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EFFECT OF LOW INTENSITY NEAR INFRARED (810 nm) LASER RADIATION ON HUMAN ERYTHROCYTE MEMBRANE ENZYMES

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Abstract

Objective: The exact mechanism of the influence of low-intensity laser radiation on living cells has not yet been explained.

The aim of the study was to investigate the effect of low-intensity infrared laser radiation (810 nm, 125 mW/cm^2) on activity of human erythrocyte acetylcholinesterase (AChE) and (Na^+, K^+, Mg^{2+}) ATPase. In this study, the dependence of activity of human erythrocyte membrane enzymes on dose of energy, density of energy, and way of dosage were performed.

Summary background data: Explanations based on the light absorption by primary endogenous chromophores have been proposed to describe biological effects of laser light on the structure of cellular membranes and are recognized to play a secondary role in the relation to those observed in mitochondria.

Methods: Human erythrocyte cell membranes were irradiated with low-intensity laser light (810 nm, 125 mW/cm^2) at different continuous light doses (0-20 J) and fractionated doses (9 J and 12 J) at 37°C as the parameters characterizing the structural and functional changes of cell membranes, the activities of Na^+-, K^+- and Mg^{2+}-ATPases as well as acetylcholinesterase activity (AChE) were measured.

Results: It was found that low-intensity, near-infrared laser radiation affects changes in the AChEase and ATPase activities in the dose - and way of dosage - dependent manner. At smaller doses of irradiation the activity of the enzymes slightly increased in comparison to control and reached a maximum for 9 J. The total and Na^+, K^+ ATPase activity after fractionated doses (3+9 J and 9+3 J) was significantly higher than for a single dose of 12 J, and significantly decreased for a dose of 3+6 J and 6+3 J with comparison to a single dose of 9 J. AChE activity after fractionated dosage of radiation

changed in the opposite way. When bigger doses were applied first they induced greater stimulation of enzyme activity.

Conclusions: Low-intensity near-infrared laser radiation (810 nm, 125 mW/cm^2) changed membrane enzymes activity in the dose- and way of dosage-dependent manner.

Fractionation of the light dose significantly altered the membrane response to laser radiation.

The differences of the effects of laser light on activities of both enzymes may have resulted from their different location in the membrane.

Key words: low power lasers, ATPase, acetylcholinesterase, fractionated dosage

Introduction

Laser devices with an output power lower than 500 mW are used in low-intensity laser therapy. This local phototherapeutic modality is characterized by its ability to induce athermic and nondestructive photobiological processes in cells and tissues (1-3). Laser therapy has been shown to be successful in the treatment of a variety of diseases (4-7). In traumatology, the stimulating effect of light in regeneration of the tissues was used (8-14). The analgesic effect of low-level laser radiation was confirmed (15).

However, the mechanisms of low-level, laser radiation–induced bio-stimulation, are still not well understood.

Various explanations based on the light absorption by primary endogenous chromophores (mitochondrial enzymes, cytochromes, flavins, porphyrins) have been proposed to describe biological effects of laser light. Protein molecule conformational transitions, enzyme activity modifications, metabolic and signal transduction pathway modulations in cells have been suggested as laser radiation-induced events (16, 17).

A variety of studies both *in vivo* and *in vitro* showed significant influence of laser irradiation on cell functional state (18-21).

These stimulatory effects are often named "biostimulation". On the other hand, numerous observations show inhibitory effects of laser light on various metabolic processes, so we suggest a more appropriate term, "biomodulation".

At the same time, another group of researchers found no detectable effects of light exposure.

However, in these studies only the wavelength and the power of the source were characterized. No information on the energy dose and both power and energy density, were provided.

The aim of the study was to study the influence of low-intensity infrared laser radiation (810 nm, 125 mW/cm^2) on the activities of human erythrocytes Na^+, K^+, Mg^{2+}ATPase, Na^+, K^+, ATPase and Mg^{2+}ATPase and acetylcholinesterase (AChE).

Also, the dependence of these enzymes activity changes on the energy density, dose of energy and a way of dosage of laser radiation was studied.

Materials and methods

Chemicals

Reagents of analytical grade were from POCH (Gliwice, Poland); 5,5'-dithiobis (2-nitrobenzoic acid) (Ellman's Reagent), acetylthiocholine iodide (AcTCh), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA) were from Sigma-Aldrich GmbH, Germany.

Blood from healthy donors was purchased from the Central Blood Bank in Lodz. Blood sample was treated with a 3,7% sodium citrate solution.

After removing plasma and the leukocyte layer, erythrocytes were washed three times with cold (4^{o}C) phosphate buffered

saline (PBS: 0.15 M NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4).

Preparation of erythrocyte membranes

Erythrocyte membranes were isolated from washed cells according to the method of Dodge et al. with some modifications (22).

The erythrocytes were haemolysed with 20 volumes of 10 mM Tris - HCl buffer, pH 7.8, containing 1 mM EDTA and 0.5 mM PMSF as proteolytic inhibitors, and centrifuged for 20 min at 4°C at 20 000 g. The ghosts were resuspended in ice-cold 5 mM Tris-HCl buffer, pH 7.4, and this process was continued until the ghosts were free of residual hemoglobin.

Laser irradiation

The suspensions of erythrocyte membranes (1 mg protein•ml^{-1}) in 5 mM Tris - HCl buffer, pH 7.4, were irradiated with near-infrared (810 nm) therapy laser CTL 1106MX (LASERINSTRUMENTS, Warsaw) in plastic tubes through uncovered surface with continuous gentle mixing at 37°C. The volume of irradiated suspensions was 0.5 ml, the diameter of a light spot was 0.8 cm^2. The power of laser device was 100 mW and the power density was equal to 125 mW/cm^2. Irradiation light dose changed from 0 to 20 J. In experiments with fractionated doses of radiation the total light dose was 9 J and 12 J. The exposure of membrane suspensions to light was made in two parts with one hour interval in several options: 3 J + 6 J; 6 J + 3 J; 3 J + 9 J; 9 J + 3 J.

Enzyme activity measurement

Total and Na^+, K^+ ATPase activity (Bonting and Canady modified method, 1964) (23).

Total and Na^+, K^+ -ATPase activity were determined in a medium containing 100 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 15 mM KCl, 85 mM NaCl, 2 mM EDTA and 2 mM ATP (final concentrations) by measurement of the difference in the liberation of inorganic phosphate in the absence and in the presence of 0.1 mM ouabain during 30-min incubation of membrane preparations at 37°C. The activity measured in the presence of ouabain was referred to as the basal Mg^{2+}-ATPase. The phosphate liberated was estimated with Malachite Green. Protein was measured according to the method of Lowry et al (24).

Acetylcholinesterase (AChEase) activity (Ellman's method, 1961) (25)

Acetylcholinesterase (AChEase) activity was determined according to the method of Ellman et al. using acetylthiocholine iodide (AcTCh). Erythrocyte membranes were suspended in 0.145 mol•l^{-1} NaCl solution containing 5 mmol•l^{-1} potassium phosphate, pH 7.9 at 22°C. AcTCh concentrations were 20-100 µmol•l^{-1} and the concentration of Ellman's reagent in the samples was 50 µmol•l^{-1}.

Measurements of reaction kinetics were made in 1 min at a wavelength of 412 nm. Lineweaver-Burk plots of the red blood cell membrane acetylcholinesterase activity before and after cell radiation were appointed. Two parameters were estimated using these graphs: V_{max}, which is the maximal rate of the enzymatic reaction, and the Michaelis-Menten constant K_m, which corresponds to the substrate concentration at which the reaction rate equals to the half of the maximum. The results of irradiated samples were compared to the unirradiated ones (controls).

Statistical analysis of results

Results were expressed as mean of fourteen independent measurements ± S.D.

The statistical analysis of results was conducted using the package: Statgraphics Plus for Windows 5.0 and Statistica 6.0 PL. The differences between results were evaluated using the following tests:

– the Shapiro –Wilk's test (for checking the normality of distribution);
– the Student's t – test (to compare two averages);
– method ANOVA
– the Bartlet's test (to compare equality of variances);
– Tukey's method (to compare many averages).

The level of significance in all cases p = 0.05 was taken.

Results

Membrane acetylcholinesterase and Na^+, K^+, Mg^{2+} ATPase enzymatic parameters, were used as a marker of membrane structural and functional changes.

Table 1 and Figure 1 show the dependence of the total red blood cell membrane ATPase activity on the different doses of energy. It was found that doses of 3 J and 6 J did not statistically change the enzyme activity. For doses of 9 J the total ATPase

Table 1. *The effect of laser light (810 nm) on the erythrocyte membrane ATPase activity (nmol P_i/mg protein/hour). Red blood cell membranes (1 mg protein•ml^{-1}) in 5 mM Tris HCl – buffer, pH 7.4, were irradiated at 37°C at density of power of 125 mW/cm^2*

Dose [J]	Density of dose [J/cm^2]	Power = 100 mW (125 mW/cm^2)		
		Mg^{2+}, K^+, Na^+ - ATPase	Mg^{2+} - ATPase	K^+, Na^+ - ATPase
Control	Control	46.5 ±2.4 100%	26.7±1.5 100%	19.8±1.0 100%
3	3.75	46.5 ±1.9 100%	27.2±1.5 100%	19.3±1.4 97%
6	7.5	46.8 ±2.9 101%	26.9±2.2 100%	19.9±1.8 101%
9	11.25	52.3 ±2.1 113%*	27.1±1.6 100%	25.1±0.6 127%*
12	15	43.8 ± 2.2 94%*	27.1±1.4 100%	16.7±1.1 85%*
15	18.75	42.4 ± 2.7 91%*	27.0±1.9 100%	15.4±1.0 78%*
20	25	41.9 ± 2.7 90%*	27.0±1.6 100%	14.9±1.3 75%*

* $p < 0.05$ in comparison to control

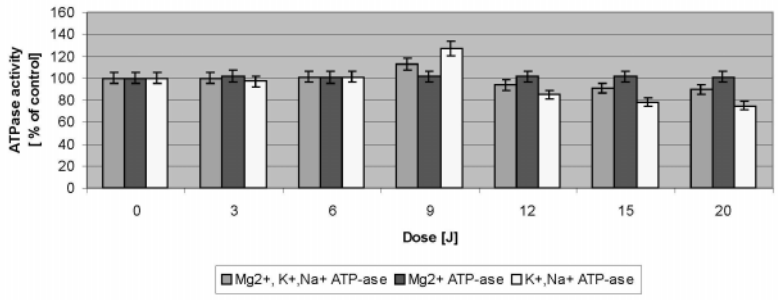

Figure 1. *Effect of continuous doses of laser radiation (810 nm, 125mW/cm^2) on ATPase activity*

Table 2. *The effect of laser light (810 nm) on the erythrocyte membrane ATPase activity (nmol P_i/mg protein/hour). Red blood cell membranes (1 mg protein•ml^{-1}) in 5 mM Tris HCl – buffer, pH 7.4, were irradiated at 37°C at density of power of 125 mW/cm^2*

Dose [J]	Density of dose [J/cm^2]	Power = 100 mW (125 mW/cm^2)		
		Mg^{2+}, K^+, Na^+ - ATPase	Mg^{2+} - ATPase	K^+, Na^+ - ATPase
Control	Control	46.5 ±2.7 100 %	26.7±1.4 100 %	19.8±1.4 100%
9	11.25	52.3±2.8 112%*	27.1±1.5 101%	25.2±1.5 127%*
3 + 6	3.75 + 7.5	44.0±2.4 95%*	26.5±1.7 99%	17.5±1.2 88%*
6 + 3	7.5 + 3.75	43.6±2.7 94%*	27.0±1.4 101%	16.9±1.3 85%*
12	15	43.6±2.8 94%*	26.9±1.6 101%	16.7±1.4 84%*
3 + 9	3.75 + 11.25	51.7±2.4 111%*	27.0±1.3 101%	24.7±1.4 124%*
9 + 3	11.25 + 3.75	46.6±2.8 100 %	26.8±1.2 100%	19.9±2.1 100%

* $p < 0.05$ in comparison to control

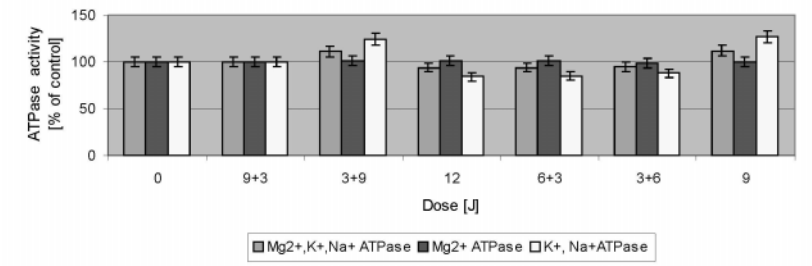

Figure 2. *Effect of fractionated doses of laser radiation (810 nm, 125mW/cm^2) on ATPase activity*

activity statistically increased (52.3 nmol P_i/mg protein/h) in comparison with control but for higher doses an inhibition of enzyme was observed.

The effect of laser light on Na^+, K^+ ATPase was similar and had a biphasic character. At smaller doses of radiation the activity of the enzyme slightly increased from 19.7 nmol P_i/mg protein/h for the control and reached a maximum for 9 J (25.5 nmol P_i/mg protein/h). After that it decreased and for energy doses higher than 12 J leveled off (15.3 nmol P_i/mg protein/h). After fractionated doses (3+9 J and 9+3 J) the activity of Na^+, K^+ATPase was significantly higher (24.6 and 19.9 nmol P_i/mg protein/h) than for a single energy dose of 12 J (16.6 nmol P_i/mg protein/h) (Table 2, Fig. 2). Similarly, a significant decrease of Na^+, K^+ ATPase activity was observed for a dose of 3+6 J and 6+3 J (17.4 and 16.8 nmol P_i/mg protein/h) with comparison to a single dose of 9 J (25.2 nmol P_i /mg protein/h).

Irradiation (810 nm, 125 mW/cm^2) of the suspension of red blood cell membranes significantly changed the kinetic parameters of AChEase enzymatic reaction. For a power density of 125 mW/cm^2 the Michaelis-Menten constant (K_m) value and V_{max} increased reaching the maximum for an energy dose of 9 J (11.25 J/cm^2). These parameters were not significantly increased for doses higher then 12 J (Table 3, Fig. 3-4).

Table 3. *The changes of kinetics parameters of AChEase reaction upon the continuous doses of laser irradiation (810 nm, 125 mW / cm^2).*

Dose [J]	Density of dose [J/ cm^2]	$K_M \cdot 10^{-4}$ [M]	K_M [% of control]	SD	p<	$V_{max} \cdot 10^{-6}$ [min·mg protein/ AcTCh]	V_{max} [% of control]	SD	p<
control	0	3.05	100			2.60	100		
3	3.75	4.48	142	33	0.03	3.63	140	30	0.02
6	7.50	4.26	145	32	0.01	4.03	142	30	0.01
9	11.25	8.67	294	31	0.0002	7.63	284	32	0.0001
12	15.00	4.87	152	30	0.02	3.82	284	33	0.01
15	18.75	3.90	120	29	ns	3.19	123	30	ns

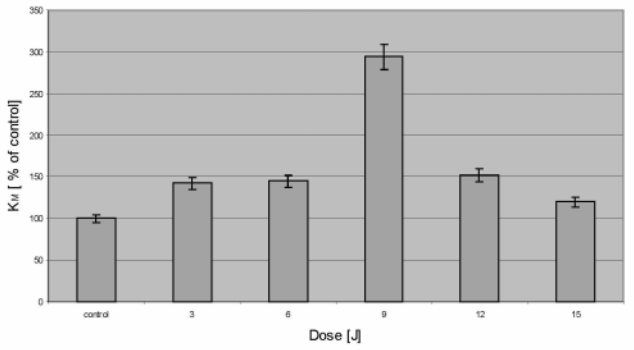

Figure 3. *The effect of continuous doses of laser radiation (810 nm, 125 mW/cm^2) on the Michaelis-Menten constant (KM) value of AChE reaction*

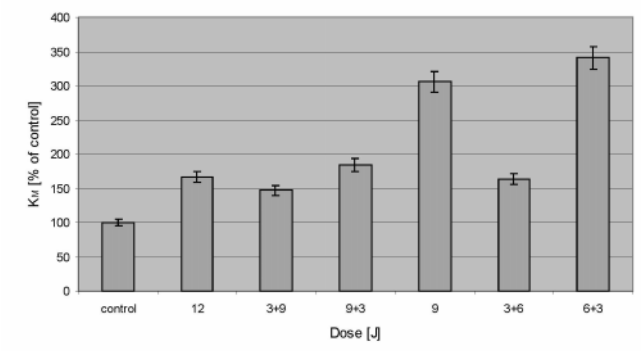

Figure 4. *The effect of continuous doses of laser radiation (810 nm, 125 mW/cm^2) on the V_{max} value of AChE reaction*

Next, the doses which were applied continuously (9 J and 12 J) were divided into two non equal parts (3 J + 6 J, 3 J + 9 J) in order to study the effect of fractionated doses of laser light on the AChE activity. The dosage was made in two different ways. In one case the smaller dose was applied first, in the other one, the bigger. The application of fractionated doses of laser radiation caused an increase of AChEase activity in comparison to control for both ways of energy dosage (Table 4, Fig. 5-6).

Table 4. *The comparison of changes of activity AChE under the continuous and fractionated doses of laser radiation (810 nm, 125 mW / cm²)*

Dose [J]	Density of dose [J/ cm²]	$K_M \cdot 10^{-4}$ [M]	K_M [% of control]	SD	p<	$V_{max} \cdot 10^{-6}$ [min·mg protein/ AcTCh]	V_{max} [% of control]	SD	p<
0	0	2.85	100			2.77	100		
9	11.25	8.94	306	31	0.0001	7.67	283	28	0.0001
3+6	3.75 + 7.5	4.64	164*	32	0.002	3.48	143*	31	0.03
6+3	7.5 + 3.75	10.05	341	30	0.0001	8.45	309	30	0.0001
12	15	5.64	166	37	0.002	4.18	171	34	0.002
3+9	3.75 + 11.25	5.10	147	44	0.04	3.42	142	44	0.03
9+3	11.25 + 3.75	5.75	184	42	0.002	4.22	182	40	0.002

*statistical significance to the continuous dose of 9 J

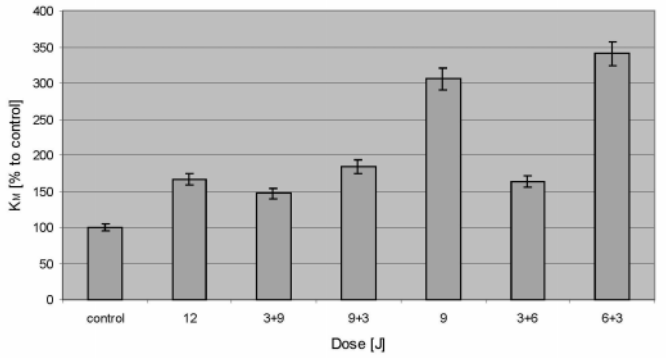

Figure 5. *The effect of fractionated doses of laser radiation (810 nm, 125 mW/cm²) on the Michaelis-Menten constant (K_M) value of AChE reaction*

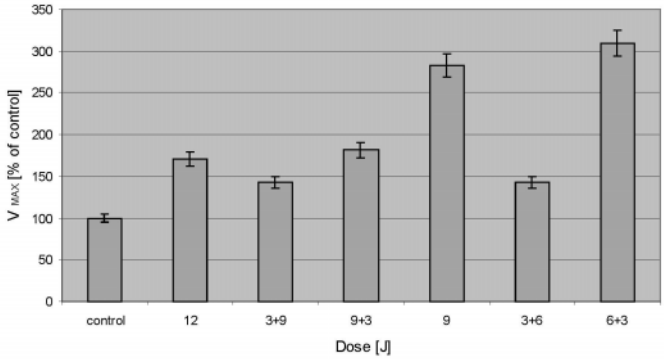

Figure 6. *The effect of fractionated doses of laser radiation (810 nm, 125 mW/cm²) on the V_{max} value of AChE reaction*

When the dose of 9 J was fractionated and the smaller part of the split dose was applied first, the significant inhibitory effect on enzyme activity in comparison to a continuous dosage was observed. When a larger part of the dose was applied first, the increase of AChE activity, although not significant in comparison to the continuous dose of 9 J, was found (Table 4).

The fractionated dose of 12 J significantly changed AChE activity in comparison to the control but not to the continuous dose. At first, the application of a smaller fractionated dose caused the inhibition of stimulating effect in comparison to the continuously applied 12 J as well as in comparison to the case when the larger dose was applied first.

Discussion

Studies have shown that the biological effects of low level laser irradiation depend on wavelength, radiation power, time exposure, and radiation dose (26, 27).

In our study, the influence of low-power near-infrared (810 nm) laser radiation on human erythrocyte membrane enzymes was observed. After irradiation of isolated erythrocyte membranes the parameters characterizing the structure and functions of cell membranes were altered.

The effect of laser light on Na^+, K^+ATPase activity was of biphasic character. While at smaller doses of irradiation the activity of the enzyme slightly increased in comparison to control and reached a maximum for 9J (25.5 nmol P_i/ mg protein/h) in the latter stage of this activity it decreased, and for energy doses higher than 12J it leveled off (15.3 nmol P_i/ mg protein/h).

Biostimulatory effect of small doses of light is not entirely clear but it is known that the modification of some enzymes activity by laser light can be the main reason for its healing action (28, 29). Friedman and coworkers (30) suggested that low doses of light induced the formation of a transmembrane electrochemical proton gradient in mitochondria, which was followed by a calcium release from mitochondria. At higher doses of light, too much calcium was released, which caused hyper-activity of Ca^{2+}-ATPase of calcium pumps and exhausted the ATP pool of the cell.

In our studies of the power of light of 100 mW (125 mW/cm^2) we observed that exceeding the dose of 9 J (11.25 J/cm^2) caused the inhibition of enzymes activity (Fig. 1 and 7). The Michaelis-Menten constant (K_m) value and V_{max} increased reaching the maximum for an energy dose of 9 J.

Similarly, Bolton et al. (31) using 860 nm diode laser showed a relationship between radiation-induced change of fibroblast proliferation and succinic dehydrogenase activity. At a dose of 2 J/cm^2 both the cell proliferation and enzyme activity were significantly increased, whereas at a dose of 16 J/cm^2, inhibition of both parameters was noted.

The analysis of the results of our studies shows that energy dose of laser radiation plays a crucial role in the efficacy of stimulation of AChEase activity.

Due to Lineweaver-Burk plots the mechanism of laser-induced changes of enzymatic activity is complex one. The activation of AChEase for some doses of radiation is accompanied by the decrease of substrate affinity when K_m is increased. Such changes of enzyme activity reflect complex membrane or enzyme transformations. In our earlier studies, where a suspension of erythrocytes was used, a different effect was observed (32). After irradiation of erythrocytes with laser light (λ = 810 nm, 200 mW) a biphasic change of the parameters of AChE reaction kinetics was found: for doses smaller than 12 J a decrease of both Michaelis-Menten constant (K_M)

and a maximal reaction rate were observed, whereas larger doses caused an increase of both reaction parameters.

In our previous experiments with near-infrared light no significant changes of erythrocyte stability, cellular redox state (reduced glutathione or lipid peroxidation levels) or cell membrane electrochemical potential (33) were observed.

Thus AChEase activity changes reflect a rather direct effect of light on the enzyme due to energy absorption. The different dependence of enzyme activity upon light, when the whole erythrocytes were irradiated, needs to be verified for 100 mW light power and further studies on molecular level to explain these differences are required.

In addition to 'normal' dose-rate behavior, there is the increasing number of reports on fractionated doses effects (34, 35). In our studies fractionated doses caused the effect on AChEase activity similar to a continuous irradiation because laser light induced an increase of AChEase activity in comparison to the control (non irradiated sample) regardless the way of its dosage. However, for the application of smaller fractionated dose as first the effect was smaller in comparison to the case when a larger dose was applied first.

In our investigation the activity of Na^+, K^+ATPase after fractionated doses (3+9 J and 9+3 J) was significantly higher than for continuous dose of 12 J, contrary to AChEase activity, which did not change significantly. The significant decrease of Na^+, K^+ATPase activity was observed for a dose of 3+6 J and 6+3 J with comparison to a continuous dose of 9 J. AChE activity for a fractionated dose of 3+6 J also significantly decreased.

Thus, we conclude that the photomodulation effect depends on the dose and dose rate, and is changed by dose fractionation.

Contrary to the radiobiology, where the normal dose rate effect of the ionizing radiation and the profitable fractionated dose effect are known (35, 36), the effects of fractionation of laser light doses and their therapeutic application still require elucidation.

Conclusions

1. Near-infrared low-intensity laser radiation changes the total ATPases activities of the membrane ion pumps in the dose- and way of dosage-dependent manner.
2. The changes of the total activity of erythrocyte membrane ATPase were mainly due to the changes of Na^+, K^+ATPase activity because Mg^{2+}ATPase activity was not significantly altered.
3. Fractionation of the light doses significantly changed the membrane enzymes response to laser radiation.
4. The differences in the effect of laser radiation on the activity of both enzymes result probably from their different location in the membrane.

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