnormalized = 0.915) at dose of 65.18 J/cm² and the third decreases to global minima (ODnormalized = 0.577) at fractional dose of 97.77 J/cm², all shown in Figure 4(e). Optical density for the decreased samples varies between -15 to -28 % (local minima) and -38 to -43 % (global minima). The α-1-antitrypsin deficiency is mainly due to genetic conditions, and can be exacerbated by environmental factors like smoke and dust. Fractionation data presented here agree with Kilik et al. experimental result which shows one common ground between Kilik’s group and this present research is incubation time after laser treatment. In Kilik et al (2019), they irradiated rats in vivo for 9 days reflecting these two research works commonality seems to suggest that incubation time lowers α-1-antitrypsin level in blood plasma post-irradiation [13].

5. Conclusion.
The research main aim was to elucidates the stimulatory effects of LLLI on the change of blood proteins (ceruloplasmin, fibrinogen (α-chain), complement C2, albumin, and α-1-antitrypsin) acting as antioxidants or anti-inflammatory agents in the human blood. The protein profiles of blood may vary in physiological and pathological conditions [13]. As blood constitutes such a variety of proteins in a dynamic and wide concentration range, 2x concentration SDS-PAGE (gel electrophoresis) was appropriately used technique for such high-complex mixtures separation. The single exposure and fractionation exposures were carried out which showed morphologies of crenated RBCs constitute insignificant effect on protein level perturbation. The single exposure results demonstrated an overall increased protein level for all blood proteins. Global maxima (8 minutes’ single exposure) optical density ranges from 20 to 315 % for ceruloplasmin, 55 to 423 % for fibrinogen (α-chain), 35 to 144 % for complement C2, 30 to 60 % for albumin, and 50 to 81 % for α-1-antitrypsin. The research findings agreed with biostimulating effect of LLLI and to Arndt-Schulz curve. The increase of the protein levels may be related to haemoglobin released from lysis of the red blood cell. The blood proteins serve as blood scavengers of the heme and haemoglobin protecting against mediated oxidative stress of heme and prevention of heme-iron damage during the acute phase responses. Just as the fibrinogen binds to platelets to help in aggregation of platelet. The fibrinogen increase is specifically related to its released from activated platelets stimulated by laser irradiation [13]. Whereas the fractionation results showed an overall decreased protein level for all five proteins. Global minima (3rd fraction) optical density ranges from -25 to -50 % for ceruloplasmin, -28 to -71 % for fibrinogen (α-chain), -27 to -38 % for complement C2, -22 to -33 % for albumin, and -38 to -43 % for α-1-antitrypsin. The decreasing trend could be incubation time motivated. The research findings also demonstrated that 2 hours’ incubation time is enough to decrease protein level. Finally, proteomic research studies allow a much wider investigation of LLLI as a cause of the oxidative stress response, comparably to the conventional biochemical procedures. However, further studies involving proteomic analysis of human blood are necessary for deeper clarification of the laser biostimulation mechanism as well as clinical trials to optimizes irradiation parameters for effective treatment method.

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