EXPERIMENTAL FACTS CONCERNING THE He-Ne LASER EFFECT ON PLATELETS MEMBRANE MICROVISCOSITY

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Abstract. There are many clinical studies reporting the biostimulatory effects of He-Ne laser on different biological processes like: wound healing, cell proliferation, tissue regeneration and hemostasis, but the exact mechanisms of these findings have never been revealed [1, 3, 7, 10, 11, 14, 16]. Thus, we have considered a fundamental approach, trying to come up with some new biophysical evidence. In our experiments we have measured the human platelets membrane microviscosity before and after laser irradiation with different doses. The steady-state fluorescence anisotropy of membranes labeled with trimethylammonio-1,6-diphenyl-1,3,5-hexatriene (TMA-DPH) has been determined in different experimental protocols. The irradiation fluence was from 1.2 to 18 J·cm^{-2}, that covers the range used in the literature [1, 3, 7, 10, 11, 16, 18]. No significant influence of He-Ne laser irradiation on membrane microviscosity has been observed. However, He-Ne laser increases the membrane fluidity of starved (near-exhausted) platelets.

Key words: He-Ne laser, fluorescence anisotropy, platelets.

INTRODUCTION

In medical practice (dentistry, surgery) any method that can stop bleeding can be very useful on patients with hemostatic disorders or increased capillary fragility [4, 18]. Using He-Ne laser for such an important medical task requires complete understanding of the biophysical mechanism of this phenomenon for revealing the real biologic efficacy of this interaction.

Here, we investigate the hypothesis that He-Ne laser activates mainly on platelets and less on other factors involved in hemostasis. An important physical property of the membrane – microviscosity – was measured. This parameter reflects very well and is sensitive to the modifications of the membrane properties and functions [5, 9, 13]. The membrane fluidity measured by steady-state fluorescence anisotropy of TMA-DPH embedded into the membrane is relatively easy to follow [9, 17]. As platelet membrane is involved in almost every stage of the hemostatic

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process (adhesion, shape change, secretion and aggregation) [12], it can be possible to obtain information about laser effect on hemostasis by evaluating the changes in platelet membrane fluidity during He-Ne laser irradiation [6].

MATERIAL AND METHODS

CELL PREPARATION AND STORAGE

Platelets were prepared as described in [2a]. This is the usual protocol for obtaining platelet concentrates up to 10^{10} cell/ml for hematological therapy. The storage buffer contains adenosine and glucose, that guaranties the viability of the platelets for no more than 5 days at 20–24 °C with gentle and continuous agitation. We washed once and resuspended to $5 \cdot 10^7$/ml using Hepes saline buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2 \times 6H_2O$, 10 mM Hepes, 10 mM $C_6H_{12}O_6 \times H_2O$, pH = 7.4) for fluorimetric measurement. The *fresh* samples contain platelets up to 4 days of storage and *near-exhausted* samples contain platelets with more than 5 days of storage in standard conditions [2b].

TMA-DPH LABELING AND SAMPLE PREPARATION

I. Dissolve TMA-DPH (Sigma, Mw = 324) in spectroscopic dimethyl-formamide (DMF) to obtain a $5 \cdot 10^{-4}$ M stock solution.
II. Inject 5 μl stock solution into 1 ml HSB with intensive stirring in a quartz cuvette, then add 1.5 ml platelets suspension (PS) to the cuvette and mix them carefully. The incorporation of the probe into the plasma membrane is very fast and saturates in approximately 5 min. The probe is located at the surface of inner leaflet (has polar headgroup region) of the plasma membrane [5] and is not internalized for several hours [9].

Three types of samples were considered (for each category *fresh* or *near-exhausted*):

1. blind = 1 ml HSB + 1.5 ml PS (for measuring the background light)
2. control = 1 ml HSB + TMA-DPH + 1.5 ml PS without laser irradiation
3. sample = 1 ml HSB + TMA-DPH + 1.5 ml PS with laser irradiation

The final cell concentration in each sample was about $3 \cdot 10^7$ cell /ml.

FLUORESCENCE ANISOTROPY MEASUREMENTS

The steady-state fluorescence anisotropy measurements were performed at room temperature and with gentle stirring using a Jobin-Yvonne spectrofluorimeter set at 355 nm for excitation and 425 nm for emission wavelength with a 10 nm slit. The measured parameter was:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \tag{1}$$

where G is an instrumental correction factor, and I_{VV} and I_{VH} are the emission intensities with polarizers, respectively parallel and perpendicular to the direction of the vertically polarized excitation light. The G factor was measured according to Lakowicz [13] at the beginning and at the end of the irradiation protocols using the control sample.

The optical setup was in "L" format and the acquisition time for the intensities mean values was 10 s at 10 Hz sampling rate. So, the time resolution for r values was up to 30 s. All the intensity mean values were corrected for background scattered light using unlabelled controls (blind samples).

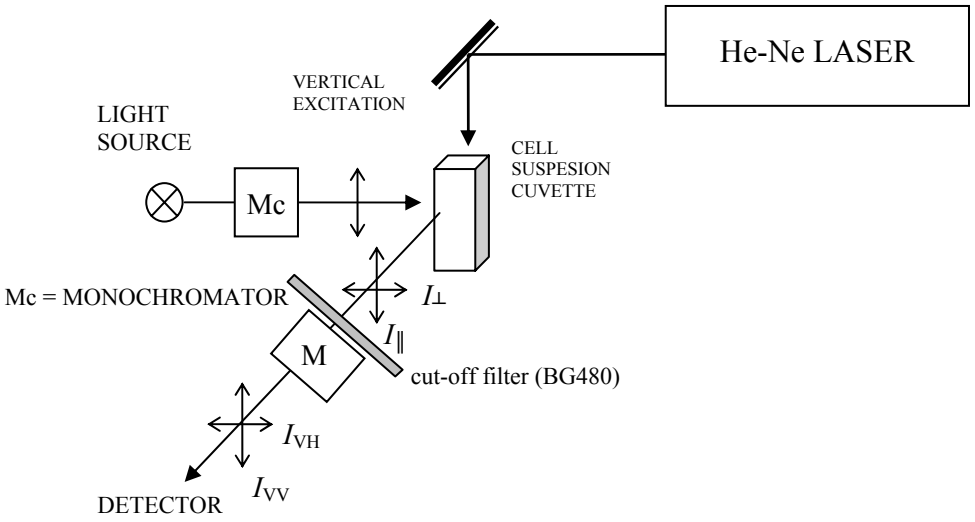

Fig. 1. – Experimental setup (optical scheme).

LASER IRRADIATION PROTOCOLS

A single mode continuous wave He-Ne laser (632.8 nm) for different irradiation times was used. Irradiation system allows the delivery of 10 mW laser power directly on the spectrofluorimeter cuvette containing 2.5 ml cell suspension, as shown in Figure 1. The cells were under continuous stirring during irradiation, to render out the inhomogeneity in dose distribution in different parts of the volume.

The fluence rate is:

$$FR = \frac{P}{s} \tag{2}$$

where $s = 2.54 \cdot 10^{-4}$ cm^2 is the area of the laser beam and P the output power of the laser. Total fluence during the irradiation is:

$$TF = FR \cdot t_r = \frac{P}{s} \cdot t_r \text{, and } t_r = t \cdot \frac{s}{S} \tag{3}$$

where t is the total irradiation time, t_r is the real irradiation time for one cell and $S = 1$ cm^2 is the section of the cell suspension volume.

That leads to the following formula for the fluence used in these experiments:

$$TF = \frac{P}{S} \cdot t \tag{4}$$

Thus, we assume that, due to the continuous stirring, the incident laser energy is uniformly distributed on the 1 cm^2 area of the cuvette. So, we obtain a fluence of 10 mW·cm^{-2} for each second [7]. For the experiments that detect fluorescence during laser irradiation we used an optical filter (BG480) to cut-off the effect of scattered laser light on the detection system.

The laser irradiation protocols and detecting periods were as follows:

1. *Laser-on protocols* – I_{VV} and I_{VH} were measured during laser irradiation by consecutive detection and repeated after different time intervals (2–5 min). The total fluence varies from 1.2 J·cm^{-2} to 7.2 J·cm^{-2}. Fluorescence intensities were also measured before laser irradiation, immediately after completing the irradiation protocol and again after a dark relaxation period (2–5 min).
2. *Laser-off protocols* – I_{VV} and I_{VH} were measured before and after laser irradiation. Cells suspensions were irradiated for different periods of time (15 min and 30 min) and, consecutively, the intensity values (I_{VV} and I_{VH}) were measured. The total fluence was 9 J·cm^{-2} and, respectively, 18 J·cm^{-2}. These procedures were consistent only for fresh platelet suspensions. TMA-DPH signal vanishes after the irradiation time in most of the starved platelets suspensions and that is consistent with the idea that a significant number of cells died during laser irradion.

RESULTS AND DISCUSSION

Our experimental protocol was designed to investigate if:

1. There is any effect on membrane microviscosity of human platelets.
2. The effect (if any) is limited only to the time of exposure or also persists for some time after removing the laser beam.
3. The effect (if any) builds-up in time or it appears rapidly and remains constant from the first moments of irradiation.
4. The effect (if any) is expressed in the same manner on *fresh* (1–4 days in standard condition storage [2] or starved (*near-exhausted)* platelets (low energetic resources – more than 5 days of storage).

For expressing the results we used the relative value of the fluorescence anisotropy:

$$r_r = \frac{r}{r_0} \tag{5}$$

where r_0 is the fluorescence anisotropy mean value of the control samples. This was obtained by averaging the fluorescence anisotropy values for all the control samples including the initial values of the probe samples. The mean value was 0.27325 ± 0.024338 (Table 1).

Table 1

Control anisotropy

No.	**Anisotropy**
1.	0.293007
2.	0.294908
3.	0.291506
4.	0.272039
5.	0.270216
6.	0.27106
7.	0.234227
8.	0.23605
9.	0.275105
10.	0.27332
11.	0.316763
12.	0.250798
r_0	**0.27325**
std	**0.024338**
std%	**8.906851**

The results can be grouped as follows:

1. On *fresh* prepared platelets: we did not find any significant modifications in the relative fluorescence anisotropy of the irradiated cells compared with non-irradiated cells, and that is consistent for all the fluence levels used (Figs. 2, 3, 4). This can be explained by tree possible ways, also described in the literature:
   - The TMA-DPH anisotropy measurements are not conclusive for platelets physiology [6].
   - Cells have enough resources (adenosine and glucose) to maintain the homeostasis under laser irradiation [10, 16] and there is no effect on membrane fluidity.
   - There is no effect of low level He-Ne laser on platelets whatsoever; other mechanisms may be involved [3], for explaining the hemostatic effect. We propose future studies of the He-Ne laser effect on platelets activation curve.

2. On starved (*near-exhausted)* platelets, with decreased adenosine and glucose concentrations (Fig. 4), we observed a marked decrease in the fluorescence anisotropy (increased fluidity) for the irradiated cells, since the anisotropy for the non-irradiated near-exhausted platelets (control) remains in the same range.
   - The effect appears rapidly and remains constant from the first moments of irradiation.
   - The effect is not reversible within minutes, but further investigation is needed to see if the microviscosity recovers itself after longer periods of time or after glucose/adenosine addition.

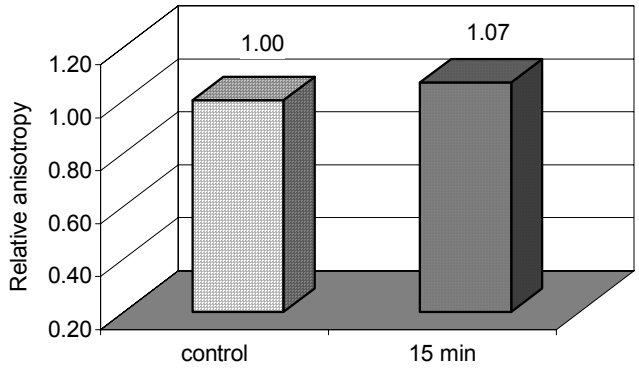

Fig. 2. – Relative anisotropy of *fresh* platelets; *laser-off* setup; detection was made few seconds before (control) and after 15 min of irradiation (fluence of 9 J·cm^{-2}).

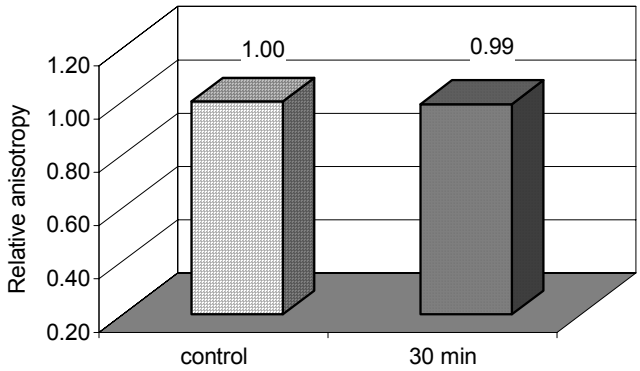

Fig. 3. – Relative anisotropy of *fresh* platelets; *laser-off* setup; detection was made few seconds before (control) and after 30 min of irradiation (fluence of 18 J·cm^{-2}).

These facts confirm the hypothesis that low level electromagnetic irradiation has certain influence if a moderate second stressor (like cell starvation) is present. It is assumed that the preexisting stressor could affect components of the same signal transduction pathways as in the laser irradiation case [8]. These findings also

sustain the hypothesis that He-Ne laser activates mitochondrial formation of ATP [7, 10, 16] that faster consumes energetic resources (glucose and oxygen). Cells seem to enter in a critical physiological state characterized by a more fluid membrane.

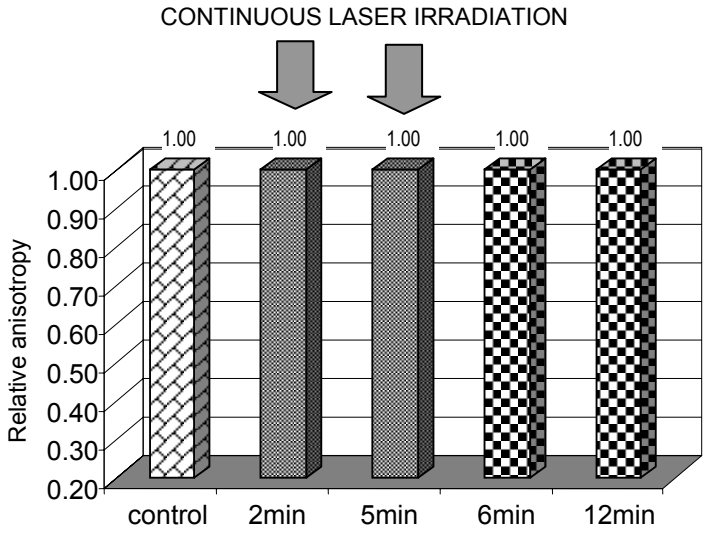

Fig. 4. – Relative anisotropy of *fresh* platelets; *laser-on* setup (total fluence was 1.2 J·cm^{-2} after 2 min and 3.0 J·cm^{-2} at the end of irradiation time, after 5 min); the *6 min* point represents the values immediately after turning off the laser.

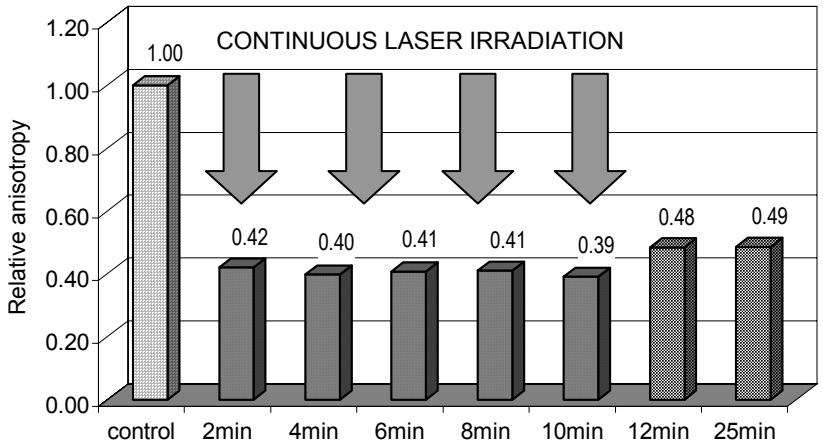

Fig. 5. – Relative anisotropy on *near-exhausted* platelets; *laser-on* setup (total fluence was 1.2 J·cm^{-2} after 2 min and 6 J·cm^{-2} at the end of irradiation time after 10 min).

We can consider two different hypotheses:

1. Laser irradiation may increase the kinetics of ATP synthesis, that very fast consumes the remaining glucose and oxygen reserves. For a starved cell this may lead instantly to a critical state that explains the observed increased fluidity of the membrane.
2. In a starved cell that is already in a critical physiological state the ATP enzymatic system becomes insensitive to the stimulatory effect of laser, so the increase in membrane fluidity may be regarded as a direct effect of laser.

CONCLUSIONS

For *fresh* platelets, a significant influence of He-Ne laser irradiation on membrane microviscosity has not been observed. This does not exclude however other possible interactions of laser with platelets function.

He-Ne laser irradiation increases the membrane fluidity of starved or *near-exhausted* platelets.

These findings cannot explain directly the laser effect on hemostasis, but can lead to other new possibilities for studying He-Ne laser effect on human platelets:

- The membrane microviscosity recovery after adenosine/glucose addition on near-exhausted platelets suspensions.
- To experimentally induce the starvation of fresh platelets and to monitorise both the ATP synthesis and the membrane fluidity upon laser irradiation.

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