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FLOW CYTOMETRIC EVALUATION OF LOW INTENSITY LASER ACTION ON HUMAN RED BLOOD CELLS (RBCs) VIABILITY STORED IN SAGM MEDIUM FOR 3 WEEKS

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Transfusion medicine relies on the possibility of storing red blood cells (RBCs) for a prolonged period of time. A better understanding of the nature of changes in stored RBCs may provide new strategies to improve the balance of benefits and transfusion's risks. In the light of recent results on the mechanism of programmed cell death of RBCs *(erythroptosis*), where numerous cellular changes are common hallmarks of the apoptotic phenomenon, we have developed new flow cytometric criteria to evaluate the viability of stored erythrocytes. In order to evaluate the possible benefic effect of low-intensity laser irradiation for increasing the blood preservation period, we irradiated the whole blood from healthy donors in specially designed bags for research purposes by MacoPharma Company containing SAGM medium, and conserved it for 3 weeks. LED (Light emitting diode) (λ=465nm, P=60mW, P/S=100mW/cm^2) and laser diode (λ=660nm, P=30mW, P/S=50mW/cm^2) in continuous wave have been used as sources of irradiation. Doses were ranged between 0 and 2J/cm^3. After that period RBCs were analyzed by flow cytometry for morphological changes (FSC/SSC), apoptosis/necrosis analysis (FITC-annexin-V labeling/PI) and viability using an original Calcein-AM method on a FACScan cytometer using CellQuest Pro software for acquisition and analysis. Complementary, the RBCs integrity was analyzed by scanning electron microscopy (SEM). Morphological changes assessment of red blood cells by flow cytometry and scanning electron microscopy showed a good preservation of the discoidal shape for the irradiated cells compared with control.

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1. Introduction

The blood transfusion is widely used in our days, in the case of a patient who suffered from acute loss of blood and when problems regarding a destruction of erythrocytes or a poor production in blood bone marrow exist[1].

Starting in 1667 with the first transfusion in humans, physicians and biologists tried to improve the transfusional act and also the storage conditions of the blood, in order to obtain better effects and to minimize the transfusion risks.

The most important issues in blood transfusion are:

- the effects of the transfusional act on human, especially on the politransfused subjects (where a massive charge of iron appears);

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- the quality of preserved blood and the clearance of the transfused RBCs, considering that after a blood transfusion, 30% of transfused RBCs disappear from the circulation after 1 day and 70% disappear after 3 days[2, 3, 4].

Erythrocytes are now stored for a period that may be for 42 days, but during the storage a lot of modifications appears, which alters the quality of the blood. These modifications have been generically called “storage lesions”[5].

The storage lesions involve the loss of (RBC’s) viability, changes in their biochemical and mechanical properties, and the oxidative damage[6].

Regarding the quality of preserved blood, many studies have been made in order to improve the quality, to minimize the storage lesions, and to find better criteria for evaluate the quality of the blood.

In the light of recent results on the mechanism of programmed cell death of RBCs *(erythroptosis*)[7], where numerous cellular changes are common hallmarks of the apoptotic phenomenon, we have developed new flow cytometric criteria to evaluate the viability of stored erythrocytes[8]. Using flow cytometry we also may observe changes in cellular morphology and density, as well as analyse the phosphatidileserine exposure as a marker for apoptosis phenomenon.

The flow cytometers functioning is based on the principle of the pluriparametrical analysis with a laser beam of every single cells which is in a cellular suspension and goes out from the cytometer injector to the analyse chamber. We obtain information based on two kind of physical phenomenon: 1) the light scattering - which provides information about the cell size, the intracellular and structural content and 2) the fluorescence emission of one or more fluorochromes fixed on the cells, under the laser excitation[9].

In order to minimize the storage lesions, to prolong the period of preservation, and to improve the quality of the stored blood, several studies have been made in various research directions like leukodepletion[10], anaerobic storage[11], adding additive solutions[12], better prelevation techniques[13, 14], blood irradiation[15, 16, 17], and so on.

The practice of blood irradiation started in 1920, by the experiments of Knott's Emmet concerning the extracorporeal blood irradiation with ultraviolet (UV) radiation. In 1923 Knott tried to treat infectious diseases of the blood using antibacterial properties already known of UV radiation. He assumed that the pharmacological effect of irradiated blood manifests itself at the reintroduction of blood in the body, by inducing secondary emission of biophotons from cells activated by UV radiation. Like other researchers, Knott found that weighted emission of biophotons stimulates the activity of white blood cells, which explains the beneficial immune response, but in large quantity this emission destroy different types of white blood cells, which explains why the emission of biophotons is effective in autoimmune diseases[18].

By the 90s in Russia began to be used intravenous blood irradiation with low power lasers by inserting a catheter into a vein containing fiber inside. This method has several advantages over the extracorporeal irradiation:

- Does not require the use of anticoagulants;
- It is easy to use and dosage can be controlled more precisely.

Stulin et al reported in 1994, a success of the treatment by intravenous irradiation of blood in schizophrenics with depressive syndrome, resistant to all types of drugs. This efficacy results from the ability of treated blood to destroy white cells metabolically active, which blocked the microcirculation in the brain. In this case the laser radiation can be seen as a substitute for glucose and thus as a suppressor of any excessive metabolic activity in any part of the body. A comparison between the effectiveness of the extracorporeal irradiation of blood and intravenous irradiation is not easy to do, because this efficiency is different from one disease to another[19].

It can be assumed that the combination of these two types of therapy by irradiation would lead to a very effective therapeutic effect[20, 21].

The literature studies show that the main effects on treated blood as a result of extracorporeal irradiation or intravenous irradiation characteristics are better oxygenation and improved blood characteristics (rheology, vasodilatation, peripheral circulation). These effects occur with unusual rapidity after transfusion with treated blood, which can lead to the disintegration of erythrocytes or platelets conglomerates to turn them into flowing and normal

diffuse networks running in a few minutes. Researchers from the ex-Soviet space reported unexpectedly good results in the treatment of cerebrovascular disorders, of the heart and legs[22, 23].

Other short-term effects of extracorporeal or intravenous irradiation are:
- changes in erythrocyte membrane, resulting in the release of substances that cause blood subsequent changes;
- structural changes in plasma proteins;
- immediate release of oxygen free radicals followed by growing of antiradical factors;
- increasing blood volume and slightly decreased hematocrit;
- decrease in blood pressure;
- degranulation of mast cells and granulocytes;
- decrease for a short period of platelet number and sometimes inhibiting their functions;
- activation of fibrinolytic factors and reduction of coagulants activity;
- increased phagocytosis.

A practice less used so far is the UV irradiation of blood collected from donors, then perform blood transfusions using irradiated blood. Not yet clearly established whether this practice has advantages over the autotransfusions with irradiated blood, though so far there has not been appearance of post-transfusion hepatitis after transfusion with blood treated with UV radiation. Thus, some researchers recommend this practice to reduce the risk of post-transfusion hepatitis transmission[24].

In choosing the experiments described in the current paper we have considered the already proven effect of low power laser radiation on the blood *in vitro*. We were particularly interested in determining changes brought by laser radiation on red blood cells and plasma proteins adsorbed on their surface[25, 26, 27, 28, 29 30].

From the practice of intravenous blood irradiation it can be concluded that the effects on autoimmune diseases could be stopped (even if it cannot restore damage already done) by suppressive action of excessive metabolic activity, as well as by the action of autoimmune response.

Some researchers have used blood irradiation during plasmapheresis and cytapheresis procedures to see if there are certain therapeutic effects of irradiated blood to treat diseases with immune deficiency. This method although slightly different (using photoactive drugs) has been used by Edelson[31].

To the anti-virus action of blood obtained from donors, mainly pursued by other researchers, we have in mind especially the therapeutic effects which blood irradiation may have on specific diseases as a result of transfusion performed with irradiated blood.

Knowledge of the molecular mechanisms of interaction between low power laser radiation and blood, currently unclear, could allow us approaching in a new and differentiated manner the problem of therapeutic effects of laser radiation. Considering the concrete parameters of blood from each patient, we could set differently for each patient the necessary laser parameters (wavelength, power density, dose, duration etc.) for an effective treatment. Influence of laser parameters on the biological effects of low power laser radiation is not fully elucidated, as well as the influence of the operating modes and treatment protocol, further studies being necessary[32, 33, 34, 22, 23].

As the irradiation of preserved blood, one of the aims is to achieve a rejuvenating effect of preserved blood (a process by which the ratio of young and old erythrocytes increases by the fact laser radiation causes an increase in hemolysis of old or broken cell). This effect would be useful in the view of making transfusions with irradiated blood to try such treatment of immune diseases or serious hematologic diseases (leukemia of various types).

To evaluate the possible benefic effect of low-intensity laser irradiation in relation to the blood preservation period we irradiated the whole blood from healthy donors in bags containing SAGM medium. The bags were special designed for research purposes by MacoPharma Company, and the blood was preserved for 3 weeks.

2 Materials and methods

2.1 Chemicals

Fluorescein conjugated annexin-V (Annexin-V-FITC), HEPES binding buffer (HEPES buffer pH 7.4 containing 2.5mM calcium chloride), were obtained from Pharmingen (San Diego, CA, USA), Calcein-AM from Sigma Aldrich (St. Louis, M0, USA) and CaspGLOW^{TM} Fluorescein active caspase-8 and caspase-3 staining kits from BioVision Research Products (Mountain View, CA, USA). The flow cytometer was a Becton-Dickinson FACScan apparatus (San Jose, CA, USA) with CellQuest Pro software for acquisition and analysis.

2.2 Blood preservation for the study of laser effect

To evaluate the effect of laser radiation on the RBCs stored in SAGM medium, blood of 2 donors was preserved in special mini-bags designed for research purposes (MacoPharma Company, France) containing RBCs obtained after deleucocytation of whole blood and stored at 4°C for maximum 3 weeks.

In order to find the best conditions for irradiation (dose and exposure time) two series of ten bags were irradiated at doses ranging gradually from 0 (A_1) to 1J/cm^3 (A_2 - A_9) using the wavelength of λ=465nm, and from 0.2 to 1.8J/cm ^{3} (B_{11} - B_{19}) (λ=660nm). Irradiation was done on days 11 th, 14 th and 21 st after collecting RBC's. Samples were prepared by resuspension of RBCs in isotonic phosphate buffered saline (PBS):Na_2HPO_4 1.8mM, KH_2PO_4 140mM, NaCl 2.7mM, KCl, pH 7.4, (10^7 cells per ml) for flow cytometric analyses and complementary techniques 12h after RBCs irradiation, and were analyzed in a period of three weeks.

Flow cytometric analyses were performed on a FACScan cytometer using CellQuest Pro software for acquisition and analysis. Cells in suspension in isotonic PBS buffer pH 7.4 were gated for the light scatter channels on linear gains, and the fluorescence channels were set on a logarithmic scale with a minimum of 10,000 cells analyzed in each condition.

2.3 Morphological changes assessment of red blood cells by light scattered measurements

Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides information about cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with cell size. The intensity of scattered light measured at a right angle from the laser beam (side scatter/SSC) correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light, being associated with cell shrinkage. RBCs in suspension in isotonic PBS buffer, pH 7.4, is gated under forward and side scatter parameters (FSC versus SSC).

2.4 Flow cytometric measurement of cell viability using Calcein-AM

Cell viability assessment was studied according to the procedure of Bratosin et al[35], which is based on the use of acetoxymethyl ester calcein (Calcein-AM), a fluorescein derivative and non-fluorescent vital dye that passively crosses the membrane of viable cells and is converted by cytosolic esterases into calcein that produces intense green (530nm) signal, and is retained by cells with intact plasma membranes. From dying or damaged cells with compromised membrane integrity unhydrolysed substrate and their fluorescent products are rapidly extruded from cells.

The membrane-permeable dye Calcein-AM was prepared as a stock solution of 10mM in dimethylsulfoxide stored at -20°C and as a working solution of 100 μm in PBS buffer pH 7.4. RBCs (4 x10^5 in 200μl PBS buffer, pH 7.4) were incubated with 10μl Calcein-AM working solution (final concentration in Calcein-AM: 5 μM) for 45min at 37°C in the dark. Subsequently the sample was diluted in 0.5ml of PBS buffer for immediate flow cytometric analysis of calcein fluorescence retention in cells. Experiments were performed at least three times with three replicates each time.

2.5 Flow cytometric analysis of phosphatidylserine exposure

Phosphatidylserine exposure on RBCs was assessed using FITC-annexin V. Erythrocytes were resuspended (10^6 cells) in HEPES buffer pH 7.4 containing 2.5mM calcium chloride with

10µl (0.1µg) of FITC-annexin-V solution and incubated for 15min at room temperature in the dark. The cells were gated for biparametric histograms FL1 (FITC fluorescence) versus FL2 (RBC autofluorescence). Experiments were carried out in triplicate.

2.6 Scanning electron microscopy (SEM) analysis

Erythrocytes were fixed for 4h with a 1.25% glutaraldehyde solution in 0.1M sodium cacodylate buffer pH 7.2 and post-fixed for 4h in 1% osmium tetraoxide in the same buffer. The suspensions were then filtered onto 0.2µ anodisc filters and dehydrated in an ethanol series. After drying with carbon dioxide by the critical point method and sputter-coating with gold, samples were examined on a 35 CF JEOL SEM.

3. Results and Discussions

3.1 Morphological changes assessment of red blood cells by flow cytometry and scanning electron microscopy

In flow cytometry, analysis of the scattered light by the mode FSC/SSC provides information about cell size and structure. The intensity of light scattered in the forward direction (FSC) correlates with the cell size. On the other hand, the intensity of scattered light measured at a right angle to the laser beam (side scattering/SSC) correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light. The cell's ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane and, in the case of apoptosis, chromatin condensation, nuclear fragmentation and shedding of apoptotic bodies. During apoptosis, the decrease in forward light scatter (which is a result of cell shrinkage) is not initially paralleled by a decrease in side scatter. A transient increase in right angle scatter can be seen during apoptosis in some cell systems. This may reflect an increased light reflectiveness by condensed chromatin and fragmented nuclei. However, in later stages of apoptosis, the intensity of light scattered at both forward and right angle directions decreases. Cell necrosis is associated with an initial increase and then rapid decrease in the cell's ability to scatter light simultaneously in the forward and right angle direction. This is a consquence of an initial cell swelling followed by plasma membrane rupture and leakage of the cell's constituents[36].

Our results presented in fig.1 show a comparative flow cytometric analysis of morphological histogram of X Geo Mean (cell size) and Y Geo Mean (granularity) values of RBCs conserved in SAGM medium for 3 weeks and irradiated with low level laser irradiation (C and D). Figures 1A and 1B are the dot-plot analysis in system FSC/SSC of RBCs before irradiation (at the point time T_0 when blood sampling). RBCs conserved for 3 weeks in SAGM medium without irradiation (A_1 and B_1 in fig.1C and 1D), for both donors were used as control. The irradiated RBCs didn't show variation in X Geo Mean values (values correlated with the cellular morphology), or an important hemolyse, which is usually expressed in decreased values for Y Geo Mean (cellular content), excepting samples B_{14}, B_{15} and B_{16}, where we may observe a little hemolyse. This little hemolyse may be due to an uneven irradiation that may appear as a consequence of the manual technique used for irradiation or to the little size of the mini bags used for blood conservation. It may also appear as a particularity of the Donor B, which is possible to have RBCs more sensible.

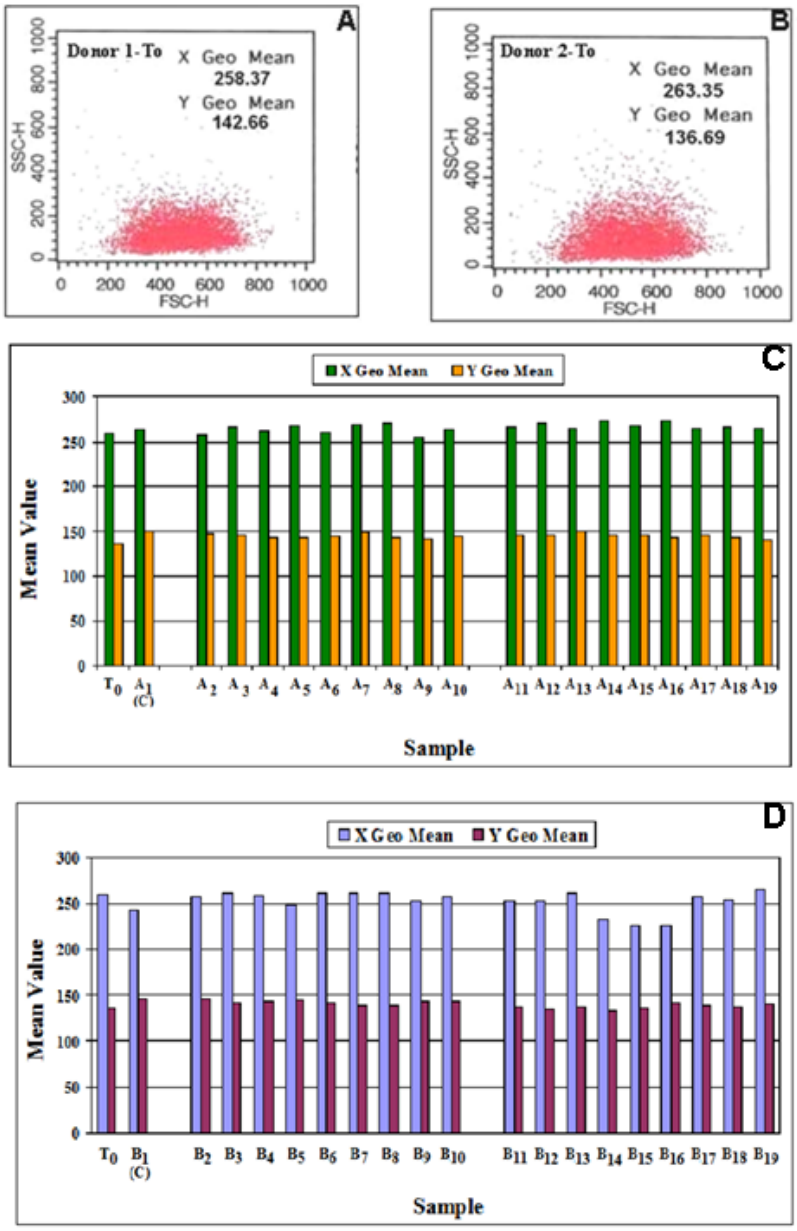

Fig. 1. Comparative flow cytometric analysis of morphological histogram of X-and Y Geo Mean values of RBCs conserved in SAGM medium for 3 weeks and irradiated with low level laser irradiation. A and B represent dot-plot analysis FSC/SSC of RBCs before irradiation (at the point time T_0 when blood sampling). Abscissa: forward scatter (cell size); ordinates: side scatter (cell density, granularity and refractiveness). C and D represent the comparative hystograms of X Geo Mean and Y Geo Mean for irradiated samples. Data are representative of three analysis giving similar results. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed. (C) under A_1 and B_1 signifies control.

Results obtained by flow cytometry are in concordance with results obtained by scanning electron microscopy (see fig.2), were we observe a better preservation of discoidal morphology in the irradiated cells (C_1 and C_2) compared with control (B_1 and B_2).

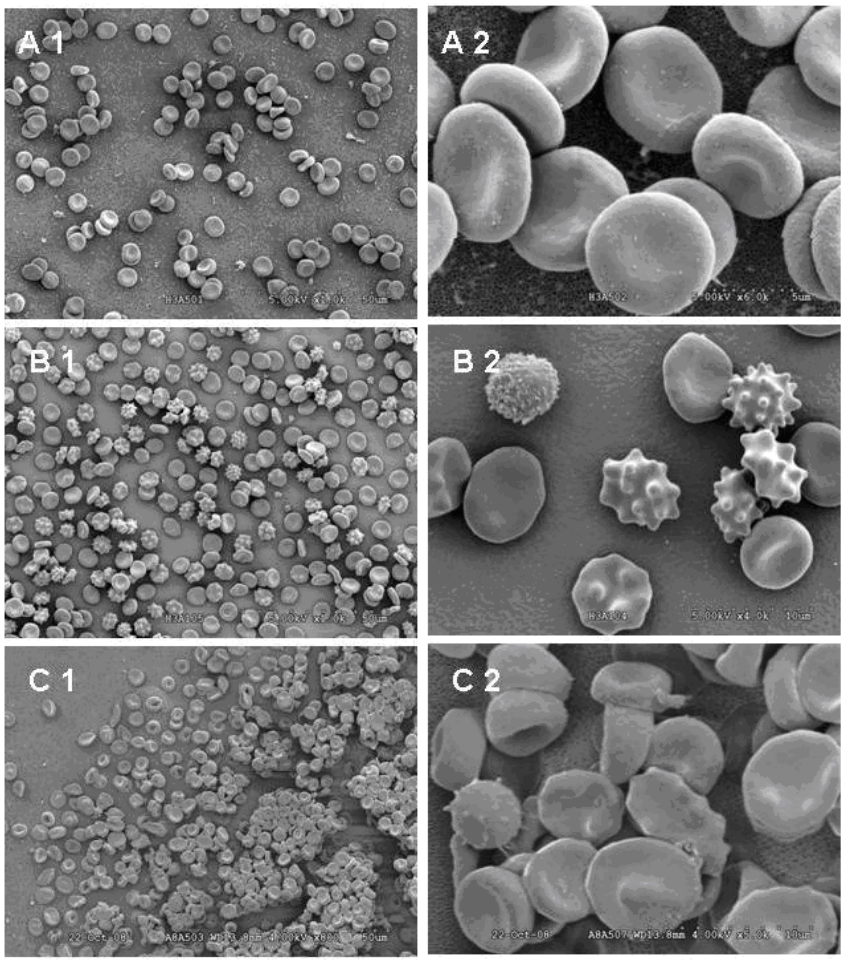

Fig. 2. SEM analyses of control RBCs from the donor D2 conserved in SAGM medium for 3 weeks (B_1, B_2) and irradiated with low level laser irradiation λ= 660 nm [1,6 J/cm^3] after 3 weeks of conservation in SAGM medium (C_1, C_2). A_1 and A_2 represent control samples of RBCs before conservation (T_0). Data are representative of three analyses giving similar results.

Note: There is no correlation between notations in Fig.1 and Fig.2 except T_0, which has the same meaning.

3.2 Flow cytometric analysis of phosphatidylserine exposure

Phosphatidylserine exposure on the outer leaflet of plasma membrane is regarded as one of the signals allowing macrophages to ingest erythrocytes. We evaluated the phosphatidilserine exposure by comparative flow cytometric analyses usig annexin-V-FITC labelling on red blood cells.

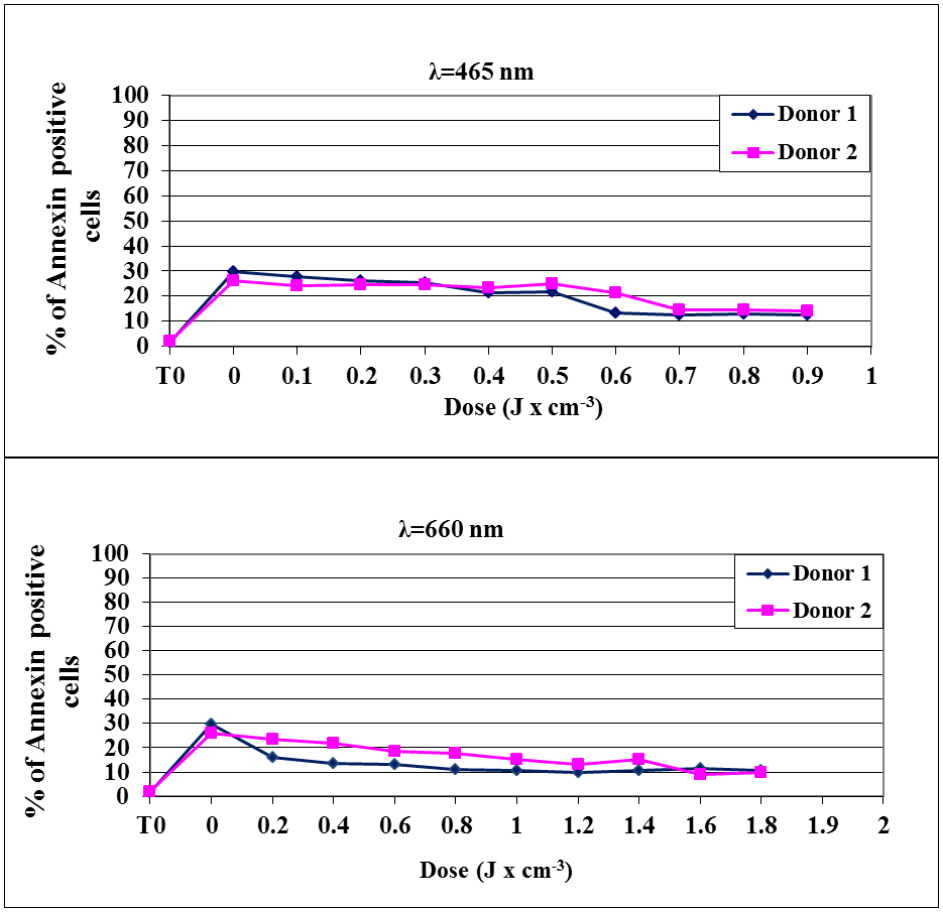

Fig. 3. Comparative flow cytometric analysis of the percentage of apoptotic erythrocytes determined with annexin-V-FITC for RBCs conserved in SAGM medium for 3 weeks and irradiated with low level laser irradiation. Abscissa: dose of irradiation (J/cm^3); Ordinate: Percentage of Annexin-V-FITC positive cells. Data are representative of three analysis giving similar results. Number of counted cells: 10,000. Results presented are from one out of three representative experiments performed.

3.3 Flow cytometric measurement of cell viability using Calcein-AM

We have applied the flow cytometric assay we previously developed for the measurement of erythrocyte viability (Bratosin & al. [17]). As described in "Materials and methods", the assay is based on the use of Calcein-AM, a non-fluorescent vital dye that passively crosses the cell membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein which is retained only by cells with intact membranes.

The beneficial effect of laser irradiation is observed in fig.4, where the overlay of the histograms reveals an increased viability for the irradiated samples compared with non irradiated control samples (97% viable cells at the dose of 0.8 J/cm^3 for λ=465 nm and 98.8% viable cells at 1.8 J/cm^3 for λ=660 nm respectively), compared with 66% for control RBCs. The results we obtained showed for every irradiated sample a better viability compared with the control.

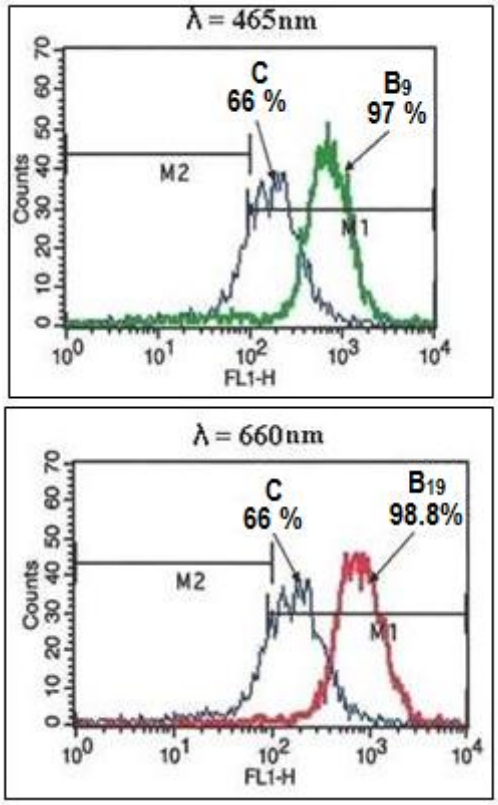

Fig. 4. Comparative flow cytometric histogram analysis of Calcein-AM cell viability of nonirradiated RBCs (C) and irradiated RBCs at λ= 465 nm [0.8 J/cm^3] and λ= 660 nm [1,8 J/cm^3] from the donor D2 conserved in SAGM medium for 3 weeks .

λ - wavelength; M_1: region of fluorescent cells with intact membranes (living cells) and M_2: region of nonfluorescent cells with damaged cell membranes (dead cells) after 3 weeks of conservation in SAGM medium. Abscissa: log scale green fluorescence intensity of calceine (FL1). Ordinate: relative cell number. Number of counted cells: 10,000. Results presented are from one out of three representative experiments performed.

5. Conclusion

Given the controversy of data presented in literature, we proposed to investigate the effects on low level laser irradiation on human RBCs viability stored in SAGM medium for 3 weeks. We had evaluated the cellular viability by measuring the level of cellular esterase with Calcein-AM, by flow cytometry. We also correlated the cellular viability with phosphatidilserine exposure and cellular shape, also determined by flow cytometry.

Our results show a markedly viability improvement on laser irradiated erythrocytes determined by Calcein-AM assay and a reduction in phosphatidilserine exposure (almost half), determined with Annexin V. Previously we found that calcein fluorescence is directly proportional to the amount of ATP, another marker of RBC senescence and apoptosis which is classically considered as an indicator of viability for RBCs stored in blood banks.

Morphological changes assessment of red blood cells by flow cytometry and scanning electron microscopy showed a good preservation of the discoidal shape for the irradiated cells compared with control. Using our new criteria for assessing RBC's viability founded on the use of flow cytometry, we clearly demonstrate the benefic effect of laser irradiation for improving RBCs preservation in blood banks[8, 35].

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References

[1] V. M. J. Novotny, Transfusion Clinique et Biologique **14**(6), 538 (2007).
[2] P. L. Mollison, Transfusion **24**(2), 93 (1984).
[3] P. L. Mollison, Transfusion **45**(12), 1985 (2005).
[4] M. Luten, B. Roerdinkholder – Stoelwinder, N. P. M. Schaap, W. J. De Grip, H.J. Bos, G. J. C. G. M. Bosman, Transfusion **48**(7), 1478 (2008).
[5] E. Beutler, Transfusion **40**(8), 893 (2000).
[6] T. Yoshida, S. S. Shevkoplyas, Blood Transfus. **8** (4), 220 (2010).
[7] D. Bratosin, J. Estaquier, F. Petit, D. Arnoult, B. Quatannens, J-P Tissier, C. Slomianny, C. Sartiaux, C. Alonso, J-J Huart, J. Montreuil and J. C. Ameisen, Cell. Death. Differ. **8**(12), 1143 (2001).
[8] L. Mitrofan-Oprea, C. Palii, J. P. Tissier, A. Héron, T. Verpoort, M. Behague, F. Goudaliez, E. Smagghe, F. Schooneman, J-J. Huart, J. Montreuil, D. Bratosin, Transfus. Clin. Biol. **14**(4), 393 (2007).
[9] D. Bratosin, Vasile Goldis University Press, Arad, 1-244 (2007).
[10] T. Lundeberg, M. Malen, Ann. Plast. Surg. **27**(6), 535 (1991).
[11] K. M. Lagan, B. A. Clements, S. McDonough, G. D. Baxter, Laser Surg. Med. **28**(1), 27 (2001).
[12] N. Kipshidze, H. Sahota, H. Wolinsky et al., Circulation **90**, 327 (1994).
[13] S. C. Haskins, J Am Vet Med Assoc. **170**(4), 429 (1977).
[14] R. F. Weir, R. S. Olick, The stored tissue issue: biomedical research, ethics, and law in the era of genomic medicine, Oxford University Press (2004).
[15] A. Piasecka, W. Leyko, E. Krajewska, M. Bryszewsk. Scand. J. Clin. Lab. Invest **60**(5), 395 (2000).
[16] D. G. Siposan, S. Bobe, Laser Therapy **19(**4), 245 (2010).
[17] J. Konawa, L. Zavodnik, I. Zavodnik, V. Buko, A. Lapshyna, M. Bryszewska, J. Clin. Laser Med. Surg. **22**(2), 111 (2004).
[18] E. K. Knott, American Journal of Surgery **76** (2), 165 (1948).
[19] J. D. Stullin et al., Zhurnal Nevropatologii i Psikhiatrii Imeni S. S. Korsakova **1,** 54 (1994).
[20] G. I. Sukhanova, Laser Therapy in the Far East, Vladivostok, Dal'nauka (1993)
[21] L. Gasparyan. "Healing Photons": Intravenous laser blood irradiation therapy http://www.geocities.com/lgasparyan
[22] N. N. Kipsidze et al., HeNe laser treatment of cardiac ischemia, Tbilisi, "AMIRANI" Publishing (1993). Translated from Russian.
[23] B. N. Zhukov, N. A. Lysov, Laser radiation in experimental and clinical angiology, Univ. of Medicine from Samara, Russia (1996). Translated from Russian.
[24] A.V. Marchenko et al., Vestnik Khirurgii Imeni II Grekova, **2**, 87 (1990).
[25] K. Iijima, N. Shimoyama, M. Shimoyama, T. Mizuguchi, Journal of Clinical Laser Medicine and Surgery; Aug; **11**(4), 185 (1993).
[26] K.Iijima, N.Shimoyama, M.Shimoyama, T.Mizuguchi, Journal of Clinical Laser Medicine and Surgery; **9**(5), 385 (1991).
[27] E. B. Zhiburt, N. B. Serebrianaia, E. N. Rozhdestvenskaia, V. I.Vashchenko, I. V. Katkova, V.V. D'iakova, M. Iu. Boitsova, Patol Fiziol Eksp Ter, **3**, 6 (1998).
[28] J. H. Theis, G. Lee, R. M. Ikeda, D. Stobbe, C. Ogata, H. Lui, D. T. Mason, Clin Cardiol., **6**(8): 396 (1983).

[29] T. I. Karu, L. V. Piatibrat, G.S.Kalendo, Biull. Eksp. Biol. Med, **115**(6), 622 (1993).
[30] M. Singh, T. M. Vatsala, Biorheology; **19**(1/2), 165 (1982).
[31] R. Edelson, Annals N. Y. Acad. Sci **11**, 636 (1998).
[32] T. Karu, The Science of Low-Power Laser Therapy, Amsterdam, The Netherlands, Gordon and Breach Science Publishers, p. 29, 189 (1998).
[33] D. G. Siposan, A. Lukacs, Journal of Clinical Laser Medicine & Surgery, **18**(4), 185 (2000).
[34] D. G. Siposan, A. Lukacs, Journal of Clinical Laser Medicine & Surgery, **19**(2), 89 (2001).
[35] D. Bratosin, L. Mitrofan, C. Palii, J. Montreuil, Cytometry A, **66**A, 78 (2005).
[36] Z. Darzynkiewicz, G. Juan, X. Li, W. Gorczyca, T. Murakami, T. Traganos Cytometry **27**(1), 1 (1997).