Proteomic Analysis of Plasma Proteins after Low-Level Laser Therapy in Rats

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Summary

The laser radiation absorbed by cells induces production of reactive oxygen species (ROS), followed by the development of oxidative stress. Proteins are major targets for ROS due to their abundance in biological systems. The aim of the present pilot study was to examine the effects of transcutaneous laser blood irradiation (TLBI), i.e., low-level laser therapy (LLLT) at 830 nm on plasma proteome in Wistar rats. Rats were irradiated in the heart area (i.e. coronary arteries) daily (i.e., for 9-day period), by commercially available GaAsAl diode laser (Maestro/CCM, Medicom Prague, Czech Republic, λ=830 nm, power density 450mW/cm^2, daily dose 60,3 J/ cm^2, irradiation time 134 sec). The comparison of blood plasma proteome from irradiated and non-irradiated rats was performed utilizing 2D electrophoresis followed by MALDI TOF/TOF mass spectrometry. LLLT led to a quantitative change in the acute phase proteins with antioxidant protection i.e., haptoglobin (log_2 fold change (FC)=3.5), hemopexin (log_2 FC=0.5), fibrinogen gamma (log_2 FC=1.4), alpha-1-antitrypsin (log_2 FC=-2.2), fetuin A (log_2 FC= -0.6) and fetuin B (log_2 FC=-2.3). In comparison to conventional biochemical methods, the changes in protein levels in blood plasma induced by LLLT offer a deeper insight into the oxidative stress response.

Key words

Low-level laser therapy ● Oxidative stress ● Acute phase proteins ● Proteomic analysis

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Introduction

Low Level Laser Therapy (LLLT) is the medical application of light produced by a low energy laser in the range of 1 to 500 mW (Huang *et al.* 2009). Unlike laser surgery, laser therapy utilizes low power laser light without significant thermal effects causing damage to irradiated tissues (Tuner and Hode 1999).

Transcutaneous laser blood irradiation (TLBI) is most commonly used to irradiate areas of the skin near large blood vessels. It is a safe method without any side effects. TLBI is based on blood modification influenced by laser light in order to promote faster and better regeneration. It is generally believed that TLBI affects blood, especially blood cells and plasma proteins (Gasparyan 1998).

The absorption of laser light leads to the production of reactive oxygen species (ROS), being a natural by-product of oxygen metabolism (Kim *et al.* 2002). They have a key role in cellular signaling pathways from mitochondria to nuclei regulating the nucleic acid synthesis, protein synthesis, enzyme activation, and cell cycle progression (Storz 2007, Brondon *et al.* 2005). An increase in ROS induces gene expression that is involved in inflammatory and acute phase reactions (APR). One important event in the acute phase reaction is the change in plasma protein molecules known as acute phase proteins (APP) (Eckersall and Bell

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2010). APPs are sensitive markers to assess the status of oxidative stress divided into two groups. Proteins, such as haptoglobin and hemopexin, represent a family of positive plasma proteins in the acute phase (Altruda *et al.* 1982, Bowman and Kuroski 1985). In contrast, fetuin A and fetuin B belong to the family of negative plasma proteins in the acute phase (Lebreton *et al.* 1979). It is believed that abundant proteins can act as physiological antioxidants by protecting other molecules from free radical-mediated oxidation.

In this pilot study, a semi-quantitative method (OD x area) to detect changes in plasma proteins (indicated by $\log_2$ fold change) was used. These changes were induced by TLBI on rats (Wistar). Among all identified proteins, the APP were statistically significant ($p \leq 0.05$) in FGG, HP and HPX which were up-regulated and AHSG, FETUB and SERPINA1 which were down-regulated.

Methods

Animal model

This experiment was approved by the Ethics Committee of the Faculty of Medicine of P. J. Šafárik University. Each animal experiment was in accordance with the European Guidelines on Laboratory Animal Care. Five-months-old male Wistar rats (n=8), were included in the experiment and randomly assigned into two groups of 4 animals, i.e., the non-irradiated control group (C) and the irradiated group (I).

Low level laser therapy

Using the trans-cutaneous laser blood irradiation, the rats were daily irradiated in the heart area (i.e., coronary arteries) after shaving the skin with a commercially available gallium–aluminum–arsenium (GaAlAs) diode laser (Maestro/CCM, Medicom Prague, Czech Republic, λ=830 nm, oval shape of beam-spot size * 1 cm^2, power density 450 mW/cm^2, total daily dose 60,3 J/cm^2, irradiation time 134 sec) for 9 days. During the laser irradiation, all rats were restrained without anasthesia in a tight Plexiglas cage fitted to rat body with a circular opening over the heart area.

Blood collection

The rats were subjected to general anesthesia [ketamine 40 mg/kg (Narkamon a.u.v., Spofa, Prague, Czech Republic), xylazine 15 mg/kg (Rometar a.u.v., Spofa, Prague, Czech Republic), tramadol 5 mg/kg (Tramadol- K, Krka, Novo Mesto, Slovenia)].

Blood samples were acquired from the heart and collected into a collection tube. Immediately after the blood collection, anticoagulant Na_2EDTA was added to prevent clotting (1 % Na_2 EDTA in saline). After the blood collection, the tube was immediately placed in an ice bath and delivered for analysis. The blood samples were centrifuged at 2000 g for 10 min at 4 °C. Blood plasma was stored at -80 °C.

Precipitation of plasma protein

The mixture of plasma (200 µl) in 10 % acetone solution of trichloroacetic acid (Sigma Aldrich, St. Louis, USA) was incubated at -20 °C for 1 h and centrifuged at 14 000 g for 5 min at 4 °C. The supernatant was removed and the ice-cold acetone (1 ml) was added to wash the pellet. The sample was incubated at 4 °C for 1h and centrifuged as described above. The acetone containing supernatant was removed and the pellet was air-dried.

Two-dimensional electrophoresis (SDS-PAGE)

The protein sample (50 µg) was dissolved in rehydratation buffer (125 µl, buffer consisting of 7 M urea, 2 M thiourea, 0.035 M Tris-HCl, 2 % w/v CHAPS, 65 mM DTT) and loaded onto IPG strip (7 cm strips, pH 4–7, Bio-Rad, Hercules, CA). The IPG strip was covered with the mineral oil and passive rehydration was permitted during next 12 h at room temperature. In the next step, the protein mixture was focused in the Protean IEF Cell (Bio-Rad, Hercules, CA) using a voltage program increasing from 0 V to 250 V over 1 h, from 250 V to 4000 V over 20 h and kept at 4000 V for 1 h. The strip was removed from the focusing tray and reduced with 130 mM DTT (Merck, Darmstadt, Germany, 2 ml) equilibration buffer (6 M urea, 20 % (v/v) glycerol, 2 % (w/v) SDS, and 0.375 mM Tris–HCl pH=8.8) for 10 min. Next, the strip was treated with iodoacetamide (Applichem, Darmstadt, Germany, 2 ml) in the equilibration buffer for another 10 min. The IPG strip was transferred onto a slab polyacrylamide gel (prepared by the polymerization of 12.5 % acrylamide solution) and overlaid with 0.5 % hot agarose (1ml) which was allowed to solidify. Electrophoresis was performed in a Protean II cell (Bio-Rad, Hercules,CA) for 1 h at 200 V and 400 mA. The gel was taken for staining in Comassie briliant blue G-250 (Bio-Rad, Hercules, CA) for 60 min followed by washing with water (proteomic grade, 3×20 minutes). The scanning of the gel was performed on Calibrated Densitometer GS-800 and

subsequently analyzed with the PDQuest software (Bio-Rad Laboratories USA).

Protein digestion

Protein spots were cut with EXQuest Spot Cutter (needle id 1.5 mm, Bio-Rad Laboratories, Inc.) and the gel pieces were transferred into the Eppendorf tubes. The color was removed from the gel by washing it with ACN (50 %)/25 mM ammonium bicarbonate (50 %) buffer (200 µl). The supernatant was discarded and ACN (Sigma Aldrich, St. Louis, USA, 100 µl) was added. The gel pieces turned white after 5 min and ACN was removed. Next, the gel pieces were air dried and rehydrated in 25 mM ammonium bicarbonate (Applichem, Darmstadt, Germany, 25 µl) containing trypsin (Promega, Medison, USA, 12.5 µg). The mixture was placed in the ice for 1 hour and the remaining trypsin solution was removed. Gel pieces were covered with 25 mM ammonium bicarbonate (20 µl) to prevent the gel from drying and the samples were incubated at 37 ºC overnight. The samples were separated by a spinning process and the supernatant was transferred to a 500 µl tube. Peptides were extracted with ACN (50 %)/TFA (1 %) (2 × 20 µl) and the organic layers were combined.

MALDI-TOF MS analysis

The solution of extracted peptides was mixed with the matrix solution (6 mg/ml α-cyano-4-hydroxycinnamic acid in acetonitrile/methanol/0.1 % TFA in water, 84/14/2 v/v/v) in a proportion of 2:1. The 1µl of peptides/matrix mixture was applied onto a MALDI target plate and allowed to dry. Samples were analyzed with the mass spectrometer MALDI TOF/TOF UltrafleXtreme (Bruker Daltonik, Germany). Spectra were acquired in the reflector mode within the range of 700 – 3500 Da. Mass calibration was performed externally with the PepCal (Bruker Daltonik, Germany) standard. The data were evaluated using the Mascot algorithm (Matrix Science Ltd., UK). Mass spectra and tandem mass spectra of peptides were compared against the NCBI database. The search terms were as follows: taxonomy Rattus norvegicus (rat), global modifications Carbamidomethyl (C), variable modifications Oxidation (M), enzyme Trypsin, the number of maximum missed cleavages 1, mass error tolerance 100 ppm in MS and 0.3 Da in MS/MS mode. False discovery rate (FDR) ≤ 1 %, minimum 2 unique peptides.

Results

Quantitative changes of the spots on the 2D gels in the irradiated group (I) compared to the non-irradiated rats, i.e., the control group (C) were identified. These quantitative changes were indicated by signal intensity per pixel, i.e., optical density (OD) multiplied by the number of pixels in the selected spot (area) (Fig. 1).

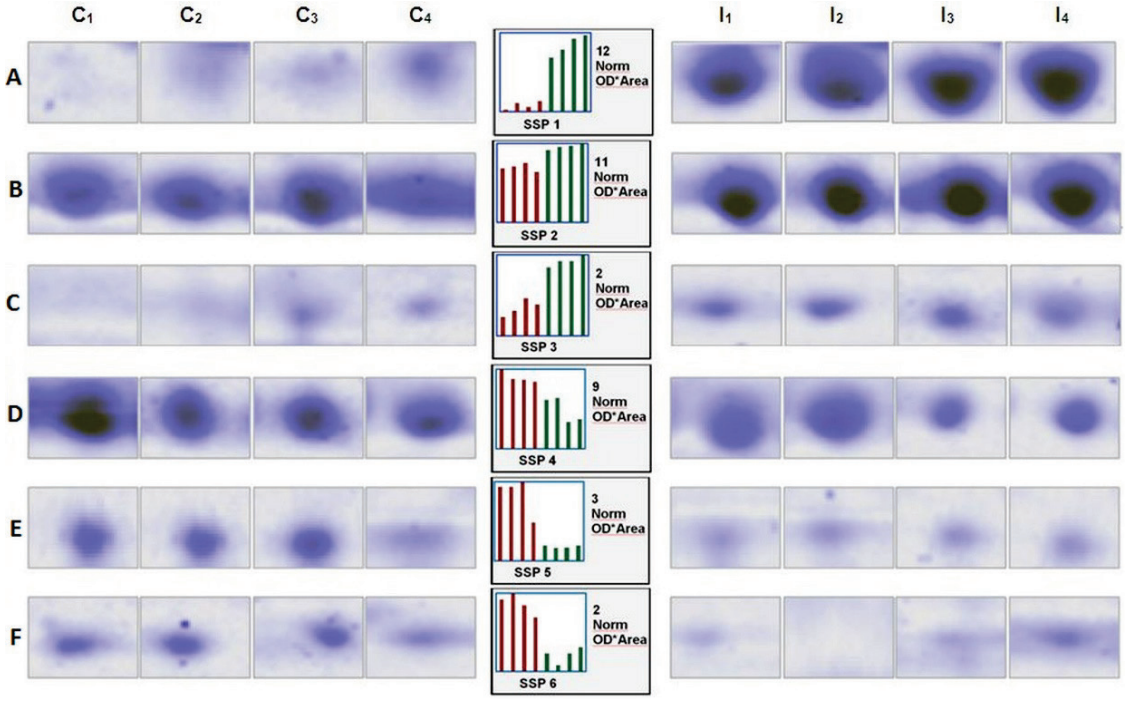

Fig. 1. Semi-quantitative changes (OD x area) of the plasma proteins for irradiated and non-irradiated rats A - haptoglobin, B – hemopexin, C – fibrinogen gamma, D – fetuin A, E – fetuin B, F – alpha-1-antitrypsin.

Table 1. Changes in plasma proteins (log_2 fold change) comparing of the non-irradiated and irradiated groups of rats (Wistar) using a semi-quantitative method (OD x area).

protein name	non-irradiation (control) group (OD*Area)				$C_{average}$±SD	irradiation group (OD*Area)				$I_{average}$±SD	log2FC	t-test p≤0.05
	C_1	C_2	C_3	C_4		I_1	I_2	I_3	I_4			
haptoglobin					0.98±0.59					10.80±1.46		
hemopexin	7.5	7.8	8.3	7	7.65±0.54	10.1	10.5	10.7	11	10.60±0.38	0.5	1.2E-04
fibrinogen gamma	0.3	0.4	0.6	0.5	0.45±0.13	1.1	1.2	1.2	1.3	1.20±0.08	1.4	6.4E-05
fetuin-A	8.3	7.3	7.2	7	7.45±0.58	5.1	5.3	2.8	3.1	4.80±1.31	-0.6	3.2E-03
fetuin-B	2.9	2.9	3.1	1.5	2.60±0.74	0.6	0.5	0.5	0.6	0.55±0.06	-2.3	1.5E-03
alpha-1-antitrypsin	1.2	1.3	1.1	0.9	1.3±0.17	0.3	0.1	0.3	0.4	0.28±0.13	-2.2	2.0E-04

SD - standard deviation, FC - fold change, OD - optical density

Subsequently, these spots were cut out of the gel and analyzed quantitatively by MALDI TOF/TOF mass spectrometer.

The calculated values of the log_2 fold change of selected blood plasma proteins from the OD x area of irradiated versus non-irradiated group $(I_1+I_2+I_3+I_4)/(C_1+C_2+C_3+C_4)$ were compared using a t-test, ($p \leq 0.05$). The following quantitative changes of proteins were identified: haptoglobin (HP, log_2fold change=3.5), hemopexin (HPX, log_2 fold change=0.5), fibrinogen gamma (FGG, log_2 fold change=1.4), alpha-1-antitrypsin (SERPINA1, log_2 fold change=-2.2), fetuin-A (AHSG, log_2 fold change=-0.6) and fetuin-B (FETUB, log_2 fold change=-2.3) (Table 1).

Discussion

The exact mechanism at the cellular level by which LLLT stimulates tissue repair and regeneration is still not fully understood. Nevertheless, past research showed evidence that the therapeutic effect depends on the settings of basic laser parameters, such as wavelength, power density, and dose (Grendel *et al.* 2011). In study of Karu *et al.* the infrared (IR) laser light at 830 nm wavelength was reported as the most efficient one in therapeutic applications (Karu *et al.* 2001). By the low-intensity (1-500 mW) IR laser exposure the protein content and myeloperoxidase activity in blood samples were increased (da Fonseca *et al.* 2011). Also, by IR laser, the cell proliferation, lipid peroxidation, conformational transitions of red blood cell (RBC) membrane were induced (Grossman *et al.* 1998, Kujawa *et al.* 2004). Thus, IR laser irradiation modulates rheological properties and improves microcirculation of blood samples (Ghadage and Kulkarni 2010).

LLLT had lower tissue penetration ability when compared with other energy forms such as ultrasound (USG) or electromagnetic (EM) radiation. However, in IR spectrum superficial chromophores show weak absorption in the window 700-1000 nm, resulting in a deeper energy penetration through the skin compared to a visible light spectrum (Karu 2007).

The invisible infrared light (Ga-As, 600-1200 nm) penetrates 2-4 mm deep in soft tissue whereas the visible light (He-Ne, 400-700 nm) penetrates only 1-2 mm (Robertson *et al.* 2006). Karu demonstrated that light of 0.01 J/cm^2 influences cellular processes (Karu 1987). Approximately six penetration depths, i.d. about 24 mm for Gallium-aluminum-arsenide laser (830 nm, 90mW, 9J/cm^2 at treatment place) are possible before the beam of light drops from 9J/cm^2 to 0,01 J/cm^2. As we applied gallium–aluminum–arsenium laser with power density 450 mW/cm^2, we suppose penetration of 0.01 J/cm^2 laser light up to 30 mm, enough for relevant irradiation of tissue and blood.

Therefore, the lasers with IR wavelengths in the above mentioned electromagnetic window are more often used in physiotherapy practice (Enwemeka 2009). It was observed that the 20 % energy penetrated over the rat skin using IR laser (810 nm, 200 mW). However, the important fact is, that this residual penetrating energy is sufficient to reach the radiation dose threshold for

stimulating tissue repair and physiological processes (Joensen *et al.* 2012). The blood cells are located deeper under the skin and subcutaneous tissue. Therefore, we suppose that the changes in acute phase proteins observed in this study are the result both TLBI and unknown changes of oxygen metabolism in subcutaneous tissues.

Reactive oxygen species (ROS) induce oxidative stress and quantitative changes in the acute phase proteins. It is known that acute phase proteins can act in the antioxidative defense mechanism. The acute phase protein is defined as a protein whose concentration increases or decreases by at least 25 % during oxidative stress (Gabay and Kushner 1999). Acute phase proteins proved to be successful in the identification of novel mechanisms of cell response against oxidative stress (Keightley *et al.* 2004).

The main aim of this experiment was to examine the stimulatory effect of LLLT on the change of acute phase proteins acting as antioxidants or anti-inflammatory agents in blood plasma. Plasma is not only the cardinal clinical specimen but represents also the largest and most varied fraction of the proteome that is present in the body. The protein profiles of plasma may vary in physiological and pathological conditions (Anderson *et al.* 2002, Rosenblatt *et al* 2004). As plasma contains such a variety of proteins in a wide and dynamic concentration range, two-dimensional gel electrophoresis (2-DE) was found to be a useful technique for the separation of such high-complex mixtures (O'Farrell *et al.* 1975).

In our study the increase of the positive APPs was observed, i.e., haptoglobin ($\log_2$FC=3.5), hemopexin ($\log_2$FC=0.5) and fibrinogen gamma ($\log_2$FC=1.4). The increase of the HP and HPX may be related to hemoglobin released from red blood cell lysis. These proteins act as plasma scavengers of hemoglobin and heme respectively. Thus, they are important for the protection against heme mediated oxidative stress as well as for the prevention of heme-iron loss during the acute phase response (Chiabrando *et al.* 2011). Fibrinogen gamma binds to platelets and is an essential requirement for platelet aggregation. The increase of the FGG relates to its release from activated platelets induced by laser exposure (da Fonseca *et al.* 2012).

In conclusion, proteomic studies allow a much broader investigation of LLLT as a cause of the oxidative stress response, as compared to the conventional biochemical methods. Nevertheless, further studies involving proteomic analysis of blood plasma are required to explain the mechanisms of laser biostimulation and clinical trials to optimize irradiation parameters before it becomes an actual method of treatment.

Conflict of Interest

There is no conflict of interest.

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